Article

From Bakery Leftovers to Brewing Sustainability: Fermentation of Spent Grain with *Yarrowia lipolytica* and *Lactobacillus acidophilus*

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Abstract: The use of bakery leftovers as a substitute for malt in brewing represents a sustainable approach that reduces costs and waste. In this paper, the fermentation of brewer's spent grain, a byproduct of beer production, is integrated with the use of non-conventional yeasts to unlock the potential of yeasts beyond the common *Saccharomyces* species. This creates a circular system where byproducts are efficiently utilized, fostering sustainability and innovation in food production. This study assesses the fermentative capabilities of the non-conventional yeast *Yarrowia lipolytica* and the lactic acid bacterium *Lactobacillus acidophilus* DSM 20079 on brewer's spent grain, a byproduct from brewing beer with old wheat bread and barley malt. Both hydrolyzed with a cell-wall-degrading enzyme complex and non-hydrolyzed brewer's spent grain were evaluated for key fermentation indicators such as the number of microbial cells, total titratable acidity, pH, reduced sugar content, and fatty acid composition. The findings reveal that *Yarrowia lipolytica* effectively fermented brewer's spent grain without prior hydrolysis, maintaining a balanced fatty acid profile. The combined action of both microorganisms provided optimal fermentation outcomes, offering a promising approach for valorizing brewer's spent grain, reducing waste, and promoting a circular economy in the brewing and food industries.

Keywords: brewer's spent grain; fermentation; non-conventional yeast; lactic acid bacterium; sustainability; old bread

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1. Introduction

The brewing industry is one of the most resource-intensive sectors, generating significant by-products during the production of beer. One of the primary by-products is spent grain, which accounts for approximately 85% of the total waste generated during brewing [1]. Brewer's spent grain (BSG) is composed of the barley husk and residual materials that remain after the brewing process extracts sugars for fermentation. With a BSG content of around 20 kg/hL of brewed beer, approximately 36.4 million tonnes of BSG are produced worldwide annually [2]. BSG has a short shelf life due to its high moisture content and susceptibility to microbial spoilage, and current production is largely limited to low-quality animal feed or landfills. Such practices are unsustainable as the supply of BSG

often exceeds the food needs of local farmers, and each tonne of BSG in landfill releases approximately 513 kg of CO_2 equivalent greenhouse gasses [3].

BSG can be dried to extend its shelf life and facilitate its use as a food ingredient. Although it is a promising source for human nutrition due to its fiber and protein content, the inclusion of BSG in food systems can have a negative impact on the technological, functional, and sensory properties of the product [4–6]. Therefore, implementing processing strategies such as enzymatic processing or fermentation can help improve the functional efficiency of BSG as a food ingredient.

Given the increasing global focus on sustainability and waste reduction, addressing the challenge of BSG disposal presents a critical opportunity for the brewing industry to improve its environmental footprint while unlocking the economic potential of this underutilized resource [2].

Meanwhile, another major contributor to food waste comes from the bakery sector, where substantial quantities of leftover baked goods end up in landfills each year. This accumulation of food waste presents a severe environmental issue, highlighting the need for innovative waste management strategies in both the brewing and bakery industries to enhance sustainability [7].

Bakery waste streams are significant sources of food loss, contributing to the overall issue of global food waste, which amounts to over 1.3 billion tons annually. Bakery waste primarily consists of unsold bread, cakes, and pastries, which are often disposed of due to their short shelf life. Despite the high caloric content and potential nutritional value of these leftovers, they are largely discarded in landfills, where they decompose and release methane, a potent greenhouse gas that contributes to climate change [8].

Efforts to repurpose these waste materials have included composting, animal feed production, and energy recovery through anaerobic digestion. However, these methods often fail to capture the full potential of the nutritional and biochemical content of bakery leftovers and BSG. The fermentation of these by-products using microorganisms offers a more sustainable and economically viable solution, as it enables the production of high-value products while reducing waste volume and greenhouse gas emissions [2].

One promising approach is the microbial fermentation of bakery leftovers and BSG using organisms such as *Yarrowia lipolytica* and *Lactobacillus acidophilus*. These microorganisms have the potential to convert BSG into high-value products such as biofuels, bioactive compounds, and functional food ingredients. This approach aligns with the principles of the circular economy, wherein waste materials are transformed into valuable resources, contributing to industrial sustainability [9]. The combined use of *Y. lipolytica* and *L. acidophilus* leverages their complementary metabolic capabilities: *Y. lipolytica* is known for its capacity to accumulate lipids and produce biofuels and other industrial chemicals, while *L. acidophilus* can enhance the nutritional profile of fermented substrates by producing lactic acid and other metabolites [10].

Microbial fermentation is a biotechnological process that can convert raw materials such as bakery leftovers and BSG into valuable products, including biofuels, organic acids, enzymes, and nutritional supplements [11]. By introducing specific microorganisms capable of metabolizing the complex carbohydrates and proteins in BSG, the fermentation process can enhance the digestibility, nutritional content, and shelf life of BSG-based products [9].

The yeast *Y. lipolytica* has attracted significant interest in the context of BSG fermentation due to its versatile metabolic capabilities. *Y. lipolytica* is an oleaginous yeast, meaning it can accumulate significant amounts of lipids, which can be used to produce biofuels, bioplastics, and other industrial products [12]. For instance, in the fermentation of BSG, *Y. lipolytica* can convert the sugars and residual proteins into microbial lipids, which can be used for biodiesel production, or into proteins for animal feed. Furthermore, *Y. lipolytica*

has been shown to produce a variety of extracellular enzymes, including lipases and proteases, which can break down the complex organic matter in BSG, making it easier for other organisms or processes to utilize the material [13]. The ability of *Y. lipolytica* to grow on complex organic waste materials with minimal pretreatment makes it an attractive option for industrial-scale fermentation processes aimed at converting food waste into biofuels or other bioproducts [14].

On the other hand, *L. acidophilus* is a well-known lactic acid bacteria (LAB) used extensively in the food industry for its probiotic properties and ability to produce lactic acid from sugars. When utilized in the fermentation of bakery waste or BSG, *L. acidophilus* can convert the fermentable sugars present in these substrates into lactic acid, which can be further purified and used as a platform chemical. By fermenting BSG with *L. acidophilus*, it is possible to enhance the nutritional value of BSG by increasing the levels of bioavailable nutrients, such as free amino acids and vitamins [15]. Additionally, lactic acid production during fermentation can lower the pH of the substrate, inhibiting the growth of spoilage microorganisms and thus extending the shelf life of BSG-derived products [16]. Moreover, the use of *L. acidophilus* in the fermentation of BSG offers additional benefits due to its ability to produce peptides, which may have antimicrobial, antioxidant, or antihypertensive properties. These bioactive peptides can enhance the nutritional value of the fermented products, potentially opening new markets for functional foods and nutraceuticals derived from waste products like BSG [17]. In the context of bakery waste, *L. acidophilus* can improve the nutritional profile of the final product by increasing the digestibility of proteins and carbohydrates. The fermentation process can break down complex sugars and fiber, making them more bioavailable and enhancing the nutritional content of the waste-derived products [18].

The combination of *Y. lipolytica* and *L. acidophilus* in the fermentation of BSG presents a synergistic approach that can optimize the valorization of this by-product. *Y. lipolytica* can break down the complex lipids and proteins in bakery leftovers and BSG, releasing simpler compounds that *L. acidophilus* can further metabolize into lactic acid and other bioactive metabolites [2]. This dual fermentation process not only improves the overall yield of valuable products, but also enhances the stability and safety of the fermented material.

Several studies have demonstrated the potential of co-culturing yeasts and LAB in the fermentation of agro-industrial by-products [19]. For instance, co-fermentation of bakery leftovers and BSG with *Saccharomyces cerevisiae* and *Lactobacillus plantarum* has been shown to increase the protein content and digestibility of the final product, making it more suitable for use in functional foods and animal feed [20]. The use of *Y. lipolytica* in combination with *L. acidophilus* is expected to yield similar, if not greater, benefits due to the unique metabolic capabilities of these organisms.

The products derived from the fermentation of bakery leftovers and BSG using *Y. lipolytica* and *L. acidophilus* have several potential applications across multiple industries. One of the most promising applications is in the production of biofuels. The lipids produced by *Y. lipolytica* can be converted into biodiesel, providing a renewable alternative to fossil fuels [12]. Additionally, the lactic acid produced by *L. acidophilus* can be used as a precursor for the production of polylactic acid, a biodegradable plastic that is gaining popularity as a sustainable alternative to conventional plastics [15].

In the food industry, fermented BSG could be used as a functional ingredient in animal feed or as a nutritional supplement in human diets. The fermentation process improves the bioavailability of essential nutrients, making bakery leftovers and BSG a more valuable food source [16]. Furthermore, the probiotic properties of *L. acidophilus* could enhance the health benefits of BSG-based food products, offering potential applications in the development of functional foods and dietary supplements [10].

The combination of *Y. lipolytica* and *L. acidophilus* in the fermentation of bakery leftovers and BSG could have synergistic effects, enhancing the overall efficiency of the bioconversion process. *Y. lipolytica* can break down complex carbohydrates and lipids into simpler components, while *L. acidophilus* can further metabolize sugars into lactic acid, creating a more diverse set of products. This multi-step fermentation process could improve the yield of biofuels, biochemicals, and other high-value products derived from waste substrates [21]. Additionally, the combination of these microorganisms can help optimize the fermentation conditions, improve microbial growth rates, and balance the metabolic pathways. For example, *L. acidophilus* could help lower the pH of the fermentation medium, promoting the growth of *Y. lipolytica* and suppressing the growth of unwanted microorganisms. Such a cooperative fermentation process could increase the overall productivity of the system, leading to more efficient waste valorization [22]. Continued research and development in this area could lead to significant advancements in industrial sustainability and circular economy practices.

In this research, the fermentative capabilities of the non-conventional yeast *Y. lipolytica* and the LAB *L. acidophilus* DSM 20079 were examined on BSG composed of old wheat bread and barley malt. The proposed solution efficacy was estimated based on the values of determined fermentation success indicators. In order to ensure reliable conclusions, the experimental results were processed statistically. To compare the mean values of the analyzed data groups, two-way analysis of variance (two-way ANOVA) was applied because it is the most commonly used technique for statistical data processing when examining the influence of two qualitative independent variables. Furthermore, post hoc analysis was performed using Duncan's multiple-range test, which is suitable for multiple comparisons in biotechnology research.

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 mixture of 1/3 leftover wheat bread, 2 days old (Mlini d.o.o., Čapljina, Bosnia and Herzegovina), and 2/3 barley malt (SLAVONIJA-SLAD d.o.o., Nova Gradiška, Croatia), which consisted of base malt (92.1%), Munich malt (3.9%), and caramel malt (3.9%). The bread and malts were ground in a mill (Matmill Klassik Basis, Kirkel, Germany), and the mixture was combined with softened water (6 °dH) in a mashing machine (Brew Monk, Brouwland, Beverlo, Belgium). During the mashing process, five rests were performed at the following temperatures: 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 this process were collected, frozen, and later utilized in the current study. Moisture content of BSG was 32.4 $\pm$ 0.3%. Moisture content was determined by drying BSG at 105 °C to constant weight according to AOAC Official Methods 920.151 [23].

2.2. Bacterial Strains and Culture Conditions

LAB (*L. acidophilus* DSM 20079) was obtained from the Kaunas University of Technology collection, and *Y. lipolytica* was obtained from the University of Zagreb collection. *L. acidophillus* DSM 20079 was grown in de Man Rogosa and Sharpe (MRS) medium (Biolife, Milan, Italy) supplemented with 0.05% L-cysteine (w/v) at 37 °C. *Y. lypolitica* was grown on Yeast Extract–Peptone–Dextrose (YPD) medium (Sigma-Aldrich, Burlington, NJ, USA) composed of yeast extract (10 g/L), peptone (10 g/L), and glucose (50 g/L). The cultures of *Y. lipolytica* were transported in a refrigerated box from Zagreb to Lithuania. Bacteria cells were collected from slants and transferred into 10 mL YPD medium (Sigma-Aldrich, Burlington, MA, USA) for 24 h at 30 °C.

2.3. Enzymatic Hydrolysis and Fermentation of BSG

BSG was enzymatically hydrolyzed using a Viscozyme® L (Cell Wall Degrading Enzyme Complex from *Aspergillus* sp., Novozyme A/S, Bagsværd, Denmark). BSG was mixed with distilled water at a ratio of 1:1.4 (w/v), and Viscozyme (0.07 mL/g BSG) was added. Enzymatic hydrolysis was conducted in a water bath at 50 °C with shaking (200 rpm) for 6 h [24,25]. The selected samples were used for fermentation with *L. acidophilus* DSM 20079 and *Y. lypolitica*. In parallel, non-hydrolyzed BSG was used as a control for the fermentation experiments. The fermentation of BSG was carried out using three different approaches to investigate the effects of LAB and yeast, individually and in combination. In the first approach, 1% (v/v) of an overnight culture of *L. acidophilus* DSM 20079 was inoculated into the BSG and fermented at 37 °C for 24 h under anaerobic conditions. In the second approach, 1% (v/v) of an overnight culture of *Y. lipolytica* was inoculated into the BSG, and the fermentation was conducted at 30 °C for 24 h under aerobic conditions. The third approach involved a sequential fermentation process. Initially, *L. acidophilus* DSM 20079 was added at 1% (v/v) to the BSG and fermented at 37 °C for 24 h under anaerobic conditions. Following this, 1% (v/v) of *Y. lipolytica* was introduced into the pre-fermented BSG, and a second fermentation stage was performed for an additional 24 h at 30 °C under aerobic conditions. Hydrolysis, fermentation, and subsequent analyses were carried out at Kaunas University of Technology.

2.4. Microbiological Analyses

LAB and yeast count in BSG was carried out according to ISO 15214:1998 and ISO 21527-2:2008 [26,27]. Briefly, 10 g of fermented BSG were homogenized in sterile 9 g/L NaCl. Cell counts were performed by serial dilutions using standard plate count technique. Number of LAB cells was calculated on the MRS agar plate after 72 h incubation at optimal temperature and was expressed as $\log_{10}$ value of CFU/g. Number of yeast cells was calculated on the YPD agar after 24 h incubation at optimal temperature and was expressed as $\log_{10}$ value of CFU/g.

2.5. pH and Total Titratable Acidity Analysis

The pH and total titratable acidity (TTA) of BSG were determined from a 10 g sample homogenized with 90 mL of distilled water. The pH was determined directly using a pH meter (WinLab® Excellent Line, Clausthal-Zellerfeld, Germany). The TTA was expressed in milliliters of 1M NaOH solution used to obtain pH = 8.5.

2.6. Determination of Reduced Sugar Content

The reducing sugar (RS) content was determined using 3,5-dinitrosalicylic acid (DNS) assay according to Miller [28], with some modifications. BSG (1 g) was mixed with distilled water (100 mL) for 10 min and centrifuged at 5000 rpm (Microcen 23, Ortoalresa, Madrid, Spain) for 15 min at room temperature. In total, 1 mL of obtained supernatant was mixed with 1 mL DNS reagent and heated for 5 min at 95 °C. After that, the mixture was cooled and diluted using 6 mL of distilled water. The absorbance was measured at 540 nm using a spectrophotometer (Model Genesys 10, Thermo Electron LED GmbH, Langenselbold, Germany). RS content was calculated from the equation of the standard curve. The standard curve was prepared using glucose solution (1 mg/mL) and distilled water to obtain final glucose concentrations in the range from 0 to 1 mg/mL.

2.7. Gas Chromatography Determination of Fatty Acid Composition

The fatty acid composition of BSG (fermented and non-fermented) lipid extract was analyzed using gas chromatography (GC) after converting fatty acids into their methyl

esters (FAMEs) using boron trifluoride (BF_3) as a catalyst according to the EN ISO 12966-4:2015 method [29]. Briefly, for triglycerides esterification and free fatty acids saponification, 0.5 ± 0.001 g of BSG lipid extract and 4 mL of methanolic NaOH (0.5 M) were poured into 50 mL round-bottomed flask and refluxed in a boiling water bath for 15–20 min until the disappearance of the fatty phase. After esterification, 5 mL of 24% boron trifluoride/methanol complex was added, the mixture was boiled for 2 min, cooled to room temperature, diluted with 5 mL n-hexane followed with the addition of NaCl, well shaken, and left still until the layers separated.

For FAMEs analysis, 100 µL of hexane phase was diluted with 900 µL of pure GC-grade hexane for compound quantification. The samples were analyzed using gas chromatograph HRGC 5300 (Mega Series, Carlo Erba, Milan, Italy) equipped with a flame ionization detector (GC-FID) and 100 m length 0.25 mm (id), 0.20 µm film thickness fused silica capillary column SPTM-2560 (Supelco, Bellefonte, PA, USA). The analysis parameters were as follows: injection temperature 220 °C; detector's temperature 240 °C; split ratio 100:1; oven temperature was programmed from 80 °C to 240 °C at 4 °C/min and held isothermal for 5 min; carrier gas–helium with a flow rate of 20 cm^3/s; and injection volume–1 µL. A standard FAME mixture of 37 fatty acids (C8–C24) was used for compound identification; the results are expressed as a percentage of the total GC-FID peak area.

2.8. Data Analysis

All experiments were performed in triplicate, and the results were averaged and are represented as mean ± standard deviation. The experimental results were processed using Two-way analysis of variance (Two-way ANOVA) followed by a post hoc Duncan's multiple-range test. For the graphical interpretation of the statistical analysis results, Box and Whisker Plots were generated. 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).

3. Results

In accordance with the aim of this research, the fermentation of non-hydrolyzed and hydrolyzed BSG composed of old wheat bread and barley malt was performed using *Y. lipolytica* and *L. acidophilus* DSM 20079. The fermentative performance of selected non-conventional yeast and lactic acid bacterium was examined both in pure and mixed cultures. Microbial cell counts, reducing sugar content, pH, TTA, and fatty acid composition were selected to evaluate the fermentation process, as they provide a comprehensive assessment of microbial activity, metabolic dynamics, and fermentation progress.

Furthermore, experimental data were statistically processed at a confidence interval of 95% to establish the effect of variation in applied microorganisms and substrate preparation on the efficacy of brewers' spent grain utilization. Additionally, the determined results of fermentation success indicators were analyzed using Duncan's multiple range test to select the combination of microorganism (*Y. lipolytica*, *L. acidophilus* DSM 20079, and their mixtures) and substrate (non-hydrolyzed and hydrolyzed BSG) for which the most significant value of the observed indicator was achieved.

3.1. Statistical Parameters

The results of the two-way ANOVA analysis for the effect of different microorganisms and substrates on the number of microbial cells, pH level, total titratable acidity, reducing sugar content, and fatty acid methyl ester (C16:0, C18:0, C18:1n9c, C18:2n6n, and C18:3n3), determined in media obtained upon completion of fermentation, are given in Table 1. The two-way ANOVA analysis of the results related to the number of microbial cells were not

performed, as the variability in the experimental data was sufficient to draw meaningful conclusions, and statistical confirmation is not required.

Table 1. Two-way ANOVA results for the effect of different microorganisms and substrates on indicators of brewers' spent grain fermentation success.

Fermentation Success Indicator	Effect	SS	DF	MS	F-Ratio	*p*-Value
pH level (1)	Microorganisms	3.665	2	1.833	9424.657	<0.000001
	Substrates	0.125	1	0.125	642.857	<0.000001
	Microorganisms and substrates	0.075	2	0.038	193.971	<0.000001
	Error	0.002	12	0.000	-	-
Total titratable acidity (mL of 1M NaOH)	Microorganisms	14.698	2	7.349	220.467	<0.000001
	Substrates	3.380	1	3.380	101.400	<0.000001
	Microorganisms and substrates	20.253	2	10.127	303.800	<0.000001
	Error	0.400	12	0.033	-	-
Reducing sugar content (mg/mL)	Microorganisms	1312.979	2	656.490	42,015.235	<0.000001
	Substrates	0.303	1	0.303	19.414	0.000856
	Microorganisms and substrates	40.305	2	20.153	1289.767	<0.000001
	Error	0.188	12	0.016	-	-
C16:0 (%)	Microorganisms	26.374	1	26.374	308.645	<0.000001
	Substrates	27.694	1	27.694	324.101	<0.000001
	Microorganisms and substrates	0.460	1	0.460	5.386	0.048865
	Error	0.684	8	0.085	-	-
C18:0 (%)	Microorganisms	0.216	1	0.216	33.751	0.000401
	Substrates	3.360	1	3.360	525.033	<0.000001
	Microorganisms and substrates	0.010	1	0.010	1.595	0.242171
	Error	0.051	8	0.006	-	-
C18:1n9c (%)	Microorganisms	90.915	1	90.915	4275.004	<0.000001
	Substrates	39.640	1	39.640	1863.935	<0.000001
	Microorganisms and substrates	14.941	1	14.941	702.555	<0.000001
	Error	0.170	8	0.021	-	-
C18:2n6c (%)	Microorganisms	114.825	1	114.825	590.535	<0.000001
	Substrates	1062.954	1	1062.954	5466.697	<0.000001
	Microorganisms and substrates	0.015	1	0.015	0.076	0.790317
	Error	1.556	8	0.194	-	-
C18:3n3 (%)	Microorganisms	778.596	1	778.596	95,241.138	<0.000001
	Substrates	860.552	1	860.552	105,266.304	<0.000001
	Microorganisms and substrates	8.201	1	8.201	1003.123	<0.000001
	Error	0.065	8	0.008		

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

The two-way ANOVA summary results presented in Table 1 indicate that *p*-values for examined factors and their interactions are much below 0.05, which is the critical value for a confidence interval of 95%. That means that applied microorganisms and utilized substrates, as well as their combinations, have a statistically significant effect on observed indicators of BSG fermentation success. This does not apply to the influence of the interactions between microorganisms and the substrate preparation procedure on the formation of C18:0 and C18:2n6c fatty acid methyl esters.

The mean square values given in Table 1 indicate that the selection of microorganisms has the strongest influence on the pH level of fermented media, the residual content of reducing sugars, and the formation of the C18:1n9c fatty acid among the analyzed effects. On the other hand, the values of the same statistical parameter for the formation of C16:0, C18:2n6c, and C18:3n3 fatty acid methyl esters suggest that the substrate preparation procedure has the most significant effect on the values of these fermentation success indicators. Furthermore, the represented values of the mean square indicate that the interaction of examined factors has the most important influence on the total titratable acidity of fermented media. Finally, statistical analysis determined that the selection of microorganisms and the preparation of substrates have an equally pronounced im-

pact on the formation of the C16:0 fatty acid when performing experiments under the applied conditions.

3.2. Number of Microbial Cells in Fermented Media

The results of the microbiological analyses are shown in Table 2.

Table 2. Microbiological analyses' results.

Microorganisms	Fermentation Time	Substrate	
		Hydrolyzed	Non-Hydrolyzed
		(log_{10} CFU/g)	
L. acidophilus	0 h	6.69 ± 0.03	6.56 ± 0.14
	24 h	8.45 ± 0.18	8.65 ± 0.04
Y. lipolytica	0 h	6.02 ± 0.01	6.06 ± 0.06
	24 h	8.18 ± 0.02	8.46 ± 0.09
Combination of *L. acidophilus* and *Y. lipolytica*:			
L. acidophilus	0 h	6.61 ± 0.04	6.59 ± 0.14
	24 h	8.45 ± 0.18	8.65 ± 0.04
	48 h	7.94 ± 0.01	8.20 ± 0.10
Y. lipolytica	0 h	-	-
	24 h	6.12 ± 0.07	6.11 ± 0.06
	48 h	7.12 ± 0.04	7.98 ± 0.22

The present study investigated the growth dynamics of yeast and LAB in a co-cultured fermentation system using BSG as a medium. The results demonstrate that yeast and LAB showed different growth patterns when used in combination, compared to their single-culture counterparts. Specifically, yeast counts remained lower in samples where LAB was first introduced, which can be attributed to several factors, including competition for available sugars, pH changes, and differences in optimal growth temperatures. Our findings are consistent with the findings of a study which demonstrated that co-culturing LAB and yeast did not yield superior fermentation results when compared to single-culture fermentation [30]. In fact, in their study, yeast performance was inhibited in the presence of LAB, which supports our hypothesis of competitive interactions between the two microorganisms for fermentable sugars in the medium.

It was observed that LAB growth did not significantly increase during the second fermentation stage (24–48 h) following the addition of yeast. One possible explanation is the suboptimal temperature for LAB growth. The fermentation process was carried out at 30 °C, which is closer to the optimal growth temperature for yeast, but lower than the optimal range for LAB, typically around 37 °C [31,32]. LAB typically thrive at temperatures between 37 °C and 42 °C, with lower temperatures such as 30 °C potentially hindering their growth [32].

Additionally, the low pH produced during LAB fermentation could have further compromised yeast growth by creating a less favorable environment for their survival and activity [22]. Moreover, the results show that the yeast population did not increase significantly after 24 h in the LAB-preconditioned medium, likely due to the fact that low-pH. *Y. lipolytica* thrives best in pH environments between 5.5 and 6.0, and acidic conditions (pH < 4.0) resulting from LAB fermentation inhibit its metabolic functions, including sugar uptake and other metabolic activities [33,34]. Acidification, particularly in values below 4.0, significantly reduces the metabolic efficiency of *Y. lipolytica*, as low pH inhibits key enzymatic pathways required for growth and metabolism [35]. This reinforces the importance of pH management in mixed-culture fermentations, especially when utilizing LAB and yeast together in a single process.

Interestingly, the LAB count also did not show a significant increase between 24 and 48 h, suggesting that LAB's growth was potentially limited by the fermentation temperature.

Given that the incubation temperature of 30 °C was optimized for yeast, LAB growth might have been compromised, preventing a further increase in the LAB population during the later fermentation stages [36].

3.3. pH Level of Fermented Media

The results of the statistical analysis for the effect of applied microorganisms on the pH levels of fermented media are shown in Figure 1a. Graphically presented results indicate that media with the highest pH value were obtained when *Y. lipolytica* is used to ferment BSG, whether hydrolyzed or not. This finding aligns with previous research, such as the study by Papanikolaou et al., where *Y. lipolytica* was shown to thrive in alkaline conditions, producing fewer acids while focusing on lipid production rather than lowering the pH of the medium [37]. In contrast, when fermentation of both substrates was performed by *L. acidophilus* DSM 20079 independently or in combination with applied non-conventional yeast, the bioprocess outcomes were media with significantly lower pH levels. This is supported by findings that LAB, including *L. acidophilus*, are key in lowering pH through the production of organic acids, primarily lactic acid, which preserves food and enhances its flavor [18].

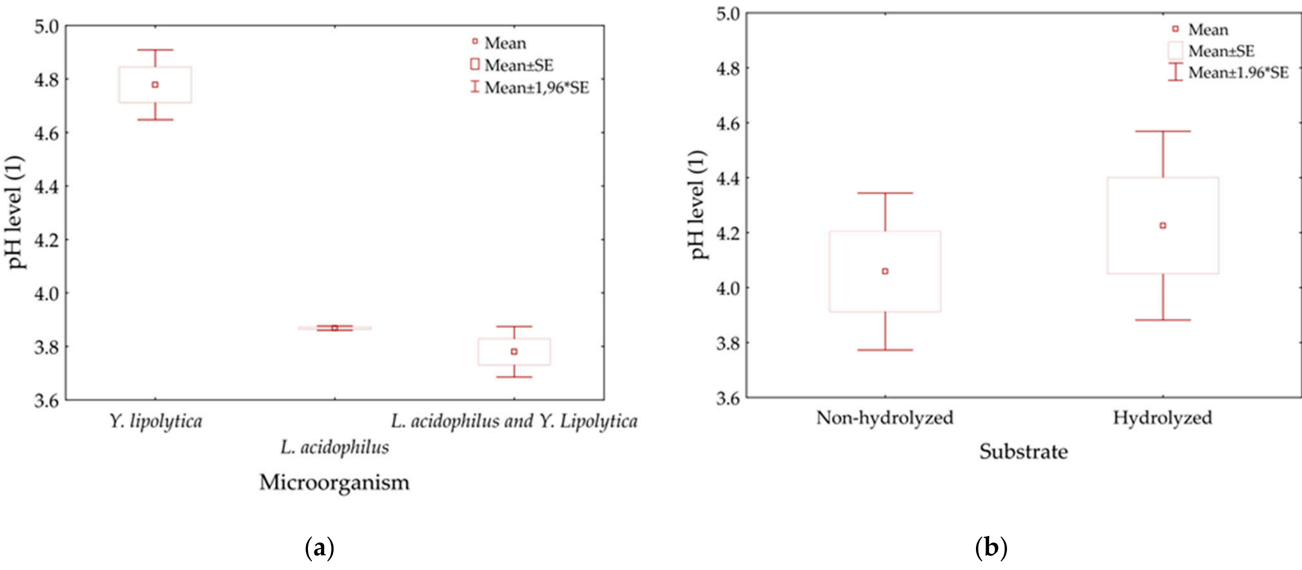

Figure 1. The effect of examined factors on pH level of fermented media: (**a**) variation in applied microorganisms; (**b**) variation in substrate preparation.

A graphical representation of the statistical analysis results for the effect of substrate preparation on the pH level of media at the end of fermentation is given in Figure 1b. It is evident that, regardless of the applied microorganism, the media with somewhat lower pH values were obtained if the preparation of BSG for fermentation did not involve the hydrolysis step. In the context of lignocellulosic biomass, cellulose and hemicellulose can be converted into fermentable sugars via enzymatic hydrolysis, which can then be used by LAB, enhancing the fermentation processes and acid production [38].

The summarized results of Duncan's multiple range test, conducted to select the combination of microorganism and substrate for which the most appropriate pH level of fermented media was achieved, are presented in Table 3. It can be seen that the highest values of this parameter were measured in media fermented using *Y. lipolytica* (4.93 ± 0.01 for hydrolyzed and 4.63 ± 0.02 for non-hydrolyzed substrate). However, there is a statistically significant difference between the pH values obtained for experiments performed using hydrolyzed and non-hydrolyzed BSG (p = 0.000172). The medium with the lowest pH level

(3.67 ± 0.02) was obtained when *L. acidophilus* DSM 20079 and *Y. lipolytica* simultaneously fermented the non-hydrolyzed substrate, while the fermentation of hydrolyzed BSG using mixed cultures resulted in medium with a significantly higher pH level (3.89 ± 0.02), which was confirmed by the *p*-value of 0.000066. It can also be noticed that almost identical pH values of media were obtained after the fermentation of hydrolyzed and non-hydrolyzed substrates by *L. acidophilus* DSM 20079 (3.86 ± 0.01 and 3.87 ± 0.01, respectively).

Table 3. Duncan's multiple range test: mean ± standard deviation for pH level of fermented media.

Microorganism	Substrate	pH Level (1) *
L. acidophilus and *Y. lipolytica*	Non-hydrolyzed	3.67 ± 0.02 [a]
L. acidophilus	Hydrolyzed	3.86 ± 0.01 [b]
L. acidophilus	Non-hydrolyzed	3.87 ± 0.01 [b]
L. acidophilus and *Y. lipolytica*	Hydrolyzed	3.89 ± 0.02 [b]
Y. lipolytica	Non-hydrolyzed	4.63 ± 0.02 [c]
Y. lipolytica	Hydrolyzed	4.93 ± 0.01 [d]

* Values marked with the same letter are not significantly different at α = 0.05.

3.4. Content of Reducing Sugars in Fermented Media

RS were analyzed in the examined substrates before and after fermentation under the applied conditions. The obtained results show that non-hydrolyzed BSG initially contained an RS of 13.07 ± 0.41 mg/mL. However, after hydrolysis carried out, as described above (Section 2.3), the initial content of RS increased to 18.63 ± 0.49 mg/mL.

The data shown in Figure 2a represent the statistical analysis results for the effect of applied microorganisms on the RS content in fermented media. Considering the mean values of this parameter for both substrates, the highest sugars concentration remained after the fermentation performed using *L. acidophilus* DSM 20079. According to the graphically presented results, it can also be noticed that fermentation of BSG, whether hydrolyzed or not, by *Y. lipolytica* resulted in significantly lower residual RS. However, the lowest RS content was detected in media that were simultaneously fermented by LAB and non-conventional yeast.

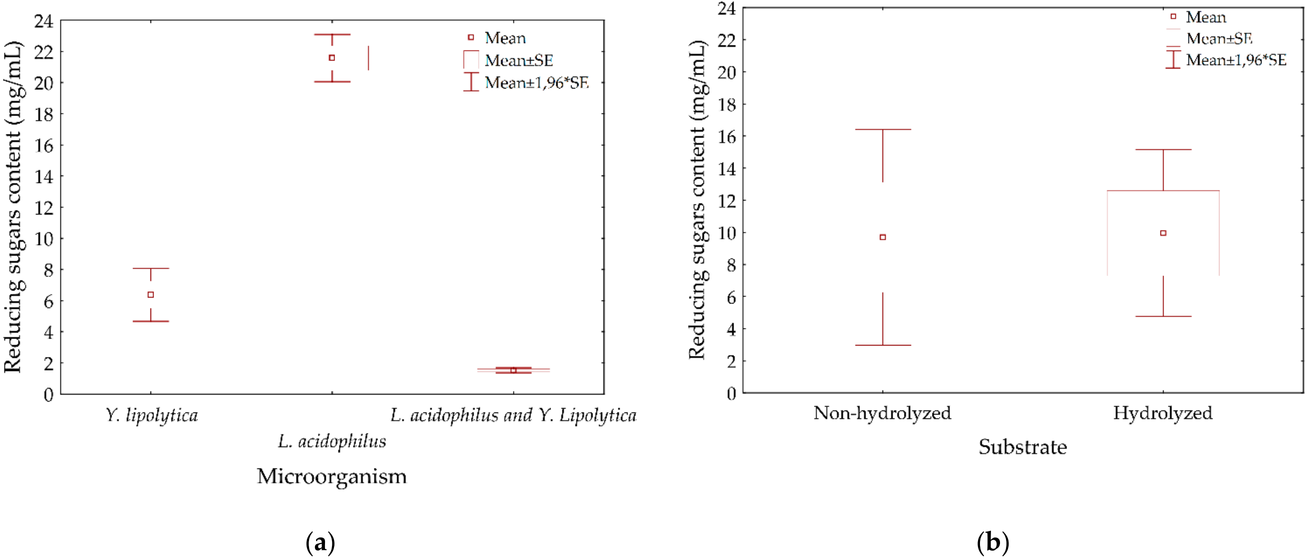

Figure 2. The effect of examined factors on reducing sugars content in fermented media: (**a**) variation in applied microorganisms; (**b**) variation in substrate preparation.

The results of the statistical analysis of experimental data that are related to the RS content, detected in examined substrates after the completion of fermentation, are graphically

represented in Figure 2b. It is evident that, regardless of the applied microorganism, there were no significant differences in the residual RS content between the obtained media.

The summary of data obtained using the post hoc analysis using Duncan's multiple range test, performed to select the combination of microorganisms and substrate for which the most appropriate RS content in fermented media was achieved, is given in Table 4. From the presented results, it can be observed that the highest concentration of residual RS was determined in media fermented by *L. acidophilus* DSM 20079 (23.30 ± 0.08 mg/mL for non-hydrolyzed and 19.85 ± 0.24 mg/mL for hydrolyzed substrate). Higher pH levels often enhance microbial activity, leading to better sugar utilization [39]. It seems that *Y. lypolitica* generates a significant amount of weak acids or non-dissociating acids, captured by the TTA measurement. However, the statistically significant difference between the RS content obtained for experiments using hydrolyzed and non-hydrolyzed BSG was established (p = 0.000172). The media with the lowest residual RS content were obtained after the fermentation of non-hydrolyzed and hydrolyzed BSG using mixed cultures (1.35 ± 0.02 mg/mL and 1.70 ± 0.12 mg/mL, respectively). However, a statistically significant difference was found between these values (p = 0.004853). When it comes to the fermentation of BSG by *Y. lipolytica*, it was noticed that lower RS content remained in the non-hydrolyzed substrate (4.43 ± 0.05 mg/mL) compared to the hydrolyzed substrate (8.30 ± 0.12 mg/mL) and that this difference was statistically significant (p = 0.000172).

Table 4. Duncan's multiple range test: mean ± standard deviation for reducing sugars content in fermented media.

Microorganism	Substrate	Reducing Sugars Content (mg/mL) *
L. acidophilus and *Y. lipolytica*	Non-hydrolyzed	1.35 ± 0.02 [a]
L. acidophilus and *Y. lipolytica*	Hydrolyzed	1.70 ± 0.12 [b]
Y. lipolytica	Non-hydrolyzed	4.43 ± 0.05 [c]
Y. lipolytica	Hydrolyzed	8.30 ± 0.12 [d]
L. acidophilus	Hydrolyzed	19.85 ± 0.24 [e]
L. acidophilus	Non-hydrolyzed	23.30 ± 0.08 [f]

* Values marked with the same letter are not significantly different at α = 0.05.

3.5. Total Titratable Acidity of Fermented Media

The data derived after the statistical analysis of experimental results related to the TTA of fermented media are graphically presented in Figure 3a. It can be observed that the media with the lowest TTA were obtained when *L. acidophilus* DSM 20079 was used to ferment BSG, whether hydrolyzed or not. Conversely, when the fermentation of both substrates was carried out by *Y. lipolytica*, independently or in combination with applied LAB, media with significantly higher TTAs were achieved.

A graphical interpretation of the results of the statistical analysis, performed to examine the effect of substrate preparation on the TTA of fermented media, is shown in Figure 3b. It is obvious that the use of examined microorganisms in the fermentation of hydrolyzed BSG resulted in media with higher acidity compared to the media obtained after the fermentation of the non-hydrolyzed substrate.

The summarized results of Duncan's multiple range test, performed to select the combination of microorganism and substrate for which the most appropriate TTA of fermented media was achieved, are given in Table 5. According to the presented results, it is evident that a certain acidity level was detected in all analyzed media samples. However, the medium with the highest TTA (9.33 ± 0.12 mL of 1M NaOH) was obtained after fermentation of hydrolyzed BSG by *Y. lipolytica*. Simultaneous usage of lactic acid bacterium and non-conventional yeast in the fermentation of non-hydrolyzed and hydrolyzed BSG resulted in the fermented media with a lower TTA (8.27 ± 0.12 mL of 1M NaOH and

6.80 ± 0.00 mL of 1M NaOH, respectively). There is a statistically significant difference between all of these acidity levels, which was confirmed by the calculated *p*-values (0.000178, 0.000095, and 0.000172). The medium with the lowest TTA (5.40 ± 0.20 mL of 1M NaOH) was obtained when *L. acidophilus* DSM 20079 fermented the non-hydrolyzed BSG, while the fermentation of the hydrolyzed substrate using the same microorganism resulted in a medium with statistically significantly higher acidity (5.80 ± 0.20 mL of 1M NaOH), which was confirmed by the *p*-value of 0.000032. In addition, it can be noticed that the acidity level of the medium, obtained after the fermentation of non-hydrolyzed substrates by *Y. lipolytica* (5.67 ± 0.31 mL of 1M NaOH), is at the same level of statistical significance as the two lowest discussed values (p = 0.099037 and p = 0.388876, respectively). Contrary to expectations based on *Y. lipolytica*'s low acid production, the TTA for this yeast is higher when fermenting hydrolyzed substrates (9.33 ± 0.12 mL of 1M NaOH) compared to non-hydrolyzed substrates (5.67 ± 0.31 mL of 1M NaOH). This can be explained by the hydrolysis process breaking down complex sugars into fermentable monosaccharides, leading to better microbial metabolism and higher overall acid production despite the focus on lipids. In the case of *Y. lipolytica*, the enhanced metabolism also supports the production of abscisic acid as part of its secondary metabolites, along with acids. The higher TTA observed in hydrolyzed samples suggests an increased acid production, which aligns with the dual metabolic activity for lipid synthesis [40] and secondary metabolite production such as abscisic acid [41]. *Y. lipolytica*, which seems to generate weak or non-dissociating acids (as indicated by TTA measurements), can influence the final pH differently than LAB. Although *Y. lipolytica* does produce acids, including citric and other organic acids, these are weak acids that do not significantly lower the pH [35]. Citric acid, for instance, is a weak acid and only partially dissociates in water, which helps in buffering the environment rather than drastically acidifying it [34]. These weak acids play a crucial role in flavor development and can modulate the fermentation process by interacting with other microorganisms. Consequently, this often leads to a higher pH, which facilitates better sugar utilization and supports the growth of yeast and other non-LAB organisms.

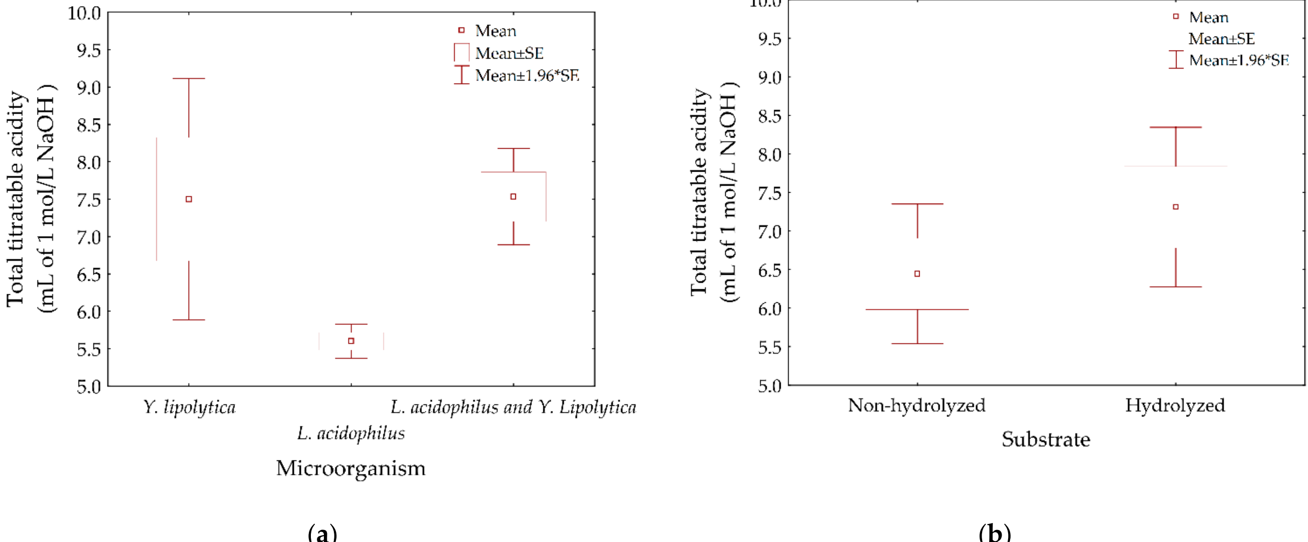

Figure 3. The effect of examined factors on total titratable acidity of fermented media: (**a**) variation in applied microorganisms; (**b**) variation in substrate preparation.

Table 5. Duncan's multiple range test: mean ± standard deviation for total titratable acidity of fermented media.

Microorganism	Substrate	Total Titratable Acidity (mL of 1M NaOH) *
L. acidophilus	Non-hydrolyzed	5.40 ± 0.20 [a]
Y. lipolytica	Non-hydrolyzed	5.67 ± 0.31 [ab]
L. acidophilus	Hydrolyzed	5.80 ± 0.20 [b]
L. acidophilus and *Y. lipolytica*	Hydrolyzed	6.80 ± 0.00 [c]
L. acidophilus and *Y. lipolytica*	Non-hydrolyzed	8.27 ± 0.12 [d]
Y. lipolytica	Hydrolyzed	9.33 ± 0.12 [e]

* Values marked with the same letter are not significantly different at $\alpha = 0.05$.

3.6. Fatty Acids Composition of Fermented Media

The fatty acid methyl esters analyzed in this study included C11:0, C13:0, C14:0, C15:0, C15:1, C16:0, C16:1, C17:0, C17:1, C18:0, C18:1n9c, C18:2n6c, C18:3n3, C20:0, C22:0, C20:3n3, C20:4n6, C23:0, C22:2, and C20:5n3. In Table 6, only the fatty acid methyl esters with the highest concentrations are presented, specifically C16:0, C18:0, C18:1n9c, C18:2n6c, and C18:3n3. The decision to statistically analyze these particular fatty acid methyl esters was based on their prominence, making them more relevant for interpretation and meaningful comparisons. By focusing on the fatty acid methyl esters with the highest concentrations, the statistical analysis is more reliable and robust, as these data points are less likely to be influenced by variability or detection limits compared to fatty acid methyl esters present in smaller amounts. Duncan's multiple-range test results, shown in Table 6, indicate that all analyzed media samples differ significantly ($p < 0.05$), suggesting a statistically significant difference in fatty acid content across the samples. Exceptionally, in the case of C16:0 fatty acid methyl ester detection, two samples were at the same significance level ($p = 0.766662$). These are media obtained by the metabolic activity of *L. acidophilus* and *Y. lipolytica* on non-hydrolyzed BSG, as well as by *Y. lipolytica* on a hydrolyzed BSG. In the study by Liu et al. [42], *Y. lipolytica* demonstrated high tolerance to long-chain fatty acids (C16–C18), which may explain its enhanced performance in fermenting hydrolyzed BSG. This tolerance allows for the yeast to effectively utilize long-chain fatty acids as carbon and energy sources, contributing to the production of both saturated (C18:0) and unsaturated fatty acids (C18:1n9c, C18:2n6c, and C18:3n3). The increased concentrations of these fatty acids in hydrolyzed BSG could be a result of the yeast's metabolic flexibility, which supports the hypothesis that *Y. lipolytica* can thrive in environments rich in long-chain fatty acids, promoting efficient fatty acid synthesis during fermentation [42]. Furthermore, *Y. lipolytica*'s ability to metabolize diverse carbon sources, such as those found in hydrolyzed BSG, further enhances its capacity to produce higher concentrations of fatty acids, supporting its use in industrial fermentation processes [43,44].

Table 6. Duncan's multiple range test: mean ± standard deviation for fatty acid methyl ester in fermented media.

Microorganism	Substrate	C16:0 (%) *	C18:0 (%) *	C18:1n9c (%) *	C18:2n6c (%) *	C18:3n3 (%) *
Y. lipolytica	Non-hydrolyzed	11.39 ± 0.20 [a]	3.21 ± 0.05 [d]	20.37 ± 0.05 [b]	25.39 ± 0.05 [a]	36.52 ± 0.12 [d]
Y. lipolytica	Hydrolyzed	14.04 ± 0.16 [b]	2.09 ± 0.04 [b]	18.97 ± 0.08 [a]	44.28 ± 0.23 [c]	17.93 ± 0.03 [b]
L. acidophilus and *Y. lipolytica*	Non-hydrolyzed	13.96 ± 0.06 [b]	2.88 ± 0.05 [c]	28.11 ± 0.04 [d]	31.65 ± 0.21 [b]	18.76 ± 0.13 [c]
L. acidophilus and *Y. lipolytica*	Hydrolyzed	17.39 ± 0.53 [c]	1.88 ± 0.14 [a]	22.24 ± 0.28 [c]	50.40 ± 0.83 [d]	3.48 ± 0.04 [a]

* Values marked with the same letter in the same column are not significantly different at $\alpha = 0.05$.

4. Conclusions

For the first time, BSG was used as a fermentation medium for *Y. lipolytica*, either alone or in combination with LAB. Inoculation strategies and fermentation temperature

(30 °C) significantly influenced the dynamics of LAB and yeast growth. LAB growth was constrained by suboptimal temperatures for its optimal range (37–42 °C), while *Y. lipolytica* growth was inhibited by the acidic conditions (pH < 4.0) created by the LAB metabolism.

Fermentation with *L. acidophilus* DSM 20079 resulted in the highest residual sugar content (23.30 ± 0.08 mg/mL for non-hydrolyzed and 19.85 ± 0.24 mg/mL for hydrolyzed BSG), indicating lower sugar consumption. In contrast, mixed cultures achieved the lowest residual sugar content, particularly in non-hydrolyzed substrates (1.35 ± 0.02 mg/mL), demonstrating their synergistic sugar utilization. Hydrolysis significantly enhanced sugar availability, improving substrate utilization by both LAB and yeast. Interestingly, *Y. lipolytica* performed better on non-hydrolyzed BSG, suggesting substrate-specific interactions.

Media fermented with *Y. lipolytica* maintained the highest pH values, consistent with its preference for alkaline conditions and lipid production rather than acid formation. In contrast, *L. acidophilus* DSM 20079 and mixed cultures produced significantly lower pH levels due to lactic acid production, aligning with LAB's role in food preservation and flavor enhancement. Hydrolyzed BSG substrates exhibited higher TTA, regardless of the microorganism applied, suggesting enhanced microbial metabolism due to the availability of simpler sugars after hydrolysis.

Y. lipolytica demonstrated efficient fatty acid synthesis. It also showed tolerance to long-chain fatty acids (C16:0, C18:0, C18:1n9c, C18:2n6c, and C18:3n3), contributing to its potential in food fermentation.

Co-cultivation of yeast and LAB in BSG media resulted in lower yeast growth compared to single-culture conditions, likely due to competition for sugars, pH reduction, and temperature constraints. These findings highlight the need for optimized fermentation parameters, including sequential inoculation timing, pH management, and temperature control, to maximize the growth and activity of both LAB and yeast in co-culture systems. This could lead to more balanced fermentation outcomes and enhanced product quality.

Further optimization of hydrolysis parameters is crucial, with future research focusing on the analysis of reducing sugars, monosaccharides, disaccharides, and polysaccharides. A particular emphasis on fiber metabolism is essential, given BSG's high fiber content, to better understand *Y. lipolytica*'s substrate preferences.

These findings demonstrate the potential for bio-transforming BSG using *Y. lipolytica* as a valuable tool to enhance LAB metabolism and produce a fermented product suitable for use as a functional food ingredient. This approach offers a sustainable pathway for utilizing BSG, contributing to both industrial applications and food innovation.

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