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Biological Pretreatment of *Cynodon* sp. Using *Trametes hirsuta*: Influence on Enzymatic Activity and Anaerobic Bioconversion

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Abstract

Garden pruning waste from *Cynodon* sp. is a lignocellulosic resource with high lignin content, which limits anaerobic digestion efficiency. While white-rot fungi can delignify biomass through solid-state fermentation (SSF), their efficacy depends on balancing lignin removal with preservation of fermentable carbohydrates. This study evaluated the effect of SSF times (8, 21, and 36 days) with *Trametes hirsuta* on enzymatic activity and subsequent biogas production. Laccase activity increased progressively, reaching 983.84 U/L at 36 days; whereas manganese and versatile peroxidases peaked at 21 days. Fungal-pretreated samples exhibited lower methane yields, a maximum of 225.32 NmL/gVS at 8 days, compared to untreated biomass (381.66 NmL/gVS). Total lignin content apparently increased across treatments, suggesting pseudo-lignin formation during autoclave sterilization, while glucose and xylose decreased. Biological pretreatment affected methane production by reducing sugar availability, potentially forming inhibitory furanic compounds and antimicrobial metabolites, thereby negating the benefits of enzymatic delignification. These results underscore the complexity of optimizing fungal pretreatment and highlight the need to balance fermentation time to preserve carbohydrates while modifying lignin structure.

Keywords: anaerobic digestion; *Cynodon* sp; white-rot fungi; solid state fermentation; laccase activity

1. Introduction

Since the end of the 19th century, society has used fossil fuels in combustion processes to produce electricity and heat required worldwide [1]. However, this practice contributes to environmental degradation by emitting greenhouse gases, leading to global warming, increased desertification, erosion, and reduced biodiversity. The International Renewable Energy Agency (IRENA) reports that European fossil gas prices averaged 4.9 times more from January to April 2022 compared to 2021, from 90 to 147 USD/MWh [2].

On the other hand, pollution resulting from the inadequate management of municipal solid waste (MSW) has become a global problem. According to [3], around 2,010,000,000 tons of MSW are generated per year, which represents an average of 0.74 Kg per capita per day. Therefore, one of the strategies has been the search for alternatives to biogas generation, such as biomethane, which is recognized as the most cost-effective and environmentally sustainable among existing biofuels and significantly contributes to reducing greenhouse gas emissions. Biomethane is produced by anaerobic digestion (AD) using different feedstocks.

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Lignocellulosic biomass has garnered significant attention as a potential feedstock for producing biofuels, biochemicals, and other value-added products contributing to achieving carbon neutrality [4,5]. For this reason, researchers are working not only on new biomass sources but also on improving process efficiency. MSW includes pruning waste from urban gardens, which is considered lignocellulosic waste. Lignocellulosic biomass is a term used to describe plant-derived materials composed of cellulose, hemicellulose, and lignin [6]. These three main components are the structural building blocks of plant cell walls, and their relative composition varies across biomass sources [7,8]. Garden pruning waste poses an environmental challenge due to its accumulation and high lignocellulose content, which hinders natural degradation [9]. These residues contain significant amounts of cellulose and hemicellulose, which could be valorized through biogas production; however, their lignin content (11 – 45% dry weight) reduces bioavailability for anaerobic microorganisms [10–13].

Lignocellulosic waste requires pretreatment to degrade lignin, a recalcitrant compound that protects the polysaccharides. White rot fungi are a viable alternative because they possess a unique enzymatic system for lignin degradation [14]. Solid state fermentation by White-Rot Fungi (WRF) enhances the porosity of lignocellulosic biomass, thereby exposing the polysaccharides to the enzymatic hydrolysis process [15]. Several of these fungi produce oxidase and peroxidase enzymes capable of degrading lignin, including laccase (Lac), manganese peroxidase (MnP), versatile peroxidase (VP), and lignin peroxidase (LiP). Polysaccharides, when hydrolyzed by enzymes such as cellulases, amylases, and others, produce monosaccharides, including glucose, mannose, xylose, cellobiose, and galactose, primarily from cellulose and hemicellulose. Solid-state fermentation with *Trametes hirsuta* has been widely recognized as an effective strategy to produce lignocellulolytic enzymes [16].

The monosaccharides released during solid-state fermentation can be subsequently converted into biofuels, such as methane, ethanol, or hydrogen, through distinct fermentative pathways. In the context of anaerobic digestion, fungal pretreatment seeks to balance two opposing phenomena: sufficient lignin removal to improve substrate accessibility and minimal consumption of easily degradable carbohydrates that would otherwise serve as substrates for methanogenesis. Therefore, the efficacy of this biological pretreatment depends critically on fermentation time, which governs both the extent of delignification and the availability of soluble sugars for subsequent anaerobic conversion.

While the conversion of sugars into biofuels is the ultimate goal, the application of fungal pretreatment to enhance anaerobic digestion has yielded mixed results in the literature. Some authors have successfully improved methane yields through fungal pretreatment on various feedstocks [17,18]. Conversely, other studies have reported adverse effects; [8] observed a lower methane yield in bean pods treated for 30 days with *T. versicolor*, likely due to the excessive consumption of easily fermentable sugars by the fungus during the extended process. Similarly, [19] highlighted the complex relationship between aeration, biomass production, and lignocellulosic degradation, demonstrating that cultivation conditions can substantially modify the availability of fermentable sugars. These findings suggest that the efficiency of fungal pretreatment depends on a delicate balance between lignin removal and the preservation of soluble carbohydrates. Therefore, it is necessary to further optimize parameters such as incubation time, aeration, and fungal species selection. Consequently, in order to explain the role of SSF as pretreatment on lignocellulosic biomass, this work evaluates the effect of solid-state fermentation time with *Trametes hirsuta* on *Cynodon* sp. residues. The focus is on the roles of simple sugar assimilation and lignin transformation, considering both biological processes and abiotic degradation, and their impact on the efficiency of anaerobic digestion for biogas production.

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2. Materials and Methods

2.1. Lignocellulose Residue

The pruning residue of *Cynodon* sp. was collected in October 2024 and dried with solar radiation. It was crushed in a hammer and knife pulverized mill model TH3000 equipped with a 7.5HP electric motor and sieved through a 5.6 mm mesh. The particles retained by this mesh were used to continue with the experimental tests. The biomass was kept in hermetically sealed bags without air and refrigerated at 4 °C.

2.2. Microorganisms Used in Fermentation

T. hirsuta carpophore was collected in the Jilotzingo municipality in the State of Mexico province in Mexico and isolated in the Escuela Nacional de Ciencias Biológicas. The strain was reactivated on malt extract agar (MEA) plates and maintained at 4 °C until used.

2.3. Solid State Fermentation

In Erlenmeyer flasks, 15 g of dry grass waste was placed, and the modified Sivakumar medium was added to reach 85% moisture. The media contained the following composition: (mg/L): KH_2PO_4, 1000; $(NH_4)_2SO_4$, 50; $CaCl_2$, 10; $MgSO_4$, 500; $FeSO_4$, 10; $MnSO_4$, 1; $ZnSO_4$, 1, and $CuSO_4$, 2 [20]. Flasks were sterilized at 120 °C for 20 min and 15 psi. Next, the flasks were inoculated under aseptic conditions using six pellets (4 mm) of mycelia from the *T. hirsuta* PDA plate, each inoculated separately. Subsequently, the flasks were incubated at 20 °C for three different fermentation times (8, 21, and 36 days).

2.4. Determination of Enzymatic Activities

To determine laccase enzyme activity, at each fermentation time, 2 grams of wet sample were added under aseptic conditions; then, 10 mL of 0.1M acetate buffer pH 4 was added in a 50 mL beaker, the grass fermented and buffer mixture were left in agitation for 24 h, 20 rpm, and kept at 4 °C. Afterward, the liquid was decanted into 20 mL bottles for enzyme activity determination. Activity of laccase, Manganese peroxidase, and versatile peroxidase was determined as described in [21]. The activity of laccase was determined by the oxidation method (ABTS) (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)). The reaction mixture (1 mL) contained 100 µL of 0.5 mM ABTS, 800 µL of 100 mM acetate buffer at pH 4.5, and 100 µL of enzyme extract, which was added to give a final volume of 1 mL. Subsequently, absorbance was determined using a HACH model DR5000 spectrophotometer at 420 nm for 4 min; a molar extinction coefficient of ε = 3.6 × 10^4 M^-1 cm^-1 was used. A unit of enzymatic activity (U) was defined as the production of 1 µmol of the ABTS radical per mL per minute. Manganese peroxidase, was determined at 610 nm, ε = 4460 abs/M cm, the reaction was carried out in 50 µL of sodium succinate buffer (20 mM) at pH 4.5 for 5 min and the reaction mixture contained 700 µL of the enzyme extract, 50 µL phenol red 0.2%, 50 µL of sodium lactate 0.5 mM, 50 µL of egg albumin 0.1%, 50 µL of manganese sulfate 2 mM and 50 µL of hydrogen peroxide 2 mM, finally was stopped by 50 µL of NaOH (2N); one U was defined as 1 µmol of product formed per min per g of dry mass. Finally, the versatile peroxidase was determined as for MnP, replacing 50 µL of manganese sulfate with 50 µL of deionized water, under the same reaction and measurement conditions.

2.5. Analytical Determination of Lignin Content and Free Sugar Composition

The structural carbohydrates and lignin contents were determined for the initial feedstock and products of solid-state fermentation and anaerobic digestion at the three selected times. A two-step acid hydrolysis method according to the National Renewable Energy Laboratory (NREL) was used [22]. The samples underwent initial hydrolysis with 3 mL of 72% sulfuric acid at 30 °C for 1 hour, then 84 mL of deionized water was added, and the mixture was autoclaved at 121 °C for 1 hour in

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pressure tubes. Then, the sample was filtered through a Gooch crucible, the solid fraction was used to quantify insoluble lignin by gravimetric analysis, and the filtrate was used to measure soluble lignin and structural sugars. The pH of the sample was adjusted to around 7 with CaCO3. Simple structural sugars were quantified by capillary electrophoresis (Beckman MDQ). The method was described by [23]. A large silica column, 60 cm long and 50 µm in internal diameter, was used. Subsequently, the sample was injected for 4 s at 0.5 psi, and 16 kV was applied to separate the sample. 130 mM NaOH—36 mM $Na_2HPO_4 \bullet 2H_2O$ was used as a buffer (pH 12.6). The peaks were measured with PDA detection at 270 nm.

2.6. Biomethane Production by Anaerobic Digestion

The inoculum used was cow manure provided by a local farmer. The biochemical methane potential (BMP) assay was carried out in 60 mL serological vials. One gram of wet fermentation product from *T. hirsuta* was added, followed by one gram of wet manure as inoculum. Then, 20 mL of media was added, prepared as described in [24]. Five solutions were prepared, the first (A) contained in g/L: NH_4Cl, 100; NaCl, 10; $MgCl_2 \bullet 6H_2O$, 10; and $CaCl_2 \bullet 2H_2O$, 5. The (B) solution was composed of 200 g/L of $K_2HPO_4 \bullet 3H_2O$. (C) solution, resazurin 0.5 g/L. Trace elements and selenite solution (D) (g/L): $FeCl_2 \bullet 4H_2O$, 2; H_3BO_3, 0.05; $ZnCl_2$, 0.05; $CuCl_2 \bullet 2H_2O$, 0.038; $MnCl_2 \bullet 4H_2O$, 0.05; $(NH_4)_6Mo_7O_{24} \bullet 4H_2O$, 0.05; $AlCl_3$, 0.05; $CoCl_2 \bullet 6H_2O$, 0.05; $NiCl_2 \bullet 6H_2O$, 0.092; ethylenediaminetetraacetate, 0.5; concentrated HCl, 1mL; $Na_2SeO_3 \bullet 5H_2O$, 0.1. The vitamin solution (E) contained in mg/L: Biotin, 2; folic acid, 2; pyridoxine acid, 10; riboflavin, 5; thiamin hydrochloride, 5; cyanocobalamin, 0.1; nicotinic acid, 5; p-aminobenzoic acid, 5; lipoic acid, 5, and DL-pantothenic acid, 5. In a 1 L volumetric flask, 975 mL of distilled water was added. After adding 10 mL of A solution, 2 mL of B solution, and 1 mL of C, D, and E, respectively, the flask was filled to the mark. Finally, 0.5 g HCl-cysteine and 2.6 g NaHCO3 were dissolved. The experiment was conducted at 35 °C and 120 rpm for 90 days. The volume of methane produced during anaerobic digestion of the solid-state fermentation product was measured using an inverted column and NaOH 3N, by volume displacement.

3. Results and Discussion

3.1. Enzymatic Activity

Laccase (Lac), Manganese Peroxidase (MnP), and Versatile Peroxidase (VP) activities were measured at 8, 21, and 36 days of solid-state fermentation. Lac activity increases throughout the fermentation period, reaching 68 ± 5.2 at 8 days, 671.2 ± 28.9 at 21 days, and 983.84 ± 107.92 U/L at 36 days of pretreatment. In contrast, MnP and VP reached maximal activity at 21 days (76.61 ± 0.7 and 51.61 ± 0.6 U/L, respectively), subsequently declining at 36 days (Figure 1).

Similar behavior was observed by [8], who obtained higher laccase activity than MnP during solid state fermentation of bean pods with *Trametes versicolor* and *Pleurotus ostreatus* as pretreatments for anaerobic digestion. Specifically with *T. versicolor*, they reached maximum laccase activity of 1588 U/L at 12 days. Conversely, when using P. ostreatus, they observed a predominance of MnP over laccase, reaching 820 U/L at 12 days. The predominance of laccase activity over peroxidases during extended fermentation periods has been associated with the oxidative capacity required for lignin depolymerization, which theoretically should enhance substrate accessibility for subsequent anaerobic digestion [16,25].

Several studies have established a positive correlation between high ligninolytic enzyme activity and enhanced methane production. For instance, [25] demonstrated that laccase pretreatment detoxified lignocellulosic substrates, and [13] increased methane generation by 25% under similar conditions. In comparison, [26] reported that *Pleurotus eryngii* strains with elevated laccase activity improved biogas yields by 19% after 30 days of pretreatment. These findings suggest that enzymatic delignification should facilitate polysaccharide accessibility, thereby increasing the availability of fermentable sugars for methanogenesis. However, the relationship between enzyme production and

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methane yield is not always linear, as fungal metabolism may simultaneously consume the liberated sugars, reducing the carbon available for anaerobic conversion [13,17].

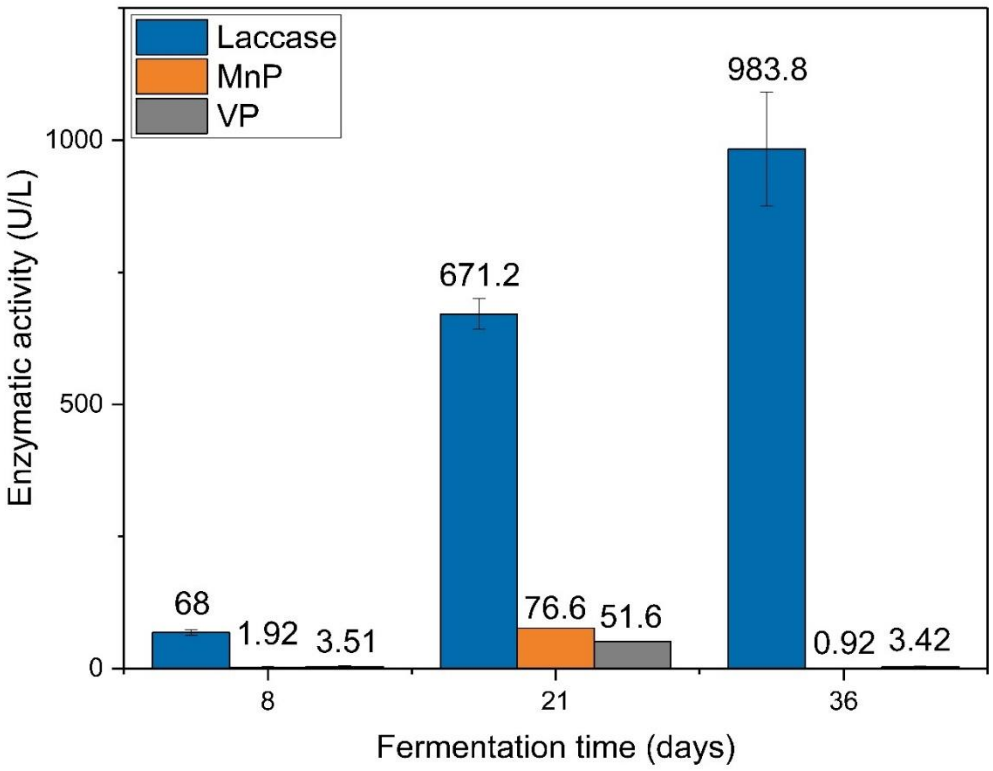

Figure 1. Enzymatic production by *Trametes hirsuta* during solid-state fermentation of *Cynodon* sp. at different times (8, 21, and 36 days): Laccase (blue), manganese peroxidase (orange), and versatile peroxidase (gray) activities (U/L).

3.2. Biogas Production

For the methane production test, *Cynodon* sp. pretreated by solid-state fermentation using *T. hirsuta* at different times was evaluated for its effect on biochemical methane potential (BMP). Cumulative biogas production kinetics are shown in Figure 2. Untreated pruning residue achieved the highest yield, 381.66 ± 24.9 NmL/gVS, followed by the sterilized controls: C 21d: 316.12 ± 31.27; C 36d: 267.72 ± 8.1; and C 8d: 266.23 ± 3.18 NmL/gVS. The pretreated samples showed the lowest methane yields, with T8d yielding the highest at 225.32 ± 29.2 NmL/gVS, followed by T36d at 207.31 ± 24.9, and finally T21d at 201.9 ± 31.9 NmL/gVS. The inoculum-only production reached 90.84 ± 6.5 NmL/gVS, serving as a baseline for BMP calculations.

This result contrasts with that reported by [17], who applied similar autoclave sterilization conditions, followed by solid fermentation with *P. ostreatus* at 75% moisture content for 20 days at 28 °C on rice straw, resulting in a significant increase in methane production (263 NmL/gVS). These same authors reported that rice straw pre-treated with *Trichoderma reesei* for 20 days achieved 214 NmL/gVS at 75% moisture content at 28 °C. Similarly, [27] demonstrated that pretreatment with *P. ostreatus* significantly accelerated the hydrolysis of lignocellulosic biomass, enhancing the methane yield of rice straw to 270 mL/gVS compared to the untreated substrate, while [28] observed significant benefits in daily and specific methane yields using fungal pretreatment on poplar biomass (205 mL d^{-1} L^{-1} and 44 mL gVS^{-1} L^{-1} for pretreated versus 98 mL d^{-1} L^{-1} and 26 mL gVS^{-1} L^{-1} for control fermenters).

Another important aspect to mention is that prolonged fermentation times generally result in lower methane yields. [12] Reported that a 10 days incubation time of SSF with *P. ostreatus* led to optimal methane production, while longer pretreatments (30 and 60 days) resulted in lower methane

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yields with corn silage as biomass. This may be attributed to the loss of organic matter during extended fungal growth, which affects the available substrate for anaerobic digestion [13].

The results with *T. hirsuta* showed that fermentation times exceeding 8 days decreased methane production. These findings are consistent with [29], who observed that a very short pretreatment (3 days) with *Trametes* sp. W-4 on barley straw increased methane production by 63.81% compared to the control (111.51 mL/gVS vs 68.07 mL/gVS). The authors concluded that decreased biogas production under longer pretreatment conditions could be attributed to nutrient consumption by the fungus or the production of phenolic compounds, as this strain exhibits high laccase activity that may inhibit methanogen growth or activity.

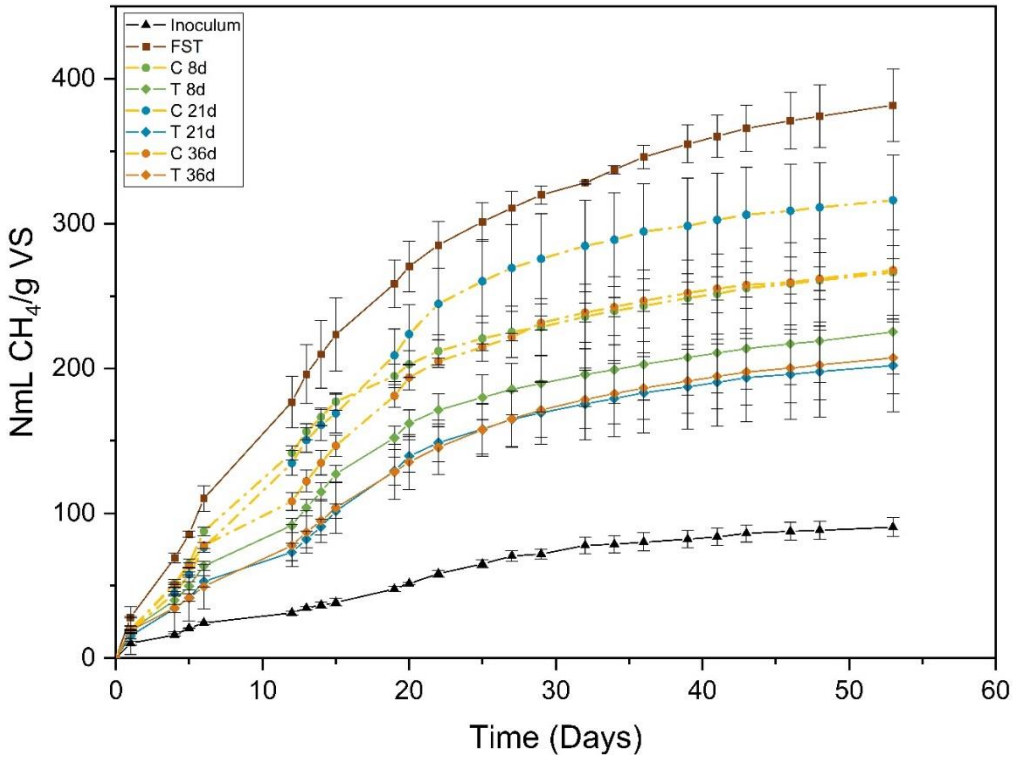

Figure 2. Cumulative biogas production from different substrates: Untreated *Cynodon* sp (FST), time-control samples (C 8d, C 21d, C 36d; yellow), and solid-state fermented substrates at 8 (T 8d; green), 21 (T 21d; blue), and 36 (T 36d; red) days.

3.3. Lignin Content and Composition

Furthermore, enzymatic activity is crucial for obtaining significant biogas yields from lignocellulosic biomass. In this study, lignin and polysaccharide degradation by *T. hirsuta* demonstrated effective hemicellulose removal, while cellulose and lignin degradation rates were consistent with previous reports [30,31]. To explain the results obtained in the present study, fermentation time negatively affected the BMP of *Cynodon* sp. residues; sugar and lignin content were also analyzed.

The laccase activity observed in this work was related to lignin content modification. Figure 3 shows that total lignin content increased across all experimental times compared to the feedstock (*Cynodon* sp. non-sterilized). The feedstock lignin content was 20.79 ± 0.78%, while C8d was 23.52 ± 0.65% and T8d was 25.2 ± 2.2%, representing increases of 2.27 and 5.4%, respectively. At 21 days, C21d (22.04 ± 0.16%) and T21d (26.32 ± 0.58%) showed increases of 1.24 and 5.52% in each. Finally, lignin content at 36 days was 29.54 ± 0.02% for C36d and 27.84 ± 0.53% for T36d, representing increases of 8.75 and 7.05% above the feedstock, respectively.

The apparent increase in lignin content suggests recalcitrant behavior due to the stability of this compound during solid state fermentation. However, this observation requires careful interpretation.

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Figure 3 presents the percentage composition of different lignin types across fermentation time points, revealing an increase in total and insoluble lignin while soluble lignin remained relatively stable. Untreated pruning residue (20.79 ± 0.78%) showed significantly lower total lignin content than all pretreated samples. Notably, sterilized controls – which underwent autoclave processing without microbial interaction – maintained higher lignin percentages than untreated samples. This reduction suggests that thermal treatment may induce autohydrolysis or degradation of fermentable sugars.

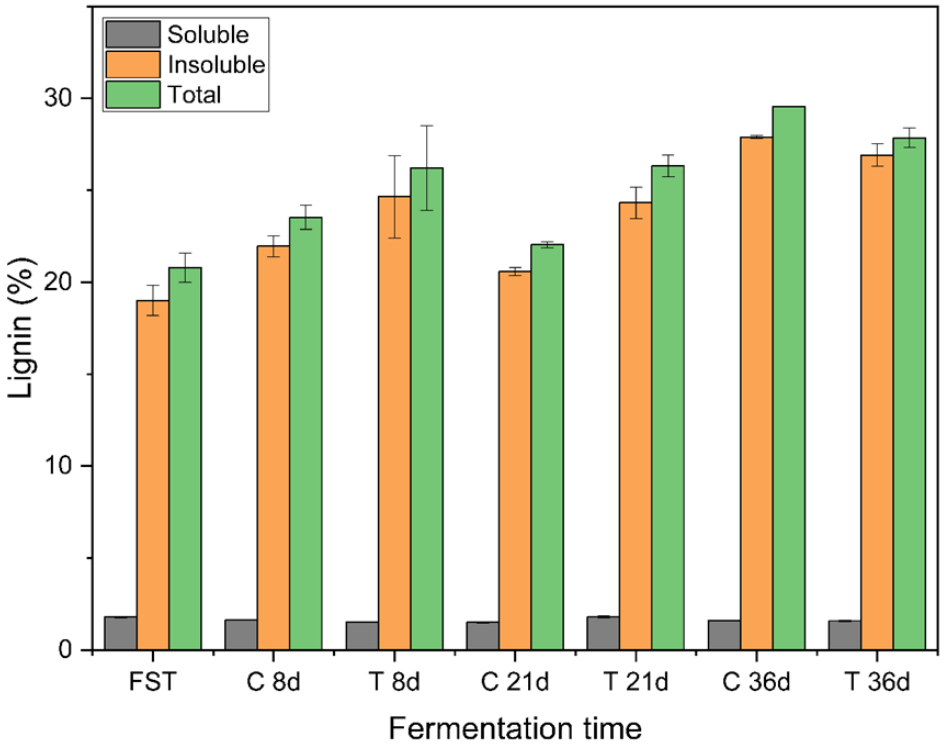

Figure 3. Soluble, insoluble, and total lignin content (%) at different pretreatment times: untreated substrate (FST), sterilized controls (C 8d, C 21d, C 36d), and fungal-treated samples (T8, T21, T36) following solid-state fermentation.

This phenomenon may be explained by the formation of pseudo-lignin during thermal processing. [32] Demonstrated that lignocellulosic biomass treated at high temperatures with water or dilute acid promotes the generation of pseudo-lignin and reduces enzymatic hydrolysis efficiency. Pseudo-lignin is an aromatic material containing hydroxyl and carbonyl functional groups that contributes to Klason lignin values but is not derived from native lignin; it arises from polymerization/condensation reactions of intermediates such as 3,8-dihydroxy-2-methylchromone and 1,2,4-benzenetriol derived from furfural (FF) and 5-hydroxymethylfurfural (5-HMF), respectively [33,34]. Furthermore, pseudo-lignin retards biological conversion through unproductive binding to enzymes/microbes and by physical hindrance by blocking active cellulose surface binding sites.

[35] Investigated lignin structural changes during degradation via physical/chemical pretreatments and *T. hirsuta* laccase action, demonstrating that chemical treatments generate furanone, apocynin, furfural, hydroxymethylfurfural, and furaldehyde, while water pretreatment followed by laccase treatment yields additional furan and phenolic degradation products. These findings align with reports by [13,16,17], who note that while high enzyme production enhances lignin degradation, it simultaneously enables fungal metabolism of simpler organic compounds into CO_2 and water, ultimately reducing fermentable sugar availability for subsequent anaerobic digestion.

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3.4. Free Sugar Composition

The concentrations of simple sugars present in *Cynodon* sp. were evaluated during the solid state fermentation process. The feedstock sugars without a sterilization process were also evaluated (Figure 4). Glucose and xylose were the predominant compounds in all samples. Unsterilized feedstock showed the highest concentrations of glucose (1.92 g/L) and xylose (0.72 g/L), and it showed significant differences ($P<0.05$) from treated samples. Control 2 exhibited a distinct behavior compared to controls 1 and 3. This variation could be due to substrate heterogeneity or uneven thermal exposure.

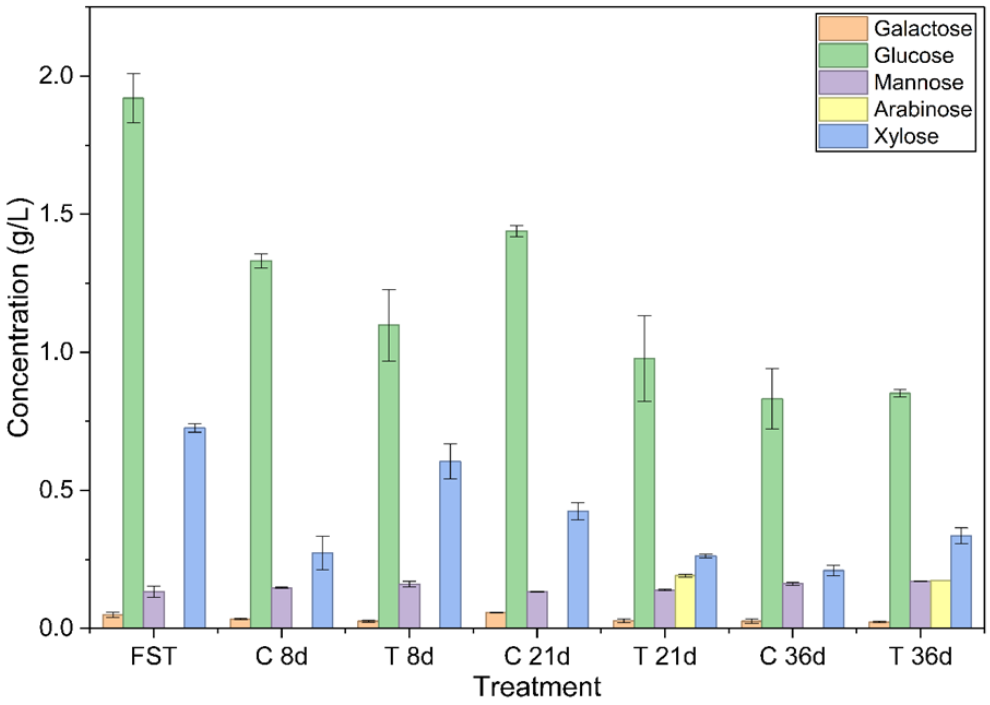

Figure 4. Changes in the composition of different sugars during SSF pretreatment at different times, along with their respective controls, including the residue without pretreatment, and their significant differences ($p<0.05$).

Glucose presented a decrease in controls and treatments, for C1 1.4 g/L and C3 0.9 g/L, while T1 was 1.1 g/L, T2 0.95 g/L, and finally T3 0.8 g/L. However, the treatments did not differ significantly from their respective controls. Xylose did not show a clear tendency between controls and treatments. The observed reduction in reducing sugar levels can be attributed to abiotic processes, particularly auto-hydrolysis phenomena induced during the sterilization stage. The plant residue of *Cynodon* sp. was subjected to heat treatment in an autoclave (120 °C, 15 psi, 20 minutes), conditions that, in the presence of moisture, favor spontaneous acid hydrolysis reactions in the absence of external catalysts. This process can release monosaccharides such as glucose and xylose but can also form secondary degradation products, including acetic acid, furfural, and 5-HMF, which compromise the availability of fermentable carbon and can inhibit anaerobic digestion at high concentrations [36,37].

Another abiotic factor, such as sunlight exposure, could induce chemical transformations affecting sugar stability. [38] Evaluated the effect of different drying methods - including shade, sun, and oven at temperatures of 30, 40, and 50 °C on the phytochemical composition of *Cynodon dactylon* leaves and rhizomes. Their study identified the formation of compounds such as 5-hydroxymethylfurfural (5-HMF) and maltol, derived from the thermal degradation of sugars during drying, confirming that even moderate thermal conditions can induce dehydration and oxidation reactions of hexoses.

The decrease in glucose levels observed during SSF can also be attributed to fungal consumption. It has been reported that *T. hirsuta* synthesizes ligninolytic enzymes during the secondary metabolism

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phase, once simple sugars are consumed; furthermore, fungal growth stages affect the synthesis and secretion of both intracellular and extracellular enzymes [39]. The fungus may metabolize liberated sugars for biomass production and enzyme synthesis, thereby reducing the fermentable substrate available for methanogenesis.

3.5. Mechanisms of Inhibition and Hypotheses

The low biogas production observed in this study can be attributed mainly to the limited availability of fermentable sugars for anaerobic digestion. However, certain assays showed sugar levels comparable to or even higher than the control values, yet this did not translate into higher methane yield. This paradox can be explained by the presence of inhibitory compounds generated during pretreatment, such as furfural and hydroxymethylfurfural, as well as lignin and hemicellulose derived phenolic derivatives identified by [35] as fermentation inhibitors. Similarly, [36] demonstrated that different concentrations of furanic and phenolic compounds can affect methanogenesis in an anaerobic mixed culture. Furthermore, mushrooms are known to synthesize various secondary metabolites with potent antimicrobial and antibacterial properties, which support their natural survival [40]. Specifically, species of the genus *Trametes* have been reported to produce high levels of bioactive compounds, including phenols, flavonoids, saponins, and anthraquinones, which exhibit recognized antimicrobial activity [41]. The presence of these metabolites could potentially suppress methanogenic activity despite the availability of sugars. Additionally,[42] reviewed the inhibition and disinhibition effects of 5-hydroxymethylfurfural in anaerobic fermentation, noting that this compound can inhibit hydrogen and methane production, interfere with microbial growth, and alter the structure of the microbial community in anaerobic sludge.

Contrary to expectations, the results obtained in this study showed that fungal pretreatment with *T. hirsuta* did not improve methane production in anaerobic digestion tests. In fact, a decrease in methanogenic biochemical potential was observed compared to untreated controls. This finding suggests that the reduction in BMP could be due to a combination of factors: (i) reduced availability of sugars due to fungal consumption during prolonged SSF, (ii) transformation of sugars into inhibitory compounds such as furfural and pseudo-lignin during thermal processing, (iii) production of phenolic compounds and secondary metabolites by *T. hirsuta* that inhibit the activity of methanogenic archaea, and (iv) formation of pseudo-lignin during autoclave sterilization, which masks the true degradation of lignin and creates additional physical barriers to enzymatic attack. Finally, another factor that can influence BMP values is the C/N ratio. This parameter is essential for obtaining significant methane yields from anaerobic digestion. Various investigations have reported optimal C/N ratios: [43] recommends a range between 20 and 30, while [44] suggests values between 20 and 30. In this study, a ratio of 25.29 was observed, within the acceptable range. However, it has been pointed out that the optimal C/N ratio depends on the specific characteristics of the substrate and inoculum [43], which may account for additional variation in the observed yield.

4. Conclusions

In conclusion, the efficacy of biological pretreatment with *T. hirsuta* depends critically on fermentation time, which governs both the extent of delignification and the availability of soluble sugars for subsequent anaerobic conversion. While high laccase activity was achieved, the theoretical benefits of lignin degradation were negated by a combination of biotic and abiotic factors: the concomitant consumption of fermentable sugars by fungal metabolism, and the formation of inhibitory compounds and pseudo-lignin during thermal sterilization. These results underscore the complexity of optimizing fungal pretreatment for anaerobic digestion and highlight the need to balance enzymatic lignin modification with the preservation of fermentable carbohydrates. Consequently, future scaling efforts and research should focus on short pretreatment times or non-sterile conditions to avoid thermal degradation and maximize biomethane yields.

5. Acknowledgment

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