How mushrooms feed on compost:

Conversion of carbohydrates and lignin in industrial wheat straw based compost enabling the growth of *Agaricus bisporus*

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This research was conducted under the auspices of the Graduate School VLAG (Advanced studies in Food Technology, Agrobiotechnology, Nutrition and Health Sciences).

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Thesis

submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Academic Board,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Friday 19 June 2015
at 4 p.m. in the Aula.

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How Mushrooms feed on compost: Conversion of carbohydrates and linin in industrial wheat straw based compost enabling the growth of *Agaricus bisporus*
156 pages.

PhD thesis, Wageningen University, Wageningen, NL (2015)
With references, with summaries in Dutch and English

ISBN: 978-94-6257-311-6

Abstract

In this thesis, the fate of carbohydrates and lignin was studied in industrial wheat straw based compost during composting and growth of *Agaricus bisporus*. The aim was to understand the availability and degradability of carbohydrates in order to help improve their utilization in the compost. The wheat straw based compost was characterized as being composed mainly of cellulose and lowly substituted xylan. During the first phase of composting, ester-bound substituents were removed from the xylan backbone and during the second phase of composting 50% of carbohydrates present in the original material where metabolized in a uniform manner. Lignin structure, however, remained unaltered during these composting stages. Over the period of *A. bisporus* mycelium growth, 20% of the original xylan became water soluble while xylan structures remained rather similar and the remaining water insoluble xylan was partially degraded. In addition, 40% of lignin was metabolized during mycelium growth with an increase in the ratio of syringyl to guaiacyl lignin units from 0.5 to 0.7 in mycelium grown compost compared to the basic compost mixture. During the fruiting body formation minor changes in lignin structure occurred, while accumulation of xylan substituents was observed for arabinosyl residues and glucuronic acid substituents. Finally, putative genes encoding carbohydrate degrading enzymes were identified in *A. bisporus'* genome. Genes involved in the pentose and hexose catabolic pathway were found to be upregulated in *A. bisporus* mycelium. *A. bisporus* was found to produce both xylan and cellulose degrading enzymes and maximum activity was observed during the formation of the 1st flush of mushrooms. But, as observed from the remaining xylan structures analyzed, *A. bisporus* lacks the enzymatic activity to degrade xylan substituted with two arabinosyl- residues and glucuronic acid substituted xylan.

List of abbreviations

1M KOHss	1M KOH soluble solids
4M KOHss	4M KOH soluble solids
AcA	Acetic acid
Ara	Arabinosyl
DM	Dry matter
DP	Degree of polymerization
DS	Degree of substitution
EX	Endoxylanase
G	Guaiacyl-like lignin units
Gal	Galactosyl
GAX	Glucurono-arabinoxylan
GC	Gas chromatography
Glc	Glucosyl
GlcA	Glucuronic acid
HPAEC	High performance anion exchange chromatography
HPSEC	High performance size exclusion chromatograpyy
Man	Mannosyl
MALDI-TOF	Matrix-assisted laser desorption/ionization time-of-flight
MS	Mass spectrometry
n.a.	Not analyzed
n.s.	Not specified
Py-GC/MS	Pyrolysis GC/MS
Rha	Rhamnosyl
RS	Reverse search
PAD	Pulsed amperiometric detection
Res	Residue after sequential extraction
S	Syringyl-like lignin units
UA	Uronyl
WAX	Wheat arabinoxylan
WSS	Water soluble solids
WSSd	Water soluble solids dialyzed
WUS	Water un-extractable solids
Xyl	Xylosyl

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Chapter 1

General introduction

1.1 Project background and aim

The white button mushroom, *Agaricus bisporus* is cultivated worldwide for its edible, nutritious fruiting bodies. For commercial purposes, mainly, the *A. bisporus* heterokaryon strain A15 is cultivated. In the last years, in the Netherlands, 250 million kg of this mushroom were produced yearly (personal communication CNC-C4C). Commercially, *A. bisporus* is grown on a compost based on wheat straw, straw bedded horse manure, chicken manure and gypsum (Gerrits, 1988).

Composting of raw ingredients and conditions needed for mushroom production are well-established. However, knowledge on the biochemical changes occurring during different stages of this process is lacking. It is not known to which extent compost components are converted and in which stage of the process. Next to the production of mushrooms also large amounts of spent compost still containing nutrients like carbohydrates remain at the end of the commercial mushroom production.

Therefore, the overall aim of the Dutch Technology Foundation (STW) project 'How mushrooms feed on sugar', of which this thesis was part of, was to understand the availability and degradability of carbohydrates in order to help improve their utilization in the compost and, hereby ultimately increase the amount of mushrooms obtained per ton of compost. The project combined research from various scientific fields, like fungal genetics and molecular biology with carbohydrate biochemistry. In this thesis, the focus is on the characterization and quantification of remaining carbohydrates in the compost after different stages of composting and mushroom growth with well-established carbohydrate analysis methods. In addition, a method for lignin characterization was optimized and also assessed for lignin quantification in compost samples. Finally, carbohydrate degrading enzymes present in mycelium grown compost were tested for their activity throughout the mushroom growth stages, and compared with the expression levels of corresponding genes of *A. bisporus.*

1.2 White button mushroom, *Agaricus bisporus*

Agaricus bisporus belongs to the family *Agaricaceae*, order *Agaricales* and subclass *Agaricomycetidae* (Kirk et al., 2001) and is a leaf litter degrading basidiomycota fungus that naturally grows in grasslands and forests. The lifecycle of A. *bisporus* consists of two phases. A vegetative mycelial phase, followed by a reproductive fruiting body forming phase. In general, the role of the vegetative mycelium is to supply nutrients for the growth of fruiting bodies, while the role of fruiting bodies is reproduction (Bonner et al., 1956).

In an industrial process, the change from the vegetative to the reproductive fruiting body formation phase occurs as a result of covering colonized compost with a layer of non-sterile peat or soil, mixed with lime, known as 'casing soil' (Fig. 1.1).

The presence of the bacteria, specifically *Pseudomonas putida*, in the casing layer is required for the development of mushroom primordia (pins) (Hayes et al., 1969; Park and Agnihotri, 1969). These bacteria are found to respond to amino acids from the exudate from *A. bisporus* mycelium (Grewal and Rainey, 1991) and stimulate hyphal extension. *P. putida* was found to markedly affect hyphal growth and colony morphology of *A. bisporus* only when growing in direct contact with the mycelium (Rainey, 1991). This suggests that the stimulus afforded by the bacterium is neither volatile nor readily diffusible. This is consistent with the hypothesis that *P. putida* induces fruiting by removal of a fruiting inhibitory substances such as CO_2 (Long and Jacobs, 1974; Wood, 1984). The covering by casing soil together with the right conditioning regarding temperature and humidity results in the formation of pins, which develop some days later into mature mushrooms.

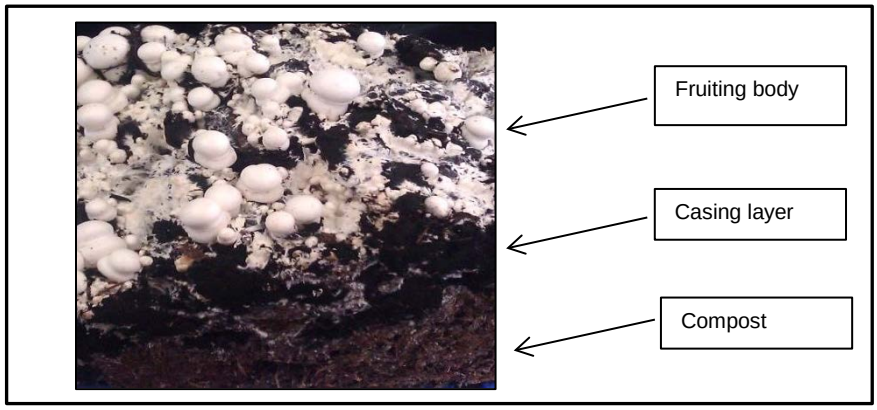

Figure 1.1. A vertical cross-section through compost with fruiting bodies, casing layer and compost. The casing layer and compost are grown with mycelium (white tracks).

A. bisporus mycelium cell walls consist of 43% (w/w) chitin, 14% (w/w) KOH-soluble α-glucan, 27% (w/w) β-glucan, 16% (w/w) protein and 1.5% (w/w) lipids of which the totals are slightly over 100%, but as such reported by Michalenko and co-authors (1976). The inner layer of the mycelium cell wall is composed of chitin microfibrils in a β-glucan matrix also containing protein, while the outer layer consists of a KOH-soluble α-glucan (Michalenko et al., 1976). Compost grown mycelium is found to grow by apical elongation, while mycelium of expanding mushrooms grows by diffuse extension over their whole wall surface. No significant differences were found in overall chemical wall composition of the two mycelium types (Mol and Wessels, 1990). The main difference regarding the composition of mycelium compared with fruiting bodies is in the accumulated amounts of mannitol in fruiting bodies, which can make up to 35% of total soluble material (Hammond and Nichols, 1976).

1.3 Compost as feedstock for mushroom growth

Commercial compost production is a process involving the bioconversion of raw materials into a substrate supporting the growth of *A. bisporus*. In different parts of the world the raw ingredients used for compost production differ. Nevertheless, they always contain a carbon and a nitrogen source. In the European composting process, compost is produced from a mixture of wheat straw (40 to 45% w/w), straw bedded horse manure (20 to 25% w/w), poultry manure (10 to 15% w/w) and gypsum (5 to 10% w/w) with varying amounts (10 to 15% w/w) of optional ingredients, such as grain, seed meals, cotton hulls and other agricultural byproducts (Iiyama et al., 1994).

The production of the compost substrate for *A. bisporus* is usually carried out in two indoor phases, Phases I and II (PI and PII, Fig. 1.2). PI is characterized by high temperatures up to 80ºC and can last 3 to 10 days. During PI, mesophilic microbiota develop and degrade the carbohydrates and proteins, which results in the release of heat and ammonia. These reactions cause the wheat straw to soften. As the temperature rises, mesophilic microbiota are replaced by thermophilic microbiota (Gerrits, 1988).

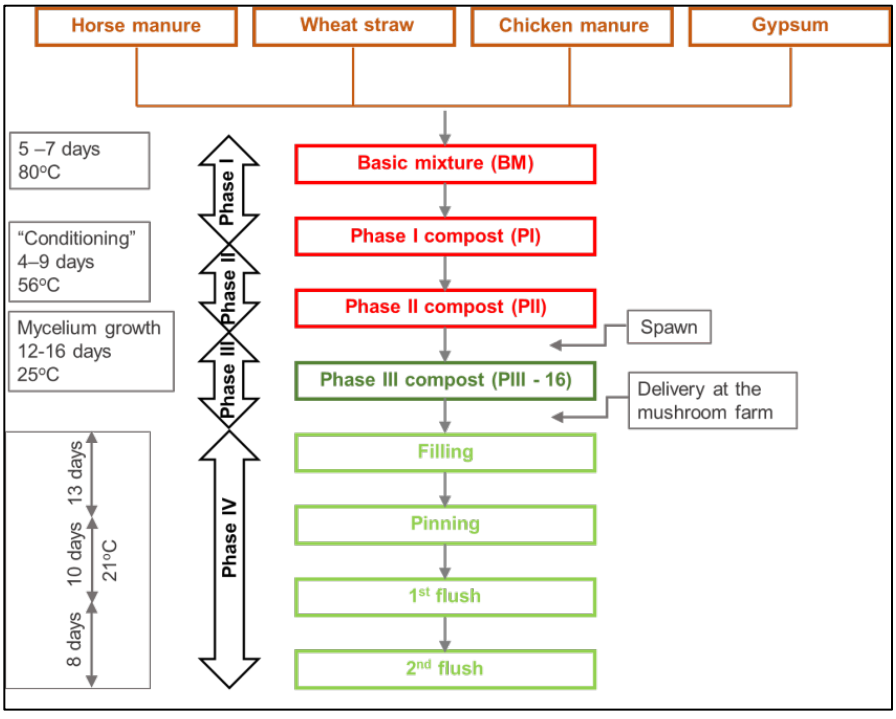

Figure 1.2. Schematic representation of the composting and mushroom growth phases.

PII conditions the compost until it is free of ammonia at a temperature up to 60ºC for 4 to 9 days. Also, the aim of PII is to inactivate undesirable microorganisms in the compost. In this phase, microorganisms, in particular actinomycetes and fungi consume part of the ammonia present (about 40%). The other part (60%) disappears as NH_3 in the air (Gerrits, 1988).

As a result of the composting process, the PII compost consists mainly of lignocellulosic components together with microbial biomass. A major part of this microbial biomass consists of the thermophilic fungus *Humicola insolens* var. *thermoidea* (*Scytalidium thermophilum*), which is important for increasing the growth rate of the mushrooms and reducing the ammonia concentration (Fermor and Grant, 1985; Straatsma et al., 1989).

At the end of PII, the temperature is lowered to about 24ºC and PII compost is inoculated with millet or rye grains colonized with *A. bisporus*. Mushroom mycelium can consume the microflora present in the compost (Fermor and Wood, 1981). However, this is not sufficient and mushrooms degrade and consume cellulose, hemicellulose and lignin from the compost in order to obtain enough nutrients. After inoculation, mycelium grows through the compost for 12-16 days after which compost is considered mature. At this point, casing layer (Fig. 1.1) is added on top of the compost to start the fruiting body formation (Gerrits, 1988). The first harvestable mushrooms appear after 18 to 21 days and after that in repeating cycles of 7- to 8- day known as flushes (Flegg and Wood, 1985). Several flushes of mushrooms can be harvested before compost is considered spent, although generally there are two harvests.

Previous studies performed on compost structure and effect of composting phases and mushroom growth are difficult to compare. This is due to different raw ingredients used for the basic compost mixture. Moreover, different composting conditions, e.g. length of composting phases, were applied for production of wheat straw compost (Durrant et al., 1991, Gerrits, 1967; Iiyama et al., 1994; Lyons et al., 2006) (Table 1.1). Mainly total carbohydrate content, lignin and inorganic constituents in the compost were investigated on a relative base and not quantified in absolute values (Gerrits, 1967, Lyons et al., 2006). Compost samples were collected at different phases and, although composting conditions were not the same, the obtained results suggest the loss of dry matter during composting and carbohydrate degradation during composting and mushroom growth (Table 1.1).

To date, the composition and content of compost throughout the composting and mushroom growth is not completely characterized for all phases in a single European industrial process. Also, the influence of composting conditions and mushroom growth on the carbohydrate and lignin structures remains unclear.

Table 1.1. Overview of wheat straw based compost composition and content in Basic mixture (BM), Phase I (PI), Phase II (PII) and spent (After 1st flush) compost based on literature.

Sample			Ash (% w/w)	% dry matter (% organic matter)			
	DM[a] (kg)	Ash (kg)		Nitrogen	Neutral carbohydrates	Lignin	
						Acetyl-bromide	Klason[b]
BM	400	84	-	-	-	-	-
PI	280	84	6/18	1.6	40.0 (44.9)	22.6 (25.4)	25.1
PII	235	84	10/23	2.3	22.4 (31.4)	22.9 (32.1)	31.5
PIII	-	-	-	-	-	-	-
Spent compost	182	82	10	-	11.1 (15.3)	23.5 (32.5)	40.3

BM: mixture of raw ingredients, PI: compost after Phase I of composting, PII: compost after Phase II of composting, PIII: mycelium grown compost.
Iiyama et al., 1994; PI: 2 weeks (outdoor), PII: 1 week (indoor), spent compost after 1st flush.
Lyons et al, 2006; PI: 0-8 days (indoor), PII:6-8 days (indoor).
[a]Based on 1000 kg fresh BM (Gerrits, 1988.).
[b]Treatment with 72% (w/w) H_2SO_4 at room temperature for 4 h and then with boiling 3% (w/w) H_2SO_4 for 2 h.

1.4 Wheat straw cell wall composition

Wheat straw provides both nutrition for the mushroom mycelium and a physical structure in the compost. The latter ensures that air spaces in the compost provide aerobic conditions for biodegradation. Manure is added to activate fermentation and act as a slow-releasing nitrogen and carbon source. Gypsum is added as a mineral source and, in addition, it precipitates suspended colloids and makes compost less greasy (Lyons et al., 2006).

Wheat straw (*Triticum spp*.) is the straw biomass remaining after the removal of the wheat grains from the plants. Wheat belongs to the family Poaceae in the plant group of Monocots.

In general, plant cell walls are composed of a primary and secondary layer, both built from various polymers, such as polysaccharides, lignin and protein. In grasses primary cell walls are mainly built from cellulose fibers (20-30% w/w), glucuronoarabinoxylan (xylan) (20-40% w/w) and are low in pectin (5% w/w) and structural proteins (1% w/w). Also, lignin is a minor component of the primary cell walls. Secondary cell walls make up to 50% (w/w) of stem cell walls and consist of cellulose (35-45% w/w), xylan (40-50% w/w/) and lignin (20% w/w) (Vogel, 2008). Xylans in wheat straw are found to be composed of xylosyl-residues in the xylan backbone with mainly arabinosyl- and glucuronic acid substituents (Sun et al.,

1996). However, detailed information on the amount of substituents and their distribution over the xylan backbone is not known.

Overall, the plant cell walls of wheat straw are mainly composed of cellulose (40% w/w), xylan (24% w/w) and lignin (22% w/w) (Kristensen et al., 2008). These three main polymers and enzymes known to degrade their structures are listed below.

1.4.1 Cellulose and cellulose degrading enzymes

Cellulose is a linear insoluble polymer composed of (1,4)-β-D-glucosyl units (Himmel et al., 1999). Cellulose macromolecules aggregate to microfibrils, which can form highly ordered crystalline structures, or amorphous, less ordered regions (Kolpak and Blackwell, 1976).

For a complete cellulose degradation, at least three classes of enzymes are shown to be relevant, preferably with a synergistic action (Himmel et al., 2007). Endo-glucanases cleave in the middle of the (1,4)-β-D-glucan chains releasing smaller oligosaccharides from the cellulose matrix and upon cleavage in the middle of a chain, making additional starting sites available for cellobiohydrolases to act on (Lopez-Casado et al., 2008). Cellobiohydrolases release cellobiose from the reducing or non-reducing ends and can also act on more crystalline regions of cellulose (Duan and Feng, 2010). Finally, β-glucosidases cleave cellobiose and oligosaccharides to glucose (Saibi and Gargouri, 2011). Recently, some proteins expected to be glycoside hydrolases (GH61) (Harris et al., 2010) were found to lack glycoside hydrolase activity, however, in the presence of various divalent metal ions they significantly enhance the degradation of lignocellulosic biomass.

1.4.2 Hemicellulose and hemicellulose degrading enzymes

In wheat straw the β-D-xylopyranosyl (Xyl*p*) units of the (1,4)-β-D-xylan backbone can be single or double substituted with α-L-arabinofuranosyl (Ara*f*) units at *O*3 and/or at *O*2 of the Xyl*p* units (Fincher, 2009). Some of the α-L-arabinofuranosyl units can be further 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) and its 4-*O*-methyl ether can be a substituent, linked to the *O*2 of Xyl*p* units of the xylan backbone (Fincher and Stone, 2004). A number of publications also report the presence of *O*-acetyl substituents in xylans from Poaceae to which wheat belongs (Ishii, 1991; Wende and Fry, 1997) (Fig. 1.3).

Xylan degrading enzymes comprise endo-acting β-1,4-xylanases and exo-acting β-xylosidases for depolymerization. For total xylan hydrolysis to xylose, of course, all substituents must be removed. Hereto, accessory enzymes, like α-arabinofuranosidases, α-glucuronidases, acetyl xylan esterases, ferulic acid esterase and p-coumaric acid esterases, are necessary.

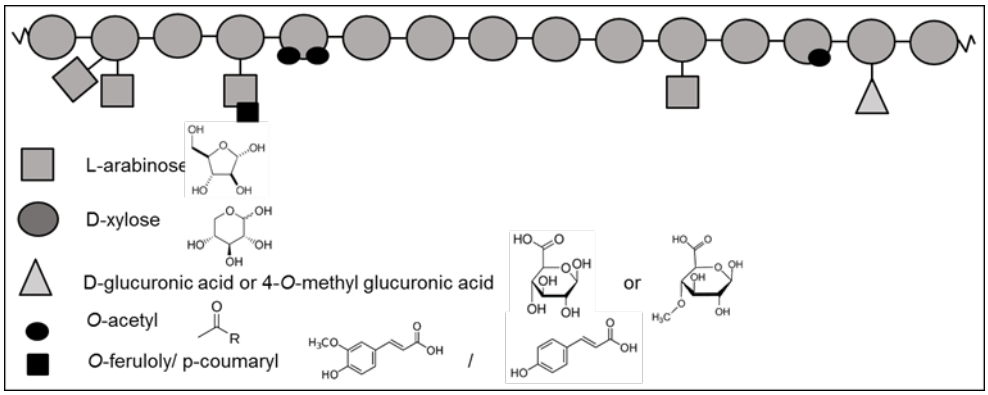

Figure 1.3. Schematic representation of wheat straw xylan.

1.4.3 Lignin and lignin degrading enzymes

Lignin is an aromatic polymer that consists of three different polypropane units: p-coumaryl, coniferyl and synapyl alcohol (Lu and Ralph, 1997). These monolignols are present in lignin as p-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) phenylpropanoid lignin units (Fig. 1.4), which are found in different ratios depending on the source (Dashtban et al., 2010). Some of the lignin units can further be covalently bound to hemicellulose to form lignin-carbohydrate complexes, in which cellulose is enwrapped (Kim and Ralph, 2010, Ralph et al., 2004). As mentioned above, these three polymers are deposited predominantly in the secondary cell walls and in Poaceae (grasses) biomass between 5-25% of lignin is present (Ralph et al., 2010). However, little is known on the structure of lignin in wheat straw to date.

Lignin protects cell wall polysaccharides from microbial degradation, thus imparting resistance to decay. Lignin limits the rate and extent of (enzymatic) hydrolysis by acting as a shield, preventing the digestible parts of the substrate to be hydrolyzed (Chang and Holtzapple, 2000). Moreover, enzyme performance is reduced during lignocellulose hydrolysis by adsorption of the enzyme proteins to lignin (Berlin et al., 2006). The degradation of lignin by either chemicals or enzymes is still a complex and unraveled area.

Lignin is synthesized by free radical condensation of p-coumaryl, coniferyl and synapyl alcohol yielding a heterogeneous, amorphous and highly branched structure. These strucures are not susceptible to enzyme hydrolysis, but depolymerization by oxidative mechanisms is required (Gold and Alic, 1993).

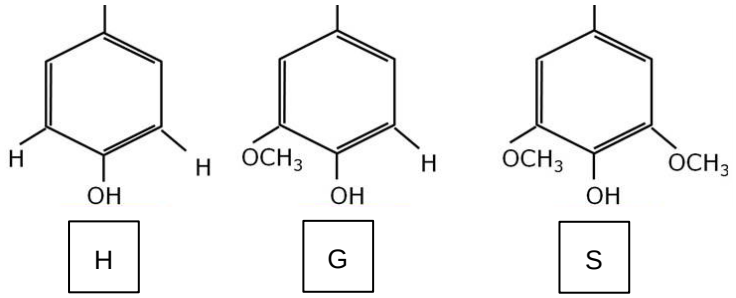

Figure 1.4. Phenylpropanoid lignin units: p-hydroxyphenyl- (H), guaiacyl- (G) and syringyl- (S).

As such, enzymes proposed to be involved in lignin degradation can be grouped into two families: phenol oxidases (e.g. laccases) and peroxidases whose activity depends on the presence of H_2O_2 (Gold and Alic, 1993). However, their role in lignin degradation is not known and mainly indirect evidences are presented to indicate lignin degradation by the action of these enzymes. Also, non-enzymatic degradation assisted by oxidation through production of free hydroxyl radicals has been shown to induce lignocellulosic degradation (Suzuki et al., 2006). It was shown that these radicals are formed by both oxidases and peroxidases. The free radicals formed by these enzymes can act on lignin as well as on polysaccharides and may enable lignocellulolytic enzymes to penetrate the plant cell wall (Call and Mücke, 1997, Wang et al., 2006).

1.5 Degradation of plant cell walls by *Agaricus bisporus*

Agaricus bisporus is known to degrade cellulose and hemicellulose during growth under the conditions used for the commercial production of mushrooms (Durrant et al., 1991, Gerrits, 1967; Gerrits, 1968; Wood and Leatham, 1983). Furthermore, it has been suggested that *A. bisporus* degrades lignin to increase the bioavailability of carbohydrates (ten Have et al., 2003). So far, mainly qualitative changes of compost composition have been reported (Gerrits, 1967; Iiyama et al., 1994) and in case of lignin degradation, indirect evidence is provided by using model (^{14}C labeled) lignin or lignin degradation is proposed by the determination of the presence of the laccase activity (Bonnen et al., 1994; Durrant et al., 1991; Waksman and Nissen, 1932; Wood and Leatham, 1983).

It has been shown, that during mycelial growth and fruiting, *A. bisporus* produces a range of extracellular enzymes involved in the degradation of the lignocellulosic polymers in the compost. Cellulase activity, in particular endo-glucanase activity, is reported to be highest at the onset of the fruiting body formation (Wood and Goodenough, 1977). Xylanase activity is found to be higher than cellulase activity, indicating the importance of xylan as a substrate for *A. bisporus* growth (Savoie, 1998). Knowledge on enzymatic activities produced by *A.*

bisporus is mainly obtained for exo-active carbohydrases under their optimal conditions with standard assays, e.g. reducing end assay, as well as, activity towards model substrates, e.g. carboxymethyl cellulose, PNP-sugars. These results can be misleading as standard assays are not always representative for the enzyme activity under composting conditions or on natural polysaccharides (Kabel et al., 2006).

A. bisporus is shown to produce both manganese peroxidase and laccase, similar to white rot fungi (Bonnen et al., 2004, Perry et al., 1993), which are expected to be involved in lignin degradation. Opposite to the carbohydrate degrading enzymes excreted, which were found to be the highest during fruiting body formation, laccase levels are found to be highest in the mycelium grown compost, and declined during fruiting body formation (Perry et al., 1993). Additionally, laccase production is found to coincide with lignin disappearance (Durrant et al., 1991, Waksman and Nissen, 1932).

With the use of the recombinant DNA technology, a large number of *A. bisporus* genes were cloned and characterized, such as genes involved in compost utilization as well as fruiting body development (De Groot et al., 1998). Specific gene expression and enzyme excretion, such as cellobiohydrolase, β-glucosidase and α-glucuronidase, were mainly studied as a result of the growth of *A. bisporus* on a specific model substrates, like cellulose or birchwood xylan (Chow et al., 1994; Puls et al., 1987; Yague et al., 1997), but not on compost. This approach can provide misleading results as different genes might be expressed and translated to enzymes differently in compost compared to model substrates. Only endoxylanase and laccase gene expression was studied and confirmed in compost samples (De Groot et al., 1998; Perry et al., 1993). Apart from the genes discussed above, the complete genome of *A. bisporus* and its carbohydrate degrading machinery is not known.

1.5.1. Carbohydrate analyses with a focus on methods applied in this thesis

Total neutral sugar content is analyzed as the sum of constituent monosaccharides after the cleavage of glycosidic bonds in a polysaccharide. These monosaccharides are released in the presence of a strong acid and heat and analyzed directly by high performance anion exchange chromatography (HPAEC) or after derivarization as their alditol acetates by gas chromatography (GC).

Uronic acids content is commonly determined as anhydro-uronic acid content by the m-hydroxyphenyl assay (Thibault, 1979) with addition of sodium tetraborate. Uronic acids react with m-hydroxydiphenyl in a strongly acidic environment to form colored complexes which can be measured by absorbance at 520 nm and further quantified by using appropriate standards, e.g. glucuronic acid.

Xylan substitutents; like ester- bound ferulic, p-coumaric and acetic acid, are usually measured by reversed phase (C18) separation making use of ultra-high performance liquid chromatography (RP-UHPLC) after saponification of the samples with alkali solutions, e.g. NaOH or KOH.

In addition, enzymatic hydrolysis is a useful tool to determine carbohydrate structures by producing oligosaccharides or specifically releasing monosaccharides. It relies on the ability of an enzyme to catalyze a specific reaction. Enzymatic methods are highly specific, usually rapid and sensitive to low carbohydrate concentrations (Brummer and Cui, 2005).

After acid or enzyme hydrolysis, released mono- and oligosaccharides can be separated by HPAEC, with optimized and for the purpose suitable separation gradients, followed by identification and quantification with the use of appropriate standards. With this method, however, information about methyl esters and acetyl groups is lost due to saponification. Therefore, for separation of highly polar oligosaccharides, hydrophylic interaction chromatography (HILIC) is preferred (Leijdekkers et al., 2011). Also, polysaccharides and oligosaccharides can be separated based on their size by size exclusion chromatography (HPSEC). HPSEC is combined with a refractive index detectors, often used alone or in combination with UV detectors.

1.5.2. Lignin analyses with a focus on methods applied in this thesis

A major difficulty in studying lignin is the complexity of the molecular structure and its' highly insoluble nature. Several methods to study lignin are present in literature. The most commonly used method for lignin analysis is empirical, the Klason lignin analysis. This method measures the insoluble residue after pre-hydrolysis with 72% (w/w) H_2SO_4 and subsequent hydrolysis with 1M H_2SO_4. After treatment, the suspension is filtered and the remaining neutralized and dried residue is considered to be Klason lignin. Also, acid soluble lignin is quantified with this method but spectrophotometric at 205 nm. This method provides mainly an indication of total lignin content (Jung et al., 1999). Klason lignin values are influenced by the presence of protein and pseudo-lignin (aromatic material that is not derived from native lignin) (Sannigrahi et al., 2011; Saura-Calixto et al., 1991). Lignin content is also studied by the non-gravimetric acetyl-bromide lignin method (Fukushima and Kerley, 2011) that is based on O-acetylation of terminal lignin monomers increasing their solubilization, which is then measured spectrophotometric at 280 nm. However, quantification of lignin can be overestimated if other chemical reactions occur (Iiyama et al., 1994). This method, similar to the Klason lignin method, provides mainly indicative information on lignin content, however it does not provide information on lignin structures. 2D-NMR is used in lignin analysis to obtain information on the degree of acetylation and inter-

unit linkages. However, 2D-NMR is a relatively expensive method to be used for a large number of samples. Further, similar to Klason and acetyl-bromide lignin, the accuracy of this method depends on the solvent used, reaction conditions and the solubility of the lignins in the solvent used.

Lignin can also be analyzed by analytical pyrolysis linked to GC-MS. Analytical pyrolysis (Py-GC/MS) provides information on structural lignin compounds by pyrolysing the native sample and introducing the volatile lignin-derived phenolic compounds into a mass spectrometer after separation by GC. So far, this method is mainly used to characterize lignin in plant materials (Ralph and Hatfield, 1991) and not for description of changes in structure or quantification of lignin after pre-treatment and/or enzymatic degradation. Py-GC/MS is independent on the solubility of lignin in the sample.

One measure of lignin composition is the content of S (syringyl-like lignin structures) and G (guaiacyl-like lignin structures) and their ratio (S:G) (Davison et al., 2006). Studies have shown that lignin, in general, is related to cell wall digestibility. Content, S:G ratio and the three–dimensional structure of lignin were found to influence the degradability of cell walls. In the paper-pulping process, a higher S:G ratio was found to be related to a better depolymerization (Rodrigues et al., 1999). In addition, in cell walls of grasses, lignin structure with a more condensed coupling of G units into branched lignin polymers was found to limit enzymatic hydrolysis of polysaccharides to a greater extent than the presence of linear lignin having more β-O-4 coupled S units (Grabber et al., 2004).

1.6. Thesis outline

As stated above (**chapter 1**), limited research of compost structure, focusing mostly on relative composition of compost constituents was performed to date. This chapter describes the background and aim of this PhD thesis. Further, it summarises the current knowledge on the characterization of xylan and lignin structures throughout composting and mushroom growth of *Agaricus bisporus*. Mapping the amounts of structural components in the compost is needed for improving the process.

Chapter 2 describes the carbohydrate composition of compost during composting and mycelium growth of *A. bisporus*. Cell wall polysaccharides were extracted using water and KOH solvents and analyzed for their carbohydrate composition and molecular structure. In **chapter 3** the mass balance of compost and its structural components throughout composting and mycelium growth is analyzed. In addition, lignin, S:G ratio, content and distribution of lignin units is analyzed by analytical Py-GC/MS in order to elucidate changes in lignin during composting and mycelium growth.

In **chapter 4**, xylan and lignin structures in compost samples during fruiting body formation of *A. bisporus* are described. Xylan is extracted from the compost and analysed for its composition and degree of substitution in order to identify the recalcitrant xylan structures. Lignin structures and S:G ratio is analysed by Py-GC/MS in order to identify changes in lignin during the reproductive stage of *A. bisporus* growth.

Chapter 5 describes the carbohydrate utilization and differentiation of carbohydrate metabolism in *A. bisporus*. Genes encoding putative enzymes for carbon metabolism are identified and their expression is studied in mycelium grown compost and fruiting bodies after the first flush of mushrooms was collected. The expression of the genes is further correlated to the soluble carbohydrates and composition of mycelium grown compost, casing layer and fruiting bodies.

In **chapter 6**, extracellular enzymes are extracted from the compost before addition of mycelium, throughout vegetative mycelium growth and during fruiting body formation of *A. bisporus*. Extracted enzymes are tested on a range of cell wall carbohydrates and assessed for their ability to degrade the carbohydrates present in the compost under compost conditions.

In **chapter 7**, the different results obtained in this thesis are summarized and discussed. The effect of each of the stages of composting and mushroom growth is discussed with respect to its' effect on the cell wall structures of wheat straw and possible applications of obtained results are evaluated.

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Chapter 2

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

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, 1M and 4M 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.

Based on: Jurak, E., Kabel, M.A., Gruppen, H., 2014. Carbohydrate composition of compost during composting and mycelium growth of *Agaricus bisporus*. Carbohydr. Polym, 101, 281-288.

2.1. Introduction

The world-wide production of the white button mushroom *Agaricus bisporus* was estimated to be about 4 million tons in 2009 (Sonnenberg, 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 microbiota, including fungi, start to decompose the BM material and cause rise of temperature (Chang, 1967) up to 80°C (personal communication CNC, Milsbeek, The Netherlands) resulting in Phase I (PI) compost (Fig. 2.1). 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, 1994). The third phase (PIII) starts after inoculation of PII compost with *Agaricus bisporus* mycelium (Fig. 2.1). 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, 1982, Straatsma, 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, 2000, Semple, 2001).

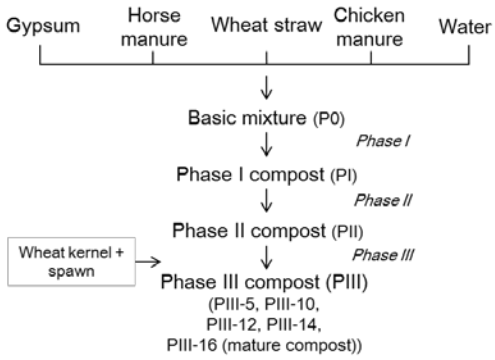

Figure 2.1. Schematic representation of the composting and mycelium growth phases.

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, 1999). Specific features of xylan, are determined by the source from which xylan is extracted (Gruppen et al. 1992). Xylan from Poaceae consist of a (1,4)-β-D-xylan backbone. The β-D-xylopyranosyl (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 and Stone, 2004). Few publications also report the presence of *O*-acetyl substituents present in xylans from Poaceae (Ishii, 1991, Wende and 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, 1967, Lyons et al., 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 (Iiyama, 1994, Durrant et al., 1991, Wood and 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.

2.2. Materials and methods

2.2.1. 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), 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) (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).

2.2.2. 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 x 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 1M KOH containing 1% (w/w) $NaBH_4$ for 18 h at room temperature under continuous stirring. After centrifugation (10 000 x g; 30 min; 20°C), the residue was washed with 200 mL 1M KOH containing 1% (w/w) $NaBH_4$ and again centrifuged. Supernatants were combined (1M KOHss). The residue was re-extracted and washed once with 4M KOH containing 1% (w/w) $NaBH_4$, as described for the 1M KOH extraction. Supernatants were combined and labeled 4M 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.

2.2.3. Hydrolysis of alkali extracts with endoxylanase

Suspensions of 1M and 4M KOHss (4 mg KOHss) in 50mM sodium acetate buffer pH5 (977 µL) were incubated with a pure endo-(1,4)-β-D-xylanase 1 (EX1) from *Aspergillus awamori* (23 µL, 0.55 µg protein) (Kormelink et al, 1993) overnight at 35°C. After inactivation of the enzyme (10 min, 100°C) digests were

analysed by HPSEC (non diluted), HPAEC (diluted 20x in water) and MALDI-TOF MS (non diluted). Wheat arabinoxylan, medium viscosity (Megazyme, Wicklow, Ireland) was treated similarly and used as a reference.

2.2.4. Analytical, chromatographic and spectrometric methods

All analyses were performed in duplicate.

2.2.4.1. 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 1M 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 1M and 4M KOHss before and after EX1 digestion, was directly hydrolyzed with 1M H_2SO_4 for 3 h at 100°C. Samples were diluted (20x) and analyzed for monosaccharide content by HPAEC (high performance anion exchange chromatography).

2.2.4.2. 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, Breda, The Netherlands). Glucuronic acid (Fluka AG, Busch, Switzerland) was used as a reference (12.4 to 200 µg mL^{-1}).

2.2.4.3. Esterified acetic acid content

Each sample (20 mg) was saponified with 1 mL of 0.4M NaOH in isopropanol/H_20 (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 x 7.8 mm) (Bio-Rad, Hercules, CA, USA) plus pre-column. Elution was performed by using 5mM 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.

2.2.4.4. Esterified ferulic and coumaric acid contents

To 10 mg of sample 1 mL of 2M 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 (6M). The amounts of free ferulic and coumaric acid present in the sample was determined by adding 1.5 mL of

premade mixture of 2M KOH (1 mL), flushed with N_2 and HCl (0.5 mL, 6M). 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 x 150 mm column with 1.9 µm particle size (Thermo Scientific, Rockford,IL, USA). The mobile phases were (A) H_20 + 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.

2.2.4.5. 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 hydrolyzed 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.

2.2.4.6. 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 insoluble extract was analysed 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).

2.2.4.7. 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.

2.2.4.8. 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 x 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 0.2M 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.

2.2.4.9. 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 x 250 mm ID) in combination with a CarboPac guard column (2 mm x 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: 0.1M NaOH and B: 1M NaOAc in 0.1M 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) 0.1M NaOH, (B) 1M NaOAc in 0.1M 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 0.5M NaOH at 0.1 ml^{-1} was performed to detect and quantify the eluted saccharides.

2.2.4.10. 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 2.8mM 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.

2.3. Results and discussion

2.3.1. 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 2.1. It should be noted that this study focussed on characterisation of components being left at the end of various phases. The total carbohydrate content (neutral carbohydrates and uronic acids) of 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% (w/w) of total carbohydrates remaining after Phase I and 22% (w/w) 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 2.1). 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 acid remained. It is expected that esters were removed due to a rather high pH of this mixture (Table 2.1). 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 utilisation 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.

Table 2.1. 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]	WS
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 (mol%)									
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	0.0	0.1	<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]BM: 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, WS: wheat straw.
[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/100mol; abbreviations: Ara, arabinosyl; GlcA, glucuronic acid, AcA, acetic acid.

2.3.2. 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 1M KOHss, 4M KOHss and residue (Res) is shown in Table 2.2.

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 2.2). 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 1M KOHss and 4M KOHss was analyzed (Table 2.2). 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 1M KOH fractions (Table 2.2). In 4M 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 et al., 2011). Unexpectedly, the amount of xylan recovered in 1M KOH fraction was rather similar as is the amount of xylan recovered in 4M KOH fraction for PI, PII and PIII-16. This indicates that the alkali extractability of xylan remained similar from PI to PIII-16.

2.3.3. 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 2.3. Of PI- xylan in both 1M and 4M 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 2.1). 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 (Table 2.1 and Table 2.3). 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, 1M KOH and 4M 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 2.2. Distribution over water-soluble (dialyzed: WSSd and non-dialyzed: WSS) and water un-extractable solids (WUS), as well as WUS over alkali extracts 1M KOHss, 4M KOHss and the residue (Res) of PI, PII and PIII-16.

	% w/w			
	Yield DM[a,b]	**Yield total sugar**	**Yield glucan**	**Yield GAX[c]**
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
1M KOHss	24	21	1	54
4M 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
1M KOHss	27	25	1	62
4M 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
1M KOHss	26	25	2	58
4M 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 uronyl, arabinosyl and xylosyl residues.
[d]PI: Phase I compost, PII: Phase II compost, PIII-16: Phase III compost after 16 days of mycelium growth.

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 2.2) were submitted to HPSEC (Fig. 2.2). 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 2.4). About 20% of the total carbohydrates in 1M KOHss and 4M KOHss of PI and PII, was found to be water-soluble, whereas for 1M 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^{-1} was found.

Table 2.4. Total carbohydrates in alkali extracts, 1M KOHss and 4M KOHss, of PI, PII and PIII-16 and carbohydrate yield of their water- soluble fraction before and after digestion with endoxylanase. DM= dry matter

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

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 et al., 2007). Xylan fractions of PIII-16 show elution in a lower molecular weight region compared to PI 1M and 4M KOHss (Fig. 2.2). 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 well characterised, 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. 2.2 (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 1M and 4M KOHss. This was confirmed by analyzing the yield of carbohydrates in the soluble fractions after enzymatic digestion. After enzyme hydrolysis of 1M KOHss from PI, PII and PIII-16 over 100% of carbohydrate yield was recovered. For 4M 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.

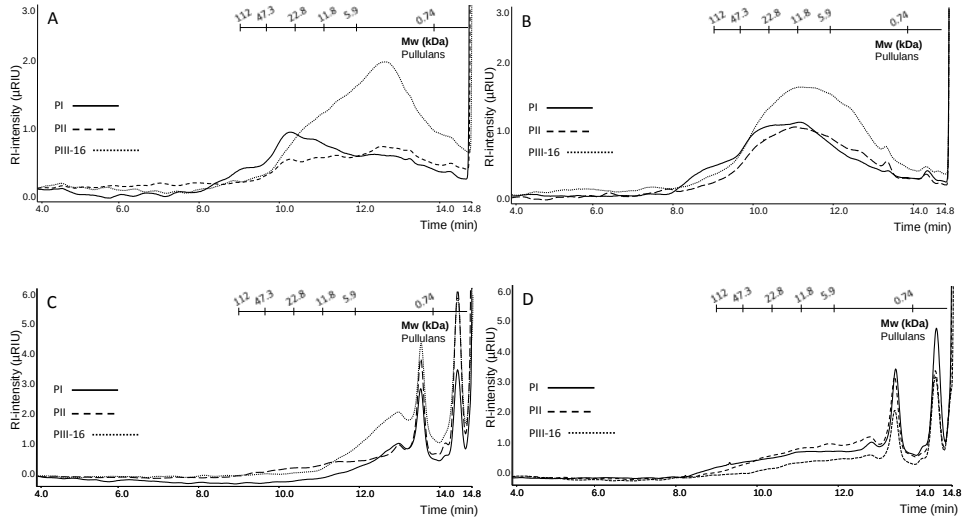

Figure 2.2. Molecular weight distribution of 1M KOHss extract PI, PII and PIII-16 before digestion (A) and endpoint digested (C); and 4M KOHss extract before digestion (B) and endpoint digested (D). A purified and well characterized endoxylanase (EX1) (Kormelink, 1993), is used for digestion.

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. 2.3) 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 1M KOHss and about 15% for 4M 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 2.3). 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. 2.2). Nevertheless, all these results added to our suggestion that xylan in the compost showed a low substitution.

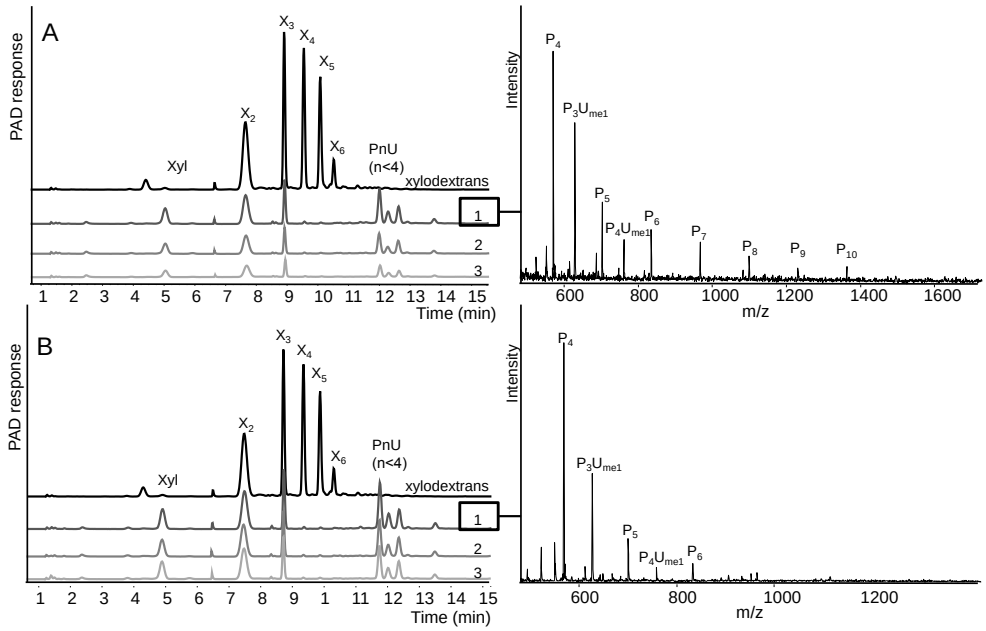

Figure 2.3. HPAEC elution pattern (left) and MALDI mass spectrum (insert at right) of the 1M (A) and 4M (B) alkali-soluble fractions of PI (1), PII (2) and PIII-16 (3) after degradation by endoxylanase (P = pentose, Xyl = xylose, X_2-X_6= xylodextrans DP 2-6, U= uronic acid, U_{me}= methyl-uronic acid).

Table 2.3. Degree of substituents 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
1M KOHss	13	6
4M KOHss	12	5
Res	3	27
PII	15	11
WSS[b]	14	5
WSSd	89	215
1M KOHss	12	6
4M KOHss	10	6
Res	41	30
PIII-16	14	13
WSS[b]	17	18
WSSd	81	160
1M KOHss	12	8
4M KOHss	10	8
Res	34	30

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

2.4. 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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Chapter 3

Fate of carbohydrates and lignin during composting and mycelium growth of *Agaricus bisporus* on wheat straw based compost

Abstract

In wheat straw based composting, enabling growth of *Agaricus bisporus* mushrooms, it is unknown to which extent the carbohydrate-lignin matrix changes and how much is metabolized. In this paper we report yields and remaining structures of the major components. During the Phase II of composting 50% of both xylan and cellulose were metabolized by microbial activity, while lignin structures were unaltered. During *A. bisporus'* mycelium growth (Phase III) carbohydrates were only slightly consumed and xylan was found to be partially degraded. At the same time, lignin was metabolized for 40% based on pyrolysis GC/MS. Remaining lignin was found to be modified by an increase in the ratio of syringyl (S) to guaiacyl (G) units from 0.5 to 0.7 during mycelium growth, while fewer decorations on the phenolic skeleton of both S and G units remained.

Jurak, E., Punt, A.M., Arts, W., Kabel, M.A., Gruppen, H.. Submitted for publication.

3.1. Introduction

In the conventional European process, compost for mushroom growth is produced from a basic mixture (BM) of straw bedded horse manure, wheat straw, poultry manure and gypsum (Iiyama et al., 1994). The BM composition and duration of composting phases can differ in different parts of the world, but compost always serves as carbon and nitrogen source for *Agaricus bisporus*' mushroom growth.

The main ingredient in the European compost, wheat straw, contains about 57% (w/w) of carbohydrates, mostly cellulose (44 mol%) and xylan (46 mol%), and 27% (w/w) of lignin (chapter 2). Cellulose is a non-branched polymer of β-1,4-linked glucosyl units. Xylan in grasses, like wheat straw, is composed of a 1,4-linked β-D-xylopyranosyl-backbone with arabinosyl, *O*-acetyl and (4-*O*-methyl-) glucuronic acid side chains (Scalbert et al., 1985). However, the exact amounts and distribution of all substituents on wheat straw xylan is not reported. Lignin is composed of three main monolignols: p-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) phenylpropanoid units. In wheat straw lignin all three units are present (H:G:S ratio of 6:64:30; del Río et al., 2012). Cellulose, xylan and lignin form together a complex, hard to degrade, network. In grasses, like wheat straw, xylan can adsorb to cellulose, but also be oxidatively cross-linked with other xylan molecules and with lignin via hydroxycinnamic acid residues (Ralph, 2010). Composting aims at opening up such a complex to facilitate release of monosaccharides, which serve as carbon source during *Agaricus bisporus'* mushroom growth (chapter 2). As such, composting has a similar goal as many other pre-treatments of lignocellulosic plant biomass aiming at an improved enzymatic release of fermentable monosaccharides to produce biofuels and chemicals from. Therefore, insights in the reactions occurring could be of use for other pre-treated plant materials.

The industrial production of compost is carried out in closed tunnels and involves three phases, described in detail elsewhere (chapter 2). In brief, meso- and thermophilic microbiota decompose BM (Phase I (PI)), causing a rise in temperature to 80°C and release of ammonia. In the next phase (PII), microorganisms, in particular actinomycetes and fungi, consume at a maximum of 60°C about 40% of the ammonia present, while the other part disappears in the air (Gerrits, 1988). As a result of two composting phases, compost has become accessible and specific for *A. bisporus* mycelium growth in the third phase at temperatures around 24°C for 16 days (PIII-16). In PIII, the *A. bisporus* mycelium is known to consume (part of) the microbiota present (Fermor and Wood, 1981). For optimal growth the *A. bisporus* mycelium needs also to degrade and consume the carbohydrates and, possibly, lignin present (chapter 2, Iiyama et al., 1994).

PIII-16 compost is considered mature and by adding a casing layer on top of this compost the fruiting body formation starts (Gerrits, 1988).

Composting is an accelerated version of natural decomposition of lignocellulose by the microorganisms present (Savoie, 1998). The activity of these microorganisms and growth of *A. bisporus* chemically alters the compost (chapter 2, Iiyama et al., 1994). Quantification of remaining components, like xylan, cellulose, lignin and protein, however, has not been reported. So far, mainly qualitative changes in compost composition have been reported, with a focus on a decrease in carbohydrate and protein as based on total dry matter (chapter 2, Gerrits, 1967, Iiyama et al., 1994). Lignin degradation has been mentioned, but only indirect evidence was shown, either by investigating whether *A. bisporus* can grow on radioactive labelled ^{14}C lignin or by determining the presence of laccase-activity and manganese peroxidase. The latter is hypothesized to be linked to lignin degradation during mycelium growth (Bonnen et al., 1994; Durrant et al., 1991). Although important, these results lack the possibility to determine absolute quantities of carbohydrates, lignin and protein metabolized.

In our research, in a tunnel-experiment at industrial scale, a mass balance was conducted for dry matter as well as for proteins, cellulose, xylan, lignin and ash. In addition, the structural changes of xylan and lignin were studied. Mapping the amounts and structures of the main components available for mushroom growth is essential for improving the process. Generally, our study contributes to the understanding how wheat straw compost is degraded.

3.2. Materials and methods

3.2.1. Composting process

At the composting company CNC-C4C (Milsbeek, The Netherlands) basic mixture (BM) was obtained by mixing on a wet basis, 63% w/w of fresh horse manure, 2% w/w of gypsum, 1% w/w of ammonium sulphate solution (20% w/v $(NH_4)_2SO_4$ in water), 17% w/w of filtered percolate water, 11% w/w of chicken manure, 4% w/w straw and 2% w/w of water. Fresh horse manure and wheat straw were collected in October 2013 and the experiment was carried out in October and November 2013. For this experiment, one tunnel was assigned for compost production from which all samples were taken from. The composting phases are described elsewhere (chapter 2). In addition to the previously described information, it should be mentioned that the PI phase lasted for 5 days, and reached 80°C under formation of ammonia, after which PI compost was obtained. To PI compost 10g kg^{-1} of PII compost was added to introduce viable microflora necessary for the conditioning phase. The duration of PII was also 5 days. To PII

compost 4.5g kg^{-1} of rye-based spawn was added and inoculated for 16 days after which PIII-16 mycelium grown compost was obtained.

3.2.2. Samples

All samples were taken from the same tunnel (35x4x4 meter), which was filled with 200 tons of BM. Of this BM 100 kg was kept apart and divided into 3 batches (A, B, C) of about 33 kg. Each batch was handled separately and placed in onion mesh bags in the same tunnel (Fig. 3.1). Per batch two bags (biological duplicates) were prepared and weighed (min 15 kg), labelled (e.g. A-1 and A-2) and placed over the length of the tunnel, about 30 cm below the surface of BM compost. After phase PI, both bags from the corresponding batch were weighed and afterwards mixed, and one sample (min 1 kg) was taken from each batch (A, B and C). After sampling, the material was again divided over two bags and placed in the tunnel for phase PII (35x4x3.62). Total compost in PII tunnel was 200 tons. The same procedure was followed for phase PIII (tunnel 35x4x3.62 meter, total compost 144 tons). So, for each sampling step (end of each phase) three samples (biological triplicates) were obtained (min 1 kg). Throughout the complete composting process, the batches (A, B and C) were weighed at the end of each phase and all the changes (addition of water, spawn) were noted. Weight of each sample (batch) was determined after sampling and after collecting, samples were immediately frozen at -18°C. First, the dry matter content was determined for each sample (100 g, 105°C overnight). Based on dry matter content and fresh weigh of each batch throughout PI, PII and PIII-16, the dry matter yield was 91.9% of PI, of PII 77.1% and of PIII-16 69.4% (average of three batches, STDEV 1.2, 1.9 and 1.9, respectively). Dried samples were milled (<1 mm) using an MM 2000 mill (Retsch, Haan, Germany) prior to further analysis. Samples were analyzed for their protein, carbohydrate, ash and lignin contents for each sample (batch). Contents of all analyzed components was summed up and compared to the dry matter content of corresponding sample and the recovery was found to be >95%. In addition, the carbohydrate and lignin composition was analyzed.

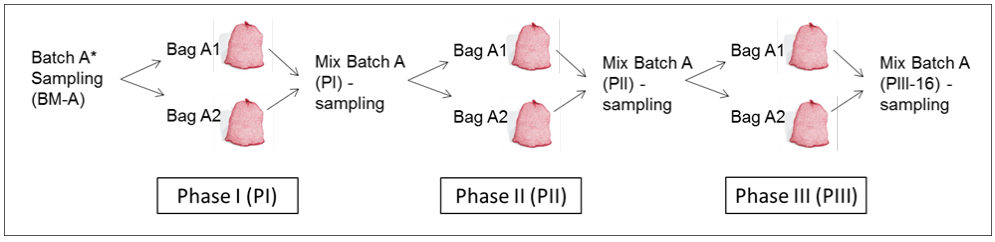


Figure 3.1. Schematic representation of sampling. *Sampling performed in this way also for batches B and C.

3.2.3. Preparation of water un-extractable solids (WUS)

For batch A, freeze dried, milled samples (5 g) of BM, PI, PII and PIII-16was suspended in water (175 mL) and boiled at 100°C for 5 min. Next, the suspension was stirred for 16 h at 21°C. The supernatant was removed after centrifugation (10 000 x g, 30 min, 20°C) and the residue was washed twice with water (60 mL and 75 mL). The final residues were freeze dried and collected as water un-extractable solids (e.g. PI-WUS). Samples were analyzed for yield, protein and dry matter contents.

3.2.4. Analytical techniques and methods

3.2.4.1. Carbohydrate content and composition

The neutral carbohydrate and uronic acid content and composition was determined in duplicate, as described in chapter 2.

3.2.4.2. Nitrogen and protein content

Samples (7-10 mg) 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. Nitrogen content in the water soluble extract was calculated by difference. Nitrogen to protein conversion factor of 6.25 was used (Jones, 1931). For PIII-16, due to the presence of *A. bisporus* mycelium in compost, protein was not specified (n.s. Table 3.2)

3.2.4.3. Ash content

Freeze dried samples (1 g) or lignin residues (200-400 mg; see 2.4.4.) were dried in the oven overnight (105°C) and weighed, then put at 575°C for 5 h. Next, samples were weighed and difference between the mass at 105°C and 575°C was taken as ash content. Additionally, samples were burned at 575°C for 16 h more and afterwards weighed. No difference in mass was observed between residue after 5 h and 21 h.

3.2.4.4. Klason lignin residue and acid soluble lignin (ASL)

To each sample of 1 g (dry matter) 10 mL of 72% w/w H_2SO_4 was added and samples were hydrolyzed for 1h at 30°C. Next, 100 mL of distilled water was added to each sample and samples were put in a boiling water bath for 3h and shaken every half hour. Next, the suspensions were filtered over G4 glass filters. The filtrate was measured for acid soluble lignin (ASL) spectrophotometrically at 205 nm. ASL was calculated according to the formula: ASL= (A * B * C)/(D * E), with A= absorption relative to 1M H_2SO_4, B= dilution factor, C= filtrate volume, D= extinction coefficient for lignin (110 g L^{-1} cm^{-1}), and E= weight of substrate (g). The residual part was washed until it was free of acid (pH paper) and dried overnight at

105°C. The final residues were corrected for ash and considered as a measure for the acid insoluble lignin (Klason) content after ash-correction. To this end, acid insoluble lignin was burned for ash. Total lignin was defined as a sum of Klason lignin residue, corrected for ash, and acid soluble lignin. For wheat straw, Klason lignin content corrected for ash was 27% (w/w) and acid soluble lignin content was 1.9% (w/w) based on dry matter.

3.2.4.5. Lignin analysis by analytical pyrolysis-GC-MS (Py-GC/MS)

Pyrolysis was performed with a 2020 microfurnace pyrolyzer (Frontier Laboratories, New Ulm, MN, USA) equipped with an AS-1020E Autoshot. Components were identified by GC-MS using a Trace GC equipped with a DB-1701 fused-silica capillary column (30m x 0.25 mm i.d. 0.25 µm film thickness) coupled to a DSQ-II (EI at 70 eV) (both Thermo Scientific, Waltham, MA, USA). The pyrolysis was performed at 500°C for 1 min. Helium was the carrier gas (1 mL min^{-1}). Samples (60-70 µg) were pyrolyzed and each measurement was performed at least in triplicate. Initial oven temperature was 70°C (2 min hold) and it increased to 230°C with a rate of 5°C min^{-1}, to 240°C by 2.5°C min^{-1} and finally to 270°C min^{-1} by 2.5°C min^{-1}. Pure compounds were used as standards (Sigma Aldrich, St. Louis, MO, USA; Brunshwig Chemie B.V., Amsterdam, The Netherlands and Fisher Scientific, Landsmeer, The Netherlands) and peak molar area was calculated as defined by del Rio (del Río et al., 2007). For wheat straw a cut-off of 1% molar area for single S (syringyl-like lignin structures) and G (guaiacyl-like lignin structures) compounds was applied and only the fate of remaining compounds (>1% molar area) was analyzed for compost samples. Compounds with a molar area >1% in wheat straw are specified in Table 3.4. For WUS, the fate of the same S and G compounds as in original compost was compared. Remaining S and G compounds were annotated as Rest S* and Rest G*. The same cut-off level was applied for phenolic furanose/pyranose (F/P) and unknown compounds based on total area of these compounds. F/ P compounds with a molar area >1% are annotated in Supplementary table 1. The remaining compounds are specified in Suppl. table 2. Amdis software (version 2.71, NIST, USA) was used for identification and deconvolution of peaks. For deconvolution the following parameters were set: adjacent peak subtraction = one, resolution = medium, sensitivity = high and shape requirements = low. For identification a target compound library (based on referents standards) was built. Referents standards were measured in order to obtain retention time (RT) information and mass spectra (Table 3.4, Suppl. tables 1 and 2). Compounds identified based on referents standards were, first, selected based on RT (± 1.0 min; or ± 0.1 min for isomers). If RT was within the selected window an annotation was given if reversed search (RS) value was higher than 80%. Finally, for all WS compounds, also the ones

identified based on Ralph and Hatfield (1991), spectra were checked manually. Total annotated area of S- and G- lignin units in wheat straw was ±80%.

3.2.4.6. Estimation of lignin quantities with analytical Py-GC/MS

To estimate absolute amounts of lignin in the samples, the areas of Py-GC/MS pyrograms were assumed to indicate amounts of lignin units present. As a base quantity, the total lignin content (sum of Klason lignin (26.5% w/w) and ASL (1.9% w/w)) of wheat straw was correlated with the area under the Py-GC/MS pyrograms of wheat straw. Areas of S- and G-units annotated in Table 4 and Supplementary table 2 were included as total area. For wheat straw about 85% of dry matter was pyrolyzed in the Py-GC/MS, based on gravimetric analysis prior and after the pyrolysis.

3.3. Results and discussion

3.3.1. Dry matter, organic matter, carbohydrate and protein mass balance during composting and mycelium growth phases

The contents based on dry matter of carbohydrates, ash, Klason lignin residue (- ash) and nitrogen were analyzed for all three batches (A, B and C) are presented on a dry matter basis in Table 3.1. From Table 3.1, batch C was found to be an outlier with respect to carbohydrate content and dry matter content of Phase I (PI). Namely, in 1000 kg of basic mixture (BM), based on the carbohydrate content, 424 kg of carbohydrates were present compared to 440 kg in PI, for batch C. While 439 kg in BM and 412 kg in PI and 449 kg in BM and 420 kg in PI of carbohydrates, were calculated for batch A and B, respectively So, only for batch C this would, impossibly, indicate a gain in carbohydrates in PI. Its carbohydrate content was analyzed at least 3 times indicating that this outlier was not due to an analytical error. Considering the correct values of PII and PIII-16, the error appeared to have occurred in the sampling after PI. Hence, after carbohydrate and ash analysis, batch C was excluded from further analysis.

The carbohydrate contents (based on dry matter) of batch A and B was found to be, on average, 44% w/w for BM, 46% w/w for PI , 26% w/w for Phase II (PII) and 27% for PIII-16. Ash content was found to be 21%, 23%, 30% and 32% for BM, PI, PII and PIII-16, respectively (w/w based on dry matter). Total nitrogen content was 1.3%, 1.4%, 2.1% and 2.2% for BM, PI, PII and PIII-16, respectively and water insoluble nitrogen content was found to be 0.8%, 0.8%, 1.6% and 2.1% for BM, PI, PII and PIII-16, respectively (w/w based on dry matter). Lastly, Klason lignin contents, corrected for ash, were 21%, 22%, 23% and 21% for BM, PI, PII and PIII-16, respectively (w/w based on dry matter).

Table 3.1. Carbohydrate, ash, nitrogen, Klason lignin and dry matter content (based on dry matter) for compost after PI, PII and PIII-16. BM: basic mixture; PI: compost after Phase I; PII: compost after Phase II; PIII-16: compost after 16 days of mycelium growth; A, B, C different batches.

	Carbohydrate content (% w/w DM)[a]	Ash content (% w/w DM)	Total nitrogen content (% w/w DM)	Water insoluble nitrogen) content (% w/w DM)	Klason lignin (-ash) content (% w/w DM)	DM (% w/w)
BM	44[b]	21[c]	1.3[d]	0.8[d]	21[f]	100
PI-A	45	24	1.4	0.8	22[f]	91.4
PI-B	46	21	1.4	0.8	n.a.	91.1
PI-C	47	22	n.a.	n.a.	n.a.	93.2
average	46	22	1.4	0.8	n.a.	91.9
(STDEV)	(1.0)	(1.5)	(0.03)[e]	(0.04)[e]		(1.2)
PII-A	25	30	2.0	1.4	23[f]	78.7
PII-B	26	29	2.1	1.7	n.a.	74.9
PII-C	27	28	n.a.	n.a.	n.a.	77.7
average	26	29	2.1	1.6	n.a.	77.1
(STDEV)	(1.1)	(0.9)	(0.03)[e]	(0.2)[e]		(1.9)
PIII-16-A	26	34	2.3	2.1	21[f]	70.8
PIII-16-B	27	30	2.2	2.1	n.a.	67.2
PIII-16-C	23	30	n.a.	n.a.	n.a.	70.2
average	26	3	2.2	2.1	n.a.	69.4
(STDEV)	(1.9)	(2.3)	(0.07)[e]	(0.5)[e]		(1.9)

[a]Each sample was analyzed in duplicate (STDEV <1).
[b]Average of dupplicates of batches A, B and C (STDEV 1.3).
[c]Average of duplicates of batches A, B, C (STDEV 1).
[d]Average of dupplicates of batches A and B (STDEV 0.4).
[e]Average of duplicates of batches A and B.
[f]Average of duplicates for batch A (STDEV BM 0.4, PI 7, PII 1.3, PIII-16 1).
n.a. not analyzed; DM dry matter.

Next, for batch A the mass balance concerning ash, protein, carbohydrates and lignin during composting and mycelium growth is presented (Table 3.2) based on a starting amount of 1000 kg dry matter BM. The totals of all analyzed components covered 95% w/w or more of the total amount of dry matter, indicating the completeness of the analyses performed. Compared to BM, a decrease of 8% w/w of dry matter was analyzed for PI, 23% w/w for PII and 31% w/w for PIII-16.

Overall, some variations in the absolute amounts of ash was observed (Table 3.2). Previously, variations in the amount of inorganic materials during composting have been reported (Iiyama et al., 1994). Ash present mainly originated from sand and stones found in the commercial compost solids. Possibly, these are introduced together with recycled process-water, and therefore present in various amounts in the different samples (personal communication CNC-C4C). Such ash-recycles may also contribute to the higher decrease in organic matter (OM) compared with DM (Table 3.2).

Total nitrogen remained rather similar in the compost during composting and mycelium growth. Given the low nitrogen values (% w/w, Table 3.1), comparison of absolute nitrogen amounts should be performed with caution. Given this, a tendency in increase of water insoluble nitrogen might be observed in PII compost compared to PI compost. During PI rise in temperature and formation of ammonia was observed (personal communication CNC-C4C) indicating microbial growth, however, no big differences in carbohydrate and lignin content were observed (Table 3.1). In contrast to this, a tendency in increase in the amount of total nitrogen and protein in PII (mass balance, Table 3.2) could be interpreted, while a decrease in organic matter was observed (18% w/w). In this phase ±50% w/w of the carbohydrates present were metabolized. The decrease was observed for both xylan and cellulose present (Fig. 3.2). Both the increase in nitrogen and decrease of carbohydrates was expected to be related to the growth of nitrogen-fixating and other viable microbiota introduced into compost at the beginning of PII (Bisaria et al., 1990; Kurtzman and Zadrazil, 1982).

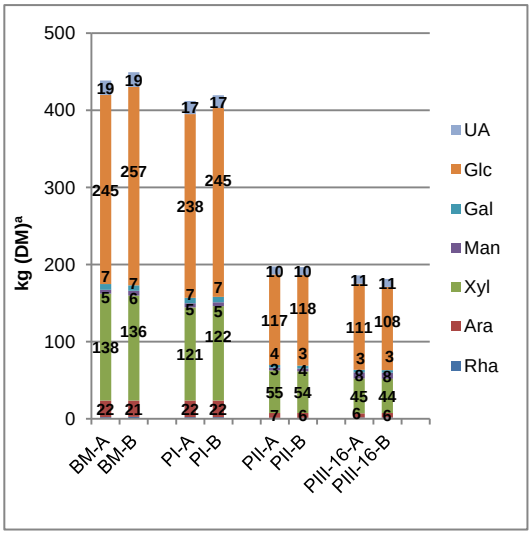


Figure 3.2. Mass balance of constituent monosaccharides during composting and mycelium growth for batch A and B (BM, PI, PII, PIII-16). BM= basic mixture, PI= compost after Phase I, PII= compost after Phase II, PIII-16= compost after 16 days of mycelium growth; Rha= ramnosyl, Ara= arabinosyl, Xyl= xylosyl, Man= mannosyl, Gal= galactosyl, Glc= glucosyl, UA= uronyl. [a]Calculation was performed based on 1000 kg of DM using values presented in Table 3.2 and 3.3.

Table 3.2. Mass balance of dry matter and organic matter and structural components (carbohydrates, nitrogen, lignin and ash) in the compost during composting and mycelium growth. BM: basic mixture; PI: compost after Phase I; PII: compost after Phase II; PIII-16: compost after 16 days of mycelium growth for batch A.

	kg												% w/w		
	Total dry matter	**Carbohydrates**		**Nitrogen**				**Lignin**			**Ash**	**Organic matter (OM)[a]**	**Loss OM relative to BM**	**Loss dry matter relative to BM**	**Recovery[b]**
		Total saccharides	Mannitol	Water insoluble nitrogen	Insoluble Protein[c]	Water sobluble nitrogen[d]	Total nitrogen	Klason lignin[e]	Acid soluble lignin	Total lignin					
BM	1000	439±9	0	8±0.3	48±2	6±0.3	14	212±44	49±23	261	199±1	801	-	-	95
PI	914	412±7	0	8±0.1	47±0.6	6±0.0	14	209±63	44±13	253	215±2	699	13	9	100
PII	787	197±16	0	11±1.4	66±8.6	5±1.4	16	188±10	64±19	252	236±1	551	31	21	95
PIII-16	708	186±1	3	15±0.5	n.s.	1±0.1	16	153±6	61±23	214	240±5	468	42	29	100

[a]Organic matter= Total dry matter – ash.
[b]Recovery= Calculated as sum(carbohydrates+protein+total lignin +ash)/total dry matter*100.
[c]Nitrogen to protein conversion factor 6.25.
[d]Calculated as difference= total nitrogen-water insoluble nitrogen.
[e]Corrected for ash.
n.s. not specified.

In PIII-16 (Table 3.2), the amount of protein increased further, mainly seen in the increase in water insoluble nitrogen, and the amount of carbohydrates decreased slightly both in xylan and cellulose (Fig. 3.2), most likely as a result of the observed mycelium growth in this phase (Akinyle and Akinyosoye, 2011; Sales-Campos et al., 2010). It should be noted that due to the formation of mycelium dry matter, partly built from glucan, the decrease in compost-glucan (cellulose; Fig. 3.2) derived from the starting material is underestimated. Namely, in our analysis, total glucan was analyzed, regardless whether it originated from plant or microbial origin. Finally, mannitol was analyzed to be present in PIII-16 compost, which is a known soluble carbohydrate in the mycelium of *A. bisporus* (Hammond and Nichols, 1976).

Overall, the molar composition of the compost carbohydrates in P0, PI, PII and PIII-16 (Table 3.3) remained rather similar. However, a decrease in xylosyl residues could be observed in PIII-16 compared to PII. In all phases, the main carbohydrate constituents were xylosyl (28-35 mol%) and glucosyl (52-56 mol%) residues, which is in agreement with previously published data (chapter 2). Recently, it was shown that during PIII compost xylan is partly degraded, thereby, making it more water soluble (chapter 2), which is expected to provide more easily accessible carbohydrates during fruiting of *A. bisporus.* In the present study, no division between water soluble and water insoluble glucans and xylan was performed.

3.3.2. Lignin mass balance during composting and mycelium growth phases

First, the Klason lignin analysis was applied to a lab-cultivated *A. bisporus* mycelium sample, which allowed us to observe the fate of mycelium in this analysis. It was shown that more than 50% of the mycelium dry matter, was collected as 'Klason lignin'. This indicated that the Klason lignin analysis in samples containing substantial amounts of mycelium, like in PIII-16, would give an overestimation of the lignin present. Also, denatured proteins are known to remain in the Klason lignin residues (Iiyama et al., 1994). Nevertheless, Klason lignin residues were analyzed for batch A (Table 3.2), allowing comparison with the scarce previously reported data on compost composition (Iiyama et al., 1994). For PI and PII values obtained for Klason lignin corrected for ash were in line with values reported by Iiyama et al. (1994).

Lignin structure and content was also analyzed as single monolignol units by analytical Py-GC/MS. Based on the correlation between the wheat straw Klason lignin content, and the area of annotated S- and G-units in the wheat straw pyrogram obtained, from the pyrogram-areas of the compost samples (in triplicate) the lignin yield in these samples was calculated, based on 1000 kg BM dry matter (Table 3.5). In general, the amount of pyrogram based lignin remained rather

similar during composting (PI and PII). In contrast, a decrease of 40% w/w in the amount of lignin, based on pyrolysis, was observed after 16 days of mycelium growth. The overall difference in kg between BM and PIII-16 is more pronounced by the GC/MS analysis than by the classical Klason lignin analysis. This also accounts for the decrease in dry matter during the PIII phase. To our opinion, the Py-GC/MS data are more representative for the lignin amounts present than the Klason lignin residue analysis, because with the former technique only lignin derived pyrolysis units were taken into account in the quantification in mycelium grown compost samples. The GC/MS analysis leads to the total lignin yield based on constituent units present after pyrolysis. Hence, this technique also provided valuable data on compositional changes during the different phases.

Table 3.3. Carbohydrate composition (mol%) and degree of substitution of xylan in different compost phases, based on dry matter. BM: basic mixture; PI: compost after Phase I; PII: compost after Phase II; PIII-16: compost after 16 days of mycelium growth.

	Mol%[a]								
	Rha[b]	Ara[b]	Xyl[b]	Man[bc]	Gal[b]	Glc[b]	UA[b]	Ara/Xyl[d]	GlcA/Xyl[d]
BM-A	1	6	36	1	2	52	4	16	10
BM-B	1	5	35	1	1	53	4	15	10
BM-C	0	6	35	1	2	52	4	16	11
Average[e]	1	6	35	1	2	52	4	16	10
STDEV[e]	0.0	0.2	0.6	0.1	0.0	0.8	0.1	0.4	0.4
PI-A	1	6	33	1	2	54	4	18	11
PI-B	1	6	33	1	2	54	3	18	10
PI-C	1	6	33	1	1	55	3	17	10
Average[e]	1	6	33	1	2	54	3	17	10
STDEV[e]	0.0	0.2	0.1	0.1	0.1	0.4	0.1	0.5	0.4
PII-A	1	4	32	2	2	56	5	12	14
PII-B	1	4	32	2	2	56	4	12	14
PII-C	1	4	31	2	2	56	4	12	14
Average[e]	1	4	32	2	2	56	4	12	14
STDEV[e]	0.0	0.1	0.4	0.1	0.0	0.5	0.1	0.1	0.0
PIII-16-A	1	3	28	4	2	57	5	12	18
PIII-16-B	1	4	28	4	2	56	5	13	18
PIII-16-C	1	4	28	4	2	56	5	13	19
Average[e]	1	4	28	4	2	56	5	13	18
STDEV[e]	0.2	0.1	0.1	0.1	0.1	0.3	0.2	0.3	0.5

[a]Molar compostition. Rha= ramnosyl, Ara= arabinosyl, Xyl= xylosyl, Man= mannosyl, Gal= galactosyl, Glc= glucosyl, UA= uronyl.
[b]Carbohydrate analysis in duplicate, average of duplicates presented, STDEV within samples in range from 0.1 to 1.
[c]Not corrected for mannitol.
[d]Ratio mol/100mol.
[e]Average and standard deviation (STDEV) of batch A, B and C.

3.3.2.1. Structural changes of lignin during composting and mycelium growth phases

The Py-GC/MS lignin-fingerprints of the BM, PI, PII and PIII-16 composts were annotated based on the fully annotated pyrogram of untreated wheat straw (Fig. 3.3A). The pattern and main annotated peaks of lignin compounds for wheat straw were in line with previously reported data (del Río et al., 2012). Due to better baseline separation and additional spectra measured from standard lignin compounds, some peaks (e.g. trans-isoeugenol, 4-methylsyringol, vanilin) were differently annotated than previously reported (Table 3.4, Supplementary tables 1 and 2) (del Río et al., 2012).

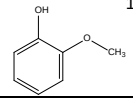
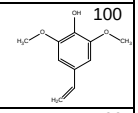
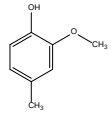
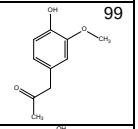
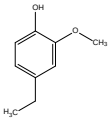
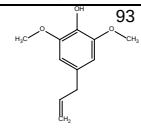
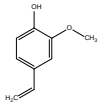
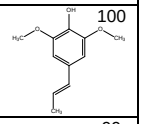
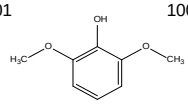
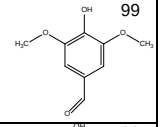
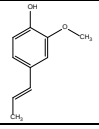
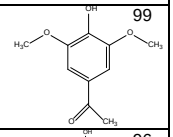
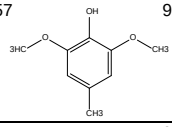
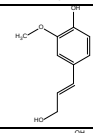
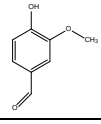
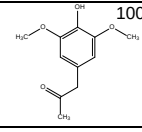
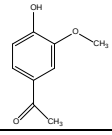
For BM the pattern and the ratios between peaks is quite similar to those of wheat straw, which was expected as lignin in BM-compost originates from wheat straw. Also, the pyrograms of PI and PII composts were majorly similar as the ones of BM and wheat straw. In Phase III, however, the ratios (in molar area) between some monolignol-units, mainly vinyl-guaiacol, guaiacol, vinyl-syringol and syringol, were very different between the BM and PIII-16 pyrograms (Fig. 3.3).

In order to understand the differences observed during mycelium growth, first, the various monolignol-units present in the pyrogram of wheat straw are discussed. As previously stated, wheat straw lignin is mainly composed of S- (syringyl-like) and G- (guaiacyl-like) lignin units, and to a minor extent of H (p-hydroxyphenyl) units. Therefore, we focused on S and G lignin units.

The S:G ratios of wheat straw and different compost samples was calculated and is shown in Fig. 3.4A. The S:G ratio in wheat straw was 0.49 (Fig. 3.4A), which is in line with the value reported by del Rio (2012), where vinyl-syringol and vinyl-guaiacol were excluded from the S:G ratio. The S:G ratio in PI and PII remained 0.51. After 16 days of mycelium growth (PIII-16), the S:G ratio changed to 0.68 (Fig. 3.4A), indicating a modification in lignin by *Agaricus* mycelium.

Changes in distribution of S and G lignin units were determined for S and G structures with molar area larger than 1% of total S+G molar area (Table 3.4). Remaining annotated compounds are presented in Supplementary tables 1 and 2 (see 2.4.5.), but not taken into account further.

Table 3.4. Identities of lignin-derived phenolic S (syringyl-like) and G (guaiacyl-like) compounds identified with Py-GC/MS and relative molar area higher than 1% in wheat straw (out of total S+G molar area).

No.	Compound	CAS No	Chemical structure	RS (for wheat straw)	No.	Compound	CAS No	Chemical structure	RS (for wheat straw)
9G[a]	Guaiacol	90051		100	34S[b]	4-vinylsyringol	28343228		100
16G[a]	4-methylguaiacol	93516		95	35G[a]	Guaiacylacetone	2503460		99
17G[a]	4-ethylguaiacol	2785899		100	36S[a]	4-allyl-2,6-dimethoxyphenol	6627889		93
19G[a]	4-vinylguaiacol	7786610		100	42S[b]	*trans*-2,6-dimethoxy-4-propenylphenol	26624135		100
24S[a]	Syringol	91101		100	43S[a]	Syringaldehyde	134963		99
26G[a]	*trans*-Isoeugenol	97541		99	46S[a]	Acetosyringone	2478388		99
28S[a]	4-methylsyringol	6638057		98	47G[a]	*trans*-Coniferyl-alcohol	458355		96
29G[a]	Vanilin	121335		99	49S[b]	Syringylacetone	19037582		100
33G[a]	Acetovanillone	498022		94					

[b]Interpretation based on Ralph and Hatfield (1991), reverse search of compound in compost or WUS versus compound in wheat straw: 34S>99%, 42S>97%, 49S>99%.

Compared to PII compost, a lower proportion of substituted vinyl-syringyol and vinyl-guaiacol lignin compounds in PIII-16 compost was present in favor of the less substituted guaiacol and syringol (Table 3.4, Fig. 3.3). This may point at cleavage of substituents on the phenolic skeleton during PIII (Fig. 3.4B). The observed modification of substituents is mainly observed in vinyl- groups leading to a relative decrease in vinyl-guaiacol and vinyl-syringol during PIII. Lignin structures analyzed by NMR in wheat straw (del Río et al., 2012) indicate that such vinyl-decorations are mainly responsible for inter-lignin linkages. Therefore, our findings suggest that *A. bisporus* is capable of cleaving larger lignin structures into smaller ones, and further remove the decorations leaving mainly the basic S and G phenolic skeletons of the lignin structures.

In previous research (del Río et al., 2012), vinyl-guaiacol and vinyl-syringol were excluded from the S:G ratio as during pyrolysis p-hydroxycinnamates are known to result in the same compounds as those derived from lignin. If these compounds were only part of xylan, it could lead to overestimation of lignin. However, in BM, due to high pH, no free and ester bound FA and very low amounts of pCA (<0.1% w/w based on dry matter) were found (chapter 2). This indicated that in BM compost less than 0.3% (w/w based on dry matter) of ester bound FA and pCA were present. Also, the amount of vinyl-guaiacol that could be formed from FA and *p*CA after pyrolysis was less than 4% of the total of vinyl-guaiacol analyzed in the wheat straw pyrogram. The remaining ether-bound FA and pCA are expected to account for less than 0.5% w/w based on dry matter (Pan et al., 1998).

Table 3.5. Relative pyrogram area (%) and Py-GC/MS pyrogram based lignin for wheat straw and different compost phases for batch A. BM: basic mixture; PI: compost after Phase I; PII: compost after Phase II; PIII-16: compost after 16 days of mycelium growth.

Sample	Relative pyrogram area (%)[a]	Lignin (pyrogram based) yield (kg)[b]
Wheat straw	100	284±4[c]
BM-A	86±7	244±28
PI-A	70±7	188±23
PII-A	90±8	264±45
PIII-16-A	45±3	128±5

[a]Total of areas of all S- and G-units annotated in Table 3.4, Suppl. tab.1.

[b]Based on 1000 kg dry matter BM; calculated based on wheat straw total lignin and pyrogram analysis.

[c]Lignin yield for wheat straw based on Klason lignin+acid soluble lignin. STDEV between duplicates.
±STDEV between triplicates.

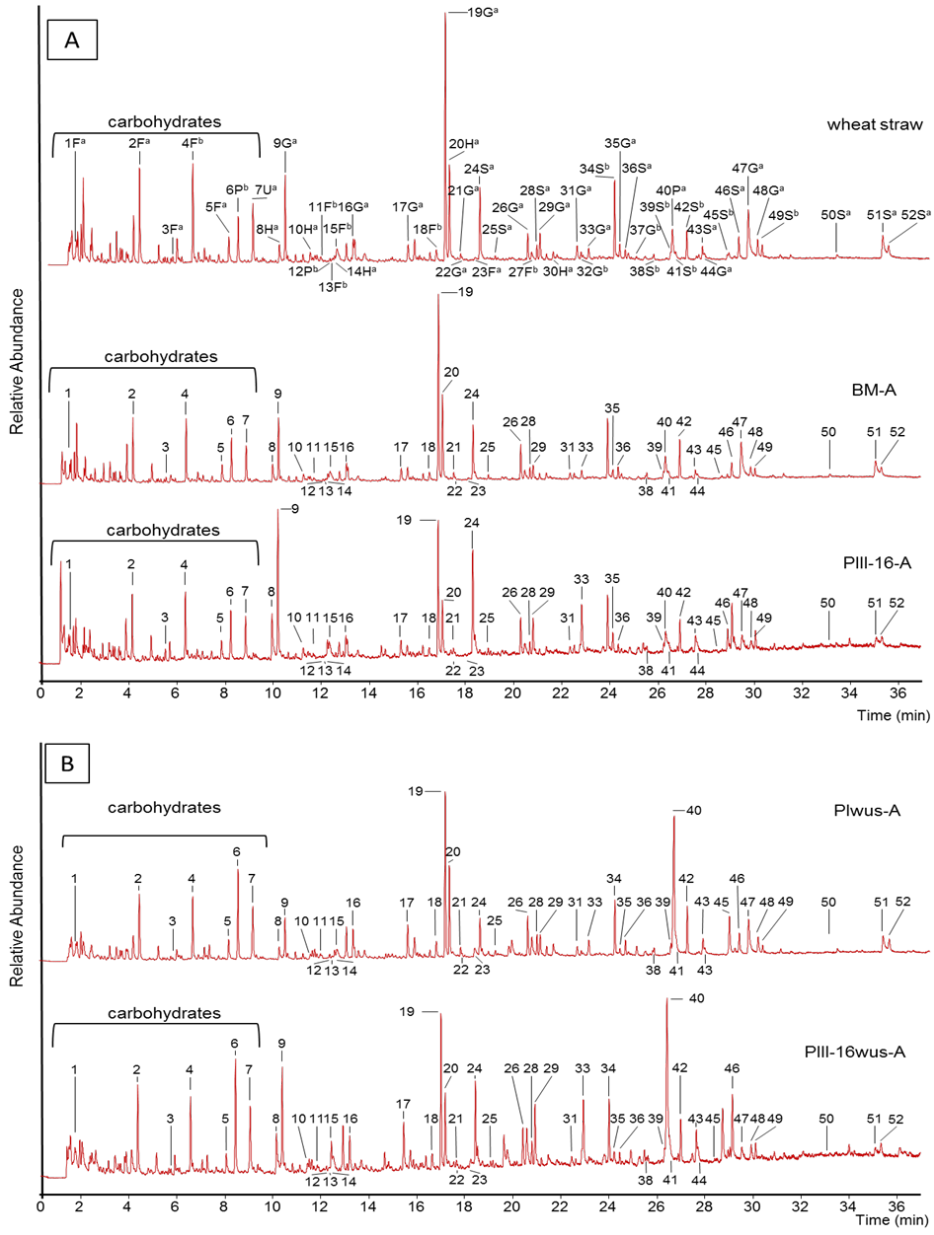


Figure 3.3. Py-GC/MS pyrograms of wheat straw, basic compost mix (BM) and compost after 16 days of mycelium growth (PIII-16) (A) and water un-extractable solids (WUS) of Phase I and PIII-16 (B) for batch A. The identities and structures of main syringyl and guaiacyl (and p-hydroxyphenyl) compounds are listed in Table 3.4, Supplementary table 1 and 2. PI: compost after Phase I: PII: compost after Phase II.

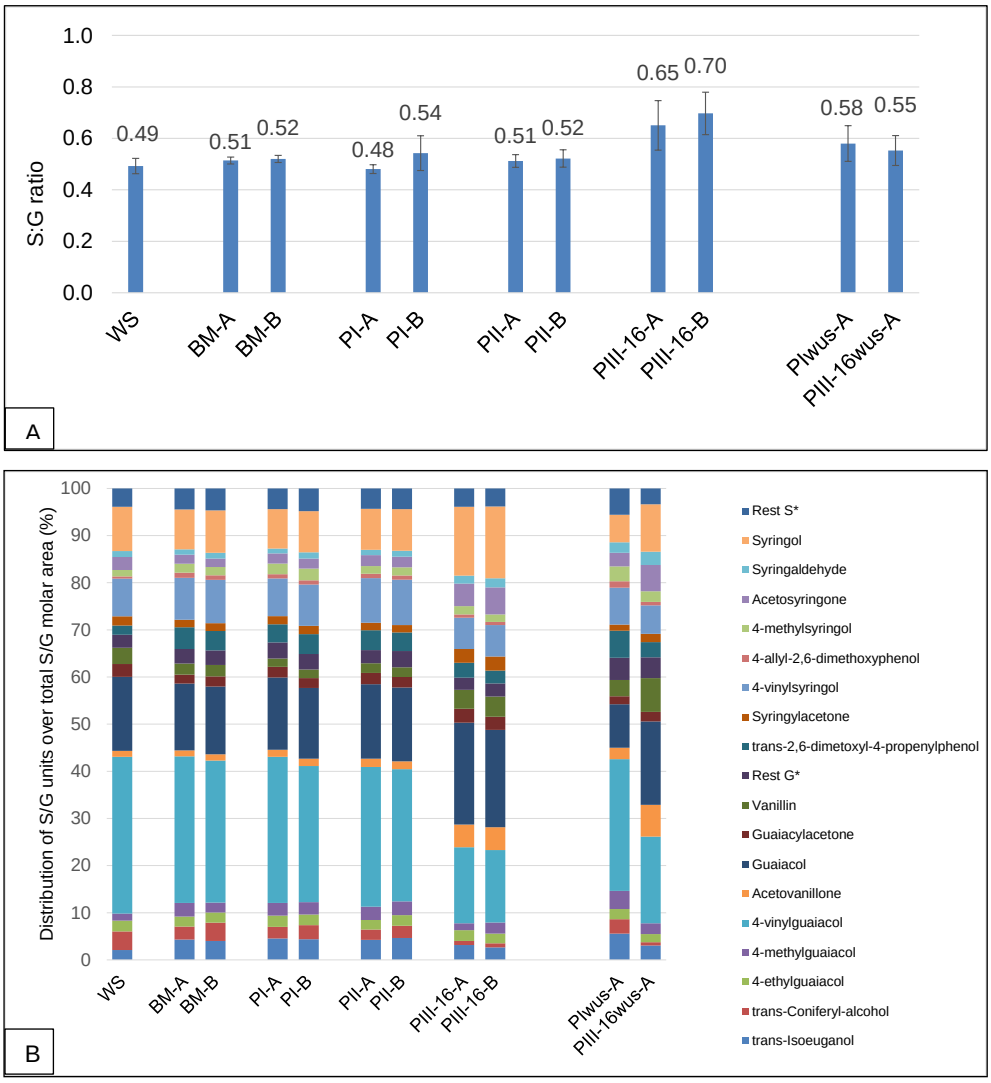


Figure 3.4. S:G ratio (A) and distribution of syringyl (S) and guaiacyl (G) units (B), based on molar area, of wheat straw (WS) and total compost samples during composting BM, PI, PII and mycelium growth PIII-16 and in water insoluble compost PIwus, PIII-16wus. Rest S* and Rest G* Supplementary table 2, BM: basic mixture; PI: compost after Phase I: PII: compost after Phase II; PIII-16: compost after 16 days of mycelium growth, A and B are biological duplicates and each sample measurement was performed in quadruplicates.

Composition and S:G ratio of water insoluble lignin in compost samples is presented in Fig. 3.4 and corresponding pyrograms are presented in Fig. 3.3. As no major compositional changes in the relative distribution of S and G lignin

compounds were observed between BM and PII (Fig. 3.4B), only for PI (PIwus) and PIII-16 (PIII-16wus) water insoluble lignin was analyzed in detail in particular for batch A. For PI the distribution of S and G compounds of PIwus-A (Fig. 3.4A) was found to be rather similar as that of the total sample (PI-A). On the contrary, in PIII-16wus a relatively lower S:G ratio was found compared to the total sample of PIII-16 indicating that part of lignin in PIII-16 compost became more water soluble (Fig. 3.4B).

Lignin modification in the compost by *A. bisporus* mycelium was previously indicated based on the degradation on ^{14}C-labelled lignin (Wood and Leatham, 1983). However, till now, this was not confirmed in compost itself. Also, during mycelium growth, high amounts of laccase activity, suspected to be involved in lignin degradation, were also found (Durrant et al., 1991; Waksman and Nissen, 1932; Wood and Goodenough, 1977). These data support our finding that *A. bisporus*, during the vegetative growth, is able to modify lignin structures. It is proposed that observed lignin degradation and modification increase the bioavailability of the carbohydrates in the wheat based compost.

To our knowledge, this is the first time that degradation and metabolization of lignin by *A. mycelium* was shown directly on the lignin structure in mycelium grown wheat straw based compost. Overall, our research provides more insights in how *A. bisporus* mycelium degraded lignocellulosic biomass for mushroom growth, and in general, give new insights in lignocellulosic plant biomass degradation.

3.4. Conclusions

During PI of composting, no changes in carbohydrates and lignin were observed in the compost. In PII, 50% of carbohydrates, both cellulose and xylan were metabolized, while lignin structure was not. During 16 days of mycelium growth (PIII-16) 40% of lignin was metabolized and the remaining lignin was modified resulting in an increased S:G ratio (0.51 to 0.68). Furthermore, from both S and G phenylpropanoid units the decorations, mainly vinyl-groups, were removed from the phenolic skeleton.

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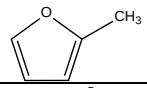
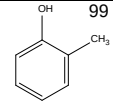
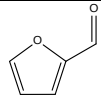
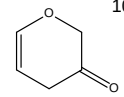
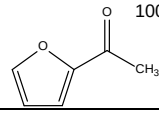
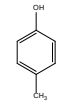
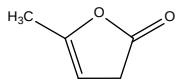
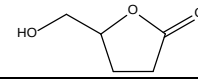
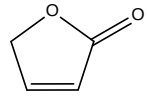
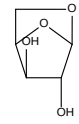
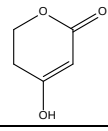
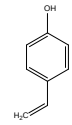
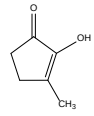
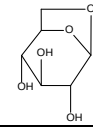
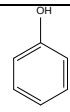
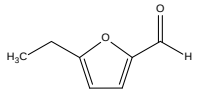
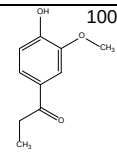
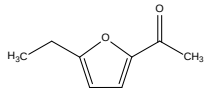
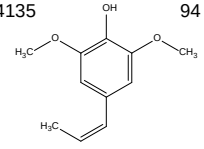
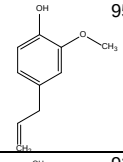
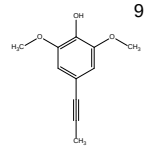
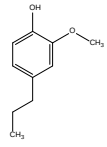
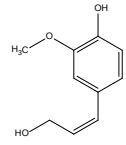
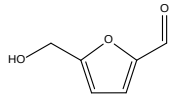
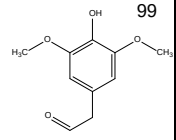
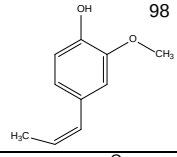
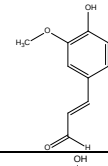
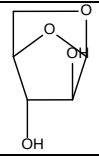
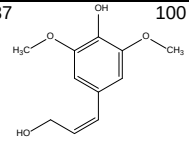
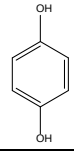
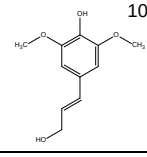
Supplementary data

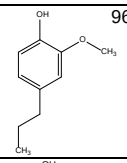
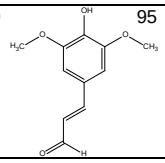
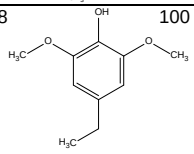
Supplementary table 1. Identities of lignin-derived phenolic F/P and unknowns compounds identified with Py-GC/MS with relative molar area higher than 1% in wheat straw (out of total F/P and unknowns compounds molar area).

No.	Compound	CAS number	Chemical structure	RS (for wheat straw)	No.	Compound	CAS number	Chemical structure	RS (for wheat straw)
1F[a]	2-methylfuran	534225		99	10H[a]	O-cresol	95487		99
2F[a]	Furfural	98011		100	12P[b]	2,4-dihydropyran-3-one	-		100
3F[a]	2-acetylfuran	1192627		100	14H[a]	P-cresol	106445		97
4F[b]	2,3-dihydro-5-methylfuran-2-one	591128		99	15F[b]	5-(hydroxymethyl) dihydro-2(3H)-furanone	3278066		100
5F[a]	2(5H)-furanone	497234		100	18F[b,c]	1,4-anhydroxylofuranose	-		100
6P[b]	4-hydroxy-5,6-dihydro-(2H)-pyran-2-one	-		100	20H[a]	4-vinylphenol	2628173		100
7U[a]	2-hydroxy-3-methyl-2cyclopenten-1-one	765708		97	40P[a]	1,6-anhydro-β-D-glucopyranose	498077		100
8H[a]	Phenol	108952		99					

[a]Interpretation based on pure compounds.
[b]Interpretation based on Ralph and Hatfield (1991), reverse search of compound in compost or WUS versus compound in wheat straw: 6P>99%, 12P>87%, 15F>89%, 18F>99%.
[c]Compound 18F and 27F has similar spectra, assignment were based on relative abundance in the sample.
RS= reverse search.

Supplementary table 2. Identities of lignin-derived phenolic S, G, F/P and unknowns compounds below 1% of relative molar area in wheat straw (for S and G out of total S+G molar area, and for F/P and unknown out of total F/P + unknowns molar area) identified with Py-GC/MS.

No.	Compound	CAS No	Chemical structure	RS (for wheat straw)	No.	Compound	CAS No	Chemical structure	RS (for wheat straw)
11F[b]	5-ethyl-2-furfural	23074104		100	37G[b]	Propiovanillone	1835149		100
13F[b]	Unknown, 2-acetoxy-5-ethylfuran?	24241847		97	38S[b]	*cis*-2,6-dimethoxy-4-propenylphenol	26624135		94
21G[a]	Eugenol	97530		95	39S[b] 41S[b]	1-(3,5-dimethoxy-4-hydroxyphenyl) propyne	-		93
22G[a]	4-propylguaiacol	2785877		92	44G[a]	*cis*-Coniferyl-alcohol	458355		99
23F[a]	5-hydroxy-methylfurfural	67470		89	45S[b]	Homosyringalde-hyde	-		99
25S[a]	*cis*-Isoeugenol	97541		98	48G[a]	*trans*-Coniferaldehyde	458366		99
27F[b,c]	1,4-anhydro-arabinofuranose	-		98	50S[a]	*cis*-Sinapyl-alcohol	537337		100
30H[a]	Hydroquinone	123319		99	51S[a]	*trans*-Sinapyl-alcohol	537337		100

31G[a]	4-propylguaiacol	2785877		96	52S[a]	*trans*-Sinapaldehyde	4206580		95
32G[b]	4-ethyl-syringol	14059928		100					

[a]Interpretation based on pure compounds
[b]Interpretation based on Ralph and Hatfield (1991), reverse search of compound in compost or WUS versus compound in wheat straw: 11F>92%, 13F>87%, 32G>97%, 37G>83%, 38S>88%, 39S/ 41S>82%, 45S>80%.
[c]Compound 18F and 27F has similar spectra, assignment were based on relative abundance in the sample.
RS= reverse search.

Chapter 4

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

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, 1M and 4M 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 1M and 4M KOHss 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.

Jurak, E., Patyshakuliyeva, A., Kapsokalyvas, D., Xing, L., van Zandvoort, M.A.M.J., de Vries, R.P., Gruppen, H., Kabel, M. A. Submitted for publication.

4.1. 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* (chapter 2, Gerrits, 1988). 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, 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 et al., 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 (chapter 2). The xylan in PIII-16 has ratios of arabinosyl and glucuronic acid residues to 100 xylosyl residues, of 14 and 13, respectively (chapter 2). In order to achieve a complete saccharification of xylan, and thereby a complete use of this carbohydrate in the compost, all the 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 (chapter 5). 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 et al., 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 et al., 1994). It coincides with the reported metabolisation and modifications of lignin in the same mycelium growth phase (chapter 3). During fruiting body formation, laccase levels are found to decline (Ohga et al., 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.

4.2. Materials and methods

4.2.1. Materials used

Compost obtained after filling of the beds with mycelium grown compost (PIII-16) compost 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.

4.2.2. 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 x 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 1M KOH containing 1% (w/w) $NaBH_4$

for 18 h at room temperature under continuous stirring. After centrifugation (10 000 x g; 30 min; 20°C), the residue was washed with 200 mL 1M KOH containing 1% (w/w) $NaBH_4$ and again centrifuged. Supernatants were combined, neutralized with acetic acid, extensively dialyzed (10-12 kDa cutoff, Medicell International, London, UK) against distilled water and freeze dried (1M KOHss). The residue was re-extracted and washed once with 4M KOH containing 1% (w/w) $NaBH_4$, as described for the 1M 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 cutoff, Medicell International, London, UK) against distilled water and freeze dried, coded as 4M KOHss and Res for the 4M KOH extracts and residues, respectively.

The yield of xylan (% w/w) over WSS, WUS, 1M KOHss, 4M 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 1M KOHss the water solubility was 75-90% (w/w) and for 4M KOH 40-60% (w/w).

4.2.3. Hydrolysis of alkali extracts with endoxylanase 1 (GH10), α-arabinofuranosidase (GH43) and α-glucuronidase (GH67)

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

The 100 µL of EX1 digested 1M and 4M KOHss of compost (2^{nd} flush) of supernatant was, subsequently, hydrolyzed (24h, head over tail) with a pure GH43 AXH-d3 arabinofuranosidase (30 µL, 2.7mg ml^{-1}) (Fig. 4.2) (Van Leare et al., 1999.). Also, of EX1 digested 1M and 4M KOHss of compost (2^{nd} flush) 100 µL was incubated with a partially purified α-glucuronidase GH67 (30 µL, 23 µg ml^{-1}) (Verbruggen et al., 1998).

4.2.4. Carbohydrate content and composition

The neutral carbohydrate and uronic acid content and composition was determined in duplicate, as described in chapter 2.

4.2.5 Pyrolysis-GC/MS

The composition of lignin was determined in triplicate, as described elsewhere (chapter 3).

4.2.6. 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 x 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 0.2M 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.

4.2.7 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 x 250 mm ID) in combination with a CarboPac guard column (2 mm x 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: 0.1M NaOH and B: 1M NaOAc in 0.1M 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.

4.2.8. 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.

4.2.9. Molecular analysis

To generate the maximum likelihood (ML) tree the amino acid sequences used were aligned using MAFFT (Katoh et al., 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 and 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).

4.3. Results

4.3.1. 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 yield of the compost obtained after Filling, Pinning, 1st flush, 2nd flush, and associated fractions, is presented in Table 4.1.

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 1M KOHss, 4M KOHss and Res was 86% or higher (Table 4.1). 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 1M and 4M 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, 1M KOHss contained 5-12 mol % and 4M 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.

4.3.2. The distribution and structural characteristics of xylan in WSS, 1M KOHss and 4M 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, 1M KOHss and 4M KOHss is presented in Table 4.2. From the total xylan in the original compost sample 80-93% 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 1M KOHss fractions, 10-12% in the 4M KOHss fractions, and 24-28% in the Res (Table 4.2).

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. 4.1A and B) as part of alkali extracted xylan became insoluble upon dialysis (see 4.2.2). For all WSS fractions similar elution profiles were observed (0.75-6 kDa). Further, similar HPSEC profiles were observed for all 1M KOHss xylan fractions, and also all the 4M 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 4.2. At Filling and at Pinning, in 1M 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. No changes in the DS were observed after 2nd flush for the 1M KOHss fraction. For the 4M 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 4M KOHss the DS Ara and UA increased to 21 and 28, respectively.

Table 4.1. 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 (1M KOHss and 4M KOHss) and residue (Res) is included.

Sample name	**Carbohydrate composition (mol%)[a]**							**Total carbohydrates (w/w % DM)**	**DM Yield (%)**
	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
1M 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
4M 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
1M 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
4M 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
1M 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
4M 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
1M 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
4M 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.

Next, the molecular weight distribution of the alkali extracts after digestion with a pure endoxylanase (EX1) (Kormelink, 1993) was analyzed on HPSEC (Fig. 4.1C 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 1M KOHss from Filling appeared to be degraded to a larger extent than 1M KOHss from 2nd flush.

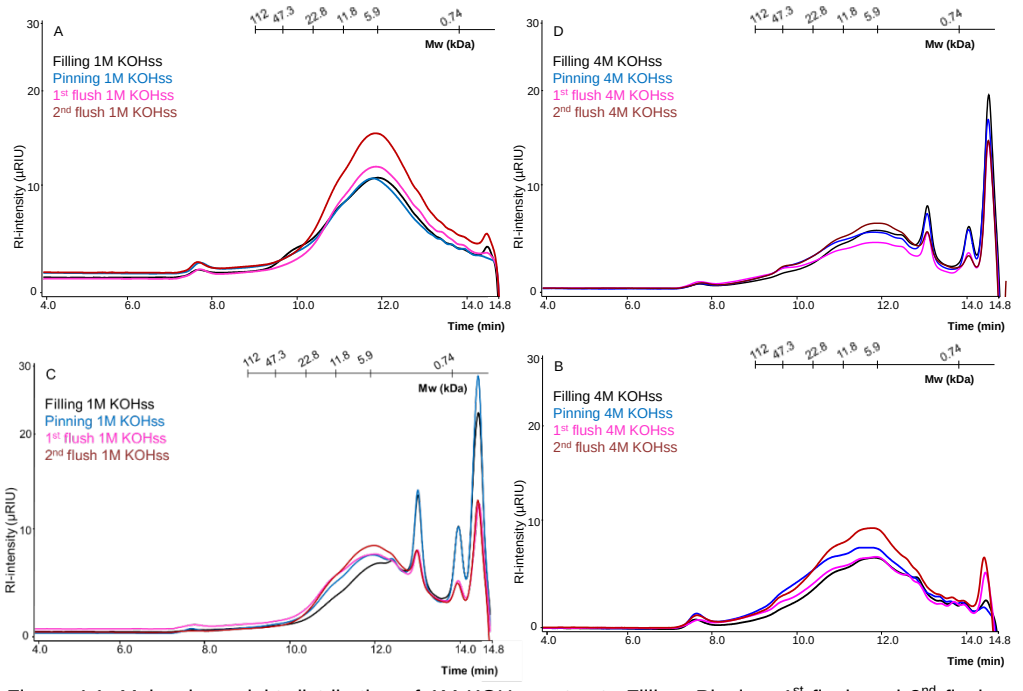


Figure 4.1. Molecular weight distribution of 1M KOHss extracts Filling, Pinning, 1st flush and 2nd flush before digestion (A) and endpoint digested (C); and 4M KOHss extract before digestion (B) and endpoint digested (D). A purified and well characterized endoxylanase (EX1) (Kormelink et al., 1993), was used for digestion.

In addition to the analyzed xylose, xylobiose and xylotriose released from the 1M and 4M KOHss of 2nd flush by the EX1 used, also substituted XOS were released (Fig 4.2). Based on elution profile and retention times of XOS from birchwood xylan and wheat arabinoxylan, using the same endo-xylanase, it was shown that the substituted XOS released from both 1M and 4M KOHss included glucuronic acid substituted XOS and XOS having one or two arabinosyl substituents per xylosyl residues (Fig. 4.2).

Table 4.2. Degree of substitution of xylan (mol/100mol 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 (1M KOHss and 4M 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
1M KOHss	11	10	54.1
4M 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
1M KOHss	11	10	52.1
4M 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
1M KOHss	17	18	47.9
4M 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
1M KOHss	16	17	47.2
4M KOHss	21	28	10.0
Res	39	67	24.1

To confirm the presence of XOS having 2 arabinosyl residues per xylosyl residue, EX1 hydrolyzed xylan fractions, 1M and 4M KOHss, from compost after 2nd flush were incubated with a pure arabinofuranosidase (AXH-d3) only able to remove arabinosyl residues from double substituted XOS with arabinosyl-residues (Van Laere et al., 1999). From 1M KOHss 2nd flush 20% of the total arabinosyl residues present was released after digestion as monomeric arabinose, and 10% from 4M KOHss 2nd flush. As AXH-d3 releases arabinosyl residues from double substituted XOS this indicated that 40% of all arabinosyl residues present in the 1M KOHss and 20% in the 4M 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 1M and 4M KOHss of 2nd flush, 9% and 2%, of total glucuronic acid present was released, respectively.

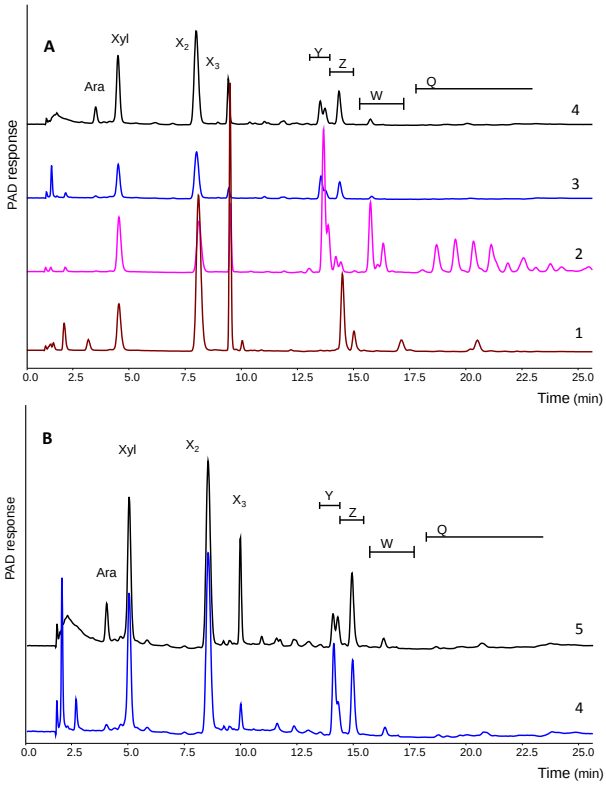


Figure 4.2. HPAEC elution profile of birchwood xylan (1) and wheat arabinoxylan (2) incubated with EX1, 1M KOHss (A) compost extracts after 2nd flush incubated with EX1 (3) and then sequentially with pure GH43 AXH-d3 arabinofuranosidase (4) and 4M KOHss (B) compost extracts after 2nd flush incubated 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).

As mentioned above, the soluble part of the alkali extracts was analyzed by HPSEC and HPAEC. The remaining insoluble xylan of the 1M KOHss and 4M 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. 4.3. The particle analysis imaging of 1M 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 1M KOHss (25 µm^2) compared to Filling 1M KOHss (50 µm^2). In 4M KOHss, more particles were present compared to the 1M KOHss, and again, smaller particles were observed for 1st flush 4M KOHss (100 µm^2) compared to Filling 4M KOHss (400 µm^2) (Fig. 4.3).

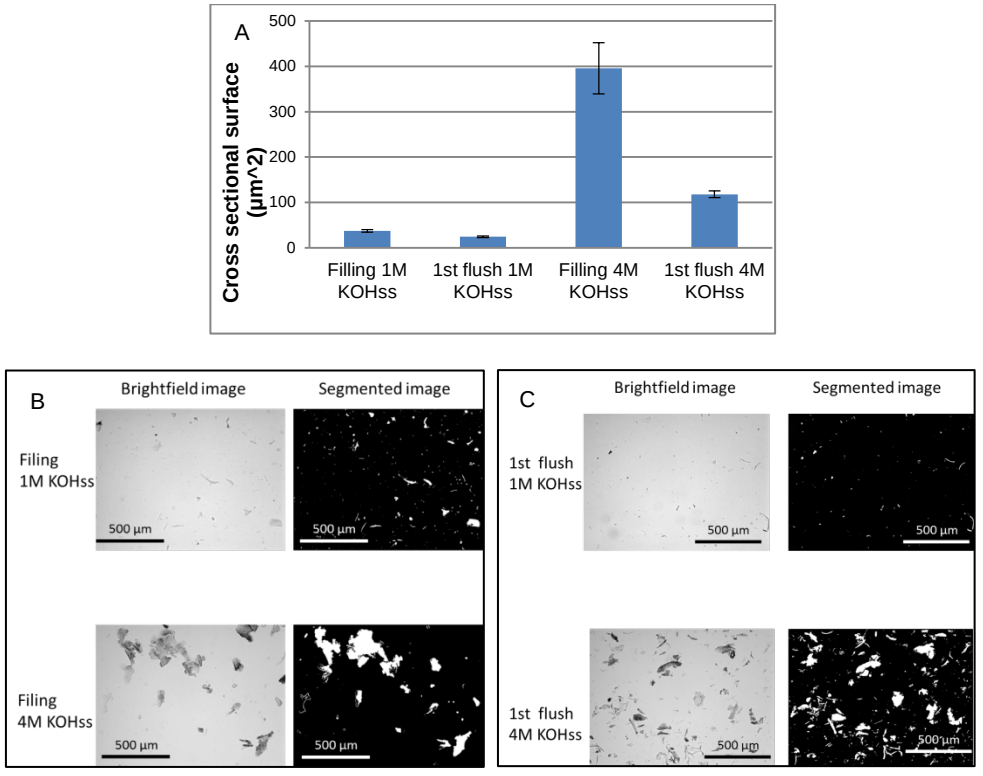


Figure 4.3. Cross sectional surface of particles in water insoluble xylan in 1M KOHss and 4M KOHss from compost after Filling and after 1st flush (A) and microscopy images of water insoluble xylan in 1M KOHss and 4M KOHss of compost after Filling (B) and after 1st flush (C).

4.3.3. 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* (Sorensen 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. 4.4A) was rooted using four GH54 arabinofuranosidase sequences and showed 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 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 (chapter 5). However, expression analysis demonstrated no- to very low expression of these genes (Fig. 4.4B).

4.3.4. 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. 4.5A).

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. 4.5B. 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.

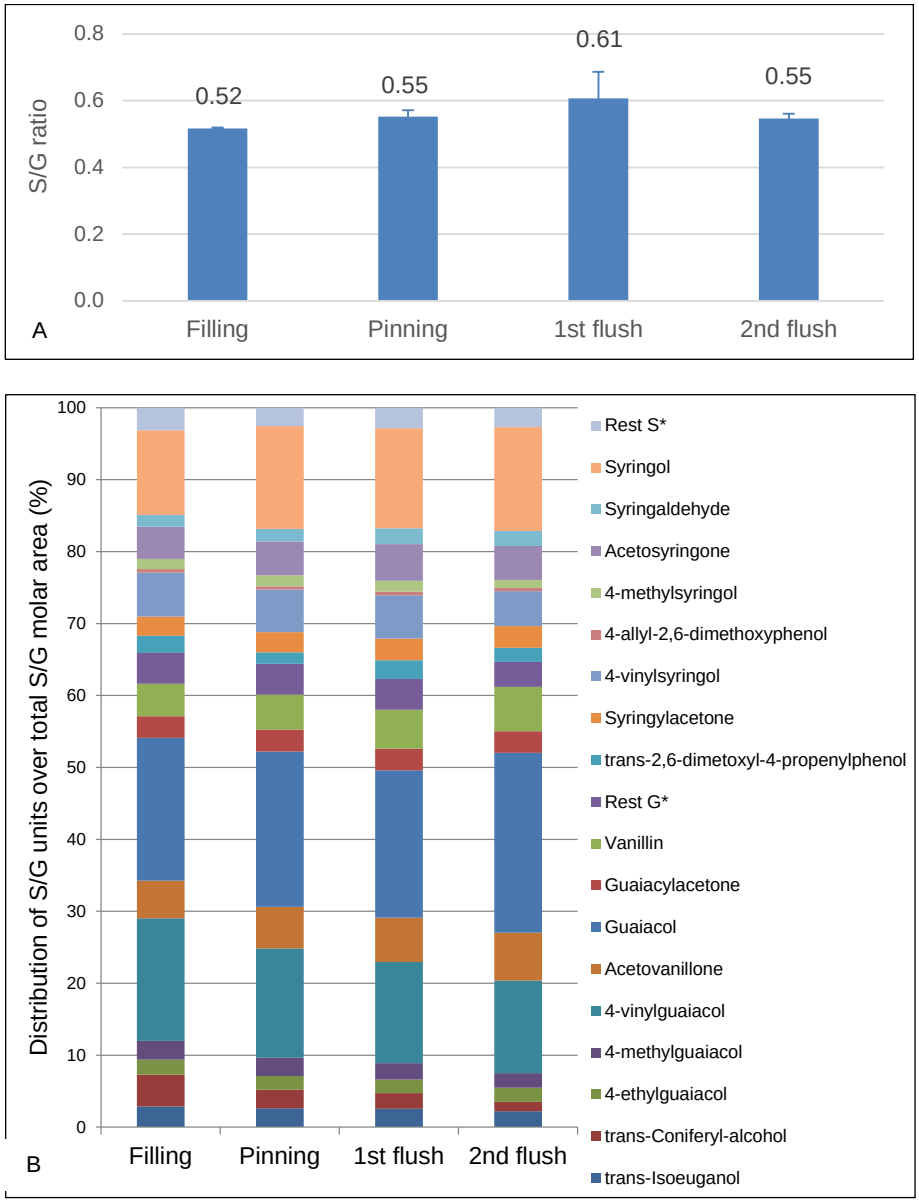


Figure 4.5. Syringyl (S) to guaiacyl (G) ratio (S:G ratio) (A) and distribution of S and G units (B), based on molar area of compost after Filling, Pinning, 1st flush and 2nd flush . Rest S* and G* compounds with <1% total molar area in wheat straw (chapter 3).

4.4. 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 *Agaricus 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 building blocks xylosyl (Xyl), arabinosyl (Ara) and uronyl (UA) residues, and of glucosyl (Glc) residues, which is in line with the reported composition of wheat straw based compost (chapter 2). 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 (chapter 5). 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* (chapter 3, Durrant et al., 1991) 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 4.1) 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 et al., 2007; Qing et al., 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 1M KOH and 4M 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 4.2). In general, water soluble carbohydrates are easier to access for enzyme degradation and hereby 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 1M KOHss of Filling, Pinning, 1st flush and 2nd flush (47-54% w/w), and 10-12% (w/w) in the 4M KOHss. The water soluble part of the alkali extracts was evaluated by HPSEC, which showed that the molecular weight distribution of the water soluble xylans in both 1M and 4M KOHss fractions of all composts analyzed during fruiting were similar and ranging from 0.5-23 kDa (based on pullulans).

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 4M KOHss 1st flush the particles' cross sectional surface was smaller compared to the 4M KOHss Filling. Also, microscopy showed that in the water insoluble part of 1M KOHss less insoluble particles were observed having a smaller cross section surface compared to the water insoluble part of 4M KOHss fractions (Fig. 4.3). In addition to xylan also lignin is extracted with KOH. The 1M and 4M KOHss were analyzed with Py-GC/MS and some differences in the obtained pyrograms of 1M and 4M 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 4M KOHss fractions analyzed relate to the presence of more or larger xylan-lignin complexes compared to 1M KOHss. So, it was assumed that during fruiting large xylan or xylan-lignin complexes were degraded, but nevertheless still partly present.

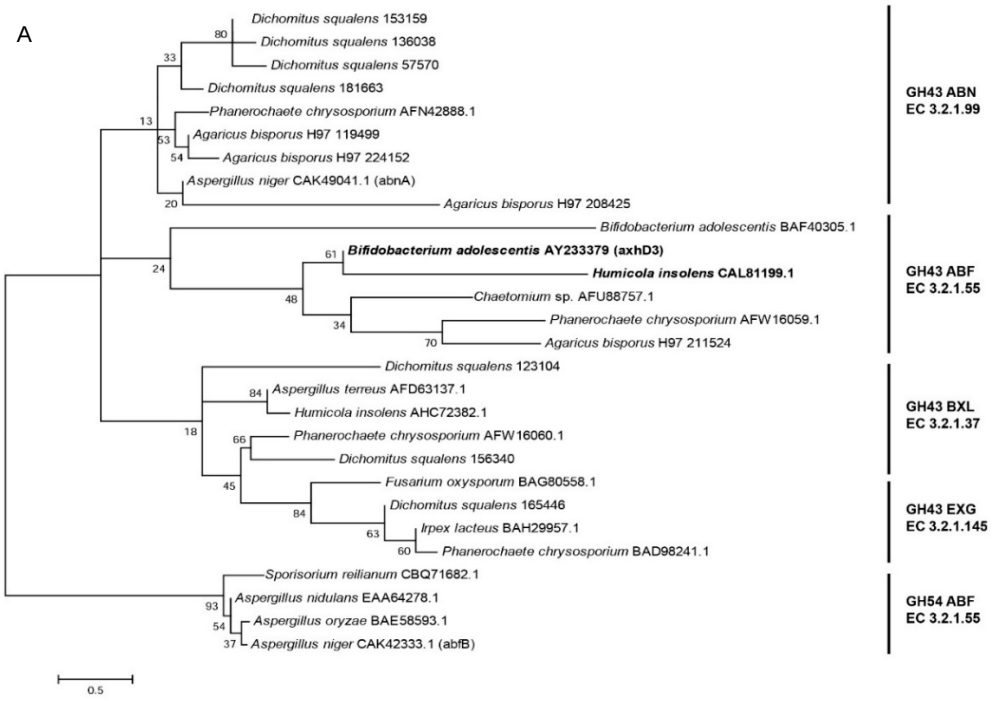


Figure 4.4. Maximum likelihood (ML) tree of selected fungal GH43 protein sequences (A) and expression profile (B) of two endoxylanases (GH10 XLN and GH11 XLN) and two α-glucuronidases from *A. bisporus* (both GH115 AGU). Enzymes in bold were confirmed to be able to release arabinose from double substituted xylosyl residues (Sorensen et al., 2006; van den Broek et al., 2005).

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, chapter 5, Pathyshakuliyeva et al., 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 4.4A), which matched with the lack of enzyme activities observed in compost enzyme extracts. Finally, neither α-glucuronidase nor α-arabinforuanosidase able to remove arabinosyl-residues from double substituted xylo oligomers were found to be active in the compost during fruiting body formation (chapter 6).

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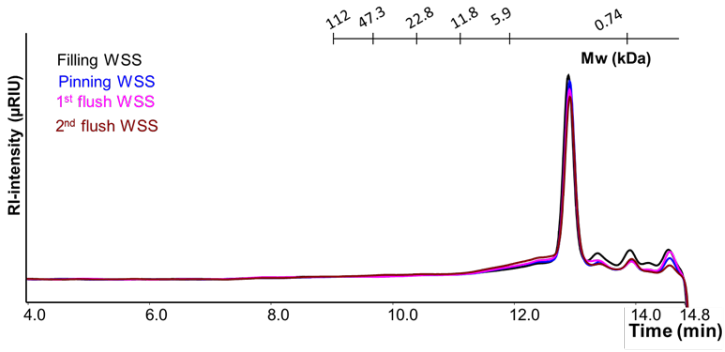
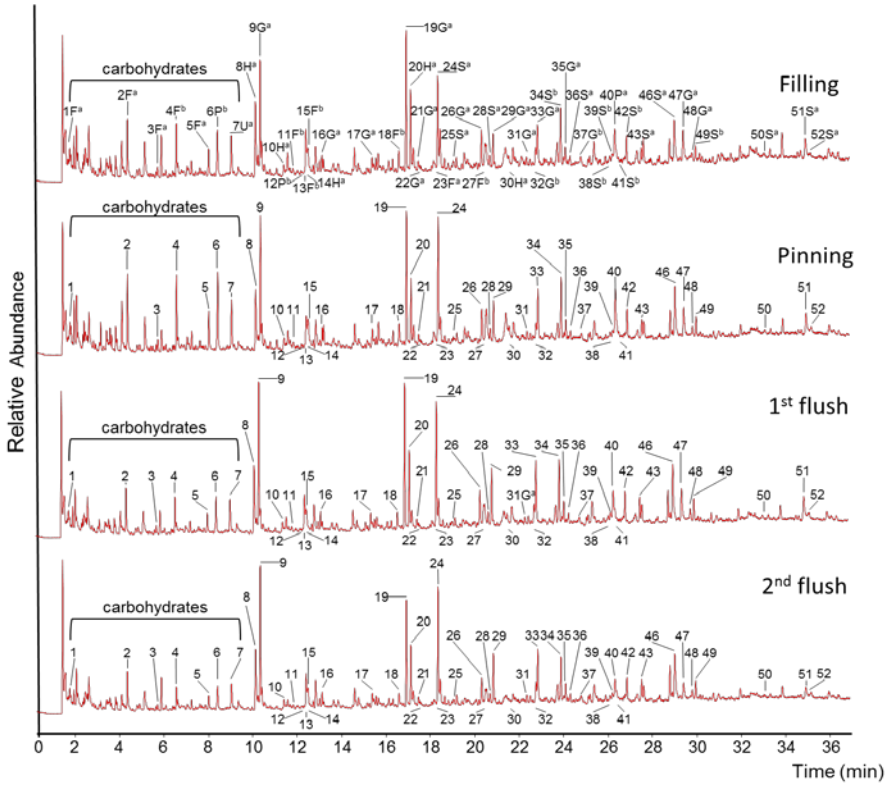
Supplementary data



Supplementary figure 1.Molecular weight distribution of WSS extract Filling, Pinning, 1st flush and 2nd flush.



Supplementary figure 2. Py-GC/MS pyrograms of compost during fruiting body formation, after Filling, Pinning, after 1st flush, after 2nd flush. The identities and structures of numbered syringyl and guaiacyl (and p-hydroxyphenyl) compounds are described in chapter 3, where the numbers presented here match with the numbers presented in chapter 3.

Chapter 5

Carbohydrate utilization and metabolism is highly differentiated in *Agaricus bisporus*

Abstract

In this study, genes encoding putative enzymes from carbon metabolism were identified and their expression was studied in different growth stages of *A. bisporus*. We correlated the expression of genes encoding plant and fungal polysaccharide modifying enzymes identified in the *A. bisporus* genome to the soluble carbohydrates and the composition of mycelium grown compost, casing layer and fruiting bodies. The compost grown vegetative mycelium of *A. bisporus* consumes a wide variety of monosaccharides. However, in fruiting bodies only hexose catabolism occurs, and no accumulation of other sugars was observed. This suggests that only hexoses or their conversion products are transported from the vegetative mycelium to the fruiting body, while the other sugars likely provide energy for growth and maintenance of the vegetative mycelium. Clear correlations were found between expression of the genes and composition of carbohydrates. Genes encoding plant cell wall polysaccharide degrading enzymes were mainly expressed in compost-grown mycelium, and largely absent in fruiting bodies. In contrast, genes encoding fungal cell wall polysaccharide modifying enzymes were expressed in both fruiting bodies and vegetative mycelium, but different gene sets were expressed in these samples.

Based on: Patyshakuliyeva, A., Jurak, E., Kohler, A., Baker, A., Battaglia, E., de Bruijn, W., Burton, K., Challen, M., Coutinho, P., Eastwood, D., Gruben, B., Makela, M., Martin, F., Nadal, M., van den Brink, J., Wiebenga, A., Zhou, M., Henrissat, B., Kabel, M.A., Gruppen, H., de Vries, R.P., 2013. Carbohydrate utilization and metabolism is highly differentiated in *Agaricus bisporus*. BMC Genomics, 14, 663.

5.1. Introduction

Carbon catabolism serves fungi with energy in the form of reducing equivalents and ATP, as well as essential precursor metabolites for biosynthesis, such as glucose-6-phosphate and fructose-6-phosphate (Chang and Miles, 2004). In nature plant biomass is the main carbon source for many fungal species. *A. bisporus* (the white button mushroom) is commercially cultivated on a composted mixture of lignocellulose-containing materials (mainly wheat straw and horse manure), which is highly selective for this fungus (Fermor et al., 1985; Tautorus and Townsley, 1984).

The major constituents of the lignocellulose fraction of compost are cellulose and the hemicellulose xylan (70% of the biomass) (Jørgensen et al., 2007) and lignin (Lawther et al., 1995; Lynch, 1993; Vane et al., 2001). Due to their diverse and complex polymeric nature, degradation of plant cell wall polysaccharides to their monomeric constituent requires a large range of enzymes (Coutinho et al., 2009; de Vries and Visser, 2001). Most of these enzymes have been divided into families in a classification system for Carbohydrate Active enZymes (CAZy, www.cazy.org) (Cantarel et al., 2009). It has been shown that during mycelial growth and fruiting *A. bisporus* produces a range of extracellular enzymes, which are involved in the degradation of the lignocellulosic fraction in compost (Fermor et al., 1991; Gerrits, 1969; Wood et al., 1991; Yague et al., 1997). A shift in fungal metabolism takes place during development of the fruiting body of *A. bisporus* that is closely linked to an increased rate of cellulose and hemicellulose degradation (Wood and Goodenough, 1977). The production of laccase and cellulase was suggested to be connected to the high rate and flow of carbon metabolism during fruiting body development (Claydon et al., 1988; Wells et al., 1987). Lignin degradation by *A. bisporus* decreases towards the end of the mushroom production cycle (Durrant et al., 1991; Waksman and Nissen, 1932; Wood and Leatham, 1983).

The major monosaccharide constituents of lignocellulose are D-glucose, D-xylose, and L-arabinose, while smaller amounts of D-galactose, D-galacturonic acid, L-rhamnose and D-mannose are also present. These monosaccharides are taken up by the fungal cell and converted through specific pathways (Battaglia, 2010). Both L-arabinose and D-xylose catabolism are part of the pentose catabolic pathway (Witteveen et al., 1989), which ends at D-xylulose-5-phosphate, an intermediate of the pentose phosphate pathway (PPP). D-Glucose can enter several biochemical pathways (de Vries and Visser, 2001; Hayashi, 1989; Timell, 1967), but can also lead to the synthesis of mannitol, trehalose and other storage compounds, such as glycogen and fatty acids (Martin et al., 1988). The minor components of polysaccharides present in compost are converted through the galacturonic acid catabolic pathway (Richard and Hilditch, 2009), the D-galactose

catabolic pathways (the Leloir pathway, the oxido-reductive pathway and the DeLey Doudoroff pathway) (Mojzita et al., 2012) and the L-rhamnose catabolic pathway (Watanabe et al., 2008).

Studies on carbon metabolism in *A. bisporus* have mainly focused on mannitol and trehalose. Synthesis of mannitol in *A. bisporus* is mediated by an NADPH-dependent mannitol dehydrogenase using fructose as substrate (Edmundowicz and Wriston, 1963). Metabolism trehalose involves either the trehalose synthase complex, (Wannet et al., 1998), or trehalose phosphorylase (EC 2.4.1.64), which catalyze the reversible hydrolysis of trehalose into glucose-1-phosphate and glucose (Wannet et al., 1998). Remarkable differences were found in carbon metabolism of fruiting body and vegetative mycelium (Beecher et al., 2001; Hammond, 1985; Hammond, 1981; Wannet et al., 1999). Mannitol functions as an osmolyte, which accumulates to high levels during fruiting body growth while after sporulation the level of mannitol decreases rapidly (Hammond and Nichols, 1976). It might also serve as a post-harvest reserve carbohydrate (Beecher et al., 2001; Hammond, 1981; Wood, 1985). Trehalose also serves as a reserve carbohydrate, which is present at lower levels than mannitol that decline during fruiting body development. It has been suggested that trehalose is synthesized in the mycelium and translocated to the fruiting body (Hammond, 1985; Wannet et al., 1999; Wells et al., 1987).

Gene expression analysis of genes encoding enzymes for polysaccharide modification and sugar metabolism offers an improved understanding of carbohydrate utilization and the metabolic fate of monosaccharides in the litter degrading fungus *A. bisporus*. Here, we identified genes encoding enzymes involved in carbon metabolism using the recently sequenced *A. bisporus* genome (Morin et al., 2012). The expression of these genes and genes encoding plant biomass degrading enzymes was analyzed during different stages of growth of *A. bisporus*, revealing significant differences between mycelium grown on plates, in compost or in casing-soil, and fruiting bodies.

5.2. Methods

5.2.1. Materials used

Compost, casing layer and fruiting bodies cultures were harvested at the first flush stage of *A. bisporus* strain A15 and were stored at -20°C (Coöperatieve Nederlandse Champignonkwekersvereniging (CNC); Milsbeek, The Netherlands). Samples (about 100 g) were collected, freeze dried and milled (<1 mm) (Mill MM 2000, Retsch, Haan, Germany). Duplicates were mixed in ratio 1:1. Wheat straw was collected as raw material and a representative sample was made by mixing 16 different freeze dried and milled samples of wheat straw in the same ratio. All

chemicals, unless stated otherwise were obtained from Sigma/Fluka (St. Louis, MO, USA) or Merck (Darmstadt, Germany).

5.2.2. Water extraction

Milled compost, casing layer and fruiting bodies (0.4 g) were suspended in millipore water (20 mL) and boiled at 100°C for 10 min to inactivate enzyme activity, shaken vigorously and filtered (0.2 µm). The filtrate was used to analyze water soluble carbohydrates.

5.2.3. Analytical and spectrometric methods

5.2.3.1. Neutral carbohydrate composition

Neutral carbohydrate composition of wheat straw, compost and casing layer was analyzed according to Englyst (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 1M H_2SO_4 for 3 h at 100°C and the constituent sugars released were derivatized and analyzed as their alditol acetates using gas chromatography (GC). The amount of neutral carbohydrates was corrected for mannitol, sorbitol and trehalose.

5.2.3.2. Uronic acid content

Uronic acids content of wheat straw, compost and casing layer was determined as anhydro-uronic acid by an automated m-hydroxydiphenyl assay (Blumenkrantz and Asboe-Hansen, 1973) using an autoanalyser (Skalar Analytical, Breda, The Netherlands). Glucuronic acid was used as a reference.

5.2.3.3. Lignin content

Samples of wheat straw, compost and casing layer were analysed for acid insoluble (Klason) lignin. To each sample of 300 mg (dry matter) 3 mL of 72% (w/w) H_2SO_4 was added and samples were pre-hydrolyzed for 1 h at 30°C. After this pre-hydrolysis, 37 mL of distilled water was added and samples were put in a boiling water bath for 3 h and shaken every half hour. Further, suspension was filtered over G4 glass filters (Duran, Mainz, Germany). The residual part was washed until it was free of acid and dried overnight at 105°C. The weight of the dried residual part was taken as a measure of the acid insoluble lignin content.

5.2.3.4. Protein content

Nitrogen content of wheat straw, compost and casing layer was analysed using the combustion (DUMAS) method on a Flash EA 1112 Nitrogen Analyser (Thermo Scientific, Rockford, IL, USA). Methionine (Acros Organics, New Jersey, USA) was used as a standard and protein content was calculated from the nitrogen content of the material, using a protein conversion factor of 6.25 (Jones, 1931).

5.2.3.5. Ash content

Samples of wheat straw, compost and casing layer (0.5 g) were dried in the oven overnight (105°C), weighed and put in the oven on 504°C overnight. Next day samples were weighed and difference between the mass at 105°C and 504°C was taken as ash content.

5.2.4. Chromatographic methods

5.2.4.1. Analysis of soluble carbohydrates, sorbitol, trehalose and mannitol

High performance anion exchange chromatography (HPAEC) was performed on an Ultimate 3000 system (Dionex, Sunnyvale, CA, USA) equipped with a CarboPac PA-1 column (2 mm x 250 mm ID) in combination with a CarboPac guard column (2 mm x 50 mm ID) and PAD detection. System was controlled by the Chromelion software (Dionex).

Separation and quantification of monosaccharides was done at a flow rate 0.4 ml min^{-1}, and the mobile phase consisted of (A) 0.1M NaOH, (B) 1M NaOAc in 0.1M NaOH and (C) H_2O. The elution profile was as follows: 0-40 min 100% C; 40.1-45.1 min from 100% A to 100% B, 45.1-50 min 100% B, 50.1-58 min 100% A, 58.1-73 min 100% C. From 0 to 40 min and from 58 to 73 min post column addition of 0.5M NaOH at 0.1 ml min^{-1} was performed to detect and quantify the eluted saccharides.

Soluble carbohydrates sorbitol, mannitol and trehalose were separated on the same system, including columns and detection. The flow rate used to separate sorbitol, mannitol and trehalose was 0.3 mL min^{-1}, and the mobile phase consisted of (A) 0.1M NaOH, (B) 1M NaOAc in 0.1M NaOH and (C) H_2O. The elution profile was as follows: 0-5 min 100% A, 5-25% 0-30% B, 25.1-30 min 100% B, 30-50 min 100% A.

Water soluble oligosaccharides were separated (0.3 mL min^{-1}) with a combination of linear gradients from two types of eluents, A: 0.1M NaOH and B: 1M NaOAc in 0.1M NaOH. The elution profile was as following: 0-35 min: 0-38% B, cleaning step 3 min 100% B and equilibration step 12 min 100% A. As a reference for xylo-oligomers with substitution, elution pattern of wheat arabinoxylan (medium viscosity, Megazyme, Bray, Ireland) digest with a pure and well described endoxylanase 1 was used (Kormelink et al., 1993; Van Laere et al., 1999), while as a standard for cellulose and xylan oligomers, cellodextrans and xylodextrans were used. Water extract of compost and casing layer were injected on the column without dilution and fruiting body water extract was diluted 20 times before injecting it on the column.

5.2.4.2. Organic acid analysis

Oxalic acid and citric acid were determined with an Ultimate system (Dionex) equipped with a Shodex RI detector and an Aminex HPX 87H column (300 mm x 7.8 mm) (Bio-Rad, Hercules, CA, USA) plus pre-column (Voragen et al., 1986). Elution was performed by using 5mM H_2SO_4 as eluent at a flow rate of 0.6 ml min^{-1} at 40°C.

5.2.4.3. Esterified acetic acid content

Samples of compost and casing layer (20 mg) were saponified with 1 mL of 0.4M NaOH in isopropanol/H_20 (1:1) for 3 h at room temperature. The acetic acid content was determined with an Ultimate system (Dionex) equipped with a Shodex RI detector and an Aminex HPX 87H column (300 mm x 7.8 mm) (Bio-Rad) plus pre-column (Voragen et al., 1986). Elution was performed by using 5mM 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 in the sample.

5.2.5. Genome annotation and comparative genomics

A. bisporus var bisporus (http://genome.jgi.doe.gov/Agabi_varbisH97_2/Agabi_varbisH97_2.home.html), *A. bisporus* var *burnetti* (http://genome.jgi.doe.gov/Agabi_varbur_1/Agabi_varbur_1.home.html), *Aspergillus niger, Aspergillus oryzae, Aspergillus nidulans, Phanerochaete chrysosporium* or *Postia placenta* and *Laccaria bicolor* S238N genomes (http://genome.jgi-psf.org/Lacbi2/Lacbi2.home.html) were used to perform genomic comparisons. Full genome clusters of orthologous genes were created by OrthoMCL (http://www.ncbi.nlm.nih.gov/pubmed/12952885) with E-value 1e-5 and sequence matching coverage 60% as the cutoff (http://www.ncbi.nlm.nih.gov/pubmed/20152020). Carbon catabolic genes of *Agaricus* and *Laccaria* were identified by extracting the orthologous clusters containing known carbon catabolic genes from *Aspergulli*, *P. chrysosporium* or *P. placenta*.

5.2.6. Transcriptome analysis

Gene expression was profiled in the commercial (heterokaryon) strain A15. *A. bisporus* strain A15 was grown in compost made from wheat straw, chicken litter and gypsum in the proportions 10:6:0.5 w/w. The first phase of composting was with regular mixing and took approximately 25 days. At phase II of composting process compost was pasteurized with steam at 70°C for 7 days. Phase II compost was inoculated with 1-2 % w/w *A. bisporus* mycelium spawn, placed in 50 kg growth trays, and incubated at 25°C, 95% relative humidity for 21 days. The colonized compost was covered by 5 cm peat-based casing layer and incubated for a further 7 days. The culture samples refer to axenic culture and the media used was compost extract medium (Calvo-Bado et al., 2000). Fresh pasteurized

compost was oven dried for 48 h at 80°C. Dried compost was boiled in distilled water (7.5 g L^{-1}) for 1 h and cooled to room temperature. After centrifugation (5000 rpm, 20 min), the supernatant was used to make the medium (Sonnenberg et al., 1988). Peptone (0.5% w/v) was added to the extract and the medium buffered to pH 7 using potassium phosphate buffer.

The fruiting body samples represent the mature mushroom stage 2 with a stretched, unbroken veil fruiting body (including the stipe, cap and pilei pellis (skin) tissues) (Hammond and Nichols, 1976). The casing samples consisted of a mixture of mycelium aggregates, undifferentiated primordia (1-2 mm circular with no differentiation between stipe and cap tissues), differentiated primordia (~7 mm diameter, oval with some evidence of cap tissue differentiation). The compost samples represent the mycelium growing in wheat straw compost. The samples for RNA extraction were collected on separate occasions from separate mushroom houses. Four biological replicates of each developmental stage were analyzed (Morin et al., 2012).

RNA was prepared from fruiting body and culture samples using a standard Trizol protocol. RNA was extracted from compost and casing samples using a method based on the FastRNA Pro Soil-Direct kit (MP Biochemicals) (Eastwood et al., 2013). RNA was quantified using a NanoDrop-1000 spectrophotometer and quality was monitored with the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

Custom arrays (Agilent ID 027120) were developed using 10,438 CDS (filtered model set) from the H97 v2 gene annotation; 5 x 60-mer oligos per CDS and the 8 x 60K randomized format were designed using the Agilent eArray software. Cyanine-3 (Cy3) labeled cRNA was prepared from 0.6 ug RNA using the Quick Amp Labelling kit (Agilent) according to the manufacturer's instructions, followed by RNAeasy column purification (QIAGEN, Valencia, CA, USA). Dye incorporation and cRNA yield were checked with the NanoDrop ND-1000 Spectrophotometer. 600 ng of Cy3-labelled cRNA (specific activity >10.0 pmol Cy3/ug cRNA) was fragmented at 60°C for 30 minutes in a reaction volume of 25 µL containing 1x Agilent fragmentation buffer and 2x Agilent blocking agent following the manufacturer's instructions. On completion of the fragmentation reaction, 25 µL of 2x Agilent hybridization buffer was added to the fragmentation mixture and hybridized to Agilent arrays (ID 027120) for 17 hours at 65°C in a rotating Agilent hybridization oven. After hybridization, microarrays were washed 1 minute at room temperature with GE Wash Buffer 1 (Agilent) and 1 minute with 37°C GE Wash buffer 2 (Agilent) then 10 seconds in acetonitrile and 30 seconds in Stabilization and drying solution (Agilent). Slides were scanned immediately after washing on the Agilent's High-Resolution C Scanner (G2505C US94100321) using one color scan setting for 8 x 60K array slides (Scan resolution 3µm). The scanned

images were analyzed with Feature Extraction Software (Agilent) using default parameters (protocol GE1_107_Sep09 and Grid: 027120_D_F_20100129) to obtain background subtracted and spatially detrended Processed Signal intensities. Features flagged in Feature Extraction as Feature Non-uniform outliers were excluded (Morin et al., 2012). Only those genes with > 2-fold differences and P-value <0.05 in gene expression between compost/casing layer/fruiting body and culture-grown mycelium were considered to be differentially expressed. Comparison of ratios of compost/culture transcript profiles was used to identify the most highly upregulated transcripts found in mycelium grown on compost during vegetative growth. The comparison of compost/fruiting body transcript profiles highlights developmental stage differences during mushroom formation (Morin et al., 2012).

The *Laccaria bicolor* S238N transcriptomes of 2 weeks free-living mycelium (FLM) and mature fruiting bodies were extracted from Gene Expression Omnibus (GEO) by series number GSE9784. Gene expression profiles were extracted, normalized and analyzed as described previously (Martin et al., 2008). Only genes with 2-fold differences and P-value <0.05 were considered significantly differentially expressed.

5.3. Results

5.3.1. Identification and expression analysis of genes encoding enzymes of central metabolism

The two sequenced genomes of *A. bisporus* var. *bisporus* H97 and var. *burnettii* JB137-s8 were analyzed to identify genes involved in central carbon metabolism. Identification was performed using the confirmed pathway genes from other fungi (Additional file 1).

Gene expression was assessed in mycelium grown on defined medium, in casing layer and in compost, and in fruiting bodies, using specific custom 60-mer Agilent microarrays (see “Methods”). Only those genes with > 2-fold differences and P-value <0.05 in gene expression between compost/casing layer/fruiting body and culture-grown mycelium were considered to be differentially expressed (Additional file 2).

5.3.2. Glycolysis & gluconeogenesis

Most genes from glycolysis were moderately upregulated in compost and casing compared to undifferentiated mycelium grown on agar medium, while their levels were similar or downregulated in the fruiting bodies (Figure 5.1, Additional files 3 and 4). In contrast, the gluconeogenic gene encoding phosphoenolpyruvate carboxykinase (PEPCK) was 8-fold upregulated in fruiting bodies.

5.3.3. Pentose phosphate pathway

Expression of most PPP genes is similar in casing, compost and fruiting bodies compared to plate grown mycelium, while only some genes are slightly up- (in compost and casing layer) or down-regulated (in fruiting bodies) (Fig. 5.1, Additional files 3 and 4). There is no consistent effect on either the oxidative or the non-oxidative part of the PPP.

5.3.4. Pentose catabolic pathway

A significant increase in expression of most of the pentose catabolic pathway genes were detected in compost and to a lesser extent in the casing layer compared to plate grown mycelium, while their expression was reduced in fruiting bodies (Additional file 2). An exception was the putative L-xylulose reductase encoding gene that had reduced expression levels in compost and casing compared to plate-grown mycelium.

5.3.5. Catabolism of D-galactose, D-galacturonic acid, L-rhamnose and D-mannose

The putative *A. bisporus* genes of galacturonic acid catabolic pathway are strongly upregulated in compost and to a lesser extent in the casing layer, while they are down-regulated in fruiting bodies (Figure 5.2). Expression of genes from the D-galactose Leloir pathway was similar or elevated in all samples compared to plate-grown mycelium (Additional file 2). In contrast, nearly all genes of the D-galactose oxido-reductive pathway were upregulated in compost and downregulated in fruiting bodies (Additional file 2). Most genes from the rhamnose and mannose catabolic pathways (Additional file 1) (Watanabe et al., 2008) were similar or upregulated in compost, casing layer and fruiting bodies, compared to plate-grown mycelium (Additional file 2).

5.3.6. Mannitol and trehalose metabolism

The mannitol-1-phosphate dehydrogenase encoding gene was similarly expressed in compost, casing layer and fruiting bodies, while the mannitol dehydrogenase encoding gene was similar in compost and downregulated in casing layer and fruiting body (Figure 5.2).

Expression of most trehalose metabolism genes was similar or upregulated in samples from compost and casing layer in comparison to undifferentiated plate-grown mycelium (Additional file 2). The exception was the gene encoding the neutral trehalase (EC 3.2.1.28), which was downregulated in compost. In samples from fruiting bodies, a gene encoding a neutral trehalase was slightly upregulated.

5.3.7. Organic acid metabolism

Oxalic acid and citric acid are among the two most commonly produced organic acids by fungi (Gadd, 1999). No specific upregulation for oxalic acid metabolic genes was observed in any of the samples. In contrast, several of the citric acid metabolic genes were expressed at higher levels in fruiting bodies than in compost or the casing layer.

5.3.8. Comparison of the expression of carbon metabolic genes between *A. bisporus and L. bicolor*

Orthologs of *A. bisporus* carbon metabolic genes were identified in the genome of a mycorrhiza species *L. bicolor* S238N (Additional file 1), with the exception of genes for L-rhamnose utilization genes for which no homologs could be found in *L. bicolor*.

The gene expression differentiation pattern of fruiting body versus mycelium was calculated for both fungi. In contrast to the prevalent gene downregulation in glycolysis, PPP and PCP pathways in *A. bisporus*, most of the genes in these pathways showed constant expression in mature fruiting bodies and free-living mycelium in *L. bicolor*.

5.3.9. Expression of genes encoding plant cell wall polysaccharide degrading enzymes

Expression of genes encoding plant cell wall degrading enzymes from *A. bisporus* active against all the major plant cell wall polysaccharides was detected (Table 5.1). These genes are expressed at significantly higher levels in compost than in the other samples. For xylan and cellulose related genes, 90% and 64%, respectively, were expressed in compost while in casing layer and fruiting bodies less than 15% of these genes were expressed. In compost, expression of genes encoding enzymes targeting other polysaccharides (e.g. starch, pectin and xyloglucan) was also observed. Some genes of families GH5 and CE4, which contain enzymes acting on both plant and fungal cell wall polysaccharides, were upregulated in either compost or fruiting bodies.

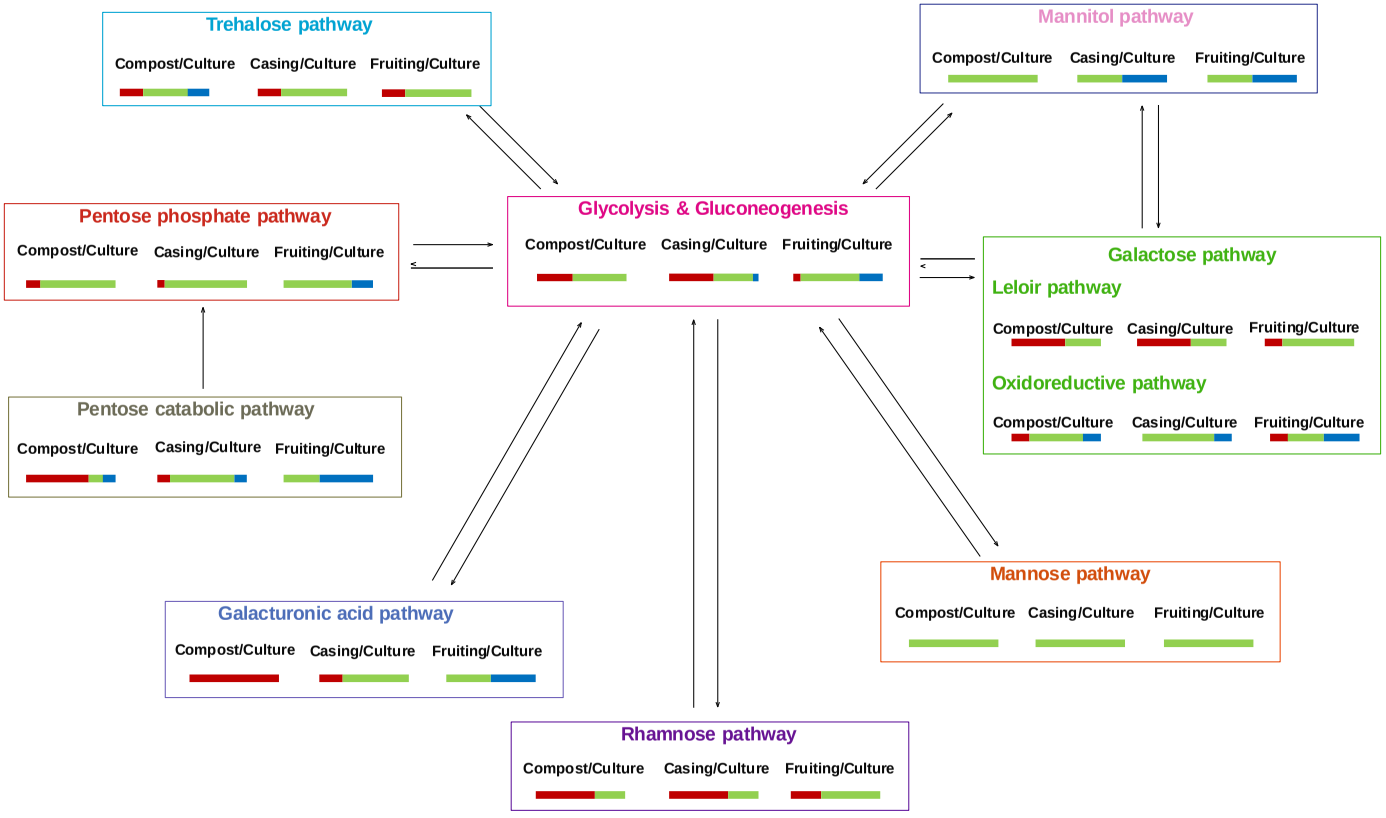


Figure 5.1. Schematic representation of the expression of genes of the different carbon metabolic pathways. Bars under the growth stages indicate the percentage of genes that are 2-fold upregulated (red), between 2-fold upregulated and 2-fold downregulated (green), and more than 2-fold downregulated (blue) in the sample compared to culture-grown mycelium.

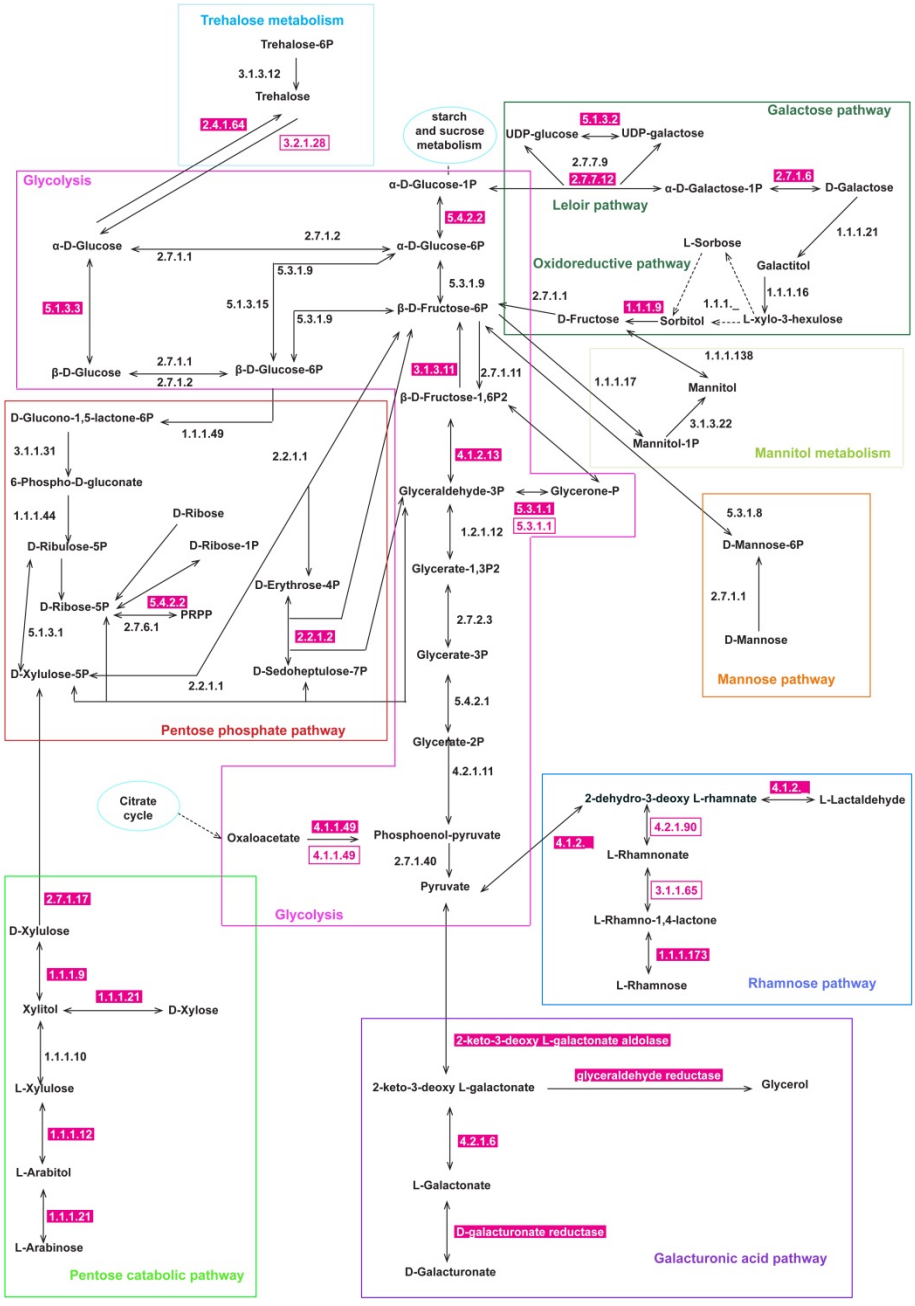


Figure 5.2. Map of the central metabolism in *A. bisporus*. Gene products contributing to these pathways are indicated. EC numbers in pink boxes indicate that genes encoding these enzymes are upregulated in compost (white numbers) or fruiting bodies (pink numbers) compared to plate-grown mycelium or fruiting bodies (pink numbers) compared to plate-grown mycelium.

5.3.10. Expression of genes encoding fungal cell wall degrading/modifying enzymes

Fungal cell wall degrading and modifying enzymes have received less attention than plant cell wall degrading enzymes, resulting in a less well defined assignment of function. During growth *A. bisporus* needs to synthesize and modify its cell wall. As growth occurs in compost, casing layer and fruiting bodies, genes encoding fungal cell wall modifying enzymes need to be expressed in all growth stages. However, as the morphology of these stages is not identical, different genes may be expressed in compost and fruiting bodies. A complete list of genes encoding putative fungal cell wall modifying enzymes can be found in Additional file 5, including their putative function. Of all genes encoding putative fungal cell wall modifying enzymes 36% were expressed in all three samples, indicating a basal set of fungal cell wall modifying enzymes. Only 20% of the genes were upregulated in the compost, while about 30% were upregulated in the fruiting bodies. None of the genes were specifically upregulated in the casing layer.

Some CAZy families related to fungal cell wall modification contain genes that were upregulated in compost as well as genes that were upregulated in fruiting bodies. This applies in particular to GH16 (endo-1,3(4)-β-glucanase), GH17 (endo-1,3-β-glucosidase) and GH18 (chitinases). Genes specifically expressed in compost were found in GH5, GH55 and GH72. Most of the genes of GH92 (α-mannosidase) are upregulated in compost. Genes specifically expressed in fruiting bodies were found in GH63 (α-glucosidase) and GT17 (glucan endo-1,3-β-glucosidase). Most genes from GT2 (chitin synthase), GT48 (1,3-β-glucan synthase), GT57 (α-1,3-glucosyltransferase) and GT15 were also upregulated in fruiting bodies.

Table 5.1. Percentage of plant degrading cell wall enzymes that are up regulated, number of genes expressed in compost, casing layer or fruiting bodies grouped by polysaccharide and their putative function. A detailed list of the genes of these CAZy can be found in Supplementary file 5.

Polysaccharide	CAZy families	No. genes	Compost	Casing layer	Fruiting bodies
Xylan	GH10,11,43,115 CE1,5,15	19	89	5	5
Xyloglucan	GH12,21,31*,74,95	5	100	0	0
Cellulose	GH1*,5*,3,6,7,9; AA9	22	64	9	14
Chitin/xylan	CE4*	11	36	9	27
Pectin	GH2,28,35,51,53,78,88,105 CE8,12 PL1,3,4	26	96	12	4
Mannan	GH1*,5*,27	5	60	40	0
Starch	GH13,15,31*	15	31	0	19

*not all genes of the family are related to designated polysaccharide.
GH: Glycoside Hydrolase, CE: Carbohydrate Esterase, PL: Polysaccharide Lyase; AA: Auxiliary Activities.
A detailed list of the genes of these CAZy can be found in Table S3.

5.3.11. Carbohydrate composition analysis of mycelium grown compost and casing layer and of fruiting bodies

Compost, casing layer and wheat straw were analyzed for lignin, ash, protein, total carbohydrates and carbohydrate composition. Results are presented in Table 5.2. When the *A. bisporus* mushrooms have matured, compost consists of lignin (41% w/w) and ash (36% w/w), carbohydrates (17% w/w) and proteins (13%). Significant amounts of sandy particles and gravel are present in the compost and casing layer and due to the Klason lignin determination method we expect that some of this sandy inorganic material remained on the filter and is included in the calculated lignin amount (Iiyama et al., 1994). The main monosaccharides released from compost by acid hydrolysis were xylose and glucose (4.4% w/w and 8.4% w/w, respectively). The composition of wheat straw was used as a reference for the composition of carbohydrates in raw compost as analysis showed that in raw compost the molar composition of carbohydrates is the same as in wheat straw (data not shown). The wheat straw composition determined in our study (Table 5.2) is in agreement with previously reported composition (Kabel et al., 2007). The molar composition of compost after mature mushrooms have been formed differs from that of wheat straw.

The casing layer is a mixture of calcium and peat that consists mainly of lignin (52% w/w) and ash (29% w/w). There are few carbohydrates present (12% w/w) and the main monosaccharides released after acid hydrolysis were xylose (1.4% w/w), mannose (0.6% w/w) and glucose (7.5% w/w) (Straatsma et al., 1991). As mentioned above, the actual lignin amount is likely to be lower than measured due to calcium and sandy particles that remain on the filter after acid hydrolysis.

Aqueous extraction of compost, casing layer and fruiting bodies revealed that more than 95% of carbohydrates are insoluble. A high performance anion exchange (HPAEC) elution pattern of water extract from mycelium grown compost, casing layer and fruiting bodies was used to analyze the extract.

Changes in free soluble monosaccharides were observed in these samples. Concentrations of arabinose, galactose and xylose were high in compost, while only traces of these monosaccharides were found in casing layer and fruiting bodies (Table 5.3). High levels of glucose were observed in all samples. Mannitol and trehalose levels were significantly higher in fruiting bodies than in compost and casing layer (Table 5.3), as were the levels of citric acid (data not shown), while no oxalic acid was detected in the samples. The very high level of sorbitol in the compost samples could suggest a role as a transportable carbon compound from the vegetative mycelium to the fruiting body (Table 5.3).

Table 5.2. Composition of wheat straw, compost and casing layer.

% w/w (based on dry matter)	Wheat straw	Compost	Casing layer
Lignin (Klason)	27	41[a]	52[a]
Total carbohydrates	57	17	12
Ash	5	36	29
Protein (%N *6.25)	3	13	7
Carbohydrate composition (molar %)			
Arabinose	6.0	5.6	1.6
Xylose	42.6	30	14
Mannose	0.9	4	6.1
Galactose	1.3	3.3	7
Rhamnose	0.8	1.4	2
Glucose	45	47	60
Uronic acids	3.9	8.6	9.2
DS Acetic acid (mol Ac/100 mol Xyl)	32	12	9

[a]Small part of inorganic material is included.

Soluble oligosaccharides were detected in the compost, while none were detected in the casing layer or fruiting bodies (Figure 5.3). The peaks detected in the compost were compared to standards of xylo- and cello-dextran oligosaccharides (DP 2-6) and the elution pattern of the well described endoxylanase (EX1) digest of wheat arabinoxylan in order to identify them (Kormelink et al., 1993). Mainly xylobiose (A in Fig. 5.3), xylotriose (C in Fig. 5.3), and presumably xylo-oligomers with attached glucuronic acid or its 4-*O*-methyl ether (E in Fig. 5.3) were found. In addition to xylo-oligomers, cellobiose was detected. The small peaks that were detected are likely xylo- and cello-oligomers of higher degree of polymerization and arabinose substituted xylo-oligomers.

Table 5.3. Concentration (mg kg^{-1}) of free (soluble) monosaccharides, trehalose, mannitol and sorbitol.

Component (mg/kg fresh material)	Compost	Casing layer	Fruiting body
Arabinose	37.4	3.0	3.5
Rhamnose	7.9	1.5	1.1
Galactose	15.9	6.1	2.3
Glucose	819.9	224.3	149.4
Xylose	221.9	11.6	5.0
Mannose	23.8	10.2	7.9
Fructose	70.4	41.7	703
Sorbitol	7654	3160	5242
Mannitol	3994	1657	20298
Trehalose	397	140	1064

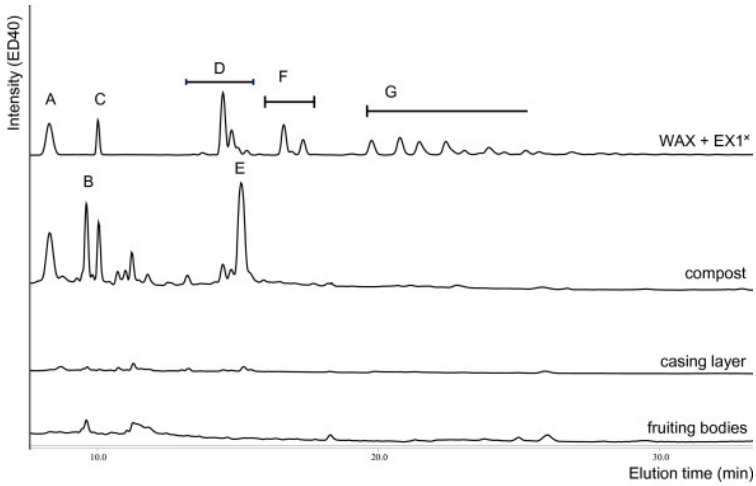


Figure 5.3. HPAEC elution patterns of the water soluble fraction of compost, casing layer and fruiting bodies (xylobiose (A), cellobiose (B), xylotriose (C), single substituted xylo-oligomers (D), likely xylo-oligomers with GlcA substituent (E), double substituted xylo-oligomers (F), multiple substituted xylo-oligomers (G)). *wheat arabinoxylan incubated with a pure endoxylanase (EX1) (Kormelink et al., 1993).

5.4. Discussion

In this study, genes encoding carbon metabolic genes were identified in the genome of *A. bisporus* and their expression in different growth stages was compared to the available carbohydrates and the expression of genes encoding carbohydrate modifying enzymes.

5.4.1. Compost is mainly focused on degrading plant biomass

Analysis of the expression of genes encoding plant and fungal polysaccharide modifying enzymes identified in the *A. bisporus* genome (Morin et al., 2012) revealed correlation between these genes and composition of carbohydrates. Expression analysis of CAZy-genes demonstrated that in compost the highest expressed genes are related to (hemi-)cellulose and pectin degradation, while also some genes related to β-1,3-glucan modification were expressed. A large decrease of carbohydrate content and, therefore, polysaccharides was revealed in the compost after growth of *A. bisporus* and fruiting body production. Expression data supports that the decrease in carbohydrates observed is partially caused by the growth of *A. bisporus*. About 90% of the genes encoding xylan degrading enzymes were upregulated in the compost. This correlates well with the detection of soluble xylo-oligosaccharides in compost. Higher proportions of arabinose and xylose in the water extracts of compost than in the water extracts of casing layer and fruiting bodies (Table 5.3)

are in good agreement with the expression of genes encoding arabinofuranosidases, endoxylanases and β-xylosidases. The presence of xylo-oligomers in compost suggests that the β-xylosidase activity may be the limiting factor in xylose release. The pentose catabolic pathway was strongly upregulated in the compost and moderately upregulated in the casing layer, while it was downregulated in the fruiting bodies. This confirms the relevance of release and conversion of these pentoses as a main carbon source for *A. bisporus* during growth in compost.

Expression of genes encoding other plant polysaccharide degrading enzymes that are not normally associated with compost, e.g. starch, pectin and xyloglucan related genes, was also detected. In nature *A. bisporus* can grow on various substrates ranging from leaf litter and soil under cypress in coastal California to manured soil, composts of plant debris, and other horticultural and agricultural situations reported in Europe (Kerrigan et al., 1998). Growth on these different substrates is likely due to the ability of *A. bisporus* to produce a wide range of plant polysaccharide degrading enzymes and it may co-express genes aimed at different polysaccharides. Such a system is well described for the ascomycete *Aspergillus niger*, in which a single regulator (XlnR) activates the expression of genes related to cellulose, xylan and xyloglucan degradation (Hasper et al., 2002; van Peij et al., 1998). For this fungus six regulators involved in plant polysaccharide degradation have been described and they usually respond to the presence of the monomeric building blocks of the polysaccharides (Battaglia et al., 2011; Gruben, 2012; Gruben et al., 2012; van Peij et al., 1998; van Kuyk et al., 2012; Yuan et al., 2008). While no homologs of these regulators have been found in basidiomycetes (Todd and de Vries, unpublished data), it is likely that basidiomycetes have developed similar systems using different regulators.

5.4.2. The casing layer serves as an intermediate phase

In the casing layer, which is a mixture of peat and lime, it is likely that the detected glucose and mannose at least partially drive from the mycelial cell wall, in the form of glucans and mannoproteins, respectively. While some genes encoding putative plant cell wall degrading enzymes were expressed in the casing layer, the level of up-regulation compared to plate-grown mycelium is much smaller than that in compost. In addition, expression of some chitinase encoding genes was detected. The casing layer seems to be an intermediate phase in which some genes related to plant biomass degradation are expressed, but also modification of the *A. bisporus* cell wall is an important process for the conversion to fruiting body morphology. The lack of soluble polysaccharides indicates that the role of the mycelium in the casing layer is mainly to supply carbohydrates to the fruiting body.

5.4.3. The fruiting body focuses on modification of fungal polysaccharides

For *A. bisporus* growth and development a basal set of fungal cell wall modifying enzymes is needed and about 36% of the genes encoding such enzymes were expressed in mycelium grown compost, casing layer and fruiting bodies. The other expressed genes encoding fungal cell wall modifying enzymes are upregulated during specific growth stages. This suggests that *A. bisporus* has specific genes for mycelium development and growth and others for fruiting body formation and modification. Some genes from GH16 (encoding endo-1,3(4)-β-glucanase), GH17 (encoding glucan endo-1,3-β-glucosidase) and GH18 (encoding chitinases) are upregulated in the compost while others from the same families are upregulated in the fruiting bodies. These results support the compositional and morphological differences found between mycelium and fruiting bodies (Hammond and Nichols, 1976). Expression of different sets of genes encoding fungal cell wall modifying enzymes has also been described for other fungi. For example, in *A. niger* different sets of genes encoding chitinases, chitin synthases and alpha-1.3-glucan synthases were expressed in the center and the periphery of plate grown cultures (Levin et al., 2007).

Enzymes from families GH5 and CE4 have several described activities, some of which are related to plant cell wall polysaccharides, while others are related to fungal cell wall polysaccharides (www.cazy.org). For some of the enzymes from these families upregulation in compost was observed, while others were upregulated in fruiting bodies. A strong correlation was observed between the putative function and the expression of genes from these families. While genes encoding putative plant biomass degrading enzymes were upregulated in compost, genes encoding putative fungal cell wall modifying enzymes were upregulated in fruiting bodies (Additional file 6).

5.4.4. Carbon metabolism is partially differentiated in *A. bisporus*

Expression analysis demonstrated that the pentose catabolic pathway and galacturonic acid pathway were strongly upregulated in compost and moderately upregulated in the casing layer, while they were downregulated in fruiting bodies. Most genes of the oxido-reductive galactose pathway were also higher expressed in compost than in fruiting bodies, which correlates with a higher galactose level in compost compared to fruiting bodies. In contrast to the pathways described above, the glycolytic pathway and PPP are active in all growth stages of *A. bisporus*. This correlates well with the presence of free glucose in all samples, suggesting that hexose catabolism is an important factor in all growth stages of *A. bisporus*. The PPP has been described as the major route of glucose catabolism in fruiting bodies of A. bisporus (Hammond and Nichols, 1976; Hammond, 1977; Hammond and Nichols, 1977) as well as *Lentinula edodes* (Tan and Moore, 1995) as a greater proportion of glucose oxidation occurs via the PPP in the fruiting body than in

vegetative mycelium, while glycolysis has been suggested to be the major pathway of sugar metabolism during fruiting body development in *Pleurotus ostreatus, Coprinus cinereus* and *Schizophyllum commune* (Chakraborty et al., 2003; Moore and Ewaze, 1976; Schwalb, 1974).

The concentration of mannitol in fruiting bodies was six times higher than in compost. However, expression of mannitol pathway genes was significantly lower in fruiting bodies than in compost, suggesting that mannitol is synthesized in the vegetative mycelium and transported to the fruiting body. Earlier studies observed that mannitol functions as an osmoregulatory compound and facilitates a continuous influx of water from compost to the fruiting body to support turgor and fruiting body development (Kalberer, 1990; Stoop and Mooibroek, 1998). This would suggest that mannitol is unlikely to be transported by diffusion from the mycelium. Therefore, it should either be transferred by active transport or alternatively, be synthesized in the fruiting body. If the latter is the case, a possible explanation for the observed expression of the genes could be that the encoded enzymes are transported into the fruiting body.

Trehalase activity was reported to be highest during the peak of each flush, while low activity was detected during the interflush period (Wells et al., 1987), which correlates well with the highest expression of a putative trehalase encoding gene in fruiting bodies of our study. In contrast, trehalose phosphorylase was found to increase during the interflush period (Wannet et al., 1999), which was also confirmed by the expression analysis in our study.

No significant differences were observed in the expression of genes related to oxalic acid metabolism in the different growth stages and the expression levels suggest that oxalic acid formation occurs in all stages. The high expression of one of the putative oxalate decarboxylase encoding genes could explain why no oxalic acid was detected in the samples as this could imply that degradation of oxalic acid occurs at least as fast as its synthesis. It should also be noted that only free oxalic acid was analyzed in this study, while oxalic acid present in the form of calcium oxalate was not included.

In contrast, several of the genes involved in citric acid metabolism are higher expressed in the fruiting body than in compost and casing layer, which correlates well with the higher levels of citric acid that were detected in these samples. As citric acid is known to have preservative properties against bacteria in food (Brul and Coote, 1999), it is tempting to speculate that the accumulation of citric acid in fruiting bodies may also be involved in the defense mechanism of the mushroom against bacteria. Another explanation may be the high respiration rates of the fruiting bodies, which requires high expression of genes associated with the citric acid/Krebs cycle and mitochondria in general (Tan and Moore, 1995). High expression of isocitrate lyase was also reported in brown-rot fungi, where this

enzyme produced succinate and glyoxylate from isocitrate (Martinez et al., 2009; Munir et al., 2001). Progressive downregulation of this gene was observed in the casing layer during the shift from vegetative mycelium to fruiting body (Eastwood et al., 2013).

5.4.5. The difference in carbon metabolism between *A. bisporus* and *L. bicolor*

Comparison of two basidiomycetes *A. bisporus* and *L. bicolor* didn't show any correlation in expression of carbon metabolic genes. This could be explained by the difference in life styles of these two species. As a saprobe, *A. bisporus* is highly dependent on obtaining carbon from its surroundings. In contrast, the mycorrhizae *L. bicolor* obtains carbon from its symbiotic partner in the form of sucrose, placing a much lower demand on a versatile carbon metabolism.

5.5. Conclusions

The data from our study demonstrates that overall there is a clear correlation between expressions of genes related to plant and fungal polysaccharides and the ability of *A. bisporus* to degrade these polysaccharides. We see a clear difference in genes expressed within mycelium grown compost and fruiting bodies supporting the hypothesis that different genes are expressed in *A. bisporus* mycelium and fruiting bodies. This supports previous results that this fungus produces different enzymes during its life cycle (Garcia Mendoza et al., 1996). However, it should also be recognized that gene expression is likely to be dynamic and here we have examined it at the time point when first flush was harvested (approximately 34 days after compost was inoculated with spawn). Large oscillations of cellulase activity in the compost have been observed which co-ordinate with mushroom fruiting body production and oscillations of activities of fruiting body metabolic enzymes (Burton et al., 1994; Claydon et al., 1988; Wells et al., 1987).

Moreover, our study demonstrates a clear correlation between the expression of genes encoding plant and fungal cell wall polysaccharides with the composition of carbohydrates in compost, casing layer and fruiting bodies. Genes encoding plant cell wall polysaccharide degrading enzymes were mainly expressed in compost-grown mycelium, and largely absent in fruiting bodies. In contrast, genes encoding fungal cell wall polysaccharide modifying enzymes were expressed in both fruiting bodies and vegetative mycelium in the compost, but different gene sets were expressed in these samples.

In the present study an in silico metabolic reconstruction of the central carbon metabolism in *A. bisporus* was performed and combined with expression analysis of the relevant genes in different growth stages of *A. bisporus*. The

analysis of metabolic pathways in *A. bisporus* may provide information about the requirements of carbon source and energy metabolism during commercial growth of *A. bisporus*. We showed that during growth in compost and casing a much larger variety of carbon sources was used by *A. bisporus* than during growth on synthetic medium. In contrast, carbon metabolism in fruiting bodies appears to be mainly aimed at hexoses. This could indicate that only these sugars are transported towards the fruiting body from the vegetative mycelium, which implies that carbon transport to the fruiting bodies is a highly regulated and selective process.

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Supplementary data

Micro array data from *Agaricus bisporus* and *Laccaria bicolor* used in this paper is available at GEO, accession number GSE39569 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE39569) and GSE32559 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE32559), respectively.

Additional files

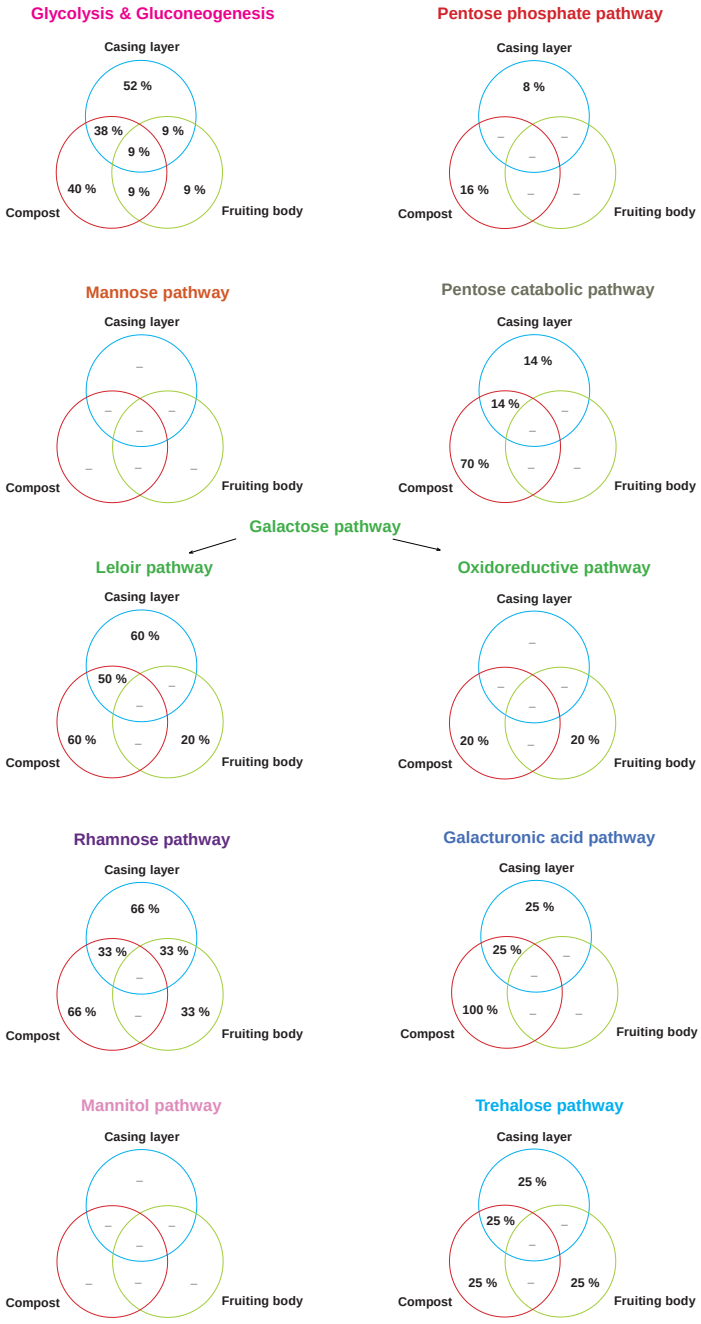
- Files do not fit in the format of this thesis.

Additional file 1 as EXCEL – Genes encoding putative enzymes of carbon metabolism in *A. bisporus* and *L. bicolor*.
Genes in *A. bisporus* var. *bisporus* were identified using orthologous clustering method based on best bi-derectional hits of all-vs-all blast to the genomes included in analysis.

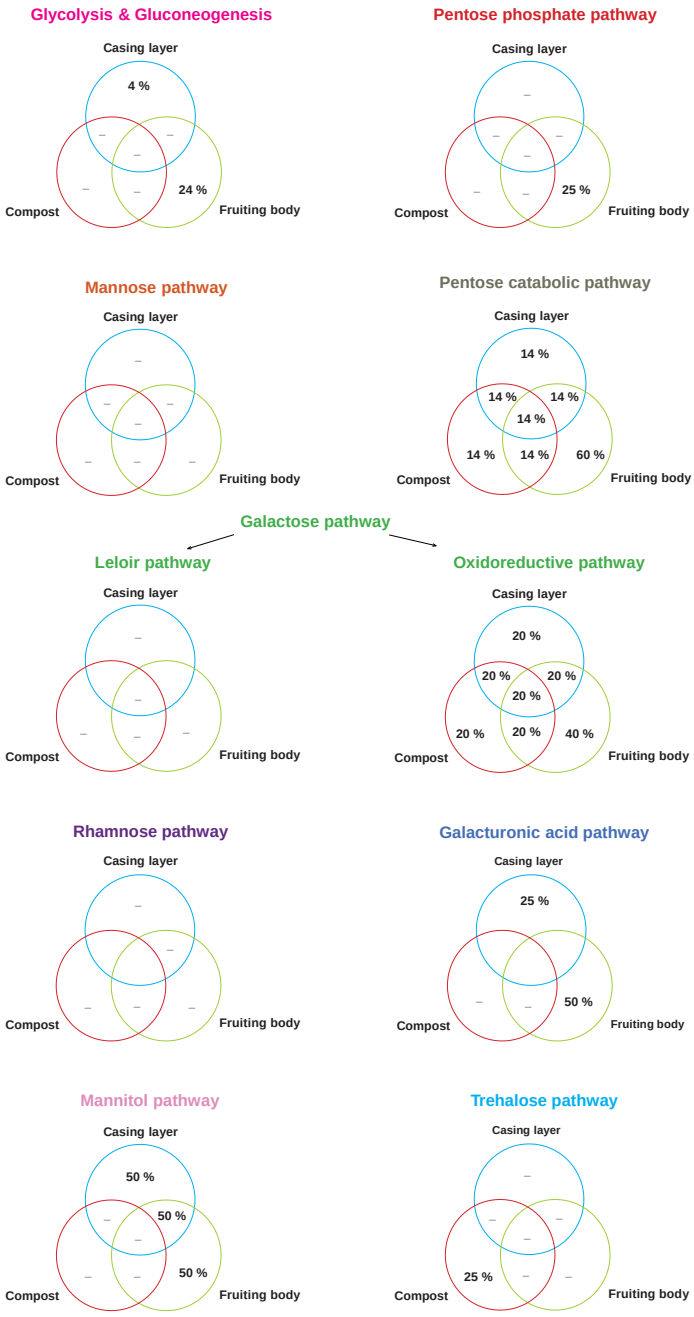
Additional file 2 as EXCEL – Expression comparison of carbon metabolic genes in different growth stages of *A. bisporus* var. *bisporus* and *L. bicolor*.
In the ratio between the values genes that are upregulated compared to culture-grown mycelium are in pink, while genes that are down regulated are in green.

Additional file 5 as EXCEL Expression of genes encoding putative fungal and plant polysaccharide modifying enzymes
Putative functions are based on CAZy family assignment and homology to characterized enzymes. The activity on plant of fungal polysaccharides is putative and not always supported biochemically. The expression levels are the average of 4 biological replicates.

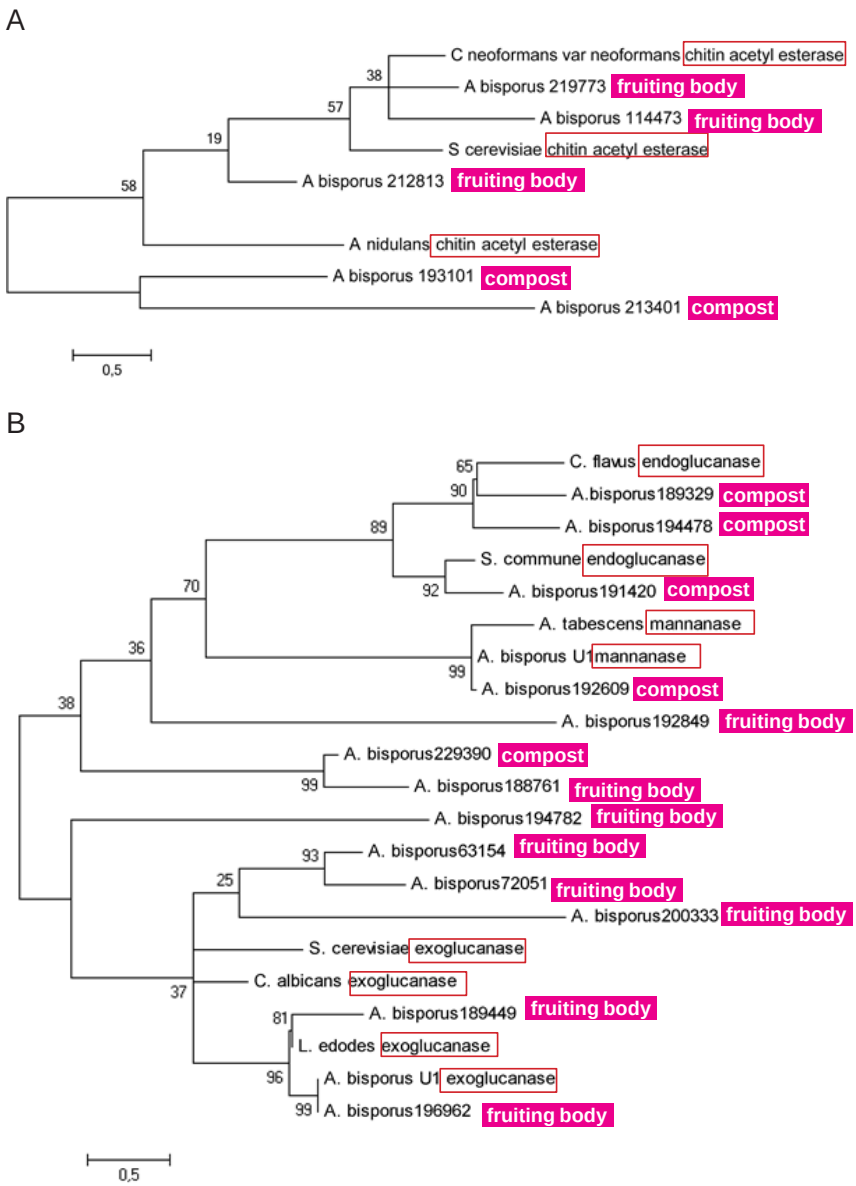
Additional file 3. Proportion of upregulated genes of the different carbon metabolic pathways in compost, casing layer and fruiting bodies.
Venn diagrams represent different carbon metabolic pathways indicating the percentage of genes that are 2-fold upregulated in the samples compared to culture-grown mycelium.



Additional file 4. Proportion of downregulated genes of the different carbon metabolic pathways in compost, casing layer and fruiting bodies. Venn diagrams represent different carbon metabolic pathways indicating the percentage of genes that are 2-fold downregulated in the samples compared to culture-grown mycelium.



Additional file 6. Maximum likelihood tree showing the correlation between plant biomass degrading and fungal cell wall modifying enzymes and upregulation of genes encoding these enzymes in compost or fruiting body.
Phylogenetic tree of the members of CE4 (A) and GH5 (B) families together with characterized enzymes was based on maximum likelihood method with 1000 bootstraps replications and WAG substitution model. Text in pink boxes shows that genes encoding indicated enzymes are upregulated in compost/fruiting body.



Chapter 6

Compost grown *Agaricus bisporus* lacks the ability to degrade and consume highly substituted xylan fragments

Abstract

The fungus *Agaricus bisporus* is commercially grown for the production of edible mushrooms. This cultivation occurs on compost, but not all of this substrate is consumed by the fungus. To determine why certain fractions remain unused, carbohydrate degrading enzymes, water-extracted from mushroom-grown compost at different stages of mycelium growth and fruiting body formation, were analyzed for their ability to degrade a range of polysaccharides. Mainly endo-xylanase, endo-glucanase, β-xylosidase and β-glucanase activities were determined in the compost extracts obtained during mushroom growth. Interestingly, arabinofuranosidase activity able to remove arabinosyl residues from doubly substituted xylose residues and α-glucuronidase activity were not detected in the compost enzyme extracts. This correlates with the observed accumulation of arabinosyl and glucuronic acid substituents on the xylan backbone in the compost towards the end of the cultivation. Hence, it was concluded that compost grown *A. bisporus* lacks the ability to degrade and consume highly substituted xylan fragments.

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6.1. Introduction

Commercially, white button mushrooms (*Agaricus bisporus*) are grown on compost, of which the carbon and nitrogen sources may differ throughout the world. In Europe, *A. bisporus* compost is based on wheat straw, and horse and chicken manure. Before mushroom mycelium is introduced two composting stages, having different conditions with respect to e.g. pH and temperature, are applied to make the compost accessible and highly specific for the growth of *A. bisporus* (chapter 2, Iiyama et al., 1994). Mycelium is introduced to the compost and grown until the compost is considered mature. This mature compost contains about 27% (w/w) of carbohydrates based on total dry matter (chapter 2). A casing layer (mixture of peat and lime) is put on top of the mature compost to induce fruiting body formation (Hayest et. al., 1969). Normally, several flushes of mushrooms can be harvested before the compost is considered spent. This spent compost still contains about 10% (w/w) of carbohydrates based on total dry matter (Iiyama et al., 1994), which include both plant and fungal biomass carbohydrates. Apparently, complete degradation and consumption of compost carbohydrates is not achieved, but why this fractions remains has so far not been investigated in detail.

In a previous study, it was shown that before mushroom mycelium is introduced to the compost, mainly lowly substituted xylan and cellulose are present as carbohydrates. This xylan (34 mol%) is substituted with arabinosyl (5 mol%) and with 4-*O*-methyl-glucuronic acid (4 mol%) residues, while hardly any ester bound substituents are present (chapter 2). Already during mycelium growth of *A. bisporus* these xylan and cellulose are partly degraded and consumed (Iiyama et al., 1994). It is likely that such modifications of the available carbohydrate structures affect their degradation during the later fruiting body formation stages.

The changes in carbohydrate structure and content can be majorly related to enzyme activities produced by *A. bisporus*, since mature compost is fully colonized with mycelium of this species. Others have already studied enzyme activities in compost during mycelium growth and fruiting body formation (Bonnen, et al., 1994, Savoie, 1998, Wood, 1980). However, the main focus of these studies was on lignin degrading enzymes or on exo-acting carbohydrases. In addition, these exo-acting enzymes were investigated at their optimal conditions with standard assays, which are not always representative for their activity under composting conditions and on compost carbohydrates (Kabel et al., 2006). In this paper we present the first thorough characterization of enzyme activities present in compost during mycelium growth and fruiting body formation of *A. bisporus*. A deeper analysis was facilitated by the availability of the *A. bisporus* genome sequence (Morin et al., 2012) and a recent study in which all genes encoding (putative) carbohydrate degrading enzymes have been identified (chapter 5). In the same study it was described which of these genes are up- or down- regulated during fruiting body

formation. Nevertheless, it is not known whether these expressed genes are translated and whether their corresponding enzymes are active in compost. Therefore, in the study presented here the aim is to investigate which enzymatic activities are present in the compost in different phases of fruiting of *A. bisporus* and compare these both to the genome information described by Patyshakuliyeva et al. (chapter 5) and to the structure of the remaining carbohydrates in the compost. This provides a deeper understanding of the carbohydrate degrading machinery of *A. bisporus* and the commercial cultivation process of this fungus. The samples used in this study correspond to different stages of the cultivation (Table 6.1).

6.2. Materials and methods

6.2.1. Compost samples

The strain used in this study is the commercial *A. bisporus* strain Sylvan A15, (CNC, Milsbeek, The Netherlands). For a first screening of enzyme activities, compost samples of PII_{end} and PIII-16 were supplied by CNC as described previously (chapter 2). For a second, more extensive screening, fresh compost samples of end of Phase II (PII_{end}), which is the compost phase just before addition of mycelium and of 16 days mature mycelium grown compost (PIII-16) were obtained. Further, composts obtained after filling of the beds with PIII-16 compost and covered with casing soil (Filling), after pinning of *A. bisporus* (Pinning), after the first flush (1^{st} flush) and after the second flush (2^{nd} flush) of mushrooms were supplied by CNC; all were from the same timeline. Sample codes and description are summarized in Table 6.1. Samples were collected (about 1 kg each) in duplicate and frozen. For carbohydrate analysis, part of the frozen samples were freeze dried and milled (<1 mm) (Mill MM 2000, Retsch, Haan, Germany). After milling, duplicates were mixed in equal ratios and the mixed samples were analyzed.

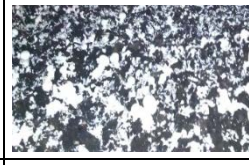
6.2.2. Extraction of enzymes

Extraction of enzymes from the compost was tested under various conditions such as pH, extraction time and temperature and, finally, a modified method from Singh et al. (2003) was used in order to achieve the highest recovery of proteins from the compost.

Frozen compost samples were defrosted and on the same day, 10 g of the sample was mixed with 100 mL distilled water in 250 mL Erlenmeyer flasks. The flasks were incubated for 1 h at 200 rpm and 4°C. Samples were then centrifuged (10 000 x g, 15 min, 4°C) and the supernatant was collected as the crude enzyme extract, which was used for PNP assays (6.2.4). For the other assays,

supernatants were filtered through 0.2 µm filters. The filtrate obtained was then filtered through a 10 kDa filter (Merck Millipore, Billerica, MA, USA) and washed twice to remove small carbohydrates. The 10 kDa retentate was mixed with millipore water to reach the starting volume and denoted as “enzyme extract" from all the phases.

Table 6.1. Compost sample codes and description.

Sample	**Description**	
PII_{end}	Compost just before addition of mushroom mycelium	
PIII-16	Mature compost, after 16 days of mycelium growth	
Filling	Compost after filling of the growth beds with PIII-16 and covered with casing layer	
Pinning	Compost after first mushroom pins appear (13 days after filling)	
1^{st} flush	Compost after first flush of mushrooms was harvested (23 days after filling)	
2^{nd} flush	Compost after second flush of mushrooms was harvested (31 day after filling)	

6.2.3. Protein content, pH, conductivity and Mw profiles

The protein content of the prepared enzyme extract was measured by using the bicinchoninic acid (BCA) assay with bovine serum albumin as standard (Pierce, Thermo Scientific, Rockford, IL, USA).

Extracellular proteins in the enzyme extracts were separated by SDS-PAGE using 12% polyacrylamide gels and stained using GelCode Blue stain reagent (Pierce, Rockford, IL, USA).The enzyme extracts were concentrated 4 times and 40 µL of concentrated extract was loaded on the gel.

6.2.4. Enzyme activity assays

Exo-enzyme activities in the enzyme extracts were measured in duplicate using p-nitrophenol (PNP)-linked substrates (4-nitrophenyl α-L-arabinofuranoside, 4-nitrophenyl β-D-glucopyranoside, 4-nitrophenyl β-D-xylopyranoside, 4-nitrophenyl β-D-cellobioside, and 4-nitrophenyl β-D-mannopyranoside; Sigma-Aldrich, St. Louis, MO, USA). In a total volume of 100 µL using 40 µL of the compost extract, 10 µL of 0.01% (w/v) p-nitrophenol-linked substrates, and 50mM sodium acetate buffer (pH 5.0) was mixed. Samples were incubated in microtiter plates for 4 h at 30°C. Reactions were stopped by addition of 100 µL 0.25M Na_2CO_3 solution. Absorbance was measured at 405 nm in a microtiter plate reader (FLUOstar Optima; BMG LabTech, Ortenberg, Germany). The activities were calculated using a standard curve ranging from 0 to 20 nmol of p-nitrophenol per assay volume. Overall, exo-enzyme activities of compost enzyme extracts were observed to be quite low (between 0.1 and 4.5 nmol pnp ml^{-1} min^{-1}) after 4 h.

Enzyme activities were tested for their overall hydrolytic activity (combined exo- and endo-activity) on various polysaccharides, and assayed by the PAHBAH reducing-end assay in duplicate (Lever, 1972). For this assay, a working solution was prepared by mixing one part of p-hydroxybenzoic acid hydrazide (5% w/w) in 0.5M HCl with four parts of 0.5M NaOH. The sample (10 µL) was mixed with 200 µL working solution and incubated at 70°C for 30 min in microtiter plate covered with aluminium foil. After cooling, absorbance was measured at 405 nm. The reducing-end concentration was quantified using xylose and glucose calibration curves (10-750 µg ml^{-1}).

Endo-enzyme activities were tested on a range of carbohydrate substrates. Wheat arabinoxylan (medium viscosity), birchwood xylan and potato galactan were obtained from Megazyme (Wicklow, Ireland). Tamarind xyloglucan was obtained from Danippon Pharmaceutical (Osaka, Japan) and carboxymethyl cellulose (low viscosity) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Rhamnogalacturonan I (apple modified hairy regions-B, saponified) were obtained as described by Schols et al. (1990); Branched sugar-beet arabinan was obtained from British Sugar (Peterborough, UK (McCleary et al., 1993)). Finally, low (DM 30, C30) and high (DM 70, C72) methylated homogalacturonan was provided by

Copenhagen Pectin A/S (Lille Skendved, Denmark, (Daas et al., 1999). These carbohydrate substrates were incubated with enzyme extracts from PII_{end} and PIII-16 in water. For incubation, 800 µL of 2.5 mg ml^{-1} of substrates was used and 200 µL of non-diluted enzyme extract was used. Incubations were performed at 35°C rotating "head over tail" for 24 h.

Substrates for the second screening were Locus bean gum (SKW Biosystems, Enschede, The Netherlands), wheat arabinoxylan (WAX, Medium viscosity, Megazyme), birchwood xylan (Megazyme), carboxymethylcellulose (Low viscosity, Sigma-Aldrich) and a well-defined digest of WAX by endo-xylanase 1 from *A. awamori* (Gruppen et al., 1992, Kormelink et al., 1993). Substrates were incubated in water with enzyme extracts from PII_{end}, PIII-16, Filling, Pinning, 1st flush and 2^{nd} flush. Incubations were performed as described above.

In order to confirm the presence of double substituted xylo-oligomers in the WAX digested with compost-extracts, WAX was first incubated with 1^{st} flush, and sequentially incubated with pure GH43 AXH-d3 arabinofuranosidase (Van Laere et al., 1999) as described above.

6.2.5. Analytical methods

Neutral carbohydrate and uronic acid content and composition were determined in duplicate, as described in chapter 2.

High Performance Size Exclusion Chromatography (HPSEC) was performed as described in chapter 2. Enzyme digests were analyzed without prior dilution. Enzyme activity was evaluated by comparing the high performance size exclusion chromatography (HPSEC) elution profiles of polysaccharides before and after enzyme hydrolysis. If after incubation with the enzyme extracts the HPSEC profile was the same as in the substrate without enzymes it was concluded that there was "no degradation". When a decrease in large molecular weight (Mw) material was observed together with the formation of some smaller Mw weight material after enzyme hydrolysis it was annotated as "partial degradation". When none of the originally high Mw material of the polysaccharide tested remained, but only small Mw material was observed after incubation with enzyme extracts, it was annotated as "complete degradation" (Table 6.2).

High Performance Anion Exchange Chromatography (HPAEC) was performed as described in chapter 2. Oligosaccharides released from WAX were identified using a profile of WAX degraded by pure, well characterized endo-xylanase was used (Kormelink et al., 1993). Enzyme digests were diluted 10 times prior to analysis.

6.3. Results and discussion

6.3.1. Preliminary screening of enzymes from extracts of PII_{end} and PIII-16

A first screening was performed for two enzyme extracts, PII_{end} and PIII-16, on nine different cell wall polysaccharides. Degradation of these polysaccharides was evaluated by comparing the high performance size exclusion chromatography (HPSEC) elution profiles of polysaccharides before and after enzyme hydrolysis. The results are summarized in Table 6.2.

Wheat arabinoxylan (WAX) was completely degraded by extracts from PII_{end} and partially by PIII-16. This was expected as xylan is, next to cellulose, the main carbohydrate source in compost used for mushroom growth (chapter 2). In addition, cellulose (CMC) was partially degraded by PII_{end}-extract, whereas not at all by PIII-16-extract. For the pectic substrates, almost no degradation was observed, apart from branched arabinan by PIII-16-extract. Further, partial degradation was observed by both extracts from PII_{end} and PIII-16 on model galactomannan and xyloglucan. Recently it was shown that the *A. bisporus* genomes encodes enzymes targeting all plant polysaccharides (chapter 5), likely due to the fact that in nature *A. bisporus* grows on a variety of (plant-based) substrates. This correlates with the observed enzyme activities towards xylan, arabinan, galactan, mannan and xyloglucan in PIII-16 (Table 6.2). However, no activity towards homogalacturonan, RGI or CMC was observed. It is likely that gene expression and production of specific polysaccharide degrading enzymes in *A. bisporus* respond to the presence of different polysaccharide-derived inducers in the growth medium, as was described for the expression of galactosidase genes in *Aspergillus niger* (de Vries et al., 1999). The absence of pectin in compost would therefore abolish the induction of pectinase activity. The absence of CMC activity could be explained by the suggested link between production of cellulases by *A. bisporus* and fruiting body development (Claydon et al., 1988), which is not yet initiated in PIII-16.

Table 6.2. First screening of extracellular enzyme activities from the compost in PII_{end} and PIII-16 analyzed by HPSEC after 24 h incubation.

Substrate	PII_{end}	PIII-16
Low methylated homo-galacturonan (DM30)	-	-
High methylated homo-galacturonan (DM70)	-	-
Sugar beet branched arabinan	-	+
Rhamnogalacturonan I (RGI)	-	-
Potato galactan	+	+
Wheat arabinoxylan (WAX)	++	+
Galactomannan	+	+
Carboxymethyl cellulose (CMC)	+	-
Xyloglucan	+	+

(- no degradation, + partial degradation, ++ complete degradation)

6.3.2. Detailed analysis of enzymes from various compost extracts

For the second, more extensive screening, only CMC and xylan were selected as substrates based on the carbohydrate composition of compost previously described (chapter 2), which showed that cellulose and xylan are the two most abundant polysaccharides present in the compost used in this study. In addition to wheat arabinoxylan (WAX), also birchwood xylan, substituted with glucuronic acid, was used as a substrate. Further, since high activity was detected towards mannan, galactomannan was added as a substrate, even though it is not a main component of compost. Screening of enzyme activities was assayed on PNP-substrates for exo-activities, as well as by PAHBAH for overall hydrolytic activity on the selected polysaccharides (see Materials and Methods). The results are discussed below for xylan, cellulose and mannan degrading enzyme activities.

The protein content of the extracts used was determined to be for PII_{end}, PIII-16, Filling, Pinning, 1st flush and 2nd flush, 6.2, 5.9, 7.1, 7.6, 9.4 and 7.8 mg protein g^{-1} dry matter of compost, respectively. The pH of the extracts was measured as 6.5, 6.2, 6.1, 6.3, 5.7 and 6.7, for PII_{end}, PIII-16, Filling, Pinning, 1st flush and 2nd flush, respectively. Further, conductivity of the extracts was measured and was found to be in a range between 3.3 and 4.7 mS cm^{-1}. In addition, the protein profiles analyzed by SDS-PAGE gel electrophoresis were similar up to Pinning and showed very intense protein bands around 75 kDa. In contrast, the extract after 1st flush, did not show the intense band around 75 kDa, but multiple less intense bands in the range of 50-75 kDa (Supplementary fig. 1).

6.3.2.1. Xylan degrading activities

The overall hydrolytic enzyme activity tested after incubation for 4h showed similar trends for WAX and birchwood xylan (Fig. 6.1). Enzyme activity on both substrates was lowest in Filling and increased over time with the highest activity in 1st flush. After that the activity tended to decline for about 20% in 2nd flush. The same trend was observed after incubation for 24 h for both substrates. After 24 h, the concentration of reducing ends released from birchwood xylan was between 51 and 547 µg Xyl ml^{-1}, and from WAX between 142 and 833 µg Xyl ml^{-1} (from 2 mg ml^{-1} of substrate). Similar to the 4h incubation, the 1st flush-extract showed the highest activity on the two xylans tested; approximately 13% (birchwood xylan) and 17% (WAX) of the reducing ends were released from the substrate. This xylan degrading activity correlated well with the lower xylan content analyzed in the compost (Table 6.3), and indicated the presence of good hydrolytic activities towards xylan in the compost during fruiting body formation. The activity of β-xylosidase and α-arabinofuranosidase was also assayed on PNP-monosaccharides after a 4 h incubation (Fig. 6.1). The β-xylosidase activity was lowest (0.1 nmol pnp ml^{-1} min^{-1}) in the extract of Filling and it increased during

fruiting body formation having the highest activity in compost collected after the first flush of mushrooms (1st flush, 0.9 nmol pnp ml^{-1} min^{-1}). During the period of fruiting body formation (Filling to 2nd flush) the α-arabinofuranosidase activity was at least 4 times higher and more constant than the β-xylosidase activity (4.1 to 4.3 nmol pnp ml^{-1} min^{-1}). Results of both overall and exo-enzyme activity demonstrated that the xylan degrading machinery of *A. bisporus* was active in the compost throughout the cultivation.

Analysis of the xylans, before and after hydrolysis with the enzyme extracts, with HPSEC and HPAEC gave more detailed information about the mode-of-action of the various enzyme extracts used (Fig. 6.2 and Fig. 6.3). The enzyme extract obtained from compost without mycelium (PII_{end}) completely degraded polymeric WAX into monomeric and small oligomeric compounds with a molecular mass <12 kDa (Fig. 6.2). Over the period of mushroom growth (PIII-16 to 2nd flush) two trends could be observed: partial and complete degradation of WAX. Partial degradation was obtained with extracts from PIII-16 and Filling, and the relatively large amounts of high molecular weight xylan that remained after the incubation indicated limited activity of endo-xylanases. This could be due to a change in the source of hydrolytic enzymes, from bacterial origin (PII_{end}) to *A. bisporus* (PIII-16 and Filling). Complete degradation of high molecular weight WAX was obtained with enzyme extracts from the period of fruiting body formation (Pinning to 2nd flush) indicating an increase in the activity of xylanases. Again, maximum activity was achieved by the 1st flush-extract. These results were in line with the results obtained by the reducing end assay (PAHBAH) after 24 h.

Table 6.3. Total carbohydrate content, carbohydrate molar composition and xylan degree of substitution of compost samples obtained at PII, PIII-16, after filling, after pinning, after 1st and after 2nd flush.

	PII[a]	PIII-16[a]	Filling[b]	Pinning[b]	1st flush[b]	2nd flush[b]
Total carbohydrates (% w/w)[c]	26±1	27±1	22±1	23±1	18±1	16±1
Carbohydrate composition (molar %)[d]						
Arabinose	5	5	6	4	6	6
Xylose	34	35	34	27	25	24
Mannose	1	3	5	6	9	8
Galactose	2	2	3	2	3	3
Rhamnose	2	2	1	1	2	1
Glucose	52	50	44	53	47	49
Uronic acid	4	5	6	6	8	8
Degree of substitution						
Ara/Xyl[e]	15	14	17	16	23	26
GlcA/Xyl[e]	11	13	18	21	31	35

[a]PII: Phase II compost; PIII-16 is Phase III compost after 16 days of mycelium growth (adapted from chapter 2).
[b]Filling: compost after filling of compost beds at the farm, Pinning: after mushroom pins appear, 1st flush: after first flush of mushroom was collected, 2nd flush: spent compost, after 2nd flush of mushrooms was collected.
[c]Weight percentage is based on dry matter of composting phases.
[d]As anhydro-sugars; STDEV < 0.5 for all samples.
[e]Ratio mol substituents/100mol of xylosyl residues; abbreviations: Ara, arabinosyl; GlcA, glucuronic acid.

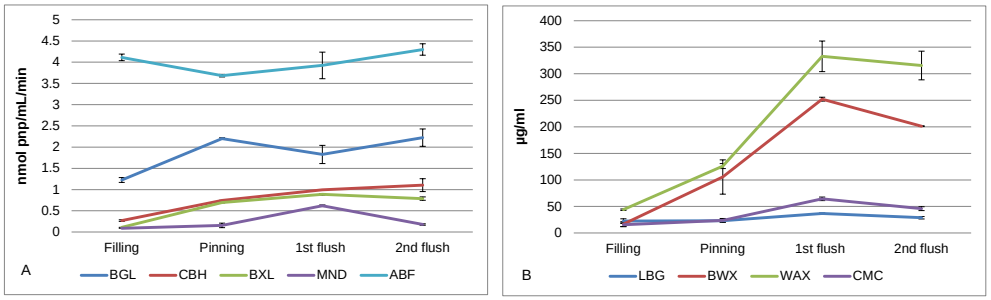


Figure 6.1. Relative enzyme activities in water extracts (4h incubation) of various stages of mushroom production. A: exo-activities (in nmol PNP per ml per min). B: total hydrolysis based on reducing sugar determination (in µg ml^{-1}). BGL= β-glucosidase, CBH= cellobiohydrolase, BXL= β-xylosidase, ABF= α-arabinofuranosidase, MND= β-mannosidase. BWX= birchwood xylan, WAX= wheat arabinoxylan, CMS= carboxymethyl cellulose, LBG= locus bean gum.

Degradation of 4-*O*-methyl-glucuronic acid substituted xylan (birchwood xylan, Fig. 6.2) followed the same trend as was observed for WAX hydrolysis, although less pronounced. However, for birchwood xylan, high molecular weight material was still present after hydrolysis with the 1st flush-extract, suggesting that the extracted enzymes were not able to completely degrade xylan substituted with 4-*O*-methyl-glucuronic acid.

The HPAEC profiles of WAX degraded by extracellular enzymes from the compost (24 h) are presented in Fig. 6.3. WAX hydrolysed with the PII_{end}-extract resulted in relatively high amounts of xylose, indicating β-xylosidase next to endo-xylanase activity. Nevertheless, some small amounts of xylobiose and xylotriose remained. Further, relatively low amounts of arabinose were released (Supplementary table 1). Together with the presence of xylo-oligomers substituted with arabinose this indicated a poor arabinofuranosidase activity in PII_{end}. The HPAEC chromatogram of WAX incubated with extracts from PIII-16 and Filling (Fig. 6.3) showed only low amounts of arabinose, no xylose and very small amounts of xylan-oligomers. This was also in line with the results obtained by HPSEC (Fig. 6.2), which showed mainly large molecular weight xylan remaining.

These results matched with previous research (chapter 2) suggesting that mainly partial degradation of xylan occurs during 16 days of mycelium growth. This increases the solubility of xylan and results in a relatively easy to degrade carbohydrate source available during fruiting body formation. Indeed, WAX degraded with extracts from Pinning, 1^{st} flush and 2^{nd} flush showed much more xylose and arabinose as well as xylobiose and xylotriose than from PII_{end}, PIII-16 and Filling (Fig. 6.3), being the result from a nearly complete WAX hydrolysis as was also detected on HPSEC (Fig. 6.2).

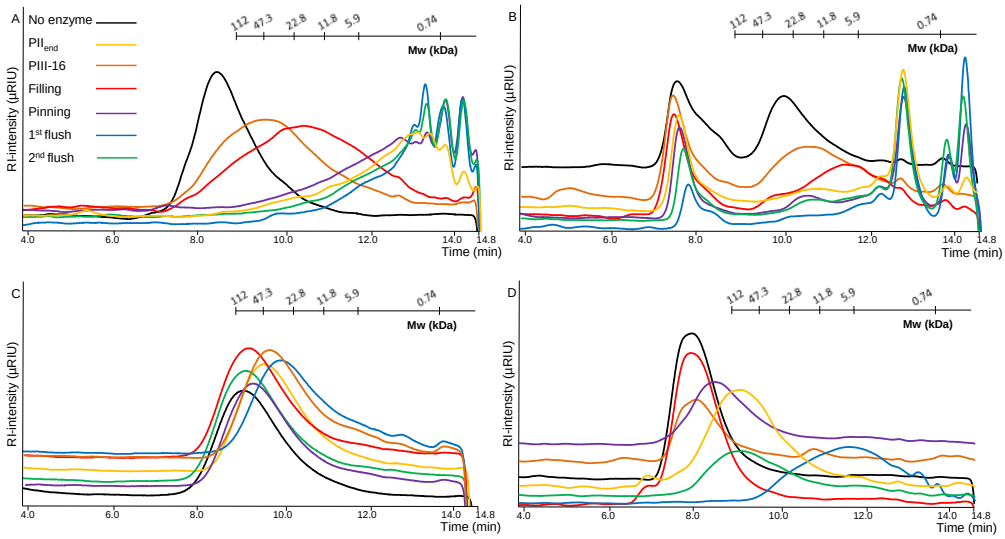


Figure 6.2. High performance size exclusion profiles of wheat arabinoxylan (A), birchwood xylan (B), carboxymethyl cellulose (C) and galactomannan (D), after degradation with enzyme extracts obtained from compost of different stages of mushroom growth (24 h incubation).

Although during the period of fruiting body formation an increase in β-xylosidase activity is observed, relatively high amounts of xylo-oligomers remained. However, no single substituted xylo-oligomers were detected. This suggested the presence of an efficient α-arabinofuranosidase in the compost in these stages that is able to release arabinosyl units present as single substituents on the xylan backbone. This xylanase and arabinofuranosidase activity correlates well with the upregulated xylanase- and arabinofuranosidase-encoding genes during fruiting body formation of *A. bisporus*, as published previously (chapter 5).

In contrast, double substituted xylo-oligomers remained (Fig. 6.4). Apparently, arabinofuranosidase activity able to release arabinosyl units from doubly substituted xylooligomers was lacking. Two of such specific arabinofuranosidases have been characterized, both belonging to the GH43 family (CAZy, www.cazy.org), one produced by *Humicola insolens* (Sørensen et al., 2006) and one by *Bifidobacterium adolescentus* (Broek et al., 2005). For *A. bisporus* (chapter 5) putative genes encoding enzymes belonging to the family GH43 were found to be up regulated in compost (1st flush), but, it is shown in a phylogeny tree that these GH43 genes are not likely to be active towards the doubly substituted xylan parts.

Fig. 6.3 shows the hydrolysis products of birchwood xylan substituted with 4-*O*-methyl-glucuronic acid analyzed with HPAEC. No free 4-*O*-methyl-glucuronic acid was detected in any of the digests. Apparently, none of the enzyme extracts

was able to release 4-*O*-methyl-glucuronic acid from xylan or xylan oligomers indicating that the α-glucuronidase activity was either not present or excreted in non-detectable amounts. Nevertheless, *A. bisporus* contains two genes predicted to encode α-glucuronidases activity, but these were both *not* upregulated in mycelium grown compost as analyzed in the same batch of samples as used in this research (chapter 4). Also, α-glucuronidase activity was previously detected after growing *A. bisporus* mycelium on beechwood xylan. This suggests that the commercial growth conditions for *A. bisporus* result in a different physiology than was previously described for this species on beechwood xylan (Puls et al., 1987).

In conclusion, substantial xylanase activity was observed (based on degradation of WAX observed on HPSEC chromatograms (Fig. 6.2) in enzyme extracts from compost grown with mycelium and fruiting bodies. For complete saccharification of xylan, more efficient β-xylosidase activity, α-glucuronidase and arabinofuranosidase activity able to release arabinosyl units from doubly substituted xylooligomers activity is needed than was produced in these samples.

6.3.2.2. Cellulose degrading activities

Overall, cellulase activity tested on CMC after 4h confirmed the activity of cellulases in the compost throughout the fruiting body formation as previously reported by Wood and Goodenough (1977) (Fig. 6.1). About 16 µg Glc ml^{-1} reducing ends were released from CMC with the Filling-extract and the activity increased slightly in compost obtained after mushroom pins appeared (Pinning, 23 µg Glc ml^{-1}). Highest cellulase activity was observed in the compost-extract obtained after the first flush of mushrooms (1st flush, 64µg Glc ml^{-1}) and the activity decreased slightly after the second flush was collected (2nd flush, 46 µg Glc ml^{-1}).

Cellobiohydrolase activity (4 h incubation) of was lowest in the extract from Filling (0.3 nmol pnp ml^{-1} min^{-1}) and increased throughout the fruiting body formation having the highest activity (1.1 nmol pnp ml^{-1} min^{-1}) in 2nd flush (Fig. 6.1). In the extract from Filling, β-glucosidase activity was the lowest (1.2 nmol pnp ml^{-1} min^{-1}), while after pinning (Pinning), activity was much higher (2.2 nmol pnp ml^{-1} min^{-1}) and it decreased again in 1st flush (1.8 nmol pnp ml^{-1} min^{-1}). In the 2nd flush extract, β-glucosidase activity was the same as after pinning.

HPSEC-chromatograms (24 h incubation) showed that all compost extracts were able to partially degrade CMC. In comparison with xylan hydrolysis CMC hydrolysis was much lower, as was observed by the rather large amount of high molecular weight (Mw) material remaining after the incubation. This could be due to the difference in structure of CMC and the cellulose present in compost. After 16 days of mycelium growth (PIII-16) cellulase activity decreased compared to PII_{end}. From filling (Filling) to the first flush (1st flush) cellulase activity increased and after the second flush (2nd flush) a slight decrease in activity was detected (Fig. 6.2).

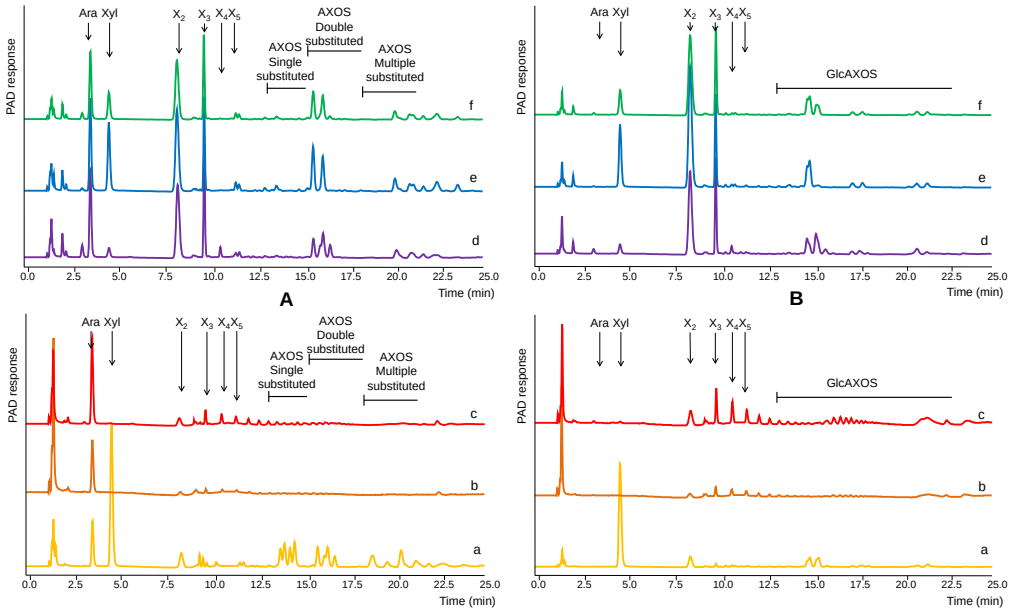


Figure 6.3. HPAEC elution profile of WAX (A) and birchwood xylan (B) incubated (24h) with a: PII_{end}, b: PIII-16, c: Filling, d: Pinning, e: 1st flush and f: 2nd flush extracellular enzymes. AXOS= oligomers substituted with arabinose. GlcAXOS= oligomers substituted with 4-*O*-methyl-glucuronic acid.

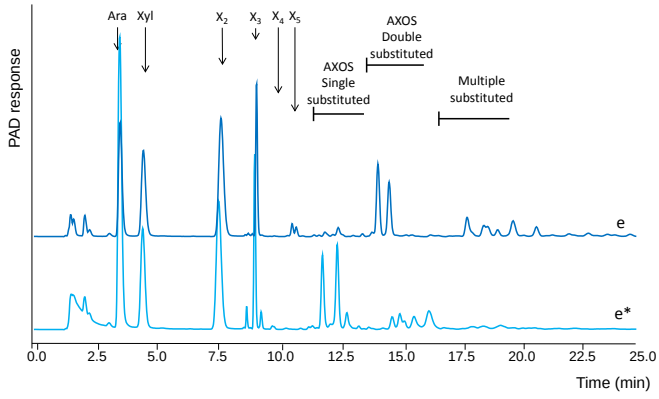


Figure 6.4. HPAEC elution profile of WAX incubated (24h) first with 1st flush (e) and then sequentially with pure GH43 AXH-d3 arabinofuranosidase (e*) (Van Laere et al., 1999). AXOS= oligomers substituted with arabinose.

Overall, the trend of CMC hydrolysis by enzyme extracts from the fruiting body phases observed with HPSEC was in line with the trend observed for the same samples analyzed by PAHBAH assay after 4 (see Fig. 6.1) and 24h (not shown). Previously, it was reported that the onset of fruiting body formation is

accompanied by an increase in cellulase activity, in particular endo-glucanase activity (Wood and Goodenough, 1977). Also, during fruiting body formation the rate of cellulose and hemicellulose degradation was analyzed to be higher compared to mycelium grown compost (Gerrits, 1968). The results of our study confirm this, since we obtained the highest cellulase and hemicellulase activity during fruiting body formation. Our data also fit with the previous transcriptomics study of *A. bisporus*, which demonstrated that cellulase encoding genes were upregulated in the compost (chapter 5). Xylanase activity was higher than cellulase activity in compost, which confirms a previous study (Savoie, 1998), and suggested that xylan is an important growth substrate for *A. bisporus*, especially during fruiting body formation. As xylan consists mainly of the pentoses xylose and arabinose, this correlates well with the upregulation of most of the pentose catabolic pathway genes, required for conversion of these pentoses, in mycelium grown compost (chapter 5).

6.3.2.3. Mannan degrading activities

Fig. 6.2 shows HPSEC profiles of galactomannan degraded by enzyme extracts (24h incubation). For both the exo-activity and the overall enzyme activity (Fig. 6.1) the same trend was observed. In extracts from Filling and Pinning very low mannanase activity was detected. Nevertheless, in 1st flush a remarkable increase in both endo- and exo-activity was observed. In 2nd flush the overall mannanase activity decreased (Fig. 6.2) and exo-activity decreased for 70% (Fig. 6.1). This trend was the same as observed for cellulases and xylanases.

6.3.3. Compost composition and carbohydrate structures

Table 6.4 shows that for all samples the main polysaccharides present consisted of xylosyl (34-24 mol%) and glucosyl (52-49 mol%) residues, as was previously described for wheat straw based compost (chapter 2, Iiyama et al., 1994).

PII compost contained about 26% (w/w DM) of carbohydrates and during the 16 days of mycelium growth, the total carbohydrate content, including both plant and fungal biomass carbohydrates, remained the same (chapter 2). This indicated that only low amounts of carbohydrates were metabolized from the compost carbohydrates by the microbial population in PIII-16, which was also concluded from the rather low enzyme activities present in this phase, being mainly endo-activity. Further, as previously mentioned, the partial degradation of compost carbohydrates in PIII-16, delivered soluble carbohydrates for *A. bisporus* needed during fruiting. For the growth of *A. bisporus*, complete saccharification of compost carbohydrates during early stages of mycelium growth is not favored, because increasing levels of monosaccharides will promote microbial growth over subsequent *A. bisporus* growth.

After compost is spent (2nd flush) total carbohydrate content decreased to about 16%. Previously, it was reported that during mycelium growth and fruiting of *A. bisporus* carbohydrate content decreases to 11% (Iiyama et al., 1994). This difference may be due to variations in the origin of raw materials (Australia versus Europe) and the composting process (e.g. mycelium was grown for 4 weeks versus 16 days, before addition of casing layer) (Iiyama et al., 1994). Comparing the molar carbohydrate composition of the samples obtained during pinning up to spend compost (2nd flush), a gradual decrease in xylosyl and an increase in arabinosyl and uronic acids, most likely glucuronic acid residues, was observed (Table 6.3). The decrease in xylosyl residues correlated with the maximum activity of xylan degrading enzymes observed. Overall, the amount of total xylan substituents in the compost increased two times from Pinning to 2nd flush (Table 6.3). This suggests the inability of *A. bisporus* to degrade xylan substituted with glucuronic acid or with two arabinosyl residues per xylosyl residues, leading to an accumulation of these recalcitrant xylan structures. Therefore, it can be speculated that in spent compost (2nd flush) mainly xylan substituted with glucuronic acid or two arabinosyl residues per xylosyl residue is present.

6.4. Conclusions

In all enzyme extracts from compost during growth of *A. bisporus* the activity of endo-xylanase and β-xylosidase activities was present and to a lesser extent of glucanase and β-glucosidase. Maximal enzymatic activity was observed after the first flush of mushrooms. In contrast, α-glucuronidase activity and arabinofuranosidase activity able to remove arabinosyl residues from doubly substituted xylose residues was absent in these extracts. As a result, the degree of substitution of xylan with both arabinosyl and glucuronic acid significantly increased during fruiting body formation of *A. bisporus*. Exploring the options to apply these missing xylan de-branching enzymes directly to the compost or developing *A. bisporus* strains that include these activities in their enzyme-machinery may improve commercial mushroom production.

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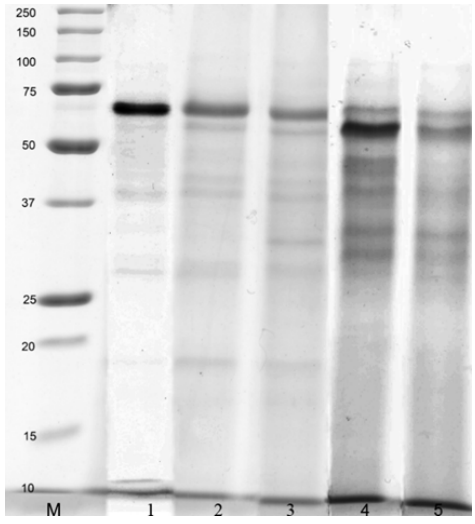
Supplementary data

Supplementary table 1. Released mono and oligosaccharides from wheat arabinoxylan and birchwood xylan after 24h digestion with extracellular enzymes from different compost phases, analyzed by HPAEC.

Concentration (µg released/mg of substrate)							
	Xyl	Ara	X_2	X_3	X_4	GlcA	Total
WAX							
PII_{end}	102	23	19	0	0	n.a	144
PIII-16	0	12	2	0	0	n.a	14
Filling	0	30	7	7	0	n.a	44
Pinning	8	57	124	106	12	n.a	307
1st flush	61	60	141	92	0	n.a	354
2nd flush	33	58	131	107	0	n.a	328
Birchwood xylan							
PII_{end}	202	n.a	36	6	0	n.f.	245
PIII-16	0	n.a	4	5	0	n.f.	8
Filling	1	n.a	12	16	17	n.f.	47
Pinning	9	n.a	153	100	11	n.f.	273
1st flush	69	n.a	255	108	0	n.f.	432
2nd flush	30	n.a	187	117	0	n.f.	335

Xyl: xylose, Ara: arabinose, GlcA: glucuronic acid, X_2: xylobiose, X_3: xylotriose, X_4: xylotetraose. n.f.: not found, n.a.: not applicable.



Supplementary figure 1. Protein profiles on SDS page. M: marker, Extracts of 1: PIII-16, 2: Filling, 3: Pinning, 4: 1st flush, 5: 2nd flush.

Chapter 7

General discussion

7.1. Motivation and short summary of the research

When it comes to obtaining economically feasible yields of good quality *Agaricus bisporus* mushrooms, the compost recipe and composting process are well established. Some flexibility in composting conditions, such as changing the duration of a phases or adding additives (personal communication, CNC-C4C, Milsbeek, The Netherlands), is common practice to maintain compost quality with the existing variations in substrates used. Finding the optimal composting conditions is, so far, mainly based on empirical experience. It is unclear how the composting conditions influence changes on a molecular level and on the final yield. Therefore, up to now conceptualized processing with the aim to improve the mushroom yield is not possible. The project of which this thesis was part of, entitled "How mushrooms feed on sugars", was set up to obtain detailed information on the content and composition of structural remaining components in the compost throughout each of the composting phases and *A. bisporus* growth, as well as on the ability of *A. bisporus* to degrade carbohydrate structures in the compost. The latter was done in collaboration with the Fungal Biodiversity Centre (CBS-KNAW) and Utrecht University, both in the Netherlands, where the *A. bisporus* genome and the level of expression of genes encoding e.g. carbohydrate degrading enzymes were investigated. The main aim of this thesis was to identify the biochemical changes in different compost samples and relate how these changes affect the degradation of carbohydrates needed for mushroom growth. In addition, it was aimed at elucidating the structures hindering the complete saccharification of carbohydrates, analyzing the lignin structures and identifying the enzyme activities present or lacking in the compost. This knowledge is expected to lead to possibilities for improving the composting process and utilization of carbohydrates.

7.2. Phase I - the effect of composting on the plant cell wall structures present

The first main finding of the research was that an almost complete removal of ester bound substituents from the xylan backbone took place during Phase I of composting (chapter 2). At the beginning of the process, the raw materials, which are wheat straw, straw bedded horse manure, chicken manure and gypsum, are mixed (BM) while water is added (chapter 1). This BM enters Phase I of composting, which is characterized by high temperatures (80ºC) and alkaline conditions (pH 8). These conditions are the result of the exothermic growth and activity of microbiota originating from the horse and chicken manure producing high amounts of ammonia (Gerrits, 1988). As said, after the first phase of composting (PI) esterified substituents were present in very low amounts (less than 0.1% w/w (based on dry matter) (chapter 2).

The release of esterified substituents, like ferulic, coumaric and acetic acid, from the xylans present in the plant cell walls, is expected to “open” the cell wall network by uncoupling lignin-carbohydrate and carbohydrate-carbohydrate complexes. Specifically, ferulic acids participate in xylan-xylan and carbohydrate-lignin binding (Pan et al., 1998, Ralph, 2010). In addition, the removal of ester-bound acetic, ferulic and coumaric acids is expected to positively impact the hemicellulose saccharification (Biely, 2012; Dodd and Cann, 2009) in the subsequent phases.

Next to the removal of ester bound substituents, the softening of wheat straw has been previously reported to be the result of PI (Gerrits, 1988), however, this was not studied in detail. Softening is mainly studied in fruits as a consequence of ripening. Since composting is very different from ripening, no comparison could be made. Nevertheless, cell wall degrading enzymes were reported to be involved in the softening of fruits (Yamaki and Kakiuchi, 1979).

Extracellular enzyme activities were tested in the PII compost, which is the substrate for mycelium growth, and in the compost collected throughout fruiting body formation in order to investigate the carbohydrate degrading machinery of *A. bisporus* (chapter 6). To complete the overview of carbohydrate degrading enzymes in the compost, also enzyme activities extracted from PI compost were tested. A thermo-tolerant endo-xylanase activity was detected in the PI-compost enzyme extract (Textbox 7.1). Overall, Phase I is expected to be important for increasing the accessibility and degradability of carbohydrates for microbial or fungal carbohydrate degrading enzymes in the later composting and mushroom growth stages.

7.3. Phase II - the effect of composting on the plant cell wall structures present

The second main finding of our work was the extensive metabolization of around 50% of both xylan and cellulose during Phase II of composting. Moreover, xylan was degraded in a uniform manner (chapter 3).

During Phase II of composting, the ammonia produced in Phase I has to be removed, because ammonia is known to inhibit growth of *A. bisporus* (Gerrits, 1988). Therefore, the compost is “conditioned” at around 50ºC for 5 days to obtain Phase II compost (PII). Compared to all phases studied, in Phase II the metabolization of carbohydrates was the highest (chapter 3).

The observed degradation of the carbohydrates is expected to be a result of the activity of mesophylic bacteria (Gerrits, 1988), which have been found to be present to on the surface of wheat straw fibers in PII-compost (Atkey and Wood, 1983). The decrease in xylan and cellulose contents is in line with both xylanase and cellulase activities observed in the enzyme-extract of PII compost (chapter 6).

The remaining xylan structures in PII were found to be similar to PI compost in terms of carbohydrate composition and degree of xylan substitution. This indicates that micro-organisms present in the compost were not hindered by specific cell wall structures of the metabolized fractions (chapter 2). The content of lignin remained constant in PII, and lignin structures analyzed by analytical Py-GC/MS were not altered in PII compost compared to PI (chapter 3).

Textbox 7.1.

In Phase I, thermo-tolerant microbiota enabled the ammonia formation. To study whether enzymes, in particular xylan degrading enzymes, were excreted by the microbiota present, a water extract of PI-compost was prepared. Enzymes present were tested for their activity on wheat arabinoxylan (medium viscosity, Megazyme, Ireland) (Fig. 7.2) (**35**ºC, at pH 5.5 using a buffer 50mM NaOAc, 24h). In addition, the enzyme extract from PI was tested on the same arabinoxylan at **75**ºC without pH/buffer adjustments (under compost conditions).

Based on the size exclusion profiles of the arabinoxylans, before and after incubation, it is concluded that endo-xylanase activity was present in PI compost, as was seen by the decrease in molecular weight of the original substrate (Fig. 7.1). Interestingly, the endo-xylanase activity was able to degrade arabinoxylan also at 75ºC, and even to a further extent at 75ºC than at 35ºC, both in 24h. These results strongly suggest the presence of a thermo-tolerant endo-xylanase in the PI enzyme extract. The extract was partially purified with size exclusion chromatography and anion exchange chromatography. Although not completely pure, endo-xylanase activity was associated with a molecular mass of ±35 kDa (SDS-PAGE), which is common for family GH10 endo-xylanases (Lombard et al., 2014).

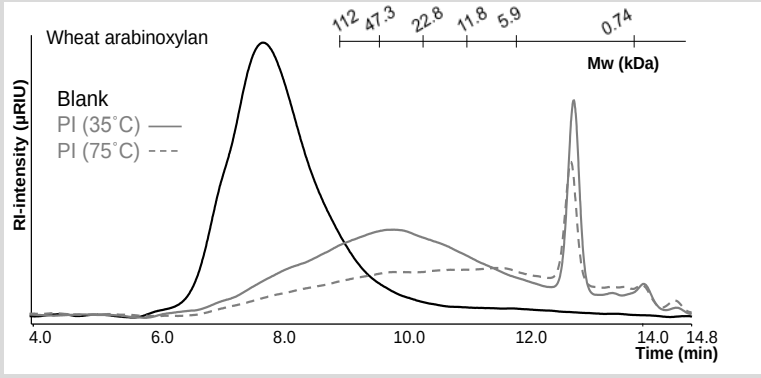


Figure 7.1. High performance size exclusion profiles of wheat arabinoxylan, before (blank) and after 24h hydrolysis with PI enzyme extract. For extraction of the enzymes and incubation conditions see chapter 6.

7.4. PIII - vegetative mycelium growth- changes in plant cell wall structures

Another main finding was that both xylan and lignin structures were altered to the largest extent during 16 days of mycelium growth in Phase III.

This finding is based on the observed changes in xylan properties and in lignin-structures. About 20% of the total remaining xylan was water soluble (chapter 2.) in PIII-16 compost, compared to 3% in PII, and PIII-16 alkali extracted xylan showed a decreased molecular weight (HPSEC) compared to PII (chapter 2). Albeit xylan was found to be degraded, the carbohydrate composition, and the degree of substitution of the xylans in PII and PIII-16 compost was rather similar (chapters 2, 3).

Also, less than 6% of xylan was metabolized during PIII. In contrast, 40% of lignin was metabolized. The remaining lignin showed a different fingerprint in PIII-16 compared to PI or PII, deducted from the Py-GC/MS pyrograms. In PIII-16, the ratio of syringyl-like and guaiacyl-like units (S:G ratio) increased and the ratios of vinyl-substituted guaiacol to guaiacol and vinyl-syringol to syringol decreased compared to PI and PII (chapter 3). Moreover, for PI the distribution of S and G compounds of PIwus was found to be similar to that of the total sample (PI). On the contrary, in PIII-16wus a relatively lower S:G ratio was found compared to the total sample of PIII-16 indicating that part of lignin in PIII-16 compost became more water soluble.

Previously, studies on lignin degrading fungi (white rot fungi) have shown that the extracellular lignin system involved in lignin degradation is induced by starvation (ten Have and Teunissen, 2001). Therefore, it can be speculated that due to the metabolization of 50% of carbohydrates in PII, only more difficult to degrade carbohydrates remain in the compost at the end of PII. But, this is not expected to be related to the degree of substitution. The increased carbohydrate recalcitrance in Phase II was also observed when comparing the degradability of PI and PII compost by a commercial enzyme cocktail. After enzymatic treatment, more monosaccharides were released from PI compost compared to PII compost (personal communication Thibaut Mouthier, Wageningen University). As a result, the lignin degrading machinery of *A. bisporus* is induced in Phase III, resulting in lignin degradation as shown in chapter 3. In grasses (wheat straw), the presence of lignin in the plant cell wall is often negatively related to the digestibility of cell wall carbohydrates in grasses (Grabber et al., 2004). Therefore, it is proposed that the observed lignin changes during mycelium growth have an important impact in improving the digestibility of carbohydrates in the later stages of mushroom growth.

The observed modification in lignin structures during mycelium growth indicated a targeted degradation. Laccase and manganese-peroxidase found to be active in the compost during mycelium growth (Bonnen et al., 1994), however, were reported to be non-specific (Wong, 2009). It could be proposed that removal

of vinyl-substituents from vinyl-guaiacol and vinyl-syringol occur as a result of depolymerisation of the lignin matrix. As lignin is analyzed as its monolignol units by Py-GC/MS, the mode of degradation of lignin remained unclear.

7.5. Fruiting body formation of *A. bisporus* - Changes in plant cell wall structures (Filling, Pinning, 1st flush and 2nd flush)

Concerning the fruiting body formation phases of *A. bisporus* (Filling, Pining, 1st flush, and 2nd flush) the main finding was the accumulation of arabinosyl- and (4-*O*-mehtyl-)glucuronic acid xylan substituents in the compost.

Next to the accumulation of xylan substituents, genome-expression analysis showed that *A. bisporus* did not have the ability to degrade this substituted xylan in the compost during the mushroom growth conditions applied. Specifically, *A. bisporus* genes encoding the enzyme activity required to degrade the accumulated structures, namely to remove (4-*O*-methyl-)glucuronic acid or arabinose from xylosyl residues having two arabinosyl residues, were not present in the genome or not expressed (chapters 4). This was confirmed by the lack of the above named enzymatic activities in water extract from compost during fruiting body formation (chapters 4).These results show the inability of *A. bisporus* to completely degrade xylan structures in the compost.

The compositional changes in xylan structure (chapter 4) do not reflect changes in absolute amounts of carbohydrates in the compost during fruiting body formation. Therefore, the decrease in dry matter amounts of compost from Filling to 2nd flush (reported by Baars and Sonnenberg, 2014, http://edepot.wur.nl/299892) for the same composting process was used in order to quantify the degradation of carbohydrates. To that end, by using the carbohydrate content values (% w/w) obtained in chapter 4, the mass balance was calculated of total carbohydrates, xylan and cellulose as shown in Table 7.1. A consumption of 40% of total carbohydrates was calculated based from Filling to 2nd flush. In more detail, xylan was found to be consumed to a larger extent (50%) compared to glucan (35%) (Table 7.1). Since glucan can be present in multiple forms, although mainly as cellulose and as glucan in mycelium cell walls, it could not be distinguished how much cellulose was consumed and how much glucan was built in mycelial cell walls.

The main focus of this PhD thesis research was to analyze the structures of the remaining carbohydrates in the compost. Nevertheless, mushrooms harvested from 1st and 2nd flush were also analyzed for their composition (Textbox 7.2). The carbohydrate contents should be interpreted with caution as chitin was shown to be incompletely degraded by the acid hydrolysis applied in the carbohydrate analysis procedure. Therefore, the carbohydrate contents are probably underestimated.

Table 7.1. Dry matter, total carbohydrates, xylan and glucan content (% w/w based on dry matter (DM)) and mass balance from Filling to after 2nd flush.

	Content (w/w% DM)		Mass balance (kg/m^2)			Loss
	Filling[a]	2nd flush[a]	Filling[b]	2nd flush[b]	Loss	relative to Filling phase
Dry matter	37	39	30.9	25.9	5	16%
Total carbohydrates	21.9	15.7	6.8	4.1	2.7	40%
Glucan	10.4	8.1	3.2	2.1	1.1	35%
Xylan	9.3	5.5	2.9	1.4	1.5	50%

[a]values based on chapter 4.
[b]based on report, Baars and Sonnenberg, 2014 (http://edepot.wur.nl/299892).

Textbox 7.2.

The compositional analysis of fruiting bodies of *A. bisporus* collected after 1st flush and after 2nd flush is shown in Table 7.2. Mushrooms harvested from the 1st flush contained 39% w/w of carbohydrates, and 22% w/w of protein, while mushrooms after 2nd flush consisted of 33% w/w of carbohydrates and 30% w/w of protein. Whether the difference in carbohydrate content of mushrooms of 1st flush and 2nd flush is related to the carbohydrate availability, transport of carbohydrates to fruiting bodies or other factors, remaines unclear.

Table 7.2. Dry matter (DM) content (% w/w); protein, carbohydrate and ash content (% w/w based on dry matter (DM), all STDEV<1) of fruiting bodies collected at 1st flush and at 2nd flush. *nitrogen to protein conversion factor 4.38 (Braaksma and Schaap, 1996). For methods see chapter 4.

	DM (w/w%)	**Content (w/w% DM)**		
		Protein	**Carbohydrates[a]**	**Ash**
Mushrooms 1st flush	8.2	21.7	38.9	11.1
Mushrooms 2nd flush	10.0	29.7	33.3	7.4

[a]Including sugar alcohols, mannitol, sorbitol, trehalose

7.6. Challenges in studying plant cell wall structures in compost

Apart from wheat straw cell walls, compost contains microbiota from chicken and horse manure and in the later stages *A. bisporus* mycelium. Hence, the analysis of the carbohydrates, xylan and cellulose, and the analysis of lignin in this complex system provided a number of challenges, which are discussed below.

7.6.1. Xylan

The main difficulty in studying xylan structures in the compost in detail (molecular weight and distribution of substituents) was the solubility. In PII 50% of both xylan and cellulose were metabolized although no differences were observed in the composition of the remaining xylan in PII compared to PI. Therefore, xylan was sequentially extracted with alkali. Again, compositional analysis after acid hydrolysis showed similar compositions of 1M KOHss and 4M KOHss xylan from PI, PII and PIII-16 (chapter 2). To study the molecular weight (Mw) of xylan extract on HPSEC, only water soluble xylan could be accessed which was found to be only 20-40% of alkali extracted xylan (no further data given). Nevertheless, differences in Mw were observed indicating that during mycelium growth the xylans present in the water un-extractable part of compost became partially degraded as seen from smaller Mw material present in both 1M KOHss and 4M KOHss from PIII-16 compared to PII (chapter 2).

As not all xylan extracted by alkali was water soluble, even after degradation with pure endoxylanase, the water insoluble xylans were investigated by microscopy. This was investigated by analysis of the 1M KOHss and 4M KOHss fraction of PI by confocal microscopy. In general, structural differences were observed for 1M KOHss and 4M KOHss xylan fraction. Shown in Fig. 7.2, for 1M KOH insoluble xylan sample, self- aggregation was observed, whereas 4M KOHss soluble xylan sample showed strong auto-fluorescence between 400-500 nm. These findings indicate that 4M KOHss insoluble xylan may not be completely purified from lignin that is known to exhibit auto-fluorescence in this range (Tobimatsu et al., 2013). Overall, microscopy could provide a useful method to study both carbohydrate structures as well as structural complexes, like carbohydrate-lignin complexes by e.g. specific dyes. In the future, with this method, we can compare also the effect of composting on the insoluble xylan part.

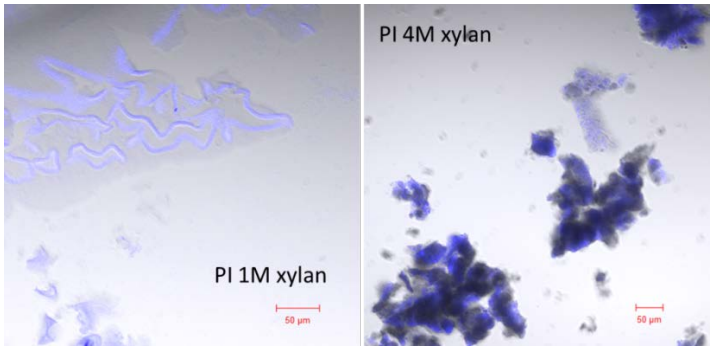


Figure 7.2. Confocal microscopy pictures of PI 1M and 4M KOHss extracted xylan (insoluble). [Kindly measured by Dr. Kinnari Shelat, The University of Queensland, Brisbane, Australia]

7.6.2. Cellulose and glucan

In the complex compost it was a major challenge to determine the origin of glucan. It turned out not to be possible, yet, to determine whether the glucan analyzed originated from plant cellulose, or from microbial and/or fungal glucan present in their cell walls. This distinction is needed to make a proper mass balance over e.g. the fruiting body stages, where due to mycelium growth, next to consumption of cellulose also accumulation of glucan occurs.

A. bisporus mycelium is composed of about 40% of glucan and 43% (w/w) of chitin (Michalenko et al., 1976). Chitin is a long chain of unbranched polysaccharides of N-acetylglucosamine residues linked through β-1,4 covalent bonds (Martínez et al., 2001). Commercially available chitin is not water soluble and not completely degradable by the sulphuric acid conditions applied in the used carbohydrate analysis (chapter 3). With the used carbohydrate analysis indeed almost no carbohydrates were detected from commercial chitin (data not shown). Till now, no solution for this is available. Therefore, it cannot be excluded that part of glucan analyzed in the compost during mycelium growth and fruiting body formation, at least in part, originates from mycelium cell wall.

After sequential extraction of composts from fruiting body formation (Filling, Pinning, 1st flush and 2nd flush) the recovery of dry matter of extracted fractions was ±90% (w/w) (chapter 4). This indicates that most of the dry matter was successfully recovered after extraction. In mycelium grown compost, however, the yield of glucan as sum of the various water un-extractable fractions was 140% (Table 7.3). It could be proposed that due to alkali treatment during sequential extraction the glucan became more susceptible to acid hydrolysis compared to the glucan in the WUS. As previously mentioned, the observed glucan "gain" is suspected to be, at least in part, the result from the presence of mycelium in the compost.

Table 7.3. Extraction yield of glucan after acid hydrolysis in the alkali fraction (1M KOHss and 4M KOHss) and residue (Res) compared to glucan analyzed in water unsoluble (WUS) fraction from the compost after 1st flush.

		Yield glucan (% w/w)
After 1st flush		
WUS		100.0
	1M KOHss	5.8±0.2
	4M KOHss	4.2±0.0
	Res	130±12.5
	gain	**40.2**±12.7

7.6.3. Lignin analysis and quantification by Pyrolysis GC/MS

Lignin is the second most abundant biopolymer on earth, after cellulose, presenting about 30% w/w of organic carbon (Boerjan et al., 2003). Therefore, while studying cell wall degradation, lignin should not be ignored. The main challenge in studying lignin structure is, similar to xylan and cellulose, the highly insoluble nature of lignin. Moreover, the many different lignin units and complex 3D structure of lignin, that is difficult to degrade cause difficulties in investigating lignin structure (Hatakka, 2005).

Lignin content is commonly analyzed gravimetrically as a residue after acid hydrolysis, so-called Klason lignin. However, in mycelium grown compost chitin was shown not be completely hydrolyzed by acid hydrolysis, which leads to overestimation of the lignin content in mycelium containing composts (chapter 4).

Also, extraction of lignin, which could aid in analysis of lignins' structure, is a challenge as lignin was shown to be only partially soluble in acid, organic solvents or alkaline solutions (Evstigneev, 2011, Iiyama et al., 1994, Wang et al., 2011). In addition, the solubility of lignin and its original chemical structure was shown to change after structural modification. Therefore, a single method for lignin extraction is not expected to be applicable for compost samples over different phases of composting and mushroom growth where lignin structures were shown to change (chapter 3).

Py-GC/MS can function as a useful tool to analyze lignin structures, also in compost, as no extraction of lignin is required. With Py-GC/MS, lignin is analyzed by its monolignol subunits (chapter 4). During the pyrolysis of plant biomass also carbohydrate pyrolysis products can be found. These are considerably more difficult to identify by MS because of their facile fragmentation to low molecular weight ions (Ralph and Hatfield, 1991). The Py-GC/MS as conducted in the research described in this thesis still has many options for improvement. Currently, pyrolysis of plant biomass is performed by pyrolyzing samples on a set temperature (single-shot, 500°C or 700°C) (chapter 3, del Río et al., 2012; Ralph and Hatfield, 1991). By this pyrolysis on one temperature, analyzed products originate both from carbohydrates as well as lignin. Nevertheless, several modifications of the existing method can be made. By profiling a sample with evolved gas chromatography (EGA, pyrolysis over a temperature range) an overall temperature pyrolysis profile might be obtained.

To this end, EGA was preliminary tested to analyze the structural profile of PI compost as well as alkali extracts and the remaining residue. Analysis of model substrates (wheat straw, wheat arabinoxylan, beechwood xylan, avicel cellulose and chitin) showed that different polysaccharides show different EGA-profiles over a pyrolysis temperature range (Fig. 7.3A).

Similarly, different xylan extracts and alkali insoluble residues from PI compost also showed different EGA-profiles (Fig. 7.3B). The exact meaning of these profiles is yet unknown. However, it can be proposed that by optimizing the pyrolysis temperature "gradient" insights into xylan-lignin and carbohydrate-carbohydrate complexes could be obtained.

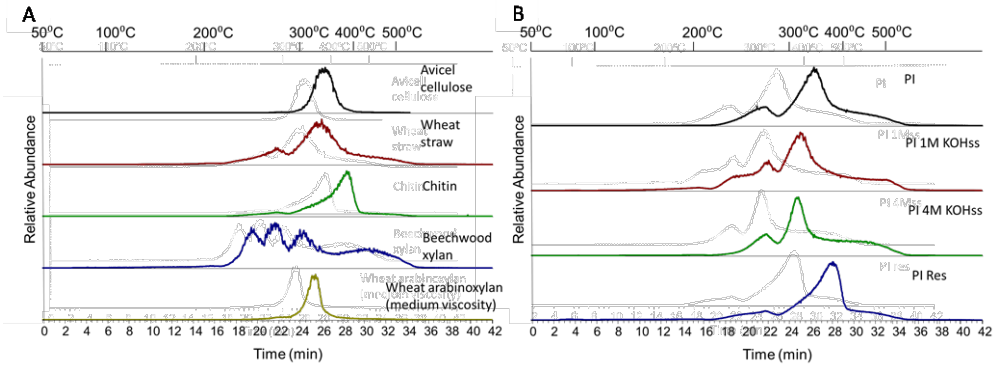


Figure 7.3. Evolved Gas Analysis pyrograms of model carbohydrate substrates and wheat straw (A) and compost at PI and corresponding alkali fractions (1M and 4M) and alkali insoluble residue (B).

An advantage of further understanding EGA would be that a specific pyrolysis temperature for single shot pyrolysis can be chosen depending on the targeted structure or complex of interest. Moreover, with double shot pyrolysis, samples can be sequentially pyrolyzed, e.g. first at 300°C, and the remaining material can be pyrolyzed again at 500/700°C and with use of a cold-trap, two GC/MS profiles can be obtained. The obvious advantages of double shot pyrolysis would be the pyrolysis of carbohydrates and lignin separately, which would enable identifying components of origin. Due to time limitations, this was not developed as part of this PhD thesis research.

Although, detailed information on lignin structures can be obtained by single-shot pyrolysis, analyzing lignin as its monolignol subunits does not provide information on inter-unit linkages in the original or modified lignin structure. In order to obtain this information, 2D-NMR method could be applied to understand the modifications of lignin in complex matrix. With this method, the information on the ratio of lignin subunits as well as main inter-unit linkages can be obtained (Heikkinen et al., 2003). However, only soluble lignin fragments can be analyzed with 2D-NMR and the challenges involved in lignin solubility are explained above.

7.7. Proposed model of conversion of wheat straw during composting and mushroom growth

A schematic overview of changes in wheat straw cell walls during composting and growth of *A. bisporus* is proposed in Fig. 7.4. In this scheme changes reported in this thesis combined with structural changes observed by electron microscopy as reported by Atkey and Wood (1983).

In wheat straw (Fig. 7.4A) primary cell walls are mainly built from cellulose fibers (20-30% w/w) and glucuronoarabinoxylan (xylan) (20-40% w/w). The secondary cell walls make up to 50% (w/w) of stem cell walls and consist of cellulose (35-45% w/w), xylan (40-50% w/w/) and lignin (20% w/w) (chapter 1).

By the alkali conditions in PI, ester linkages are expected to be broken as was seen by the loss of ester-bound substituents (chapter 2). This, in turn, is proposed to cleave, in part, carbohydrate-carbohydrate and carbohydrate-lignin complexes and “open up” the cell wall Fig. 7.4B.

We reported that in PII 50% of carbohydrates are metabolized (chapter 2, Table 7.4). In addition, electron microscopy of wheat straw during composting for mushroom production reported (Atkey and Wood, 1983) that during PII, bacteria are present on the surface of wheat straw cell walls as well as within the cells. Also, separation along middle lamella has been observed, which is expected to additionally reduce the rigidity of the connected cells (Atkey and Wood, 1983). Both the separation of the middle lamella and the carbohydrate metabolization is taken into account in Fig. 7.4D.

After the mycelium was fully grown through the compost (PIII-16) xylan was found to become more water soluble and partially degraded next to metabolization of 40% of lignin and modifications of remaining lignin structures (chapter 2, 3). In the endoxylanase digests (Kormelink et al., 1993) of 1M KOHss and 4M KOHss of PIII-16, carbohydrates were 100% water soluble. Therefore, after quantification of linear xylo-oligomers it was concluded that in 1M KOHss xylan ±40% was linear and only 10% of 4M KOHss xylan was linear (chapter 2). As more linear xylo-oligomers were released from 1M KOHss than from 4M KOHss while DS were the same. Moreover, knowing that endoxylanase used is hindered by xylan substituents, it is proposed that xylan substituents could be distributed in a more block wise manner in 1M KOHss compared to 4M KOHss. Finally, less bacteria were found to be present on the wheat straw cell wall surface compared to PII. Mycelium was thinly spread over the surface of the straw, but was not shown within the cell walls (Atkey and Wood, 1983) (Fig. 7.4D).

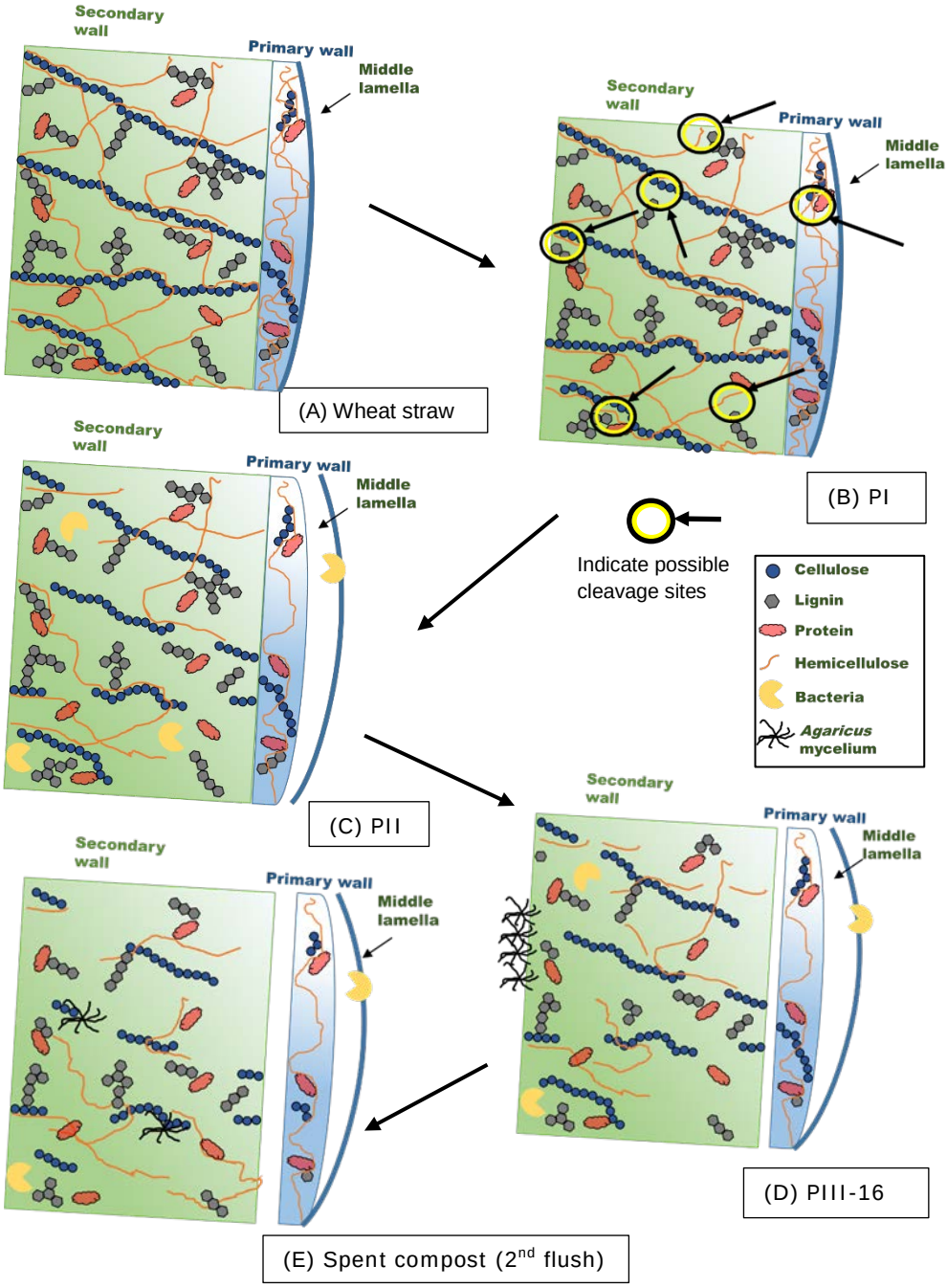


Figure 7.4. Proposed cell wall model of wheat straw (adapted from Achyuthan et al. (2010)) and modifications in PI, PII, PIII-16 and 2nd flush compost.

After the 2nd flush of mushrooms was collected, compost was considered spent and remaining cell wall structures are expected to be severely disrupted (Fig. 7.4E.). Overall 20% of carbohydrates are expected to be degraded in spent compost compared to compost at Filling (see Table 7.1 and Table 7.4). Remaining xylan in the spent compost was found to have a higher degree of substitution compared to PIII-16 compost indicating that mainly linear xylan was degraded during fruiting body formation (chapter 4). Secondary cell wall were found to be separated from the middle lamella indicating the loss of cell wall integrity. In addition, mycelium hyphae were found to penetrate into the cellulose fibrils (Atkey and Wood, 1983).

Next to structural changes in wheat straw cell wall, an overall quantitative degradation of xylan, glucan and lignin in the compost throughout composting and mushroom growth phases is presented in Table 7.4 based on 1000 kg of BM.

Both xylan and cellulose are mainly degraded during PII of composting (7.3) and during fruiting body formation (7.5) and based on Py-GC/MS quantification lignin was degraded during mycelium growth (PIII-16). A deviating value for lignin (Table 3.5) yield was obtained for PII (108% w/w), which is expected to be a result of the not fully optimized pyrolysis method, as discussed previously. Further, we mention in chapter 5 minor changes in lignin structure and S:G ration, which may correspond to the slightly decreased lignin amounts during fruiting body formation (2nd flush). These results should be viewed with caution as the DM content was obtained with a different experiment (Baars and Sonnenberg, 2014). Nevertheless, this might point out that lignin degradation continues slightly during fruiting body formation.

Table 7.4. Yield of xylan, glucan and lignin throughout composting and mushroom growth phases in the compost based on 1000 kg dry matter.

Sample	**DM (kg)**	**Yield (% w/w)**		
		Xylan*	Glucan	Lignin
BM	1000	100	100	100
PI	914	89	96	77
PII	787	41	48	108
PIII-16	708	35	43	52
2nd flush	595	23	22	44

*Xylan= sum of xylosyl, arabinosyl and uronyl; residues.
Based on chapters 3 and 4 and Baars and Sonnenberg, 2014 (http://edepot.wur.nl/299892).

7.8. Recommendations and future perspectives

7.8.1. Methodology

While studying carbohydrate structures in the compost, “top to bottom” analysis was shown to be a good approach. Indications of changes in carbohydrates were observed already at the level of total carbohydrate content. In large sample sets this can be used as a good “screening” before fractionation of the cell wall populations in a systems such as compost.

Detailed analysis of compost would benefit from developing specific extraction methods for extraction of *A. bisporus* mycelium from the compost. As mentioned, growing microbiota and *A. bisporus* mycelium, provides difficulties in distinguishing the origin of carbohydrate structures. As an example, *A. bisporus* carbohydrates will be partially analyzed as total carbohydrates in the compost during mushroom growth stages as well as partially extracted during sequential extraction of xylan. Also, chitin will be partially included in the Klason lignin residue.

For identification of changes of carbohydrate structures enzyme methods were preferred as they were found to provide most detailed structural information. Degradation of cell wall fractions with pure enzymes or characterized enzyme cocktails could provide both contents of certain xylan fragments as well as structural details such as the degree and pattern of distribution. Recommendations for improving the analysis of lignin were discussed previously, see 7.6.3.

7.8.2. Analysis *in situ*

A. bisporus mycelium was shown to produce different enzymes when grown on different substrates (chapter 6, Savoie, 1998). As an example, when grown on model beechwood xylan *A. bisporus* was shown to produce α-glucuronidase (Puls et al., 1987) whereas it did not in the compost (chapter 4). Moreover, laboratory grown mycelium is expected to behave differently than compost grown mycelium; e.g. different production of enzymes; different growth rate or production of fruiting bodies. The same could be expected from the microbiota. Therefore, chemical analysis should be performed under actual growth conditions while accessing the compost as system.

7.8.3. Improving the compost for higher mushroom yields

The main aim of this PhD project was to analyze the changes in carbohydrate structures during composting and mushroom growth in the wheat straw based compost in order to improve their utilization. To this end, results obtained in this thesis and discussed above were used to propose several approached to improve the degradation of carbohydrates in the compost.

7.8.3.1. Shorter PII by faster removal of ammonia

The main two purposes of Phase II are i) the removal of ammonia and ii) to make the compost specific for the growth of *A. bisporus* in order to prevent growth of spoilage organisms. During composting 50% of carbohydrates (chapter 3) are metabolized which can be considered as a rather negative side effect of the current PII-process as these carbohydrates are not available for mushroom growth.

For this reason, it is suggested that by applying a faster ammonia removal by faster air circulation and air filters, PII can be shortened. Consequently, more carbohydrates will be available for mycelium growth in Phase III. However, if the lignin degrading machinery is indeed triggered by starvation, as suggested earlier in this chapter, it should be determined if shortening of PII will not affect a good mycelium growth in PIII, and that still mushroom yields remain good. The observed lignin degradation in PIII-16 was analyzed at the end point. Therefore, it is still not known if the degradation of lignin is taking place over all 16 days of mycelium growth. This information could be obtained by the analysis of lignin degradation at several time points of PIII. Moreover, analysis of several time points of PIII could be helpful in understanding how a shorter PII phase, providing more carbohydrates present in PII compost, would affect the overall degradation of carbohydrates and lignin during PIII.

7.8.3.2. Shorter period of mycelium growth (PIII)

During 16 days of mycelium growth lignin was shown to be degraded, which is proposed to help the accessibility of carbohydrates in the compost during fruiting body formation (chapter 3). A faster mycelium growth could help to shorten the PIII. This could possibly achieved by 1) chopping of the straw 2) using an improved *A. bisporus* strain. The degradation of wheat straw was proposed to start at the chopped ends of wheat straw (Atkey and Wood, 1983). Therefore, it could be proposed that better chopping of the wheat straw and/or straw bedded horse manure into smaller pieces could provide more access points for mushroom mycelium. Furthermore, a faster degradation of lignin and carbohydrates could be achieved by an adapted *A. bisporus* strain, either by breeding or GMO, with better expression of genes involved in degradation of these structures.

7.8.3.3. Addition of enzymes at Filling

After 16 days compost is fully grown with mycelium and is considered mature. Upon addition of the casing layer on top of the PIII-16 compost, to induce mushroom growth, water is sprayed over the compost filled beds. At this point, enzymes to improve carbohydrate degradation could be added.

Addition of enzymes, in particular, enzymes removing the remaining xylan substituents, namely α-glucuronidases and GH43 arabinofuranosidase (AXHd3), may increase the carbohydrate degradation during fruiting. Whether increased

carbohydrate degradation will lead to higher yields of mushrooms or to shorter time between flushes is evidently unknown.

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Summary

The white button mushroom, *Agaricus bisporus*, is commercially grown for the production of edible fruiting bodies. This cultivation occurs on wheat straw based compost, but not all of this substrate is consumed by the fungus. The aim of this thesis is to elucidate the carbohydrate structure in the compost throughout composting and all growth phases of mushroom growth. Moreover, the availability and degradability of carbohydrates is studied under industrial growth conditions in order to help improve their utilization during growth of *A. bisporus.* In addition, lignin structures in the compost are studied as well as the activity of water extracted carbohydrases from compost of various phases.

In **chapter 1** an overview of information provided in literature is given. *A. bisporus* is known to degrade both xylan and cellulose during its growth in the compost. However, the research present in literature focusses mainly on the composition of compost constituents rather than taking into account their quantities or detailed molecular structures. Further, it has been proposed that *A. bisporus* is able to degrade lignin.

In this thesis, first, the carbohydrates present in the industrial wheat straw based compost from the first three phases of composting were studied. Hereto, representative samples from Phase I and Phase II of composting were analyzed as well as compost during mycelium growth (Phase III). The composition of these samples was studied as well as the structures of the xylan present were characterized (**chapter 2**). At the beginning of the composting process 34% (w/w) of the dry matter of the compost was composed of carbohydrates, which decreased to 27% (w/w) after 16 days of mycelium growth. The carbohydrate composition analysis showed that in the compost of the first three phases mainly cellulose and lowly substituted xylan were present. The type and degree of substituents of xylan was the same in all phases studied. During mycelium growth, however, while the xylan structures remained rather similar, the water solubility of xylan increased from 11% at the beginning of Phase III to 22% (w/w) at the end of Phase III and the remaining xylan was partially degraded.

To be able to quantify the changes in carbohydrates and lignin during composting and mycelium growth, a mass balance experiment was conducted in which compost samples were obtained from the same timeline (same industrial tunnel). The exact amounts of dry matter were followed over the first three phases (**chapter 3**). Characterization of the carbohydrate contents in these samples revealed that 50% of carbohydrates present in the original material were metabolized during Phase II of composting in an uniform manner, while lignin structures remained unaltered. During the mycelium growth in Phase III, less carbohydrates were used, seen from a decrease with 5-6% (w/w) of total carbohydrates present in the compost at the end of Phase III compared to at the beginning of this phase whereas 40% of lignin was metabolized. Moreover, the

ratio of syringyl (S) to guaiacyl (G) lignin-units increased from 0.5 to 0.7 during mycelium growth and fewer decorations on the phenolic skeleton of both S and G units remained. These modifications in lignin structures during mycelium growth were analyzed by using analytical pyrolysis-GC/MS.

In **chapter 4**, xylan from compost samples during fruiting body formation of *A. bisporus* was analyzed for its composition and degree of substitution. Based on the decrease in total carbohydrate content in compost from Filling to 2nd flush (30% w/w based on dry matter (DM)), both xylan (40% w/w) and glucan (23% w/w), it is indicated that carbohydrates were metabolized during the fruiting of *A. bisporus* in the compost. The degree of substitution (DS) of the alkali extracted xylan fractions increased considerably during fruiting. The accumulation of xylan substituents was observed for arabinosyl residues, which were substituted on the xylan backbone as one or two arabinosyl residues per xylosyl residue. Also, glucuronic acid substituents accumulated in the alkali extracted xylan fractions. In the 1M and 4M KOHss of spent compost, the DS was 33 and 49 (out of 100 xylosyl residues), respectively, compared to Phase I compost where the DS was 19 and 17 (out of 100 xylosyl residues) in 1M and 4M KOHss, respectively. To understand this accumulation of xylan substituents during fruiting, in addition, the genetic ability of *A. bisporus* to degrade such accumulated structures was studied. On the one hand, *A. bisporus'* genes putatively encoding α-glucuronidase activity were not expressed in mycelium present in the analyzed compost. On the other hand, *A. bisporus'* genes encoding arabinofuranosidase activity able to remove arabinose from xylosyl residues having two arabinosyl substituents as present in xylans, were not even present. The latter was based on the performed maximum likelihood tree.

As carbohydrates were found to be degraded during fruiting body formation, the expression of genes of *A. bisporus,* which putatively are involved in carbon metabolism, were identified and their expression was investigated in the compost during the formation of the first flush of mushrooms (**chapter 5**). It was shown that in mycelium the genes involved in both the hexose and the pentose catabolic pathway were upregulated, however, in fruiting bodies mainly the hexose catabolic pathway was upregulated. Moreover, the expression of genes encoding for plant polysaccharide modifying enzymes in the *A. bisporus* genome was correlated to the soluble carbohydrates and the composition of mycelium grown compost, casing layer and fruiting bodies. It was found that that only hexoses or their conversion products were transported from the vegetative mycelium to the fruiting body, while pentoses likely provided energy for growth and maintenance of the vegetative mycelium. Overall, genes encoding for plant cell wall polysaccharide degrading enzymes were mainly expressed in compost-grown mycelium, whereas genes encoding fungal cell wall polysaccharide modifying enzymes were expressed in both fruiting bodies and vegetative mycelium. Nevertheless, different

gene sets for fungal cell wall polysaccharide modifying enzymes were expressed in fruiting bodies compared to in mycelium.

In **chapter 6**, extracellular enzymes were extracted from the compost (same compost samples as used in chapter 4) of all the stages of mushroom growth and characterized for their ability to degrade various carbohydrates. Overall, endo-xylanase, endo-glucanase, β-xylosidase and β-glucanase activities were determined in the compost extracts obtained during mushroom growth. The maximum enzyme activities were found after the 1st flush of mushrooms, for both xylanases and cellulases. However, activities needed to release arabinose from xylosyl residues having two arabinosyl residues as well as to release (4-*O*-methyl-) glucuronic acid from xylan were not detected. This correlated with the observed accumulation of arabinosyl and glucuronic acid substituents on the xylan backbone in the compost towards the end of the cultivation (chapter 4).

Chapter 7 provides an overview of the findings described in the previous chapters and proposes changes in the cell wall structures of wheat straw during composting and growth of *A. bisporus* in the compost. In addition, possible strategies to improve the utilization of carbohydrates in the compost are proposed.

In conclusion, xylan is for a major part degraded and metabolized during PII of composting (48% w/w), while most of the lignin is degraded and metabolized during mycelium growth in PIII (40% w/w). In the fruiting body formation phases another 12% (w/w) of the xylan was metabolized and 23% (w/w) of xylan remained present in the spent compost. Nevertheless, during fruiting, the remaining xylan became increasingly substituted with arabinosyl and glucuronic acid residues during fruiting, while only minor structural changes in lignin were observed.

Samenvatting

Witte champignons worden op industriële schaal geteeld. Het zijn de paddenstoelen van de schimmel *Agaricus bisporus* (champignon). Ze worden op stro gebaseerde compost gecultiveerd, waarvan niet alle componenten volledig worden benut door de schimmel. Het doel van het in dit proefschrift beschreven promotie onderzoek was de opheldering van de structuurveranderingen te bestuderen die de koolhydraten in de compost ondergaan tijdens de verschillende groeistadia van *A. bisporus*. Niet alleen zijn de moleculaire structuren van de koolhydraten geanalyseerd, maar ook de hoeveelheid overgebleven koolhydraten in de compost. Dit is gedaan om uiteindelijk meer inzicht te krijgen in hoe het verbruik van het substraat tijdens de groei van de champignon verbeterd kan worden. Lignine is een andere belangrijke component uit compost en daarom is ook aandacht besteed aan de structuur analyse van de in de compost aanwezige lignine. Tenslotte is de activiteit van de in water-extraheerbare carbohydrases afkomstig uit de compost in verschillende groeistadia onderzocht.

Hoofdstuk 1 geeft een overzicht van de literatuur. Het is bekend dat *A. bisporus* de uit de compost afkomstige xylanen en celluloses afbreekt tijdens de groeifase in de compost. In de literatuur wordt vooral aandacht besteed aan de globale samenstelling van de compost. De kwantificering van de afzonderlijke compost koolhydraten en lignine structuren (“units”) wordt nauwelijks besproken, al wordt wel gesuggereerd dat *A. bisporus* lignine kan afbreken. Ook ontbreekt een gedetailleerde opheldering van de koolhydraat en lignine structuren.

In de volgende hoofdstukken van dit proefschrift is allereerst bestudeerd welke koolhydraten aanwezig zijn in de eerste drie fases van het industriële composteringsproces, wat is gebaseerd op tarwestro. Representatieve compost monsters zijn geanalyseerd van de composteringsfases I en II en van de mycelium-groei fase (Fase III). De analyses resulteerden in een weergave van de samenstelling van de compost monsters, en van de aanwezige xylan-structuren (**hoofdstuk 2**). Aan het begin van het composteringsproces bestond 34% (w/w) van het drooggewicht van de compost uit koolhydraten. Dit was afgenomen tot 27% (w/w) in compost na 16 dagen mycelium groei. Door middel van suikersamenstellingsanalyse werd geanalyseerd dat de koolhydraten aanwezig in compost van Fase I, II en III voornamelijk aanwezig waren als cellulose en laag-gesubstitueerd xylaan. Het type substituent en verdeling ervan over de xylaan-ketens was gelijk voor alle bestudeerde compost van Fase I tot en met III. Echter, tijdens de mycelium groei in Fase III nam wel de wateroplosbaarheid van de xylanen toe. Namelijk, aan het begin van Fase III was slechts 11% van alle aanwezige xylanen in de compost water oplosbaar, terwijl aan het eind van Fase III dit 22% was. Ook werd gevonden dat de aanwezige overgebleven xylanen gedeeltelijk afgebroken waren.

Om de afname in koolhydraten en lignine tijdens compostering en myceliumgroei te kwantificeren, werd een massabalans bepaald. Voor dit experiment werden compost-samples gevolgd tijdens compostering en mycelium groei in een industrieel tunnel-proces. De exacte hoeveelheden drooggewicht werden gemeten na iedere Fase (**hoofdstuk 3**). Analyse van de concentraties koolhydraten in de compost toonde aan dat 50% van de koolhydraten werd gemetaboliseerd tijdens Fase II. Er werd geen specifieke fractie met voorkeur gemetaboliseerd. Lignine structuren bleven in deze fase onveranderd. In Fase III, de myceliumgroei, werden minder koolhydraten verbruikt, namelijk aan het eind van Fase III werd 5-6% minder koolhydraten teruggevonden vergeleken met het begin van deze Fase, (w), terwijl 40% van de aanwezige lignine werd gemetaboliseerd. Daarbij nam de ratio van lignine syringyl- (S) en guaiacyl-units (G) toe van 0.5 naar 0.7. Verder werd een afname van decoraties op de fenolische ringstructuur van zowel de S- en G-units geconstateerd. Deze structuurmodificaties van lignine tijdens de myceliumgroei werden geanalyseerd met behulp van analytische pyrolyse-GC/MS.

In **hoofdstuk 4** is de samenstelling en xylaan-substitutie beschreven van compost uit de groeifase van de *A. bisporus* champignons. De hoeveelheid koolhydraten in de compost verminderde met 30% (van 22% w/w naar 16% w/w op basis van droge stof) van het vullen van de compost tot aan de tweede vlucht champignons; zowel voor xylanen (vermindering van 40%) als voor glucanen (vermindering van 23%). Deze afname geeft aan dat compost-koolhydraten werden gemetaboliseerd tijdens vruchtlichaamvorming van *A. bisporus*. De substitutiegraad van de met loog geëxtraheerde xylaan-fracties werd opvallend hoger tijdens de champignon-groei. Met name meer arabinosyl-residuen, één of twee arabinosyl-residuen per xylosyl-residue, als ook meer glucuronzuur-residuen werden aangetroffen aan de xylaan-ketens in de met KOH-geëxtraheerde xylaanfracties vanuit compost van het eind van de groeifase vergeleken met die van het begin. In het water-oplosabare deel van de 1M en 4M KOH extracten (1M en 4M KOHss) van compost aan het eind van de tweede vlucht, was de substitutiegraad 33 en 49 (per 100 xylosylresiduen). Ter vergelijking, in Fase I was de substitutiegraad 19 en 17 (per 100 xylosylresiduen) voor de 1M en 4M KOHss fracties. Om te kunnen begrijpen of deze accumulatie van xylaan substituenten tijdens vruchtlichaamvorming overeen kwam met de verwachte *A. bisporus* enzymactiviteiten werd het genoom van *A. bisporus* onderzocht op de eigenschappen om zulke structuren af te breken. In mycelium aanwezig in compost tijdens de vruchtlichaamgroei werden potentiële α-glucuronidase kandidaat genen niet tot expressie gebracht. Genen die coderen voor enzymen die arabinose afsplitsen van xylosyl-residuen met twee arabinosyl-substituenten werden niet

waargenomen in het genoom van *A. bisporus*. Het laatste werd gebaseerd op een verwantschapsstamboom.

Omdat tijdens de vruchtlichaamvorming koolhydraten in de compost werden afgebroken, werd de expressie van genen betrokken in koolhydraatmetabolisme, geïdentificeerd in *A. bisporus,* gemeten in mycelium in de compost en in champignons tijdens de eerste champignonvlucht (**Hoofdstuk 5**). Er werd geconcludeerd dat de expressie van myceliumgenen betrokken bij de hexose en pentose katabolisme was verhoogd, alhoewel in champignons vooral de expressie van hexose-metabolisme verhoogd was. Bijkomend, werd de expressie van genen voor enzymen die planten-polysachariden kunnen modificeren, aanwezig in het genoom van *A. bisporus* bepaald. Deze gen expressie werd vervolgens gecorreleerd aan de hoeveelheid oplosbare koolhydraten en aan de samenstelling van het mycelium-begroeide compost, de deklaag en de champignons. Hierbij werd gevonden dat alleen hexoses of omzettingsproducten daarvan werden getransporteerd vanuit het vegetatieve mycelium naar het vruchtlichaam. Pentoses zorgden, daarentegen, waarschijnlijk voor de energievoorziening voor groei en onderhoud van het vegetatieve mycelium. In het algemeen werden genen coderend voor enzymen die planten-celwand-polysachariden afbreken tot expressie gebracht in compost begroeid met mycelium. De genexpressie voor enzymen die plantencelwand polysachariden modificeren werd vooral aangetroffen in zowel de vruchtlichamen als in het vegetatieve mycelium. Echter,, in de vruchtlichamen werden andere sets van genen coderend voor enzymen die schimmelcelwand polysachariden modificeren tot expressie gebracht dan bij de expressie in het mycelium.

In **hoofdstuk 6** zijn de eigenschappen beschreven van extracellulaire enzymen geëxtraheerd uit de compost (dezelfde compostmonsters als in **hoofdstuk 4**) afkomstig uit alle stadia van de champignongroei. Deze enzym-extracten werden gescreend op hun potentiele koolhydraat-afbrekende eigenschappen, met name endo-xylanase, endo-glucanase, β-xylosidase en β-glucanase activiteit werd bepaald. De hoogste enzymactiviteit werd gevonden na de eerste champignonvlucht, dit voor zowel xylanases als cellulases. Enzymactiviteiten gelieerd aan het afsplitsen van (4-*O*-methyl-)glucuronzuur van xylaan werden niet gedetecteerd. Dit komt overeen met de gevonden accumulatie van arabinosyl- en glucuronzuur-substituenten van de xylaan-ketens in de compost op het einde van de champignon groei fase (**hoofdstuk 4**).

In **hoofdstuk 7** wordt een overzicht gegeven van de resultaten die zijn gevonden in dit onderzoek. Verder wordt schematisch voorgesteld welke structuurveranderingen in celwanden van tarwestro optreden tijdens compostering en tijdens de groei van *A. bisporus* in de compost. Ook worden verschillende

strategieën besproken die de benutting van koolhydraten in de compost zouden kunnen verbeteren.

Samenvattend, xylanen worden voor het grootste gedeelte afgebroken en gemetaboliseerd tijdens Fase II van de compostering (48% w/w), terwijl dit voor lignine het geval is tijdens de myceliumgroei in Fase III (40%). Tijdens de champignon groei werd nog eens 12% (w/w) van het xylaan in de compost gemetaboliseerd. Aan het eind van de champignon-groei bleef echter nog 23% (w/w) achter in de uiteindelijke compost. Arabinosyl- en glucuronzuur-substituenten accumuleerden aan het resterende xylaan, terwijl de ligninestructuur slechts in kleine mate werd gemodificeerd.

Acknowledgements

So, this adventure started 4 years ago with moving to a new country, new job, new people and culture and is now finishing with the good stuff. Remembering all the nice people and experiences. First of all, thanks for giving me the opportunity to bring some of Croatian spirit to the Laboratory of Food Chemistry as a first Croatian in the group! To my opinion, work atmosphere makes the work possible. So, if not for all of you, there would not be a thesis to write. Big thank you for that.

Mirjam, “thank you” seems like an understatement after four years together ☺. You were/are both my supervisor and mentor. I learned a lot from you and not to become too cheesy, one of the big things I learned is who I want to be when I grow up.

Harry, another paragraph of compliments for you to digest so bear with me. Thank you! For giving me this opportunity and for challenging me and teaching me how to be critical, fair and how get my point across as well as how to pick my battles. Moreover, thank you for caring about your group and every single person in it!

Henk, thanks for believing I could do this. Guess you were not far off as I am writing these last pages of my thesis. And you were right, working in the Zoological garden does help ☺.

Big thank you to Jolanda for all the help and friendship. Until things go badly (not in our laboratory, mind you) I guess we do not even realize how much work and energy goes into making things run smoothly. Well, a lot! Thank you!

My BSc and MSc thesis students, Wouter, Ci, Lia, Marleen, Roan and Thessa, thank you for your interest in my project. I learned a lot from all of you!

Rene, Margaret and Arjen, thank you for helping out with my research and bouncing ideas!

Maxime and Marijn, simply thank you for being there basically throughout my study period, exactly when it mattered the most!

My “new” office ladies: Annewieke, Alexandra and Suzanne. We are all fairly different, but we make it work. Thank you for cutting down on bday decorations for me! But mostly, thank you for being there during the last months and listening to whatever I had to get out of my system. Now it is your turn!

Annewieke ❀, I really appreciate you want to stand next to me at the end of this journey!

Red and Surender, thank you for being there from the beginning while we were finding our way around FCH and our research. Thank you for bringing a bit of India and Taiwan to my life.

Peter, thank you so very much! More often than not you had a direct link to the chaos in my head and were always there for support and help from the beginning of my PhD to standing next to me on my defense!

Tibo, a big HUG! We had some great times/talks and although you might not remember them, thank you for that!

Food Chemistry, lots of people were around the last four years and we had some good discussions and great times so thank you all for that!

Of course, next to colleagues there were a lot of people who made this whole experience awesome! Thank you to my Dutch friends, Merthe, Bart, Casper and all the musicians ☺! Rudy and Carla, thank you!

Niveska, čak i s tisuću kilometara razlike znam da te mogu nazvat kad treba i da si tu. Hvala ljubac! Žac, jedva čekam da vidim što nam život nosi i da to dijelimo skupa. Filipe, stari frendu, fala kaj si bil tu kad je trebala muška ruka. Anja, definitivno ne bi bilo ove teze da nema tebe. Hvala sto me potičeš/gurneš kad zatreba. Baš si „mama bear". Hvala! Nika i Maja, mice, mislim da sve znate... Hvala!

Mamac, ne bi ja bila tko jesam da nije bilo tebe i najvjerojatnije ne bi danas završavala doktorat. Hvala za svu podršku i potporu i posebno za pakete iz Hrvatske. Puno volim. Gogo, uvijek voljim! Fala kaj si uvijek bila tu za mene.

Yannick ♡

"There's so many different worlds
So many different suns
And we have just one world
But we live in different ones"

Pusa, *Edita*

About the author

Curriculum Vitae

Edita Jurak was born on 19th of September 1985 in Zagreb, Croatia. She obtained her master's degree in biology with a specialization in ecology at the Faculty of Science at University of Zagreb in 2010. Her MSc thesis was performed at Division of Botany with the focus on the physiology of carnivorous plant, *Drosera rodundifolia,* in relation to light stress. During her studies at the university, she was an active member of the Board of European Students of Technology (BEST) and worked as an educator in Zoological garden, Zagreb, Croatia. In February, 2011 she started her PhD at the Laboratory of Food Chemistry within the project "How mushrooms feed on sugars" of which the results are presented in this thesis. During her PhD study period Edita supervised three BSc and three MSc student thesis projects and was an active member of VLAG PhD council. Currently, Edita continues working at the Laboratory of Food Chemistry as a researcher.

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List of Publications

Patyshakuliyeva, A., **Jurak, E.**, Kohler, A., Baker, A., Battaglia, E., de Bruijn, W., Burton, K., Challen, M., Coutinho, P., Eastwood, D., Gruben, B., Makela, M., Martin, F., Nadal, M., van den Brink, J., Wiebenga, A., Zhou, M., Henrissat, B., Kabel, M.A., Gruppen, H., de Vries, R.P., 2013. Carbohydrate utilization and metabolism is highly differentiated in *Agaricus bisporus*. BMC Genomics, 14, 663.

Jurak, E., Kabel, M.A., Gruppen, H., 2014. Carbohydrate composition of compost during composting and mycelium growth of *Agaricus bisporus*. Carbohydr. Polym. 101, 281-288.

Tkalec, M., Doboš, M., Babić, M., **Jurak, E.** 2015. The acclimation of carnivorous round-leaved sundew (*Drosera rotundifolia* L.) to solar radiation. Acta Physiol. Plant. 37, 1-9.

Jurak, E., Punt, A.M., Arts, W., Kabel, M.A., Gruppen, H. Fate of carbohydrates and lignin during composting and mycelium growth of *Agaricus bisporus* on wheat straw based compost. Submitted.

Jurak, E., Patyshakuliyeva, A., Kapsokalyvas, D., Xing, L., van Zandvoort, M.A.M.J., de Vries., R.P., Gruppen, H., Kabel, M.A., Accumulation of recalcitrant xylan in mushroom-compost is due to a lack of xylan substituent removing enzyme activities of *Agaricus bisporus*. Submitted.

Jurak, E., Patyshakuliyeva, A., de Vries, R.P., Kabel, M.A., & Gruppen, H. Compost grown *Agaricus bisporus* lacks the ability to degrade and consume highly substituted xylan fragments. Submitted.

Patyshakuliyeva, A., Post, H., Zhou, M., **Jurak, E.**, Heck, A.J.R., Hildén, K.S., Kabel, M.A., Mäkelä, M. R., Altelaar, A.F.M., de Vries, R.P. Uncovering the abilities of *Agaricus bisporus* to degrade plant biomass throughout its life cycle. Submitted.

Overview of completed training activities

Discipline specific activities

7th International conference on mushroom biology, Arcachon, France, 2011
Food and biorefinery enzymology[1] (VLAG), Wageningen, The Netherlands, 2011
Advanced Food Analysis[1] (VLAG), Wageningen, The Netherlands, 2013
EPNOE international polysaccharide conference[2], Nice, France, 2013
Summer course glycosciences[1] (VLAG), Groningen, The Netherlands, 2012
Mini symposium lignin, Wageningen, The Netherlands, 2011
Plant cell wall biology conference[1], Cairns, Australia, 2014

General courses

PhD introduction week (VLAG), 2011
Project and time management (WGS), 2012
Techniques for writing and presenting a scientific paper (WGS), 2013
Effective behavior in your professional surroundings (WSG), 2013
Career perspectives (WSG), 2014

Additional activities

Preparation PhD research proposal, 2011
Food Chemistry PhD-trip[1,2], Singapore and Malaysia, 2012
Food Chemistry PhD-trip[1,2], Germany, Denmark, Finland and Sweden, 2014
Food Chemistry seminars, 2011-2015
Food Chemistry colloquia, 2011-2015
VLAG PhD council member, 2013-2015

[1]Poster presentation
[2]Oral presentation

The work described in this thesis was performed at the Laboratory of Food Chemistry, Wageningen University, The Netherlands. This research was financially supported by the Technology Foundation STW (11108), applied science division of NWO and the technology programme of the Ministry of Economic Affairs (The Netherlands) and by CNC Grondstoffen B.V., Walkro International B.V., Sylvan Inc. and DSM Food Specialities.

Financial support from Wageningen University for printing this thesis is gratefully acknowledged.

Cover design by Edita Jurak and Terry van Waesberghe

Printed by Gildeprint, Enschede, The Netherlands