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Research article

Enzymes Involved in Lignocellulose Decomposition and Effects of Fungal Pretreatment During Solid-state Fermentation of Lignocellulosic Agricultural Residues: The Case of *Trametes versicolor*

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Abstract

Ligninolytic fungi are considered useful for different biotechnological uses such as lignocellulose pretreatment for biorefinery applications, composting, mushroom production, or bioremediation purposes. Enzymes contributing to lignocellulose breakdown represent valuable biocatalysts. *Trametes versicolor* is a well-known ligninolytic white-rot basidiomycete capable of growing on different lignocellulosic materials. In the present study, the use of *T. versicolor*, which secretes several enzymes capable of degrading lignocellulosic biomass, was evaluated from two biotechnological aspects including fungal crude extracts and effects on enzymatic saccharification of wheat straw. We used 20-day-old wheat straw cultures of this fungus to characterize its lignocellulose-decomposing multi-enzyme complex. At the end of the cultivation period, losses of approximately 41% in lignin and 30% in total dry mass were observed relative to their initial values. A total of 17 enzyme activities, each acting differently on the polysaccharide components of the lignocellulosic substrate, and lignin-modifying enzyme activities (laccase, peroxidases) were investigated in aqueous extracts of fungal cultures. The activities of carboxylesterase (substrate: 4-nitrophenyl valerate), endo-1,4-β-D-glucanase (substrate: 2-hydroxyethylcellulose), and laccase were found to predominate. In summary, fungal crude extracts containing different enzyme activities may be promising for various biotechnological purposes. *Trametes versicolor* effectively pretreated wheat straw, significantly increasing reducing sugar levels compared to controls.

Keywords: crude extract; fungal pretreatment; ligninolytic fungi; lignocellulose; *Trametes versicolor*

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1. Introduction

Tremendous amounts of lignocellulosic biomass arise from human activities in the form of agricultural by-products, which represent a very attractive potential feedstock for biotechnological solid-state fermentation (SSF) processes. SSF has been described as a process where microorganisms grow in an environment with the absence or near absence of free water (Lizardi-Jiménez & Hernández-Martínez, 2017; Soccol et al., 2017). It is well established that filamentous fungi, which are well able to grow in a low-water-activity environment, represent those organisms being most well suited for SSF applications (Soccol et al., 2017). SSF mimics the natural environment of various lignocellulose-decaying fungi, allowing for the complete utilization of the complex biochemical systems of lignocellulose degraders (Lizardi-Jiménez & Hernández-Martínez, 2017; Cerda et al., 2019). In this regard, ligninolytic fungi are considered useful for the conversion of lignocellulosic by-products from agriculture or forestry into various useful products in SSF-based biotechnological processes such as lignocellulose pretreatment for biorefinery applications, composting, mushroom production, or bioremediation purposes (Hölker et al., 2004; Lizardi-Jiménez & Hernández-Martínez, 2017; Gong et al., 2018; Kumla et al., 2020; Meyer et al., 2020; Tišma et al., 2021).

Among ligninolytic fungi, *Trametes versicolor* (syn. *Coriolus versicolor* and *Polyporus versicolor*) is a well-known ligninolytic white-rot basidiomycete capable of growing on different lignocellulosic materials (Kumla et al., 2020; Tišma et al., 2021). This fungus shows promise for various applications through the utilization of a variety of lignocellulosic waste biomass sources such as brewers' spent grain (Tišma et al., 2018), corn silage (Bucić-Kojić et al., 2017), corncobs (Asgher et al., 2018), tea residues (Xu et al., 2020), parthenium, sugarcane leaves, wheat straw, and rice straw (Singh et al., 2019). Following treatment with *T. versicolor*, lignocellulosic biomass can be used for various applications, including animal feed, food products, or biofuel production (Tišma et al., 2021). Additionally, the enzymes involved in the breakdown of lignocellulose can serve as valuable biocatalysts, e.g., crude enzyme extracts can be utilized for environmental remediation or biotransformation processes (Tišma et al., 2021). They offer several advantages, including cost-effectiveness, preservation of activity during concentration, and the potential for synergistic interactions among enzymes (Kovacs et al., 2009; Gottschalk et al., 2010; Hu et al., 2011; Kostylev & Wilson, 2012; Visser et al., 2013; Cardoso et al., 2018a,b).

In the present study, we considered two biotechnological aspects of employing *T. versicolor* in SSF-based processes, utilizing wheat straw as a primary lignocellulosic agricultural residue on a global scale:

1. Which enzyme activities are predominant in aqueous extracts of *T. versicolor* wheat straw cultures?

2. Can there be any enhancing effects of (pre)treatment with *T. versicolor* on the enzymatic saccharification of wheat straw?

For these purposes, we used 20-day-old wheat straw cultures of *T. versicolor* to characterize its lignocellulose-decomposing multi-enzyme complex. Enzyme activities investigated in aqueous extracts of fungal cultures included α-amylase (substrate: amylopectin), α-amylase (substrate: amylose), carboxylic ester hydrolase (carboxylesterase) (substrate: 4-nitrophenyl valerate), chitosanase (substrate: chitosan), dextranase (substrate: dextran), endo-1,3-β-D-glucanase (substrate: curdlan), endo-1,3-β-D-glucanase (substrate: pachyman), endo-1,3-β-D-glucanase (substrate: β-glucan from barley), endo-1,3-β-D-glucanase (substrate: β-glucan from yeast), endo-1,4 β-mannanase

(substrate: galactomannan), endo-1,4-β-D-glucanase (substrate: 2-hydroxyethylcellulose), endo-1,4-β-galactanase (substrate: pectic galactan), endo-1,4-β-xylanase M3 (substrate: xylan), endo-1,5-α-arabinanase (substrate: arabinan), overall cellulase activity (substrate: chromogenic wheat straw), pullulanase (substrate: pullulan), and xyloglucanase GH74 (substrate: xyloglucan). Laccase and peroxidase activities were also determined. Furthermore, the effects of (pre)treatment with *T. versicolor* on enzymatic saccharification of wheat straw using a mixture of commercial enzyme preparations were evaluated.

2. Materials and Methods

2.1 Chemicals and other materials

All chemicals used in this study were of analytical grade, with chromatography solvents being of gradient grade unless otherwise specified. The compound 2,2'-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS, purity > 98%) was sourced from AppliChem in Darmstadt, Germany. The remaining chemicals were procured from Merck, Sigma-Aldrich, and Th. Geyer GmbH located in Renningen, Germany. The enzyme mixtures Celluclast 1.5L and Viscozyme L were obtained from Sigma-Aldrich (Merck Group, Darmstadt, Germany). Celluclast 1.5L, a cellulase derived from *Trichoderma reesei*, exhibits an enzymatic activity of 756 glucanase units (GU) per gram and was supplied at a concentration of 1.22 g/mL. On the other hand, Viscozyme L, a commercial cellulolytic enzyme blend from *Aspergillus* species, contains beta-glucanases, pectinases, hemicellulases, and xylanases. According to the manufacturer (Novozymes Corp.), its primary enzymatic activity is beta-glucanase at 108 GU per gram, with a concentration of 1.21 g/mL.

For the determination of carbohydrate active enzyme (CAZy) and esterolytic enzyme activities, multi-colored chromogenic polysaccharide hydrogel (CPH) substrate assays based on 96-well plates (Kračun et al., 2015) and commercial enzymes served as references were obtained from Glycospot (Søborg, Denmark) and Megazyme or Sigma-Aldrich, respectively. CPH substrates, commercial enzymes used for calibration, and enzyme suppliers are detailed in Table 1. Enzyme activities were reported based on the activity unit definitions provided by their respective suppliers, with the exception of carboxylesterase activities, which were calculated using a molar extinction coefficient of $6.52 * 10^6$ cm^2/mol (Kademi et al., 2000).

2.2 Source and maintenance of fungal strain

Trametes versicolor was sourced from the strain collection at the Department of Applied Microbial Ecology, Helmholtz Centre for Environmental Research - UFZ in Leipzig, Germany. Additionally, this species is available from the German Collection of Microorganisms and Cell Cultures (DSMZ; Braunschweig, Germany) under the designation *T. versicolor* DSM 11269. The fungal strains were preserved on 2% (w/v) malt extract agar plates, which contained 1.5% agar and were maintained at a pH of 5.7 and incubated at 28°C in darkness.

2.3 Fungal cultivations on wheat straw and sample preparation for analytical procedures

Fungal cultivation on wheat straw, along with the extraction of water-soluble compounds, was thoroughly detailed in the studies by Duong et al. (2022a,b). Briefly, agar plugs taken from the edges of fungal colonies growing on malt agar plates were homogenized in 2% malt extract medium using an Ultra-Turrax homogenizer (IKA-Werke GmbH & Co. KG, Staufen, Germany), with one agar plug per one mL of medium. From the resulting fungal suspension, 0.5 mL was used to inoculate 0.5 g of milled, autoclaved wheat straw. Following a total incubation period of 20 days at 28°C, the vials containing the fungal-cultivated wheat straw were collected and stored at -20°C. Subsequently, a 0.1 M McIlvaine buffer solution (McIlvaine, 1921) at pH 7.0 was utilized to extract water-soluble compounds from the solid substrate. The resulting aqueous supernatants from these extractions were also preserved at -20°C for further analyses, which included assessments of enzyme activities, total reducing sugars using the dinitrosalicyclic acid (DNSA) method, and total sugars following acidic hydrolysis via the phenol-sulfuric acid method (Duong et al., 2022a,b; Duong et al., 2024). The residual solids from the extraction process were analyzed for total dry mass and lignin content.

2.4 Enzymatic digestion of solid samples after fungal (pre)treatment

To assess the impact of *T. versicolor* pretreatment on the enzymatic saccharification of wheat straw, enzymatic digestion of the solid samples was conducted as outlined by Duong et al. (2024). The enzymatic hydrolysis utilized commercial enzyme preparations, specifically Celluclast 1.5 L and Viscozyme L, in mixture at concentrations of 121 and 15.8 GU/g dry lignocellulosic solid, respectively, based on previously published data (López-Gutiérrez et al., 2021). The lignocellulosic samples were mixed at a concentration of 2.5% (w/v) in a 0.1 M sodium citrate buffer (pH 4.8), supplemented with 0.2 g/L tetracycline to inhibit bacterial growth during the reaction. The incubation conditions were set at 150 rpm and maintained at 40°C for a duration of 18 h. Following enzymatic digestion, the resulting aqueous supernatants were stored at -20°C for subsequent analyses, which included measuring total reducing sugars using the dinitrosalicylic acid (DNSA) method and determining total sugars after acidic hydrolysis via the phenol-sulfuric acid method.

Three important sugar fractions were identified: "readily bioavailable sugar", "total bioaccessible sugars", and sugars resulting from enzymatic digestion (digestibility). A schematic overview of aqueous extractions and enzymatic digestion of *T. versicolor* wheat straw cultures and the sugar fractions derived thereof is illustrated in Figure 1. Detailed methodologies regarding these sugar fractions can be found in Duong et al. (2024), which elaborates on the various aqueous extraction steps and enzymatic digestion processes employed.

2.5 Further analytical procedures

All further analytical methods aimed at the substrate characteristics, fungal activity, and corresponding sample preparations were carried out as previously described (Duong et al., 2022a,b; Duong et al., 2024). These included the determinations of: (i) total dry masses; (ii) lignin contents using Fourier transform mid-infrared (FT-MIR) spectroscopy; (iii) enzyme activities in aqueous extracts of solid substrates; (iv) total reducing sugars based on the DNSA method; and (v) total sugars after acidic hydrolysis based on the phenol–sulfuric acid method.

Table 1. CPH substrates, commercial enzymes used for calibration, and enzyme suppliers for the determination of carbohydrate active enzyme (CAZy) and esterolytic enzyme activities

CPH Substrate	Commercial Enzyme used for Calibration	Enzyme Supplier
2-hydroxyethylcellulose	Endo-1,4-β-D-glucanase from *Aspergillus niger*	Megazyme
Amylopectin	α-Amylase from *Aspergillus oryzae*	Megazyme
Amylose	α-Amylase from *A. oryzae*	Megazyme
Arabinan	Endo-1,5-α-arabinanase from *A. niger*	Megazyme
Chitosan	Chitosanase from *Streptomyces* sp. N174	Sigma-Aldrich
Curdlan	Endo-1,3-β-D-glucanase from *Trichoderma* sp.	Megazyme
Dextran	Dextranase from *Chaetomium erraticum*	Sigma-Aldrich
Galactomannan	Endo-1,4 β-mannanase from *A. niger*	Megazyme
Pachyman	Endo-1,3-β-D-glucanase from *Trichoderma* sp.	Megazyme
Pectic Galactan	Endo-1,4-β-galactanase from *A. niger*	Megazyme
Pullulan	Microbial pullulanase	Sigma-Aldrich
Xylan	Endo-1,4-β-xylanase M3 from *Trichoderma longibrachiatum*	Megazyme
Xyloglucan	Xyloglucanase GH74 from *Paenibacillus* sp.	Megazyme
β-glucan from barley	Endo-1,3-β-D-glucanase from *Trichoderma* sp.	Megazyme
β-glucan from yeast	Endo-1,3-β-D-glucanase from *Trichoderma* sp.	Megazyme
Wheat straw (complex biomass substrate kit)	Cellulase (Celluclast® 1.5L) from *Trichoderma reesei*	Sigma-Aldrich
4-nitrophenyl valerate	None*	

*Instead of calibrating enzyme activities on the basis of commercial enzymes, carboxylesterase activities using 4-nitrophenly valerate were calculated using a molar extinction coefficient of 6.52 * 10^6 cm^2/mol (Kademi et al., 2000).

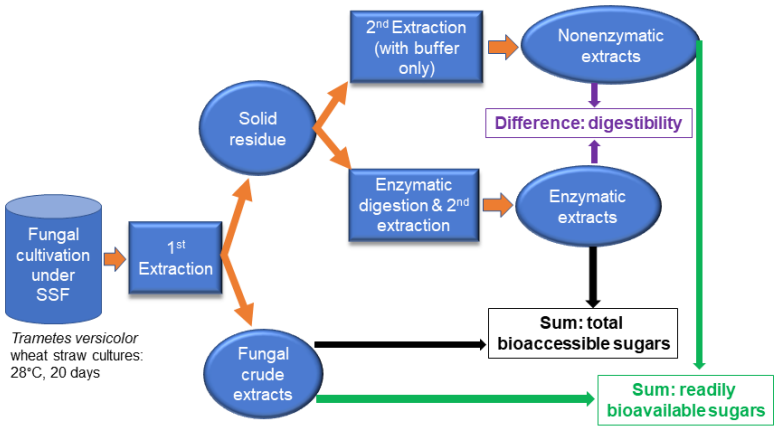


Figure 1. Schematic overview of aqueous extractions and enzymatic digestion of *T. versicolor* wheat straw cultures and the sugar fractions derived thereof

2.6 Statistical analyses

Unpaired two-sample (two-sided) Student's t-tests were performed using Microsoft® Excel® 2019 (version 16.0.10416.20073). The data included means and standard deviations derived from triplicate cultures. Where applicable, standard deviations were calculated according to the Gaussian error propagation rules.

3. Results and Discussion

Figure 2 depicts the effects of *T. versicolor* colonization on total dry mass and lignin contents of wheat straw over a total cultivation period of 20 days. At the end of the cultivation period, losses of approximately 30% in total dry mass and 41% in lignin were observed, relative to their control values.

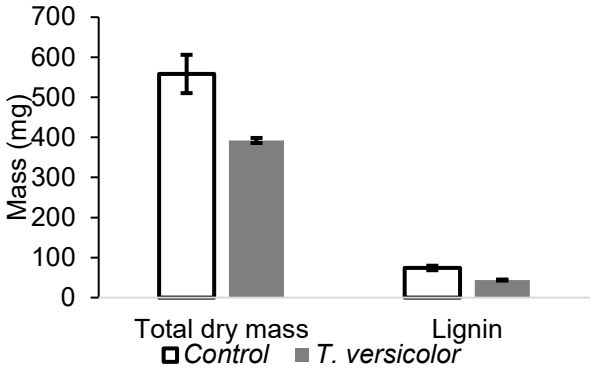


Figure 2. Total dry mass and lignin contents of the autoclaved wheat straw without fungal treatment (control) and the 20-day-old wheat straw cultures of *T. versicolor*. The data include means and standard deviations derived from triplicate cultures, represented by symbols and error bars, respectively.

Enzymes involved in lignocellulose decomposition were investigated in aqueous extracts of wheat straw cultures of *T. versicolor* (Table 2 and Figure 3). Among the investigated enzymes, the activities of carboxylesterase, endo-1,4-β-D-glucanase and laccase were found to predominate. It is worthy of note that carbohydrate active enzyme activities were determined using different substrates and different commercial enzymes that may have differed from those of *T. versicolor* in their respective catalytic properties. For example, endo-1,4-β-D-glucanase from *A. niger* was used for calibration on 2-hydroxyethylcellulose, while the overall activity on complex wheat straw was measured using the Complex Biomass Substrate kit with modified (chromogenic) wheat straw as a substrate and Celluclast® 1.5L from *T. reesei* as a reference (please refer to the materials and methods section for the details of substrates and commercial enzymes used as references). In addition, enzyme activities were only determined at the end of cultivation. Therefore, overall enzyme activity on complex wheat straw might well have been present during the cultivation period and then declined below its detection limit at the end of cultivation, whereas the activity of endo-1,4-β-D-glucanase was still detectable. Similarly, α-amylase, which was not detectable in *T. versicolor* but previously clearly found in *Stachybotrys chlorohalonata* (Table 2 and Figure 3), may have been produced only at certain time period of cultivation in either fungus.

Fungal crude extracts containing different enzyme activities may be promising for various biotechnological purposes. For example, carboxylesterases (EC 3.1.1.1) are prominent lipolytic enzymes that efficiently break down a wide array of structurally diverse compounds containing functional groups such as carboxylic acid esters, amides, and thioesters (Bornscheuer, 2002; Franklin, 2007). Their robust catalytic activity across various substrates has made them a focus of research for numerous biotechnological applications, including pesticide biodegradation, biosensor development, plastic depolymerization, and paraffin wax degradation (Johan et al., 2021). Endo-1,4-β-D-glucanases (EC 3.2.1.4) play a vital role in the breakdown of cellulose, which is essential for biomass conversion and numerous industrial applications, including those in the textile, brewing and wine, animal feed, laundry, and pulp and paper sectors (Bhat, 2000), while laccases (benzenediol oxygen oxidoreductase, EC 1.10.3.2), which are copper-containing oxidases, have given a favorable vision in meeting the needs for several industrial processes, such as pulp delignification, textile bleaching, phenolic removal, and biosensors (Wang et al., 2019).

Enzyme activities in 20-day-old wheat straw cultures of *T. versicolor* (this study) were further compared with those in 32-day-old wheat straw cultures of *Stropharia rugosoannulata* and *Stachybotrys chlorohalonata*, respectively (Table 2). The comparison revealed that *T. versicolor* was the highest laccase producer. This might be the reason why laccase is the most well-known and widely utilized enzyme among the enzymes of *T. versicolor* documented in the literature (Tišma et al., 2021). Numerous investigations of *T. versicolor* crude laccase's ability to remove dyes from wastewater (Dauda & Erkurt, 2020; Tišma et al., 2020), to degrade bisphenol A (Daâssi et al., 2016), to remove lignin or lignin-related compounds (Kapoor et al., 2015; Asgher et al., 2018), and to produce polyaniline (Giacobbe et al., 2019) were conducted. The potential of various fungal crude enzyme extracts to hydrolyze lignocellulosic biomass has been evaluated and compared with commercial enzyme preparations. For example, saccharification of alkaline pretreated sugarcane bagasse with a crude enzyme extract from the plant pathogenic fungus *Chrysoporthe cubensis* led to a higher production of glucose and xylose, compared to a similar assay employing a commercial cellulase (Falkoski et al., 2013). Cardoso et al. (2018a) compared crude extracts produced by white rot fungi (including *T. versicolor*) with a purified commercial enzyme preparation and found that the crude extract was more efficient for hydrolyzing pretreated pulps of sorghum straw.

Table 2. Enzyme activities in 20-day-old wheat straw cultures of *T. versicolor* (this study), compared with those in 32-day-old wheat straw cultures of *Stropharia rugosoannulata* and *Stachybotrys chlorohalonata*, respectively

Enzyme	Enzyme Activity (mU)					
	***T. versicolor* (this study)**		***Stropharia rugosoannulata***		***Stachybotrys chlorohalonata***	
	Mean	**S.D.**	**Mean**	**S.D.**	**Mean**	**S.D.**
Endo-1,4-β-D-glucanase (substrate: 2-hydroxyethylcellulose)	281	162	490	253	1450	139
α-Amylase (substrate: amylopectin)	0	0	4	5	129	14
α-Amylase (substrate: amylose)	3	5	0	0	33	7
Endo-1,5-α-arabinanase (substrate: arabinan)	168	149	196	125	808	132
Chitosanase (substrate: chitosan)	37	38	0	0	0	0
Endo-1,3-β-D-glucanase (substrate: curdlan)	0	0	1	1	50	8
Dextranase (substrate: dextran)	0	0	0	0	1780	41
Endo-1,4 β-mannanase (substrate: galactomannan)	0	0	0	0	176	47
Endo-1,3-β-D-glucanase (substrate: pachyman)	0	0	5	5	154	8
Endo-1,4-β-galactanase (substrate: pectic galactan)	35	20	82	36	108	11
Pullulanase (substrate: pullulan)	0	0	0	0	35	5
Endo-1,4-β-xylanase M3 (substrate: xylan)	0	0	23	18	210	33
Xyloglucanase GH74 (substrate: xyloglucan)	0	0	1071	1305	8770	1411
Endo-1,3-β-D-glucanase (substrate: β-glucan from barley)	0	0	53	47	172	22
Endo-1,3-β-D-glucanase (substrate: β-glucan from yeast)	6	9	1	2	37	1

Table 2. Enzyme activities in 20-day-old wheat straw cultures of *T. versicolor* (this study), compared with those in 32-day-old wheat straw cultures of *Stropharia rugosoannulata* and *Stachybotrys chlorohalonata*, respectively (continued)

Enzyme	Enzyme Activity (mU)					
	***T. versicolor* (this study)**		***Stropharia rugosoannulata***		***Stachybotrys chlorohalonata***	
	Mean	**S.D**	**Mean**	**S.D**	**Mean**	**S.D**
Overall activity on complex wheat straw	0	0	353	216	2474	141
Carboxylesterase (substrate: 4-nitrophenyl valerate)	558	102	363	103	2725	433
Laccase	398	123	27	16	0	0
Mn-peroxidases	0	0	0	0	0	0
Mn-independent peroxidases	0	0	0	0	0	0

Note: Data for *S. rugosoannulata* and *S. chlorohalonata*, which were previously reported (Duong et al., 2022a), were re-arranged to meet the requirements of the current Table 2. Please refer to the materials and methods section for the details of substrates and commercial enzymes used as references.

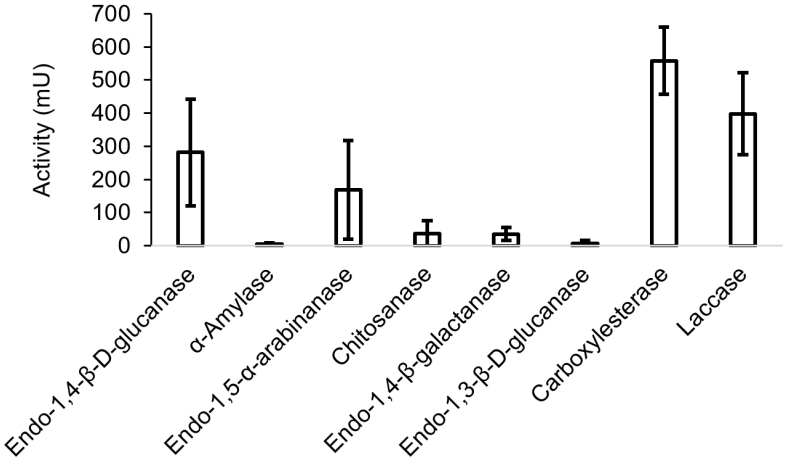


Figure 3. Detectable enzyme activities in the 20-day-old wheat straw cultures of *T. versicolor*, including endo-1,4-β-D-glucanase (substrate: 2-hydroxyethylcellulose), α-amylase (substrate: amylose), endo-1,5-α-arabinanase (substrate: arabinan), chitosanase (substrate: chitosan), endo-1,4-β-galactanase (substrate: pectic galactan), endo-1,3-β-D-glucanase (substrate: β-glucan from yeast), carboxylesterase (substrate: 4-nitrophenyl valerate), and laccase. Undetectable enzyme activities were excluded (i.e. the enzyme activities with the mean value of zero in Table 2). The data include means and standard deviations derived from triplicate cultures, represented by symbols and error bars, respectively.

The effects of fungal pretreatment on sugar contents in different aqueous extracts were evaluated and are illustrated in Figure 4 (please see Figure 1 and Duong et al. (2024) for details regarding sugar fractions obtained through different extraction steps and enzymatic digestion). It was observed that enzymatic hydrolysis consistently led to higher total bioaccessible sugar yield compared to the yield of readily bioavailable sugars when enzymatic hydrolysis was not conducted. As expected, the DNSA method yielded lower sugar amounts than the phenol-sulfuric acid method. This can be explained by the fact that the phenol-sulfuric acid method measures the total concentration of sugars after acidic hydrolysis, capturing monomers released from larger carbohydrate structures (Dubois et al., 1956), while the DNSA method only detects free reducing ends of sugars, leaving bound monomeric units undetected (Bailey, 1988; Gonçalves et al., 2010). The results align with previous findings where seven filamentous fungi were employed for fungal pretreatment during solid-state fermentation of wheat straw (Duong et al., 2024). Considering the sugar amounts determined using the phenol-sulfuric acid method, pretreatment with *T. versicolor* followed by enzymatic digestion resulted in a significant increase in both readily bioavailable and total bioaccessible sugars - approximately 40% and 38%, respectively - compared to control samples from autoclaved wheat straw without fungal treatment (Figure 4A). However, when assessed using the DNSA method, no significant differences in readily bioavailable sugars and total bioaccessible sugars were found between the fungal pretreatment with *T. versicolor* and control (Figure 4B). This study underscores the importance of both fungal pretreatment and subsequent enzymatic hydrolysis in enhancing sugar recovery from wheat straw, providing insights into optimizing biomass conversion processes for biofuel production.

The effects of fungal pretreatment on the enzymatic digestibility of wheat straw were assessed (Figure 5). The digestibility was calculated as the difference between sugar quantities in aqueous extracts with and without enzymatic digestion, respectively. As illustrated in Figure 5, the enzymatic digestibility values obtained through the DNSA method were consistently lower than those derived from the phenol-sulfuric acid method. Notably, the phenol-sulfuric acid method indicated that fungal pretreatment with *T. versicolor* resulted in the highest enzymatic digestibility of approximately 197 mg/g. This finding underscores the enhancement of lignocellulosic substrate quality, improving the accessibility and usability of residual sugars following fungal treatment with *T. versicolor*.

Considering the economic efficiency of a pretreatment process, the total bioaccessible sugar fraction deserves particular consideration as it represents the sugars that can potentially be utilized following fungal pretreatment in subsequent processes. Regarding this, we further compared the total bioaccessible sugars from treatment with *T. versicolor* (this study) with those from the seven previously investigated fungi, respectively (Table 3). Based on the comparison, *T. versicolor* demonstrated the highest sugar yield (138% of the control value), suggesting efficiency in the production of total bioaccessible sugars compared to the other fungi. The sugar yield increase of about 38% observed with *T. versicolor* was also higher than that of approximately 20% observed in red pine chips pretreated with *Stereum hirsutum* before enzymatic hydrolysis, compared to the untreated lignocellulosic material (Lee et al., 2007).

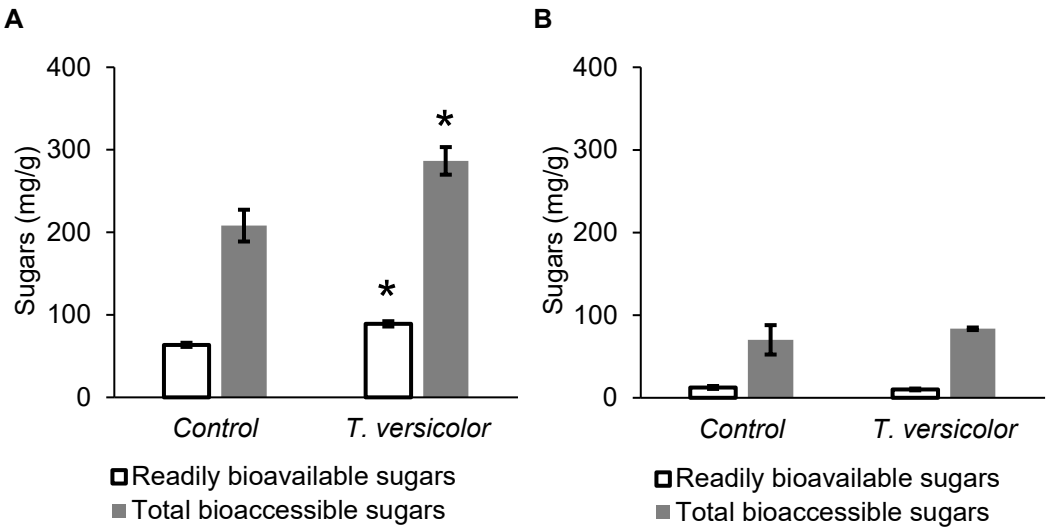


Figure 4. Readily bioavailable and total bioaccessible sugars from autoclaved wheat straw, which serves as the control, and autoclaved wheat straw subjected to treatment with *T. versicolor*. The sugar quantities are expressed per 0.5 g of dry wheat straw initially used (mg/g). The analysis of sugar content was performed using two methods: phenol-sulfuric acid (A) and DNSA (B). The data include means and standard deviations derived from triplicate cultures, represented by symbols and error bars, respectively. Significant differences between the fungal-treated samples and the control (autoclaved wheat straw) are indicated by asterisks, based on unpaired two-sample (two-sided) Student's t-tests with a significance level of $P<0.05$ (n=3).

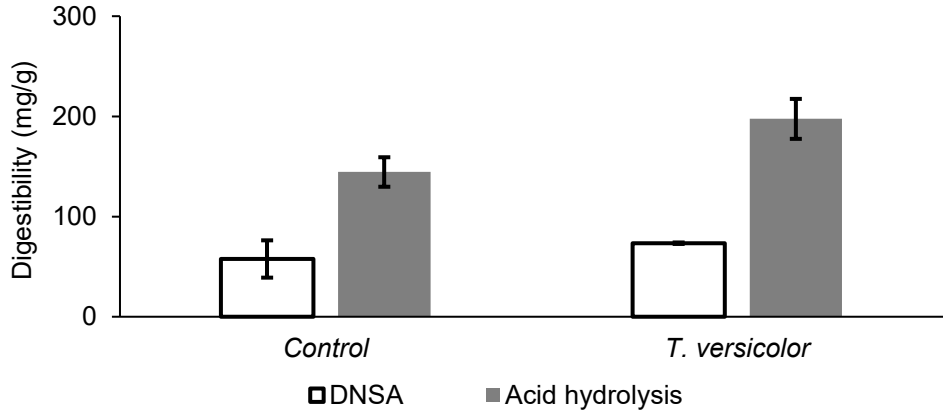


Figure 5. Digestibility of autoclaved wheat straw, which serves as the control, and autoclaved wheat straw subjected to treatment with *T. versicolor*. The sugar quantities are expressed per 0.5 g of dry wheat straw initially used (mg/g). The analysis of sugar content was performed using two methods: phenol-sulfuric acid and DNSA, respectively. The data include means and standard deviations calculated according to Gaussian error propagation rules from triplicate cultures, represented by symbols and error bars, respectively.

Pretreating lignocellulosic biomass is essential for effectively accessing its polysaccharide content, which can be converted into fermentable sugars for subsequent bioenergy and chemical production (Wan & Li, 2012; Singh et al., 2014; Duong et al., 2024). However, many conventional pretreatment methods are characterized by high energy and/or chemical requirements, making them economically unfeasible (Mosier et al., 2005; Baruah et al., 2018). In contrast, fungal pretreatment of lignocellulosic agricultural residues presents an environmentally friendly alternative that offers several benefits, including milder reaction conditions, specificity in enzymatic hydrolysis, absence of inhibitor formation, and reduced energy consumption (Singh et al., 2014; Sindhu et al., 2016; Bhatia et al., 2017; Baruah et al., 2018). In this study, the use of *T. versicolor* resulted in effective fungal pretreatment of wheat straw, significantly enhancing the yield of reducing sugars compared to control treatments. The figures for *T. versicolor* and control treatment of around 287 and 208 mg/g, respectively, with regard to total bioaccessible sugars, were found (Figure 4 and Table 3).

Table 3. Total bioaccessible sugars from autoclaved wheat straw, which serves as the control, and autoclaved wheat straw subjected to treatment with *T. versicolor* (this study), compared with those from the seven previously investigated fungi, respectively.

Total Bioaccessible Sugars	Sugars (mg/g)		Sugars (% of Control)	
	Mean	S.D	Mean	S.D
Autoclaved wheat straw	208	19	100	13
T. versicolor (this study)	287	17	138	15
Schizophyllum commune	277	7	133	13
Gongronella butleri	205	46	98	24
Gloeophyllum trabeum	154	27	74	15
Trichoderma reesei	208	66	100	33
Penicillium chrysogenum	190	33	91	18
Stropharia rugosoannulata	254	28	122	18
Stachybotrys chlorohalonata	170	25	82	14

Note: Data for *S. commune, G. butleri, G. trabeum, T. reesei, P. chrysogenum, S. rugosoannulata,* and *S. chlorohalonata*, which were previously reported (Duong et al., 2024), were re-arranged to meet the requirements of the current Table 3. The sugar quantities are expressed per 0.5 g of dry wheat straw initially used (mg/g), and relative to corresponding control values derived from autoclaved wheat straw (% of control). The analysis of sugar content was performed using phenol-sulfuric acid. The data include means and standard deviations (calculated according to the Gaussian error propagation rules) derived from triplicate cultures.

Despite the clear advantages demonstrated by fungal pretreatment at the laboratory scale, its commercialization faces significant challenges. These include extended residence times, low sugar yields, large spatial requirements, and the necessity for feedstock sterilization (Pallín et al., 2024). These factors have been identified in previous technoeconomic studies as major economic bottlenecks for scaling up fungal

pretreatment processes (Vasco-Correa & Shah, 2019). Additionally, analyses have shown that capital investment and production costs for fungal pretreatment-based facilities are higher compared to conventional pretreatment methods, further hindering its large-scale application (Baral & Shah, 2017; Vasco-Correa & Shah, 2019). To be more specific, Olughu et al. (2023) presented a comprehensive evaluation of the economic feasibility of producing cellulosic ethanol using fungal pretreatment on switchgrass. The study concluded that facility-dependent costs significantly impacted the overall production cost of cellulosic ethanol. These costs could be reduced by implementing a low-severity fungal pretreatment, particularly by shortening fermentation times. The profitability of switchgrass-based ethanol production, measured by net present value, was highly sensitive to variations in feedstock costs and glucose and xylose yields. Key challenges in achieving cost-effective cellulosic ethanol production using fungal pretreatment included low glucose yields and the substantial equipment requirements associated with this process. The authors also emphasized the need for process optimizations to enhance profitability. On the other hand, it is claimed that fungal pretreatment is insufficient as a standalone method for achieving maximum conversion of the cellulose fraction into glucose (Kandhola et al., 2017; Vasco-Correa & Shah, 2019). In addition, sugar yields obtained upon fungal pretreatment of lignocellulosic substrates were shown to be strongly dependent on the respective incubation time applied (Shirkavand et al., 2016; Hermosilla et al., 2018); hence, an optimization of the process length is crucial to the economic success of the pretreatment (Vasco-Correa & Shah, 2019). For this, the combination of fungal pretreatment with other pretreatment methods may be beneficial in addressing the challenges of fungal pretreatment (Shirkavand et al., 2016, 2017). For example, the synergistic effects of pretreatment with *T. versicolor* combined with organosolv pretreatment on loblolly pine chips were demonstrated (Kandhola et al., 2017). This combination increased the saccharification yield and resulted in higher amounts of lignin-enriched fractions (Kandhola et al., 2017). Furthermore, the combination of fungal pretreatment with mild treatments such as alkali (Salvachúa et al., 2011; López-Abelairas et al., 2013) or an organosolv pretreatment (Canam et al., 2011) resulted in yields of about 90-100% of the theoretically available glucose. Last but not least, modern circular bioeconomy concepts targeting the bioprocessing of lignocellulosic wastes within biorefinery approaches go beyond bioenergy production and consider profitable products such as biopesticides, bioplastics, biosurfactants, or biostimulants (Artola et al., 2024; Sánchez, 2024).

4. Conclusions

In the present study, the use of ligninolytic white-rot basidiomycete *T. versicolor*, which secretes several enzymes capable of degrading lignocellulosic biomass, was evaluated in two biotechnological aspects including fungal crude extracts and effects on enzymatic saccharification of wheat straw. Solid-state fermentation of wheat straw using *T. versicolor* produced fungal crude extracts containing different enzyme activities which may be promising for various biotechnological purposes. The activities of carboxylesterase (substrate: 4-nitrophenyl valerate), endo-1,4-β-D-glucanase (substrate: 2-hydroxyethylcellulose), and laccase were found to predominate in the crude extracts. Furthermore, *T. versicolor* effectively pretreated wheat straw, significantly increasing reducing sugar levels compared to controls. Future work should be aimed at optimizing the pretreatment process duration and investigating the benefits of combining fungal pretreatment with other pretreatment methods.

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6. Authors' Contributions

HLD and DS contributed to conception and design of experiments, and analysis and interpretation of data. HLD conducted the experiments, wrote the first draft of the manuscript, and edited the manuscript. DS supervised the work, and reviewed and edited the manuscript. All authors read and approved the manuscript.

7. Conflicts of Interest

All authors declare that they have no conflicts of interest.

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