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Accumulation of recalcitrant xylan in mushroom-compost is due to a lack of xylan substituent removing enzyme activities of *Agaricus bisporus*

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ABSTRACT

The ability of *Agaricus bisporus* to degrade xylan in wheat straw based compost during mushroom formation is unclear. In this paper, xylan was extracted from the compost with water, 1 M and 4 M alkali. Over the phases analyzed, the remaining xylan was increasingly substituted with (4-*O*-methyl-)glucuronic acid and arabinosyl residues, both one and two arabinosyl residues per xylosyl residue remained. In the 1 M and 4 M KOH soluble solids of spent compost, 33 and 49 out of 100 xylosyl residues, respectively, were substituted. The accumulation of glucuronic acid substituents matched with the analysis that the two *A. bisporus* genes encoding for α-glucuronidase activity (both GH115) were not expressed in the *A. bisporus* mycelium in the compost during fruiting. Also, in a maximum likelihood tree it was shown that it is not likely that *A. bisporus* possesses genes encoding for the activity to remove arabinose from xylosyl residues having two arabinosyl residues.

1. Introduction

The commercial production of wheat straw based compost for growth of *Agaricus bisporus* is a process consisting of two indoor phases, in which the mixture of raw materials is converted into a substrate supporting the growth of *A. bisporus* (Gerrits, 1988; Jurak, Kabel, & Gruppen, 2014). At the end of the second composting phase (PII), compost is inoculated with *A. bisporus* mycelium, which is grown in the compost for a period of about 16 days after which compost is considered mature (PIII-16). Next, compost is distributed over mushroom growth beds (Filling) and covered with a casing layer, which is a mixture of peat and lime, to induce fruiting body formation (Hayes, Randle, & Last, 1969). In an industrial process, usually two flushes of mushrooms are collected and after mushrooms are harvested, compost is considered spent. This spent compost still contains about 11% (w/w) of carbohydrates (2.6% w/w xylosyl and 7.6% w/w glucosyl residues) and 21% (w/w) lignin based on dry matter (Iiyama, Stone, & Macauley, 1994). The exact structures of the remaining carbohydrates in spent compost are not known. Therefore, analysis of these remaining structures is the aim of this research. It is expected that detailed elucidation of the remaining structures will help to find process improvements, e.g. for an increase in the amounts of mushrooms per flush or more flushes.

In wheat straw xylan, the xylosyl residues can carry one or two arabinosyl residues and also (4-*O*-methyl-)glucuronic acid (Fincher, 2009). The exact level and distribution of these substituents, however, is not known. In PIII-16 compost, 11% xylan (w/w) is present, next to 14% glucan (w/w), both as mycelial glucan and cellulose, and 33% (w/w) lignin (Jurak et al., 2014). The xylan in PIII-16 has ratios of arabinosyl and glucuronic acid residues to 100 xylosyl residues, of 14 and 13, respectively (Jurak et al., 2014). In order to achieve a complete saccharification of xylan, and thereby a complete use of this carbohydrate in the compost, all the

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substituents need to be removed. Genes encoding for (putative) enzymes from the carbon metabolism of *A. bisporus* were recently identified. It was shown that in compost during 1st flush, *A. bisporus* mycelium expresses a variety of genes known to be involved in the degradation of both xylan and cellulose (Patyshakuliyeva et al., 2013). However, the expression of genes encoding for (putative) enzymes degrading xylan substituents was not analyzed in detail, and only analyzed for the compost obtained at 1st flush.

In addition to the degradation of xylan and cellulose, it has been shown that *A. bisporus* is able to degrade radioactively labeled lignin (Durrant, Wood, & Cain, 1991). During the mycelium growth of *A. bisporus* (PIII) laccase activity is reported to be the highest compared to other growth stages of this fungus (Bonnen, Anton, & Orth, 1994). It coincides with the reported metabolization and modifications of lignin in the same mycelium growth phase (Jurak, Punt, Arts, Kabel, & Gruppen, 2015). During fruiting body formation, laccase levels are found to decline (Ohga, Smith, Thurston, & Wood, 1999.), which may indicate that lignin is not further degraded. Since lignin is known to inhibit enzymes, like cellulases and xylanases the remaining lignin in the compost probably inhibits saccharification of the compost carbohydrates (Berlin et al., 2006). No knowledge, however, on the lignin content and structures during fruiting body formation in the compost is available to date.

In order to elucidate the recalcitrant xylan structures in the compost throughout the fruiting body formation, xylan was extracted from the compost and analyzed for its structural characteristics. In addition, lignin was analyzed in the compost by Py-GC/MS. The genome of *A. bisporus* was assessed to identify genes, which encode for xylan substituent degrading enzymes. The expression of these genes in compost during fruiting was taken into account to discuss the observed recalcitrant and remaining xylan structures in the same samples. This research is expected to contribute to the understanding of the carbohydrate utilization in compost during fruiting of *A. bisporus*, which may help to improve the process further.

2. Materials and methods

2.1. Materials used

Compost obtained after filling of the beds with mycelium grown compost (PIII-16) and covered with casing soil (Filling), after pinning of *A. bisporus* (Pinning; 13 days after filling), after the first flush (1st flush; 23 days after filling) and after the second flush (2nd flush; 31 day after filling) of mushrooms were supplied by CNC (CNC-C4C, Milsbeek, The Netherlands). All compost samples were from the same batch and timeline. About one kg of compost, in duplicate, was collected and frozen and for this research 100 g of frozen sample was further freeze dried and milled (<1 mm) (MM 2000, Retsch, Haan, Germany). Duplicates were mixed in ratio 1:1 in order to obtain one representative sample.

2.2. Water and alkali extracts from compost

Freeze dried, mixed and milled compost samples (6–12 g dry matter (DM)) were suspended in millipore water (ratio DM:liquid = 1:8.3) and boiled at 100 °C for 5 min. Next, suspension were stirred for 3 h at 4 °C under continuous stirring. After centrifugation (10,000 × *g*; 30 min; 20 °C), the residue was washed twice with millipore water (100 mL per wash). Supernatants were combined (WSS) and part of the supernatant was dialyzed and collected as dialyzed water soluble solids (WSSd). The residues were recovered as water un-extractable solids (WUS). The yield of the non-dialyzed water extracts was calculated as the weight difference between the original freeze dried compost sample and the corresponding WUS. Next, 5 g of WUS was suspended in 200 mL 1 M KOH containing 1% (w/w) $NaBH_4$ for 18 h at room temperature under continuous stirring. After centrifugation (10,000 × *g*; 30 min; 20 °C), the residue was washed with 200 mL 1 M KOH containing 1% (w/w) $NaBH_4$ and again centrifuged. Supernatants were combined, neutralized with acetic acid, extensively dialyzed (10–12 kDa cut-off, Medicell International, London, UK) against distilled water and freeze dried (1 M KOHss). The residue was re-extracted and washed once with 4 M KOH containing 1% (w/w) $NaBH_4$, as described for the 1 M KOH extraction. Supernatants were combined and the final residue was collected. The 4 M KOH supernatants and residues were neutralized with acetic acid, extensively dialyzed (10–12 kDa cut-off, Medicell International, London, UK) against distilled water and freeze dried, coded as 4 M KOHss and Res for the 4 M KOH extracts and residues, respectively.

The yield of xylan (% w/w) over WSS, WUS, 1 M KOHss, 4 M KOHss and Res was calculated based on the total xylan content as sum of the content of arabinosyl, xylosyl and uronyl-residues, and the recovery of total dry matter in the various fractions.

Fractions were re-suspended in water and after centrifugation the water soluble part was analyzed by HPSEC and HPAEC. For 1 M KOHss the water solubility was 75–90% (w/w) and for 4 M KOH 40–60% (w/w).

2.3. Hydrolysis of alkali extracts with endoxylanase I (GH10), α-arabinofuranosidase (GH43) and α-glucuronidase (GH67)

Suspensions of 1 M and 4 M KOHss (20 mg KOHss) in 50 mM sodium acetate buffer pH 5 (977 μL) were incubated overnight at 35 °C, while rotating, with a pure endo-(1,4)-β-D-xylanase 1 (EX1) from *Aspergillus awamori* (115 μL, 2.75 μg protein) (Kormelink, Gruppen, Viëtor, & Voragen, 1993). After inactivation of the enzyme (10 min, 100 °C) digests were analyzed by HPSEC (non diluted), HPAEC (diluted 20× in water). Wheat arabinoxylan (medium viscosity) and birchwood xylan, medium viscosity (Megazyme, Wicklow, Ireland) were treated similarly and used as a reference.

After inactivation of the enzyme (10 min, 100 °C) 100 μL of EX1 digested 1 M and 4 M KOHss of compost (2nd flush) of supernatant was, subsequently, hydrolyzed (24 h, incubated in the oven at 55 °C while rotating) with a pure GH43 AXH-d3 arabinofuranosidase (30 μL, 2.7 mg mL^{-1}) (Van Laere et al., 1999). Also, of EX1 digested 1 M and 4 M KOHss of compost (2nd flush) 100 μL was incubated with a partially purified α-glucuronidase GH67 (30 μL, 23 μg mL^{-1}) (Verbruggen, Baldman, & Voragen, 1998).

2.4. Carbohydrate content and composition

The neutral carbohydrate and uronic acid content and composition was determined in duplicate, as described by Jurak et al. (2014).

2.5. Pyrolysis-GC/MS

The composition of lignin was determined in triplicate, as described elsewhere (Jurak, Punt, et al., 2015).

2.6. HPSEC

High-performance size-exclusion liquid chromatography (HPSEC) was performed on an Ultimate 3000 HPLC system (Thermo Scientific, Sunnyvale, CA, USA) equipped with three TSK-gel columns (6.0 mm × 15.0 cm per column) in series (SuperAW4000, SuperAW3000, SuperAW2500, Tosoh Bioscience, Stuttgart, Germany) in combination with a PWX-guard column (Tosoh Bioscience, Stuttgart, Germany). HPSEC was controlled by the Chromelion software (Thermo Scientific, Sunnyvale, CA, USA). Elution took place at 40 °C with 0.2 M sodium nitrate at a flow rate

of 0.6 mL min^{-1}. The eluate was monitored using a refractive index (RI) detector (Shodex RI-101, Kawasaki, Japan). Calibration was performed by using pullulans (Polymer Laboratories, Union, NY, USA) with a molecular weight in the range of 0.18–788 kDa.

2.7. HPAEC

High-performance anion exchange chromatography (HAPEC) was performed on a Dionex ICS-5000 unit (Dionex, Sunnyvale, CA, USA) equipped with a CarboPac PA-1 column (2 mm × 250 mm ID) in combination with a CarboPac guard column (2 mm × 50 mm ID) and PAD detection. The system was controlled by Chromelion software (Thermo Scientific, Sunnyvale, CA, USA). Elution and quantification of mono- and oligosaccharides (0.3 mL min^{-1}) was performed with a combination of linear gradients from two types of eluents, A: 0.1 M NaOH and B: 1 M NaOAc in 0.1 M NaOH. The elution profile was as following: 0–35 min: 0–38% B, cleaning step 3 min 100% B, equilibration step 12 min 100% A.

2.8. Microscopy imaging and image analysis

Samples were dissolved in water and mounted on a coverglass for imaging. A coverslip was placed on top. For particle analysis imaging was performed with an upright Microscope (Axioskop, Carl Zeiss, Oberkochen, Germany) in brightfield mode with an 10X, 0.3 NA dry objective (Carl Zeiss AG, Oberkochen, Germany). A low magnification objective was selected in order to have a relatively big field of view (1.4 × 1 mm^2). Images were processed with ImageJ (National Institutes of Health, Bethesda MD, USA). First a flat field correction filter was applied. Afterwards the images were segmented using a simple threshold method. The triangle threshold method of ImageJ was applied and the threshold value was optimized for each image.

The cross sectional surface of the particles in the images was measured with the 'Particle analysis' plugin of ImageJ. 10 images were analyzed for each case. Particles that were on the border of the images were excluded from the analysis.

2.9. Molecular analysis

To generate the maximum likelihood (ML) tree the amino acid sequences used were aligned using MAFFT (Katoh, Kuma, Toh, & Miyata, 2005) and manually corrected in MEGA 5 (Tamura et al., 2011). Biochemically characterized GH43 proteins of different functionality were included to allow separation based on function. In addition to the four GH43 proteins present in the genome of A. bisporus, all GH43 proteins from the white rot basidiomycete Dichomitus squalens were included for comparative purposes. Four fungal GH54 proteins were used as outgroup. The ML statistical method was performed using the WAG amino acid substitution model (Whelan & Goldman, 2001) with gamma distributed site rates and an invariable site category, as this model provided the best fit to the data.

The phylogenetic analysis, RNA extraction, cDNa library preparation and RNA seq was performed as described by Patyshakuliyeva et al. (2015).

3. Results

3.1. Carbohydrate content and composition of compost and associated fractions

The total carbohydrate content (% w/w, based on dry matter (DM)), molar carbohydrate composition and dry matter extraction

Table 1
Total carbohydrates content (% w/w) based on dry matter (DM) and molar carbohydrate composition (mol%) of compost and associated fractions obtained after Filling, after Pinning, after 1st flush and after 2nd flush. Also, the yield of dry matter in the various fractions, water soluble solids (WSS), water un-extractable solids (WUS), alkali soluble solids (1 M KOHss and 4 M KOHss) and residue (Res) is included.

Sample name	Carbohydrate composition (mol%)[a]							Total carbohydrates (w/w % DM)	Yield DM (%)
	Rha	Ara	Xyl	Man*	Gal	Glc	UA		
Filling	1 ± 0.2	6 ± 0.6	34 ± 1.9	5 ± 0.6	3 ± 0.3	44 ± 4	6 ± 0.4	21.9 ± 2.0	100
WSS	3 ± 0.3	6 ± 0.0	12 ± 0.2	25 ± 0.3	10 ± 0.0	33 ± 0.1	11 ± 0.1	8.8 ± 0.3	23.1
WSSd	6 ± 0.0	9 ± 0.3	17 ± 0.1	10 ± 0.0	13 ± 0.4	26 ± 0.3	19 ± 0.2	17.1 ± 0.3	X[b]
WUS	1 ± 0.2	5 ± 0.6	31 ± 1.9	2 ± 0.1	2 ± 0.1	54 ± 2.4	6 ± 0.5	27.1 ± 1.5	70.8
1 M KOHss	4 ± 0.9	8 ± 0.1	74 ± 1.4	1 ± 0.0	1 ± 0.0	5 ± 0.4	7 ± 0.1	25.2 ± 0.7	18.1
4 M KOHss	2 ± 0.0	8 ± 0.3	53 ± 0.9	2 ± 0.0	4 ± 0.2	24 ± 2.5	8 ± 0.4	20.7 ± 0.7	6.3
Res	1 ± 0.1	4 ± 0.4	9 ± 1.2	3 ± 0.3	2 ± 0.2	78 ± 2.5	5 ± 0.4	35.5 ± 3.1	36.7
Pinning	1 ± 0.4	4 ± 0.3	28 ± 0.9	6 ± 0.6	2 ± 0.2	53 ± 2.5	6 ± 0.2	22.8 ± 1.2	100
WSS	4 ± 0.1	7 ± 0.3	14 ± 0.1	26 ± 0.5	6 ± 0.1	27 ± 0.2	16 ± 0.3	8.3 ± 0.2	22
WSSd	5 ± 0.2	9 ± 0.3	15 ± 0.5	8 ± 0.0	10 ± 0.1	32 ± 0.4	21 ± 0.4	15.9 ± 0.1	X[b]
WUS	1 ± 0.1	4 ± 0.1	28 ± 0.6	3 ± 0.3	2 ± 0.0	57 ± 0.4	6 ± 0.3	29.2 ± 0.4	72.9
1 M KOHss	1 ± 0.0	8 ± 0.1	73 ± 0.2	1 ± 0.2	2 ± 0.1	8 ± 0.3	7 ± 0.5	22.7 ± 0.2	20.3
4 M KOHss	1 ± 0.2	7 ± 0.1	55 ± 2.3	3 ± 0.1	3 ± 0.1	24 ± 2.8	8 ± 0.9	26.0 ± 0.8	4.3
Res	1 ± 0.1	2 ± 0.1	7 ± 0.1	3 ± 0.1	1 ± 0.2	82 ± 0.2	4 ± 0.0	41.1 ± 0.9	40.6
1st flush	2 ± 0.4	6 ± 0.1	25 ± 0.2	9 ± 0.2	3 ± 0.3	47 ± 0.3	8 ± 0.2	17.6 ± 0.1	100
WSS	4 ± 0.1	7 ± 0.0	15 ± 0.3	26 ± 0.5	6 ± 0.0	28 ± 0.3	14 ± 0.3	10.2 ± 0.3	28.7
WSSd	5 ± 0.1	10 ± 0.1	18 ± 0.0	8 ± 0.0	10 ± 0.0	29 ± 0.4	20 ± 0.2	14.6 ± 0.2	X[b]
WUS	1 ± 0.1	6 ± 0.1	27 ± 0.2	4 ± 0.1	3 ± 0.3	52 ± 0.8	8 ± 0.2	17.0 ± 0.6	69.5
1 M KOHss	5 ± 4.1	10 ± 0.1	60 ± 0.3	2 ± 0.2	3 ± 0.1	11 ± 0.2	11 ± 3.6	14.3 ± 0.4	21.6
4 M KOHss	8 ± 4.7	8 ± 0.4	43 ± 4.4	5 ± 0.2	3 ± 0.2	26 ± 1.0	7 ± 2.4	20.0 ± 1.5	4.7
Res	1 ± 0.0	3 ± 0.2	8 ± 0.0	6 ± 0.3	2 ± 0.1	76 ± 0.9	4 ± 0.3	30.0 ± 2.6	36.2
2nd flush	2 ± 0.0	6 ± 0.0	24 ± 0.2	8 ± 0.7	3 ± 0.1	49 ± 0.4	8 ± 0.3	15.7 ± 0.5	100
WSS	6 ± 0.5	11 ± 0.9	19 ± 0.5	18 ± 2.1	8 ± 0.4	20 ± 0.1	18 ± 0.3	7.7 ± 0.0	26.5
WSSd	8 ± 0.4	13 ± 0.2	21 ± 0.4	8 ± 0.3	10 ± 0.2	19 ± 01	21 ± 0.3	12.8 ± 0.4	X[b]
WUS	2 ± 0.3	6 ± 0.2	26 ± 0.9	5 ± 0.7	3 ± 0.1	52 ± 0.9	8 ± 0.3	17.0 ± 0.9	65.6
1 M KOHss	2 ± 0.2	10 ± 0.2	61 ± 3.3	2 ± 0.4	2 ± 0.2	12 ± 2.9	10 ± 0.2	13.3 ± 1.1	17.2
4 M KOHss	2 ± 0.3	9 ± 0.2	40 ± 0.1	5 ± 0.0	4 ± 0.1	29 ± 0.4	11 ± 0.3	16.4 ± 0.0	8.3
Res	1 ± 0.2	4 ± 0.1	9 ± 0.6	6 ± 0.4	2 ± 0.1	72 ± 0.4	6 ± 0.1	22.9 ± 1.5	31.7

[a] Rha = ramnosyl, Ara = arabinosyl, Xyl = xylosyl, *Man = mannosyl = mannitol, Gal = galactosyl, Glc = glucosyl, UA = uronyl.
[b] WSSd not included in the yield. WSS – non dialyzed, WSSd – dialyzed.

yield of the compost obtained after Filling, Pinning, 1st flush, , 2nd flush, and associated fractions, is presented in Table 1.

The total carbohydrate content of Filling is 22% (w/w), of Pinning is 23% (w/w), of 1st flush 18% (w/w) and of 2nd flush 16% (w/w) based on dry matter. The recovery after the water extraction of dry matter in WSS and WUS was 91% (w/w) or higher. Next, the total carbohydrate content in WSS was 9% (w/w), 8% (w/w), 10% (w/w) and 8% (w/w) and for WUS 27% (w/w), 29% (w/w), 17% (w/w) and 17% (w/w), for compost at Filling, Pinning, 1st flush and 2nd flush, respectively. Overall, after water extraction most of the material (65% or more (w/w)) was recovered in the WUS fractions. The recovery after the alkaline extraction from WUS in 1 M KOHss, 4 M KOHss and Res was 86% or higher (Table 1). These yields showed that only less than 15% of the total dry matter was not recovered in one of the fractions, most likely, due to sample handling.

For all compost samples, the main carbohydrates present were constituted of xylosyl (24–34 mol%) and glucosyl (44–53 mol%) residues. The WSS constituted mainly of glucosyl 20–33 mol%), mannosyl (18–25 mol%) and xylosyl (12–19 mol%) residues. High mannose content is expected to originate from mannitol produced by *A. bisporus* mycelium (Patyshakuliyeva et al., 2013). The 1 M and 4 M KOHss were enriched in xylosyl (Xyl) residues, 60–74 mol% and 40–55 mol%, respectively, next to 7–10 mol% arabinosyl (Ara) and 7–11 mol% uronyl (UA) residues. In addition, 1 M KOHss contained 5–12 mol % and 4 M KOHss 24–29 mol% of glucosyl residues. The residues (Res) were mainly composed of glucosyl residues, 72–82 mol%, next to 7–9 mol%, 2–4 mol% and 4–6 mol% of xylosyl, arabinosyl (Ara) and uronyl (UA) residues, respectively.

3.2. The distribution and structural characteristics of xylan in WSS, 1 M KOHss and 4 M KOHss

The distribution of total xylan, calculated as the sum of Xyl, Ara and UA residues, assumed to be all building blocks of xylan, from the compost samples at Filling, Pinning, 1st flush and 2nd flush over WSS, WUS, 1 M KOHss and 4 M KOHss is presented in Table 2. From the total xylan in the original compost sample 81–93% was recovered in WUS and 8–19% in the WSS. Overall, throughout fruiting body formation the amount of water soluble xylan increased, from Filling to 2nd flush 7.5% and 19.2%, respectively. However, the majority of xylan remained in WUS, hence more difficult to access. Following, from the total xylan in WUS, 48–54% was recovered in the 1 M KOHss fractions, 10–12% in the 4 M KOHss fractions, and 24–28% in the Res (Table 2).

To look further into the structural characteristics of the xylans, first, the WSS, and the water soluble part of the alkali extracts was analyzed on HPSEC (Supplementary Fig. 1, Fig. 1A and B) as part of alkali extracted xylan became insoluble upon dialysis (see Section 2.2). For all WSS fractions similar elution profiles were observed (0.75–6 kDa). Further, similar HPSEC profiles were observed for all 1 M KOHss xylan fractions, and also all the 4 M KOHss xylan fractions showed a similar elution pattern.

Next, the degree of substitution (DS) of xylan with either Ara or UA residues was evaluated for xylan present in Filling, Pinning, 1st flush, 2nd flush, and associated fractions and is presented in Table 2. At Filling and at Pinning, in 1 M KOHss, the DS Ara and DS UA were 11 and 10, respectively. However, after 1st flush, the DS Ara and UA increased to 17 and 18, respectively. Similar DS values were observed after 2nd flush for the 1 M KOHss fraction. For the 4 M KOH fractions the DS remained rather similar at Filling, Pinning and 1st flush (12-17 DS Ara and 15-15 DS UA), however, after 2nd flush in the 4 M KOHss the DS Ara and UA increased to 21 and 28, respectively.

Next, the molecular weight distribution of the alkali extracts after digestion with a pure endoxylanase (EX1) (Kormelink et al., 1993) was analyzed on HPSEC (Fig. 1C and D). For all fractions, a large part of material was found not to be degraded and a small amount of oligomers were observed. The 1 M KOHss from Filling appeared to be degraded to a larger extent than 1 M KOHss from 2nd flush.

From the 1 M and 4 M KOHss of 2nd flush hydrolyzed with EX1 used xylose, xylobiose and xylotriose were released. In addition, substituted XOS were released (Fig. 2). Based on elution profile and retention times of XOS from birchwood xylan and wheat arabinoxylan, using the same EX1, it was shown that the substituted XOS released from both 1 M and 4 M KOHss included glucuronic acid substituted XOS and XOS having one or two arabinosyl substituents per xylosyl residues (Fig. 2).

To confirm the presence of XOS having 2 arabinosyl residues per xylosyl residue, EX1 hydrolyzed xylan fractions, 1 M and 4 M KOHss, from compost after 2nd flush were incubated with a pure arabinofuranosidase (AXH-d3) only able to remove only arabinosyl residues from double substituted XOS with arabinosyl-residues, specifically terminal arabinosyl residues (Van Laere, Beldman, & Voragen, 1997; Van Laere et al., 1999). From 1 M KOHss 2nd flush 20% of the total arabinosyl residues present was released after digestion as monomeric arabinose, and 10% from 4 M KOHss 2nd flush. As AXH-d3 releases arabinosyl residues from double substituted XOS this indicated that at least 40% of all arabinosyl residues present in the 1 M KOHss and at least 20% in the 4 M KOHss of 2nd flush were present as double substitutions in XOS. In addition, to confirm the presence of XOS having (4-*O*-methyl-)glucuronic acid substituents, EX1 digested xylan fractions were incubated with a purified α-glucuronidase (GH67). Only glucuronic acid was released and quantified. From 1 M to 4 M KOHss of 2nd flush, 9% and 2%, of total glucuronic acid present was released, respectively.

Table 2
Degree of substitution of xylan (mol/100 mol Xyl) and normalized yield of xylan (as sum of arabinosyl (Ara), uronyl (UA) and xylosyl (Xyl) residues) in different fractions of compost obtained after Filling, Pinning, 1st flush and 2nd flush. Water soluble fraction (non-dialysed: WSS, dialyzed WSSd), water un-extractable solids (WUS), alkali soluble solids (1 M KOHss and 4 M KOHss) and residue (Res).

	Degree of substitution (mol/100 mol)		Normalized yield xylan (%)
	Ara/Xyl	UA/Xyl	
Filling	16	18	100.0
WSS	52	96	7.5
WSSd	95	110	6.0
WUS	16	18	92.5
1 M KOHss	11	10	54.1
4 M KOHss	14	15	11.5
Res	40	54	27.6
Pinning	15	21	100.0
WSS	50	116	11.0
WSSd	58	139	8.9
WUS	15	21	89.0
1 M KOHss	11	10	52.1
4 M KOHss	12	15	11.0
Res	33	64	26.6
1st flush	21	31	100.0
WSS	47	92	18.2
WSSd	57	111	12.2
WUS	21	31	81.8
1 M KOHss	17	18	47.9
4 M KOHss	17	16	10.1
Res	41	48	24.5
2nd flush	22	29	100.0
WSS	60	95	19.2
WSSd	58	95	9.9
WUS	22	29	80.8
1 M KOHss	16	17	47.2
4 M KOHss	21	28	10.0
Res	39	67	24.1

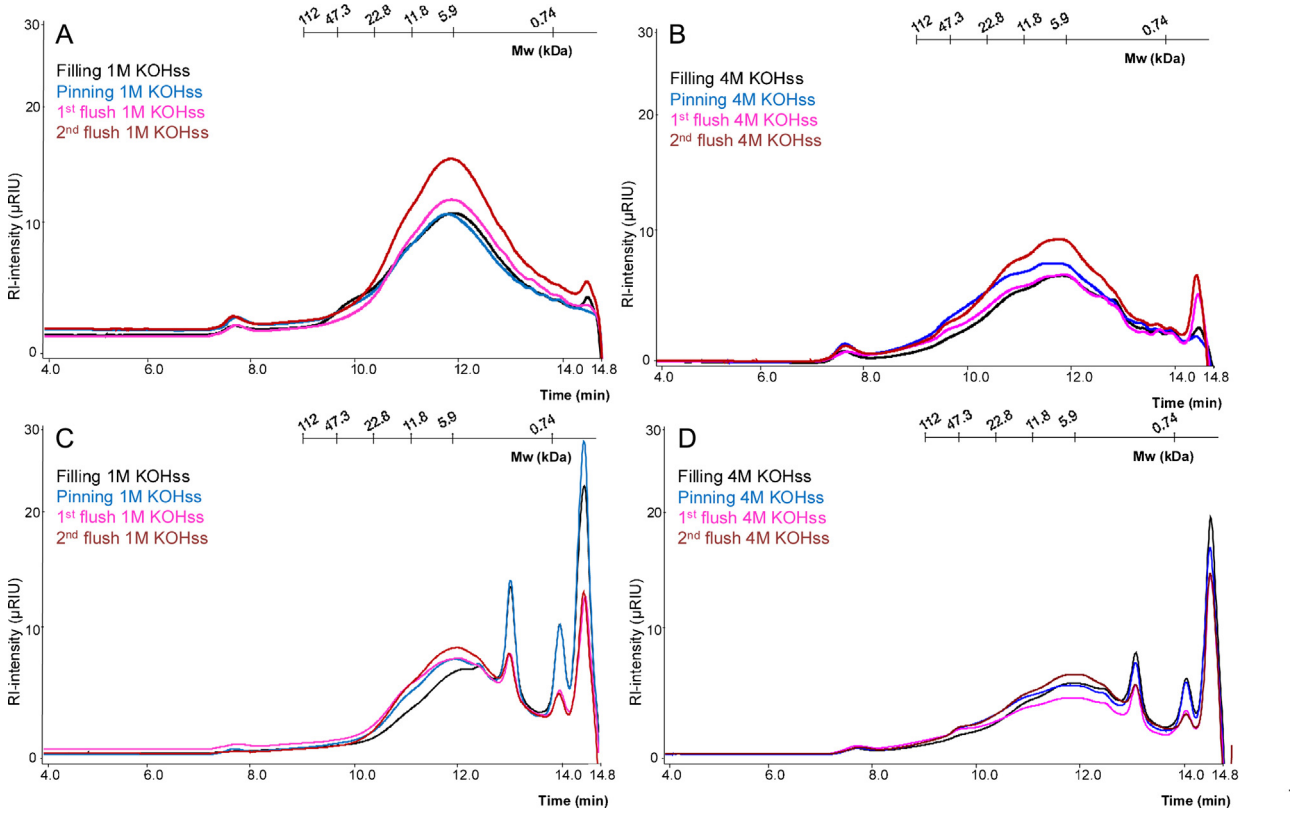

Fig. 1. Molecular weight distribution of 1 M KOHss extracts Filling, Pinning, 1st flush and 2nd flush before digestion (A) and endpoint digested (C); and 4 M KOHss extract before digestion (B) and endpoint digested (D). A purified and well characterized endoxylanase (EX1) (Kormelink et al., 1993), was used for digestion.

As mentioned above, the soluble part of the alkali extracts was analyzed by HPSEC and HPAEC. The remaining insoluble xylan of the 1 M KOHss and 4 M KOHss from compost obtained at Filling and at 1st flush was analyzed by microscopy and corresponding images of the insoluble particles are presented in Fig. 3. The particle analysis imaging of 1 M KOHss Filling and 1st flush showed material, although only a low amount of particles were visible in the sample due to a relatively high water solubility of this material. The observed particles sizes, seen from the segmented image and corresponding cross sectional surface analysis, were smaller for 1st flush 1 M KOHss (25 μm^2) compared to Filling 1 M KOHss (50 μm^2). In 4 M KOHss, more particles were present compared to the 1 M KOHss, and again, smaller particles were observed for 1st flush 4 M KOHss (100 μm^2) compared to Filling 4 M KOHss (400 μm^2) (Fig. 3).

3.3. *Phylogenetic analysis of* A. bisporus *GH43 protein sequences to assign function and expression of the* A. bisporus *genes encoding α-glucuronidase*

A phylogenetic analysis was performed on the amino acid sequence of the genes from family GH43 of *A. bisporus* to determine if any of them was likely to encode an α-arabinofuranosidase able to remove arabinosyl-residues from the double substituted xylo-oligomers. Therefore, two reference sequence of which the enzymes have been shown to release arabinose from xylosyl residues having 2 arabinosyl residues, namely AXH-d3 of *B. adolescentis* and of *H. insolens* (Sørensen et al., 2006; van den Broek et al., 2005), were included. In addition, other biochemically verified reference sequences were included encoding other GH43 enzyme functions, as well as all genes from the white rot basidiomycete *D. squalens*, to provide a larger set of basidiomycete sequences. This was needed to avoid a bias in the tree because of the larger number of ascomycete sequences. The resulting maximum likelihood tree (ML) (Fig. 4A) was rooted using four GH54 arabinofuranosidase sequences and showed that three of the *A. bisporus* GH43 genes encode most likely endoarabinanases as they are located in that branch of the tree. The fourth *A. bisporus* gene falls in the arabinofuranosidase branch, but is more similar to two enzymes acting on single substituted residues. Therefore, we conclude that *A. bisporus* is unlike to have arabinofuranosidases acting on xylo-oligomers having two arabinosyl-residues.

The *A. bisporus* genome contains two genes from family GH115, which are expected to encode α-glucuronidases able to remove (4-*O*-methyl-)glucuronic acid from xylans (Patyshakuliyeva et al., 2013)). However, expression analysis demonstrated no- to very-low expression of these genes (Fig. 4B).

3.4. *Structural changes of lignin during composting and mycelium growth phases*

The Py-GC/MS lignin-pyrograms of the compost obtained at Filling, Pinning, 1st flush and 2nd flush were annotated and presented in Supplementary Fig. 2. The pattern and the ratio between the peaks in all pyrograms are similar. The ratio of syringly-like to guaiacyl-like lignin units (S:G ratio), was 0.52 (±0.00) and 0.61 (±0.08), at Filling and 1st flush, respectively (Fig. 5A).

The molar distribution of all annotated S (syringyl) and G (guaiacyl) residues for lignin in the compost after Filling, Pinning, 1st flush and 2nd flush are presented in Fig. 5B. Overall, small differences between the distribution of S and G compounds were observed in the lignin during fruiting body formation. The relative amounts of vinyl-guaiacol and vinyl-syringol decreased slightly in favor of the less substituted guaiacol and syringol seen from the comparison of lignin from Filling with 2nd flush.

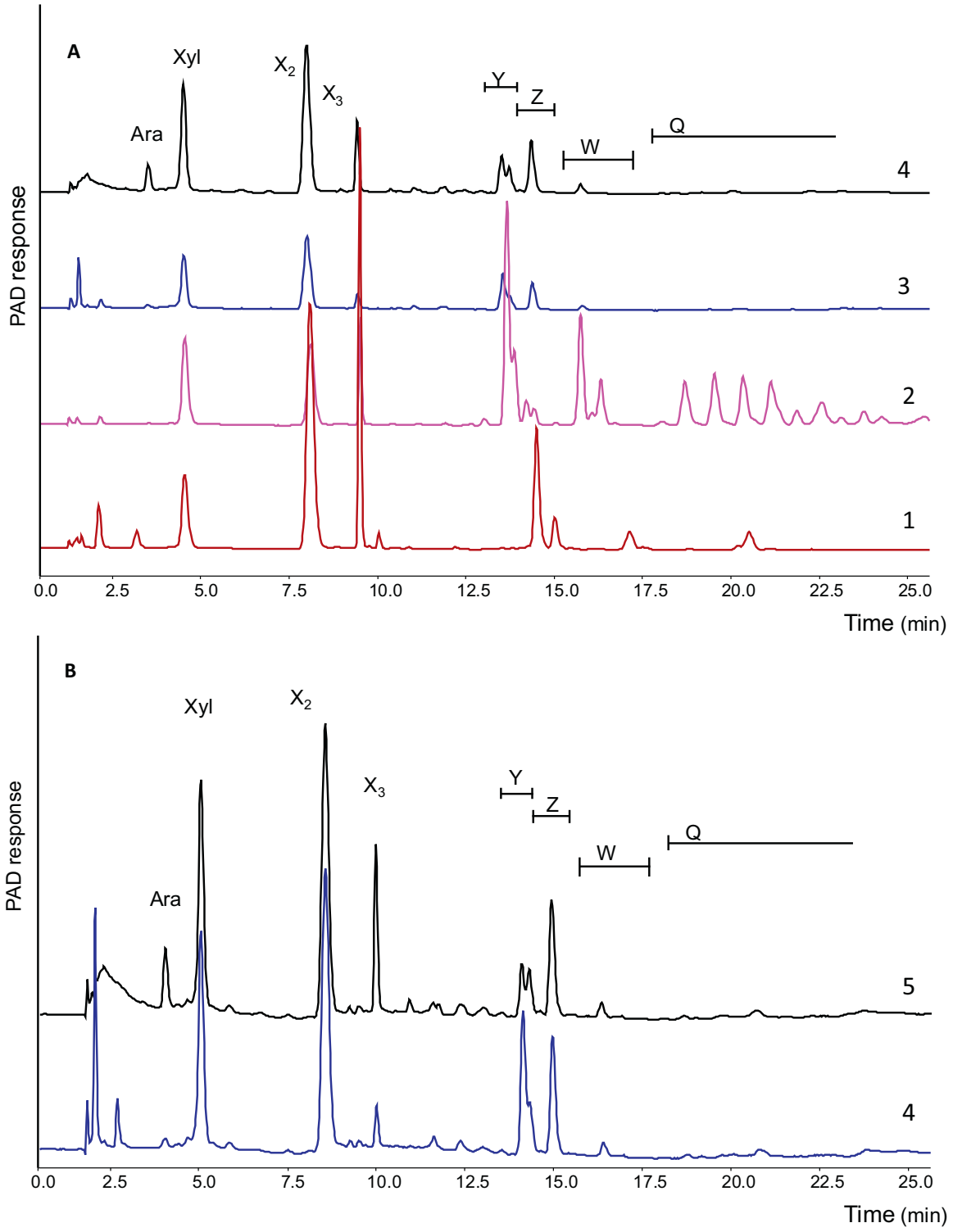

Fig. 2. HPAEC elution profile of birchwood xylan (1) and wheat arabinoxylan (2) incubated with EX1, 1 M KOHss (A) compost extracts after 2nd flush incubated with EX1 (3) and then sequentially with pure GH43 AXH-d3 arabinofuranosidase (4) and 4 M KOHss (B) compost extracts after 2nd flush incubated with EX1 (4) and then sequentially with pure GH43 AXH-d3 arabinofuranosidase (5). *Y* = single substituted Ara-XOS. *Z* = GlcA-XOS, *W* = double substituted Ara-XOS, *Q* = multiple substituted XOS (Ara + GlcA).

4. Discussion

This paper is the first to describe remaining compost structures, with a focus on remaining xylan and lignin, in different fruiting stages of the white button mushroom *A. bisporus* cultivated in compost. It has already been presented that after harvesting of two flushes of mushrooms around 11% (w/w) of carbohydrates remain and 20% (w/w) of lignin remain based on dry matter (Iiyama et al., 1994). But, how *A. bisporus* utilized its substrate in a controlled environment during the various stages of fruiting, and what polymeric compost structures were recalcitrant, is not known.

Overall, the carbohydrates analyzed in the compost at Filling, Pinning, 1st flush and 2nd flush were mainly composed of the xylan

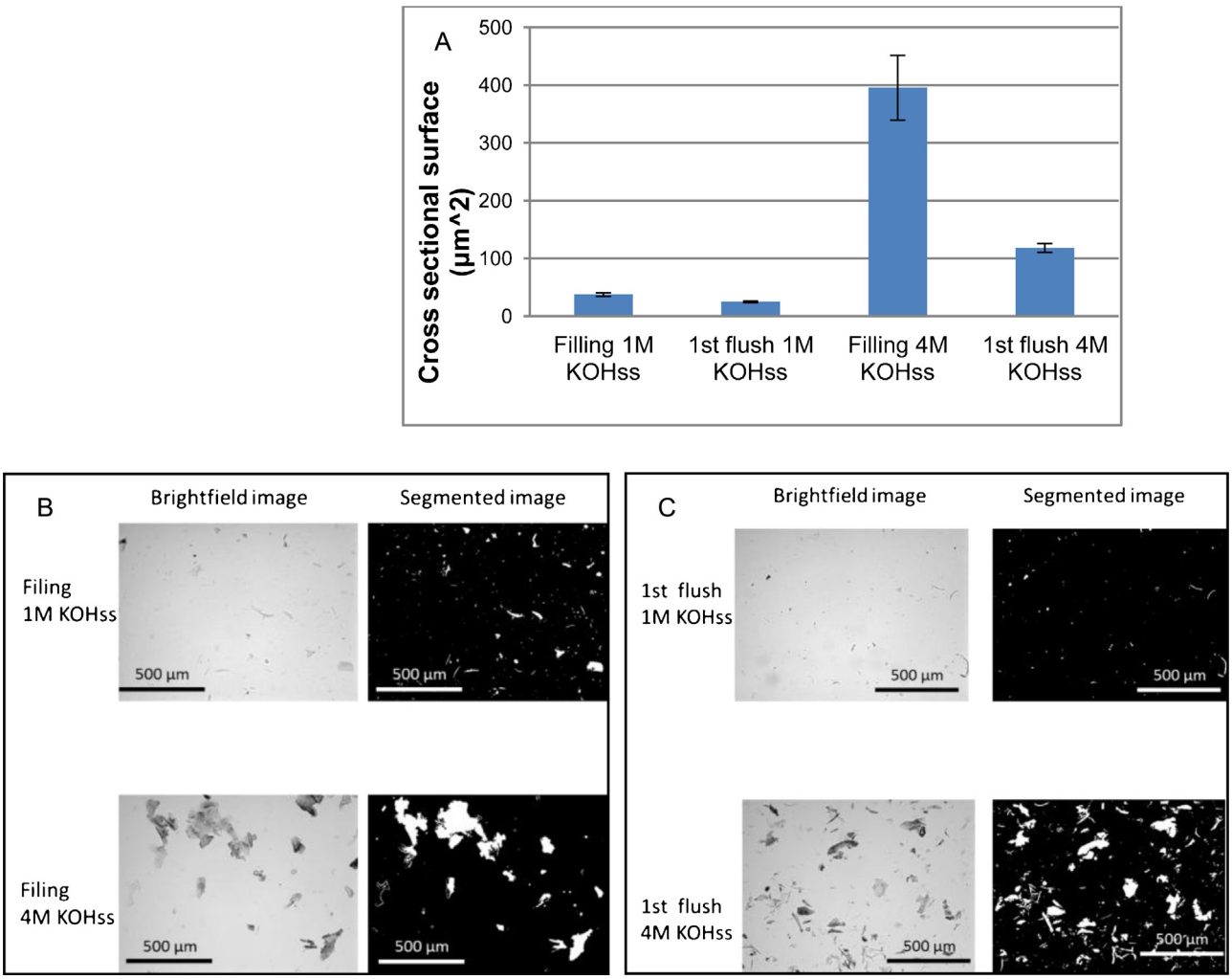

Fig. 3. Cross sectional surface of particles in water insoluble xylan in 1 M KOHss and 4 M KOHss from compost after Filling and after 1st flush (A) and microscopy images of water insoluble xylan in 1 M KOHss and 4 M KOHss of compost after Filling (B) and after 1st flush (C).

building blocks xylosyl (Xyl), arabinosyl (Ara) and uronyl (UA) residues, and of glucosyl (Glc) residues, what is in line with the reported composition of wheat straw based compost (Jurak et al., 2014). As expected, a decrease in total carbohydrate content (30% w/w based on dry matter (DM)), both xylan (40% w/w) and glucan (23% w/w), was observed in compost from Filling to 2nd flush, indicating that carbohydrates were metabolized during the fruiting of *A. bisporus*. These results were in agreement with gene expression levels of *A. bisporus* during fruiting body formation showing that both the hexose and pentose catabolic pathway were upregulated (Patyshakuliyeva et al., 2013). Previously, it was reported that if compost of PII was compared with 1st flush of mushrooms, which also includes carbohydrate consumption during mycelium growth (16 days (PIII)), a decrease of 50% (w/w DM) of carbohydrates was reported (Iiyama et al., 1994).

In contrast to the carbohydrates present, lignin was not altered during fruiting body formation, seen from the constant ratio of syringyl-like (S) to guaiacyl-like (G) lignin units (S:G ratio 0.52–0.55; Fig. 5A). Only, a slight decrease of vinyl-guaiacol and vinyl-syringol in favor of the less substituted guaiacol and syringol was observed. Earlier research has already showed that lignin degradation mainly occurs during mycelium growth of *A. bisporus* (Durrant et al., 1991; Jurak, Punt, et al., 2015) and enzymes possibly involved in lignin degradation, e.g. laccases and manganese-peroxidases, are hardly active during fruiting (Bonnen et al., 1994).

The main question in this research was which carbohydrates, in total 16% (w/w) of compost of 2nd flush, were resistant for *A. bisporus* during fruiting. Based on the carbohydrate composition (Table 1) it was concluded that the resistant carbohydrates throughout the fruiting were mainly xylan and glucan. The total glucan analyzed was the sum of cellulosic compost structures and mycelial glucan. Since cellulose has a well-studied structure composed of linear β-1,4-glucan chains, our research was dedicated to analyzing the resistant xylan structures in compost. Moreover, accumulated xylan could inhibit further cellulose degradation, either by binding to cellulose and/or inhibition of cellulose degrading enzymes (Kabel, van den Borne, Vincken, Voragen, & Schols, 2007; Qing, Yang & Wyman, 2010).

An important finding was that the degree of substitution (DS) of xylan increased in compost from Filling till 1st flush. The increase was analyzed both for the DS Ara and DS UA, from 16 and 18 mol% in Filling, respectively, to 21 and 31 mol% in 1st flush. To look into how and to which extent these substituents were linked to xylan, the various compost samples were fractionated in a water soluble part, and the water unextractable part (WUS) was sequentially fractionated in 1 M KOH and 4 M KOH soluble fractions. Most of the xylan (81–93% w/w) was recovered in the WUS, although, at the end of the fruiting a larger part of resistant xylan was water soluble (19% w/w in 2nd flush compared to 8% in Filling; Table 2). In general, water soluble carbohydrates are easier to access for enzyme degradation

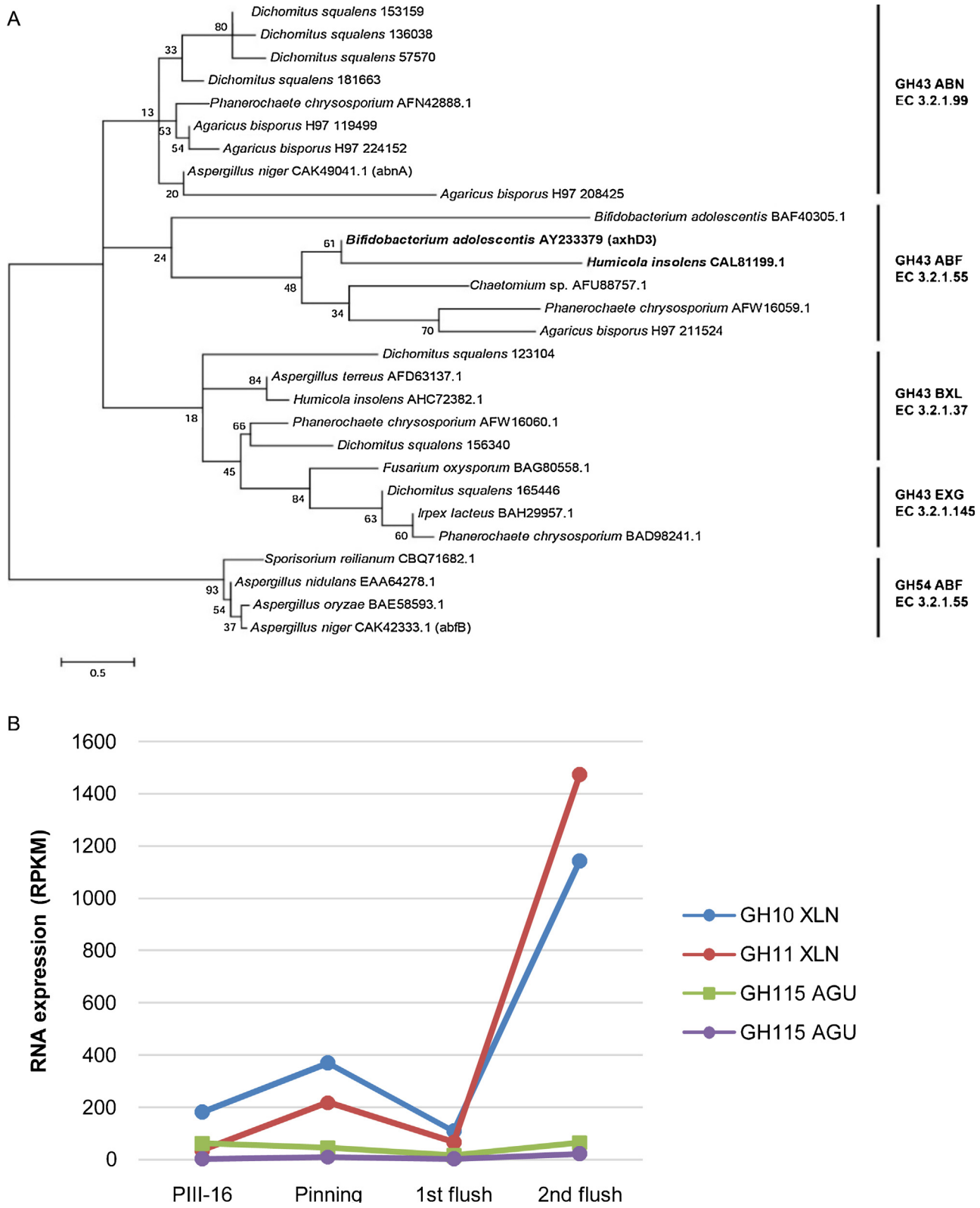

Fig. 4. Maximum likelihood (ML) tree of selected fungal GH43 protein sequences (A) and expression profile (B) of two endoxylanases (GH10 XLN and GH11 XLN) and two α-glucuronidases from *A. bisporus* (both GH115 AGU). Enzymes in bold were confirmed to be able to release arabinose from double substituted xylosyl residues (Sørensen et al., 2006; van den Broek et al., 2005). ABN: endoarabinanase, ABF: α-arabinofuranosidase, BXL: β-xylosidase, EXG: exogalactanase, XLN: endoxylanase, AGU: α-glucuronidase.

and hence for consumption. But, it is unknown whether these water soluble carbohydrates have to be considered as recalcitrant for *A. bisporus* degradation or that they could be degraded if longer times were allowed in between the flushes.

The water insoluble xylans were considered to be the most difficult for *A. bisporus* to degrade, of which half was recovered in the 1 M KOHss of Filling, Pinning, 1st flush and 2nd flush (47–54% w/w and 10–12% (w/w) in the 4 M KOHss. The water soluble part of

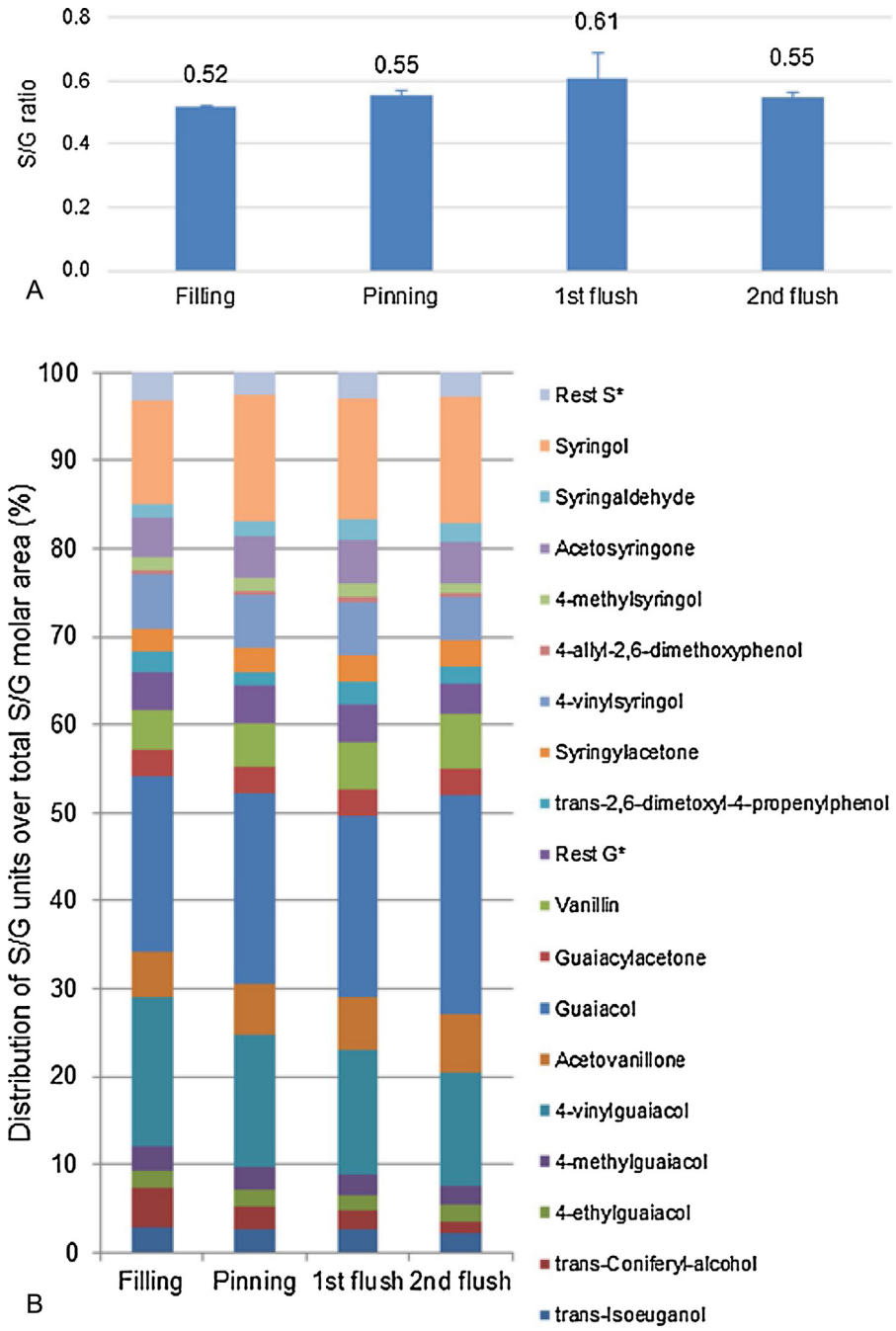

Fig. 5. Syringyl (S) to guaiacyl (G) ratio (S:G ratio) (A) and distribution of S and G units (B), based on molar area of compost after Filling, Pinning, 1st flush and 2nd flush. Rest S* and G* compounds with <1% total molar area in wheat straw (Jurak, Punt, et al., 2015).

the alkali extracts was evaluated by HPSEC, which showed that the molecular weight distribution of the water soluble xylans in both 1 M and 4 M KOHss fractions of all composts analyzed during fruiting were similar and ranging from 0.5–23 kDa (based on pullulans calibration).

The water insoluble parts of the alkali fractions of Filling and 1st flush could not be studied by using chromatographic analysis, but, microscopy showed that, in particular, in the 4 M KOHss 1st flush the particles' cross sectional surface was smaller compared to the 4 M KOHss Filling. Also, microscopy showed that in the water insoluble part of 1 M KOHss less insoluble particles were observed having a smaller cross section surface compared to the water insoluble part of 4 M KOHss fractions (Fig. 3). In addition to xylan also lignin is extracted with KOH. The 1 M and 4 M KOHss were analyzed with Py-GC/MS and some differences in the obtained pyrograms of 1 M and 4 M KOHss were observed (data not shown). However, it cannot be excluded that the observed differences were due to the KOH-treatment performed rather than due to differences in lignin structure present still, it is tempting to conclude that the larger and more insoluble particles present in both 4 M KOHss fractions analyzed relate to the presence of more or larger xylan-lignin complexes compared to 1 M KOHss. So, it was assumed that during fruiting large xylan or xylan-lignin complexes were degraded, but nevertheless still partly present.

Comparing the alkali fractions, which were enriched in xylans, it was again concluded that the DS increased during fruiting. More specifically, both arabinosyl and (4-*O*-methyl-)glucuronic acid substituents accumulated during fruiting, which was not only

indicated by the carbohydrate composition analyzed, but also the outcome of fingerprinting the alkali extracted xylans by using a pure endo-xylanase, subsequently digested by either AXH-d3 or α-glucuronidase. Analysis of the enzyme digested alkali-fractions of 2nd flush compost showed that next to xylose, xylobiose and xylotriose, mainly substituted XOS were released having either 1 or 2 arabinosyl substituents per xylosyl residues, in addition to (4-*O*-methyl-)glucuronic acid substituted XOS. Apparently, *A. bisporus* did not degrade these remaining substituted xylan fragments. That conclusion was supported by the result that the two genes annotated as putative α-glucuronidases (both GH115, Patyshakuliyeva et al., 2013, 2015) were not expressed in the same compost samples as used to analyze the resistant substituted xylans. In addition, *A. bisporus* genes from family GH43 were assessed for similarity to two genes known to encode biochemically characterized AXH-d3 activity featuring the release of arabinose from xylosyl residues doubly substituted with arabinosyl residues. No similarity was found to them (Fig. 4A), which matched with the lack of enzyme activities observed in compost enzyme extracts. Finally, neither α-glucuronidase nor α-arabinofuranosidase able to remove arabinosyl-residues from double substituted xylo oligomers were found to be active in the compost during fruiting body formation (Jurak, Patyshakuliyeva, de Vries, Kabel, & Gruppen, 2015).

5. Conclusion

During fruiting body formation of *A. bisporus* xylan and glucan in the compost were degraded whereas lignin structures remained rather similar. Over the phases analyzed, the remaining xylan was increasingly substituted with (4-*O*-methyl-)glucuronic acid and arabinosyl residues, both one and two arabinosyl residues per xylosyl residue remained. Moreover, *A. bisporus* genome was found to not contain the genes encoding α-arabinforuanosidase able to remove arabinosyl-residues from double substituted xylo-oligomers, and genes encoding α-glucuronidases present were not expressed. Overall, the observed accumulation of xylan substituents is expected to hinder the complete degradation of xylan in the wheat straw based compost during fruiting body formation of *A. bisporus*.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carbpol.2015.06.065

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