

Efficient biomass Pretreatment using the White-rot Fungus *Polyporus brumalis*

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Abstract

Implementation of cheap and eco-friendly biomass pretreatment processes is necessary to develop sustainable biorefineries. In nature, white-rot basidiomycetes are able to degrade lignin efficiently and selectively and are thus of great interest in such bioprocesses. In this study, five basidiomycetes strains were evaluated for their ability to pretreat wheat straw under solid state fermentation. Fungal pretreatments were carried out in glass-column reactors under operating conditions approaching industrial practices. The pretreatment efficiency was evaluated through the quantification of dry weight losses and subsequent hydrolysis of the carbohydrate fraction by enzymatic cocktails. The highest lignin to cellulose losses ratio was obtained using a strain of *Polyporus brumalis* which exhibited high ligninolytic capabilities. This selectivity along with the low dry weight loss makes the pretreatment profitable by enhancing cellulose and hemicellulose conversion yields. Therefore *P. brumalis* can be viewed as a promising strain to pretreat lignocellulosic biomass for biorefinery applications.

Keywords: Fungal pretreatment; *Polyporus brumalis*; White rot fungi; Wheat straw; Solid State Fermentation (SSF)

Abbreviations AVI: Avicel; Bir_X: Birch xylan; CMC: Carboxymethyl cellulose; dm: Dry matter basis; FPU: Filter paper unit; GAL: Galactomannane; Man: Mannane; MiP: Manganese-independent peroxidase; MnP: Manganese peroxidase; SSF: Solid state fermentation; WHE_XI: Wheat xylan insoluble; WHE_X: Wheat xylan; WS: Wheat straw

Introduction

To anticipate the inevitable depletion of petroleum-based fuels and contribute to sustainable development, interest in producing renewable energy and chemicals is increasing [1]. Lignocellulosic biomass is known as an abundant, low cost and widely available feedstock. It consists of a complex biopolymer of cellulose and hemicelluloses embedded in a matrix of lignin. It has been considered an attractive carbohydrate source for green chemistry applications, such as bioenergies. However, recovery of sugars from holocellulose (cellulose and hemicelluloses) is limited by the recalcitrant structure of lignin. As a consequence, the first step common to any green process is the pretreatment of the feedstock to disrupt the lignocellulosic network [2]. Pretreatment can be mechanical, physico-chemical, biological or a combination of these. Since a decade, biological pretreatments have attracted more attention as they offer an environmental-friendly alternative to current industrially used physico-chemical processes. White rot fungi, mainly basidiomycetes, are widely studied because they are the only ones capable of mineralizing lignin efficiently [3].

Several studies involving lignocellulose decaying fungi pretreatment on various feedstocks have been reported [4-7]. Among them, the selective lignin degrading fungi exhibiting the ability to remove lignin with minimum loss of carbohydrates are of great interest. This selectivity varies among fungal species, with feedstock nature and with culture conditions. In this frame, 63 fungal strains were previously

screened on solid-state fermentation (SSF) using a new multi-well plates method to select the most efficient candidates to pretreat wheat straw [8]. Among them, five strains were selected to be studied in a scaled-up process.

In this study, wheat straw was pretreated by *Trametes ljubarskii*_BRFM957, *Polyporus brumalis*_BRFM985, *Leiotrametes sp.*_BRFM1048, *Trametes menziesii*_BRFM1369 or *Trametes pavonia*_BRFM1554 for 21 days on SSF. To better control the culture parameters, 250 ml glass column systems were used to perform the fungal wheat straw pretreatment. Quantification of fungal biomass, enzymatic activity profile, dry weight loss, and carbohydrates preservation were investigated after 21 days of culture. Furthermore, the effectiveness of the pretreatment was evaluated by enzymatic saccharification in terms of digestibility and net carbohydrate conversion yields.

Material and Methods

Fungal strains and substrates

The five strains of basidiomycetes fungi used in the present study were obtained from the "Centre International de Ressources Microbiennes", fungal collection dedicated to filamentous fungi of biotechnological interest (CIRM-CF; https://www6.inra.fr/cirm_eng/CIRM-CF) of National Institute of Agricultural Research (INRA), Marseille, France. All of them were white-rot fungi selected from a previous study [8]: *Trametes ljubarskii*_BRFM957, *Polyporus brumalis*_BRFM985, *Leiotrametes sp.*_BRFM1048, *Trametes menziesii*_BRFM1369 and *Trametes pavonia*_BRFM1554. The fungi were maintained on 2% malt extract, 2% agar (BD Difco, France) slants at 4°C. Naturally dried wheat straw (Haussmann soft wheat) was obtained from Vivescia (Reims, France) and chopped (\approx 4 mm, Cutting Mill SM 100, Retsch®, Germany).

Inocula preparation

Fungal strains were cultured for 7 days at 30 °C on 2% malt extract, 2% agar (BD Difco, France) plates. Five 5 mm-disks picked from the growth front of the plates were used to inoculate sterile Roux flasks containing 200 mL of medium (2% malt extract). Inoculated Roux flasks were maintained 10 days at 30 °C. Afterwards, the mycelia mats were collected on Miracloth (Calbiochem, USA) and blended with sterile deionized water at 9500 rpm for 60 s using an Ultraturrax blender. The fungal suspensions (12±1 mg (dm) mycelia/ml) were used as inocula for SSF experiments.

Solid state fermentation and fungal biomass quantification

Experiments were performed in glass columns (20 cm × 4 cm, Legallais, France) filled with wheat straw impregnated with nutrient solution and inoculated with fungal suspension. The SSF system was designed in previous work and renewed for this study [9]. Briefly, twenty grams of dry chopped wheat straw wetted with 30 mL of deionized water were sterilized at 110 °C for 30 min in autoclave bag and cooled at room temperature. Afterwards, 25 ml of sterile nutrient solution (20 g/L of glucose and 2 g/L of ammonium tartrate dibasic) and 10 mL of inoculum suspension obtained as described in the previous section was directly added to the bag containing wheat straw. After homogenization by manual blending, the content of the bag was aseptically emptied in the sterile glass column and incubated in a controlled-temperature water bath at 28 °C. For each column, the air stream was filtered (0.2 µm) and wetted through a washing flask containing sterile deionized water before being distributed at a 0.5 v.v⁻¹.m⁻¹ flow rate. Regulation was done using a needle valve and flow meter floating ball (R2-15-AA, Brooks). Non-inoculated wheat straw was incubated under the same conditions and referred to control. Assays were performed in triplicate.

Biopretreated and control wheat straw were collected after 21 days of culture. For each replicate, one piece of sample was harvested to be flash-frozen in liquid nitrogen and stored at -80 °C for quantification of fungal biomass by qPCR method [10]. The remaining sample from the three replicates were pooled and homogenized for further analysis.

Dry matter quantification and cell wall composition analysis

One gram (wet basis) sample from the pooled replicates was dried at 105 °C overnight to measure dry mass content and to estimate weight loss. The mean values (n = 3) are reported.

Cellulose, hemicellulose, and Klason lignin content of biopretreated and control wheat straw were determined in duplicate according to the NREL method [11].

Quantitative assays of ligninocellulolytic enzymes

Extracellular proteins were extracted from 2 g (dm) aliquots of pretreated straw with deionized water (5% w dm/v) for 1 h at 4 °C under stirring. The extracts were recovered by filtration through GF/F filters (Whatman) and were stored at 4 °C before analysis. Enzyme activities were measured in the water extracts and expressed in

international enzyme units per gram of dry pretreated wheat straw (U/g). As previously described by Zhou and co-workers [8], the laccase and peroxidase activities were determined using 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) and 2,6-dimethoxyphenol as substrates, respectively. Complex substrates were used to assay the glycosyl hydrolase activities as described by Couturier and co-workers [12]. All analyses were performed in triplicate.

Enzymatic hydrolysis of pretreated wheat straw

Mild alkali treatment and enzymatic hydrolysis were performed in situ with a Tornado™ Overhead Stirring System (Radleys Discovery Technologies, United Kingdom). 6 g (dm) of biopretreated or control wheat straw were subjected to alkali treatment with 0.1% sodium hydroxide at a 6% (w dm/v) consistency at 50 °C and 700 rpm for 1 h. pH was then adjusted to 4.8 by addition of citrate phosphate buffer (100 mM, pH 4.4) resulting in consistency decrease to 3% (w dm/v). The suspension was further supplemented with 12 FPU/g substrate (dm) of commercial cellulases GC220 from *Trichoderma reesei* (Genencor Danisco, NY, USA) and 60 U/g substrate (dm) of β-glucosidase from *Aspergillus niger* (Novozyme SP188). Tetracycline (150 mg/l) and cycloheximide (40 mg/l) were added to prevent any microbial contamination. The reaction was carried out at 50 °C and 500 rpm for 96 h. 1 ml of samples were taken from the reaction mixture at convenient time points (0, 2, 4, 24, 48, 72 and 96 h), centrifuged at 5000 rpm for 5 min and filtered. The released glucose and reducing sugars were respectively quantified using the Glucose RTU kit (Biomérieux, Marcy-l'Étoile, France) and the dinitrosalicylic acid method. The digestibility and net carbohydrate conversion yields were calculated according to the following equations:

$$\text{Digestibility (\%)} = \frac{\text{glucose or xylose (g) released /g pretreated WS}}{\text{glucose or xylose (g) contained /g pretreated WS}} \times 100$$

$$\text{Net carbohydrate conversion yield (\%)} = \frac{\text{glucose or xylose (g) released /g pretreated WS} \times (100 - \% \text{weight loss})}{\text{glucose or xylose (g) contained /g initial WS}}$$

Results and Discussion

Fungal growth on wheat straw

Wheat straw was biopretreated with the white rot fungi *T. ljubarskii*_BRFM957, *P. brumalis*_BRFM985, *Leiotrametes sp.*_BRFM1048, *T. menziesii*_BRFM1369 or *T. pavonia*_BRFM1554 in a controlled SSF system. During cultivation in 250-ml glass columns, the fungal growths were first evaluated by visual examination. White mycelial growths have been observed since the second day of incubation for all the studied fungi. Once the mycelium had fully colonized the substrate in one week, the fungi showed extensive growth with rather thick and dense mycelial biomass. After 21 days of cultivation, the fungal biomasses were estimated by qPCR and varied from 19.8 up to 73.1 mg per gram (dm) of wheat straw (Table 1).

Fungal Strains	Weight loss (%)	Fungal biomass (mg/g dm WS)	Component loss (%)			Selectivity
			Cellulose	Hemicelluloses	Lignin	
<i>P. brumalis</i> BRFM985	16.7 ± 1.1	49.5 ± 2.8	14.4 ± 0.5	25.7 ± 0.7	38.9 ± 0.4	2.71
<i>Leiotrametes</i> sp. BRFM1048	17.9 ± 2.4	48 ± 8.9	15.9 ± 1.0	18.8 ± 2.7	32.1 ± 4.2	2.02
<i>T. pavonia</i> BRFM1554	18.2 ± 0.9	19.8 ± 2.2	21.8 ± 0.9	21.0 ± 3.1	32.4 ± 1.7	1.48
<i>T. menziesii</i> BRFM1369	23.5 ± 2.4	63.5 ± 4.2	25 ± 0.6	25.4 ± 1.5	33.9 ± 1.6	1.36
<i>T. ljubarskii</i> BRFM957	31.3 ± 2.5	73.1 ± 5.6	38.6 ± 0.8	41.9 ± 0.5	50.4 ± 1.6	1.31

Table 1: Weight loss, fungal biomass, cell-wall component changes and selectivity in 21-days-old fungal pretreated wheat straw.

The glucose and tartrate diammonium supplements in low concentrations might have stimulated fungal growth by providing more easily assimilable nutrients than those provided by the polymers from wheat straw. Indeed, the degradation of lignocellulose requires the production of fungal cell-wall degrading enzymes. Hence, the rate and extent of this degradation depend on the diversity and activity levels of the secreted enzymes. The set of lignocellulolytic enzymes mainly consists of oxidative and hydrolytic activities acting synergistically on the different cell-wall polymers. The main cellulose-, hemicellulose-, and lignin-degrading enzyme activities in the water-soluble extracts from each 21-days-old culture were quantified on model substrates (Table 2). Despite the fact that the pattern and the levels of cell wall degrading enzyme activities have been reported to change during fungal growth [13,14], this snapshot reflecting lignocellulolytic activities provides insight into the mechanism involved in the breakdown of wheat straw.

Cellulose degrading activities were estimated by the quantification of carboxy-methyl-cellulase and avicel-cellulase activities. All the fungi showed carboxy-methyl-cellulase activities with low or absence of avicel-cellulase activities. Among hemicellulases, the xylanase activities on soluble xylan are much higher than mannanase and galactomannanase ones which reflect the typical abundance and chemical composition of wheat straw hemicelluloses. Indeed, wheat straw hemicelluloses consist mainly of arabinoxylans substituted by α -L-arabinofuranose, 4-O-methylglucuronic acid and acetyl groups [15]. It contains also small amounts of other constituents such as arabinan, mannan and galactan.

With regard to ligninolytic activities, most of the studied fungi were capable of producing the major ones, i.e., laccase and peroxidases. The former being generally produced in relatively higher levels than laccase. In the case of *T. pavonia*_BRFM1554, no peroxidase activities were detected in 21-days-old pretreated wheat straw but only laccase, as already reported by Saparrat and co-workers [16]. In studies with different white-rot fungi, MnP activities were predominant [6,17] and there was a general increase along the incubation period. Our results also showed a general predominance of this enzymatic activity among

ligninolytic ones. It is known that the presence of metal ions enhances metalloenzyme activities, and wheat straw is naturally rich in manganese that could promote this enzymatic activity [14, 17].

Cell-wall component and dry matter losses from pretreated wheat straw

The component and dry weight losses from wheat straw pretreated with white rot fungi are shown in Table 1.

The studied fungi grew on wheat straw by attacking lignin and holocellulose whose degradation was balanced between cellulose and hemicelluloses. Cellulose, hemicelluloses and lignin losses ranged from 14-39%, 19-42% and 32-50%, respectively. White rot fungi with high selectivity for lignin degradation with minimal holocellulose loss are essential for a successful pretreatment. The selectivity of a fungal pretreatment, defined as the lignin to cellulose losses ratio, is commonly used to evaluate the selective lignin-degrading ability in defined culture conditions [18,19]. The higher the selectivity value is, the more effective the process is. As shown in Table 1, values ranged from 1.31 to 2.71 indicating that all the studied fungi had selective lignin-degrading ability. The wide range of selectivity values obtained within the *Trametes* genus pointed out the strain specificity at the specie level as reported in the literature [7]. The lowest selective strain, namely *T. ljubarskii*_BRFM957, differed from the others in the extent of cellulose, hemicellulose and lignin degradation exhibiting the highest loss values (39%, 42% and 50% respectively). This is consistent with both glycosyl hydrolase and ligninolytic activities which were the highest ones.

*P. brumalis*_BRFM985 had the greatest selectivity with high lignin loss of 39% accompanied with the lowest cellulose loss of 14% which can also be related to its enzymatic profile (Table 1). Furthermore, the enzyme production when expressed per g of dry fungal biomass stressed *P. brumalis*_BRFM985 as the best-adapted fungus based on its potential to produce high ligninolytic activities along with low glycosyl hydrolase ones (Table 2).

	(U/g dm wheat straw)						(U/g dm fungal biomass)					
		<i>P. brumalis</i> BRFM985	<i>Leiotrametes</i> sp. BRFM1048	<i>T. pavonia</i> BRFM1554	<i>T. menziesii</i> BRFM1369	<i>T. ljubarskii</i> BRFM957	<i>P. brumalis</i> BRFM985	<i>Leiotrametes</i> sp. BRFM1048	<i>T. pavonia</i> BRFM1554	<i>T. menziesii</i> BRFM1369	<i>T. ljubarskii</i> BRFM957	
GlycosylHydrolases (GH)	Cellulases	CMC	0.17	1.11	0.55	1.58	1.15	3	23	28	25	16
		AVI	0	0.39	0.42	0	0	0	8	21	0	0
	Hemicellulases	BIR_X	1.06	1.25	0.93	1.52	3.2	21	26	47	24	44
		WHE_X	1.88	1.88	1.12	3.6	4.18	38	39	56	57	57
		WHE_XI	0.08	0.62	0.55	0	0.58	2	13	28	0	8
		MAN	0.09	0.43	0.54	0	3.67	2	9	27	0	50
		GAL	0.18	0.39	0.67	0	6.33	4	8	34	0	87
Auxiliary Activities (AA)	Laccase	0.6	0.11	0.23	0	0.09	12.2	2.2	11.5	0	1.2	
	MiP	0.4	0.12	0	0.07	0.91	8	2.5	0	1.1	12.4	
	MnP	1.34	0.28	0	0.11	0.61	27.1	5.8	0	1.7	8.3	

Table 2: Cell wall degrading enzyme activities determined in 21-days-old fungal pretreated wheat straw.

Besides high selectivity, dry weight loss is a critical criterion for a profitable pretreatment (Table 1). It should be noted that the weight loss of control wheat straw was around 3.5%. This value agreed with the one reported by Wan and Li [20]. As suggested by Pensupa and co-workers [21], sterilization through autoclaving of the substrate can act as a mild hydrothermal pretreatment resulting in auto-hydrolysis of biomass. Almost all the fungal pretreatments resulted in moderate dry weight losses ranging from 17 to 24%. The sole exception is the pretreatment with *T. ljubarskii*_BRFM957 which led to 31% of dry weight loss. As this pretreatment is associated with the highest produced fungal biomass, one may wonder if weight loss could be related to fungal biomass production (Table 1). Indeed, regardless of the fungal strain, weight losses were correlated with the amounts of produced fungal biomasses ($r = 0.74$). The extended degradation of lignocellulose associated with a high dry weight loss obtained with *T. ljubarskii*_BRFM957 suggests a pretreatment time too long with the conditions of culture used. In contrast, for the other strains, 21 days of pretreatment seems to be appropriate to achieve significant reduction of lignin content over carbohydrates with moderate weight losses.

Enzymatic saccharification of fungal pretreated wheat straw

To evaluate the potential of fungal pretreatment of wheat straw for green chemistry applications in terms of cellulose and hemicellulose accessibility improvement, enzymatic hydrolysis of the carbohydrate fractions was investigated. It was performed with the commercial cellulases products GC220 and SP188 after a mild alkaline treatment. This mild alkaline step was expected to remove fungal hyphae from lignocellulosic material surface [6,22,23]. This step was soft enough to avoid any release of carbohydrates from pretreated wheat straw (data not shown).

The cellulose and hemicellulose digestibility of fungal pretreated wheat straw varied from 30% to 54% and from 31% to 50% respectively (Figure 1).

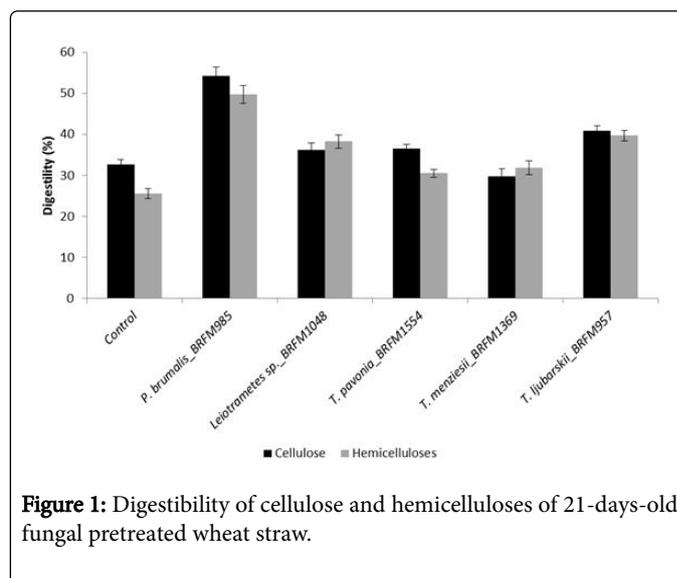


Figure 1: Digestibility of cellulose and hemicelluloses of 21-days-old fungal pretreated wheat straw.

As compared to the control, *P. brumalis*_BRFM985 and *T. ljubarskii*_BRFM957 stood out due to their ability to increase both the digestibility of cellulose (54% and 41%, respectively vs 33%) and hemicelluloses (50% and 40%, respectively vs 26%). To a lesser extent, *Leiotrametes* sp._BRFM1048, *T. pavonia*_BRFM1048 and *T. menziesii*_BRFM1369 were also able to increase the digestibility of hemicelluloses (38%, 32% and 31%, respectively). Despite the fact that some fungal strains improved the digestibility of carbohydrates, the effectiveness of the whole process required to be evaluated by net carbohydrate conversion yields. While digestibility is calculated on the basis of the remaining sugars after fungal pretreatment, which differ from one sample to another; net carbohydrate conversion yields are calculated on the basis of the initial ones (maximal theoretical

releasable sugars). Hence, the extents of the enzymatic saccharification of the fungal pretreated wheat straw can be compared to each other and give an overall view of the pretreatment performances. The net cellulose and hemicellulose conversion yields varied from 23% to 47% and from 23% to 37% respectively (Figure 2). Due to their losses in dry matter (Table 1), four fungal strains over the five studied were no longer efficient to improve both net carbohydrate conversion yields (Figure 2).

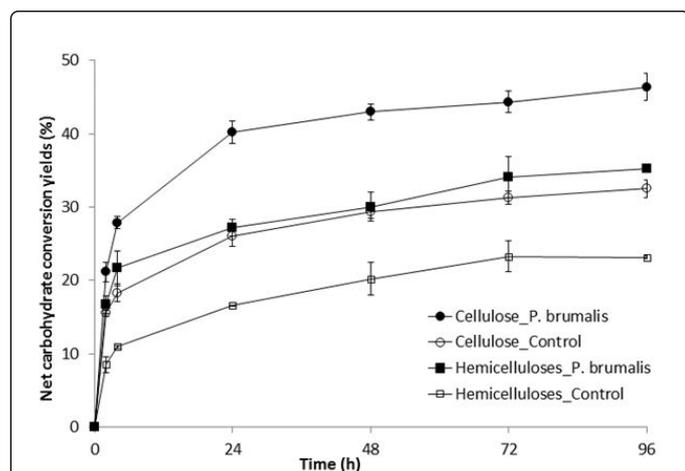


Figure 3: Time course of net carbohydrate conversion yields of 21-days-old *P. brumalis* pretreated wheat straw.

It is well illustrated by *T. ljubarskii*_BRFM957 pretreatment with 31% of dry weight loss which induced 41 and 40% of cellulose and hemicellulose digestibility, respectively, against 25 and 23% of net cellulose and hemicellulose conversion yields, respectively. It is well known that cellulose hydrolysis seemed tightly linked to hemicellulose and lignin degradation. The presence of hemicelluloses and lignin reduces the accessibility of hydrolytic enzymes to cellulose [2,24]. For green chemistry applications, it is better to preserve both carbohydrate fractions that could be hydrolysed by adapted micro-organisms or by designed enzymatic cocktails. The main remaining bottleneck is the presence of lignin. In terms of fungal pretreatment impact on enzymatic hydrolysis, more than lignin loss, the lignin to cellulose ratio and the selectivity (lignin/cellulose losses ratio) are well-adapted parameters to take into consideration for digestibility and net conversion yields, respectively. A correlation analyse was carried out and showed, as expected, cellulose and hemicellulose digestibilities were highly and negatively correlated to the lignin to cellulose ratio ($r = -0.90$ and -0.99 , respectively); while net cellulose and hemicellulose conversion yields were highly and positively correlated to the selectivity ($r = 0.94$ and 0.99 , respectively).

*P. brumalis*_BRFM985, the sole effective fungus for enhancing enzymatic hydrolysis performance, was the most selective fungus (2.7) in this study and led to 47% and 37% of net cellulose and hemicellulose conversion yields, respectively (Figure 2). The time course of net carbohydrate conversion yields from control or wheat straw pretreated with *P. brumalis*_BRFM985 during a 96 h enzymatic hydrolysis is shown on Figure 3. The shape of the kinetics was the same regardless of the sample or the carbohydrate fraction. A rapid initial increase of net carbohydrate conversion yields up to 4 h was observed, followed by rate decrease and a plateau. Nevertheless, the initial rates and the final

yields of both carbohydrate fractions were higher for fungal pre-treated samples than control ones.

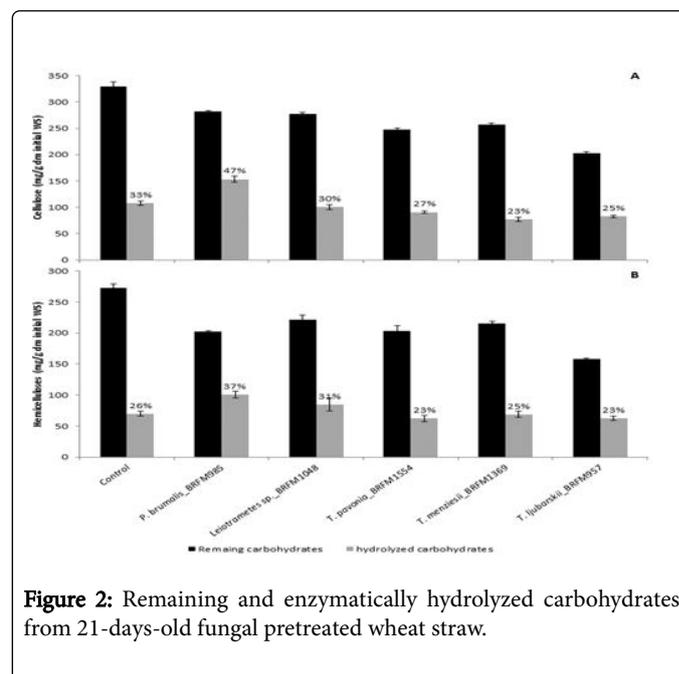


Figure 2: Remaining and enzymatically hydrolyzed carbohydrates from 21-days-old fungal pretreated wheat straw.

Biological pretreatment of lignocellulosic biomasses with white rot fungi have been more extensively studied in the last decade and was reviewed by Wan and Li [7] and more recently by Moreno and co-workers [25] highlighting the wide variety of strains, biomasses and experimental conditions studied. Some strains stood out such as *Irpex lacteus* [6, 17] or *Ceriporiopsis subvermispora* [20,26] which have been widely studied. From this literature, the outstanding highlight is the discrepancy in fungal pretreatment efficiency from one work to another. Indeed, differences in (i) fungal species or even strains (ii) fungal treatment conditions, (iii) post treatments, (iv) experimental parameters for the hydrolysis step as well as in (v) enzyme diversity and ratio in the enzymatic cocktails made hard the comparison of hydrolysis yields from the various studies [19]. As a consequence and despite the genera *Trametes* and *Polyporus* have already been studied for fungal pretreatment of various biomasses [5,7,18,27-29] it is hard to compare our data with the literature. Nevertheless, it is worth to note, to our knowledge, *P. brumalis* was for the first time shown as a promising fungus to pretreat wheat straw for green chemistry applications.

Conclusion

Build-up of successful biotechnological process for a green chemistry application consists of implementing successive steps, from down-scale to fully-industrial process, to select a well-adapted strain/substrate/process trio. With the present process conditions and among the five fungal strains previously preselected at smaller scale, *P. brumalis*_BRFM985 was proven to be the best adapted strain to pretreat wheat straw in a controlled SSF system. The relevant critical criteria to do such a selection: mass losses, selectivity and net hydrolysed carbohydrate yield, should be jointly improved to determine optimal windows of operating parameters such as temperature, duration and humidity.

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