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Sustainable Valorization of Brewer's Spent Grain via Submerged Fermentation Using *Talaromyces stollii* for Laccase and Phenolic Compounds Production

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Abstract

Brewer's spent grain (BSG) is the main solid byproduct of the brewing industry, generated in large quantities worldwide. Its high organic content and availability make it an attractive substrate for biotechnological valorization and recycling within a circular economy framework, contributing to the recovery and reuse of agro-industrial residues. This study investigates the potential of *Talaromyces stollii* I05.06 to simultaneously produce laccase and release phenolic compounds through submerged fermentation (SmF) using BSG as the sole carbon source. Initial SmF trials confirmed the fungus's capacity to metabolize BSG. Subsequent fermentations with phosphate buffer supplementation (100 mM) significantly enhanced laccase activity (1535 $\pm$ 151.6 $U \cdot L^{-1}$ on day 5) and phenolic content (6.28 $\pm$ 0.07 mg GAE per 100 g on day 1 with 50 mM buffer). However, the addition of typical laccase inducers (Cu^{2+} and Mn^{2+}) led to inhibitory effects. The results highlight *T. stollii* I05.06 as a promising microorganism for the integrated valorization of BSG, contributing to sustainable agro-industrial waste management and the development of value-added bioproducts.

Keywords: biomass valorization; brewers' spent grain; *Talaromyces stollii*; laccase; phenolic compounds

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Academic Editor: Salustiano Mato De La Iglesia

Received: 28 July 2025
Revised: 17 August 2025
Accepted: 20 August 2025
Published: 21 August 2025

Citation: Lima, E.C.S.; Nascimento, A.C.B.d.; Nascimento, R.P.d.; Itabaiana, I., Jr. Sustainable Valorization of Brewer's Spent Grain via Submerged Fermentation Using *Talaromyces stollii* for Laccase and Phenolic Compounds Production. *Recycling* **2025**, *10*, 166. https://doi.org/10.3390/recycling10040166

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1. Introduction

Beer is the most common alcoholic beverage consumed in the world and, when compared to non-alcoholic beverages, it ranks third. In 2023, global beer production reached 1.88 billion hectoliters, with the leading producing nations being China, the USA, and Brazil, respectively [1,2]. In 2024, the brewing industry global market size was valued at USD 851.15 billion with a compound annual growth rate of 4.1% [3].

Brewers' spent grain (BSG) is a byproduct of the beer manufacturing, with thousands of tons generated annually. Brewing generates a substantial volume of BSG, estimated at 20 kg per 100 L of beer produced. Given the global production of 2024, roughly 37.6 million tons were generated in that year. This residue can account for up to 85% of the total waste in the brewing industry, with the remainder consisting of hot trub and residual brewer's yeast [4,5]. Considering this biomass's high nutritional protein and fiber content, approximately 70% is used as livestock feed, about 10% is allocated to biogas production, and the remaining 20% is discarded as waste in landfills [6,7].

Brazil, a country with an economy strongly driven by agribusiness and focused on agricultural products and derivatives [8], has a notable example of contribution to the national economy in its beer industry. Accounting for 1.6% of the Gross Domestic Product (GDP), the industry annually produces 452,827 tons of barley and 14.1 billion liters of beer. It generates revenue of USD 19.38 billion while contributing USD 3.8 billion in taxes [9–11]. With population growth and increased agricultural activity, particularly in the beer industry byproducts, the generated waste tends to grow substantially. Consequently, the improved utilization of this still-underused residual biomass represents a growing interest for a country like Brazil.

BSG, as a lignocellulosic biomass, comprises three distinct polymeric organic fractions: cellulose (14–40.9%), hemicellulose (14.9–40%), and lignin (3.8–17.8%). The lignin fraction is highly recalcitrant, forming a rigid structure that limits enzymatic and microbial access to cellulose and hemicellulose. Therefore, pretreatment is essential to break down and disrupt the lignin fiber structure, thereby improving the accessibility and subsequent conversion of the carbohydrate fractions [4,12,13]. Although lignocellulosic biomass such as BSG presents a highly recalcitrant and difficult-to-dispose matrix, this structural complexity and chemical richness offer promising opportunities for valorizing its fractions into value-added products. This characteristic has increasingly drawn academic attention as a potential route to address waste management challenges in the brewing industry [14]. The use of surplus residual biomass from a growing sector, to reintegrate this material into the production chain through more sustainable methodologies, aligns with the Sustainable Development Goals (SDGs) proposed by the United Nations [15], such as promoting sustainable agriculture and encouraging industry, innovation, and infrastructure—as well as with the core principles of the circular economy.

The primary pretreatment methods currently employ physicochemical processes such as steam explosion, AFEX (ammonia fiber explosion), and wet oxidation [13,16,17]. With the gradual consolidation of green chemistry principles and circular economy concepts, the search for cleaner and more environmentally friendly processes [18,19] has focused attention on organisms capable of degrading this biological matrix. Ligninolytic microorganisms can express enzymes in their genetic framework that can act on the lignocellulosic matrix by depolymerizing it, representing an alternative to other classical pretreatment methods. Filamentous fungi are among the microorganisms capable of performing such enzymatic hydrolysis degradation [13,16,20]. Many biotechnological research studies have focused on Fermentations using such microorganisms [14].

Among these fungi, those of the phylum *Basidiomycota*, class *Agaricomycetes*, also known as White-Rot Fungi, stand out for their high capacity to produce enzymes such as laccases, lignin peroxidases, and manganese peroxidases. These enzymes act synergistically towards lignin degradation, enabling the microorganism to access the cellulose and hemicellulose fractions and proceed with the matrix hydrolysis [13]. However, filamentous fungi of the phylum *Ascomycota* are also known as ligninolytic enzyme producers [21,22]. Among these, members of the *Talaromyces* genus have been investigated for their ability to produce such enzymes [23,24].

This enzyme, expressed by fungi known as laccase or fungal laccases (EC 1.10.3.2), is an oxireductase-class enzyme belonging to the multicopper oxidase family and is intrinsically involved in lignin depolymerization. These enzymes are powerful and versatile biocatalysts due to: (1) their non-specific catalytic activity on phenolic substrates and lignin polymers; (2) requiring only oxygen to catalyze reactions while producing water as the sole byproduct (without redox mediators), making the process more sustainable; and (3) often exhibiting high redox potential [25,26]. Consequently, laccases stand out as the oxireductases with the highest number of reported applications across various sectors, including: the paper

industry [27], textile industry [28,29], pharmaceutical industry [30], food industry [31,32], and have applications in bioremediation [33].

In this enzymatic hydrolysis process of the lignocellulosic matrix, particularly concerning lignin depolymerization by laccase, some chemical intermediates of interest are phenolic compounds [34]. The phenolic compounds released during lignin depolymerization by laccases are aromatic structures characterized by one or more hydroxyl groups attached to a benzene ring. Their industrial relevance stems from three key properties: (1) antioxidant activity, neutralizing free radicals through electron donation [35]; (2) antimicrobial action, inhibiting pathogens via interaction with cell membranes [36]; and (3) chelating capacity, which enhances their application as preservatives in food and nutraceuticals [31].

Currently, the literature on BSG biovalorization using *Talaromyces* fungi remains scarce, and to date, no studies have reported *Talaromyces stollii* as a platform for fungal laccase production; In addition, considering the current underutilization of residual brewery biomass globally and its potential to generate higher value-added compounds with potential applications in various industrial sectors, this study investigates the potential of *Talaromyces stollii* as a fermenting agent for spent brewer's grain (SBG) as a lignocellulosic substrate to produce laccases and phenolic compounds of interest, proposing a promising and straightforward methodology for valorization.

2. Results and Discussion

2.1. BSG Characterization

This work commenced with the physicochemical characterization of BSG. Table 1 presents the average values and their respective standard deviation of the mean for the composition of the total fraction (weight%, dry weight basis) of the BSG sample.

Table 1. Chemical composition of brewers' spent grain (BSG).

Components	% (% Dry wt)
Cellulose	14.74 ± 1.04
Hemicellulose	22.13 ± 0.41
Lignin	15.13 ± 1.47
Ash	5.36 ± 0.57
Crude Protein	20.54 ± 0.28

The compositional analysis of our sample reveals consistent trends with prior studies on brewer's spent grain (BSG). The cellulose and hemicellulose contents of the sample are similar to those reported by Massardi et al. [37] (15.99% and 29.92%) and Mussatto et al. [38] (16% cellulose and 28.4%), suggesting minimal variation in polysaccharide content in BSG sample used in this study. In contrast, lignin content results show the most significant divergence in the literature; however, the values obtained in this work are closer to those reported by Kanauchi et al. [39] and Silva et al. [40], with lignin contents of 11.9% and 16.9%, respectively. The ash content resembles those described by Mainali et al. [41] (4.71%), with other literature values being similarly close, such as Qin et al. [42] (3.54%) and Kanauchi et al. [39] (2.4%). The results in this work show slight variation regarding the crude protein content reported in the literature for similar BSG samples. Massardi et al. [37] and Qin et al. [42] report 21.16% and 22.44%, respectively.

The variations in cellulose, hemicellulose, lignin, and other discussed components reported in the literature, whether pronounced or subtle, can be attributed to some variables in the process of brewing like grain treatment during the malting process. Due to different temperatures used during malting, enzyme activity over the grains may result in different final compositions from each batch [43]. The specific pH and temperature conditions

used during wort production, which vary according to beer recipes, may also impact the chemical characterization of BSG [44]. Additionally, regional abiotic factors such as soil characteristics and climatic conditions may also cause different growth rates on barley, significantly influencing the composition of these organic fractions on BSG [45].

2.2. Preliminary Submerged Fermentations (PSmF)

This section discusses the relevant results obtained from spectrophotometric analyses of the fermented extracts from the preliminary fermentation trials conducted in this study. This initial screening fermentation assessed the suitability of BSG in distilled water as a standalone medium for fungal laccase and phenolic compound production without nutritional supplementation. The laccase activity profile in the enzymatic extract during the 7-day fermentation is shown in Figure 1.

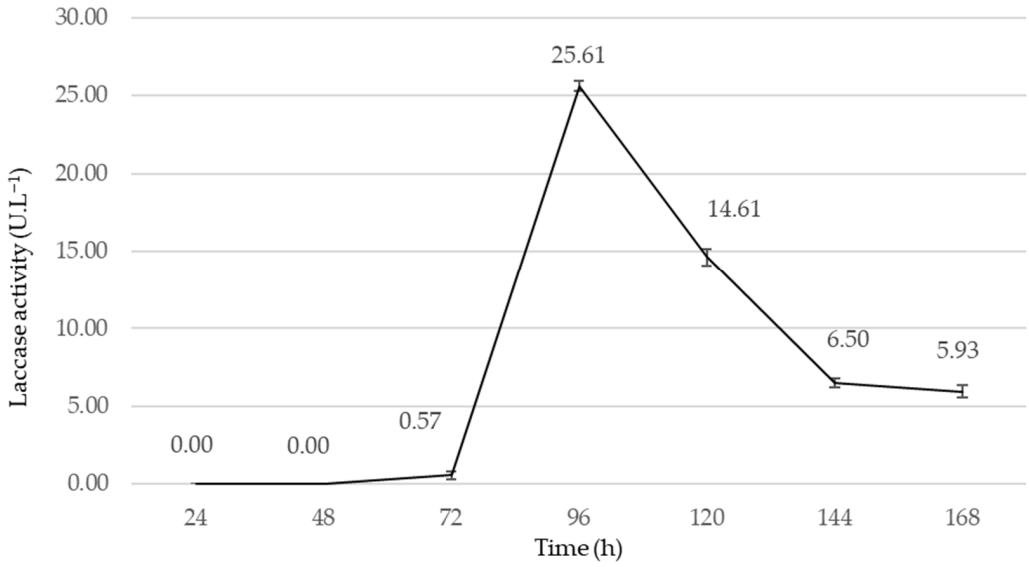

Figure 1. Laccase activity in fermentative extract throughout 7-day PSmF.

The plotted values reveal a laccase activity peak on day 4 of fermentation with an activity of 25.61 ± 0.33 U·L^{-1}, followed by a decline to 14.61 ± 0.52 U·L^{-1} on day 5. During the final fermentation days, activity decreased to 6.50 ± 0.29 U·L^{-1} and 5.92 ± 0.39 U·L^{-1} on days 6 and 7, respectively.

Forootanfar et al. [46] achieved laccase activity of 65 U·L^{-1} after 9 days of fermentation using the ascomycete *Paraconiothyrium variabile* cultivated with SDB medium. Fonseca et al. [47] conducted experiments with *Ganoderma applanatum* (44.89 U·L$^{-1)}$ and *Peniophora sp.* (81.32 U·L^{-1}), both cultivated with 12.7 g·L^{-1} malt extract and 5 g·L^{-1} corn steep liquor.

Compared to these studies that employed culture media with supplementation for their initial screening for laccase production, the enzymatic activity obtained from *Talaromyces stollii* was slightly different. However, it showed that the strain I05.06 could produce laccase and assimilate the biomass in a medium composed only of BSG and distilled water.

In cited studies and the present work, a decline in activity was observed following the recorded peaks. This phenomenon could be attributed to increased medium viscosity caused by fungal growth, whether through pellet formation or dispersed filamentous mycelia [48]. The mass transfer capacity between gas and liquid phases in a fermentation system is intrinsically related to medium viscosity. Consequently, oxygen supply in the

fermentation system may become compromised with increasing viscosity, particularly during later stages, which could explain the observed decline [49].

To further assess and understand the capacity of *Talaromyces stollii* to degrade the BSG matrix, the phenolic compound content released was evaluated during the fermentation. Figure 2 displays the pattern of phenolic compound content measured throughout the 7-day fermentation period.

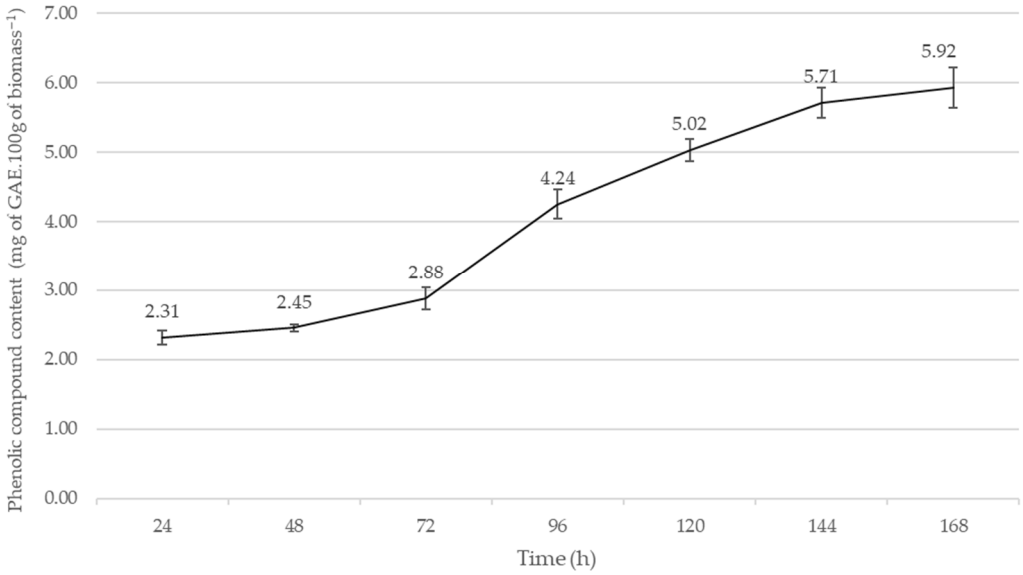

Figure 2. Phenolic compound content in fermentative extract during 7-day PSmF.

Analysis of the obtained values revealed a continuous increase in total phenolic compound concentration within the fermented extracts throughout the incubation period. Lower concentrations were observed between days 1–3 with 2.31 ± 0.9, 2.45 ± 0.5, and 2.88 ± 0.15 mg GAE 100 g biomass, respectively. From days 4–6, a marked increase in phenolic compound concentration was recorded with 4.24 ± 0.20, 5.02 ± 0.16, and 5.71 ± 0.21 mg GAE·100 g biomass^{-1}, respectively, with peak concentration reached on day 7 with 5.92 ± 0.28 mg GAE 100 g biomass.

In a study by Silva et al. [50] using *Rhizopus oryzae* as the fermenting agent, a phenolic yield of 5.37 mg GAE·100 g biomass^{-1} was obtained after 2 days of solid-state fermentation, and in another study employing the basidiomycete *Phanerochaete chrysosporium*, Moella & Bosco [51] reported a yield of 5.8 mg GAE·100 g biomass^{-1} following 48 h of solid-state fermentation.

2.3. Submerged Fermentation with Supplemented Medium

After evaluating the capability of *Talaromyces stollii* I05.06 to grow and produce laccase and phenolic compounds only using BSG and distilled water as medium, results showed that medium supplementation was necessary for higher yields of products. A 50 mM potassium/phosphate buffer was used as a supplement. Figure 3 shows the laccase activity values obtained over the 7-day fermentation period for enriched medium fermentations using 50 mM potassium phosphate buffer (pH 5.8).

The results demonstrate a substantial increase in laccase activity for the fermentation supplemented with 50 mM potassium phosphate buffer compared to the preliminary trial. The intermediate timepoint of 82 h marks the onset of enzymatic activity with 94.4 U·L^{-1} ± 10.74—already exceeding the peak value from previous fermentations. A prominent rise follows, with activities reaching 317.8 ± 13.70 U·L^{-1} (96 h) and

448.3 ± 13.33 U·L^{-1} (106 h), culminating in a peak activity of 628.3 ± 23.3 U·L^{-1} at 120 h. Subsequently, activity declined to 559.4 ± 34.07 U·L^{-1} (144 h) and 375.0 ± 16.66 U·L^{-1} (168 h).

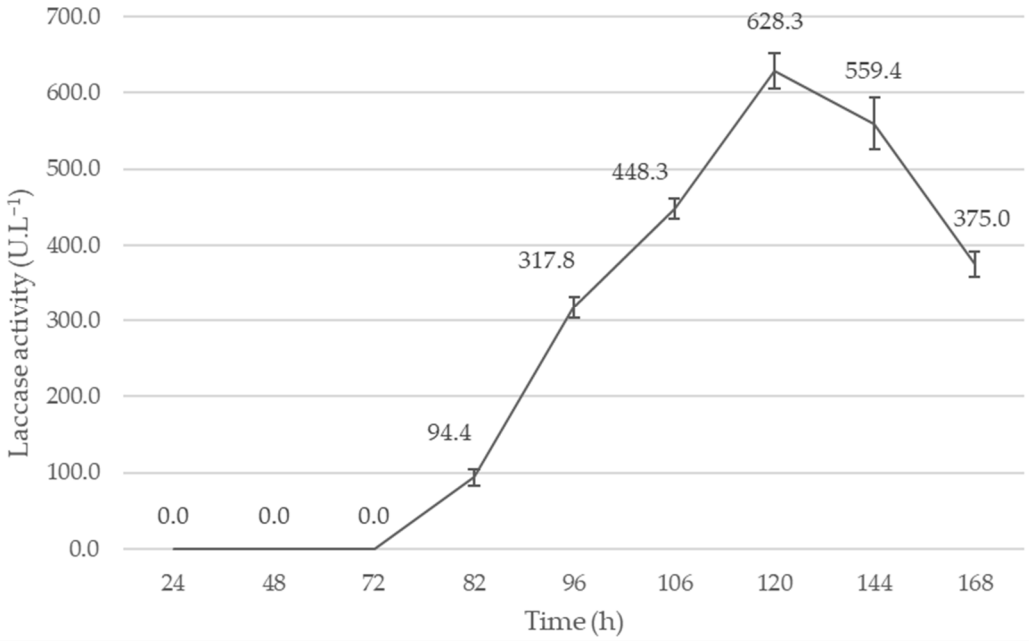

Figure 3. Laccase activity during the 7-day fermentation period in supplemented medium.

The laccase activity achieved in this assay may be considered satisfactory when compared to other laccase-producing *ascomycetes* under supplementation conditions, as demonstrated by Othman et al. [52] using a strain of *Trichoderma harzianum* S7113 under submerged fermentation with supplemented medium containing glucose (15 g·L^{-1}), beef extract (5 g·L^{-1}), and ammonium chloride (4 g·L^{-1}), authors were able to produce 355 U·L^{-1} of laccase. Forootanfar et al. [46] achieved laccase activity of 512 U·L^{-1} after 9 days of fermentation using the *P. variabile* in fermentation with copper inducers. In separate studies, Chakroun et al. [53] reported 661 U·L^{-1} activity after 7 days of submerged fermentation with *Trichoderma atroviride* in optimized glucose-based synthetic medium.

SmF with two more buffer concentrations (25 mM and 100 mM) were tested to further investigate the influence of varying potassium phosphate buffer concentrations on laccase yield. Table 2 presents the enzymatic activity results from the 5th day of the fermentation conducted with different buffer concentrations.

Table 2. Laccase activity vs. different buffer concentrations.

Potassium/Phosphate Buffer (mM)	K (g·L^{-1}) *	P (g·L^{-1}) *	Lac (U·L^{-1}) *
25	1.03	0.77	782.5 ± 29.1
50	2.06	1.54	983.3 ± 28.3
100	4.13	3.09	1535 ± 151.6

* K—potassium concentration in different buffer concentrations was used; P—phosphorus concentration in different buffer concentrations was used; Lac—laccase activity was achieved under these conditions.

The data presented in the table demonstrate that buffer concentration significantly influences laccase production, showing a positive correlation between phosphate concentration and enzymatic activity, higher concentrations yielded greater activity. This observed activity increase could be attributed to phosphate and potassium ion supplementation. These elements play fundamental roles in microbial basal metabolism, particularly in ATP synthesis [54].

The total phenolic content was measured after the SmF with supplemented medium to investigate whether the increase in the laccase yield also enhanced the phenolic compounds in the fermentation extract. Figure 4 displays the total phenolic compound concentrations measured throughout the 7-day Smf with supplemented medium, including intermediate timepoints at 82 and 106 h.

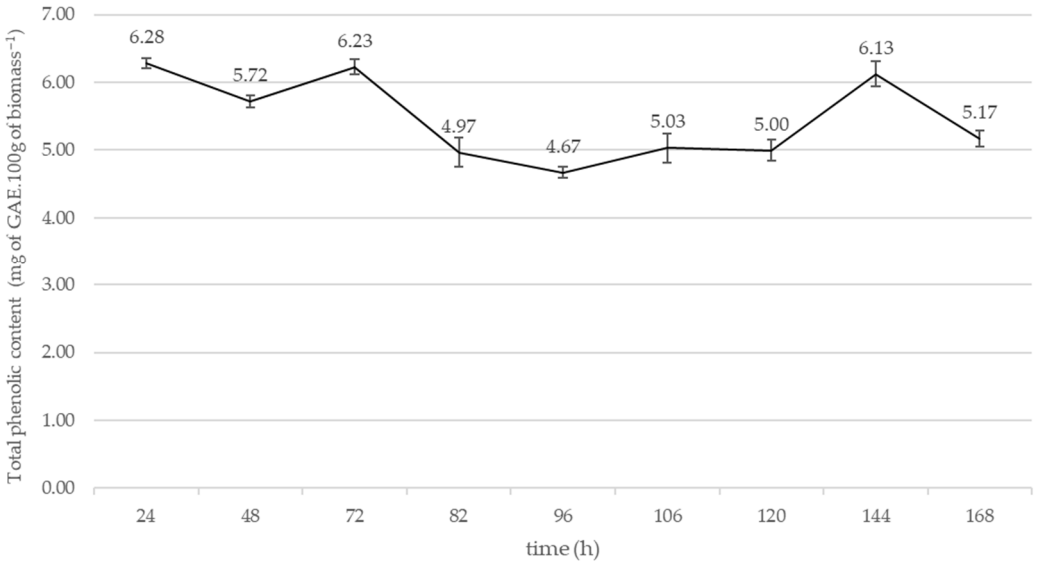

Figure 4. Profile of TPC throughout 7-day SmF with supplemented medium.

The graph analysis reveals significant fluctuations in phenolic compound content within the fermented extracts throughout the fermentation period. The highest concentration occurred on day 1 (6.28 ± 0.07 mg GAE·100 g biomass^{-1}), followed by day 2 (5.72 ± 0.08 mg GAE·100 g biomass^{-1}) and day 3 (6.23 ± 0.11 mg GAE·100 g biomass^{-1}), representing the second-highest concentration point. A prominent decline in concentration persisted from the mid-point at 82 h (4.97 ± 0.21 mg GAE·100 g biomass^{-1}) through 120 h (5.00 ± 0.15 mg GAE·100 g biomass^{-1}). On day 6, phenolic content reached its third-highest level (6.13 ± 0.18 mg GAE. 100 g biomass^{-1}) but decreased again to 5.17 ± 0.12 mg GAE·100 g biomass^{-1} by day 7.

According to França et al. [55], in similar work using submerged fermentation with enriched medium, coffee pulp as lignocellulosic substrate, and the fungus *Lentinus crinitus*, a yield of 24.6 mg GAE·100 g biomass^{-1} 100 g was obtained after 5 days of incubation. Maia et al. [56] demonstrated the efficiency of fungi from the genera *Aspergillus oryzae* and *Aspergillus terreus* in increasing the release of phenolic compounds from BSG in solid-state fermentation, resulting in 8.45 mg GAE·100 g biomass^{-1} and 7.90 mg GAE·100 g biomass^{-1}, respectively.

Although an increase was observed on the first and third days compared to preliminary fermentations, this enhancement was not considered significant, particularly compared to other studies. The varying potassium phosphate buffer concentrations (25 mM, 50 mM, and 100 mM) did not significantly influence total phenolic content, with 25 mM yielding slightly lower values and 100 mM producing results nearly identical to 50 mM. Despite the significant increase in laccase levels in the medium, this stagnation in total phenolic concentration may be explained by the absence of other ligninolytic enzymes in the fermentative extract. In this study, lignin peroxidase activity was also measured, but no significant results were detected. Thus, the presence of laccase alone allows the fungus to partially disrupt the lignocellulosic matrix partially, enabling access only to the cellulose and hemicellulose fractions [57,58].

2.4. Submerged Fermentation with Copper and Manganese Inducers

Given the influence of laccase activity inducers, such as copper and manganese salts, and other chemical compounds described in the literature, the study aimed to modulate the activity by using copper and manganese ions in combination with the previously tested buffer. Table 3 presents the laccase activity results after 5 days of fermentation using culture medium supplemented with inducing salts (copper sulfate and manganese sulfate) at varying concentrations, with the previously tested buffer at its optimal 100 mM concentration (which showed the highest laccase activity).

Table 3. Laccase activity after 5-day SmF with inducers.

Cu^{2+} ($g \cdot L^{-1}$)	Mn^{2+} (mM)	Lac ($U \cdot L^{-1}$)
0.025	0	992.5 ± 99.1
0.05	0	879.1 ± 25.8
0.075	0	291.6 ± 62.2
0	1	775 ± 46.6
0	2	665 ± 30.1
0	3	80 ± 8
0.025	1	0
0.05	2	0
0.075	3	0

Both inducers exhibited a concentration-dependent inhibitory effect on enzyme activity. Under 100 mM buffer conditions, the addition of low concentrations of Cu^{2+} (0.025 $g \cdot L^{-1}$) and Mn^{2+} (1 mM) resulted in reduced enzyme activities of 992.5 ± 99.1 $U \cdot L^{-1}$ and 775 ± 46.6 $U \cdot L^{-1}$, respectively, when compared to the inducer-free control (1535 ± 151.6 $U \cdot L^{-1}$). Notably, no detectable enzymatic activity was observed when both inducers were applied simultaneously in the same fermentation, suggesting a possible synergistic inhibitory interaction.

According to Mussatto et al. [59], BSG may naturally contain minerals such as copper and manganese. Jin et al. [60] reported that their brewer's spent grain sample contained Mn (47.3 ± 0.6 $mg \cdot kg^{-1}$) and Cu (21.6 ± 0.4 $mg \cdot kg^{-1}$). The XRF analysis results for Cu^{2+} and Mn^{2+} in the BSG sample from this study are shown in Table 4.

Table 4. BSG mineral composition for Mn^{2+} and Cu^{2+} screening.

Component	wt %
Mn^{2+}	39.0016
Cu^{2+}	60.9984

A distribution of 61% copper and 39% manganese was assessed, qualitatively confirming the pre-existence of these metals in the sample. This outcome of SmF with inducers may be due to the high concentration of these minerals in the medium, making it toxic to fungal metabolism, given that the biomass already contains significant minerals.

3. Materials and Methods

3.1. BSG Characterization

This subsection will address and describe the methodologies used for conducting the physicochemical analyses of the biomass employed in this work. All analyses were performed using the unground grain (BSG) biomass. The BSG was dried in a 7lab oven (SSE-21 L) at 60 °C for 48 h and ground in a Wiley knife mill (TE-680) using a 30 mesh (595 µm) separation sieve, resulting in a fine, flour-like powdered biomass.

3.1.1. Determination of Moisture Content

The moisture content of the BSG was determined using a Shimadzu moisture balance (MOC63u, Montevideo, Uruguay). The moisture balance is an instrument that combines a precision scale with a controlled heating system. The sample is heated at an adjustable temperature ranging from 40 °C to 200 °C, and the mass loss is monitored in real time. The moisture percentage is automatically calculated by the difference between the initial and final weight after drying, employing the thermogravimetric principle [61]. For this analysis, 0.5 g was weighed into a tray suitable for use with the equipment. The sample is heated to 105 °C and maintained at this temperature until the mass variation does not exceed 0.05% for 30 s; the result can be recorded at this point.

3.1.2. Determination of Crude Protein Content

Thus, 0.2 g of 30-mesh ground biomass and 0.7 g of catalyst (copper sulfate and sodium sulfate, 1:5 $w{\cdot}w^{-1}$) were weighed onto filter paper. The filter paper was then folded to retain its contents and placed in a digestion tube. The reaction was incubated using a digestion block programmed as described in Table S1.

After incubation, the sample was allowed to cool, then 10 mL of distilled water and three drops of phenolphthalein were added before the digestion tube was connected to the nitrogen distiller (TECNAL TE-0364). The distiller's measuring cup was filled with 40 mL of 40% NaOH. The distillation was performed into a 250 mL Erlenmeyer flask containing 10 mL of boric acid and four drops of mixed indicator. After collecting 100 mL of distillate in the flask, its content was titrated against 0.1 N HCl until the endpoint of the indicator. The crude protein percentage of the sample was calculated according to Equation (1) [37,62].

$$\%\ \text{of Crude protein} = \left(\frac{N_{HCl} * V_{HCl} * 0.014 * 100}{\text{wt biomass}} \right) * \text{F} \quad (1)$$

where N_{HCl} stands for the normality of HCl solution used in the titration; V_{HCl} represents the total volume of HCl consumed during the titration; F indicates the nitrogen-to-protein conversion factor (6,25); wt biomass express the initial measured biomass weight.

This experiment was conducted in triplicate (n = 3). Data are presented as mean ± standard deviation (SD).

3.1.3. Determination of Extractives Content

Extractives were removed using Soxhlet extraction to determine the lignin and sugar contents more accurately. The extraction can occur in two stages: one using water, and a subsequent extraction using ethanol. The biomass can then be dried, and the extractives content obtained through the weight difference in the material, as described in protocol NREL/TP-510-42619 by Sluiter et al. [63]. The experiment was conducted in triplicate.

3.1.4. Determination of Cellulose, Hemicellulose, Lignin, and Ash Content

Once the biomass has undergone the extraction process, one can proceed with the characterization of cellulose and hemicellulose content. In this stage, the sample must be subjected to acid hydrolysis to depolymerize these fractions, and it is broken down into more easily quantifiable components to quantify the percentage of the desired organic fractions. The acid hydrolysis converts polymeric carbohydrates into soluble monomers that can be quantified. At the end of the hydrolysis, the sample is filtered to separate the solid residual fraction, which will be used for the total lignin content determination protocol, from the liquid fraction that can be prepared for injection into the HPLC (High Performance Liquid Chromatography) system and properly quantified [64]. The HPLC analysis was performed using a Bio-Rad Aminex HPX-87P column (South Granville

NSW 2142, Australia), with an injection volume of 10 µL, a flow rate of 0.6 mL/min, and the temperature maintained at 80–85 °C.

The values of Acid-Insoluble Residue (AIR) and ash content in the sample must be considered for lignin content determination. The acid-soluble lignin (ASL) must be analyzed to determine total lignin. Thus, the insoluble fractions are assessed through gravimetric analysis, while the soluble portion is subjected to UV-Vis spectroscopy as described by SLUITER et al. [64]. The chromatograms generated in this study are available in the Supplementary Material, Figure S1. This experiment was conducted in triplicate (n = 3). Data are presented as mean ± standard deviation (SD).

3.2. Microorganism

The filamentous fungus *Talaromyces stollii* I05.06 used in this work was isolated from Itatiaia National Park and selected from the culture collection of the Laboratory of Ecology and Microbial Processes (LEPM) at the Federal University of Rio de Janeiro's Technology Center. The microorganism, stored in penicillin bottles using the Castellani method, was reactivated by transferring a mycelial slot to Petri dishes containing BDA medium at pH 5.5 and maintained at 28 °C for 7 days. After this period, strain maintenance through continuous subculturing was performed using 30% glycerol solution and sterile swabs [65–67]. Figure 5 displays the morphological characteristics of the fungus after growth in cultivation medium.

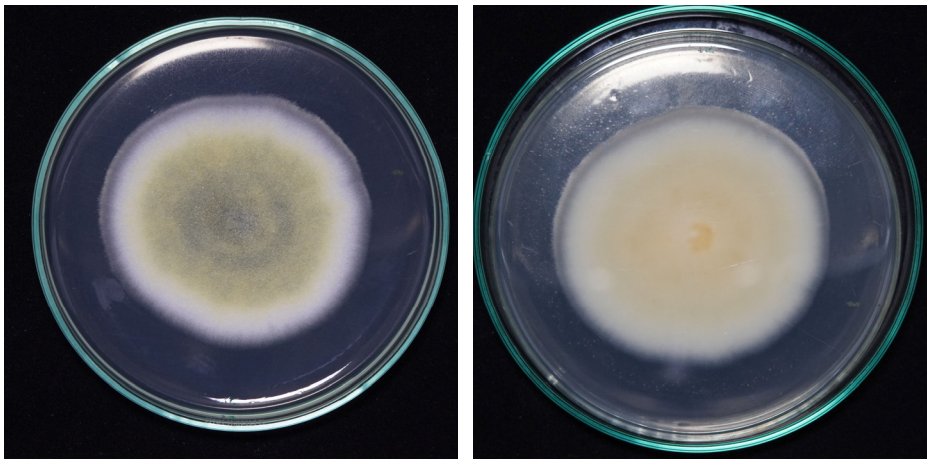

Figure 5. (**Left**) and (**Right**) sides of a Petri dish containing *Talaromyces stollii* I05.06 after 7 days of growth in PDA medium, pH 5.5, incubated at 28 °C.

3.3. Spore Suspension

A standardized spore suspension was prepared to initiate fermentation. I05.06 was cultivated in an Erlenmeyer flask containing 60 mL of BDA medium at pH 5.5, followed by 7-day incubation at 28 °C. The mycelial surface was washed with PBS buffer solution containing 0.1% TWEEN 80. Spore counting was performed using a Neubauer chamber. This study consistently used a fixed inoculum load of 4×10^5 spores/mL for all fermentations [68,69].

3.4. Preliminary Submerged Fermentations

Initial screening fermentations assessed the suitability of BSG in distilled water as a standalone medium for fungal laccase and phenolic compound production without nutritional supplementation. The preliminary submerged fermentations were performed in 250 mL Erlenmeyer flasks containing 50 mL of distilled water and 1 g of biomass, corresponding to a 2% ($w{\cdot}v^{-1}$) ratio between substrate and fermentation medium. This

weight/volume ratio was fixed for all subsequent fermentations. The established experimental conditions included a temperature of 28 °C, initial pH adjustment to 5.5, agitation at 200 rpm, and 7 days, using an incubator shaker (SOLAB SL-223) [65]. The experiment controls consisted of a system containing only BSG and distilled water.

For fermentative extract preparation of all fermentations in this work, aliquots of the fermentation medium were transferred from Erlenmeyer flasks to Falcon tubes and centrifuged at 3000 rpm for 10 min. The resulting supernatant was carefully collected and stored at 4 °C in a refrigeration unit for subsequent analytical procedures conducted in this study.

3.5. Submerged Fermentations with Enriched Medium

After the initial fermentations, potassium and phosphorus ion supplementation were introduced to the fermentation medium to optimize laccase and phenolic compound production. For this purpose, a 50 mM potassium phosphate buffer (pH 5.8) was used as the medium, providing ion concentrations of 2.06 $g \cdot L^{-1}$ of potassium and 1.54 $g \cdot L^{-1}$ of phosphate. Additionally, two intermediate timepoints were collected between days 3–4 and days 4–5. To further evaluate the buffer's observed influence, a fifth-day endpoint fermentation was conducted with three different potassium phosphate buffer concentrations: 25 mM, 50 mM, and 100 mM.

3.6. Submerged Fermentations with Inducers

The literature describes how copper ions and other metals can beneficially influence laccase production in fermentation processes. Lorenzo et al., (2006) [69] discuss in their work that the induction of laccase production caused by toxic metals would occur due to this enzyme's involvement in synthesizing pigments that prevent the uptake of these metals. Thus, to evaluate this modulation in laccase production by strain I05.06 using BSG as substrate, fermentations were conducted with the addition of copper sulfate (Cu^{2+}) and manganese sulfate (Mn^{2+}) at different concentrations, both jointly and separately, in different experiments. The response of these ions was also observed against the buffer concentrations previously used. The schematization of each ion's concentrations and the different buffer concentrations used in each fermentation assay can be observed in Table 5.

Table 5. Fermentation scheme showing inducers vs. Buffer concentration.

Experiment	Cu^{2+} ($g \cdot L^{-1}$)	Mn^{2+} (mM)	Buffer Concentration (mM)
1	0.025	0	25
2	0.05	0	50
3	0.075	0	100
4	0	1	25
5	0	2	50
6	0	3	100
7	0.025	1	25
8	0.05	2	50
9	0.075	3	100

3.7. Determination of Laccase Activity

The laccase activity was determined through oxidation of a standard substrate, ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate). This Spectrophotometric methodology is based on the enzyme's ability to oxidize the substrate, promoting its conversion from the cationic to anionic form. This process results in a color change in the medium from light blue to dark blue. This color alteration can be monitored via spectrophotometer readings at a specific wavelength to determine enzymatic activity.

The reaction occurred in 3 mL cuvettes, where 2 mL of 0.4 mM ABTS, 990 µL of 0.1 mM citrate/0.2 mM phosphate buffer (McIlvaine buffer) adjusted to pH 4.5, and 10 µL of fermented extract were added. Absorbance readings were monitored between 0 and 5 min at 30 s intervals using a spectrophotometer (KASVI K37-VIS, João Pessoa, State of Paraíba, Brazil) at a wavelength of 420 nm [24]. The enzymatic activity was calculated using the equation described by Agrawal & Verma [70].

$$Lac\ activity = \frac{\Delta abs * 10^6\ Vt}{\varepsilon.v} \quad (2)$$

where Δabs represents the absorbance difference; ε is the molar extinction coefficient of ABTS equal to 36,000 $M^{-1} \cdot cm^{-1}$ at 420 nm; V is the enzymatic extract volume (L); Vt is the total reaction volume (L); and T is the reaction time (min). This experiment was conducted in triplicate (n = 3). Data are presented as mean ± standard deviation (SD). Error bars in the figures indicate the standard deviation of the mean.

3.8. Determination of Total Phenolic Content

The spectrophotometric methodology using Folin–Ciocalteu reagent was employed to quantify total phenolic compounds in the fermented extract. This colorimetric assay is based on the interaction between reducing substances in the sample and the reagent, changing the color from yellow to blue [71].

The assay was conducted in 96-well plates. 10 µL of fermented extract and 190 µL of Folin–Ciocalteu reagent (previously diluted 1:10 in distilled water) were added for each well. After 3 min of incubation, the reaction was stopped by adding a 20% (w/v) sodium carbonate solution. Readings were then taken using a microplate reader (Molecular Devices, SpectraMax M2e, LLC., San Jose, CA, USA) at 765 nm wavelength [72,73]. The calibration curve was constructed from a 1 mg/mL gallic acid stock solution to generate points with different standard concentrations. The standard curve data points and the curve are provided in Table S2 and Figure S2. This experiment was conducted in triplicate (n = 3). Data are presented as mean ± standard deviation (SD). Error bars in the figures indicate the standard deviation of the mean.

3.9. Determination of the Presence of Copper and Manganese

For the qualitative analysis of the presence of ion species of copper (Cu^{2+}) and manganese (Mn^{2+}) in the biomass, a methodology of X-ray fluorescence (XRF) was applied [74]. The analysis was performed using XRF at the Hydrogen Technology Laboratory (LabTecH) of the School of Chemistry/UFRJ, employing a Rigaku Primini model spectrometer equipped with a palladium tube, operating at 40 kV and 1.25 mA.

4. Conclusions

Following preliminary fermentation experiments, results showed that brewer's spent grain (BSG) with distilled water as the fermentation medium alone lacks all necessary components for relevant laccase production. Laccase production was significantly improved by employing an enriched medium supplemented with potassium and phosphate ions through potassium phosphate buffer at varying concentrations. Under optimized conditions (5-day fermentation, initial pH 5.8, $4 \cdot 10^5$ cells$\cdot mL^{-1}$, 200 rpm, and 100 mM buffer containing 4.03 K and 3.5 P), the highest activity observed in this study was 1535 ± 151.6 $U \cdot L^{-1}$. Subsequent studies on inducer effects (copper and manganese ions) revealed that these metals caused an overall decrease in enzymatic activity even at low concentrations. When used simultaneously, no detectable activity was observed. Regarding the phenolic compounds obtained through the fermentations, the highest yields

were also encountered during the fermentation with the enriched medium, being day 1 (6.28 ± 0.07 mg GAE·100 g biomass^{-1}), followed by day 3 (6.23 ± 0.11 mg GAE·100 g^{-1}). Showing that despite the high yield in laccase production, the enzyme cannot fully depolymerize lignin in the matrix.

BSG is an abundant lignocellulosic biomass worldwide, particularly in agroindustry-driven countries like Brazil. This biomass remains underutilized, and improved waste management strategies are urgently needed. This study is the first report in the literature describing the use of this specific biomass for laccase production by this fungal species has successfully demonstrated that *Talaromyces stollii* can produce laccase at high yields when cultivated in a supplemented medium using brewer's spent grain (BSG) as its substrate.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/recycling10040166/s1, Table S1: Heating ramp program for the crude protein quantification digestion block; Figure S1: Chromatograms of the monomeric sugar fractions (glucose, xylose, and arabinose) derived from the biomass.; Table S2: Concentration distribution for each point in the gallic acid standard curve; Figure S2: Standard curve of gallic acid.

Author Contributions: Conceptualization, R.P.d.N. and I.I.J.; methodology, E.C.S.L. and A.C.B.d.N.; validation, E.C.S.L., A.C.B.d.N., R.P.d.N. and I.I.J.; formal analysis, E.C.S.L. and A.C.B.d.N.; investigation, E.C.S.L. and A.C.B.d.N.; resources, R.P.d.N. and I.I.J.; data curation, E.C.S.L.; writing—original draft preparation, E.C.S.L. and I.I.J.; writing—review and editing, E.C.S.L. and I.I.J.; visualization, E.C.S.L. and I.I.J.; supervision, R.P.d.N. and I.I.J.; project administration, R.P.d.N. and I.I.J.; funding acquisition, R.P.d.N. and I.I.J. All authors have read and agreed to the published version of the manuscript.

Funding: This study was funded by FAPERJ—Fundação Carlos Chagas de Amparo a Pesquisa do Estado do Rio de Janeiro/Brazil grant number: E26/201.367/2022; CNPq—Conselho Nacional de Desenvolvimento Científico e Tecnológico—BR, grant number 315160/2021-7.

Data Availability Statement: The data presented in this study are available in the article and Supplementary Materials.

Acknowledgments: Authors would like to thank the Hydrogen Technology Laboratory (LabTecH) of the School of Chemistry/UFRJ to the XRF analysis as well CAPES-BR, CNPq-BR and FAPERJ-BR for the financial support.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

BSG	Brewers' Spent Grain
Lac	Laccase
TPC	Total Phenolic Content
GAE	Gallic Acid Equivalent
SmF	Submerged Fermentation
PSmF	Preliminary Submerged Fermentation
XRF	X-Ray Fluorescence

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