WHITE-ROT FUNGAL PRETREATMENT OF WHEAT STRAW: EFFECT ON ENZYMATIC HYDROLYSIS OF CARBOHYDRATE POLYMERS

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The aim of the study was the comparative analysis of degradation of wheat straw lignin by white-rot fungi and its implications on the efficiency of enzymatic hydrolysis of holocellulose. Peroxidases were found to be predominantly responsible for lignin degradation, even though high laccase activities were detected, especially in the initial stages of fungal culturing. The studied fungal species showed various ability to degrade lignin in wheat straw, which further affected the release of reducing sugars during enzymatic saccharification. The highest rate of lignin, hemicelluloses and cellulose degradation was noticed in the sample pretreated with *Irpex lacteus*. Among all the tested species, only *Ganoderma resinaceum* was found as a suitable lignin degrader, with 2-fold higher hydrolysis yield (51.1 ± 4.7%) than in the control. A key mechanism that enhances convertibility of carbohydrates is the selective lignin removal from biomass. Operating time, holocellulose loss and unpredictable fungal response to culturing conditions are the main challenges in fungal pretreatment of lignocellulosic feedstock.

Keywords: lignin degradation, wheat straw, white-rot fungi, biological pretreatment, enzymatic hydrolysis

INTRODUCTION

The global demand for energy today is considerably dependent on fossil fuels, but the opportunities for exploitation of renewable energy sources are increasingly growing. From this perspective, the conversion of biomass as a renewable energy resource into second generation biofuels is a promising approach in green energy technologies, bio-based economy and society devoted to sustainable development.[1]

Lignocellulose is a major component of plant biomass and it is primarily composed of cellulose, hemicelluloses and lignin, which form a complex composite structure.[2] The chemical composition of this feedstock recommends it for utilization in technological processes based on the sugar platform, where cellulose or holocellulose is hydrolyzed to monosaccharides, which are further converted into bioethanol or other chemicals.[3] However, lignin as a highly complex aromatic polymer with a three-dimensional structure is mostly responsible for the recalcitrance of this potentially valuable raw material, being an obstacle that hinders an economically feasible conversion of holocellulose to fermentable sugars.[4,5] Thus, delignification is a crucial, but also a critical step in lignocellulose processing, rendering the cellulose and hemicelluloses more exposed to enzymatic saccharification.[6]

Recently, several bio-refinery concepts have been established for converting renewable biomass to fuels, but the pretreatment of plant residues still remains mostly physical, physico-chemical and chemical. Despite the undoubted advantages, such as simple and easy operation, large volumes of handled biomass, increased bulk and energy densities, *etc*., high power consumption, additional pretreatment steps, high cost of chemicals, corrosion related issues and formation of inhibitory substances are recognized as the main disadvantages.[7] In contrast, biological

pretreatment deserves much more attention, as it is safe, environmentally friendly and less energy intensive, compared to other pretreatment methods. This approach consists in using different microorganisms to remove lignin with a minimal loss of carbohydrate polymers. The advantages of biological pretreatment are also simple equipment, efficient degradation of both cellulose and hemicelluloses, and applicability for high or low biomass moisture content.[7] However, biological pretreatment is characterized by a slightly degraded lignin, it is time consuming, requires a large space and the rate of hydrolysis reaction is very low, requiring a great improvement to be commercially applicable.[7,8]

Although several groups of bacteria are capable of degrading lignin, white-rot fungi are considered as the most effective lignin-degrading organisms.[9] Fungal degradation of lignin is an extracellular oxidative process catalyzed by ligninolytic enzymes, mostly laccase, lignin peroxidases and Mn-oxidizing peroxidises.[10] Some white-rot fungi degrade lignin non-selectively, depolymerising both cellulose and hemicelluloses, so holocellulose loss could range between 17 and 50%, resulting in decreased yield of saccharification.[11] On the other hand, fungal species that exhibit selective lignin degradation have higher affinity for lignin and degrade it faster than holocellulose. The relation between lignin degradation and holocellulose loss is a crucial parameter affecting the efficiency of biological pretreatment of lignocellulose, and varies among species and even among strains of the same species.[11,12]

Biological pretreatment of wheat straw based on the action of fungal enzymes can be a promising alternative for complementary mild treatments.[13-16] Previously, it has been shown that wheat straw can be considered as a promising substrate for ligninolytic enzymes production by cultivating various white-rot fungal species.[13,16-18] However, the effects of fungal culturing as a pretreatment method in lignocelluloses processing are not yet well understood. The aim of this study was to assess the white-rot fungal delignification mechanisms crucial to enhance the efficiency of enzymatic saccharification of wheat straw after solid-state culturing.

EXPERIMENTAL

Chemicals

All reagents and chemicals were of analytical grade and were purchased either from Sigma-Aldrich (St. Louis, MO, USA) or from Merck Millipore (Darmstadt, Germany), unless otherwise stated.

Organisms

Fungal isolates used in the study were obtained from the culture collection of the Institute of Botany, Faculty of Biology, University of Belgrade (BEOFB) or from the culture collection of the Institute of Evolution, University of Haifa, Israel (HAI): *Cyclocybe cylindracea* BEOFB1210 (MW176087, 100%), *Ganoderma resinaceum* BEOFB440 (MW176072, 100%), *Irpex lacteus* BEOFB1910 (MH671316, 99.83%), *Pleurotus ostreatus* HAI592 (MW176092, 91.99%), *Trametes versicolor* BEOFB321 (MW176038, 99.64%). The identity of fungal species was confirmed by *ITS* gene sequencing and the PCR amplification of this region was conducted as described by Savković *et al*.[19] Gene bank accession numbers and homology percentages are given in brackets for each fungal isolate.

Preparation of lignocellulosic material

Wheat straw (*Triticum aestivum* L.) was washed with warm distilled water (T=50 °C), dried in a heating oven (Binder ED53) at 65 °C, to constant weight, ground in the laboratory mixer (Waring 8010 S), and sieved to obtain pieces of 0.5-2.0 cm. The thus-prepared wheat straw was subjected to biological and chemical pretreatment.

Biological pretreatment

Inoculum preparation

Inoculum was prepared for each tested species by inoculating 100 mL of synthetic medium (glucose, 10.0 g L^{-1}; NH_4NO_3, 2.0 g L^{-1}; K_2HPO_4, 1.0 g L^{-1}; $NaH_2PO_4 \times H_2O$, 0.4 g L^{-1}; $MgSO_4 \times 7H_2O$, 0.5 g L^{-1}; yeast extract, 2.0 g L^{-1}; pH 6.5) and incubation, as previously described.[20]

Cultivation conditions and extraction of ligninolytic enzymes

Solid-state culturing was carried out at 25 °C in 250 mL flasks containing 6.0 g of wheat straw and 30.0 mL of the modified synthetic medium (without glucose). Inoculation was done with 9.0 mL of inoculum per flask). Samples were harvested after 5, 10, 15 and 19 days of culturing and further used for ligninolytic enzyme extraction, according to previously established conditions.[20] The residues were used for determination of hemicelluloses, cellulose and lignin contents and for enzymatic saccharification.

Ligninolytic enzymes were extracted by stirring samples with 150.0 mL of cold dH_2O on a magnetic stirrer at 4 °C for 10 min. The extracts were filtrated using a laboratory sieve, the liquid fractions were centrifuged (4 °C, 3000 rpm, 10 min) and the supernatants obtained were used for determining laccase and Mn-oxidizing peroxidases activities spectrophotometrically (CECIL CE2501 (BioQuest)).

The solid phase remaining after the extraction was dried in a drying oven at 65 °C to constant weight.

Enzyme activity assay and determination of total protein content

Laccase (EC 1.10.3.2) activity was determined by monitoring the A_{436} change related to the rate of oxidation of 50.0 mM 2,2'-azino-bis-[3-ethylthiazoline-6-sulfonate] (ABTS) (ε_{436} = 29300 M^{-1} cm^{-1}) in 0.1 M phosphate buffer (pH 3.5) at 35 °C. The reaction mixture (V_{tot} = 1.0 mL) contained buffer, ABTS and sample.[21]

Mn-oxidizing peroxidases (Mn-dependent peroxidase [EC 1.11.1.13; MnP] and Mn-independent peroxidase [EC 1.11.1.6; MnIP]) activities were determined with 3.0 mM phenol red (ε_{610} = 22000 M^{-1} cm^{-1}) as a substrate, in a succinate buffer pH 4.5. The reaction mixture (V_{tot} = 1.0 mL) contained buffer, sample, 2.0 mM H_2O_2 and phenol red, with or without 2.0 mM $MnSO_4$ (for MnP and MnIP, respectively). The reaction was stopped by adding 2.0 M NaOH.[21]

Enzymatic activity of 1 U is defined as the amount of enzyme that transforms 1.0 μmol of substrate per min.

Determination of polymer content in wheat straw

Hemicellulose content determination

The content of hemicelluloses was determined by the fibre analysis method of Van Soest[22] by removing soluble sugars, proteins, pectin, lipids, and vitamins from the sample using neutral detergent and Na_2SO_3.[22,23] Thus, neutral detergent fibers (NDF) were treated with acid detergent solution to obtain acid detergent fibres (ADF). The hemicellulose content was then expressed as NDF – ADF.

Cellulose and lignin content determination

ADF were used for determining cellulose and lignin content using the Klason or 72% H_2SO_4 method.[24] ADF were treated with 72% H_2SO_4 and the Klason lignin residues were collected. The lignin content (LC) was expressed as percentage of the original sample. The cellulose content is presented as the difference in weights of the samples treated with ADF and LC.

Enzymatic hydrolysis

Pretreated wheat straw was ground in the laboratory mixer and sieved to separate the large fraction (L, 5-8 mm), medium fraction (M, 2-5 mm) and small fraction (S, 0.2-0.5 mm). Enzymatic saccharifications were carried out in 250-mL flasks containing 2 g of pretreated straw in 60 mL of acetate buffer (pH 4.8), with the addition of streptomycin sulphate (500 mg L^{-1}) to prevent bacterial growth, and commercial cellulase (cellulase from *Trichoderma reesei*, C2730-50ML, 700 U g^{-1}; Sigma-Aldrich) in loadings of 15, 30 and 60 U g^{-1} of solid substrate. Incubation was carried out in a shaking incubator with a horizontal rotary plate (Stuart SI500) (180 rpm, 50 °C for 72 h). Immediately after hydrolysis, the samples were immersed in boiling water for 5 min to inactivate the enzymes and further used for determination of reducing sugar content.[25]

Autoclaved untreated wheat straw was used as a negative control, while chemically pretreated wheat straw was used as a positive control. Chemical pretreatment of wheat straw was performed with 1% (w/v) NaOH at 121 °C for 90 min. The samples were then washed with dH_2O till a pH of 6.8-7.5 was achieved and the solid phase was further dried to constant weight and used for enzymatic hydrolysis.[26]

Reducing sugars content determination

The content of reducing sugars was measured colorimetrically using 1,4-dinitrosalycilic acid, according to Miller's method.[27] Sampling of the liquid fraction of the hydrolysate was performed periodically (after 6, 12, 24, 36, 48 and 72 h). Samples were centrifuged (15 000 rpm, 30 °C, 10 min) and the supernatant was further used for quantitative determination of reducing sugars. The reaction mixture containing 1 mL of DNSA solution (aqueous solution of 1,4-dinitrosalic acid, 10.0 g L^{-1}; potassium sodium tartarate tetrahydrate, 30.0 g L^{-1}; 2 M NaOH, 200 mL/L) and 1 mL of sample was incubated at 90 ± 0.5 °C in a water bath for 5 min and cooled till room temperature (22 ± 2 °C). After that, the mixture was diluted with 5 mL of dH_2O and the amount of reducing sugars was determined spectrophotometrically by monitoring the absorbance at 540 nm against a blank (containing 1 mL of acetate buffer (pH 4.8) instead of sample). Reducing sugars were expressed as g of glucose equivalent (gGE) per L of hydrolysate using the equation of calibration curve for glucose.

The yield of enzymatic hydrolysis was calculated as follows:

Hydrolysis yield (%) = Reducing sugars in hydrolysate (g) × 0.9 × 100 / Cellulose in sample (g) (1)

The correlation factor of 0.9 corresponding to hexoses was used in the calculations to compensate for the addition of a water molecule during hydrolysis of each glycosidic bond.[28]

To determine the impact of fungal cultivation on an overall wheat straw conversion to glucose, the coefficient of conversion (K_c) from the maximal theoretical value (1) of an initial sample was calculated as follows:

$K_c = m_f \times 0.9$ / Cellulose in an initial sample (g) (2)

where factor m_f corresponds to the amount of reducing sugars (g) obtained after saccharification of entire pretreated sample.

Analysis of sugars

Sugars (glucose, arabinose, cellobiose and xylose) were analyzed by high performance anion exchange chromatography. All aqueous solutions were prepared using Ultrapure TKA deionised water. The analysis

was performed according to a previously published procedure.[29]

Statistical analyses

The assays were carried out in three replicates and results were expressed as mean ± standard error. Normal distribution (Shapiro-Wilks test) and homogeneity of variance (Levene's test) were evaluated prior to further analyses, where certain groups based on different quantitative parameters were compared to see whether there are significant differences between them. If data followed normal distribution and homogeneity of variance was met, one-way analysis of variance (ANOVA) was performed to test any significant differences among means, since the number of groups was always higher than two. If data followed normal distribution, but the assumption of equal variances was violated, Welch Test or Welch ANOVA was considered for the analyses. Together with one-way ANOVA, the pairwise comparison option that runs Tukey's HSD (Honestly Significant Difference) post-hoc test was applied in some cases. Statistical significance was declared at $P < 0.01$. All statistical analyses were performed using Microsoft Excel, the statistical package XLSTAT[30] and software STATISTICA, version 6.0 (StatSoft, Inc., Tulsa, USA).

Principal component analysis (PCA) showing the relationship between the degradation of lignin and ligninolytic enzyme activities of all taxa was performed, for each enzyme separately. The values of lignin degradation by selected fungal species were used as response variables, while the enzyme activity of the taxa and time variable showing the number of days after which the measurements were performed were used as supplementary ones. Multivariate analyses were done using Canoco 5 software.[31]

RESULTS AND DISCUSSION

Activity of ligninolytic enzymes

The studied five species of fungi have shown different levels of MnP, MnIP and laccase activities after 5, 10, 15 and 19 days of solid-state cultivation on wheat straw as a substrate (Fig. 1). Based on the results of ligninolytic enzyme activities, it can be noticed that the levels of enzyme activities varied during the fungal cultivation.

The highest level of MnP activity was detected in *Pleurotus ostreatus* (Fig. 1d) with the maximum recorded after 10 days of cultivation (996.2 ± 69.1 U L^{-1}). The maximum value of MnIP activity was also noted in *P. ostreatus* (984.2 ± 84.7 U L^{-1}) after 10 days of cultivation (Fig. 1d). All the studied species, except *Irpex lacteus*, were good producers of laccase with activities much higher in comparison with Mn-oxidizing peroxidases. High laccase activities characterized the initial stages of fungal cultivation, between day 5 and day 10, while the activities of this enzyme were considerably lower on day 19. Even though the highest level of laccase was detected in *P. ostreatus* (15051.3 ± 1186.9 U L^{-1}) (Fig. 1d), *Ganoderma resinaceum* and *Cyclocybe cylindracea* were better producers of this enzyme, keeping the high laccase production till day 15 of fungal cultivation (Fig. 1b and 1a, respectively). *I. lacteus* was the only studied species that did not synthesize laccase under the experimental conditions used (Fig. 1c). *Trametes versicolor* showed persistent activity of both MnP and MnIP during the whole period of cultivation, except at the beginning (Fig. 1e).

By comparing four groups that refer to the data (all species included) obtained after 5, 10, 15 and 19 days, for each enzyme separately, the most significant difference was observed for laccase ($P<0.01$) according to one-way ANOVA.

Wheat straw was the substrate used for conversion to carbohydrates in this study, after application of fungal culturing as the method of pretreatment. So far, considerable efforts have been made in applying white-rot fungal species to pretreat lignocellulosic feedstock, even during submerged or solid-state cultivation.[32-34] The most frequently used species were from genera *Bjerkandera*, *Ceriporiopsis*, *Ganoderma*, *Irpex*, *Phanerochaete*, and *Trametes*.[34-37] Even though all these species are recognized as potent producers of ligninolytic enzymes during the cultivation on various substrates, significant variations in enzyme activities may occur depending on substrate chemical composition and physical properties. That is why, it is important to elaborate a physiological response on different types of carbon source for every single species or even strain used for pretreatment.[38]

Previous studies have shown that wheat straw is a suitable substrate for production of ligninolytic enzymes, indicating that the rate of delignification depends on specific enzymes production and the activity level.[12,18,39] Generally, peroxidases are predominantly responsible for lignin degradation and the level of their activity has to be significantly related to the rate of lignin degradation.[40] The results of this study are in accordance with previous ones, where higher peroxidase activity, stimulated by inducers, was related to efficient delignification.[12]

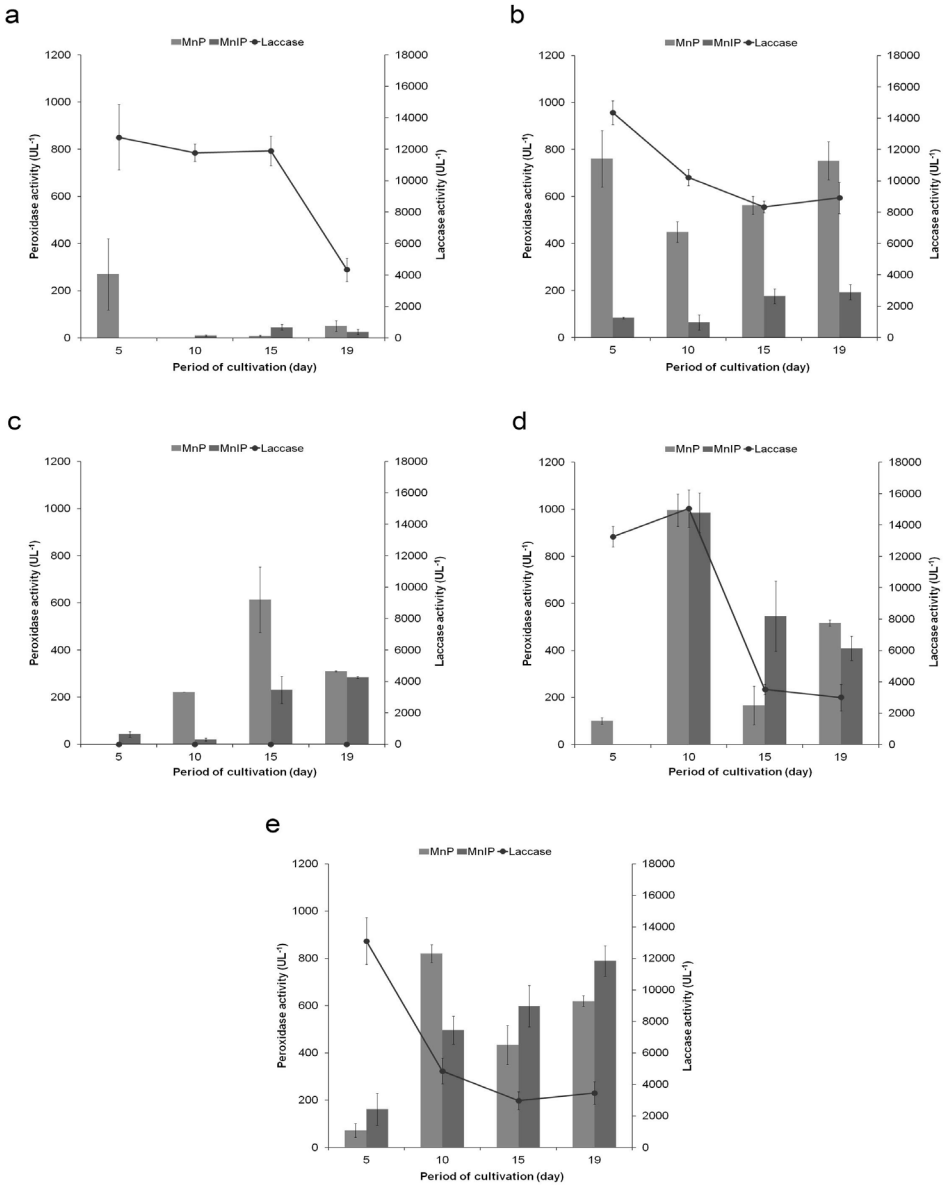

Figure 1: Dynamics of ligninolytic enzyme activity during solid-state culturing on wheat straw: a. *Cyclocybe cylindracea*; b. *Ganoderma resinaceum*; c. *Irpex lacteus*; d. *Pleurotus ostreatus*; e. *Trametes versicolor*

This is specifically attributed to the initial phase of wood degradation, when laccase has limited diffusion into non-degraded plant cell wall primarily due to molecule size. The species used for pretreatment of wheat straw in this study exhibited various capacities to produce ligninolytic enzymes and the contribution of individual enzymes in the enzymatic pool was evident. High peroxidase activity in *G. resinaceum*, *P. ostreatus* and *T. versicolor* was expected and is in line with the results reported by other authors.[41,34,12] Furthermore, peroxidase activities (MnP and MnIP) detected for *P. ostreatus* in our study were much higher (ranged up to 996.2 U L^{-1}) than those reported by Stajić *et al*.[41] (ranged up to 60.5 U L^{-1}). If not absent, laccase activities in all cases were higher at the beginning of cultivation, expressing the trend of decrease in activity levels after day 10 or day 15, which was also observed in other researches.[39,42] This can be highly related to intensive degradation of small molecular weight compounds by laccase in the initial phase of fungal growth.[43] Comparing all studied species, it can be observed that *I. lacteus* was the only species without laccase activity. According to Dias *et al*.,[33] laccase activity was not detected during wheat straw pretreatment with *I. lacteus* as well. MnP has been considered the main

ligninolytic enzyme in this fungus, which is in agreement with a previous study.[34]

Wheat straw polymers degradation during biological pretreatment

To evaluate the effect of fungal culturing on degradation of wheat straw, the changes in the content of the three main polymers were determined.

The contents of lignin, hemicelluloses and cellulose in the wheat straw were 9.0 ± 0.4%, 32.8 ± 0.6% and 47.6 ± 1.1%, respectively. The pretreatment with the studied fungal species caused significant variations in the total dry matter weight loss, bringing about changes in the content of the main polymers ($P<0.01$) (Table 1). Polymer degradation occurred continually during the cultivation period, so the maximum degradation rates were noted after 19 days. The maximum dry matter weight loss was noted after the pretreatment with *I. lacteus*, and the minimum was detected after cultivation of *C. cylindracea*. Statistically significant difference between the studied fungal taxa, when dry matter weight loss is considered as a quantitative variable (taking into account all the data from the beginning to the end of the experiment), was confirmed by one-way ANOVA ($P<0.01$). According to the performed Tukey post-hoc test, in terms of dry matter weight loss, significant differences ($P<0.01$) were observed between *I. lacteus* and all other studied species, except *T. versicolor*.

The rates of polymer degradation in wheat straw differed among the studied species (Table 1). On some occasions (*T. versicolor*, *P. ostreatus* and *G. resinaceum*), the capacities of the studied species to degrade hemicelluloses were significantly higher in comparison with their abilities to degrade cellulose. The highest rate of lignin, hemicelluloses and cellulose degradation was noted after the pretreatment with *I. lacteus*. The lowest rate of lignin and cellulose degradation was noted after the pretreatment with *P. ostreatus*, while the minimum hemicelluloses degradation was achieved after the pretreatment with *C. cylindracea*. Compared with other species, *G. resinaceum* achieved the maximum difference between the level of lignin and cellulose degradation (44.5 ± 5.4% *vs*. 34.1 ± 2.5%), which characterized this species as the most selective in delignification among all studied fungal species. One-way ANOVA was used to assess the difference between all fungal species, considering the rates of degradation, separately for lignin, hemicelluloses and cellulose. A significant difference was confirmed between fungal species toward all three polymers ($P<0.01$). Tukey's post-hoc test was also performed for lignin, and significant differences ($P<0.01$) were observed between *P. ostreatus* and all the other studied fungal species, except *C. cylindracea*, as well as between *C. cylindracea* and *I. lacteus*. Considering hemicelluloses, significant differences are observed between *C. cylindracea* and *I. lacteus*, *C. cylindracea* and *T. versicolor*, *I. lacteus* and *P. ostreatus*, *P. ostreatus* and *T. versicolor*. For cellulose, *I. lacteus* stands out, considering the degradation of this polymer, because a significant difference is observed between this and all the other tested species.

Three principal component analyses (PCAs) were done separately for MnP (Fig. 2a), MnIP (Fig. 2b) and laccase (Fig. 2c). PCAs demonstrated the relationship between the degradation of lignin by the five examined species (response variable, blue arrows), enzymes activities of the same five species (supplementary variables, red dotted arrows) and the stages of the experiment – the number of days after which the measurements were performed, D5, D10, D15 and D19 (supplementary variables). Vectors referring to the degradation of lignin by fungal species (blue arrows) are oriented toward the left side of the ordination diagram in all PCAs and correlate to the final stages of the experiment (D19 and D15). However, vectors referring to enzymes activities of studied species (red arrows) had different positions on first, second and the third PCA diagram due to different enzymes (MnP, MnIP and laccase).

The degree of lignin degradation by fungal taxa and their MnP activities were positively, negatively or non-correlated depending on the species (Fig. 2a). For *I. lacteus*, *T. versicolor* and *G. resinaceum*, the positive correlation between these two parameters was observed. The correlation was almost non-existent for *P. ostreatus*, while negative correlation was observed when *C. cylindracea* was considered. The MnP activity of *C. cylindracea* was highest after D5, and MnP activities of *I. lacteus*, *T. versicolor* and *P. ostreatus* were higher in the later stages of the experiment.

Table 1
Effect of biological pretreatment with selected white-rot fungi on degradation of wheat straw polymers

Species/ Code of strain	Period of cultivation (day)	Total dry matter weight loss after pretreatment (%)	Polymer content in sample (mg g^{-1})			Degraded fibres (%)		
			Lignin	Hemicelluloses	Cellulose	Lignin	Hemicelluloses	Cellulose
Cyclocybe cylindracea/ BEOFB1210	5	10.0 ± 0.1	80.0 ± 2.6	293.0 ± 2.9	443.0 ± 5.8	20.5 ± 2.6	19.6 ± 0.8	16.2 ± 0.1
	10	10.9 ± 0.2	79.7 ± 2.4	285.7 ± 1.8	403.0 ± 3.5	21.6 ± 2.4	22.5 ± 0.4	24.6 ± 0.7
	15	13.9 ± 0.7	76.7 ± 1.9	288.0 ± 4.0	409.3 ± 3.4	27.1 ± 2.3	24.4 ± 1.6	26.0 ± 0.0
	19	16.1 ± 0.6	71.0 ± 2.1	286.3 ± 8.2	384.7 ± 10.9	34.3 ± 2.1	26.8 ± 2.6	32.3 ± 1.7
Ganoderma resinaceum/ BEOFB440	5	9.8 ± 0.6	77.3 ± 0.3	321.0 ± 5.0	426.0 ± 8.4	23.0 ± 0.8	11.8 ± 1.6	19.3 ± 1.2
	10	13.4 ± 0.4	79.0 ± 1.5	268.0 ± 3.6	382.3 ± 9.2	24.5 ± 1.6	29.3 ± 0.7	30.4 ± 1.9
	15	17.3 ± 0.4	68.0 ± 0.6	243.0 ± 8.3	381.0 ± 4.5	37.9 ± 0.7	38.8 ± 2.0	33.8 ± 0.9
	19	19.6 ± 0.9	62.7 ± 6.4	228.7 ± 1.2	390.0 ± 11.1	44.5 ± 5.4	44.0 ± 0.3	34.1 ± 2.5
Irpex lacteus/ BEOFB1910	5	14.9 ± 0.7	82.3 ± 1.7	302.3 ± 6.0	377.7 ± 4.2	22.6 ± 1.6	21.6 ± 2.0	32.5 ± 0.3
	10	23.4 ± 0.9	71.3 ± 3.0	276.0 ± 7.6	362.0 ± 10.1	39.8 ± 1.8	35.6 ± 2.4	41.7 ± 2.3
	15	32.2 ± 0.4	68.7 ± 0.3	266.3 ± 7.5	349.7 ± 3.5	48.6 ± 0.5	45.0 ± 1.3	50.2 ± 0.3
	19	40.1 ± 2.0	74.3 ± 5.8	253.0 ± 4.5	300.7 ± 16.8	50.9 ± 4.1	53.9 ± 1.1	62.0 ± 3.3
Pleurotus ostreatus/ HAI592	5	7.4 ± 0.3	83.0 ± 3.1	299.7 ± 3.4	446.3 ± 2.7	15.2 ± 2.9	15.5 ± 0.7	13.2 ± 1.7
	10	14.5 ± 0.9	90.0 ± 1.0	299.7 ± 5.4	409.0 ± 10.3	15.1 ± 0.5	22.0 ± 0.6	26.5 ± 2.4
	15	15.7 ± 0.9	83.0 ± 4.2	277.7 ± 5.0	415.7 ± 6.6	22.8 ± 3.6	28.7 ± 2.0	26.4 ± 1.8
	19	17.5 ± 1.0	80.3 ± 0.9	263.7 ± 2.0	406.0 ± 6.2	26.8 ± 1.7	33.7 ± 0.3	29.6 ± 1.6
Trametes versicolor/ BEOFB321	5	6.2 ± 1.2	75.0 ± 1.2	266.7 ± 7.2	466.0 ± 2.5	22.3 ± 1.1	23.7 ± 1.9	8.2 ± 1.2
	10	15.9 ± 1.7	69.7 ± 2.2	270.3 ± 5.0	400.7 ± 13.2	35.3 ± 2.6	30.7 ± 2.2	29.1 ± 3.7
	15	26.2 ± 2.1	71.0 ± 1.2	251.0 ± 7.0	375.0 ± 7.2	42.2 ± 2.0	43.5 ± 3.1	42.0 ± 0.7
	19	32.7 ± 1.1	73.0 ± 4.0	225.7 ± 4.9	368.7 ± 5.2	45.8 ± 3.1	53.7 ± 3.1	47.9 ± 1.4
Untreated wheat straw	-	-	90.6 ± 4.3	328.2 ± 5.5	476.0 ± 11.4	-	-	-
NaOH pretreated wheat straw	-	36.0 ± 0.0	60.7 ± 1.3	160.0 ± 2.5	699.3 ± 4.9	57.1 ± 0.9	68.8 ± 0.5	6.0 ± 0.7

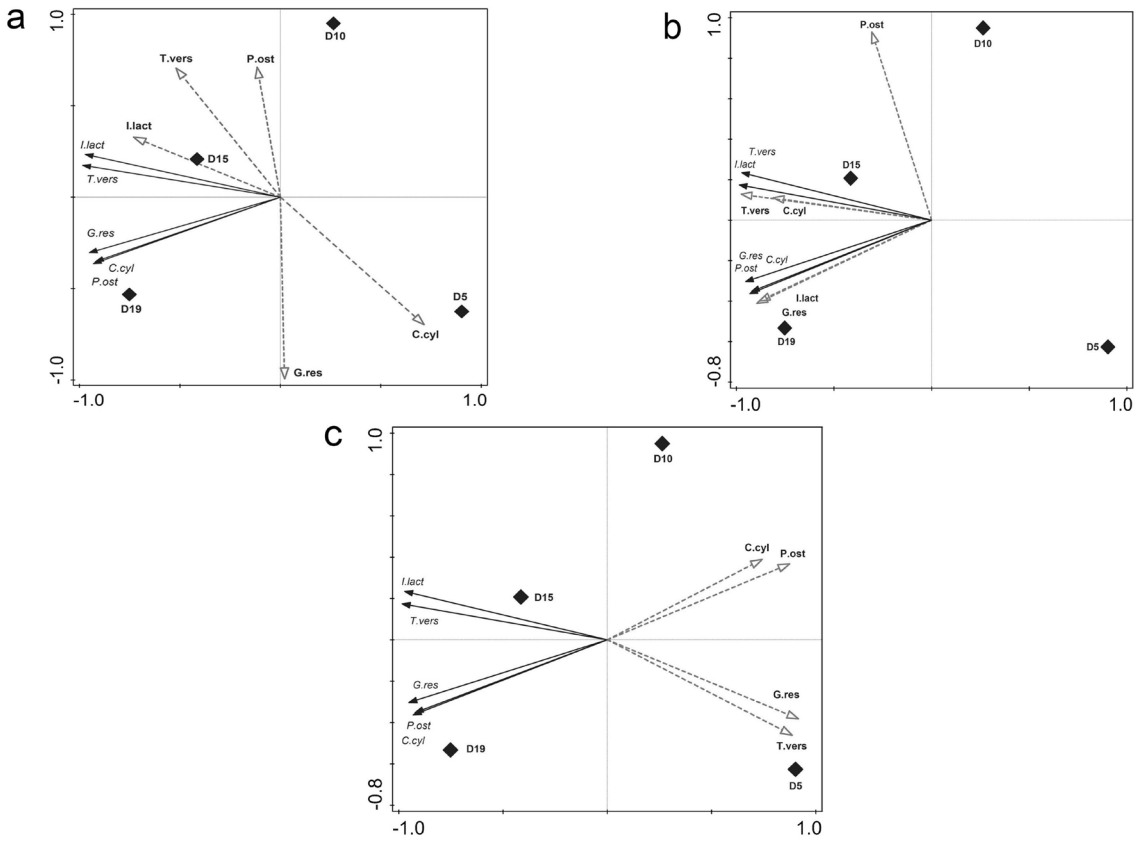

Figure 2: PCA representing the relationship between the degradation of lignin and enzyme activities of *Cyclocybe cylindracea*, *Ganoderma resinaceum*, *Irpex lacteus*, *Pleurotus ostreatus* and *Trametes versicolor*, for each enzyme separately: a. MnP; b. MnIP; c. laccase (*C.cyl – Cyclocybe cylindracea*; *G.res – Ganoderma resinaceum*; *I.lact – Irpex lacteus*; *P.ost – Pleurotus ostreatus*; *T.vers – T. versicolor*; D5, D10, D15, D19 – variables referring to the number of days after which the degree of lignin degradation and enzyme activity were measured)

For all the species, except for *P. ostreatus*, the degree of lignin degradation was positively correlated with their MnIP activities, which were the highest after D15 and D19 (Fig. 2b). The correlation of *P. ostreatus* MnIP activity with its lignin degradation was almost non-existent (the angle between two vectors is almost 90 degrees) and, as seen, MnIP activity of *P. ostreatus* was the highest after D10.

In contrast to MnP and MnIP, laccase activity was negatively correlated with the degradation of lignin for each species (Fig. 2c). Vectors referring to laccase activities of all fungal species are oriented toward the right side of the ordination diagram, confirming high laccase activities in the initial stages of fungal cultivation – D5 or D10 (Fig. 2c).

Degradation of lignin during fungal growth on the lignocellulosic substrate is an inevitable process that enables access to both cellulose and hemicelluloses. This is a key assumption that recommends this mechanism as a promising tool to be used in lignocelluloses pretreatment for bioethanol production.[44] Contrary to the biodegradation processes, which involves extracted ligninolytic enzymes targeting the lignin and its derivatives only, fungal delignification in the studied samples was followed by the loss of carbohydrates to some extent. This phenomenon was expected as a result of carbohydrate consumption by fungi.[34,45] Furthermore, this study documented the negative effect of fungal culturing on the yields of reducing sugars for the majority of the tested fungal species. However, fungal species that

selectively degrade lignocellulose have higher affinity for lignin and degrade it faster than carbohydrate components.[46] Thus, *G. resinaceum*, the most selective fungal species from our study, was characterised by a hydrolysis yield in the range of previously reported results for the most selective *Pycnoporus cinnabarinus* by Hatakka[46] (51.1 vs. 54.6%, respectively). Another study showed that the approach of using selective lignin degraders could be of a great importance in biotechnology, since most of holocellulose after the pretreatment remains in the substrate, and is available for saccharification.[36]

Enzymatic hydrolysis of wheat straw

Two crucial factors affecting the hydrolysis process with predefined conditions, enzyme loading and particle size, were investigated to determine the optimal parameters for further enzymatic hydrolysis of pretreated wheat straw. Reducing sugars are expressed as g of glucose equivalent (gGE) per L of hydrolysate.

As shown in Figure 3, the reducing sugars production profiles in all the investigated samples were similar, *i.e.* a continual increase occurred during the hydrolysis. Using the different dosage of commercial cellulase (15; 30 and 60 U g^{-1} of solid substrate) to determine the optimal concentration of enzyme required for wheat straw saccharification, a continual increase in reducing sugars concentration in the hydrolysate was noticed during the period of 72 h (Fig. 3a). The hydrolysis rate of untreated wheat straw depended on the cellulase concentration to a certain level, so the lowest value of released sugars was detected at an enzyme loading of 15 U g^{-1} (5.3 ± 0.2 gGE L^{-1}). The highest content of reducing sugars was achieved at enzyme loading of 30 U g^{-1} (6.0 ± 0.1 gGE L^{-1}) after 72 h, and did not further increase with the enzyme loading of 60 U g^{-1} (Fig. 3a).

The obtained results showed that the particle size of wheat straw used for enzymatic hydrolysis significantly affected the rate of reducing sugars release ($P<0.01$). Correspondingly, the highest content of reducing sugars in the hydrolysate was detected after 72 h in samples containing *S* fraction (6.0 ± 0.1 gGE L^{-1}), while the minimum was noticed in samples with *L* fraction (2.5 ± 0.0 gGE L^{-1}) (Fig. 3b).

Fungal culturing was further conducted with *S* fraction to investigate the effect of ligninolytic enzymes secretion by selected white-rot fungi on the release of reducing sugars during enzymatic hydrolysis of pretreated wheat straw. The pretreatment of wheat straw with selected white-rot fungi affected differently the rate of reducing sugars release during the enzymatic hydrolysis (Fig. 4a). The maximum amount of reducing sugars was released from wheat straw pretreated with *G. resinaceum* (7.3 ± 0.5 gGE L^{-1}), while the minimum was detected after pretreatment with *C. cylindracea* (1.3 ± 0.1 gGE L^{-1}) (Fig. 4a). The results indicate that, among all tested fungal species, only the pretreatment with *G. resinaceum* increased the total sugar yield, which was 21.7% higher compared with the control. Wheat straw processing with other tested species brought to lower sugar yields during saccharification.

Enzymatic hydrolysis of chemically pretreated wheat straw led to a significantly higher amount of reducing sugars compared to biologically pretreated straw, and the maximum amount was achieved after 72 h (18.2 ± 0.2 gGE L^{-1}) (Fig. 4b). Significant differences considering time, as well as fungal species, were confirmed by one-way ANOVA ($P<0.01$) when reducing sugars are used as a quantitative variable. Significant differences between all species pairs were assessed using Tukey's post-hoc test, according to which *C. cylindracea* and *G. resinaceum* were identified as the species with opposite effects on the concentration of reducing sugars.

The efficiency of the pretreatment was evaluated by enzymatic convertibility of the substrate expressed through hydrolysis yield and coefficient of conversion. The highest hydrolysis yield characterized chemically pretreated samples (70.2 ± 0.8%) (Table 2). The obtained results also showed that differences in hydrolysis yields occurred depending on the fungal species used for the pretreatment. Among pretreated samples, maximum convertibility was determined for the samples pretreated with *G. resinaceum* with the highest hydrolysis yield (51.1 ± 4.7%), which was considerably higher than in the untreated wheat straw (34.2 ± 0.3%).

In all other samples lower values of hydrolysis yields were achieved. ccharification of the samples pretreated with *C. cylindracea* (9.4 ± 0.5%). Even though high hydrolysis yield was achieved after the pretreatment with *G. resinaceum*, the coefficient of conversion (K_c) that refers to a starting sample indicates that the difference in enzymatic convertibility between this sample and the control is found negligible (0.335 and 0.341, respectively).

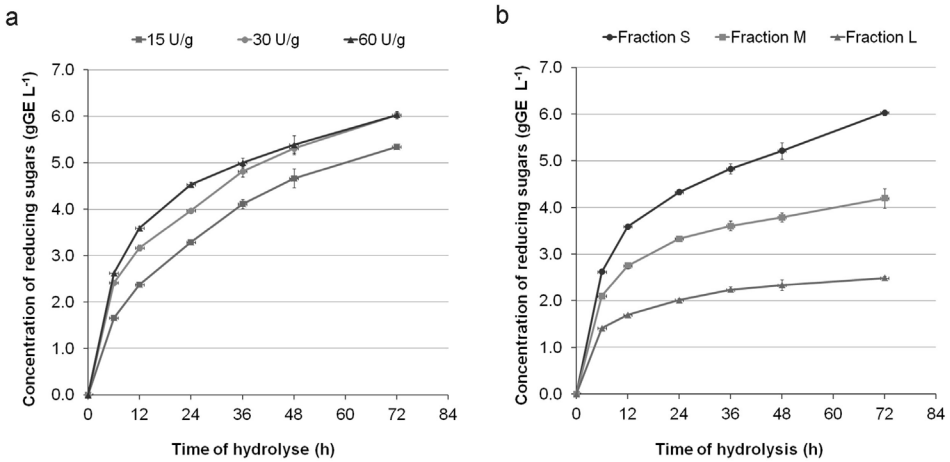

Figure 3: Time course of reducing sugars release from wheat straw during enzymatic saccharification. (a) Effect of enzyme loading presented as U g^{-1} of solid substrate; (b) Effect of particle size (Fraction S (0.2-0.5 mm), Fraction M (2-5 mm), Fraction L (5-8 mm) at enzyme loading of 60 U g^{-1})

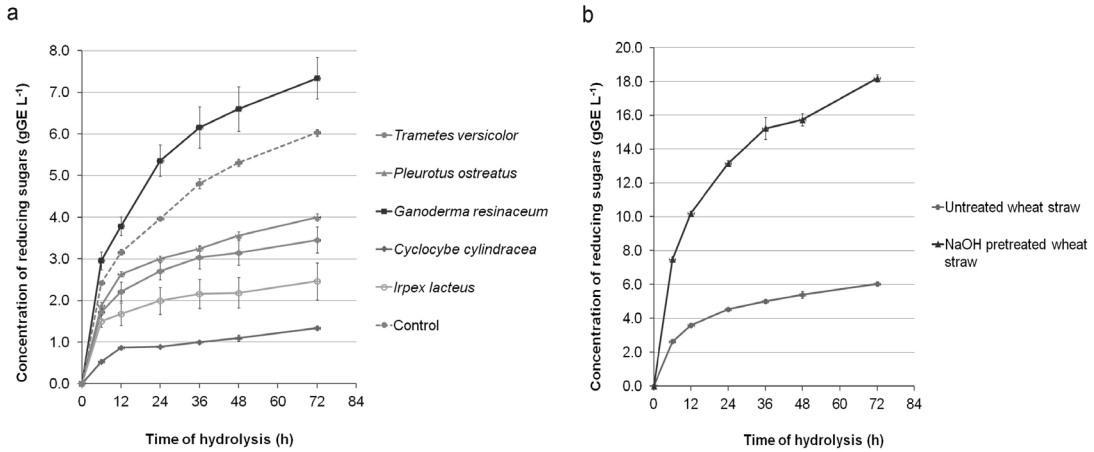

Figure 4: Time course of reducing sugars release from (a) biologically pretreated wheat straw and (b) NaOH-pretreated wheat straw, during enzymatic saccharification at enzyme loading of 60 U g^{-1} of solid substrate

The minimum was noticed after saThe highest K_c of chemically pretreated wheat straw (0.660 from the theoretical maximum value) distinguishes this method as the most effective in comparison with pretreated samples (Table 2).

Samples of the enzymatic hydrolysates of pretreated wheat straw were further analyzed by liquid chromatography to quantify the amount of solubilised sugars. As shown in Table 3, both hexoses and pentoses, as well as disaccharides, were detected in hydrolysates of the samples pretreated with selected white-rot fungi. Data show that arabinose and xylose are the predominant monosaccharides in the enzymatic hydrolysates of biologically pretreated samples. The significant amount of glucose was also detectable in the samples, but in the form of disaccharide cellobiose. The detection of pentoses in the hydrolysates of samples indicates that a significant residual amount of

hemicelluloses is still present in the samples after biological pretreatment. In addition, those sugars became available even after enzymatic hydrolysis conducted by cellulases. Finally, after simultaneous consumption of both cellulose and hemicelluloses during biological pretreatment of wheat straw, significant differences in the amounts of released sugars can be observed. Thus, according to sugar analysis, the pretreatment of wheat straw with *T. versicolor* caused the release of the highest amount of sugars (including hexoses and pentoses) during enzymatic hydrolysis (Table 3).

As can be seen from Table 3, the pretreatment with NaOH was strictly selective to lignin and hemicellulose degradation, most of the cellulose remaining undegraded. On the other hand, enzymatic hydrolysis of untreated wheat straw resulted in the highest amount of xylose.

Besides lignocellulosic feedstock preparation methods and physical parameters, the factor that strongly affects the enzymatic hydrolysis of cellulose is the proper amount of applied cellulolytic enzymes.[47] The obtained results clearly showed that an increase of cellulase concentration over an optimal level of loading cannot cause a higher rate of reducing sugars release, which is supported by the study of Tsegaye *et al*.[47] The main reason for this is that cellulose accessibility limits the effectiveness of minimum cellulase loading on the hydrolysis efficiency, leaving part of enzyme molecules unassociated to the substrate and with no effect on the hydrolysis yield.[48]

Table 2
Hydrolysis efficiency of wheat straw (WS)

Sample	Hydrolysis yield (%)	Coefficient of conversion (K_c)
WS after *Cyclocybe cylindracea* cultivation	9.4 ± 0.5 E[a]	0.064 E[a]
WS after *Ganoderma resinaceum* cultivation	51.1 ± 4.7 B	0.335 B
WS after *Irpex lacteus* cultivation	21.8 ± 2.9 D	0.084 F
WS after *Pleurotus ostreatus* cultivation	26.6 ± 1.0 D	0.187 C
WS after *Trametes versicolor* cultivation	25.3 ± 2.0 D	0.132 D
Untreated WS	34.2 ± 0.3 C	0.342 B
NaOH pretreated WS	70.2 ± 0.8 A	0.660 A

[a] Means with different letters within a column are significantly different (P<0.01)

Table 3
Sugar composition of enzymatic hydrolysates from pretreated wheat straw

Pretreatment	Sugar concentration (g L^{-1})			
	Glucose	Cellobiose	Arabinose	Xylose
Cyclocybe cylindracea	1.5 ± 0.0	4.1 ± 0.1	1.2 ± 0.0	3.7 ± 0.0
Ganoderma resinaceum	5.5 ± 0.0	13.9 ± 0.2	7.2 ± 0.1	12.8 ± 0.3
Irpex lacteus	3.6 ± 0.2	14.8 ± 0.2	6.3 ± 0.0	12.4 ± 0.2
Pleurotus ostreatus	4.7 ± 0.1	13.3 ± 0.1	8.7 ± 0.0	10.3 ± 0.1
Trametes versicolor	3.1 ± 0.1	19.2 ± 0.5	18.0 ± 0.3	17.9 ± 0.2
NaOH	18.4 ± 0.2	4.0 ± 0.1	0.6 ± 0.0	0.6 ± 0.0
Control	7.2 ± 0.0	14.4 ± 0.1	4.6 ± 0.0	42.7 ± 1.2

Apart from enzyme concentration, the particle size of lignocellulosic feedstock is also a factor that influences the rate and efficiency of enzymatic hydrolysis.[49] The results obtained in our study are similar to those reported by Khullar *et al*.,[49] who showed that particle size less than 1.0 mm gave the highest yields of sugars after saccharification. This is mainly related to porosity and also substrate accessibility, that as has been discussed in the

literature for increased cellulosic conversions. Additionally, smaller particles of lignocellulose feedstock are more accessible to enzyme molecules due to lower degree of crystallinity and increased surface area.[50] Finally, higher conversion can be attributed to improved mass and heat transfer in samples containing smaller particles.[49]

With previously defined optimal conditions for enzyme concentration and particle size, partial lignin degradation with white-rot fungi was investigated in this study from the aspect of hydrolysis efficiency and overall convertibility. The effect of fungal pretreatment on enzymatic saccharification was evident according to obtained results. The studied fungal species exhibited various effects on hydrolysis yields, which was mostly expected due to different physiological response of each fungus, influenced by different physical and chemical factors during the cultivation period.[51] Thus, fungal culturing cannot always increase the hydrolysis yield, and the positive effect depends on fungal species, the range of cultivation conditions and the substrate properties.[52] Besides our study, the positive effect of pretreatment with white-rot fungi has also been observed by other authors.[33,34,52] The increase of hydrolysis yields is directly correlated to better accessibility to polysaccharides after fungal delignification. Pinto *et al.*[34] also reported that their strains of *G. resinaceum* and *T. versicolor* were the most effective among all tested species, increasing saccharification about 2-fold. The results of biologically pretreated wheat straw clearly indicate that the higher rate of lignin in lignocellulosic feedstock decreases the efficiency of enzymatic hydrolysis, which is in accordance with previous findings.[53] However, the results of sugar analysis lead us to assume that this can be compensated with a higher cellulose amount in the feedstock after biological pretreatment, where hemicelluloses are predominantly degraded, as observed in the case of *P. ostreatus* HAI592.

It is well known that different chemical methods used to process lignocellulose promote the formation of various inhibitory compounds, which are the main obstacle in subsequent fermentation with yeasts.[54] Thus, it is crucial to find the optimal pretreatment process not only to obtain the highest possible amount of fermentable sugars, but also to avoid the formation of inhibitors as much as possible. Therefore, the effectiveness of biological pretreatment was examined in comparison with that of chemical pretreatment as predominantly used for lignocellulosic feedstock processing. Earlier researches pointed out that chemical pretreatment of lignocellulose is more effective compared with biological pretreatment. This was also the case in our study, where hydrolysis yields in samples containing NaOH pretreated wheat straw were higher than those obtained for wheat straw pretreated with the most efficient *G. resinaceum*, of 70.2 and 51.1%, respectively. This is mainly based on the partial loss of holocellulose, which is consumed by fungi during their growth and is used as a carbon source.[55,56]

Even though carbohydrates loss is expected during pretreatment with white-rot fungi, the relative carbohydrate enrichment of the biomass, *i.e.* by comparing the carbohydrates:lignin ratio in pretreated wheat straw was relatively lower. Previous research also showed that the decrease in wheat straw lignin, as the result of fungal culturing, produced a low enhancement of the carbohydrate component by increasing the glucan ratio in the biomass.[44] The pretreatment of lignocellulosic biomass with white-rot fungi, such as *I. lacteus* and *Ceriporiopsis subvermispora*, have been previously studied.[57-59] As in the case of *G. resinaceum* in our study, an efficient enzymatic conversion of pretreated lignocellulosic material was achieved, but the increased conversion came at the expense of considerable loss of holocellulose during the pretreatment. Furthermore, a positive effect of delignification has not always been seen. These inequalities occurred either because of the weak delignification rate or as a result of substantial total solids loss.[59] Although higher coefficient of conversion was not achieved in biologically pretreated samples, the approach still remains justified, as the results of this study indicate that the crucial moment can be the period of fungal culturing in which the effect of a higher concentration of carbohydrates should be stopped after partial delignification, to avoid substantial sugar consumption by fungi.

Comparing with biologically pretreated wheat straw after chemical pretreatment with NaOH, a higher amount of fermentable sugars was released. Considerably higher amounts of glucose and cellobiose, and negligible presence of pentoses in the hydrolysate of alkali pretreated wheat straw can be explained by a selective removal of lignin from biomass.[60] The process was followed by the removal of acetyl and uronic acid groups on hemicelluloses and hydrolysis of ester linkages between xylan and hemicelluloses residues. All in all, this largely improved cellulose digestibility, exhibiting minor cellulose loss comparing with fungal pretreated samples.[60] Previous studies also showed that chemical pretreatment is more efficient than biological pretreatment due to the partial loss of carbohydrates, which are the carbon source for fungal growth.[56] Another impact of enzymatic hydrolysis of pretreated wheat straw was the accumulation of cellobiose. This was due to limitation in beta-glucosidase activity in the enzymatic solution applied for saccharification. However, the presence of cellobiose in the hydrolysate cannot be considered disadvantageous, because this disaccharide can easily be converted to glucose either enzymatic or by using engineered yeast strains.[53]

CONCLUSION

The results of this study show the considerable potential of white-rot fungi in pretreating lignocellulosic biomass for efficient production of fermentable sugars and further industrial scale processing to ethanol. Different fungal species have various abilities to degrade lignin in wheat straw and this process is characterized by high laccase activity in the initial stages of fungal culturing. Increasing of commercial cellulase concentration to some extent enhances cellulose convertibility, reaching the plateau of efficiency at an optimal concentration. Mechanical preparation of the solid fraction is also significant, resulting in higher convertibility of smaller particle size. A key mechanism that enhances the conversion of carbohydrates is a selective lignin removal from the biomass. Among the tested species, only *Ganoderma resinaceum* deserves to be considered as a promising lignin degrader, with the total sugar yield increasing to for 21.7%. After biological pretreatment, a significant residual amount of hemicelluloses remains in the solid fraction, while the pretreatment with NaOH is strictly selective to lignin and hemicellulose removal. Long operating time, low sugar yields because of continual holocellulose consumption by fungi, and unpredictable fungal response to culturing conditions are the main challenges of the fungal pretreatment process in the conversion of lignocellulosic biomass. Future studies should be based on more selective fungal strains, including strategies that avoid holocellulose loss.

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