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Carbohydrate composition of compost during composting and mycelium growth of *Agaricus bisporus*

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ABSTRACT

Changes of plant cell wall carbohydrate structures occurring during the process to make suitable compost for growth of *Agaricus bisporus* are unknown. In this paper, composition and carbohydrate structures in compost samples collected during composting and mycelium growth were analyzed. Furthermore, different extracts of compost samples were prepared with water, 1 M and 4 M alkali and analyzed. At the beginning of composting, 34% and after 16 days of mycelium growth 27% of dry matter was carbohydrates. Carbohydrate composition analysis showed that mainly cellulose and poorly substituted xylan chains with similar amounts and ratios of xylan building blocks were present in all phases studied. Nevertheless, xylan solubility increased 20% over the period of mycelium growth indicating partial degradation of xylan backbone. Apparently, degradation of carbohydrates occurred over the process studied by both bacteria and fungi, mainly having an effect on xylan-chain length and solubility.

1. Introduction

The world-wide production of the white button mushroom *Agaricus bisporus* was estimated to be about 4 million tons in 2009 (Sonnenberg et al., 2011). In the Netherlands, for commercial production *A. bisporus* is grown on compost, which is based on wheat straw, horse and chicken manure, gypsum and water (Gerrits, 1988, http://www.cnc.nl/). In other parts of the world the raw materials can differ although they are always carbon and nitrogen based. In the commercial production of compost three phases are present. At the start of the (European) composting process the basic mixture of raw materials (BM) is watered and mixed. Thermophilic micro-organisms, including fungi, start to decompose the BM material and cause rise of temperature (Chang & Hudson, 1967) up to 80 °C (personal communication CNC, Milsbeek, The Netherlands) resulting in Phase I (PI) compost (Fig. 1). In the second phase the compost is conditioned at 45–50 °C until it is free of ammonia after which temperature is lowered to about 25 °C (Phase II (PII) compost). PI and PII make the compost accessible and highly specific for *A. bisporus* growth (Iiyama, Stone, & Macauley, 1994). The third phase (PIII) starts after inoculation of PII compost with *A. bisporus* mycelium (Fig. 1). Temperatures are maintained at around 25 °C and mycelium grows from the inoculum (wheat grain kernels) throughout the compost. Within 16 days (PIII-0 to PIII-16) mature compost overgrown with mushroom mycelium is obtained. By the end of composting about 1.5% of the dry weight of compost is fungal and actinomycete mycelia (Sparling, Fermor, & Wood, 1982; Straatsma et al., 1989). Addition of a mixture of peat and lime (casing layer) on top of the matured compost incubated with mushroom mycelium and another 17 days under same conditions are needed for fruiting bodies formation and maturation. Finally, each ton of mushrooms produced leaves at least an equivalent amount of spent compost containing about one third of carbohydrates from the original compost (Chiu, Law, Ching, Cheung, & Chen, 2000; Semple, Reid, & Fermor, 2001).

The composting process, described above, is aimed at opening the plant cell wall matrix to facilitate the release of monosaccharides for *A. bisporus* to grow on. As such, this process could be aligned with other treatments of plant biomasses aiming at the enzymatic release of monosaccharides as fermentable building blocks for fuels and chemicals.

As stated above, wheat straw is the main ingredient of the compost and it belongs to the family Poaceae in the plant group of Monocots. The plant cell walls of wheat straw are mainly composed of cellulose, lignin and hemicellulose (xylan). Cellulose is a polymer of (1,4)-β-D-glucan (Himmel, Ruth, & Wyman, 1999). Specific features of xylan, are determined by the source from which xylan is extracted (Gruppen, Hamer, & Voragen, 1992). Xylan from Poaceae consist of a (1,4)-β-D-xylan backbone. The β-D-xylopyranosyl

Abbreviations: DP, degree of polymerization; BM, basic mixture; PI, Phase I compost; PII, Phase II compost; PIII-0, Phase III compost with added spawn; PIII-5, PIII-10, PIII-12, PIII-14 and PIII-16, Phase III compost after 5, 10, 12 days and 16 days of mycelium growth, respectively.

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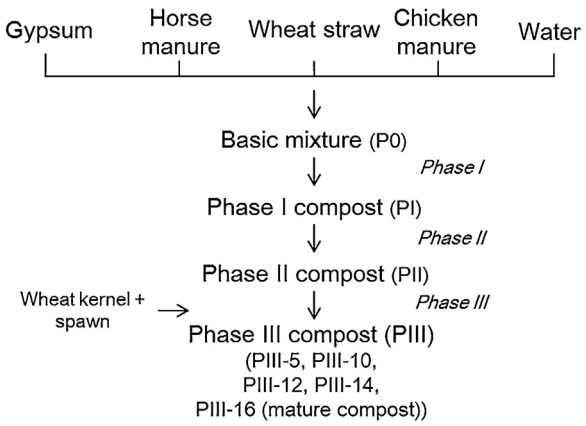

Fig. 1. Schematic representation of the composting and mycelium growth phases.

(Xyl*p*) units of the xylan backbone can be single or double substituted with α-L-arabinofuranosyl (Ara*f*) at *O*3 or at *O*2 of the Xyl*p* units (Fincher, 2009). α-L-Arabinofuranosyl units can be in turn substituted at *O*5 with ferulic acid residues and to a lesser extent, its non methoxylated analog p-coumaric acid. Also, D-glucuronic acid (GlcA*p*) and its 4-O-methyl ether is a substituent, linked to the *O*2 of Xyl*p* units of the xylan backbone (Fincher & Stone, 2004). Few publications also report the presence of O-acetyl substituents present in xylans from Poaceae (Ishii, 1991; Wende & Fry, 1997).

Earlier studies on compost based on various ingredients for mushroom growth were done on the gross composition at various stages during composting and mycelium growth (Gerrits, Bels-Koning, & Muller, 1967; Lyons, Sharma, Kilpatrick, Cheung, & Moore, 2006). However, studies on the compost from same raw ingredients used in this study focused on the total carbohydrate content and inorganic constituents, but not on constituent monosaccharides present. Further, mainly phases after inoculation with mushroom mycelium were investigated (Durrant, Wood, & Cain, 1991; Iiyama et al., 1994; Wood & Leatham, 1983). So far, the composition, use and exact nature of (hemi-) cellulosic components were not studied within the various compost and mycelium growth stages.

The aim of the present study was to determine the composition of compost after the three composting phases (BM, PI and PII) and during mycelium growth (PIII). The focus was on the analysis of carbohydrate-structures present. This knowledge about the remaining carbohydrate structures, can help to find solutions to further degrade these carbohydrates thereby improving mushroom production yield and levering the amount of the spent compost.

2. Materials and methods

2.1. Materials

Wheat straw, basic mixture of raw materials (BM) and compost samples from the end of Phase I (PI), Phase II (PII) and PIII (5–16) were obtained from CNC within the same week (Coöperatieve Nederlandse Champignonkwekersvereniging (CNC); Milsbeek), The Netherlands. Wheat straw and BM, not being in the tunnels yet, were sampled in 8 and 28 samples, respectively. PI and PII samples were collected each from 4 different tunnels from the same time point. For PI 16 samples were obtained and mixed together, while for the more homogeneous PII in total 4 samples were obtained. Samples (about 1 kg each) were collected, frozen, freeze dried and milled (<1 mm) (Retsch Mill MM 2000, Retsch, Haan, Germany). To obtain a representative samples of wheat straw, BM, PI and PII samples collected were mixed in equal ratios. Due to the increased homogeneity in the composting process mixing of PIII samples was not required (PIII-5, 10, 12, 14, 16).

2.2. Water and alkali extracts

Freeze dried, mixed and milled compost sample (10 g) was suspended in millipore water (100 mL) and extracted for 18 h at 65 °C under continuous stirring. After centrifugation (10,000 × *g*; 30 min; 20 °C), the residue was washed twice with millipore water (150 mL and 100 mL, respectively). Supernatants were combined, dialyzed and collected as dialyzed water soluble solids (WSSd). The corresponding residue was recovered as water un-extractable solids (WUS). The yield of non-dialyzed fraction was calculated as the weight difference between original sample and WUS. Next, 5 g of WUS was suspended in 200 mL 1 M KOH containing 1% $NaBH_4$ 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% $NaBH_4$and again centrifuged. Supernatants were combined (1 M KOHss). The residue was re-extracted and washed once with 4 M KOH containing 1% $NaBH_4$, as described for the 1 M KOH extraction. Supernatants were combined and labeled 4 M KOHss. The final residue was collected (Res). Alkali fractions and residues were neutralized with acetic acid, extensively dialyzed (10–12 kDa cutoff, Medicell International, London, UK) against distilled water and freeze dried.

2.3. Hydrolysis of alkali extracts with endoxylanase I

Suspensions of 1 M and 4 M KOHss (4 mg KOHss) in 50 mM sodium acetate buffer pH 5 (977 μL) were incubated with a pure endo-(1,4)-β-D-xylanase I (EX1) from *Aspergillus awamori* (23 μL, 0.55 μg protein) (Kormelink, Gruppen, Viëtor, & Voragen, 1993) overnight at 35 °C. After inactivation of the enzyme (10 min, 100 °C) digests were analyzed by HPSEC (non diluted), HPAEC (diluted 20× in water) and MALDI-TOF MS (non diluted). Wheat arabinoxylan, medium viscosity (Megazyme, Wicklow, Ireland) was treated similarly and used as a reference.

2.4. Analytical, chromatographic and spectrometric methods

All analyses were performed in duplicate.

2.4.1. Neutral sugar composition and content

Neutral sugar content and composition was determined by gas chromatography according to Englyst and Cummings (1984), using inositol as an internal standard. Samples were treated with 72% (w/w) H_2SO_4 (1 h, 30 °C) followed by hydrolysis with 1 M H_2SO_4 for 3 h at 100 °C and the constituent sugars released were analyzed as their alditol acetates using gas chromatography (Focus-GC, Thermo Scientific, Waltham, MA, USA). Total carbohydrate content was calculated as a sum of neutral carbohydrates and uronic acids. Water soluble part of 1 M and 4 M KOHss before and after EX1 digestion, was directly hydrolysed with 1 M $H_2SO_4$for 3 h at 100 °C. Samples were diluted (20×) and analyzed for monosaccharide content by HPAEC (high performance anion exchange chromatography).

2.4.2. Uronic acid content

Uronic acid content was determined as anhydro-uronic acid content by an automated m-hydroxydiphenyl assay (Thibault, 1979) with addition of sodium tetraborate using an autoanalyser (Skalar Analytical BV, Breda, The Netherlands). Glucuronic acid (Fluka AG, Busch, Switzerland) was used as a reference (12.4–200 μg mL^{-1}).

2.4.3. Esterified acetic acid content

Each sample (20 mg) was saponified with 1 mL of 0.4 M NaOH in isopropanol/H_2O (1:1 v/v) for 3 h at room temperature. The acetic acid was determined with an Ultimate 3000 system (Thermo Scientific, Rockford, IL, USA) equipped with a Shodex RI detector and an Aminex HPX 87H column (300 mm × 7.8 mm) (Bio-Rad, Hercules, CA, USA) plus pre-column. Elution was performed by using 5 mM H_2SO_4 as eluent at a flow rate of 0.6 mL min^{-1} at 40 °C. The level of acetic acid substituents was corrected for the free acetic acid present in the sample.

2.4.4. Esterified ferulic and coumaric acid contents

To 10 mg of sample 1 mL of 2 M KOH (flushed with N_2) was added. Samples were put under N_2 overnight in the dark at room temperature. Next day, the pH was adjusted (3) by adding 500 μL HCl (6 M). The amounts of free ferulic and coumaric acid present in the sample was determined by adding 1.5 mL of premade mixture of 2 M KOH (1 mL), flushed with N_2 and HCl (0.5 mL, 6 M). Samples were put under N_2 overnight in the dark at room temperature and analyzed. Analysis was performed on an Acella UHPLC system (Thermo Scientific, Rockford, IL, USA) equipped with a photodiode array detector coupled to an LTQ XL ion trap mass detector equipped with an electrospray ionization source (Thermo Scientific, Rockford, IL, USA). The system was controlled by Xcalibur software. Analysis was performed on a Hypersyl GOLD 1.9i.d. mm × 150 mm column with 1.9 μm particle size (Thermo Scientific, Rockford, IL, USA). The mobile phases were (A) H_2O + 1% (v/v) acetonitrile + 0.2% (v/v) acetic acid and (B) acetonitrile + 0.2% (v/v) acetic acid. The flow rate was 0.4 mL min^{-1}, and the column temperature was kept at 30 °C. The elution profile was: 3 min isocratic 4% B; linear from 4% to 40% B; 3–21 min, linear from 40 to 100% B; 21–21.5 min, isocratic at 100% B; 21.5–23 min, linear from 100% to 4% B, followed by reconditioning of the column for 7 min. Spectral data were collected from 200 to 600 nm, and quantification was performed at 320 nm. Ferulic and coumaric acid contents were identified and quantified on the basis of authentic standards. MS data were collected in the negative mode with an ion spray voltage of 3 kV, a sheath gas flow rate was 20 arb, aux gas flow rate was 10 arb, and a capillary temperature of 300 °C. Full MS scans were made within the range m/z 150–1500, and MS^2 fragmentation spectra data of the most intense ions were obtained.

2.4.5. Insoluble (Klason) lignin content corrected for ash

To each sample of 300 mg (dry matter) 3 mL of 72% w/w H_2SO_4 was added and samples were hydrolysed for 1 h at 30 °C. After this pre-hydrolysis, 37 mL of distilled water was added to each sample and samples were put in a boiling water bath for 3 h and shaken every half hour. Next, the suspension was filtered over G4 glass filters. The residual part was washed until it was free of acid and dried overnight at 105 °C. Final residues were corrected for ash and considered as a measure for the acid insoluble lignin content.

2.4.6. Nitrogen and protein content

Samples were analyzed for nitrogen content in duplicate using the combustion (DUMAS) method on a Flash EA 1112 Nitrogen Analyzer (Thermo Scientific, Sunnyvale, CA, USA). Methionine (Acros Organics, Geel, Belgium) was used as a standard. Samples PI, PII and PIII-16 were extracted with water, and nitrogen amount in water soluble extract after dialysis plus nitrogen in the water unsoluble extract was analyzed and added up for the calculation of protein content in the original samples. Nitrogen to protein conversion factor of 6.25 was used (Jones, 1931).

2.4.7. Ash content

Freeze dried samples (0.5 g) or lignin residues were dried in the oven overnight (105 °C) and weighed then put at 575 °C for 4 h. Next samples were weighed and difference between the mass at 105 °C and on 575 °C was taken as ash content.

2.4.8. HPSEC

High-performance size-exclusion liquid chromatography (HPSEC) was performed on an Ultimate 3000 HPLC system (Thermo Scientific, Sunnyvale, CA, USA) three TSK-gel columns (6.0 mm × 15.0 cm per column) in series (SuperAW4000. SuperAW3000, SuperAW25000, 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 made by using pullulan series (Polymer Laboratories, Union, NY, USA) with a molecular weight in the range of 0.18–788 kDa.

2.4.9. HPAEC

High-performance anion-exchange chromatography was performed on a Dionex Ultimate 3000 system (Thermo Scientific, 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. System was controlled by the Chromelion software (Thermo Scientific, Sunnyvale, CA, USA). Elution of 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%, equilibration step 12 min 100% A. Separation and quantification of monosaccharides was performed at flow rate 0.4 mL min^{-1}, combining three mobile phases: (A) 0.1 M NaOH, (B) 1 M NaOAc in 0.1 M NaOH and (C) H_2O. The elution profile was as follows: 0–40 min 100% C, 40–45 min from 100% A to 100% B, 45–50 min 100% B, 50–58 min 100% A, 58–73 min 100% C. From 0 to 40 min and from 58 to 73 min post column addition of 0.5 M NaOH at 0.1 mL min^{-1} was performed to detect and quantify the eluted saccharides.

2.4.10. MALDI-TOF MS

Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight mass spectrometry (MALDI-TOF MS) was performed using an Ultraflex3 instrument (Bruker Daltonics, Bremen, Germany) equipped with a nitrogen laser of 337 nm and operated in the positive mode. The system was controlled by FlexAnalysis software. Calibration was performed with a mixture of maltodextrins 480–3000 Da (AVEBE, Veendam, The Netherlands). After a delayed extraction time of 130 ns, positive ions were accelerated with 25 kV voltage and detected using reflector mode. The samples were desalted with resin (AG 50 W-X8 Resin; Bio-Rad, Hercules, CA, USA). The matrix solution was prepared by dissolving 10 mg of 2,5-dihydroxybenzoic acid (Bruker Daltonics, Bremen, Germany) in a 1 mL mixture of acetonitrile and 2.8 mM sodium acetate (300 μL:700 μL). On the MALDI-TOF Plate 1 μL of sample and on top 1 μL of matrix was put (Bruker Daltonics, Bremen, Germany) and allowed to dry under a constant stream of air.

3. Results and discussion

3.1. Composition of compost samples

The composition of compost samples at the end of Phase I, Phase II and over 5, 10, 12, 14 and 16 days of Phase III is presented in Table 1. It should be noted that this study focussed on characterization of components being left at the end of various phases. The total carbohydrate content (neutral carbohydrates and uronic acids) of

Table 1
Content, composition and pH of compost samples (BM, PI, PII, PIII-5, PIII-10, PIII-12, PIII-14, PIII-16) from different composting and mycelium growth phases, based on dry matter.

	BM[a]	PI[a]	PII[a]	PIII-5[a]	PIII-10[a]	PIII-12[a]	PIII-14[a]	PIII-16[a]	Wheat straw
Content (%w/w)[b]									
Lignin (Klason)[c]	25	28	26	26	23	25	26	23	27
Total carbohydrates	34	38	26	26	23	21	22	27	57
Ash	25	26	31	27	36	29	28	27	5
Total nitrogen	2	1	2	2	3	2	2	2	0.4
Protein[d]	n.a.	6	10	n.a.	n.a.	n.a.	n.a.	10	3
pH	8	8	7	7	7	6	6	6	n.a.
Carbohydrate composition (molar %)[e]									
Arabinose	7	6	5	5	5	6	6	5	6
Xylose	41	37	34	35	36	35	35	35	43
Mannose	1	1	1	2	3	3	3	3	1
Galactose	2	2	2	2	2	2	2	2	1
Rhamnose	1	1	2	2	2	2	1	2	1
Glucose	44	50	52	51	48	48	48	50	44
Uronic acid	4	4	4	4	4	4	5	5	4
Esters (% w/w)[b,f]									
Acetic acid	0.7	0.1	0.1	0.2	0.3	0.2	0.2	0.2	2
Ferulic acid	0	0	0	0	0	<0.1	<0.1	<0.1	0.2
Coumaric acid	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	0.2
Degree of substitution									
Ara/Xyl[g]	16	17	15	15	15	16	16	14	14
GlcA/Xyl[g]	10	10	11	10	12	12	15	13	7
AcA/Xyl[g]	17	3	6	9	12	9	9	9	32

[a] P0, basic mixture compost; PI, Phase I compost; PII, Phase II compost; PIII-5, PIII-10, PIII-12, PIII-14, PIII-16 is Phase III compost with spawn added and grown for 5, 10, 12, 14 or 16 days.
[b] Weight percentage is based on dry matter of composting phases; n.a. = not analyzed.
[c] Corrected for ash.
[d] Nitrogen to protein conversion factor of 6.25 was used; protein content does not include N-containing salts.
[e] As anhydro-sugars.
[f] Esters corrected for free HAc or FA.
[g] Ratio mol/100 mol. Ara, arabinose; GlcA, glucuronic acid, AcA, acetic acid.

BM is 34% (w/w), of PI is 38% (w/w), of PII 26% (w/w) and of PIII-16 27% (w/w) based on dry matter. Previously, Iiyama et al. (1994) reported 40% of total carbohydrates remaining after Phase I (w/w) and 22% after Phase II, based on dry matter, which is rather similar to our data. Small differences may be due to variation in the origin of raw materials and composting process.

For all samples, the main polysaccharides present are constituted of xylosyl (41–35 mol%) and glucosyl (52–44 mol%) residues, as expected, indicating at the presence of xylan and cellulose originating from wheat straw (Table 1). In wheat straw small amounts of ester bound acetic, ferulic and coumaric acid were found, however in BM only very small amount of esterified acetic acid remained. It is expected that esters were removed due to a rather high pH of this mixture (Table 1). It has been reported that the two hydroxycinnamic acids, e.g. present as ester-linkages to xylan and/or lignin (Fincher, 2009; Jeffries, 1990) negatively impact the hydrolysis of xylans to monomeric sugars and biodegradation as described by Dodd and Cann (2009). Also, acetylation of xylans is reported to hinder enzymatic saccharification of plant hemicelluloses to monosaccharides (Biely, 2012). For optimal utilization of plant biomass all polysaccharides need to be degraded and for the complete degradation of cell wall polysaccharides the synergistic action of a spectrum of enzymes is needed.

Therefore, the lack of esterified acetic, coumaric and ferulic acid could have a beneficial effect in degradation of the xylan backbone in later stages of composting, mycelium growth and fruiting body formation.

3.2. Yield and composition of dry matter and carbohydrates in water and alkali fractions

In order to determine whether changes can be observed in the xylan structures during composting and mycelium growth hemicellulose-populations were extracted with water- and alkali and analyzed from samples obtained at the end each out of three composting phases (PI, PII, PIII-16). Water and alkali extracted xylan is not described in literature for any of the compost phases. Distribution of dry matter, total sugars, glucans and glucuronoarabinoxylans (GAX) between WSS and WUS fraction, as well as for WUS over alkali extracts 1 M KOHss, 4 M KOHss and residue (Res) is shown in Table 2.

The yield of total soluble carbohydrates (WSS) in PI and PII was found to be low (3%), whereas the amount of soluble carbohydrates in PIII-16 was 22% (w/w). Yield of glucan in WSS of PI and PII is 1% and of PIII-16 20% (w/w) indicating an increase in solubility of glucan during mycelium growth. GAX yield in WSS is 3%, 11% and 22% (w/w) for PI, PII and PIII-16, respectively (Table 2). In WSSd of PI, PII and PIII-16 1,2 and 3% (w/w), respectively, of carbohydrates were recovered indicating low amounts of soluble carbohydrates of higher molecular weight (Mw > 15 kDa). This pointed at the surprising conclusion that about a quarter of water insoluble polysaccharides present in PII, both glucan and xylan were degraded into smaller water soluble units during mycelium growth (PIII-16).

From the WUS-fractions, the yield of DM, total carbohydrates, glucan and xylan (GAX) collected in 1 M KOHss and 4 M KOHss was analyzed (Table 2). Yield of glucuronoarabinoxylan (GAX) was calculated as the sum of arabinosyl, uronic acids and xylosyl residues. For all phases (PI, PII and PIII-16) more than half of the xylan originally present in the corresponding WUS was collected in the 1 M KOH fractions (Table 2). In 4 M KOH fraction about 25% GAX for all phases was further recovered. About 20% (w/w) GAX remained in the Res next to the majority of glucan. Similar recoveries were previously shown for cell walls of corn cobs and stover (Van Dongen, Van Eylen, & Kabel, 2011). Unexpectedly, the amount of xylan recovered in 1 M KOH fraction was rather similar as is the amount

Table 2
Distribution over water-soluble (dialysed: WSSd and non-dialysed: WSS) and water-insoluble solids (WUS), as well as WUS over alkali extracts 1 M KOHss, 4 M KOHss and the residue (Res) of PI, PII and PIII-16.

	Yield DM[a,b] (%w/w)	Yield total sugar (%w/w)	Yield glucan (%w/w)	Yield GAX[c] (%w/w)
PI[d]	100	100	100	100
WSS (WSSd)	18 (3)	3 (1)	1 (1)	3 (1)
WUS	82	98	100	97
WUS	100	100	100	100
1 M KOHss	24	21	1	54
4 M KOHss	8	10	1	24
Res	62	64	82	21
PII[d]	100	100	100	100
WSS (WSSd)	22 (3)	3 (2)	1 (1)	11 (3)
WUS	78	97	99	89
WUS	100	100	100	100
1 M KOHss	27	25	1	62
4 M KOHss	9	11	2	28
Res	55	69	97	21
PIII-16[d]	100	100	100	100
WSS (WSSd)	24 (6)	22 (3)	20 (1)	22 (4)
WUS	76	78	80	78
WUS	100	100	100	100
1 M KOHss	26	25	2	58
4 M KOHss	9	12	2	27
Res	85	68	95	20

[a] DM, dry matter.
[b] Gain is expected to be the result of salts.
[c] Glucuronoarabinoxylans (GAX) calculated as the sum of uronic acids, arabinosyl and xylosyl residues.
[d] PI, Phase I compost; PII, Phase II compost; PIII-16, Phase III compost after 16 days of mycelium growth.

of xylan recovered in 4 M KOH fraction for PI, PII and PIII-16. This indicates that the alkali extractability of xylan remained similar from PI to PIII-16.

3.3. Structural characteristics of alkali extracted xylan

The type and degree of xylan substituents of alkali extracted xylan from PI, PII and PIII-16 was compared. Results are presented in Table 3. Of PI-xylan in both 1 M and 4 M KOHss only about 20 out of 100 xylosyl units were substituted, which indicates a rather low xylan substitution, which is in line with the low xylan-substitution of wheat straw (Table 1). Interestingly, regardless the decrease in amount of total GAX from BM to PII, based on the dry matter, the number of substituents per 100 xylosyl unit changed very little (Tables 1 and 3). Therefore, the consumption of xylan during composting, PI and PII, is suggested to be in a uniform manner and not hindered by the substituents present. This statement is also supported by a similar water solubility and a similar alkali-extraction yield of these phases. Over the period of mycelium growth (PIII) the amount of substituents also remains the same as in PI and PII, for the WSS, 1 M KOH and 4 M KOH fractions. Taking into account the large increase in water- solubility in PIII it could be hypothesized that during the period of mycelium growth xylan chains are partially degraded, but without the release of substituents.

Table 3
Degree of substitutents in each stage of mushroom compost expressed as moles of substituent per 100 mol of xylosyl residues.

	Ara/Xyl[a]	GlcA/Xyl[a]
PI	16	11
WSS[b]	135	129
WSSd	79	93
1 M KOHss	13	6
4 M KOHss	12	5
Res	3	27
PII	15	11
WSS[b]	14	5
WSSd	89	215
1 M KOHss	12	6
4 M KOHss	10	6
Res	41	30
PIII-16	14	13
WSS[b]	17	18
WSSd	81	160
1 M KOHss	12	8
4 M KOHss	10	8
Res	34	30

[a] Ratio mol/100 mol. Ara, arabinose; GlcA, glucuronic acid.
[b] Calculated as a difference between total sample and corresponding WUS.

In addition to the degree of substitution, the molecular weight (Mw) distribution of xylans in the various phases may be affected as well. To study the molecular weight distribution, all alkali fractions (Table 2) were submitted to HPSEC (Fig. 2). It should be remarked that the molecular weight distribution of the alkali extracted xylan fractions could only be determined for the water soluble part of these fractions. The amount of water-soluble carbohydrates was quantified (Table 4). About 20% of the total carbohydrates in 1 M KOHss and 4 M KOHss of PI and PII, was found to be water-soluble, whereas for 1 M KOHss of PIII-16 40% was water-soluble.

For HPSEC the area under the curve is an indication for the amount of water-soluble carbohydrates, since for all samples the same dry matter versus water ratio was used for HPSEC elution. Due to the low amounts of water-soluble carbohydrates in the xylan fractions a relatively low RI-response, compared to a standard WAX of 4 mg/mL was found. The relatively low water-solubility was matching with our finding that these xylan fractions have low amount of substituents, because linear xylans are shown to possess low water-solubility most likely caused by self-aggregation (Kabel, Van den Borne, Vincken, Voragen, & Schols, 2007). Xylan fractions of PIII-16 show elution in a lower molecular weight region compared to PI 1 M and 4 M KOHss (Fig. 2). This supported again our suggestion that xylan chains in PIII became (partially) degraded.

To further study the structural characteristics of the alkali extracted xylan from PI, PII and PIII-16 of compost, a purified and

Table 4
Total carbohydrates in alkali extracts, 1 M KOHss and 4 M KOHss, of PI, PII and PIII-16 and carbohydrate yield of their water-soluble fraction before and after digestion with endoxylanase 1. DM = dry matter.

Sample	Total carbohydrates (mg/4 mg DM)	Total water-soluble carbohydrates (mg/4 mg DM)		Yield of water-soluble carbohydrates (%w/w)	
		Blank	Digest	Blank	Digest
PI					
1 M KOHss	1.8	0.4	2.0	24	111
4 M KOHss	2.7	0.6	1.5	22	56
PII					
1 M KOHss	1.5	0.3	1.7	23	115
4 M KOHss	1.9	0.5	1.6	23	82
PIII-16					
1 M KOHss	1.3	0.7	1.7	42	113
4 M KOHss	1.6	0.4	1.9	22	122

well characterized, endoxylanase (EX1) (Kormelink et al., 1993) was used to digest extracted xylan. This approach was shown to be successful in giving more structural information for other xylans as well (e.g. wheat flour, corn stover, wood, cobs, wheat bran) (Gruppen et al., 1992; Van Dongen et al., 2011). Molecular weight distributions of the digests is shown in Fig. 2(C and D). After endoxylanase digestion the area under the RI-pattern increased, indicating an increase in water solubility of digested xylan fraction for both 1 M and 4 M KOHss. This was confirmed by analysing the yield of carbohydrates in the soluble fractions after enzymatic digestion. After enzyme hydrolysis of 1 M KOHss from PI, PII and PIII-16 over 100% of carbohydrate yield was recovered. For 4 M KOHss of PI 56%, PII 82% and PIII-16 122% of total carbohydrates are soluble after enzymatic degradation. Observed "gain" of carbohydrates can be explained by difference in analytical techniques used for analysis of original sample and soluble xylan fraction.

Further, alkali extracted xylan digests were analyzed with HPAEC and MALDI TOF-MS in order to detect the oligosaccharide structures released by EX1. HPAEC chromatograms of all xylan digest were similar (Fig. 3) and showed that for all xylan fractions linear xylo-oligomers were released (DP 1–3) after EX1 digestion. About 30% of the total amount of xylan in 1 M KOHss and about 15% for 4 M KOHss was released as linear xylo-oligomers (DP 1–3) for all the three phases. Apart from linear, some substituted xylo-oligomers were identified (HPAEC and MALDI TOFMS) to be xylo-oligosaccharides low substituted with only one 4-O-methyl uronic acid. We presumed this to be 4-O-methyl-glucuronic acid as in most Poaceae xylans (Timell, 1967). After EX1 digestion, no arabinose substituted xylo-oligomers were observed on HPAEC chromatogram, although some were expected based on the Ara/Xyl-ratios of these alkaline extracts (Table 3). The released xylo-oligosaccharides substituted with arabinosyl residues were most likely too large to be separated with HPAEC. In the corresponding HPSEC-chromatograms, indeed, larger Mw-material was eluted (Fig. 2). Nevertheless, all these results added to our suggestion that xylan in the compost showed a low substitution.

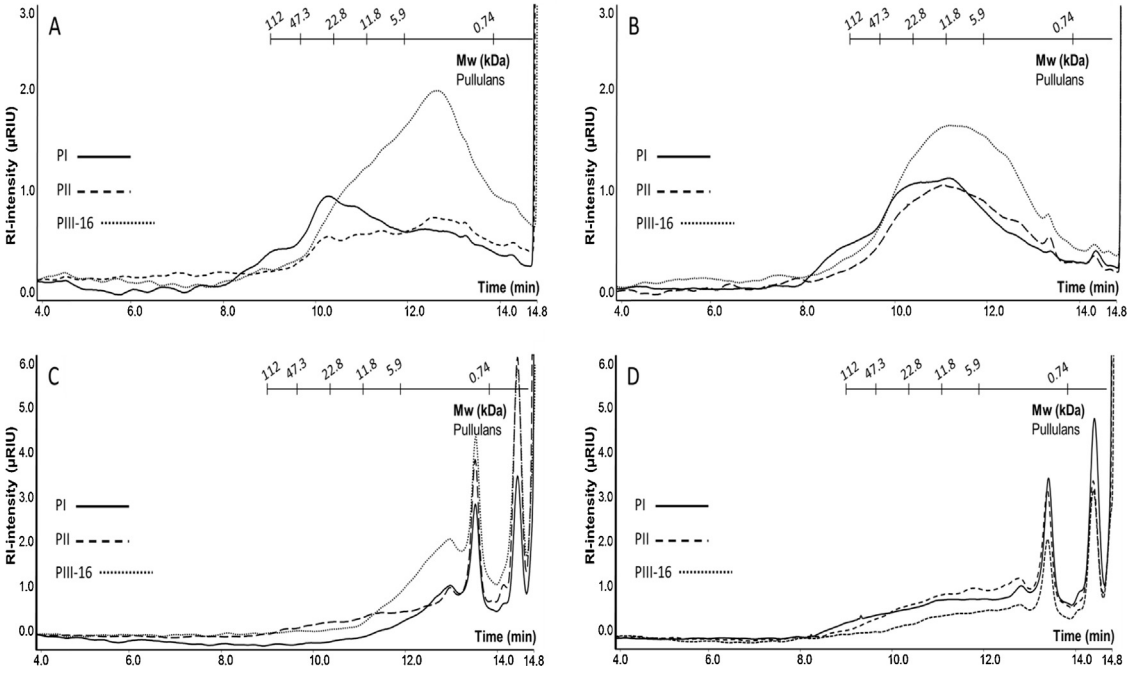

Fig. 2. Molecular weight distribution of 1 M KOH extract PI, PII and PIII-16 before digestion (A) and endpoint digested (C); and 4 M KOH extract before digestion (B) and endpoint digested (D). A purified and well characterized endoxylanase (EX) (Kormelink & Voragen, 1993), is used for digestion.

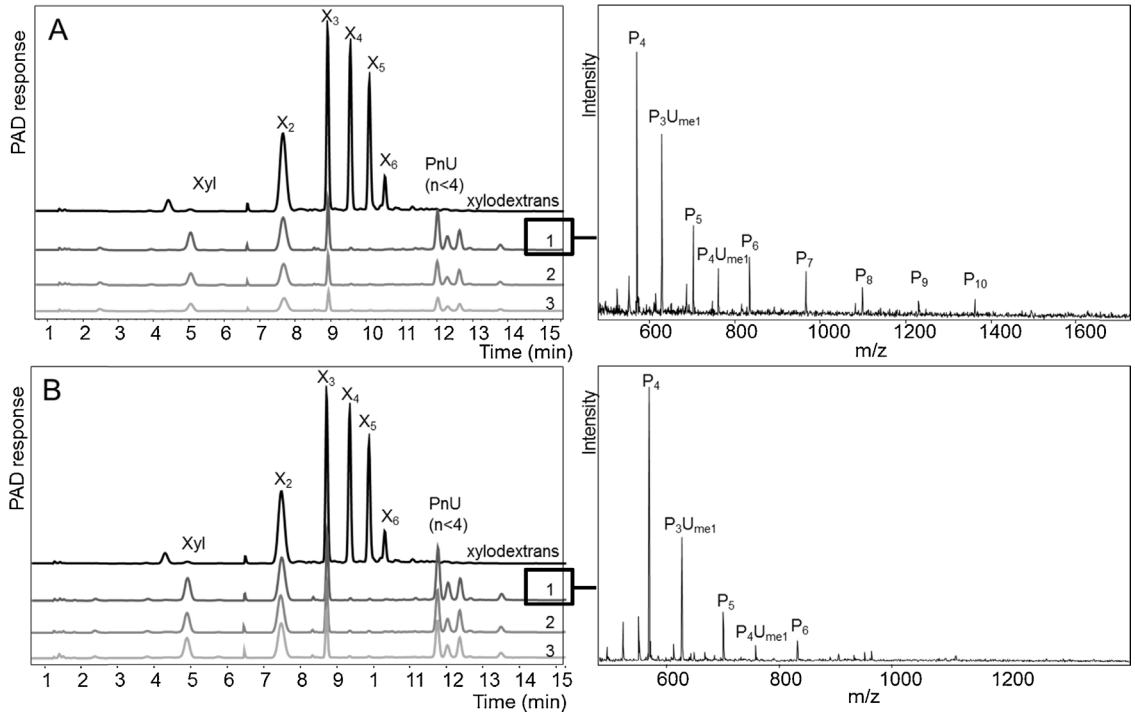

Fig. 3. HPAEC elution pattern (left) and MALDI mass spectrum (insert at right) of the 1 M (A) and 4 M (B) alkali-soluble fractions of PI (1), PII (2) and PIII-16 (3) after degradation by endoxylanase I (P = pentose, Xyl = xylose, X_2–X_6 = xylodextrans DP 2–6, U = uronic acid, U_{me} = methyl-uronic acid).

4. Conclusions

The xylan present was shown to be poorly substituted as present in remainings of all compost phases studied and no changes in the amount of substituents were observed. Due to the rather high pH of BM, no ester-linked acetic, coumaric or ferulic acid was observed, probably making the xylans present more susceptible for enzyme degradation. Degradation and consumption of carbohydrates in this phases is suspected to occur in a uniform manner, degrading as much substituents as xylosyl residues from the backbone. Over the period of mycelium growth xylan chains became partially degraded and their water-solubility increased. Also, glucans' solubility in water increased. It can be hypothesized that these partially degraded water-soluble xylan and glucan structures are well accessible and are the first source of carbohydrates for *A. bisporus* in later growth stages (e.g. fruiting body formation). To confirm this hypothesis, further research will study compost samples during mushroom fruiting body formation.

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