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ORIGINAL RESEARCH PAPER

# Combining analytical approaches for better lignocellulosic biomass degradation: a way of improving fungal enzymatic cocktails?

**Roxane Raulo · Egon Heuson · Renato Froidevaux · Vincent Phalip**

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**Abstract**
*Purpose* In this study, a combinatory approach was undertaken to assay the efficiency of fungal enzymatic cocktails from different fermentation conditions to degrade different lignocellulosic biomasses with the aim of finely characterizing fungal enzymatic cocktails.
*Methods* Enzymatic assays (AZO and pNP-linked substrates and ABTS) were used to assess the composition of the fungal enzymatic cocktails for cellulase, xylanase and laccase activities. Comparisons were made with a new range of chromogenic substrates based on complex biomass (CBS substrates). The saccharification efficiency of the cocktails was evaluated as a quantification of the sugar monomers released from the different biomasses after incubation with the enzymatic cocktails.
*Results* The results obtained showed striking differences between the AZO and pNP-linked substrates and the CBS substrates for the same enzymatic cocktails. On AZO and pNP-linked substrates, different hydrolysis profiles were observed between the different fungi species with *Aspergillus oryzae* being the most efficient. However, the results on CBS substrates were more contrasted depending on the biomass tested. Altogether, the results highlighted that assessing laccase activities and taking into account the complexity of the biomass to degrade were key in order to provide the best enzymatic cocktails.
*Conclusion* The complementary experiments performed in this study showed that different approaches needed to be taken in order to accurately assess the ability of an enzymatic cocktail to be efficient when it comes to lignocellulosic biomass degradation. The saccharification assay proved to be essential to validate the data obtained from both simple and complex substrates.

**Keywords** Enzyme · Lignocellulosic biomass · Fungal enzymatic cocktails · Lignin · Saccharification

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## Introduction

The European Commission has set a long-term goal to develop a competitive, resource efficient and low carbon economy by 2050. The transition toward bioeconomy will rely among other contributions on strong advancements in technology for an efficient degradation of lignocellulosic biomasses. This will include the improvement of a range of processes, the achievement of a breakthrough in terms of technical performances and an increase in cost effectiveness. It

will also depend on the availability of sustainable biomass. The use of biomasses hard to degrade such as wood or coming from polluted environments would also be an asset to a successful transition.

Lignocellulosic biomasses are particularly attractive as raw material since they allow the production of a large panel of chemical intermediates due to their complex composition (Jing et al. 2020; Lee et al. 2019). They are renewables and their production have been estimated at about 140 billion tons per year (Ulber and Sell 2007). The bio-based chemical intermediates or "synthons" concern a wide variety of industrial products, namely bioplastics (packaging), materials (biopolymers), fine chemicals, biopesticides, glues… It is from the use of these synthons that green chemistry really finds its field of application. The concept of biorefinery emerges from the use of renewable biomasses to produce such panel of chemicals and energy to maximize the plant material richness, particularly carbon (Menon and Rao 2012). The increase in value generated by this variety of applications still needs investments in research and processes to be done in order to replace their petrosourced counterparts.

Plant cell wall constitute the large majority of lignocellulosic materials: cellulose (35–50% dry weight), hemicelluloses (20–50% dry weight) and lignin (10–20% dry weight) (Dence 1992). Sustainable solutions for degrading these complex biomasses are based on chemical and biological processes such as microbial and/or enzymatic degradation with a predominance for the latter. However, the recalcitrance of lignocellulosic biomass to enzymatic degradation is an important barrier to large-scale production and commercialisation of biofuels and agro-chemicals derived from plant biomass. Plant cell wall is indeed complex and variable. A large diversity of enzymes is needed: cellulases, pectinases, hemicellulases, and lignin-degrading enzymes, acting in endo, exo-modes, desubstituting and debranching ones.

A recent review on biorefinery stated that the "enzymatic hydrolysis represents the second main operational cost, after the biomass production; in 2nd generation (2G) it is ∼ 25–30% of the operational costs, whereas in 1st generation (1G) it is below 3%" (Álvarez et al. 2016). Such a difference comes from the higher level of requirement in enzymatic cocktails needed for 2G (lignocellulosic biomass) production and a solution could come from the improvement of these cocktails. Several possible options exist to overcome this issue, (i) reduce the enzyme loading by improving the enzyme activity through genetic engineering using high-performance mutagenesis strategies coupled with massive tracking systems, (ii) reduce the cost of protein through better production methodologies and (iii) increase the overall hydrolysis yield by adapting the enzyme cocktail performance to the process conditions and the targeted feedstock. Improving the efficiency of enzymatic saccharification has been an active area of research during the last decade, with efforts dedicated towards the discovery and characterization of novel saccharolytic enzymes (Banerjee et al. 2010; Phalip et al. 2005), understanding mechanistic and structural aspects of enzymes catalysis (Carapito et al. 2009; Van Dyk and Pletschke 2012), and developing formulation strategies to maximize monomers release (Carapito et al. 2009; Meyer et al. 2009).

However, this research effort towards better enzymatic degradation has not yet trickled down to industry at a large scale, and one of the main reasons for this is the high cost of purified enzymes. The most promising methods mimic natural processes using the more diverse enzymatic cocktails that are produced by microorganisms, especially fungi. The widely used fungus *Trichoderma reesei*, for example, is known to produce substantial amounts of cellulases (essentially cellobiohydrolases) but is relatively poor in the other enzymes required for biomass bioconversion (Martinez et al. 2008). Thus, other fungi have been investigated for their ability to produce more diverse and efficient cocktails or to complement *T. reesei* enzymes (Gottschalk et al. 2010) with a limited number of resulting enzymatic cocktails available on the market to date.

However, profitable biomass conversion processes are highly dependent on the use of efficient enzymes for lignocellulose degradation; on the cost of producing these enzymes and is highly variable depending on the biomass used. Álvarez et al. (2016) tried to valorise poplar residues with a mixture of cellulases and hemicellulases coming from commercial cocktails. The authors described a 100% conversion rate from 2% wood material (w/v, lab scale) but the yield decreased to 84% with 20% (w/v) of biomass. Similarly, Gonçalves et al. (2015) complemented a commercial cocktail obtained from *Trichoderma* species by other enzymes and got twice more reducing

sugars after digestion using this complemented mixture. Indeed, it has been described by stakeholders that the available cocktails were not always sufficient to degrade their substrate of interest and it is widely known that the composition of the enzymatic cocktail secreted by *T. reesei* is not optimal for the industrial degradation of cellulosic biomass in terms of cellulase activity (Pryor and Nahar 2015). Many publications report the efficient and quantitative recovery of sugars, the most frequently by using microbial enzymes. Nevertheless, in most of the cases, these publications describe years of work on a single given biomass in laboratory and small-scale conditions.

In this study, a combinatory approach was undertaken to assay the efficiency of fungal enzymatic cocktails from different fermentation conditions. Enzymatic assays (AZO and pNP-linked substrates) were used to assess the composition of the fungal enzymatic cocktails for both cellulase and xylanase activities, while the ABTS assay was used to quantify oxidase activities that are important for lignin degradation. As a complementary assay, the saccharification efficiency of the cocktails was evaluated as a quantification of the sugar monomers released from the different biomasses after incubation with the enzymatic cocktails (Fig. 1). The complementary experiments performed in this study showed that different approaches needed to be taken in order to accurately assess the ability of an enzymatic cocktail to be efficient when it comes to lignocellulosic biomass degradation.

## Material and methods

### Strains and growth conditions on solid media

The *Aspergillus niger* (MUCL 745), *Trichoderma harzianum* (MUCL 29797) and *Trametes versicolor* (MUCL 1011) strains used in this work were ordered from the Belgian Co-ordinated Collection of Microorganisms (BCCM). To obtain spores, *A. niger*, *T. harzianum* and *T. versicolor* were cultured on potato dextrose agar (PDA) (Oxoid) plates for 5 to 10 days at 25 °C.

These strains were chosen for their abilities to produce predominantly different types of enzymes: *A. oryzae* for hemicellulases, *T. harzianum* for cellulases and *T. versicolor* for laccases. Wheat straw was chosen as a biomass most likely to suit *A. oryzae* regarding its hemicellulose content while corn stover that is richer in cellulose may be a better substrate for *T. harzianum*. Finally, pine, which is known to be recalcitrant to degradation because of high lignin content seemed a good substrate for *T. versicolor*. Additionally, the biomasses were chosen according to the CBS substrates available at Glycospot so that the enzymes produced on the biomasses could be related to the activities measured on the CBS plates. As no pine needle CBS plates were available, the study was performed using another resinous tree, i.e., spruce.

### Preparation of M3 minimal medium and cultivation conditions

M3 minimal media composition was based on the minimal medium described by Mitchell et al. (1997). For cultures of *A. niger*, *T. harzianum* and *T. versicolor* in the Biolector® (m2p labs, Germany), spores were washed with 0.01% (v/v) Tween-80 solution and $5 \times 10^5$ spores per mL in suspension were inoculated into each well of the FlowerPlate® (m2p-labs, Germany) filled with 1.4 mL M3 medium (Mitchell et al. 1997) containing 1% (w/v) glucose or 1% unpretreated milled biomass (Wheat Straw (WS), Corn Stover (CS) or Pine Needle (PN)) (w/v, 0.25–1 mm particles size). The plate was incubated for 7 days at 25 °C at 800 rpm in a BioLector® device (m2p-labs, Germany) for submerged fermentation (i.e., suspension with biomass particles). Supernatants were collected at day 7 after inoculation for enzymatic analysis by collecting the full content of the well. Media were sterilized prior experiments at 117 °C for 30 min instead of 121 °C to limit the caramelising of sugars. All experiments were conducted in biological triplicates.

### Enzymatic assays

The capability of the strains used in this study to degrade the different biomasses was assayed using a microbioreactor for the implementation of high-throughput filamentous fungi cultures on complex biomasses followed by the high-throughput screening of enzyme activities. Culture supernatants collected from BioLector® (m2p-labs, Germany) wells were assayed using AZO-dyed and pNP substrates as well as a new generation of versatile chromogenic

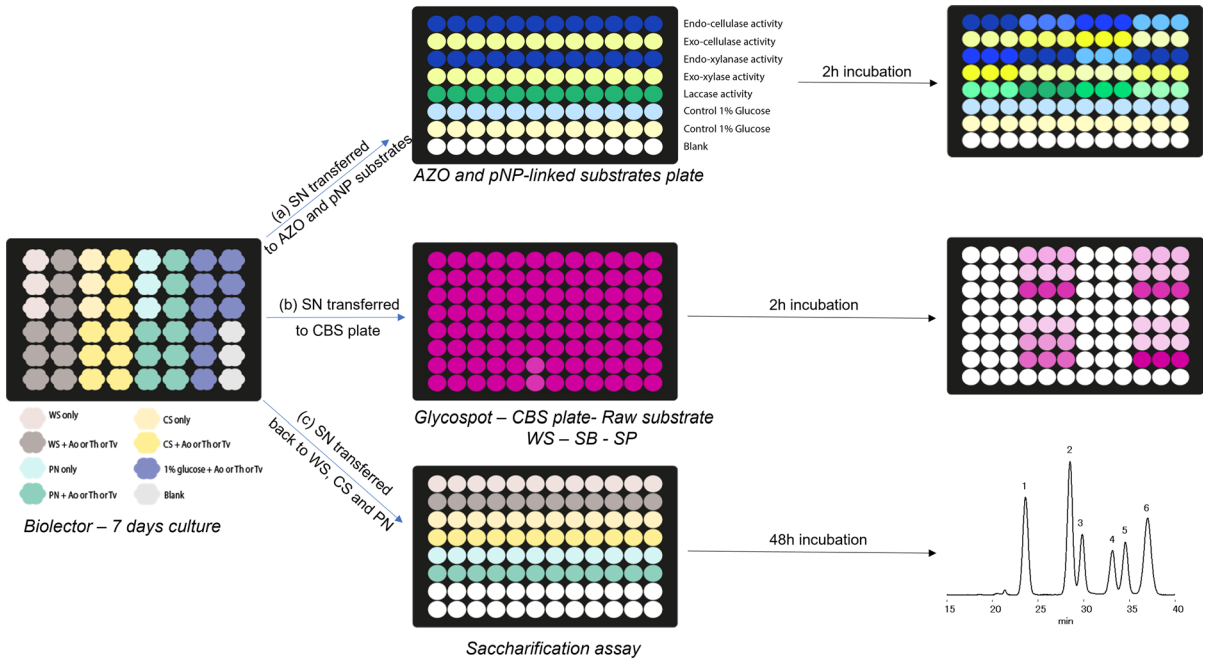



**Fig. 1** Workflow of the experimental set-up performed throughout the study. Enzymatic cocktails were produced on four different carbon sources: wheat straw (WS), corn stover (CS), pine needles (PN) and 1% glucose (w/v) in the BioLector®. The resulting supernatants (SN) were tested for enzyme activities on (a) AZO and pNP-linked substrates, (b) CBS substrates and (c) for their ability to release sugar monomers from their interactions with the biomasses

substrates (Kračun et al. 2015) (GlycoSpot IVS, Farum, Denmark).

AZO-xylan and AZO-cellulose were used to assay endo xylanases and cellulases activities (Megazyme, Ireland) while two pNP-linked substrates: 4-Nitrophenyl-β-d-glucopyranoside (pNP-β-Glc) and 4-Nitrophenyl-β-d-xylopyranoside (pNP-Xyl) (Sigma Aldrich, UK) were used to measure exo cellulases and xylanases activities. In a 96 U-well plate, 30 µL of the culture supernatants were assayed with 150 µL of 0.5% (w/v) final concentration of the substrate, 75 µL of 100 mM sodium acetate pH 5.0 in a total volume of 300 µL. The reaction mixtures were incubated at room temperature for 6 h with 5 min of initial shaking. The reactions were then stopped by adding 50 µL of the reaction mixture to 200 µL of ethanol (95% v/v) (AZO-dyed substrates) or trisodium phosphate pH 12 (2% w/v) (pNP-glucopyranoside and pNP-xylopyranoside). The remaining substrates were precipitated by centrifugation at 2120*g for 10 min. 100 µL of the supernatants were transferred to a flat-bottom 96-well reading plate and the optical densities were measured at 590 nm and 405 nm, AZO-substrates and pNP-substrates, respectively, with a plate reader (SpectraMax i3, Molecular Devices, USA).

For the assessment of the diversity and complexity of the enzymatic cocktails gathered from the interaction of the fungi and the biomasses, Complex Biomass Substrates (CBS®, GlycoSpot, Denmark) were used (Kračun et al. 2015). Briefly, three CBS were evaluated: CBS Wheat Straw (CBS-WS), CBS Sugarcane Bagasse (CBS-SB) and CBS Spruce (CBS-SP). The CBS substrates were used in a 96-well filter plate, where the solid CBS were activated by adding 200 µl of sterile water and incubating for 15 min. Then, the water was removed by centrifugation (2700*g, 10 min), and the plate washed again with water to remove free dye. The reaction mixture consisted of 150 µl of 100 mM sodium acetate buffer (pH 5.0) and 50 µl of each supernatant. Three biological replicates (wells) of each fungus – biomass culture was assayed. The plastic lid was put on top of the reaction plate and incubated for 2 h and 24 h at room temperature and 700 rpm on a Peltier shaker. At the given timepoint, the supernatants were transferred by centrifugation into the collection plate. The absorbances at 517 nm

(red) were determined using a plate reader (SpectraMax i3, Molecular Devices, USA). Positive controls for each substrate were also set up using commercial enzymes (Sigma). In addition, sterilized water was used as a negative control. Semi-quantitative data were obtained based on the absorbance values.

In order to measure oxidase activity, 2,2′-Azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) was used as the substrate for the oxidation reaction. The reaction mixture consisted of 50 µL of culture supernatant, 50 µL of ABTS (1 mg/ml) and 50 µL of 100 mM Acetate Buffer pH 5 in a final volume of 200 µL. The absorbances at 405 nm were determined using a plate reader (SpectraMax i3, Molecular Devices, USA).

Quantification of the total protein concentration was performed by the Bradford assay, 5 µL of supernatants were added to 195 µL of Bradford Reagent (Sigma). Absorbances were read at 595 nm and concentrations were calculated according to a calibration curve obtained from known concentration of Bovine Serum Albumin protein.

### Saccharification assay

The saccharification assays to quantify glucose, xylose, arabinose and galactose (the latter three are inducers of CAZymes) released from the hydrolysis of wheat straw, corn stover and spruce used to predict the flux of sugars in the 24 h cultures were performed in 2 ml 96-well plates in a total volume of 1 ml with 15 mg (w/v) of washed wheat straw, volumes of the concentrated 7-day culture filtrates and 100 mM sodium acetate buffer pH 5. Reactions were incubated for 48 h at 30 °C with moderate shaking. Samples were then collected, and the enzymes were inactivated by heating at 100 °C for 5 min followed by centrifugation to pellet the solids. The monosaccharides in the heat inactivated reactions were quantified with an Ultra-fast HPLC-RI.

### HPLC sugars detection

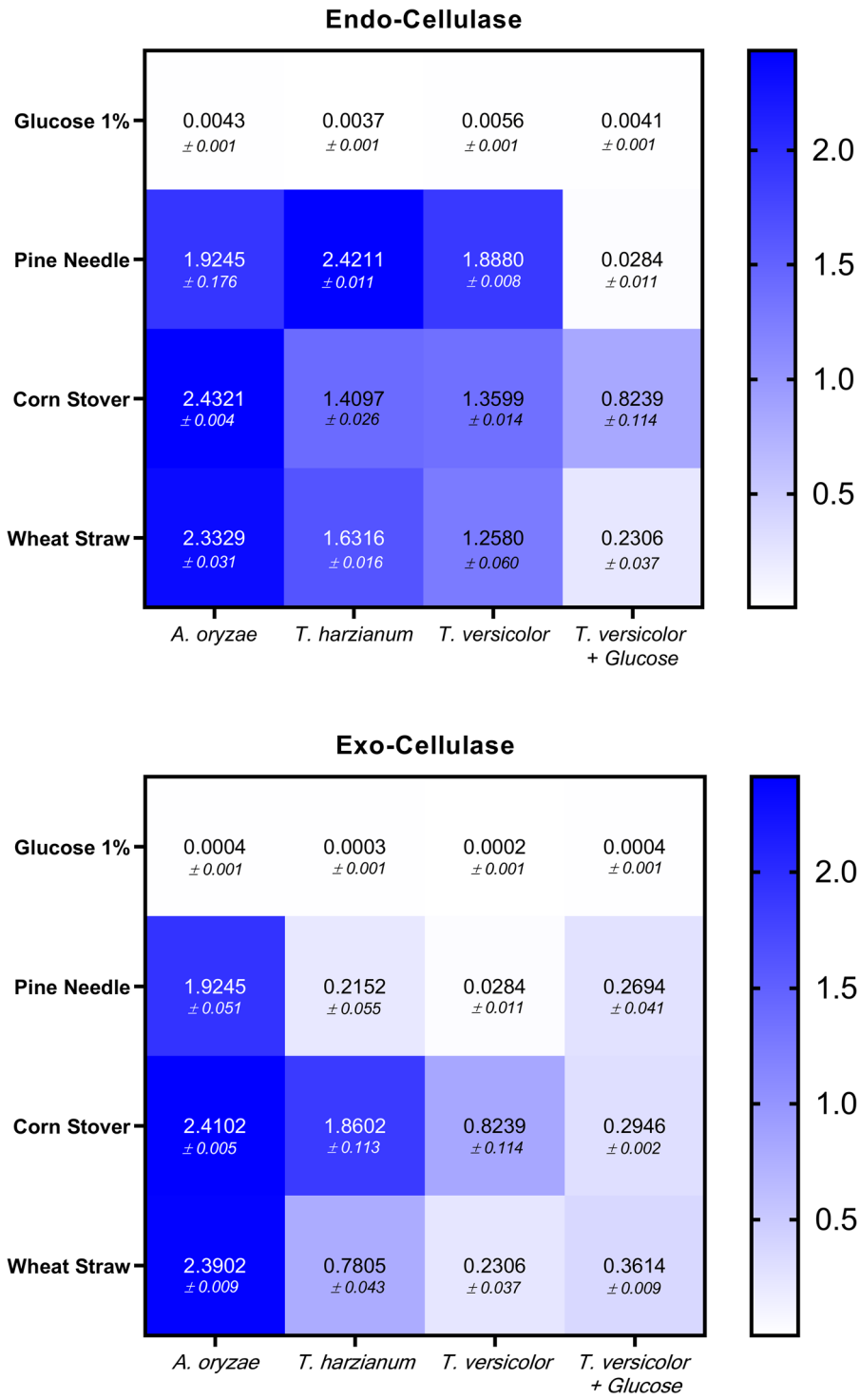
The saccharification samples analyses were performed on an Ultra-fast HPLC-RI (Shimadzu, Japan), using a Biorad Aminex (2.5 × 300 mm) cartridge column. Injection volumes of 10 µL were used for all the samples. The mobile phase consisted of 5 mM sulfuric acid. Elution was carried out at 0.4 mL.min$^{-1}$, with a 40 °C oven temperature for the column. The saccharification products were detected using the RI detector.

## Results and discussion

### Enzymatic activities on AZO and pNP substrates

Enzymatic activities were assayed after 7 days of cultures of *A. oryzae*, *T. harzianum* and *T. versicolor* as this time has been shown to be the best to assess for enzymatic activities after cultivation in the BioLector® (Raulo et al. 2019). For *T. versicolor*, a second culture was set up supplemented with 1% (w/v) glucose for laccase induction. AZO and pNP-linked substrates were used to assess endo- and exo-hydrolase activities, respectively. Regarding cellulases activities, an increase in enzymatic activities was observed between the supernatant collected on the different biomasses for all the fungal strains and compared to the glucose as sole carbon source (1% w/v) (Fig. 2). The supernatants collected from the culture of *T. versicolor* supplemented with 1% glucose (w/v) showed lower activities measurements for endo-cellulase activities suggesting that the addition of glucose led to Carbon Catabolic Repression (CCR) of the genes encoding cellulases in *T. versicolor* (Kubicek et al. 2009; de Vries and Visser 2001), while exo-cellulase activities were similar (wheat straw) or higher (pine needle) in the culture supplemented with glucose compared to *T. versicolor* only. *A. oryzae* had the highest enzymatic activities measured for both endo- and exo-cellulase activities while *T. harzianum* supernatants showed higher endo-cellulase activity, except for supernatants collected on corn stover.

Similarly, to cellulase activities, xylanase activities were measured using both AZO and pNP-linked substrates specific for these activities (Fig. 3). On wheat straw and corn stover, supernatants from *A. oryzae* displayed higher levels of endo-xylanase activities especially when grown of corn stover. Interestingly, on supernatants collected from pine needle cultures, none of the fungi seemed to produce endo-xylanases as the activities measured were strongly decreased compared to the values obtained for the wheat straw and corn stover cultures. In the *T. versicolor* culture supplemented with glucose, endo-xylanase activities were lower than for the culture




without glucose as already observed for endo-cellulase activities. The profiles obtained when measuring exo-xylanase activities were similar for all fungi and all biomasses except for *A. oryzae* on corn stover which showed a threefold increase on average compared to the other conditions tested. As for cellulase activities,

◀**Fig. 2** Cellulase activities measured using AZO-substrates and pNP-linked substrates after 7 days of cultures in the micro-bioreactor for *Aspergillus oryzae*, *Trichoderma harzianum* and *Trametes versicolor*. Each fungus was grown on three different lignocellulosic biomasses wheat straw, corn stover and pine needle as well as on 1% glucose (w/v) as sole carbon source. Endocellulase activites were measured on AZO-cellulose while exocellulase activities were measured using the pNP-glucopyranoside substrate. Results are displayed as absorbances ± the standard deviation around the mean of three biological triplicates

an increase in enzymatic activities was observed for all the supernatants collected on the different biomasses compared to the glucose as sole carbon source (1% w/v) suggesting that CCR is also active for gene encoding xylanases expression (Kubicek et al. 2009; de Vries and Visser 2001).

## ABTS assay for laccase activity

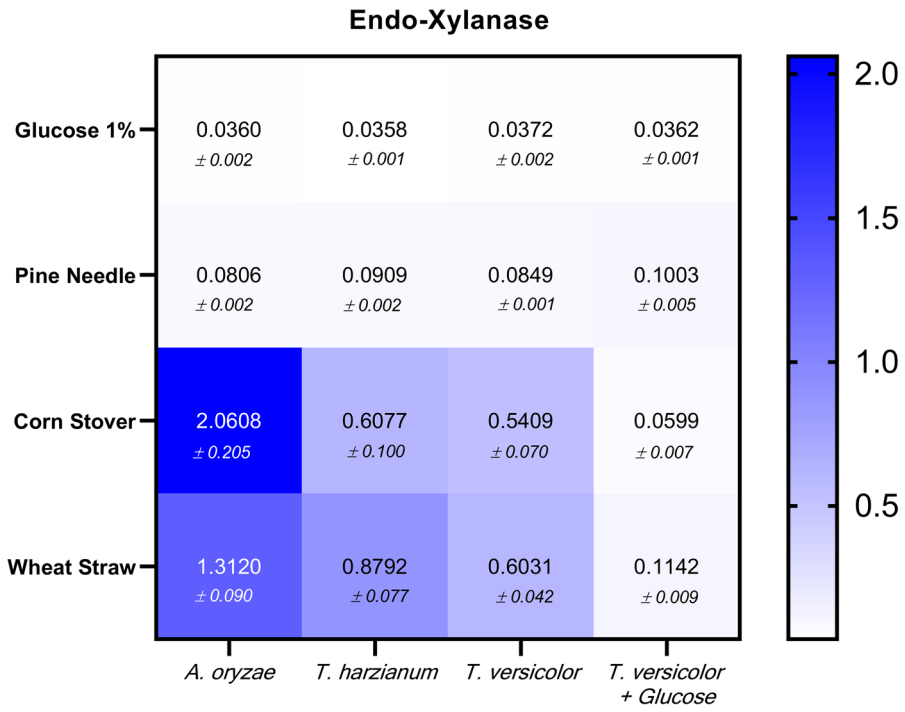
To test for oxidase activities, and especially laccases, ABTS was used as a substrate as its oxidation lead to the production of a green pigment for which absorbance can be assessed at 405 nm. *T. versicolor* was used as a model as many studies have reported that addition of glucose to the culture was inducing laccase production in this fungal species (Galhaup et al. 2002; Periasamy and Palvannan 2010). When looking at the data, overall, supernatants collected from *T. versicolor* cultures (with and without addition of glucose), displayed more laccase activities than the two other fungi especially on wheat straw and corn stover (Fig. 4). The addition of glucose to the culture led to a 4.7-fold and to a 3.2-fold increase on wheat straw and corn stover respectively between *T. versicolor* grown without glucose and with the addition of glucose to the culture. This is consistent with studies that have shown that the addition of 10 g/L of glucose to the fermentation was needed in order to induce the expression of genes encoding laccases in *T. versicolor* (Galhaup et al. 2002; Periasamy and Palvannan 2010). The supernatants collected from *A. oryzae* and *T. harzianum* cultures on the different biomasses did not show higher levels of oxidase activities than for *T. versicolor*. This is consistent with *A. oryzae* genome analysis which showed that this species only has two genes encoding laccases. The *A. oryzae* cultures showed a 12 and 14-fold decrease in the laccase activity measured on wheat straw and corn stover, respectively, compared to *T. versicolor* cultures supplemented with 1% glucose (w/v) grown on the same two biomasses. Adding glucose to the culture of *A. oryzae* and *T. harzianum* was also tested but did not lead to an increase in oxidase activities (data not shown).

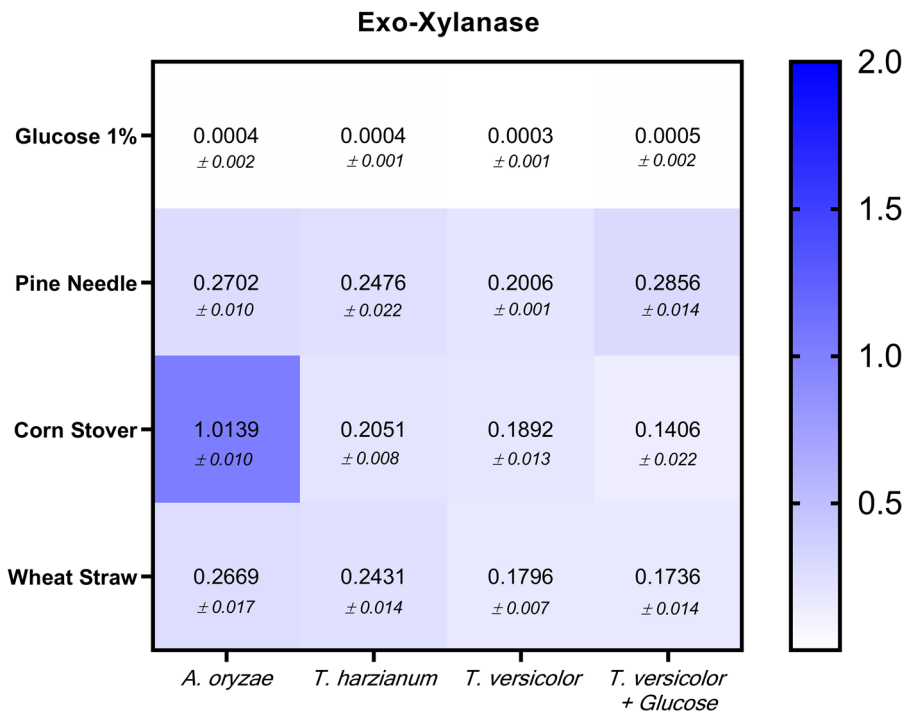
## Enzymatic activities on CBS complex substrates

In order to validate the activities measured on AZO and pNP-linked substrates, the same supernatants were tested on a new range of colorimetric substrates. CBS substrates address complex mixtures of cell wall components often encountered in industrial processes of biomass breakdown. These substrates are designed to evaluate polysaccharide availability for enzymes and provide information about how to effectively optimize degradation cocktails for more efficient lignocellulosic biomass hydrolysis.

When comparing the results obtained for enzymatic activities on the CBS substrates to the one gathered on the AZO and pNP-linked substrates, different profiles were observed for the three CBS substrates tested (Fig. 5). The enzymatic activities measured on CBS-WS were similar across all the conditions tested, ranging between 0.54 $OD_{405nm}$ for *T. harzianum* and 0.37 $OD_{405nm}$ for *T. versicolor* supplemented with glucose. This is in contrast with the results obtained on AZO-cellulose and pNP-glucopyranoside where the activities measured for *T. harzianum* were 7.08 time and 2.17 time higher than for *T. versicolor* with glucose, respectively on both substrates. Again, the supernatants of *A. oryzae* grown on 1% glucose (w/v) did not lead to an increase in the absorbances measured. The same results were obtained for *T. harzianum* grown on 1% glucose (w/v) (data not shown).

Regarding the efficiency of the enzymatic cocktails to degrade the CBS-SB substrate, a more similar profile for endo-hydrolase activities was obtained with *A. oryzae* performing the best and *T. versicolor* with glucose the least. This may be due to the composition of sugarcane bagasse compared to wheat straw with more easily accessible, though degradable, cellulose by the hydrolases in the supernatants which is consistent with the enzymatic arsenals of *A. oryzae* and *T. harzianum*. Cellulose accounts for 45% (dry weight) in sugarcane bagasse (Canilha et al. 2012)


Endo-Xylanase
Glucose 1%
Pine Needle
Corn Stover
Wheat Straw
0.0360 ± 0.002
0.0358 ± 0.001
0.0372 ± 0.002
0.0362 ± 0.001
0.0806 ± 0.002
0.0909 ± 0.002
0.0849 ± 0.001
0.1003 ± 0.005
2.0608 ± 0.205
0.6077 ± 0.100
0.5409 ± 0.070
0.0599 ± 0.007
1.3120 ± 0.090
0.8792 ± 0.077
0.6031 ± 0.042
0.1142 ± 0.009
A. oryzae
T. harzianum
T. versicolor
T. versicolor + Glucose
2.0
1.5
1.0
0.5


Exo-Xylanase
Glucose 1%
Pine Needle
Corn Stover
Wheat Straw
0.0004 ± 0.002
0.0004 ± 0.001
0.0003 ± 0.001
0.0005 ± 0.002
0.2702 ± 0.010
0.2476 ± 0.022
0.2006 ± 0.001
0.2856 ± 0.014
1.0139 ± 0.010
0.2051 ± 0.008
0.1892 ± 0.013
0.1406 ± 0.022
0.2669 ± 0.017
0.2431 ± 0.014
0.1796 ± 0.007
0.1736 ± 0.014
A. oryzae
T. harzianum
T. versicolor
T. versicolor + Glucose
2.0
1.5
1.0
0.5

◀**Fig. 3** Xylanase activities measured using AZO-substrates and pNP-linked substrates after 7 days of cultures in the microbioreactor for *Aspergillus oryzae*, *Trichoderma harzianum* and *Trametes versicolor*. Each fungus was grown on three different lignocellulosic biomasses wheat straw, corn stover and pine needle as well as on 1% glucose (w/v) as sole carbon source. Endoxylanase activites were measured on AZO-xylan while exoxylanase activities were measured using the pNP-xylopyranoside substrate. Results are displayed as absorbances ± the standard deviation around the mean of three biological replicates

while only 30.4% in wheat straw (Fang et al. 2011) and hemicelluloses for 25.8% and 21.3% respectively (Canilha et al. 2012; Fang et al. 2011).

Softwood like spruce is known to be especially recalcitrant to enzymatic hydrolysis, having even been called a "worst-case scenario" as a feedstock (Lu et al. 2002). The high lignin content (30% dry weight) (Pan et al. 2005) and its great degree of cross-linking (Chandra et al. 2007) are a major obstacle for the disintegration of the wood structure. In addition, lignin that had been isolated from pre-treated softwood was observed to exhibit stronger enzyme-lignin interactions and inhibitory effects than lignin from other sources (Nakagame et al. 2010). These observations are consistent with the results displayed in this study on spruce where little hydrolase activities were detected on CBS-SP.

CBS substrates are not showing activities like oxidases (i.e., laccases) and therefore still lack the potential to assay these activities and enzymatic cocktails for their abilities to degrade lignin-rich substrates.

## Free glucose and total protein concentrations

In addition to enzyme activities, residual glucose and total protein concentration were measured in all samples (Fig. 6a and b). No significant differences were observed in the total concentration of proteins in the different supernatants showing that the enzymatic activities measured were actually due to a specific enzyme activity rather than more proteins in the supernatants (Fig. 6a). Free glucose concentration was measured in the samples as a potential effect of CCR. In addition to induction by mono- and oligo-saccharides, genes encoding CWDEs involved in plant cell wall deconstruction in filamentous fungi can be repressed during growth in the presence of easily metabolised carbon sources, such as glucose. CCR is

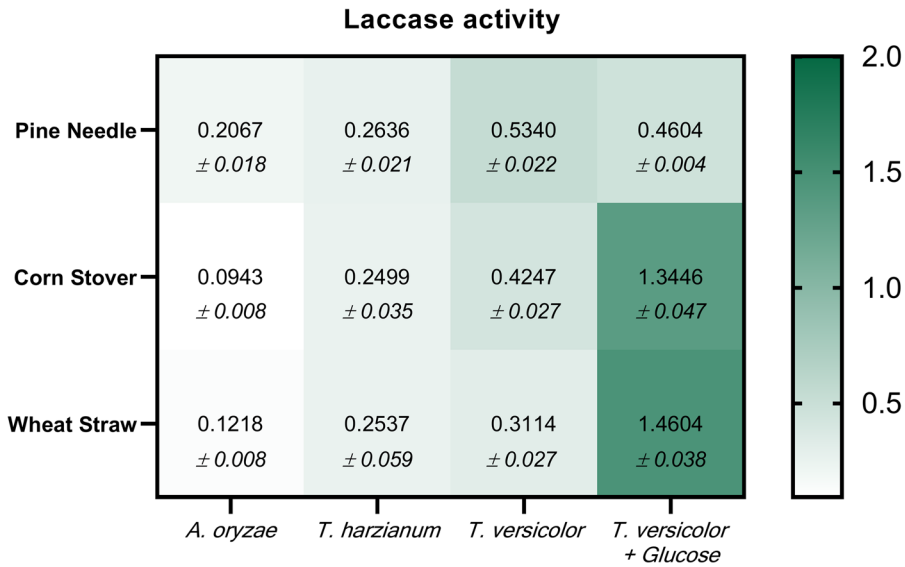



**Fig. 4** Oxidase activity measured using ABTS substrate after 7 days of cultures in the microbioreactor for *Aspergillus oryzae*, *Trichoderma harzianum* and *Trametes versicolor*. Each fungus was grown on three different lignocellulosic biomasses wheat straw, corn stover and pine needle. Results are displayed as absorbances ± the standard deviation around the mean of three biological replicates

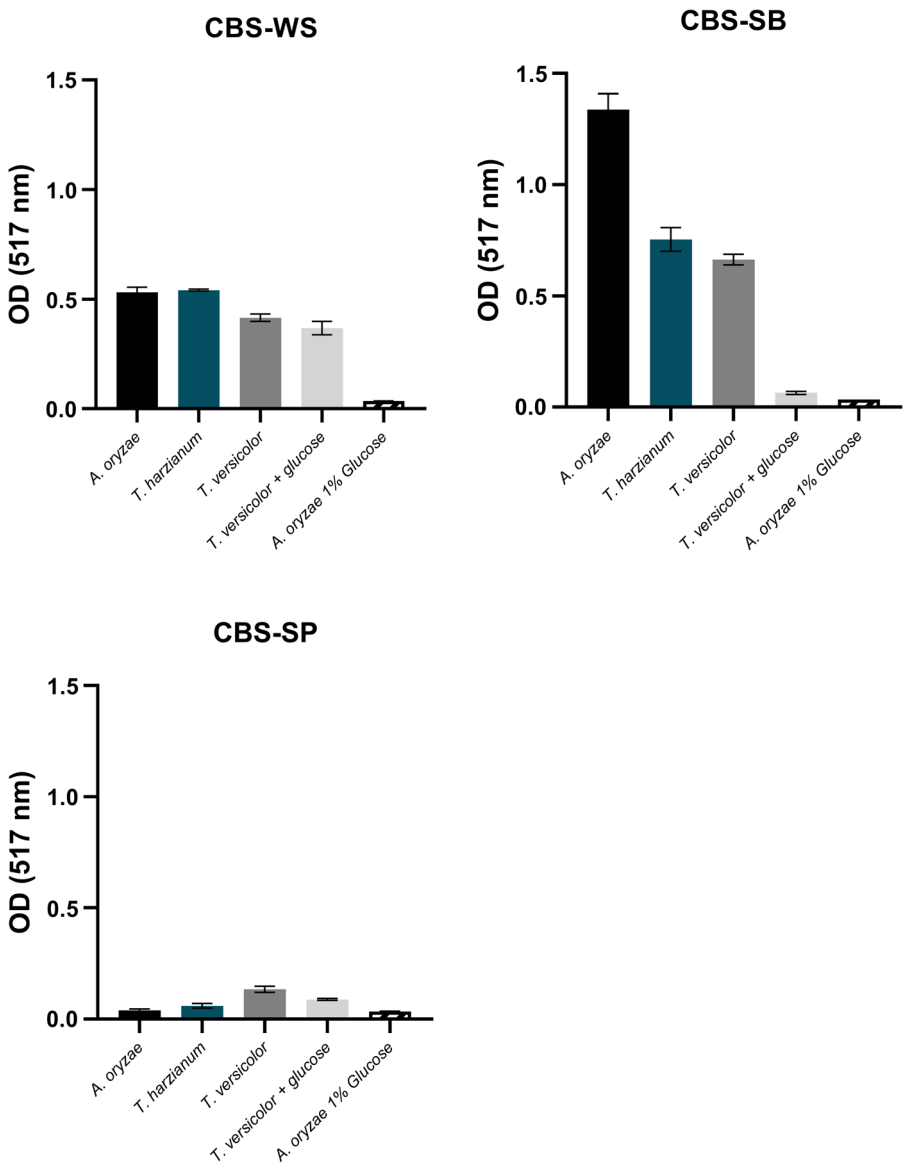



**Fig. 5** Enzyme activities measured using CBS substrates on the supernatants collected after 7 days of cultures in the micro-bioreactor for *Aspergillus oryzae*, *Trichoderma harzianum* and *Trametes versicolor*. Each fungus was grown on three different lignocellulosic biomasses wheat straw, corn stover and pine needle. *A. oryzae* was grown on 1% glucose (w/v) as sole carbon source as a measure of Carbon Catabolite Repression. Results are displayed as absorbances ± the standard deviation around the mean of three biological replicates

an important mechanism to repress the production of CWDEs during growth on preferred carbon sources (Kubicek et al. 2009; de Vries and Visser 2001). Free glucose was measured in the samples to assess if CCR may be in place in some samples where activities were lower (Fig. 6b). In the supernatants assayed in this study, free glucose appeared to be residual only, ranging between 0.25 mM for *T. versicolor* grown on WS and 0.8 mM for *A. oryzae* grown on CS. However, the apparent increase in free glucose for *A. oryzae* on CS corresponds to 0.126 g/L of glucose, which is less than 1% glucose (10 g/L) needed to induce CCR.

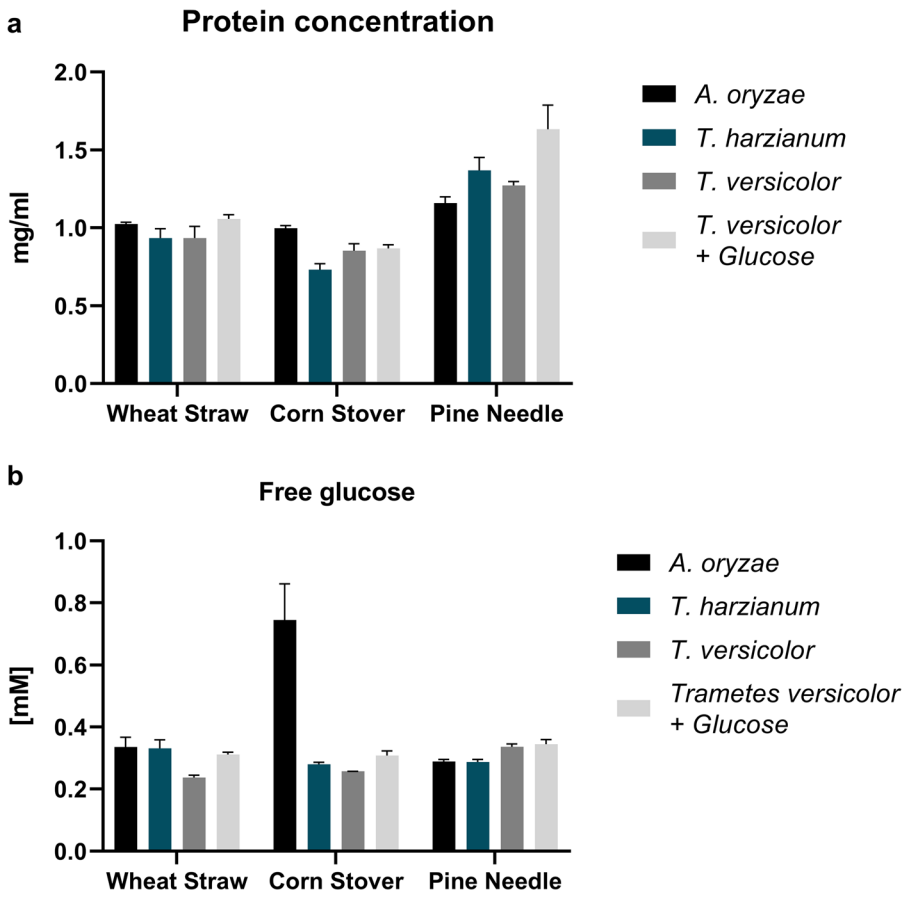



**Fig. 6** **a** Total proteins concentration secreted in the supernatants collected after 7 days of cultures in the microbioreactor for *Aspergillus oryzae*, *Trichoderma harzianum* and *Trametes versicolor*. **b** Measurement of the glucose concentration in the supernatants collected after 7 days of cultures in the microbioreactor for *Aspergillus oryzae*, *Trichoderma harzianum* and *Trametes versicolor*

Saccharification assay

In order to determine which of the activities measured using the AZO and pNP-linked substrates and the CBS substrates were the most accurate, a saccharification assay was performed by transferring the supernatants collected from the fungal submerged fermentations (for enzymes production) back on the same biomasses to assess the hydrolysis abilities of the supernatants to release carbohydrates from the biomasses. All substrates share many of the same structural molecules albeit in a different ratio and thus should share a core set of hydrolytic enzymes expressed by a given fungus species, e.g., *A. oryzae*. However, different ratios of structural components can determine the ease of degradation for hydrolytic enzymes. The difference in the amount of lignin between the different biomasses could for example contribute to the difference in difficulty to degrade one of the substrates compared to the others. Using a saccharification assay gives a complete overview of all the enzymatic activities (both exo- and endo- hydrolases) as well as any other accessory proteins that may be involved in the lignocellulosic biomass degradation taking into account the biomass composition.

The supernatants of the three fungi grown on a given biomass were put back on the same biomass in order to measure the concentrations in glucose, xylose and arabinose released from the interaction of the enzymatic cocktails with the biomass. The hydrolysis of corn stover was the most efficient, followed by the one of spruce and wheat straw hydrolysis was the least efficient in regards with the measurement of sugar monomers (Fig. 7). On corn stover, the proportions of

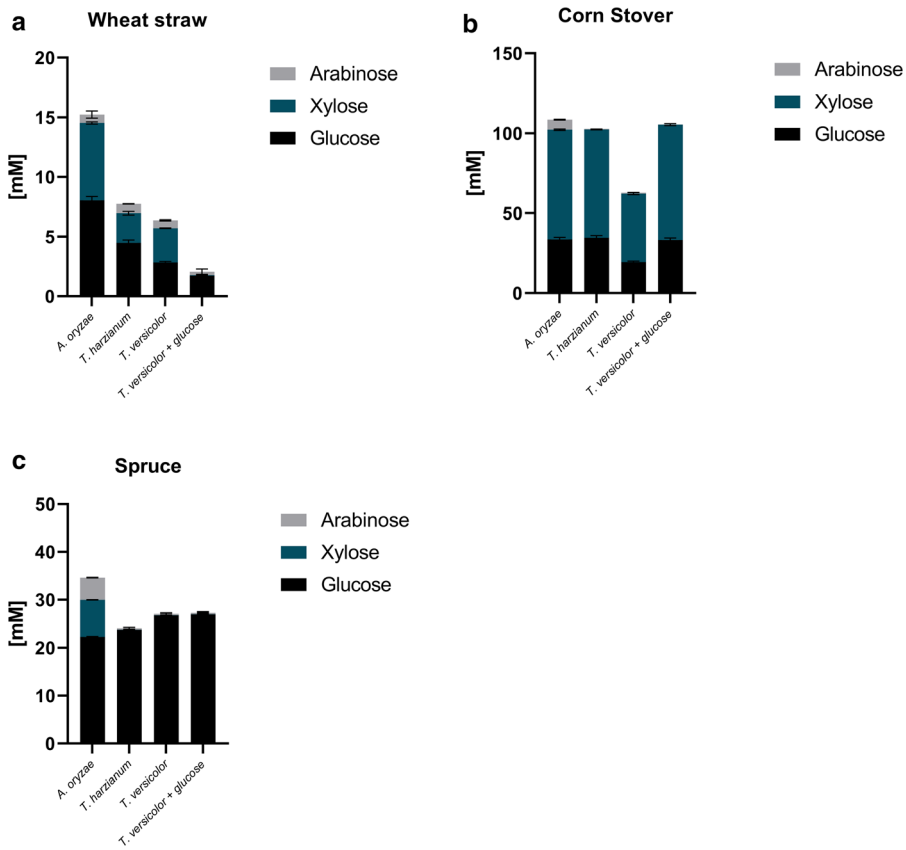



**Fig. 7** Proportion of sugar monomers glucose, xylose and arabinose released from the interaction of the supernatants collected after 7 days of cultures in the microbioreactor for *Aspergillus oryzae, Trichoderma harzianum* and *Trametes versicolor* with the different lignocellulosic biomasses wheat straw (**a**), corn stover (**b**) and spruce (**c**). Results are displayed as sugar monomers concentrations (mM) for three biological replicates

glucose and xylose were similar for most of the fungi (33.9 mM for glucose and 69.5 mM for xylose) except for the culture of *T. versicolor* with no glucose where glucose and xylose concentrations were 59% lower in average. Among the three fungi tested, only enzymes gathered from *A. oryzae* supernatant were able to release arabinose from corn stover. Walker et al. (2018) have shown that the composition of untreated corn was 38.48% glucose, 24.35% xylose and 5.07% arabinose. In the results presented here, xylose concentration is higher than for glucose, which does not confirm the results obtained with the enzymatic assays performed in this study using the different substrates. The supernatants of the three fungi displayed higher cellulases activities compared to xylanases. This divergence may be due to the difference in the complexity of the different substrates used. The saccharification assay reveals the real complexity of the biomass to degrade. As compositions vary between the different biomasses, so are the assemblies of the different components of these feedstocks. The variety of bonds, branching and interactions between the components may impair the ability of an enzymatic cocktail to efficiently hydrolyse the biomasses to sugar monomers. On the contrary, AZO and pNP-linked substrates are either cellulose or xylan polymers attached to a chromophore for which the bond is easily accessible for cleavage by the enzymes. CBS substrates rely on the same concept, but the bonds may be more difficult to reach for the enzymes given that the chromophore is directly attached to the lignocellulose. While AZO and pNP-linked substrates were useful to link back the enzyme activities measured to the substrate composition, CBS substrates were helpful to more accurately measure the real activity on a complex biomass. In addition, the different contents in

lignin for the different biomasses may have impaired the access of hydrolytic enzymes to the cellulose and hemicellulose components making the saccharification assay the best to precisely assess the hydrolysis performance of an enzymatic cocktail.

Regarding the hydrolysis of wheat straw, while the three sugars were detected, concentrations varied between the different fungi with *A. oryzae* enzymes performing the best. The main sugar released was glucose, followed by xylose and arabinose. This is consistent with the results of Fang et al. (2011) that showed that the sugar composition of polysaccharides of untreated wheat straw was made of 38.8% glucose, 13.4% xylose and 4.7% arabinose. However, the concentrations measured on wheat straw were 10 times lower (in average) than the one measured on corn stover. This may be resulting from a difference in lignin composition between the two biomasses. Wheat straw has been shown to have more lignin (20% dry weight) (Liu et al. 2013) than corn stover (13.8% dry weight) (Walker et al. 2018) and structural assembly of the different lignocellulosic components have been shown to be different too (Jia et al. 2013). In corn stover, lignin is concentrated in the corner of the cell wall which makes cellulose and hemicellulose more accessible to the enzymes (Zeng et al. 2017). Zeng et al. (2017) used stimulated Raman scattering microscopy to image xylan, cellulose, and lignin following xylanase digestion. Cell wall morphology and distribution of lignin, cellulose, and xylan in the same cell walls was compared before and after xylanase treatment. In contrast to lignin and cellulose Raman channels, dramatic concentration loss was observed for xylan. When comparing the saccharification assay results for wheat straw to the enzymatic assays, the same trends were observed with *A. oryzae* and *T. harzianum* performing the best on both types of substrates tested.

The hydrolysis of pine resulted in a completely different profiles than those obtained for wheat straw and corn stover. Its hydrolysis resulted mainly in glucose with the exception of *A. oryzae* enzymes, which released some xylose and arabinose from the substrate. This may be explained by the composition of pine that is very different to the one of the two other biomasses. Várnai et al. (2010) have showed that spruce, as pine, consisted of 45–47% cellulose, 24–26% hemicelluloses (among which 5.90% of xylan and no arabinan), 27–28% lignin and 0.4–0.9% extractives with the sugar composition of polysaccharides of untreated spruce being 64.9% glucose, 9% xylose and 2% Arabinose. This was correlated with high cellulase activities measured with AZO-cellulose substrate and, on the contrary, the low xylanase activities obtained on AZO-xylan but consistent with a high cellulose and low xylan contents in spruce. Surprisingly, given the high content in lignin, glucose concentrations were higher than for wheat straw. Mezule and Civzele (2020) have shown that the use of wood chips, which has a high lignin content such as pine, decreased the efficiency of enzymatic cocktails production on such substrates. This was due to the high lignin content as well as the potential presence of inhibitors (resin) in these biomasses. These findings are consistent with what was observed in this study, with low enzyme activities detected on the CBS-SP, as well as the predominance of one sugar monomer after saccharification which may highlight the lack of some type of enzymes being produced during the fermentation step on such biomasses.

Finally, when comparing the saccharification efficiency of the different supernatants on the other biomasses (i.e., the supernatants obtained on spruce tested for the hydrolysis of corn stover), the glucose concentrations obtained were quite high especially when spruce supernatants were tested on wheat straw and corn stover (Fig. 8). Interestingly, the supernatants of wheat straw cultures were more efficient for pine hydrolysis (for *T. harzianum* and *T. versicolor*) than wheat straw itself. This may highlight the fact that the presence of a type of enzyme activity may be more important that the amount of activity measured. This was studied by Peciulyte et al. (2017) when the authors complemented the commercial cocktail Celluclast with the supernatants of fungal cultures grown on different substrates. The authors concluded that the presence of some specific types of enzymes in the supernatant of *A. niger* (β-glucosidase, accessory activities and oxidase activities) could play an important role in improving the hydrolysis of cellulose. This may suggest that the use of a single species to produce efficient enzymatic cocktails may narrow down the potential of the lignocellulosic biomass degradation. The enzymatic hydrolysis of lignocellulosic substrates does not appear to be linked to a specificity between a given fungal species and a given biomass. Debeire et al. (2014) performed a proteomics study on four different lignocellulosic biomasses with *Fusarium*

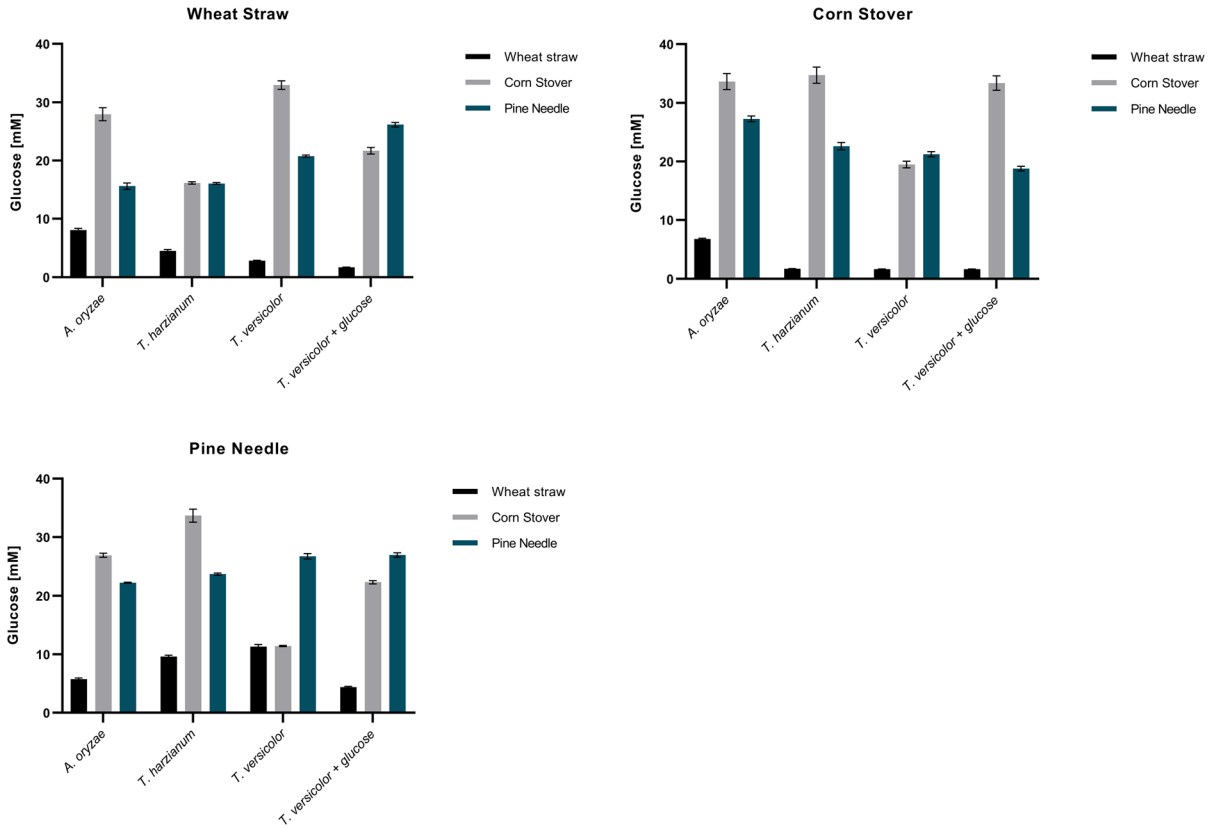



**Fig. 8** Proportion of sugar monomers glucose, xylose and arabinose released from the interaction of the supernatants collected after 7 days of cultures in the microbioreactor for *Aspergillus oryzae, Trichoderma harzianum* and *Trametes versicolor* with the different lignocellulosic biomasses wheat straw, corn stover and pine needle. Results show the saccharification efficiency of the supernatants of each fungus grown on the different biomasses to release sugar monomers from a given biomass e.g., saccharification of wheat straw with *A. oryzae* corn stover supernatant. Results are displayed as sugar monomers concentrations (mM) for three biological replicates

*graminearum*. The authors showed that the polysaccharide-degradation activities were different depending on the cocktail and the polysaccharide used. *F. graminearum* strongly modified the enzymatic cocktail it secreted as a function of the biomass used for growth. In order to improve enzymatic cocktail design for efficient biomass degradation, combining both growth on different biomasses as well as supernatants from different fungi/biomasses interaction may be the way to improve the degradation of recalcitrant biomasses.

## Conclusion

When taken altogether, the complementary experiments performed in this study showed that different approaches needed to be taken in order to accurately assess the ability of an enzymatic cocktail to be efficient when it comes to lignocellulosic biomass degradation. The susceptibility of the lignocellulosic substrates towards enzymatic hydrolysis is highly dependent on their structure, morphology and composition. The saccharification assay proved to be essential to validate the data obtained from both simple and complex substrates. While AZO and pNP-linked substrates were useful to link back the enzyme activities measured to the substrate composition, CBS substrates were helpful to more accurately measure the real activity on a complex biomass. However, when looking at oxidase activity, the CBS substrates failed to show any difference in profiles. This may be suggesting that CBS substrates allow the measurement of hydrolase activities without assessing accessory

activities, which is a limitation when thinking of developing more efficient enzymatic cocktails. Additionally, novel sources of enzymes (e.g., from Basidiomycetes), new approaches (mix of fungal supernatants for degradation), as well as non-chemical pretreatment may be of interest.

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