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Research paper

Chemical changes and increased degradability of wheat straw and oak wood chips treated with the white rot fungi *Ceriporiopsis subvermispora* and *Lentinula edodes*

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ABSTRACT

Wheat straw and oak wood chips were incubated with *Ceriporiopsis subvermispora* and *Lentinula edodes* for 8 weeks. Samples from the fungal treated substrates were collected every week for chemical characterization. *L. edodes* continuously grew during the 8 weeks on both wheat straw and oak wood chips, as determined by the ergosterol mass fraction of the dry biomass. *C. subvermispora* colonized both substrates during the first week, stopped growing on oak wood chips, and resumed growth after 6 weeks on wheat straw. Detergent fiber analysis and pyrolysis coupled to gas chromatography/mass spectrometry showed a selective lignin degradation in wheat straw, although some carbohydrates were also degraded. *L. edodes* continuously degraded lignin and hemicelluloses in wheat straw while *C. subvermispora* degraded lignin and hemicelluloses only during the first 5 weeks of treatment after which cellulose degradation started. Both fungi selectively degraded lignin in wood chips. After 4 weeks of treatment, no significant changes in chemical composition were detected. In contrast to *L. edodes*, *C. subvermispora* produced alkylitaconic acids during fungal treatment, which paralleled the degradation and modification of lignin indicating the importance of these compounds in delignification. Light microscopy visualized a dense structure of wood chips which was difficult to penetrate by the fungi, explaining the relative lower lignin degradation compared to wheat straw measured by chemical analysis. All these changes resulted in an increased in *in vitro* rumen degradability of wheat straw and oak wood chips. In addition, more glucose and xylose were released after enzymatic saccharification of fungal treated wheat straw compared to untreated material.

1. Introduction

Cellulose in lignified plant cell walls can be a source of energy in applications such as animal nutrition and biofuel production. However, the utilization of cellulose in lignocellulosic biomass cannot directly be used for these purposes because of the presence of lignin. This recalcitrant polymer is difficult to degrade and several chemical and/or physical methods have been developed to selectively remove lignin [1]. Fungal treatment of lignocellulosic biomass can be a relatively inexpensive and environmental friendly technology to decrease lignin mass fraction of the dry biomass and to increase the accessibility of cellulose [2,3]. In particular, the white-rot fungi *Ceriporiopsis subvermispora* and *Lentinula edodes* have proven to selectively degrade lignin, leaving a substrate enriched in cellulose after 6–12 weeks of treatment [4–7]. The fungal treated biomass showed increased *in vitro* rumen degradability, demonstrating that the cellulose becomes available for rumen microbes. Most studies investigating the fungal treatment of lignocellulose focus on changes in the end product and the consequences for further downstream processing [3–7]. The analyses

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of the intermediate products formed during the treatment might help to understand how fungi degrade lignocellulose and this knowledge might be used to further improve the technology. To this end, in the present work we studied in detail the structural changes occurring in the lignocellulosic matrix during 8 weeks incubation with two different fungal species grown on two different substrates. Wheat straw was used as a reference substrate and oak wood chips as a substrate high in lignin. Changes in chemical composition/as mass fraction of the dry biomass were studied using the detergent fiber method [8] and structural changes, especially in the lignin moiety, were studied using pyrolysis coupled to gas chromatography and mass spectrometry (Py-GC/MS). Light microscopy was used to visualize tissue integrity, degradation of lignin and changes in the availability of cellulose. Furthermore, the growth of the fungus was studied, both microscopically and by measuring ergosterol mass fraction of the dry biomass.

2. Material and methods

2.1. Fungal strains and spawn preparation

C. subvermispora (strain MES 13094, CBS 347.63, Westerdijk Fungal Biodiversity Institute) and *L. edodes* (strain MES 11910, CCBAS 389, Culture Collection Basidiomycetes, Institute of Microbiology, Academy of Science of the Czech Republic, Prague) were preserved in liquid nitrogen at Wageningen UR Plant Breeding. Initial culturing of the fungi was done on malt extract agar plates (pH ≈ 5.5) at 24 °C (297 K) until mycelium was covering most of the plate surface. Spawn was prepared by adding pieces of colonized agar culture to sterilized sorghum grains followed by incubation at 24 °C (297 K) until all grains were colonized by mycelium. The resulting spawn was kept at 4 °C (277 K) until further use.

2.2. Substrate preparation

Wheat straw (particles of ~3 × 0.3 cm length x diameter, harvested in the Netherlands, entire plants without the grains were air-dried and chopped) and oak wood chips (particles of ~2 × 0.5 cm length x thickness, harvested in the Netherlands, whole branches without leaves were chopped into pieces) were used as substrates. An excess of water was added to the substrates and left for 3 days to allow the water to fully penetrate the material. After removal of excess of water by draining, 50 g (on dry matter basis) of wheat straw and 100 g (on dry matter basis) of oak wood chips were weighed into 1.2 dm^3 polypropylene containers with a filter cover (model TP1200 + TPD1200 XXL Combiness, Nazareth, Belgium). Two containers of wheat straw represented one sample to correct for weight. The material was sterilized by autoclaving for 1 h at 121 °C (394 K), and the containers with sterilized substrate were kept at 20 °C (293 K) until use. A sample of the autoclaved material was collected (untreated control).

2.3. Inoculation of substrate

Spawn was added to the substrates (0.1 g wet weight of mycelium covered spawn per g dry matter of substrate) using sterile spoons and tweezers and mixed aseptically, to equally distribute the spawn through the substrate under sterile conditions. The samples were incubated at 24 °C (297 K) and a relative humidity of 70% in a climate controlled chamber. All treatments were tested in triplicate, i.e. three containers per fungus-substrate at each incubation time.

2.4. Sampling

Samples were taken every week during a period of 8 weeks. Each time samples were taken, fungal treatment was discontinued and samples were no longer treated aseptically. The samples were mixed by hands wearing gloves to ensure that a representative sample could be taken. Approximately 90% of the substrate was air-dried at 70 °C (343 K) for chemical analysis, *in vitro* gas production measurement and enzymatic saccharification. The remaining (~10%) part was freeze-dried and used to determine ergosterol mass fraction of the dry biomass and used for Py-GC/MS analyses. For microscopy, samples (fresh) of each treatment were taken only before and after 8 weeks of incubation.

The dried and freeze-dried wheat straw was ground to pass a 1 mm sieve, using a Peppink 100 AN cross beater mill (Peppink, Deventer, The Netherlands). The dried and freeze-dried oak wood chips were ground to pass a 1 mm sieve using a Retch SM2000 cutting mill (Retch, Haan, Germany), which was followed by a Retch ZM 100 centrifugal mill (Retch, Haan, Germany). Freeze-dried material was kept frozen (−80 °C, 193 K) and in the dark until and during processing.

2.5. Ergosterol

Ergosterol mass fraction was determined as described by Niemenmaa et al. [9]. In brief, ground, freeze dried material (200 mg) was saponified with 3 cm^3 10% mass fraction of KOH in methanol for 1 h at 80 °C (353 K). Ergosterol was extracted by adding 1 cm^3 water and 2 cm^3 hexane, and the hexane phase was collected in glass tubes after shaking and centrifuging for 10 min at 66.7 Hz (4000 rpm). This step was repeated for optimal extraction and both hexane phases were mixed. Hexane was evaporated under vacuum (10 min at 15 kPa, 10 min at 10 kPa, 40 min at 0 Pa) for 60 min at 30 °C (303 K) and ergosterol was dissolved in methanol. The extraction efficiency was calculated on the basis of recovery of the internal standard cholecalciferol (vitamin D3) (9.6 μg added) (Sigma Aldrich, St. Louis, Missouri, USA).

Ergosterol was analyzed using an HPLC fitted with a reversed phase C18 column (250 × 4.6 mm, Phenomex aqua 5 μm, Torrance, California, USA). The liquid phase was 90% methanol and 10% 2-propanol/hexane (1:1, v/v). Areas under the peak were corrected for the extraction efficiency based on the internal standard using Empower 2 software (Waters Corporation, Milford, Massachusetts, USA).

To calculate a conversion rate of the amount of ergosterol measured to dry weight of mycelium, mycelium of each fungus was grown on malt extract agar with cellophane. When fully colonized, the mycelium was scraped of the cellophane and weighed before and after freeze drying. Known amounts of dry mycelium were subjected to ergosterol extraction. For each fungus, the amount of ergosterol per mg mycelium was calculated.

2.6. Light microscopy

The fresh samples were fixed in a mixture of 3% (v/v) paraformaldehyde, 0.1% (v/v) glutaraldehyde, 0.3 mol/m^3 Triton™ X-100 Surfact-Amps™ (ThermoFisher, Bleiswijk, the Netherlands) dissolved in 50 mol/m^3 PIPES buffer (piperazine-N,N'-bis(2-ethanesulfonic)acid); (pH 6.9). Increased fixative penetration was achieved by bringing samples in low pressure (down to 20 kPa) until samples submerged. Fixed samples were dehydrated in an ethanol series until full dehydration was reached in 100% ethanol. Dehydrated samples were stepwise infiltrated with Technovit 7100 (Heraeus Kulzer Benelux, Haarlem, The Netherlands). Polymerization of the Technovit 7100 monomers was done for at least 1 h at

37 °C (310 K). Resin embedded samples were cut at 5 μm with a Microm (Adamas Instr, Rhenen, the Netherlands) rotary microtome. The collected sections were stretched on a water surface and baked to glass slides at 80 °C (353 K) for at least 10 min.

Staining of the sections for lignin and cellulose was based on the procedure described by Srebotnik and Messner [10]. In brief, sections were stained for 1 min in 24 mol/m^3 Safranin O (S2255, Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands) dissolved in 15% ethanol. Excess of dye was removed by washing for 1 min in water followed by two times 5 min in 30% ethanol (v/v) and 5 min in 15% ethanol (v/v). After another minute in water to remove the ethanol, sections were stained for 3 min in 8.4 mol/m^3 Astra blue (Marker Gene Technologies Inc., Eugene, Oregon, USA) dissolved in 5% ethanol. Excess dye was removed by washing four times for 3 min in clean water.

Fungal hyphae were discriminated in sections of treated samples with 1% Toluidine Blue in 1% sodium tetra borate. Sections were stained during 5 min in Toluidine Blue and excess of dye was removed during 30 min in water.

2.7. Detergent fiber analysis

Detergent fiber analysis was performed on air-dried, ground material according to the method described by Van Soest et al. [8] using Ankom fiber analyser 2000 (ANKOM Technology, Macedon, New York, USA). Acid detergent fiber (ADF) was subtracted from neutral detergent fiber (NDF) to calculate the hemicelluloses mass fraction of the dry biomass. Acid detergent lignin (ADL) was subtracted from ADF to calculate the cellulose mass fraction of the dry biomass. For dry matter determination air-dried material was dried at 103 °C (376 K) until constant weight. Ash mass fraction of the dry biomass was determined after combustion for 3 h at 550 °C (823 K) in a muffle furnace. Dry matter loss data were used to calculate the amount of dry matter remaining. Using the mass fractions of nutrients in the dry biomass, the absolute amounts of nutrients in the remaining dry matter was calculated.

2.8. Pyrolysis-gas chromatography/mass spectrometry (Py-GC/MS)

Py-GC/MS of duplicate samples (3.2 mg) were performed with a 3030 micro-furnace pyrolyzer (Frontier Laboratories Ltd., Fukushima, Japan) connected to an Agilent 7820A GC using a DB-1701 fused-silica capillary column (60 m × 0.25 mm, 0.25 μm film thickness) and an Agilent 5975 mass selective detector (EI at 70 eV) (Agilent Technologies, Santa Clara, California, USA). The pyrolysis was performed at 500 °C (773 K). The oven temperature of the gas chromatograph was programmed from 100 °C (373 K) (4 min) to 280 °C (553 K) (8 min) at 3 °C min^{-1} (3 K min^{-1}). Helium was the carrier gas (1 cm^3 min^{-1}). The compounds were identified by comparing their mass spectra with those of the Wiley and NIST libraries and those reported in the literature [11–13]. Peak molar areas were calculated for the carbohydrate and lignin-degradation products, the summed areas were normalized, and the data for replicate samples were averaged and expressed as percentages.

2.9. In vitro gas production (IVGP)

Determination of IVGP was performed according to the procedure described by Cone et al. [14]. Rumen fluid of rumen fistulated non-lactating cows fed a grass silage based diet was collected. To each 60 cm^3 buffered rumen fluid 500 mg air dried sample was added. During 72 h of incubation at 39 °C (312 K) the amount of gas produced by anaerobic fermentation was measured. Total gas production was related to organic matter mass fraction of the samples. No differentiation between methane, CO_2 or other gasses produced by the rumen microbes was made.

2.10. Enzymatic saccharification

Enzymatic saccharification was tested for wheat straw treated with *C. subvermispora* or *L. edodes* for 8 weeks. Two g of wheat straw (air-dried, ground to 1 mm) were mixed with 19 cm^3 sodium citrate buffer (50 mol/m^3, pH 5.3) and 1 cm^3 of enzymes, i.e. mixture of mainly cellulases (CMAX3, Dyadic Nederland BV, Wageningen, The Netherlands). The mixtures were incubated at 50 °C (323 K) in a rotary shaker (10 Hz). Samples of the supernatant were taken after 0, 4, 8, 12, 24, 48 and 72 h of incubation to measure the amount of released glucose and xylose. The amount of released glucose was measured using a D-glucose kit (D-glucose assay kit (GOPOD format), Megazyme, Bray, Ireland). The amount of released xylose was measured using a D-xylose kit (D-xylose assay kit, Megazyme, Bray, Ireland).

2.11. Statistical analysis

The results of detergent fiber analysis, Py-GC/MS, IVGP and enzymatic saccharification at different incubation times of the fungal treatment of each substrate were compared using the generalized linear model (GLM) analysis in SAS software version 9.3 (SAS Institute Inc., Cary, North Carolina, USA). Post-hoc multiple comparison with Tukey's significant test at a level of $\alpha = 0.05$ was performed to determine the significance of differences between the treatments. The following model was used:

$$Y_{ij} = \mu + \alpha_i + \omega_{ij}$$

in which Y_{ij} is the observation j at incubation time i; μ is the overall mean; α_i is the fixed effect of incubation time i; ω_{ij} is the random error.

Correlations between IVGP, enzymatic saccharification and ADL were subjected to correlation analysis using SAS software version 9.3. Correlations are provided as the Pearson correlation coefficient r.

2.12. Disclaimer

This work was performed on substrates of unknown provenance, for which the chain of custody is not known. The species and the cultivars cannot be specified and while the authors BELIEVE that this work exemplifies the difference between fungal strains - there is a reasonable concern that there may be substrate or other uncontrolled factors that influence the results obtained.

3. Results

3.1. Fungal growth

The changes in ergosterol mass fraction in the substrate during incubation were measured as an indication for fungal growth (Table 1 wheat straw and Table 2 wood chips). The ergosterol mass fraction in wheat straw and oak wood chips showed a steady increase during 8 weeks of *L. edodes* treatment indicating a continuous growth of this fungus during the whole incubation period. The ergosterol mass fraction of the *C. subvermispora* treated substrates showed a different pattern. Ergosterol mass fraction of both wheat straw and oak wood chips increased in the first week of treatment. In the next 7 weeks, the ergosterol mass fraction in wood chips did not change significantly. In wheat straw, however, the ergosterol mass fraction resumed its increase after 6 weeks of incubation.

Visual, macroscopic, observations confirmed the growth

Table 1
Ergosterol content and chemical composition of wheat straw before (0 weeks) and after treatment with *C. subvermispora* and *L. edodes*.

Time (weeks)	*C. subvermispora*				*L. edodes*			
	Ergosterol (mg kg^{-1} fungal mass)	ADL in DM (g kg^{-1})	HC in DM (g kg^{-1})	Cell in DM (g kg^{-1})	Ergosterol (mg kg^{-1} fungal mass)	ADL in DM (g kg^{-1})	HC in DM (g kg^{-1})	Cell in DM (g kg-1)
control	90.1^{c}	75.3^{b}	321.5^{a}	440.3^{d}	90.1^{f*}	75.3^{a}	321.5^{a}	440.3^{e}
1	183.2^{bc}	84.9^{a}	299.1^{a}	443.1^{d}	140.4^{ef*}	77.1^{a*}	301.4^{b}	442.7^{e}
2	180.1^{bc}	75.6^{ab}	243.0^{b}	439.8^{d}	174.9^{def*}	83.1^{a}	269.2^{c*}	449.9^{e}
3	192.3^{abc}	56.7^{c}	221.8^{b}	448.2^{cd}	202.6^{cde*}	65.3^{b}	232.8^{d}	464.0^{d*}
4	176.8^{bc}	39.7^{d}	171.7^{c}	457.2^{bcd}	267.3^{bcd*}	61.8^{b*}	219.2^{d*}	466.5^{d}
5	185.9^{bc}	27.6^{e}	135.4^{cd}	467.3^{bc}	295.5^{abc*}	50.7^{c*}	187.9^{e*}	470.1^{d}
6	241.6^{ab}	24.0^{ef}	117.4^{de}	473.8^{ab}	284.9^{bc*}	45.4^{cd*}	180.6^{e*}	485.3^{c}
7	294.8^{a}	20.6^{ef}	104.6^{de}	475.8^{ab}	365.7^{ab*}	37.6^{de*}	153.8^{f*}	501.8^{b*}
8	272.6^{ab}	17.4^{f}	87.2^{e}	490.2^{a}	403.5^{a*}	29.4^{e*}	132.8^{g*}	518.6^{a*}
Time (weeks)	DM loss (%)	ADL (g)	HC (g)	Cell (g)	DM loss (%)	ADL (g)	HC (g)	Cell (g)
control	–	6.0^{ab}	25.7^{a}	35.2^{a}	–	6.0^{a}	25.7^{a}	35.2^{a}
1	4^{d}	6.5^{a}	23.0^{b}	34.1^{a}	4^{e}	5.9^{a}	23.1^{b}	33.8^{abcd}
2	8^{d}	5.6^{b}	17.9^{c}	32.4^{ab}	5^{e} *	6.4^{a*}	20.6^{c*}	34.4^{ab*}
3	15^{c}	3.9^{c}	15.2^{d}	30.7^{bc}	8^{de*}	4.8^{b*}	17.0^{d}	33.9^{abc*}
4	16^{c}	2.7^{d}	11.6^{e}	30.9^{bc}	11^{de*}	4.4^{b*}	15.7^{d*}	33.5^{abcd*}
5	18^{c}	1.8^{e}	8.9^{f}	30.8^{bc}	15^{cd}	3.4^{c*}	12.8^{e*}	31.9^{bcde}
6	20^{bc}	1.5^{ef}	7.5^{fg}	30.3^{bc}	19^{bc}	2.9^{cd*}	11.7^{ef*}	31.3^{cde}
7	25^{ab}	1.2^{ef}	6.3^{gh}	28.5^{c}	22^{ab}	2.3^{de*}	9.5^{fg*}	30.9^{de}
8	29^{a}	1.0^{f}	5.0^{g}	28.1^{c}	26^{a}	1.7^{e*}	7.9^{g*}	30.9^{e}

Values with different superscripts within column and component are significantly ($P < 0.05$) different. Values with * are significant different from those after *C. subvermispora* treatment.
IVGP = *in vitro* gas production, DM = dry matter, ADL = acid detergent lignin, HC = hemicellulose, Cell = cellulose.

Table 2
Ergosterol content and chemical composition of wood chips before (0 weeks) and after treatment with *C. subvermispora* and *L. edodes*.

Time (weeks)	*C. subvermispora*				*L. edodes*			
	Ergosterol (mg kg^{-1} fungal mass)	ADL in DM (g kg^{-1})	HC in DM (g kg^{-1})	Cell in DM (g kg^{-1})	Ergosterol (mg kg^{-1} fungal mass)	ADL in DM (g kg^{-1})	HC in DM (g kg^{-1})	Cell in DM (g kg^{-1})
control	22.7^{c}	176.9^{a}	197.1^{a}	402.6	22.7^{e}	176.9^{a}	197.1^{a}	402.6
1	118.5^{b}	176.5^{a}	170.9^{ab}	417.7	85.0^{e}	177.7^{a}	170.3^{ab}	409.2
2	142.8^{ab}	144.9^{b}	161.5^{ab}	420.8	144.6^{de}	154.4^{ab}	166.1^{b}	410.4
3	152.5^{ab}	125.7^{bc}	140.2^{b}	431.3	236.1^{cd}	155.0^{ab*}	155.0^{bc}	393.2*
4	147.4^{ab}	123.5^{bc}	133.5^{b}	402.6	310.1^{bc}	144.1^{bc}	149.2^{bc}	385.1
5	179.6^{ab}	109.8^{c}	138.4^{b}	429.0	440.4^{ab}	135.0^{bcd}	144.0^{bc}	382.3*
6	181.3^{ab}	119.7^{c}	134.1^{b}	407.2	492.8^{a}	121.9^{cd}	144.7^{bc}	384.1
7	160.8^{ab}	116.5^{c}	129.0^{b}	426.2	539.7^{a}	113.8^{cd}	143.4^{bc}	385.4*
8	192.8^{b}	106.7^{c}	127.7^{b}	432.2	564.9^{a}	106.0^{d}	130.8^{c}	384.5*
Time (weeks)	DM loss (%)	ADL (g)	HC (g)	Cell (g)	DM loss (%)	ADL (g)	HC (g)	Cell (g)
control	–	14.2^{a}	15.8^{a}	32.2^{a}		14.2^{a}	15.8^{a}	32.2^{a}
1	11^{d}	13.0^{a}	12.5^{ab}	30.7^{ab}	7^{g}	13.6^{a}	13.0^{b}	31.3^{ab}
2	15^{cd}	10.2^{b}	11.4^{bc}	29.6^{abc}	13^{fg}	11.1^{b}	11.9^{bc}	29.4^{b}
3	18^{bc}	8.5^{bc}	9.5^{bcd}	29.1^{abcd}	18^{ef}	10.5^{bc*}	10.5^{cd}	26.7^{c*}
4	22^{ab}	8.0^{c}	8.7^{cd}	26.1^{cd}	22de	9.3^{bcd}	9.6^{de}	24.8^{cd}
5	23^{ab}	7.0^{c}	8.8^{cd}	27.3^{bcd}	27^{cd}	8.2^{cde}	8.7^{def}	23.1^{de*}
6	24^{a}	7.5^{c}	8.4^{cd}	25.5^{d}	31^{bc*}	6.9^{def}	8.2^{ef}	21.9^{ef*}
7	25^{a}	7.2^{c}	8.0^{cd}	26.4^{cd}	34^{ab*}	6.2^{ef}	7.8^{ef}	21.0^{ef*}
8	25^{a}	6.6^{c}	7.8^{d}	26.6^{cd}	36^{a*}	5.6^{f*}	6.9^{f}	20.2^{f*}

Values with different superscripts within column and component are significantly ($P < 0.05$) different. Values with * are significant different from those after *C. subvermispora* treatment.
IVGP = *in vitro* gas production, DM loss = dry matter loss, ADL = acid detergent lignin, HC = hemicellulose, Cell = cellulose.

patterns by *C. subvermispora* and *L. edodes* on both wheat straw and wood chips (data not shown).

3.2. Microscopy

Safranin O and Astra Blue staining of untreated wheat straw showed an intact structure with few intercellular spaces and mainly red stained walls (Fig. 1a). The Toluidine Blue stained wheat straw in Fig. 1b demonstrates the absence of fungal hyphae in the control. The lignified walls of the xylem and sclerenchyma vessel cells of the vascular bundle sheets are predominantly stained. After 8 weeks of *C. subvermispora* treatment most of the structure has disappeared (Fig. 1c). *C. subvermispora* degraded most of the thin walled parenchymatic cells and only some remnants of lignified sclerenchyma are still visible (Fig. 1c). While the Safranin O staining remained present in only thick lignified cell walls, the Astra Blue staining increased during degradation, since more lignin was degraded from the cell wall matrix, which increases the accessibility for Astra Blue to cellulose. Toluidine Blue staining revealed *C. subvermispora* hyphae (arrows) to be omnipresent in wheat straw

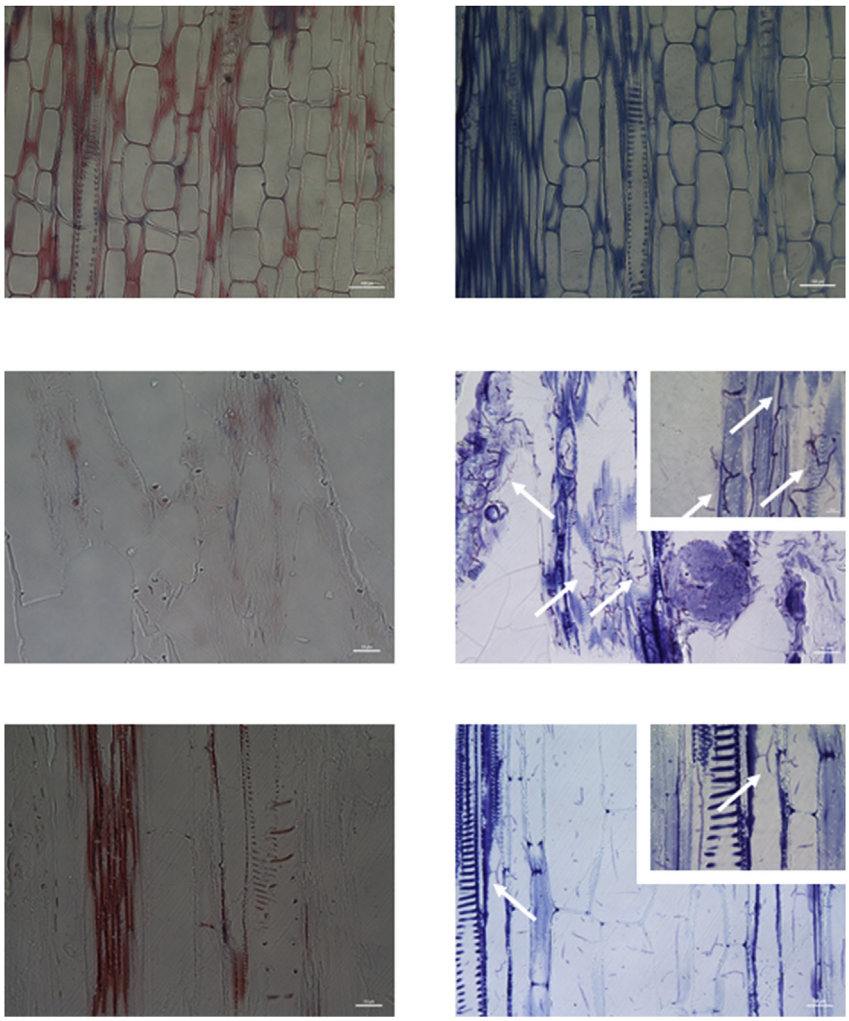

Fig. 1. Light microscopy of untreated and fungal treated wheat straw. a) autoclaved, uninoculated wheat straw stained with Safranin O and Astra Blue, b) autoclaved, uninoculated wheat straw stained with Toluidine Blue, c) *C. subvermispora* treated wheat straw for 8 weeks stained with Safranin O and Astra Blue, d) and e) *C. subvermispora* treated wheat straw for 8 weeks stained with Toluidine Blue, f) *L. edodes* treated wheat straw stained with Safranin O and Astra Blue, g) and h) *L. edodes* treated wheat straw for 8 weeks stained with Toluidine Blue. Arrows indicate fungal hyphae.

after 8 weeks of treatment (Fig. 1d and e).

Safranin O and Astra Blue staining showed that after 8 weeks of *L. edodes* treatment a clearly visible structure was left, which was stained red for lignin (Fig. 1f). Toluidine Blue staining showed an overall presence of *L. edodes* hyphae (arrows) throughout the remaining structures (Fig. 1g and h).

Sections of untreated oak wood chips showed a dense structure consisting of xylem vessels (Fig. 2a (Safranin O and Astra blue staining) and 2b (Toluidine Blue staining)). This dense structure was still present after 8 weeks of *C. subvermispora* or *L. edodes* treatment. Safranin O – Astra Blue stained fungal treated oak wood chips still showed large parts containing lignin (Fig. 2c and f). In contrast to untreated oak wood chips, the vascular bundles stained blue, showing more cellulose became accessible for Astra Blue staining due to the degradation of lignin (Fig. 2c and f). Toluidine Blue staining showed the presence of fungal hyphae that had invaded the (physically accessible) structures (Fig. 2d and g). The fungal hyphae were not omnipresent as found in wheat straw, which was most clear in *L. edodes* treated oak wood chips. In Fig. 2g, hyphae of *L. edodes* were only visible in the part of the sample showing wider vessel diameters, whereas the dense vessels were still fully intact.

3.3. Chemical composition of fungal treated material

3.3.1. Detergent fiber analysis

Tables 1 and 2 show the changes in the composition of wheat straw and oak wood chips during the fungal treatment, using the detergent fiber analysis method [8]. *C. subvermispora* incubation of wheat straw led to a decrease of dry matter of 29% after 8 weeks. The detergent fiber composition of wheat straw changed significantly only during the first 5 weeks of treatment with a continuous decrease in ADL and hemicelluloses mass fraction of the dry biomass whereas the cellulose mass fraction of the dry biomass did not change significantly. Despite the fact that the concentration of fibers did not change significantly between 5 and 7 weeks, the dry matter loss in wheat straw increased continuously upon *C. subvermispora* treatment. Expressed as absolute amounts, also a steady degradation of ADL and hemicelluloses was observed. For cellulose, however, the absolute amount in wheat straw only changed significantly in the first 3 weeks of incubation with *C. subvermispora* (Table 1).

The 8 weeks of treatment of wheat straw by *L. edodes* led to a loss of 26% dry matter, a decreased ADL and hemicelluloses mass fraction of the dry biomass and an increased cellulose mass fraction

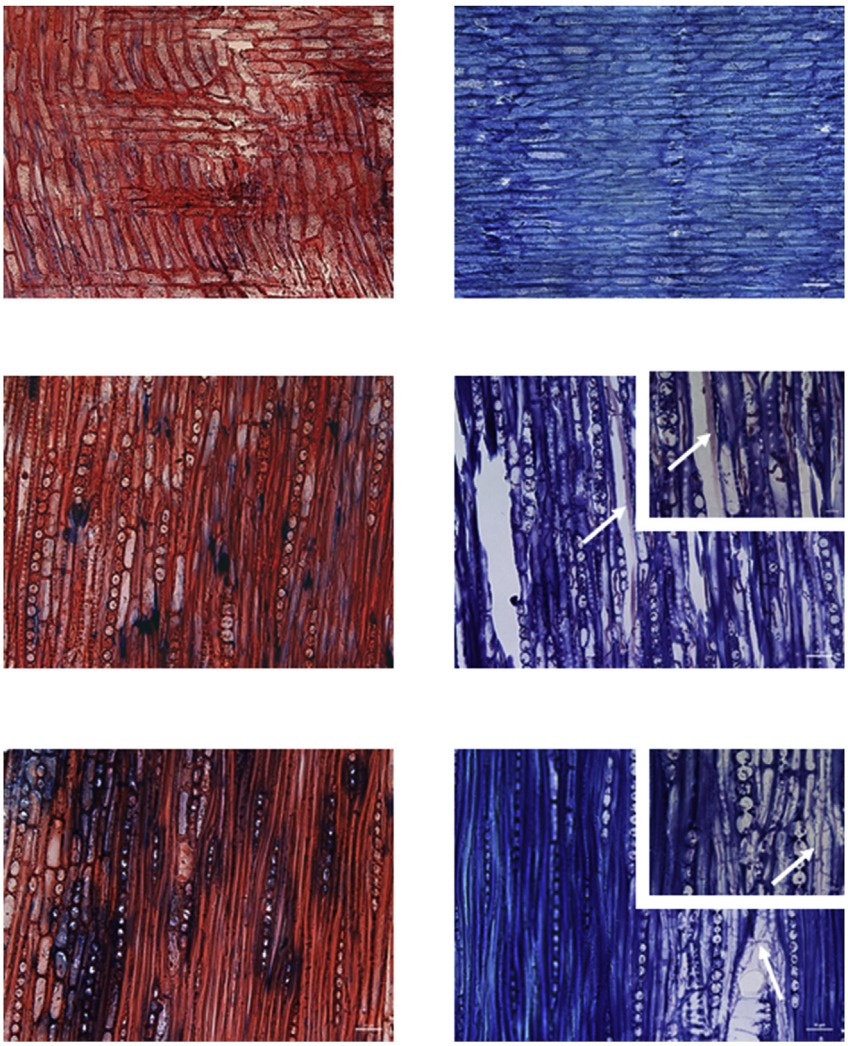

Fig. 2. Light microscopy of untreated and fungal treated oak wood chips (20× magnification). a) autoclaved, uninoculated oak wood chips stained with Safranin O and Astra Blue, b) autoclaved, uninoculated oak wood chips stained with Toluidine Blue, c) *C. subvermispora* treated oak wood chips for 8 weeks stained with Safranin O and Astra Blue, d) and e) *C. subvermispora* treated oak wood chips for 8 weeks stained with Toluidine Blue, f) *L. edodes* treated oak wood chips stained with Safranin O and Astra Blue, g) and h) *L. edodes* treated oak wood chips for 8 weeks stained with Toluidine Blue. Arrows indicate fungal hyphae.

of the dry biomass (Table 1). Absolute amounts confirmed the ADL and hemicelluloses degradation, but also showed that *L. edodes* degraded ($P < 0.05$) part of the cellulose in wheat straw (Table 1).

In oak wood chips, *C. subvermispora* degraded 25% of the dry matter in 8 weeks. Hemicelluloses and ADL mass fractions of the dry biomass changed only significantly during the first 3 weeks of treatment (Table 2), which corresponded with the obtained dry matter loss. From 4 weeks onwards no changes in the mass fractions of the main components (ADL, hemicelluloses and cellulose) of the dry biomass were observed in oak wood chips. *C. subvermispora* did not change the cellulose mass fraction in the dry biomass significantly in oak wood chips during the 8 weeks of treatment. Nevertheless, absolute amounts showed that oak wood chips treated for 4 weeks or longer with *C. subvermispora* contained a lower amount of cellulose compared to untreated biomass, resulting in a total degradation of 5.6 g cellulose after 8 weeks of treatment (Table 2).

L. edodes caused a dry matter loss in oak wood chips of up to 36%. The composition of the oak wood chips did not change significantly after 5 weeks of *L. edodes* treatment. At the end of 8 weeks of *L. edodes* treatment, the oak wood chips showed a lower ($P < 0.05$) ADL and hemicelluloses and a higher ($P < 0.05$) cellulose mass fraction of the dry biomass than the control before treatment (Table 2). Absolute amounts of detergent fiber fractions showed a degradation ($P < 0.05$) of all compounds including cellulose by *L. edodes* in oak wood chips (Table 2).

3.3.2. Py-GC/MS

The composition of the fungal treated samples was analyzed by Py-GC/MS, as shown in Figs. S1 and S2 for wheat straw and Figs. S3 and S4 for oak wood chips. The identities and relative abundances of the compounds released by Py-GC/MS are shown in Tables S1 and S2 for wheat straw and Tables S3 and S4 for oak wood chips. The most important results obtained by Py-GC/MS of untreated and fungal treated wheat straw and oak wood chips are shown in Table 3.

In the case of wheat straw, the lignin to carbohydrate (L/C) ratio estimated upon Py-GC/MS varied from 2.2 in the untreated material to 0.4 and 0.5 after 8 weeks of treatment with *C. subvermispora* and *L. edodes*, respectively (Table 3). In oak wood chips, a decrease in L/C

Table 3
Composition of lignocellulose in wheat straw and wheat straw before and after treatment with *C. subvermispora* or *L. edodes* as determined by Py-GC/MS.

Substrate	Time (weeks)	*C. subvermispora*					*L. edodes*			
		L/C ratio	S/G ratio	Ph-C0-2/Ph-C3 ratio	Cα-oxidized lignin (%)	Alkylitaconic acids (%)	L/C ratio	S/G ratio	Ph-C0-2/Ph-C3 ratio	Cα-oxidized lignin (%)
Wheat straw	control	2.2^{a}	0.7^{a}	7.6^{c}	4.4^{e}	0	2.2^{a}	0.7^{a}	7.6^{d}	4.4^{d}
	1	1.9^{a}	0.7^{a}	7.3^{c}	4.8^{de}	0	1.9^{a}	0.7^{a}	7.8^{d}	4.5^{d}
	2	1.1^{b}	0.8^{a}	8.7^{bc}	7.6^{cd}	0.2	1.4^{b}	0.7^{a}	8.4^{cd}	6.3^{cd}
	3	0.9^{b}	0.6^{a}	10.7^{abc}	8.3^{bc}	0.5	1.0^{c}	0.6^{a}	9.5^{bcd}	7.4^{bc}
	4	0.5^{c}	0.5^{b}	13.0^{a}	10.7^{ab}	1.3	0.9^{cd}	0.6^{a}	10.0^{bc}	8.7^{abc}
	5	0.5^{c}	0.4^{bc}	11.9^{ab}	12.1^{a}	1.8	0.9^{cd}	0.5^{b}	10.3^{ab}	9.2^{ab}
	6	0.4^{c}	0.4^{bc}	12.3^{ab}	12.4^{a}	2.9	0.7^{de}	0.5^{bc}	10.6^{ab}	9.4^{ab}
	7	0.4^{c}	0.4^{bc}	13.3^{a}	12.3^{a}	3.3	0.7^{de}	0.4^{cd}	10.0^{bc}	10.1^{a}
	8	0.4^{c}	0.3^{c}	12.3^{a}	10.1^{abc}	4.8	0.5^{e}	0.4^{d}	12.1^{a}	9.0^{ab}
Wood chips	control	1.7^{a}	1.2^{a}	3.3^{c}	4.3	0.0^{d}	1.7^{a}	1.2^{a}	3.3^{c}	4.3^{ab}
	1	1.3^{ab}	1.0^{ab}	4.3^{bc}	5.3	0.0^{d}	1.4^{abc}	0.9^{ab}	3.9^{bc}	2.9^{c}
	2	1.1^{bc}	0.9^{abc}	4.4^{bc}	3.7	0.3^{cd}	1.5^{abc}	0.9^{ab}	5.3^{abc}	4.9^{a}
	3	0.9^{bc}	0.7^{bc}	6.7^{abc}	3.9	0.7^{bcd}	1.1^{bc}	0.7^{b}	5.5^{abc}	3.6^{abc}
	4	0.8^{bc}	0.7^{bc}	7.6^{abc}	4.4	1.0^{bcd}	1.2^{bc}	0.7^{b}	5.7^{abc}	3.9^{abc}
	5	0.9^{bc}	0.6^{bc}	7.2^{abc}	3.9	2.2^{ab}	1.2^{bc}	0.6^{b}	6.7^{abc}	3.4^{bc}
	6	0.9^{bc}	0.6^{c}	7.4^{abc}	3.6	2.1^{ab}	1.0^{c}	0.6^{b}	7.1^{ab}	3.1^{bc}
	7	0.8^{bc}	0.6^{c}	8.0^{ab}	3.9	2.0^{abc}	1.1^{bc}	0.6^{b}	7.4^{ab}	3.2^{bc}
	8	0.6^{c}	0.5^{c}	10.7^{a}	3.6	3.2^{a}	1.0^{c}	0.6^{b}	7.8^{a}	3.4^{bc}

Values with different superscripts within column and substrate are significantly ($P < 0.05$) different.
L/C ratio: lignin to carbohydrate ratio, S/G ratio: S-lignin to G-lignin ratio, Ph-C0-2/Ph-C3 ratio: ratio between phenolic compounds with 0–2 C-atoms in the side chain to phenolic compounds with 3 C-atoms in the side chain.

ratio was found from 1.7 in the untreated material to 0.6 and 1.0 after 8 weeks of treatment with *C. subvermispora* and *L. edodes*, respectively (Table 3). The main change observed in the carbohydrate fraction of both wheat straw and wood chips was the increase in the relative content of levoglucosane (peak **38**), a compound originating from cellulose.

Analysis of the lignin-derived compounds indicated that the syringyl (S) units were preferentially degraded over guaiacyl (G) units, as observed by the decrease ($P < 0.05$) of the S/G ratio in wheat straw from 0.7 in the untreated material to 0.3 and 0.4 after 8 weeks of treatment with *C. subvermispora* and *L. edodes*, respectively (Table 3). The S/G ratio in oak wood chips decreased ($P < 0.05$) from 1.2 to 0.5 and 0.6 after 8 weeks of treatment with *C. subvermispora* and *L. edodes*, respectively (Table 3). Moreover, the treatment with *C. subvermispora* and *L. edodes* resulted in the formation of intermediate degradation products of lignin, as shown by the increased ratio of phenolic compounds bearing 0 to 2 C-atoms (Ph-C0-C2) and the intact phenolic compounds bearing 3 C-atoms (Ph-C3) in the side chain (PhC0-2/Ph-C3 ratio). In both wheat straw and oak wood chips the Ph-C0-2/Ph-C3 ratio increased. In wheat straw, the side chains were not only degraded, but also the fungi oxidized the Cα-atom, as shown by the increasing ($P < 0.05$) percentage in Cα-oxidized lignin compounds upon fungal treatment. The percentage of Cα-oxidized lignin significantly decreased during *L. edodes* treatment of wood chips, whereas *C. subvermispora* treatment did not result in an increasing amount of Cα-oxidized lignin compounds (Table 3). In wheat straw, both the side chain degradation and modification were highly related to each other (*C. subvermispora*: $r = 0.90$, $P < 0.0001$; *L. edodes*: $r = 0.79$, $P < 0.001$), whereas this was less obvious in wood chips (*C. subvermispora*: $r = -0.55$, $P = 0.0179$; *L. edodes*: $r = -0.39$, $P = 0.11$). In the case of *C. subvermispora*, the major changes in the lignin polymer occurred during the first 5 weeks of treatment, while no significant changes in L/C ratio, S/G ratio, Ph-C0-2/Ph-C3 ratio and percentage of Cα-oxidized lignin occurred after 5 weeks of treatment of wheat straw. This result corresponds with the stabilizing ADL, hemicelluloses and cellulose mass fraction of the dry biomass after 5 weeks of treatment according to the detergent fiber analysis. Upon L. *edodes* treatment a gradual decrease ($P < 0.05$) in L/C ratio and a gradual increase ($P < 0.05$) in the Ph-C0-2/Ph-C3 ratio was found during the 8 weeks of treatment. However, during the first 4 weeks of *L. edodes* treatment of wheat straw, the S- and G-lignin units were degraded simultaneously and also Cα-oxidized lignin compounds were formed. After 5 weeks, a preferential S-unit degradation started, but the production of Cα oxidized lignin compounds did not increase anymore. In wood chips, the main changes in L/C ratio, S/G ratio and Ph-C0-2/Ph-C3 ratio were found after 4 weeks of *C. subvermispora* and *L. edodes* treatment. Lignin composition in oak wood chips did not significantly change anymore in the last 4 weeks of the fungal treatment.

Three alkylitaconic acids were identified in both substrates treated with *C. subvermispora* including, tetradecylitaconic acid (peak **43**) and *cis*-7-hexadecanylitaconic acid (peak **44**) which increased after 1 week in treated wood chips and 2 weeks in treated wheat straw. Hexadecylitaconic acid (peak **45**) increased after 2 weeks in treated wood chips and 3 weeks in treated wheat straw, also increasing in time during the incubation period (Figs. S1 and S3). The ratio between the different alkylitaconic acids did not change from 3 weeks up to the end of the incubation period. An increasing amount of alkylitaconic acids were produced (Table 3), composed of 23% tetradecylitaconic acid (peak **43**), 65% *cis*-7-hexadecanylitaconic acid (peak **44**) and 12% hexadecylitaconic acid (peak **45**) after 3 weeks until the end of *C. subvermispora* treatment of wheat straw (Fig. S1). In oak wood chips the production of alkylitaconic acids stabilized from 5 weeks of *C. subvermispora* treatment (Fig. S3, Table 3). Alkylitaconic acids were not detected in wheat straw (Fig. S2) or oak wood chips (Fig. S4) treated with *L. edodes*.

3.4. In vitro rumen degradability

Total IVGP started at 223.4 $cm^3\ g^{-1}$ OM for the untreated wheat straw and increased 34.6% to 300.7 $cm^3\ g^{-1}$ OM after 8 weeks of *C. subvermispora* treatment and 27.7% to 285.3 $cm^3\ g^{-1}$ OM after 8 weeks of *L. edodes* treatment (Fig. 3a). During the first 4–5 weeks, the IVGP of wheat straw increased ($P < 0.05$) to remain similar after this period during both the *C. subvermispora* and *L. edodes* treatment (Fig. 3a).

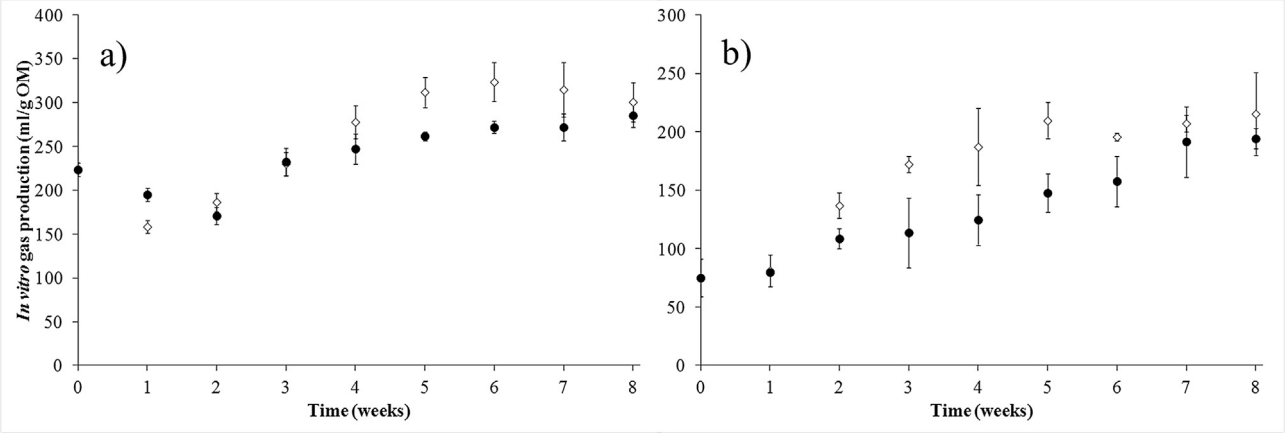

Fig. 3. *In vitro* gas production of *C. subvermispora* and *L. edodes* treated wheat straw and oak wood chips. a) wheat straw, b) oak wood chips. ◊ *C. subvermispora* ● *L. edodes*. Error bars represent standard deviations (n = 3).

The total IVGP started at 75.0 $cm^3\ g^{-1}$ OM for the untreated oak wood chips and increased 187.2% to 215.4 $cm^3\ g^{-1}$ OM after 8 weeks of *C. subvermispora* treatment and 158.8% to 194.1 $cm^3\ g^{-1}$ OM after 8 weeks of *L. edodes* treatment (Fig. 3b). The IVGP of oak wood chips increased ($P < 0.05$) during the first 3 weeks of *C. subvermispora* treatment and during the first 4 weeks of *L. edodes* treatments, after which the IVGP did not change significantly anymore (Fig. 3b).

3.5. Enzymatic saccharification

As a proof of principle, the enzymatic saccharification was only measured for wheat straw. Enzymatic saccharification with a mixture of cellulases (CM) for 72 h released most ($P < 0.05$) glucose in *L. edodes* treated wheat straw compared to *C. subvermispora* treated wheat straw (Fig. 4a). More ($P < 0.05$) glucose was released from fungal treated wheat straw compared to untreated wheat straw (Fig. 4a).

The amounts of xylose released per g of biomass were not significantly different between untreated and fungal treated materials after 72 h enzymatic saccharification (data not shown). The hemicelluloses left in the wheat straw was better accessible for enzymatic saccharification by CM, since more ($P < 0.05$) xylose was released from fungal treated material compared to untreated wheat straw after 72 h of enzymatic saccharification (Fig. 4b).

3.6. Correlations between IVGP and enzymatic saccharification

The IVGP of wheat straw correlated strongly to the glucose release by CM ($r = 0.92$, $P = 0.0004$), the xylose release by CM ($r = 0.78$, $P = 0.0128$). Strong negative correlations were found between the ADL mass fraction of the dry biomass and the IVGP of wheat straw ($r = -0.97$, $P < 0.0001$), the ADL mass fraction of the dry biomass and the glucose release by CM ($r = -0.94$, $P = 0.0001$), the ADL mass fraction of the dry biomass and the xylose release by CM ($r = -0.85$, $P = 0.0036$, expressed per g hemicelluloses). Also strong correlations were found between S/G ratio and IVGP or enzymatic saccharification. Correlations were found between the S/G ratio and the IVGP ($r = -0.99$, $P = 0.0002$), the S/G ratio and the glucose release by CM ($r = -0.97$, $P = 0.0016$), the S/G ratio and the xylose release by CM ($r = -0.91$, $P = 0.0121$, expressed per g hemicelluloses).

4. Discussion

Fungal degradation of wheat straw can be divided into two phases. The first phase can be defined by the degradation of easily accessible components such as starch, pectin and easily accessible hemicelluloses. Wheat straw contains, for example 3–4% starch [15]. Additionally, sorghum grains, which have a high starch mass fraction of the dry biomass, were used for fungal inoculation.

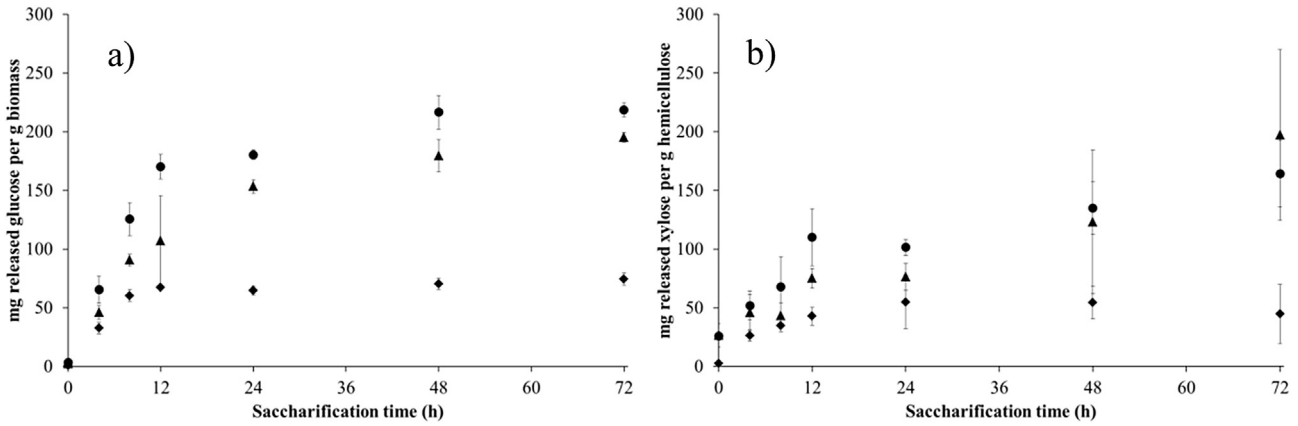

Fig. 4. Enzymatic saccharification of untreated, *C. subvermispora* and *L. edodes* treated wheat straw. a) glucose, b) xylose. ◆ untreated wheat straw, ▲ wheat straw treated for 8 weeks with *C. subvermispora*, ● wheat straw treated for 8 weeks with *L. edodes*. Error bars represent standard deviations (n = 3).

During fungal treatment, sorghum grains are also degraded, therefore, it is not possible to distinguish between the chemical composition or the effects on IVGP or enzymatic saccharification from sorghum or the substrates. The amount of sorghum grains was, however, low compared to the substrate. The degradation of these easily accessible compounds, either in small amounts originating from wheat straw or from the sorghum grains, might not be hindered by the presence of lignin and thus also easily accessible by the ruminant microflora. Removal of these compounds will thus lead to a decrease in IVGP compared to the untreated material as seen for wheat straw treated for 1 week with *C. subvermispora* or 2 weeks with *L. edodes*. During the first phase usually no significant lignin degradation occurs as shown in the fiber analysis (no decrease in ADL). However, the Py-GC/MS showed a significant decrease of the L/C ratio in fungal treated already during the first 2 weeks. In the first weeks, lignin degradation/modification in wheat straw started by oxidation of the Cα of lignin by *C. subvermispora*, which is indicated by the increase in acetovanillone (peak **30**).

The second phase can be defined as the delignification phase resulting in an increased IVGP. Significant delignification of wheat straw, as measured by the detergent fiber method, starts after 3 weeks of treatment for both fungi. This process, accompanied by a decrease in L/C ratio as measured by Py-GC/MS, continues until 5 weeks of *C. subvermispora* treatment and 8 weeks of *L. edodes* treatment. The continuous lignin degradation by *L. edodes* resulting in a continuous increase in IVGP of wheat straw was also described by Tuyen et al. [5]. Delignification starts by simultaneous degradation of S- and G-lignin units in wheat straw. However, a preferential degradation of S- over G-lignin units occurred after 4 weeks of *C. subvermispora* treatment and after 5 weeks of *L. edodes* treatment, as observed by the decrease in the S/G ratio from that point onwards. This is in line with the observation that G-units are more recalcitrant towards fungal attack because of a high condensation degree [16]. Preferential S-unit degradation shows that the fungi degrade mainly β-*O*-4-ether-linkages, the predominant linkages in S-units. The formation of degradation products, i.e. increase in side chain degradation represented by the Ph-C0-2/Ph-C3 ratio, indicates that also Cα-Cβ oxidative cleavage of lignin side chains took place during the fungal treatment from 4 weeks onwards. The increase in Ph-C0-2/Ph-C3 ratio is mainly determined by the decrease in Ph-C3 products such as eugenol (peak **21**), *cis*-isoeugenol (peak **24**), *trans*-isoeugenol (peak **25**), 4-allyl-syringol (peak **33**), *cis*-propenylsyringol (peak **35**) and *trans*-propenylsyringol (peak **36**). The fact that Ph-C0-2 products decrease in time suggests that upon fungal treatment these products are further metabolized. The negative correlation between S/G ratio on one hand and IVGP, glucose and xylose release on the other hand suggests that G-units are also more recalcitrant toward degradation by the fungi. However, in the current study, the changes in lignin composition are a direct result of lignin degradation. The real effect of changes in lignin composition on further processing cannot be separated from the effect of changes in lignin mass fraction of the dry biomass.

Delignification is accompanied by degradation of hemicelluloses by both fungi. Hemicelluloses are needed as an energy source for the fungi to grow and to produce enzymes. The ergosterol mass fraction increase in wheat straw throughout the *L. edodes* treatment indicates that this fungus might use hemicelluloses as source of energy. The hemicelluloses mass fraction of the dry biomassr of *C. subvermispora* treated wheat straw continuously decreased, while the ergosterol mass fraction only significantly increased after 6 weeks of treatment. Nevertheless, the hemicelluloses mass fraction of the dry biomass correlated significantly with the ergosterol mass fraction ($r = -0.73$) during the *C. subvermispora* treatment of wheat straw. An explanation for this correlation can be that this fungus produces xylanases and degrades/solubilizes the main structures of hemicelluloses, but not to xylose [17,18]. Solubilized hemicelluloses will dissolve in neutral detergent reagent and thus not be measured in the detergent fiber analysis as hemicelluloses, i.e. the hemicelluloses degradation is overestimated. Similarly, *Agaricus bisporus* lacks also enzymes to remove arabinosyl residues from doubly substituted xylose [19], which results in more solubilized hemicelluloses. Consequently, double substituted xylose cannot be used as an energy source by the fungus to grow. Degradation products of hemicelluloses are not included in the NDF minus ADF fraction. An extensive carbohydrate analysis should be done to ensure that hemicelluloses are degraded and to see whether monosaccharides originating from hemicelluloses are used by the fungus. It is not yet understood why *C. subvermispora* degrades hemicelluloses without using it for growth. Possibly hemicelluloses degradation cannot be avoided in the process of lignin degradation due to the tight (ether) bonds between the two components [20]. Hemicelluloses are described to hamper the enzymatic degradation of cellulose, therefore, hemicelluloses degradation might be required to increase cellulose accessibility [21]. Cellulose accessibility in wheat straw increased after 4 weeks of *C. subvermispora* treatment and after 5 weeks of *L. edodes* treatment, as indicated by the increased IVGP. On the other hand, the absolute amount of cellulose decreased after 3 weeks of *C. subvermispora* treatment and 5 weeks of *L. edodes* treatment. This means that although cellulose was degraded, the remaining cellulose after fungal treatment was better accessible for rumen microbes and enzymes than before treatment. The current study shows a decrease in the cellulose mass fraction of the dry biomass. However, literature shows that *C. subvermispora* has less genes for cellulases compared to other white rot fungi [22]. As a result *C. subvermispora* has an incomplete cellulose degrading system [18,23]. This may indicate that the ADF minus ADL fraction does not represent the whole cellulose mass fraction of the dry biomass. Similarly, underestimation of the carbohydrate fraction is also inherent to the Py-GC/MS method. Also, both the detergent fiber analysis and Py-GC/MS are generally calculated through ratios. This means that a more detailed, quantitative carbohydrate analysis of fungal treated material is recommended.

The Py-GC/MS data show the production of a series of alkylitaconic acids by *C. subvermispora*, despite the fact that ergosterol data do not show growth. The termination of production of secondary metabolites after active growth, as observed for *C. subvermispora*, has also been observed in other fungi [24]. Alkylitaconic acids were not found in the *L. edodes* treated wheat straw. The production of alkylitaconic acids started after 2 weeks of *C. subvermispora* treatment of wheat straw, similar as described for treatment of eucalyptus [11,25]. It is interesting to note that alkylitaconic acids are produced continuously throughout the treatment period, contrary to what occurs with ergosterol.

Alkylitaconic acids are involved in lignin degradation through a mechanism of lipid peroxidation by manganese peroxidase [25,26]. During this process, lipid radicals are formed that attack the most difficult to degrade, non-phenolic parts of the lignin molecule. Another theory states that alkylitaconic acids suppress hydroxyl radicals, which attack cellulose, that are released upon lignin degradation [27]. The cellulose mass fraction of the dry biomass did not significantly decrease during the first 5 weeks of *C. subvermispora* treatment, simultaneous to the increased production of alkylitaconic acids. Nevertheless, the cellulose mass fraction of the dry biomass was significantly lower after 8 weeks of *C. subvermispora* treatment compared to the control, while the production of alkylitaconic acids continued. The chemical changes in wheat straw may suggest that alkylitaconic acids are both involved in lipid peroxidation and preventing cellulose

degradation. This is the first time that the production of alkylitaconic acids has been studied in relation to fungal growth and fungal delignification of lignocellulose. The ergosterol measurements indicate that *C. subvermispora* grows mainly in the first week, stops growing in the next 4 weeks and might resume growth in the last 3 weeks. Unfortunately, the ergosterol measurements were insufficiently accurate in the last weeks. In these last weeks, the fungus formed some clusters of dense tissue (data not shown) which may have caused the variation in ergosterol measured. Nevertheless, the measurements show that the production of alkylitaconic acids and the degradation of lignin, measured as Cα-oxidation and Ph-C0-2/Ph-C3 ratio, both increase independent of the fungal growth.

Preferential lignin degradation by the fungi was also confirmed by microscopy using a combination of Safranin O and Astra Blue staining based on the method of Srebotnik and Messner [10]. Astra blue only stains cellulose in the absence of lignin and is an indirect measure for selective lignin degradation [10]. This indicates how lignin levels in the cell wall matrix can mask cellulose and prevent its staining. The degradation of plant cell walls from the cytoplasmic side of the cells is often seen in fungi that selectively degrade lignin [28]. In the current study Safranin O and Astra Blue staining did not stain fungal hyphae. Toluidine Blue showed the presence of fungal hyphae in treated wheat straw and oak wood chips. The presence of subcellular details inside hyphae indicates that the aldehyde fixation and material processing was done proper.

Enzymatic saccharification was conducted on wheat straw after 8 weeks of fungal treatment, although enzymatic saccharification would also be interesting for wood chips or wheat straw at different time points. After 8 weeks of fungal treatment, most lignin and hemicellulose was degraded in wheat straw, creating the largest contrast with the initial material, and the low amount of lignin increased the possibilities for the enzymes to reach the cellulose. As shown by the chemical analysis and the IVGP measurements, *C. subvermispora* reached an optimal IVGP and lignin degradation after 5 weeks of treatment, after which the fungus started to degrade cellulose. As a result, less cellulose was available for enzymatic saccharification after 8 weeks of treatment, resulting in a lower glucose release upon CM incubation than in *L. edodes* treated wheat straw. Interestingly, the mixture of cellulases contained some xylanase activity, since xylose was released after CM incubation. Here the terms glucose and xylose release are used, while in other studies the term sugar yield is used. Sugar yield is the amount of released sugar from the total amount of sugar present in the biomass [29]. During enzymatic saccharification, glucose is released from glucan and xylose from xylan. However in the current study the initial amounts of glucan and xylan were not measured such that sugar yields could not be calculated. In the scientific literature, only a 2–25% increase in glucose yield and a 10% increase in xylose yield were reported in wheat straw after 35 days of *C. subvermispora* treatment [29,30]. In the current study, *C. subvermispora* treatment resulted in 163% more glucose and 341% more xylose compared to untreated wheat straw, indicating that *C. subvermispora* made relatively more cellulose accessible for enzymes compared to untreated wheat straw. However, comparison with the scientific literature is difficult, since in the current study different enzymes and batches of wheat straw were used and sugar yields could not be calculated. In the current study, fungal treatment showed an increased release of glucose and xylose. Based on this result it is advised to measure enzymatic saccharification throughout the entire fungal treatment period.

Accessibility to cellulose and hemicelluloses can be increased by degradation of lignin, or breaking the bonds between lignin and carbohydrates. The strong correlations between the IVGP, the enzymatic saccharification and ADL confirm that the accessibility can be increased for both rumen microbes as enzymes by the same fungal treatment. Likely, the same theory about accessibility of carbohydrates can be applied to oak wood chips.

Unlike wheat straw, oak wood chips probably did not contain easily accessible nutrients, since the IVGP did not decrease in the first week. In addition, ADL degradation by *L. edodes* and *C. subvermispora* started already after 2 weeks of treatment and the L/C ratio numerically decreased already after 1 week of fungal treatment. Also, hemicelluloses degradation only started after 2 weeks of treatment, while in wheat straw it started in the first week. This suggests that hemicelluloses are less accessible in oak wood chips. The lower accessibility in oak wood chips can be explained by incomplete fungal colonization due to the dense structure of oak wood chips as observed by microscopy. Colonization, and delignification, not only requires physical space for the fungus to grow, but also oxygen within the tissue. The fact that the percentage of Cα-oxidized lignin in wood chips does not clearly increase upon fungal treatment, suggests that the availability of oxygen is the limiting factor. Indeed, *L. edodes* mycelium does not grow well where oxygen is limited, and when it grows actively the O_2 demand becomes even much higher than that of other fungi. O_2 and CO_2 are important factors in the cultivation of mushrooms [31]. To increase the availability of oxygen, the wood structure has therefore first to be degraded to allow oxygen entry before further degradation and growth can occur. This stepwise “delignification and colonization” requires a longer treatment time for biomass with a dense structure like oak wood. A strategy to decrease treatment time is to increase the surface to volume ratio of dense biomass to allow for more entry points for fungi.

The second phase in the fungal treatment of oak wood chips was characterized by little changes in composition of the substrate after 4 weeks of treatment. Ergosterol data showed that *C. subvermispora* only grew during the first week of colonization. On oak wood chips, in contrast to wheat straw, no further growth of the fungus was observed. Similarly, Messner et al. [32] showed a plateau in ergosterol development during *C. subvermispora* treatment of oak wood chips. The fungal growth stopped between 6 and 14 days to continue again afterwards [32]. These authors confirmed this observation by the temperature development, as the temperature did not change during the plateau period in ergosterol. Messner et al. [32] suggested that lignin degradation should take place before carbohydrates can be degraded by the fungus. Lignin is degraded by *C. subvermispora* without growing. The production of alkylitaconic acids shows that the fungus is producing secondary metabolites without active growth (as measured by ergosterol mass fraction). The fungus cannot grow until the carbohydrates are accessible, meaning that lignin should be degraded first. Messner et al. [32] described manganese peroxidase activity to be high during the plateau in the ergosterol data. This indicates that lignin degradation is the first step in the degradation process by *C. subvermispora*. However, in the current study *C. subvermispora* degraded lignin and hemicelluloses without changing the cellulose mass fraction of the dry biomass in both wheat straw and oak wood chips during the plateau period in ergosterol.

5. Conclusions

The present experiments show various aspects of fungal delignification that occur in parallel and their combined results give a better understanding of the lignocellulose biodegradation in general. The white rot fungi *C. subvermispora* and *L. edodes* preferentially degrade lignin, without changing the cellulose mass fraction of the dry biomass, during growth on wheat straw and oak wood chips. Most chemical changes occurred during the first 4 weeks of fungal treatment, and on both substrates, *C. subvermispora*

degraded more lignin than *L. edodes*. Both fungi have a different strategy in degrading the lignocellulosic materials. *L. edodes* continuously grows and degrades lignin during the growth, while *C. subvermispora* colonizes the material predominantly during the first week and degrades lignin and hemicelluloses without growing. The density of biomass seems to limit the infiltration by fungi. As a result of the selective lignin degradation, the IVGP and the sugars released upon enzymatic saccharification increases.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biombioe.2017.07.003.

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