UNRAVELING THE MYSTERY OF COMMERCIAL CULTIVATION OF *AGARICUS BISPORUS*: PLANT BIOMASS UTILIZATION AND ITS EFFECT ON MUSHROOM PRODUCTION

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Unraveling the mystery of commercial cultivation of *Agaricus bisporus*: plant biomass utilization and its effect on mushroom production

Het mysterie van de commerciële teelt van *Agaricus bisporus* ontrafelen: gebruik van plantaardige biomassa en het effect daarvan op de teelt van champignons

(met een samenvatting in het Nederlands)

Раскрытие тайны промышленного культивирования *Agaricus bisporus*: использование растительной биомассы и ее влияние на продуктивность шампиньонов

(с кратким изложением на русском языке)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. G.J. Van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen
op woensdag 16 september 2015 des middags te 12.45 uur

door

Aleksandrina Patyshakuliyeva
geboren op 16 oktober 1988 te Balkanabat, Turkmenistan

Promotor:
Prof. dr. ir. R. P. de Vries

"Science, my lad, is made up of mistakes, but they are mistakes which it is useful to make, because they lead little by little to the truth."

- Jules Verne, *A Journey to the Centre of the Earth*

"I have not failed. I've just found 10,000 ways that won't work."

- Thomas A. Edison

"From dead plant matter to nematodes to bacteria, never underestimate the cleverness of mushrooms to find new food!"
"I love a challenge and saving the Planet seems like a good one. "

- Paul Stamets

To my loving parents and my dear brother

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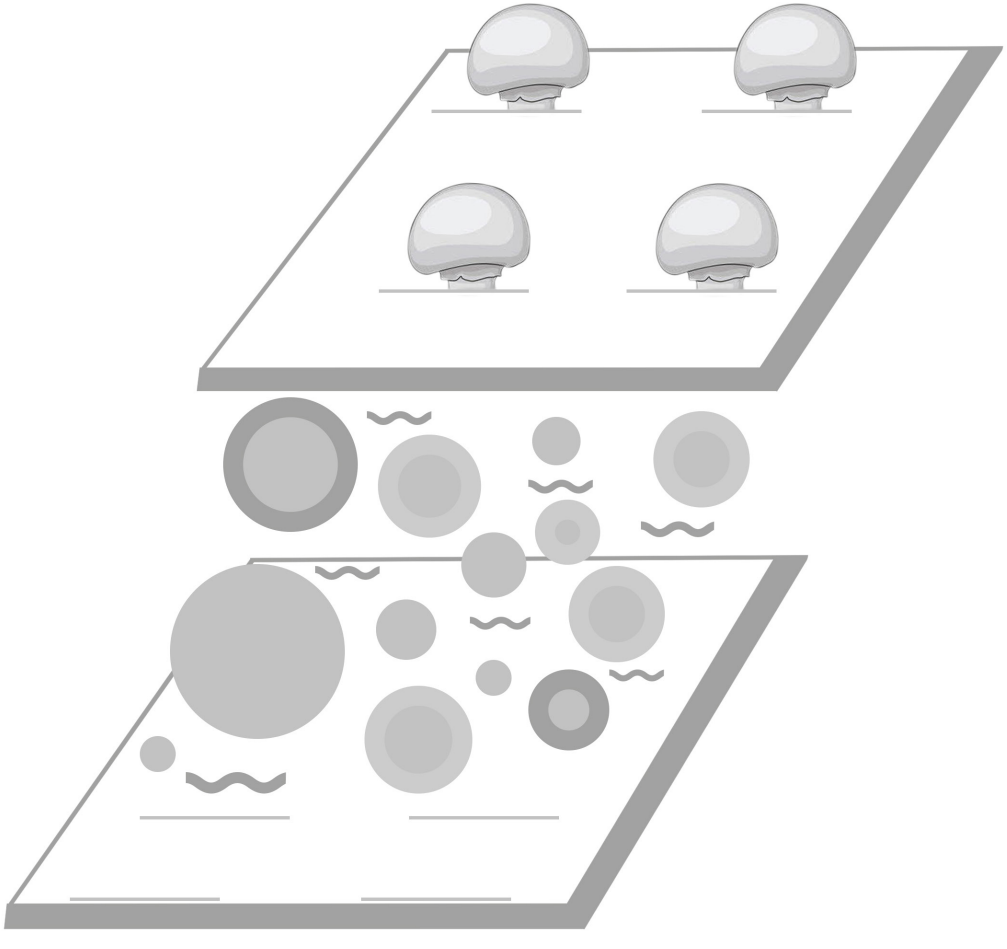

CHAPTER 1

General Introduction

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THE COMMON EDIBLE MUSHROOM *AGARICUS BISPORUS* is a basidiomycete that thrives on decaying plant material in the forests and grasslands of North America and Europe. It is adapted to forest litter and contributes to global carbon recycling, degrading cellulose, hemicellulose and lignin in plant biomass to oligomers and monomers. *A. bisporus* is also an edible mushroom that is widely cultivated and economically important. But the process of growing *A. bisporus* in compost and utilization of this substrate is poorly understood. Gaining a deeper insight into the carbon nutritive needs of this fungus, its abilities to degrade plant biomass and the underlying mechanisms of this phenomenon are the subject of this thesis.

Mushroom-forming fungi and their lifestyles

Agaricomycetes, commonly recognized as mushroom-forming fungi, is a class of the phylum Basidiomycota (Basidiomycetes) that contains around 21000 described species, which is one-fifth of all known Fungi (Kirk et al., 2008), with new taxa continually being described (Hibbett et al., 2011). This group of fungi occupies many niches in the environment and participates in decomposition of plant matter in terrestrial ecosystems, and plays a critical step in the carbon balance (Eastwood et al., 2011; Fernandez-Fueyo et al., 2012; Floudas et al., 2012; Morin et al., 2012).

Woody biomass, and above- and belowground plant litter form the major portion of terrestrial carbon and contain enormous reservoirs of lignocellulose. Wood / plant cell walls represent a complex and recalcitrant substrate and are mainly composed of cellulose, hemicellulose and lignin. With technology breakthroughs, fungal research has evolved and provided a much deeper insight into the mechanisms of fungi related to plant biomass degradation. Efficient wood degradation is typically related to Agaricomycetes (Figure 1), and the two basic forms are white rot fungi, which completely degrade all components of the plant cell wall, and brown rot fungi, which rapidly depolymerize cellulose, but leave lignin as a modified, mostly polymeric, residue (Hatakka & Hammel, 2011). White rot fungi can be distinguished based on the presence of ligninolytic peroxidases (absent in brown rot fungi), which are able to oxidase lignin, as well as the abundance of enzymes acting directly on crystalline cellulose (reduced in brown rot fungi). The first basidiomycete genomes, of the white rot fungus *Phanerochaete chrysosporium* and brown rot fungus *Postia placenta*, revealed a large set of genes and enzymes involved in lignocellulose degradation with respect to their approach of wood decay (Martinez et al., 2004; Martinez et al., 2009). Sequencing additional wood decayers suggested that brown rot fungi have evolved from the white rot lifestyle several times, while all white rot fungi are connected by the common ancestral white rot fungus (Floudas et al., 2012). According to the recent studies the existing model which divides white rot and brown rot fungi based on lignin-related peroxidases can be questioned as it does not take into account the entire diversity of wood decay mechanisms (Riley et al., 2014; Floudas et al., 2015).

Beyond the wood-decay Agaricomycetes, saprotrophic litter-inhabiting fungi (Figure 1) such as *Coprinopsis cinerea*, *Volvariella volvacea* and *Agaricus bisporus* contribute significantly to the global carbon cycle. Certain litter-decomposing fungi (*Gymnopus erythropus*, *Hypholoma fasciculare*) likely play a crucial role in the formation, transformation and degradation of humic substances, a major fraction of soil organic matter (Steffen et al., 2002; Grinhut et al., 2007; Šnajdr et al., 2010).

Another critical component of the diversity and function of terrestrial ecosystems is represented by ectomycorrhizal fungi such as *Laccaria bicolor* and *Piriformospora indica* forming beneficial mycorrhizal relationships with plant roots (Figure 1) (Martin et al., 2008; Sun et al., 2014). These fungi obtain carbon from plant hosts, but under some conditions, they can act as facultative saprotrophs by at least partially degrading soil organic matter (Baldrian, 2009; Cullings & Courty, 2009). This is supported by ectomycorrhizal genomes with an extreme reduction in the number of genes encoding enzymes involved in plant cell wall degradation (Martin et al., 2008; Vaario et al., 2012), while transcriptome and spectroscopic analyses suggest mechanisms by which soil organic extracts could be degraded by the ectomycorrhizal fungus *Paxillus involutus* using a trimmed brown rot mechanism (Rineau et al., 2012).

Overall, a considerable diversity of lifestyles is therefore found in Agaricomycetes. The progress in genomics during the past decade has significantly upgraded the list of potential genes, enzymes, pathways and hosts involved in ecological processes of terrestrial ecosystem.

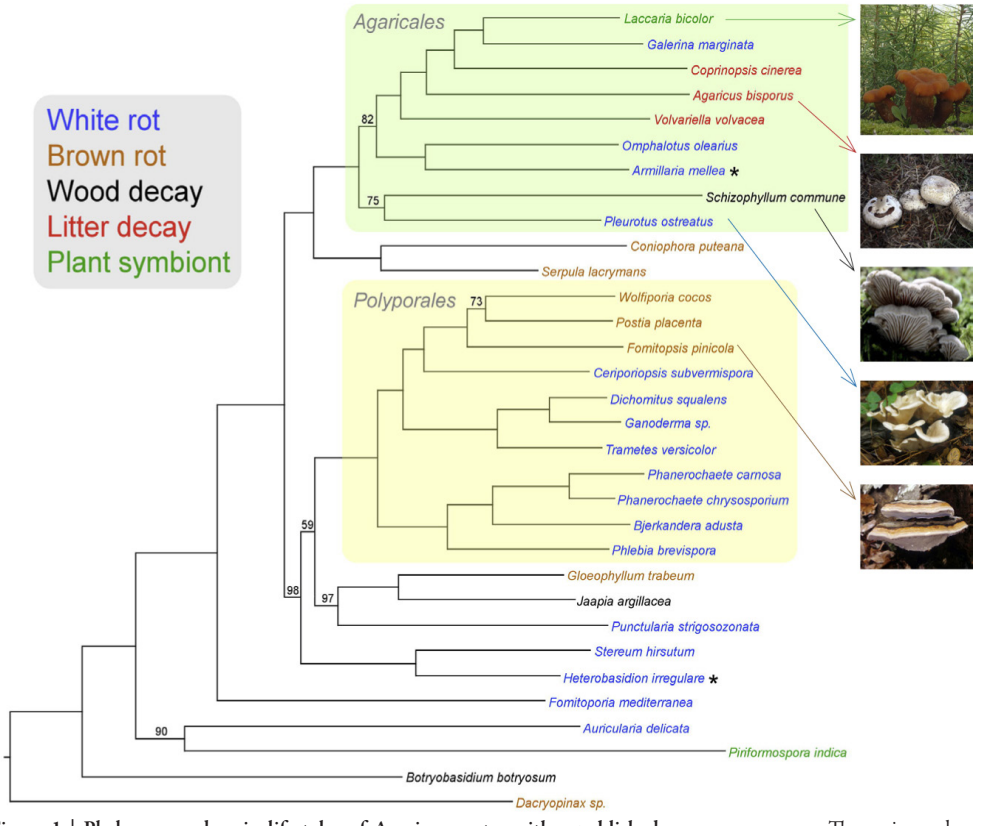

Figure 1 | Phylogeny and main lifestyles of Agaricomycetes with a published genome sequence. The major orders Agaricales and Polyporales are indicated. The majority of these species is wood decayers and can be further classified as either white rot fungi (which degrade all components of the plant cell wall) or brown rot fungi (which modify lignin, but do not break it down to a large extent). *Schizophyllum commune*, *Jaapia argillacea* and *Botryobasidium botryosum* are also wood decayers, but cannot be easily classified as either white or brown rot fungi. *Coprinopsis cinerea*, *Agaricus bisporus* and *Volvariella volvacea* are saprotrophs growing on non-woody substrates. The ectomycorrhizal fungus *Laccaria bicolor* and the endophyte *Piriformospora indica* both form interactions with plant roots. Species with an asterisk (*) are predominantly plant pathogens. Modified from (Ohm et al., 2014).

Agaricus bisporus

Beyond the importance of Agaricomycetes in forestry, many members of this class play significant role in agriculture, medicine and industrial bioconversion processes and bioremediation. In addition, some members of the Agaricomycetes, for instance *Amanita phalloides* and *Galerina autumnalis* are toxic and can cause life-threatening poisoning of humans (Benjamin, 1995). Nonetheless this class contains most of the known edible mushrooms, such as *A. bisporus* (champignon or white button mushroom), *Pleurotus ostreatus* (oyster mushroom), and *Lentinula edodes* (shiitake), and wild-collected ectomycorrhizal species, such as *Boletus edulis* (porcini), *Cantharellus cibarius* (chanterelle), and *Tricholoma matsutake* (matsutake).

Although edible mushrooms represent an important agricultural product worldwide, only a few of them (*Agaricus*, *Lentinula*, *Pleurotus*, *Auricularia*, *Volvariella*, *Flammulina*, *Tremella* and a few others) can be cultivated (Chang, 1999). *Auricularia auricula* (wood ear) was the first cultivated

mushroom (600 A.D.) followed by *Flammulina velutipes* (enokitake, 800 A.D.) and *L. edodes* (1000 A.D.) (Chang & Miles, 2004). The most significant progress in mushroom cultivation occurred in France in the seventeenth century when *A. bisporus* was grown in a composted substrate (Chang & Miles, 2004). Moving from an easy back yard crop in the early days of cultivation, to a large money maker, the mushroom industry began to grow widely. The white button mushroom, *A. bisporus*, is the world's most produced and consumed mushroom and has a high economic value (Horgen, 1992). The cultivation process is complex but highly efficient with up to 9 crops per year at modern mushroom farms, resulting in an annual crop value of about $4.7 billion worldwide (Sonnenberg et al., 2011). China, the United States and the Netherlands are the largest mushroom producers in the world and a large part of the produced mushrooms is exported.

Besides the enormous commercial importance, *A. bisporus* has a natural life style as a leaf litter degrading fungus with a wide geographical distribution from the boreal region of Alaska (Geml et al., 2008) to the equatorial climate of Congo and from coastal dunes to mountains (Kerrigan, 1995; Xu et al., 1997; Callac et al., 2002) (Table 1). *A. bisporus* plays an ecologically significant role participating in processes (including terrestrial carbon, nitrogen, phosphorus and potassium cycling) which occur in the forest floor by growing and degrading partially degraded leaf/needle litter (Kerrigan et al., 1998).

Table 1 | Natural habitats and geographical distribution of *A. bisporus*.

Habitat type	Vegetation type	Location
Temperate forest	*Cupressus* (Cypress)	California, USA; Mexico; continental Greece and Crete; Italy; France
	Picea (Spruce)	Alberta, Canada; Washington, USA
Mixed Montane forest	*Eucalyptus*	Israel, Morocco, Congo New Mexico
Boreal forest	*Picea* (Spruce) *Populus* (Poplar) *Betula* (Birch)	Alaska
Arid places	*Prosopis* (Mesquites) *Tamarix* (Salt cedar)	Sonoran Desert, California, USA
Coastal dunes	*Cupressus* (Cypress) *Poaceae* (True grasses)	France
Pastoral land use area (plant wastes and manure)		UK, France, Russia, Portugal, China, Australia, Argentina, Tasmania

Varieties of *A. bisporus*

The most typical features of Basidiomycetes are that they carry sexual spores externally on structures called basidia. Three varieties of *A. bisporus* can be distinguished by the average number of spores carried by their basidia and their life cycle (Savoie et al., 2013). *A. bisporus* is an amphithallic species with secondarily homothallism or heterothallism depending on the ploidy level of the spores, which can be homokaryotic or heterokaryotic respectively (Largeteau et al., 2011). All the traditional cultivated and most of the wild strains belong to *A. bisporus* var. *bisporus* which has predominantly a pseudohomothallic (secondarily homothallic) life cycle characterized by the feature that most of the basidia are bisporic and produce heterokaryotic spores (Raper et al., 1972). Most of the basidia of *A. bisporus* var. *burnettii* are tetrasporic and its amphithallic life cycle is predominantly heterothallic

(Kerrigan et al., 1994). *A. bisporus* var. *burnettii* was found in the Sonoran Desert, California, USA (Callac et al., 1993). *A. bisporus* var. *eurotetrasporus* is a rare tetrasporic variety found in France and Greece (Callac et al., 2003) and employs a homothallic life cycle when homokaryotic sporophores produce homokaryotic spores giving a rise to fertile homokaryons (Callac et al., 2003). This variety is characterized by mainly tetrasporic basidia as in var. *burnettii* while its mean size of spores is similar to var. *bisporus*.

Mushroom cultivation: Where Art and Science come together

A. bisporus var. *bisporus* is used for the large scale commercial mushroom production which expanded tremendously in the past few decades. The technique employed in mushroom cultivation involves composting and pasteurization of the substrate, a pure culture of spawn, adding a casing layer to the substrate and careful regulation of growing conditions (Figure 2). Growth of *A. bisporus* needs a substrate produced by the composting of animal manure (usually horse and chicken), wheat straw, gypsum, water and different additives (Fermor et al., 1985; Gerrits, 1988). Composting is an accelerated version of the natural recycling process for plant biomass (decomposition and humification) performed by microorganisms.

Commercial mushroom cultivation always starts with the production of suitable mushroom compost. The remark which Gene Longsdon made about compost production in 1989 (Logsdon, 1989): "In the not-so-distant future, compost making may well be as much an artful science as wine making" says a lot about the market and the management of the process to produce a high quality compost. The procedure to generate the compost substrate for *A. bisporus* is usually done in two stages. Phase I involves regularly mixing, wetting and heating of compost ingredients (Fermor et al., 1985; Gerrits, 1988). The essential aspect of this phase is to expose raw ingredients to aerobic thermophilic fermentation until it is well decomposed. A succession of microorganisms, especially thermophilic bacteria and fungi take part in decomposing organic matter and each group is dominant at different stages of the process (Waksman et al., 1939; Strom, 1985; Straatsma et al., 1989). The internal temperature of a compost pile can reach up to 80°C. Phase I composting lasts from 6 to 14 days, depending on the nature of the material at the start and the efficiency of the process. It is considered complete when the raw ingredients have become soft and are capable of holding water. During phase II final conditioning of compost takes place and pasteurization for 3-6 hours (60°C) is accomplished to relieve the compost of mushroom pests and pathogens that may be present in the compost (Fermor et al., 1985).

The prepared compost is inoculated with millet or rye grains colonized with *A. bisporus* mycelium, a process called spawning (Figure 2) (van Griensven, 1987). During the spawning stage the temperature of compost is approximately 25°C. Complete colonization of the compost usually requires 10 to 16 days, depending on the spawning rate and environmental conditions. The colonized substrate is covered with the casing layer, which is usually a mixture of peat and limestone on which the mushrooms eventually form (van Griensven, 1987). The casing layer acts as a water reservoir, allows appropriate gaseous exchange and supports microbial population able to release hormone-like substances which are very likely involved in stimulating the initiation of primordia (Eger, 1972; Hayes, 1981). *A. bisporus* mycelium colonizes the casing layer in about ten days, after which the temperature is lowered to 16-18°C and a relative high humidity (~ 90%) with heavy watering promotes primordia which develop further into the fruiting bodies (van Griensven, 1987). The first harvestable mushrooms appear 18 to 21 days after the addition of the casing layer and continue to develop in a rhythmic cycles known as flushes (Flegg & Wood, 1985).

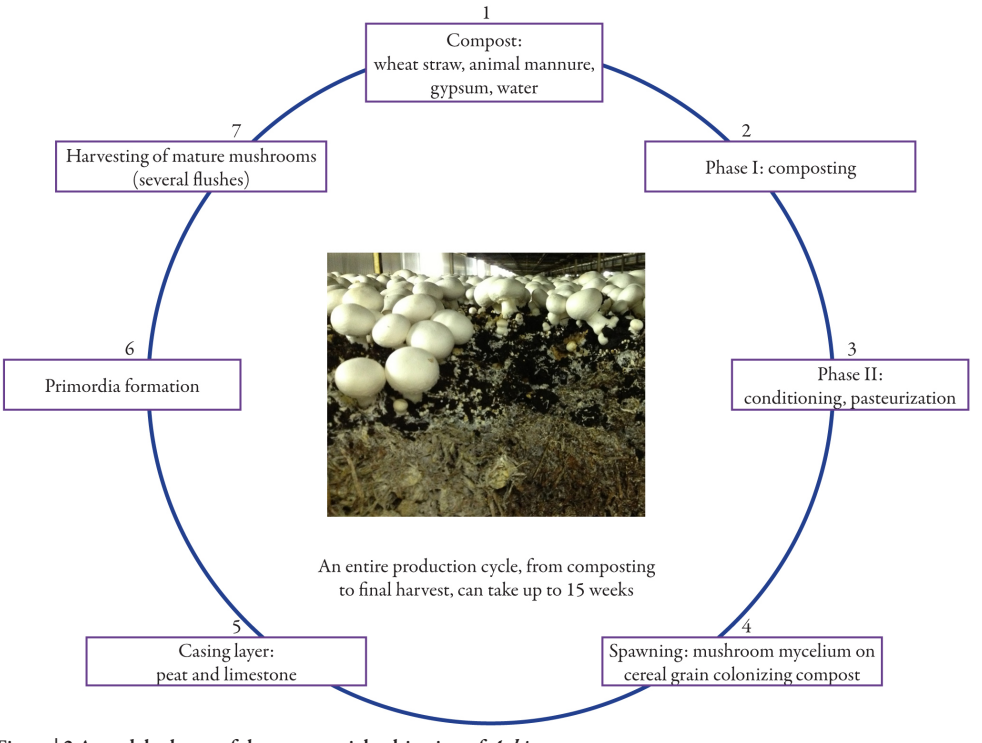

Figure | 2 A model scheme of the commercial cultivation of *A. bisporus*.

Overall, *A. bisporus* requires two different substrates to form fruiting bodies: the compost in which it grows vegetatively and the nutrient poor casing layer in which the suitable physical, chemical and biological conditions stimulate the initiation process and fruiting body production (Segula et al., 1989).

The core of the compost and how to break it apart

Wheat straw (*Triticum aestivum*) is the main ingredient of the commercial mushroom compost and belongs to the plant group of Monocots and the family Poaceae. The plant cell wall of wheat straw mainly consists of cellulose, hemicellulose (xylan) and lignin (Figure 3). Cellulose consists of linear β-1,4-linked D-glucopyranose chains that are linked together by hydrogen bonds to form microfibrils (Frey-Wyssling, 1954). Degradation of this polymer involves at least endo-β-1,4-glucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91) and β-glucosidases (EC 3.2.1.21) (Figure 4A), and probably additional hydrolytic and/or oxidative activities. Endoglucanases hydrolyze cellulose chains randomly to β-1,4-gluco-oligosaccharides. Cellobiohydrolases remove cellobiose units from cellulose chains, which are degraded by β-glucosidases to D-glucose.

One of the most complex components of hemicellulose presented in straw-derived compost is xylan. It consists of a β-1,4-linked D-xylopyranose backbone which can be substituted with a large number of carbohydrate residues (e.g. L-arabinose, D-galactose, D-glucuronic acid, acetyl), depending on its origin (Ebringerova & Heinze, 2000). The main enzymes involved in the conversion of xylan to monomeric xylose are endoxylanases (EC 3.2.1.8) and β-xylosidases (EC 3.2.1.37).

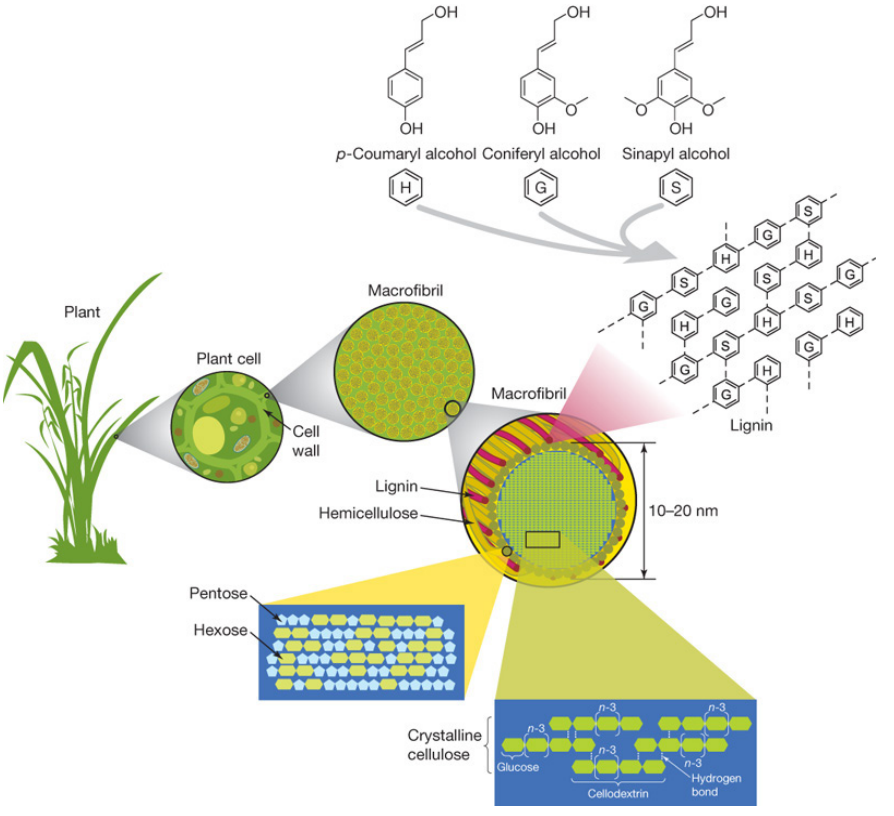

Figure 3 | Plant cell wall structure. The main component of lignocellulose is cellulose, a (1–4)-linked chain of glucose molecules. Hydrogen bonds between different layers of the polysaccharides contribute to the resistance of crystalline cellulose to degradation. Hemicellulose, the second most abundant component of lignocellulose, is composed of various 5- and 6-carbon sugars such as arabinose, galactose, glucose, mannose and xylose. Lignin is composed of three major phenolic components, namely *p*-coumaryl alcohol (H), coniferyl alcohol (G) and sinapyl alcohol (S). Lignin is synthesized by polymerization of these components and their ratio within the polymer varies between different plants, wood tissues and cell wall layers. Cellulose forms structures called microfibrils, which are organized into macrofibrils that mediate structural stability in the plant cell wall. Taken from (Rubin, 2008).

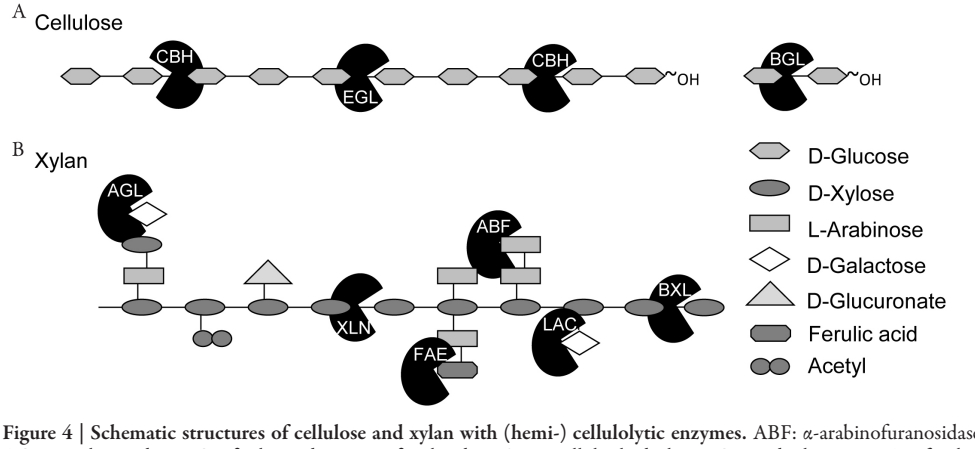

Figure 4 | Schematic structures of cellulose and xylan with (hemi-) cellulolytic enzymes. ABF: α-arabinofuranosidase, AGL: α-galactosidase, BGL: β-glucosidase, BXL: β-xylosidase, CBH: cellobiohydrolase, EGL: endoglucanase, FAE: feruloyl esterase, LAC: β-galactosidase, XLN: endoxylanase. Taken from (Klaubauf, 2015).

Endoxylanases cleave the xylan backbone to small xylo-oligosaccharides, which are converted to D-xylose by β-xylosidases. Other classes of enzymes involved in the removal of arabinosyl, glucuronosyl, acetyl and feruloyl residues from the xylan backbone are α-L-arabinofuranosidases (EC 3.2.1.55), arabinoxylan arabinofuranohydrolases, α-glucuronidases (EC 3.2.1.131), acetyl xylan esterases (EC 3.1.1.72) and feruloyl esterases (EC 3.1.1.73) (Figure 4B) (de Vries & Visser, 2001).

The β-1,4-linked D-xylopyranose backbone of xylan from the family Poaceae can be single or double decorated with α-L-arabinofuranosyl units which can be further substituted with ferulic acid and *p*-coumaric acid (Fincher, 2009). The presence of *O*-acetyl, D-glucuronic acid and its 4-*O*-methyl residues in xylan backbone of wheat straw was reported as well (Ishii, 1991; Wende & Fry, 1997; Fincher & Stone, 2004).

Wheat straw is the major lignin-containing ingredient of mushroom compost (Iiyama et al., 1994). The aromatic polymer lignin interacts with cellulose and forms covalent cross-links with hemicellulose creating an intricate and hard-to-degrade network of polymers, which is a poor substrate for most microorganisms, but not for *A. bisporus* (De Groot et al., 1998b). Due to this native association of three components of plant cell wall, cellulose and hemicellulose are not readily available as carbon source unless the lignin is modified or removed by chemical and/or biological methods (Freer & Detroy, 1982). Therefore the partial degradation of lignin plays an important role for (hemi-) cellulose utilization.

Fungal oxidative enzymes, such as heme-including peroxidases (manganese (EC 1.11.1.13), lignin (EC 1.11.1.14) and versatile (EC 1.1.11.16) peroxidases; heme-thiolate peroxidases and dye-decolorizing peroxidases (EC 1.11.1.19)), laccase-type multicopper oxidases, glyoxal oxidases, copper radical oxidases and cytochrome P450 monooxygenases play an important role in activating and decomposing lignin heteropolymers and smaller compounds originating from lignin and other aromatic compounds (Hofrichter et al., 2010; Lundell et al., 2010; Hatakka & Hammel, 2011).

Enzymes involved in degradation and modification of plant biomass are classified in the Carbohydrate Active enZyme families (CAZy, www.cazy.org) based on their amino acid sequence: Glycoside Hydrolases (GHs), Glycosyl Transferases (GTs), Polysaccharide Lyases (PLs), Carbohydrate Esterases (CEs), and Auxiliary Activities (AAs) (Levasseur et al., 2013; Lombard et al., 2014).

Plant biomass degradation during growth of *A. bisporus* in compost

After Phase I and Phase II composting are completed, mushroom compost mainly contains lignocellulose as a source of nutrients for growth of *A. bisporus* (De Groot et al., 1998b; Whiteford et al., 2000). After Phase I and Phase II, 38% - 40% and 20% - 26% of total carbohydrates remain, respectively (Iiyama et al., 1994; Jurak et al., 2014), and the main polysaccharides present are composed of xylosyl and glucosyl residues (Jurak et al., 2014).

A. bisporus secretes a wide range of lignocellulolytic enzymes to degrade plant polymers and to satisfy its nutritive needs (Wood, 1989; Fermor et al., 1991; Wood & Thurston, 1991; Yague et al., 1997). Some insight into the molecular basis of *A. bisporus* related to plant biomass degradation was obtained through isolation of genes encoding the related enzymes.

A. bisporus produces complete set of cellulolytic enzymes and four cellulose-growth specific genes have been isolated (*cel1*, *cel2*, *cel3*, and *cel4*) and their products have been characterized (Raguz et al., 1992; Chow et al., 1994; Yague et al., 1994; Yague et al., 1997). Cel2 and Cel3 are similar

to fungal cellobiohydrolase I and cellobiohydrolase II. Cel2 has homology to GH7 while Cel3 belongs to GH6. Cel4 has similarity to fungal β-mannanases belonging to glycosyl hydrolase family 5 (GH5). However no homology to cellulose degrading enzymes was detected for Cel1 although it did have homology to fungal cellulose binding domains (Armesilla et al., 1994; Yague et al., 1994).

A GH10 endoxylanase encoding gene, *xlnA*, was isolated to analyze the hemicellulolytic activities during cultivation of *A. bisporus* on compost (De Groot et al., 1998a). It was shown that *xlnA* was highly expressed in vegetative mycelium grown on sterilized compost, but no *xlnA* expression was detected in fruiting bodies of *A. bisporus* (De Groot et al., 1998a). Also no expression of *xlnA* was detected in mycelium of *A. bisporus* that was grown on compost supplemented with glucose or xylose. However, after transfer of the glucose-grown mycelium to medium containing cellulose, xylan or xylose a high level of *xlnA* expression was detected for a short time (De Groot et al., 1998a). For *cel3* a similar pattern of expression was found, suggesting that *xlnA* and *cel3* are co-regulated and that their induction occurs by compost-specific factors (De Groot et al., 1998a). The *cel2* and *cel4* genes were shown to be co-expressed with *cel3* (Yague et al., 1997).

It has been also demonstrated that *cel3* had the highest expression during the first mushroom flush (veil break) compared to other stages of growth of *A. bisporus* in compost (Ohga et al., 1999). Cellulase and endoxylanase activities could be detected throughout growth of *A. bisporus* in compost, but starting from the stage when the first primordia (pins) were visible, these activities increased and reached the highest level at the postharvest stage after the first flush mushrooms were harvested (Wood & Goodenough, 1977; Claydon et al., 1988; Whiteford et al., 2000). The detected mannanase activity correlated with the protein level of Cel4 throughout the life cycle of *A. bisporus* (Tang et al., 2001). Its protein level remained low until the first flush (veil break) reaching a maximum at the stage after the first flush mushrooms have been harvested (postharvest stage) (Tang et al., 2001).

Lignin degradation by *A. bisporus* grown in straw-based compost takes place during vegetative growth, decreasing from the spawning stage until the appearance of the first primordia (Wood & Leatham, 1983; Durrant et al., 1991). Two ligninolytic enzymes, manganese peroxidase (MnP) and laccase were secreted by *A. bisporus*, while no lignin peroxidase was found (Bonnen et al., 1994). Two laccase genes, *lcc1* and *lcc2*, were identified and high expression levels were detected for them in compost during mycelial colonization (Ohga et al., 1999). During fruiting body development no expression was detected, but after harvesting and during the second flush the expression level of laccase genes increased (Ohga et al., 1999). An MnP encoding gene (*mnp1*) has also been identified (Lankinen et al., 2005) and MnP activity has been observed in compost during the spawning and pinning stage and decreased at the onset of *A. bisporus* fruiting body formation, which correlated with developmentally regulated laccase activity (Bonnen et al., 1994).

Overall, studies on lignocellulolytic genes as well as enzymes involved in degradation of the substrate showed that they are developmentally regulated during the growth of *A. bisporus* in compost. Laccase and MnP increased during compost colonization of *A. bisporus* but declined at the onset of fruiting body formation while cellulase and xylanase increased from this moment onwards.

Thesis outline

Degradation of plant biomass is an important aspect of the cultivation process of edible mushrooms, but the mechanisms behind it are still largely a black box. The commercial cultivation of *A. bisporus* offers a unique opportunity to study the interaction of a fungus with its substrate. Compost cultivation of this fungus has a long history and the fungus is well adapted to this. In addition, each ton of produced mushrooms leaves almost the same amount of spent compost which contains approximately one third of carbohydrates from the original compost (Chiu et al., 2000; Semple et al., 2001). Therefore the aim of this PhD thesis was to obtain a deeper understanding of how *A. bisporus* grows in compost and degrades its substrate, and to study the molecular mechanisms underlying this phenomenon. Getting an insight into these mechanisms not only answers fundamental scientific questions but has also applied aspect as it may lead to new strategies to improve mushroom cultivation.

Whole genome and transcriptome sequencing and analysis are important tools to understand the unique mechanisms of lignocellulose degradation and conversion by wood and litter decay fungi. **Chapter 2** describes the genome sequence of bisporic *A. bisporus* var. *bisporus* which employs predominantly a pseudothallic life cycle and tetrasporic *A. bisporus* var. *burnettii* which employs predominantly a heterothallic reproductive cycle (Figure 5). The transcriptome of the commercial *A. bisporus* strain cultivated in compost was also analyzed. Both genome and transcriptome analyses deepened our understanding of the biology and ecology of this fungus. In **Chapter 2** it was also assessed whether the genome and transcriptome of *A. bisporus* shows the adaptation of this fungus to humic rich partially decomposed leaf material.

The availability of genome sequences for *A. bisporus* as well as transcriptome analysis also enabled us to understand in more detail the process of polysaccharide degradation and carbon utilization by this fungus (Figure 5). In **Chapter 3** transcriptome data of *A. bisporus* grown on compost obtained in Chapter 2 was used to analyse the expression of genes involved in the catabolism of different sugars. Clear differences in the expression of genes from different catabolic pathways were observed between mycelium grown in compost or in casing layer, and in fruiting bodies, suggesting a high level of specialization. Also expression of genes encoding plant and fungal polysaccharide modifying enzymes was studied to explore whether there are differences in the expression of these genes between compost- casing layer-grown mycelium and fruiting bodies.

To gain more knowledge and better understanding of plant biomass degradation by *A. bisporus*, the fungus was grown in compost under commercial cultivating conditions and samples were taken from various growth stages of *A. bisporus* starting from spawning stage until the entire second flush of mushrooms was harvested. Analysis of the transcriptome throughout life cycle of *A. bisporus* required isolation of high quality and quantity of RNA. Isolation of fungal RNA from plant biomass cultures (including compost grown cultures) is often a bottleneck in the analysis of fungal physiology on lignocellulosic substrates. **Chapter 4** describes a highly reproducible protocol for isolation of RNA from complex plant biomass substrates (compost, straw, spruce and microcrystalline cellulose Avicel) which resulted in high integrity and quantity RNA and was very suitable for RT-qPCR and transcriptomics (Figure 5).

Chapter 5 describes changes in plant biomass degradation by *A. bisporus* throughout its life cycle (including spawning, pinning, the first flush, the second flush stages and when entire first and second flushes were harvested growth stages). We performed a wide analysis of genes encoding plant biomass degrading enzymes using high throughput sequencing (RNA-seq) and lignocellulolytic enzymes secretion by *A. bisporus* grown in compost under commercial conditions (Figure 5). Clear correlations were observed between secreted extracellular plant biomass degrading enzymes, the expression of the corresponding genes and the composition of compost which is rich in plant components such as cellulose and hemicellulose.

The ability of *A. bisporus* to degrade plant polysaccharides and to explore unutilized fractions which remain in compost in the end of entire commercial cultivation process, water-extracted carbohydrate degrading enzymes from mycelium-grown compost of different growth stages were assessed in **Chapter 6** (Figure 5). Mostly cellulolytic and xylonolytic enzymatic activities were detected in the compost extracts and arabinosyl and glucuronic acid substituents of the xylan backbone were found to be accumulated in the compost towards the end of the mushroom cultivation.

The results are summarized and discussed in **Chapter 7**.

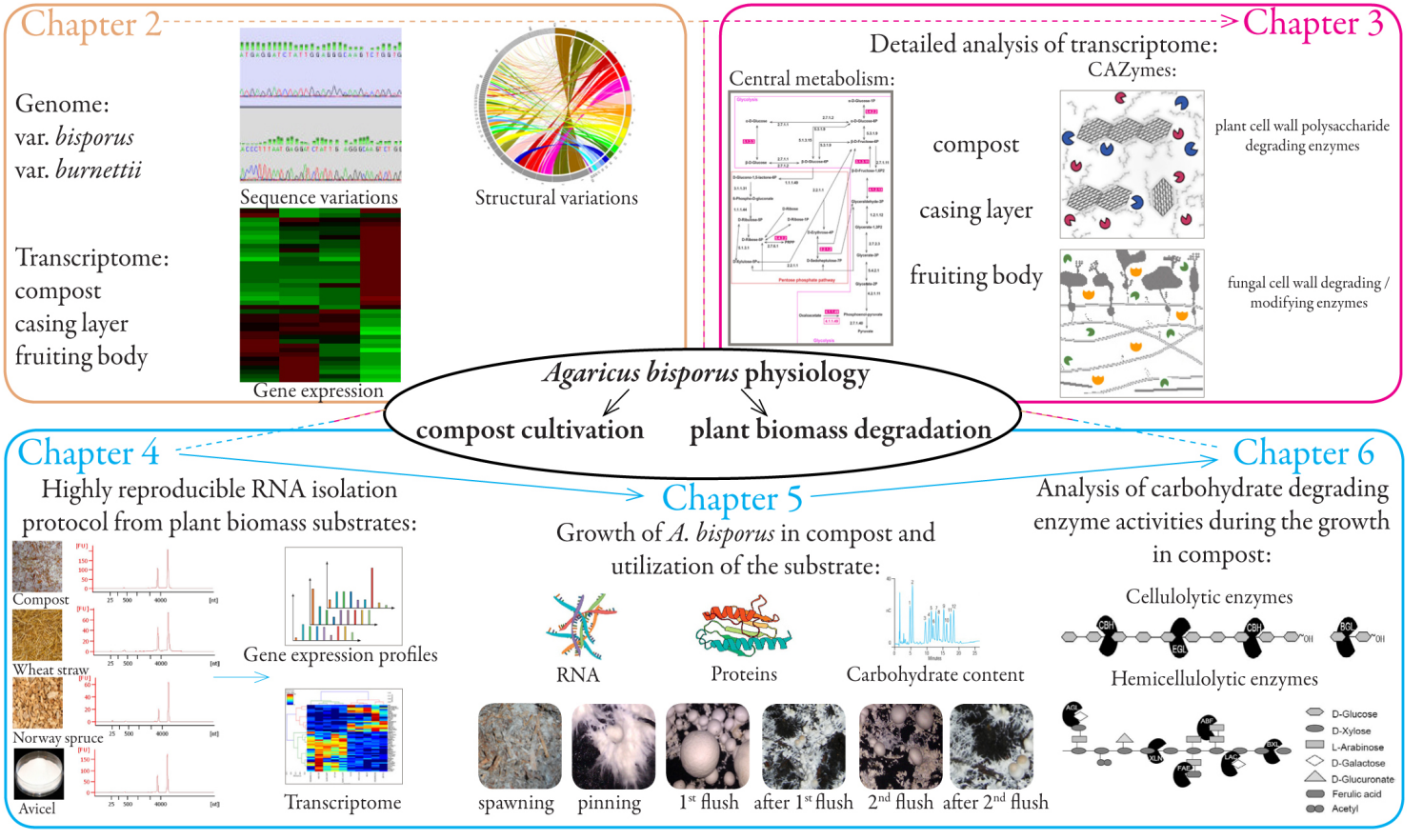

Figure 5 | Schematic outline of the thesis.

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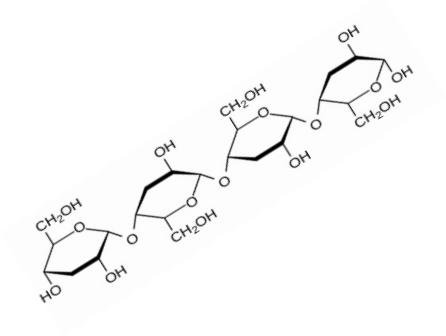

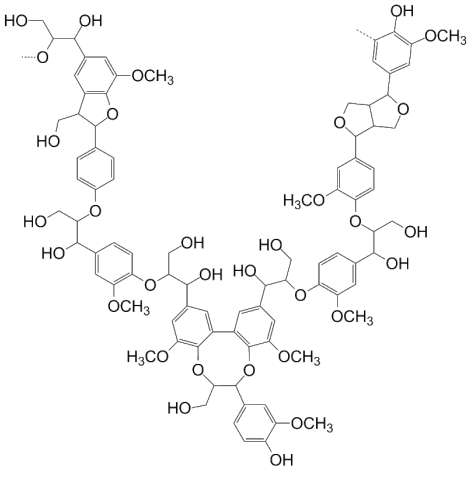

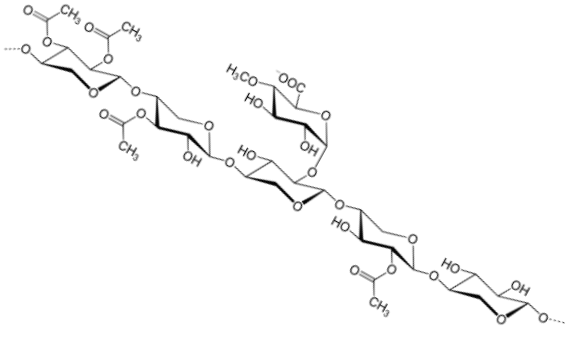

CHAPTER 2

Genome sequence of the button mushroom *Agaricus bisporus* reveals mechanisms governing adaptation to a humic-rich ecological niche

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2

AGARICUS BISPORUS IS THE MODEL FUNGUS for the adaptation, persistence, and growth in the humic-rich leaf-litter environment. Aside from its ecological role, *A. bisporus* has been an important component of the human diet for over 200 years and worldwide cultivation of the "button mushroom" forms a multibillion dollar industry. We present two *A. bisporus* genomes (*A. bisporus* var. *bisporus* H97 and *A. bisporus* var. *burnettii* JB137-S8), their gene repertoires and transcript profiles of commercial strain A15 on compost and during mushroom formation. The genomes encode a full repertoire of polysaccharide-degrading enzymes similar to that of wood-decayers. Comparative transcriptomics of mycelium grown on defined medium, casing-soil, and compost revealed that genes encoding enzymes involved in xylan, cellulose, pectin, and protein degradation are more highly expressed in compost. The striking expansion of heme-thiolate peroxidases and β-etherases is distinctive from Agaricomycotina wood-decayers and suggests a broad attack on decaying lignin and related metabolites found in its humic acid-rich environment. Similarly, up-regulation of these genes together with a lignolytic manganese peroxidase, multiple copper radical oxidases, and cytochrome P450s is consistent with challenges posed by complex humic-rich substrates. These observations reveal genetic and enzymatic mechanisms governing adaptation to the humic-rich ecological niche formed during plant degradation, further defining the critical role such fungi contribute to soil structure and carbon sequestration in terrestrial ecosystems. The genome sequence will expedite mushroom breeding for improved agronomic characteristics.

Introduction

Lignocellulose is the most abundant organic compound in the terrestrial environment, consisting of three main components: cellulose, hemicellulose, and lignin (Sánchez, 2009). These polymers are decayed primarily by wood and litter decomposers from the Agaricomycotina (mushroom-forming fungi). Comparative analyses of the "white-rot" fungus *Phanerochaete chrysosporium* and the "white-rot" like fungus *Schizophyllum commune* (Martinez et al., 2004; Ohm et al., 2010; Riley et al., 2014), the "brown-rots" *Postia placenta* and *Serpula lacrymans* (Martinez et al., 2009; Eastwood et al., 2011), and the coprophilous *Coprinopsis cinerea* (Stajich et al., 2010) has provided considerable insight into the evolution of the wood-decomposition machinery in fungi. Much less is known about fungal decomposition of partially degraded plant material, particularly leaf litter, and about adaptation to humic-rich environments. Improving knowledge of processes governing this adaptation is critical to improving carbon management and predictive modeling of terrestrial carbon cycling.

The biomass of nonwoody litter in temperate woodlands can be four- to five times greater than that of woody litter (Boddy, 1983). Concentrations of lignin and (hemi)cellulose in leaf litters of hardwood species range from 33–43% and 23–29%, respectively (Sariyildiz & Anderson, 2005). After a succession of microbial colonizations, the litter is substantially modified by the removal of readily available carbon, nitrogen, and minerals, and the formation of humic substances. Humic substances originate from the decay of modified lignin and other recalcitrant aromatic compounds and microbial activity. These substances are chemically heterogeneous, complex, and difficult to define, consisting of relatively small molecules forming supramolecular associations by hydrophobic and hydrogen bonds, and sequester proteins (Piccolo, 2002; Tomaszewski et al., 2011). Humic substances comprise up to 70–80% of organic compounds in mineral soils and their properties strongly influence the physical properties and structure of soil (Piccolo, 2002).

The basidiomycete *Agaricus bisporus* (Lange) Imbach is the favored model for adaptation, persistence, and growth in this humic rich environment, where nutrition is not readily available to primary degrading fungi (Fermor & Wood, 1981; Burton et al., 1997). The ability to use humic proteins gives the fungus an advantage over other saprobes in this complex substrate. The machinery used by *A. bisporus* to exploit the diverse mixture of nutrient resources is, however, poorly understood.

Aside from its ecological roles, *A. bisporus* is widely cultivated to produce mushrooms (Figure 1) and is the basis of a multibillion dollar industry. This cultivation involves the large-scale (megaton) biotechnological conversion of agricultural lignocellulosic wastes to high-value food with obvious extrapolations for bioenergy and biorefining. In commercial production for mushroom cultivation, the humic-rich substrate is typically derived from composted wheat straw supplemented with gypsum and nitrogen rich materials, such as chicken and horse manures.

Our hypothesis has been that metabolic strategies and niche adaptations that might not be seen in the white-rot and brown rot wood-decomposing fungi, nor in coprophilous fungi, such as *C. cinerea*, may have evolved in humicolous species, such as *A. bisporus*. Among the "detritophiles," they may have a distinctly different deployment of substrate conversion enzymes or regulatory regimens in adaptation to their ecological niche, the partially degraded and humified plant litter.

Here, we report a draft 30-Mb genome of *A. bisporus* H97 and transcriptome sequence of *A. bisporus* A15, a European isolates obtained from a historically cultivated stock of var. *bisporus*, considered to represent a member of the "adapted" European population of the bisporic var. *bisporus* now associated with agricultural environments. We also sequenced the genome of the strain JB137-S8, belonging to the tetrasporic var. *burnettii* Kerrigan & Callac that is known only from the

Sonoran desert of California, where it is associated with leaf litter in native stands of woody species. To identify *A. bisporus*-specific traits, we compared the H97 and JB137-S8 genomes with those of diverse fungi, including 12 newly sequenced species of white- and brown-rot Agaricomycotina (Floudas et al., 2012). We focused annotation on gene families likely to be involved in litter decomposition, and transcript profiling to reveal adaptation processes for growth on humic-rich substrates.

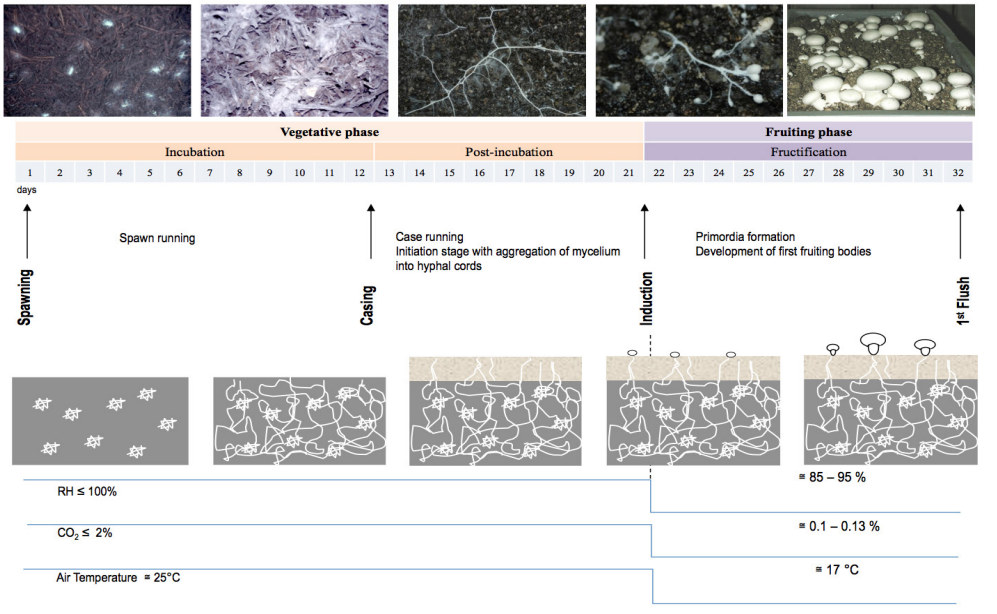

Figure 1 | Developmental stages of *A. bisporus* during the successive steps of its cultivation: spawning, casing, induction, and first fruiting-body flush. The vegetative (incubation, postincubation) and fructification phases are shown. Inoculation of compost is done with wheat kernels overgrown with mycelium (spawning). In 2 weeks, mycelium has grown throughout the compost and induction of fruiting bodies is taking place 22 days after a change in aeration and addition of the casing layer. The first flush of fruiting bodies is observed at 32nd day, with a switch from mycelium extension to the production of primordia (pinning). Key physicochemical factors (relative humidity, RH%, CO_2 concentration and air temperature, T °C) are given at the bottom of the figure.

Materials and Methods

Genome sequencing and assembly

A. bisporus var. *bisporus* H97 (homokaryon) was sequenced using Sanger sequencing on ABI 3730XL capillary machines. Three different sized libraries were used as templates for the plasmid subclone sequencing process and both ends were sequenced. 191,136 reads from the 2.9 kb sized library, 179,424 reads from the 6.4 kb sized library, and 36,192 reads from a 39.6 kb fosmids library were sequenced. A total of 406,752 reads were assembled using a modified version of Arachne (Jaffe et al., 2003) v.20071016 with parameters maxcliq1=100, correct1_passes=0 and BINGE_AND_PURGE=True. This produced 33 scaffold sequences, with L50 of 2.4 Mb, 18 scaffolds larger than 100 kb, and total scaffold size of 30.4 Mb. Two scaffold breaks were made based on integration of a genetic map. Each scaffold was screened against bacterial proteins, organelle sequences and GenBank using megablast against Genbank NR and blastp against a set of known microbial proteins.

No scaffolds were identified as contamination. We classified additional scaffolds as unanchored rDNA (18), mitochondrion (1), and small repetitive (3). Additional scaffolds were removed if they consisted of greater than 95% 24mers that occurred 4 other times in the scaffolds larger than 50kb or if the scaffold contained only unanchored rDNA sequences. We also excluded 2 scaffolds smaller than 1 kb. The final assembly contains 29 scaffolds that cover 30.0 Mb of the genome with a contig L50 of 262.5 kb and a scaffold L50 of 2.3 Mb (Table S1).

A. bisporus var. *burnettii* JB137-S8 (homokaryon) genome was sequenced using a combination of 454 (Roche) and Illumina sequencing platforms. All general aspects of library construction and sequencing can be found at the JGI website (http://jgi.doe.gov). The Illumina reads were quality trimmed to 57 bp based on an average quality per base position plot, randomly subsetted to 50X coverage depth, assembled as unpaired data with the Velvet assembler version 0.7.34 (Zerbino & Birney, 2008) with a hash length of 31, and the resulting contigs greater than or equal to 800bp in length were shredded into 1000 bp chunks, if possible, with 800 bp overlap. Reverse complemented shreds were also created at contig ends. 454 data and shredded Velvet assembled Illumina consensus, screened for contamination, were assembled with the Newbler assembler version 2.3-PreRelease-10/20/2009 to produce a draft with a final assembled coverage of 46X. The assembly was improved using one round of automated gap closure using gap Resolution (DOE Joint Genome Institute http://jgi.doe.gov), which resulted in 131 closed gaps. The consensus of the closed gaps was stitched into the assembly and it was further improved using synteny to *A. bisporus* H97_2 using Nucmer and mummerplot (Kurtz et al., 2004). 15 joins were identified and manually made with 600 bp of gap in between each manual join. This resulted in a final improved assembly with 2016 scaffolds with an N/L50 of 8/1.23 Mb, up from 2030 scaffolds with an N/L50 of 14/0.56 Mb (Table S1).

The genome sequences have been deposited in the GenBank database [accession nos. AEOK00000000 (*A. bisporus* var. *bisporus* H97) and AEOL00000000 (*A. bisporus* var. *burnettii*)].

Genome annotation

Both genomes were annotated using the JGI annotation pipeline, which takes multiple inputs (scaffolds, ESTs, and known genes) and runs several analytical tools for gene prediction and annotation, and deposits the results in the JGI Genome Portal (http://jgi.doe.gov/Agaricus) for further analysis and manual curation. Genomic assembly scaffolds were masked using RepeatMasker (Smit et al., 1996–2010) and the RepBase library of 234 fungal repeats (Jurka et al., 2005). tRNAs were predicted using tRNAscan-SE (Lowe & Eddy, 1997). Using the repeat-masked assembly, several gene prediction programs falling into three general categories were used: 1) *ab initio* - FGENESH (Salamov & Solovyev, 2000); GeneMark (Isono et al., 1994), 2) *homology-based* - FGENESH+; Genewise (Birney & Durbin, 2000) seeded by BLASTx alignments against GenBank's database of non-redundant proteins (NR: http://www.ncbi.nlm.nih.gov/BLAST/), and 3) EST-based - EST_map (http://www.softberry.com/) seeded by *A. bisporus* H97 EST contigs. Genewise models were extended whenever possible using scaffolds data to find start and stop codons. EST BLAT alignments (Kent, 2002) were used to extend, verify, and complete the predicted gene models. The resulting set of models was then filtered for the best models, based on EST and homology support, to produce a non-redundant representative set. This representative set was subject to further analysis and manual curation. Measures of model quality include proportions of models complete (with start and stop codons) (>87% of models), consistent with ESTs (>61% of models covered over ≥75% of exon length), supported by similarity with proteins from the NCBI NR database (>83% of models). Quality metrics for gene models are summarized in Table S2.

All predicted gene models were functionally annotated using SignalP (Nielsen et al., 1997), TMHMM (Melen et al., 2003), InterProScan (Zdobnov & Apweiler, 2001), BLASTp (Altschul et al., 1990) against GenBank's database of non-redundant proteins, and hardware-accelerated double affine Smith-Waterman alignments (deCypherSW; http://www.timelogic.com/decypher_sw.html) against SwissProt (http://uniprot.org), KEGG (Kanehisa et al., 2008), and KOG (Koonin et al., 2004). KEGG hits were used to assign EC numbers (http://enzyme.expasy.org/), and Interpro and SwissProt hits were used to map GO terms (http://www.geneontology.org/).

Manual curation of the automated annotations was performed trough by using the web-based interactive editing tools of the JGI Genome Portal to assess predicted gene structures, assign gene functions, and report supporting evidence.

Secreted proteins were identified using a custom pipeline including the TargetP (http://www.cbs.dtu.dk/services/TargetP/) and SignalP (http://www.cbs.dtu.dk/services/SignalP/) algorithms (Nielsen et al., 1997; Emanuelsson et al., 2000). The secreted peptidases were identified in the genome using the MEROPS database (Rawlings et al., 2012) (http://merops.sanger.ac.uk).

EST sequencing, clustering, and assembly

Total RNA of *A. bisporus* var. *bisporus* strain U1 (heterokaryon) was used to extract PolyA+ RNA using oligo (DT) magnetic beads (Absolutely mRNA™ Purification kit, Stratagene). PolyA RNA was reversed transcribed using Superscript III (Invitrogen) using a dT15VN2 primer. Second strand cDNA was synthesized by nick translation with *E. coli* DNA ligase, *E. coli* DNA polymerase I, and RNase H and blunt end repaired using T4 polymerase (Invitrogen). The dscDNA was fragmented and 300-800 base pair fragments were gel purified using a 2% agarose gel. The purified fragments were then used to create the 454 single stranded cDNA library as described below (454 library preparation kit, Roche).

The fragment ends were polished using T4 ligase and T4 polynucleotide kinase (Roche). Adaptors containing primer sequences and a biotinylated tag were ligated to the fragment ends (Roche). The fragments with properly ligated adapters were immobilized onto magnetic streptavidin-coated beads (Roche). Nicks or gaps between the adapters and the dscDNA fragments were repaired using the fill-in polymerase (Roche). The non biotinylated strands of the immobilized dscDNA fragments were melted off to generate the single stranded cDNA library for 454 sequencing.

The ESTs were evaluated for the presence of polyA tails (which if present were removed) then evaluated for length, removing ESTs with fewer than 50 bases remaining. Additionally, ESTs consisting of more than 50% low complexity sequence were removed from the final set of ESTs. For clustering, ESTs were evaluated with malign (JGI tool), a kmer based alignment tool, which clusters ESTs based on sequence overlap (kmer = 16, seed length requirement = 32 alignment ID >= 98%). EST clusters were then each assembled using CAP3 (Huang & Madan, 1999) to form consensus sequences.

Three separate RNA samples were used to generate libraries: vegetative, undifferentiated mycelium on casing and compost substrates, and fruiting bodies. They produced, respectively, 442,365, 291,949, and 405,357 ESTs. Together with 470 ESTs from external sources, they all were clustered and assembled into 79,271 consensus sequences and 42,693 singletons. ESTs have been submitted and released by NCBI Short Read Archive: Accession n° SRA037617.

Transcriptome analysis

Gene expressions were profiled in the commercial (heterokaryon) strain A15. Casing, compost and fruit body samples were grown in standard conditions. The casing sample consisted of a mixture

of mycelium aggregates and primordia. The culture samples refer to axenic culture and the media used was compost extract medium in agar plates (Calvo-Bado et al., 2000). The 'Fruiting bodies' sample represents the mature mushroom fruit body (including the stipe, cap and pilei pellis (skin) tissues). The 'Compost' sample represents the mycelium growing in wheat straw compost. This mycelium is both fine (likely to be nutritional) and strands or cords (some secondary structure and thicker (clearly visible to the naked eye). The 'Casing' sample consists of fine and stranded/corded mycelium, and stages to fruit body primordia (including aggregates and hyphal knots, undifferentiated and differentiated primordia). The main components of compost are wheat straw, horse and chicken manure, gypsum and soy meal. It originates from a pre-treatment that includes bacterial pre-digestion and also the presence of other fungi during cultivation. The samples for RNA extraction were collected on separate occasions from separate mushroom houses. Four biological replicates of each developmental stage were analyzed.

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). RNA was quantified using a NanoDrop-1000 spectrophotometer and quality was monitored with the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA).

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 randomised 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). 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 (ID027120) 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 3um). 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.

Raw array data were Robust multichip average normalized using the ARRAYSTAR software (DNASTAR, Inc. Madison, WI, USA). A Student t-test with false discovery rate (FDR) multiple testing corrections were applied to the data using the ARRAYSTAR software (DNASTAR). Transcripts with a significant p-value (<0.05) were considered as differentially expressed. The complete expression dataset is available as series GSE39569 at the Gene Expression Omnibus at NCBI (http://www.ncbi.nlm.nih.gov/geo/). In addition a supplemental table summarizing the array data is available for download on http://mycor.nancy.inra.fr/Agaricus/.

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. To assess the effect of a humic environment on gene expression the ratio of humic/non-humic

expression was calculated as: expression in compost/ (average expression in culture, casing and fruit bodies). The comparison of compost/fruit body transcript profiles highlights developmental stage differences during mushroom formation.

Growth and utilization of carbohydrates

Growth of *A. bisporus* was performed using minimal medium with the carbon sources as indicated in the text and at www.fung-growth.org. Minimal medium consisted of 20.5 mM MOPS, 2 mM KH_2PO_4, 1 mM $MgSO_4$, 0.5 mM $CaCl_2$, 0.134 mM EDTA, 25 μM FeO_4, 5 μM $ZnSO_4$, 5 μM $MnSO_4$, 4.8 μM H3BO_3, 2.4 μM KI, 52 nM Na_2MoO_4, 4 nM $CuSO_4$, 4 nM $CoCl_2$, 0.5 μM thiamine.HCL, 0.1 μM D(+) biotine and 20 mM NH_4CL and was set at pH 6.8. *C. cinerea* was grown on minimal medium consisted of 2 mM $MgSO_4$*7H_2O, 3.4 mM KH_2PO_4, 5.7 mM K_2HPO_4, 0.4 mM thiamine, 15 mM L-asparagine, 74 μM adenine. Basic media for the other fungi (*S. commune*; *P. chrysosporium=Aspergillus niger* minimal medium; *Laccaria bicolor=Rhizoctonia solanii* minimal medium) were as described at www.fung-growth.org. Carbon sources were added to a final concentration of 25 mM for monosaccharides and 1% for polysaccharides.

To check if humic acids affect growth and utilization of carbohydrates, *A. bisporus* was grown on minimal media (see above) with the carbon sources and with adding humic extracts. Humic extracts were presented as a pine (cypress) soil extract and compost (phase II) extract and they were added to a final concentration of 0.1%.

Prediction of CAZymes and lignolytic oxidoreductases

To determine whether *A. bisporus* sugar-cleaving capabilities resemble those of other fungi, we have undertaken a comparison of the glycoside hydrolases (GH) and polysaccharide lyases (PL) repertoires of 25 completely sequenced fungi (Figure S1). For each fungus we have listed the number of representatives of each CAZy family (families defined in the CAZy database; www.cazy.org) (Cantarel et al., 2009) and then performed a double clustering based on Bray-Curtis distances (i) between organisms according to their family distribution and (ii) between families according on their distribution pattern in the different genomes. Distances were computed using GINKGO (De Caceres et al., 2007) and the distance trees were constructed with FastME (Desper & Gascuel, 2002).

Then, we searched *A. bisporus* var. *bisporus* and *A. bisporus* var. *burnettii* proteomes for 27 gene families encoding oxidoreductases and carbohydrateactive enzymes (CAZymes) that are known to be implicated in wood decay (Floudas et al., 2012) (Table S3). To estimate the significance of the observed family size differences among taxa, we performed an analysis using the CAFE program (De Bie et al., 2006) with a p-value <0.001. To assess if *A. bisporus* is sharing the same distribution for these 27 gene families with brown rot or white rot fungi, we carry out a Correspondance Analysis, with R package FactoMineR (Figure S2).

Results and Discussion

Genome assembly and gene content

We sequenced and compared the genomes of the homokaryotic (haploid) strains H97 and JB137-S8. Sanger sequencing of the genomic DNA of strain H97 with 8.29× coverage resulted in a 30,387,844 base-pair genome assembly (Table S1). Ninety-two genetic marker sequences selected along the 13 linkage groups (chromosomes) of *A. bisporus* (Sonnenberg et al., 1996; Foulongne-

Oriol et al., 2011) were mapped on the H97 genome assembly to validate order and orientation of the 19 largest scaffolds (> 50 kbp) (Figure S3). The ratio of physical lengths to genetic distances averaged 33 kbp/cM.

The second genome, of *A. bisporus* var. *burnettii* strain JB137-S8, was sequenced using 454 pyrosequencing and Illumina HiSeq. The final assembly contained 52 scaffolds > 50 kbp (Figure S4 and Table S1). We estimated 10,438 and 11,289 protein-coding genes in the H97 and JB137-S8 genomes, respectively, by combining both homology-based and ab initio methods (Tables S2 and S4), along with 1,140,000 expressed sequence tags.

Genome comparisons between A. bisporus var. bisporus and A. bisporus var. burnettii

These two varieties differ in their ecology, modes of reproduction, and morphological characteristics. Understanding their genome structural differences could potentially help us to reveal the genetic basis for such differences and help to guide future hybridization and breeding programs. Segmental duplications were found for 54 genes and 31 genes in 15 and 9 blocks respectively for the var. *bisporus* and var. *burnettii* strains, with maximum 6 and 5 genes per block in the two strains. However, the amounts of tandem repeats were larger than segmental duplications. In the *A. bisporus* var. *bisporus* strain H97, 1083 genes were included in 363 tandems with 15 genes maximum for an individual repeat. A similar number was found in the var. *burnettii* strain with 1032 genes in 352 tandems with 9 genes maximum for an individual repeat. The tandem repeats include cytochromes P450, aldo/keto-reductases, hydrophobins, coesterases, methyltransferases, GMC_oxred, Cuoxidases, Ricin_B_lectin (6-12 genes each). Several domains were found duplicated in only one of the two strains (eg, var. *burnettii*-specific NADH-ubiquinone/plastoquinone oxidoreductases and LAGLIDADG endonucleases). Over 390,000 SNPs were detected between the two strains with 40% in protein coding regions.

We compared the genome structures between strains H97 and JB137-S8 based on their respectively assembled 29 and 2016 scaffolds. Because of the large difference in the number of scaffolds between the two assembled genomes (Figure S4) and their incomplete correspondence with chromosomes and linkage groups, inferences about inter-chromosomal translocations between the genomes are not possible at present. Instead, our analysis is focused on comparing the unambiguously assembled scaffolds to identify the inversions and translocations between homologous scaffolds of the two strains. In this analysis, we first used each individual scaffold of strain H97 as a query to identify homologous sequences in strain JB137-S8. The corresponding scaffold(s) in strain JB137-S8 that matched each queried scaffold of strain H97 was recorded. Each matching scaffold pair between these two strains was then compared using the "Dot Plot" tab of the "Synteny" function at the JGI website to identify inversions and translocations.

A total of 74 one-to-one matching scaffold block pairs ranging from ~4.5kb to over 2.9 Mbp were identified between the two genomes. Within the 74 pairs of blocks, 12 were completely syntenic with no inversion or translocation, 30 contained both inversions and translocations, 5 contained only inversions, and 27 contained only translocations. The numbers of inversions and translocations between the two sequenced genomes of *A. bisporus* were significantly greater than those found between closely related strains in other species (Sun & Xu, 2009). Chromosomal size polymorphisms due to chromosomal rearrangements have also been observed among strains within the same variety, *A. bisporus* var. *bisporus* (Royer et al., 1992). Such rearrangements likely contribute to the relatively low rate of recombination and low viability of haploid meiotic spores from crosses involving parental strains from either within the same variety (Kerrigan et al., 1993) or between the two different varieties (Foulongne-Oriol et al., 2010).

Genes involved in lignocellulose decomposition

A. bisporus is a very poor competitor on fresh nondegraded plant wastes but competes well on partially decomposed plant litter on forest floors and grassland soils rich in humic substrates. *A. bisporus* is adapted to growing in this ecological niche, where it and other species of Agaricaceae can occur abundantly and even predominate based upon observed fructifications. To identify the genomic traits enabling *A. bisporus* to adapt to its biotope and to efficiently complete its life cycle, we have identified the repertoire and expression of genes known to be involved in organic matter degradation [carbohydrate - acting enzymes (CAZymes), lignin-related oxidoreductases, secreted proteases] and compared this arsenal to that of the white and brown wood-rotters, the coprophilous *C. cinerea* and the mycorrhizal symbiont *L. bicolor* (Martinez et al., 2004; Martin et al., 2008; Martinez et al., 2009; Ohm et al., 2010; Stajich et al., 2010; Eastwood et al., 2011; Floudas et al., 2012). We also used custom microarrays to compare gene expression at four developmental stages, defined by mycelial cultures on agar-medium, mycelium colonizing the casing-soil layer, or the compost (a proxy for humic-rich composted material) and mature fruiting bodies (Figure 1). Among the most highly up-regulated transcripts found in mycelium grown on compost were CAZymes, cutinases, oxidoreductases and secreted proteases (Figure S5 and Table S5).

Carbohydrate-acting enzymes

A. bisporus is a generalist with respect to polysaccharide degradation when grown in laboratory culture on minimal medium and specific carbon sources (Figure 2). *A. bisporus* grows better (relative to glucose) on xylan than do other basidiomycetes, such as *S. commune* and *L. bicolor* (Figure 2). Xylan is the second most abundant polysaccharide in plant cell walls (PCW), comprising 7–12% of plant dry mass (Fry, 1988), and in wheat straw (Iiyama et al., 1994; Lawther et al., 1995). Although *A. bisporus* grows well on cellulose, it is less efficient than *S. commune* and *C. cinerea* (Figure 2).

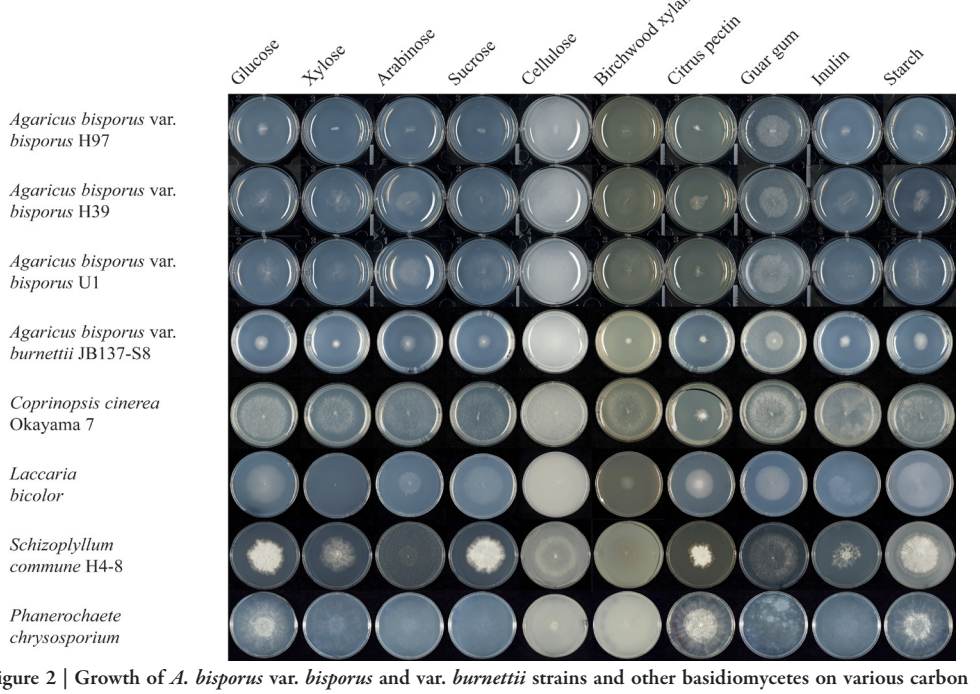

Figure 2 | Growth of *A. bisporus* var. *bisporus* and var. *burnettii* strains and other basidiomycetes on various carbon sources.

This catabolic ability concurs with the presence of a large set of genes encoding CAZymes (Cantarel et al., 2009) acting on plant, fungal, and bacterial cell wall polysaccharides [including 188 glycoside hydrolases (GH), 59 polysaccharide lyases (PL), and 10 carbohydrate esterases (CE)]. Clustering of CAZyme profiles in a large set of sequenced fungi, including white- and brown-rots, plant and animal parasites, and an ectomycorrhizal symbiont showed that the CAZyme profiles deviate from species phylogeny. The total CAZyme repertoire for *A. bisporus* is similar to that of white- and brown-rot basidiomycetes (Figure S2) rather than the more closely taxonomically related *C. cinerea* and *L. bicolor*. This pattern likely reflects the adaptation of *A. bisporus* to PCW polysaccharide-rich leaf litters (Sariyildiz & Anderson, 2005). The up-regulation of transcripts with high similarity to PCW-degrading GHs (e.g., GH5, GH6, GH7, GH12, AA9 (formerly GH61), GH105) from basidiomycetes implicated in wood decay confirms that *A. bisporus* has the generic potential to break down PCW polymers by deploying a complete suite of enzymes degrading crystalline cellulose and xylans (Figure S1, S6, and Table S3). Notably, families GH6 and GH7 (Figure S6A), which include cellobiohydrolases that are involved in the attack of crystalline cellulose (Coughlan & Hazlewood, 1993; Baldrian & Valaskova, 2008), are present in all white-rot lineages and *A. bisporus*, but they are absent in brown-rot lineages (except Boletales) and *L. bicolor* (Figure S1).

Profiling of CAZyme transcripts from mycelium growing in compost demonstrated that 115 (51%) of GHs, PLs, and CEs present in *A. bisporus* were up-regulated (from 10- to 1,450-fold) in compost, contrasting sharply with only 17% up-regulated transcripts in both the differentiating casing-soil mycelium and the fruiting body (Figure S5A and Table S6). Four genes (two CE5 acetyl xylan esterases and two GH12 cellulases) showed the highest upregulation compared with agar-grown mycelium (Table S6). A rhamnogalacturonyl hydrolase (GH105) was induced 836-fold. Notably, growth on compost was accompanied by the up-regulation of all 16 genes encoding cellulose-binding motif 1-containing proteins. Although significantly up-regulated, the pectin digestion machinery was comparatively less prominent than that for cellulose and xylan (Figure S5A and Table S6). The transcript profile of the mycelium growing on compost is therefore compatible with a prevailing substrate preference for xylan and cellulose, a medium activity on pectin and a slight activity on mannan. This digestion pattern is well-matched to the known composition of grasses and straw (Fry, 1988; Lawther et al., 1995) and sequential changes in carbohydrates during composting and mushroom growth (Iiyama et al., 1994).

Lignin-converting oxidoreductase genes

To gain access to cellulose, wood-decaying white-rots use fungal class II lignolytic peroxidases (PODs) to degrade lignin (Floudas et al., 2012). We searched sequenced Agaricomycotina genomes for 27 gene families encoding oxidoreductases and CAZymes that have been implicated in wood decay (Figure S2). *A. bisporus* has a distinctive pattern that is not seen in white- and brown-rotters, *C. cinerea* and *L. bicolor*. Comparative analysis of the distribution of genes encoding lignolytic PODs [lignin peroxidase, manganese peroxidase (MnP), and versatile peroxidase] revealed that *A. bisporus* retains a limited POD machinery to degrade lignin (Table S7). Of the genes encoding lignolytic PODs, which have been shown to be important for lignin degradation in wood decayers (Baldrian, 2008; Hofrichter et al., 2010; Floudas et al., 2012), only two MnP genes were found in the *A. bisporus* genome. In compost, transcript levels of one of these MnPs (MNP1, JGI ID#221245) were significantly up-regulated relative to agar medium (Figure S5B). Thus, the repertoire of *A. bisporus* lignolytic PODs differs from *P. chrysosporium* and other sequenced white-rot fungi, which feature 6–26 POD genes (Figure S7) (Floudas et al., 2012). The number and types of lignolytic PODs is similar to brown-rot lineages and *C. cinerea* having a single POD (Floudas et al., 2012) (Table S7).

In contrast, the *A. bisporus* genome contains the largest set (24 members) of HTP genes, including aromatic peroxygenases (APOs) and classic chloroperoxidases (CPOs), a significant expansion relative to wood-decay fungi (Figure 3 and Table S7) (Floudas et al., 2012). Typical APOs and CPOs are secreted, versatile enzymes with multiple catalytic activities with organic hydrocarbons and lignin-like aromatic compounds, including peroxidative oxidation, epoxidation, hydroxylation, and oxygen transfer reactions (Hofrichter et al., 2010). Moreover, 16 of these HTP genes were significantly up-regulated in compost relative to defined medium, including an APO-induced 1,492-fold (Figure S5B and Table S8). Several transcripts coding for β-etherases, one of the *A. bisporus* expanding family, were also significantly up-regulated in compost relative to agar medium (Figure S5B). Possibly, the number and expression patterns of *A. bisporus* HTPs and β-etherases are related to the heterogeneous nature of humic-rich lignocellulosic materials, such as compost (Iiyama et al., 1994; Baldrian & Valaskova, 2008). To estimate patterns of duplication and loss of genes encoding HTPs in the organismal phylogeny, we performed gene-tree/species-tree reconciliation analyses using Notung (Vernot et al., 2008). This analysis suggests that the ancestor of the Agaricomycotina possessed six HTP gene copies, and that the number of HTP paralogs has been more or less steady throughout the evolution of most Agaricomycetes (Figure 3). However, an abrupt expansion of HTPs is reconstructed in the lineage leading from the common ancestor of *Agaricus*, *Coprinopsis*, and *Laccaria*, which is reconstructed as having seven HTP gene copies, and *Agaricus*, which has 24 HTP gene copies (Figure 3). Expansion of HTPs could have been an adaption for decomposition of nonwoody plant matter and humic substances in soil, which is a common substrate for species of Agaricaceae.

Substrate complexity may also explain the presence of three compost-induced glyoxal oxidase-encoding genes. These copper radical oxidases are thought to play a role in production of extracellular H_2O_2 from simple aldehydes, such as glyoxal and methylglyoxal, and they are typically associated with class II PODs (Tables S7 and S8). Catalytically distinct from the copper radical oxidases, at least 12 laccases *sensu stricto* were also confidently predicted (Table S7). Three *A. bisporus* laccase-encoding genes were significantly up-regulated (>10-fold, $P < 0.05$) in compost relative to agar medium (Figure S5B and Table S8). Other potential H_2O_2-generating extracellular enzymes include various glucose-methanolcholine oxidoreductases that include a likely aryl alcohol oxidase (JGI ID#185801) and a methanol oxidase (JGI ID#195553). The gene coding for the latter enzyme is highly up-regulated (Figure S5B and Table S8).

Intracellular metabolism of lignin metabolites and related compounds is poorly understood but cytochrome P450s (CYPs) are generally thought to play an important role. The *A. bisporus* genome contains a relatively low number of CYPs (109 genes) (Table S7), but many were highly expressed and regulated. Specifically, six CYP64-encoding genes were up-regulated >10-fold in compost relative to defined medium (Table S8).

Growth and utilization of carbohydrates and an effect of humic acids

A. bisporus var. *bisporus* H97, H39 and their dikaryon U1 and *A. bisporus* var. *burnettii* JB137-s8 were grown on minimal medium containing 25 mM of the carbon sources as indicated in Figure 4. The commercial heterokaryon U1 was similar to H39 on most monosaccharides. U1 and H39 showed more dispersed growth on D-glucose, D-mannose, L-rhamnose, and better growth on D-xylose, L-arabinose and D-galacturonic acid that H97. U1 grew best on D-galactose, while less dispersed growth was observed for H97 and no growth for H39. *A. bisporus* var. *burnettii* JB137-s8 differed significantly from the other strains in that it grew best on D-glucose and D-mannose, with slower growth on D-galactose, D-xylose and L-arabinose, and only residual growth on L-rhamnose. It was not able to grow on D-galacturonic acid (Figure 4).

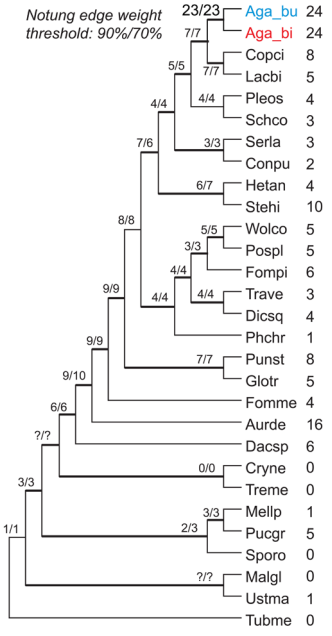

Figure 3 | The expansion of HTPs in *A. bisporus*. HTP copy numbers at internal nodes in the Agaricomycetes as reconstructed by gene-tree species-tree reconciliations in Notung (Vernot et al., 2008) under two edge-weight threshold values. Numbers after species names denote the extant copy numbers of HTPs found in the genomes of these species.

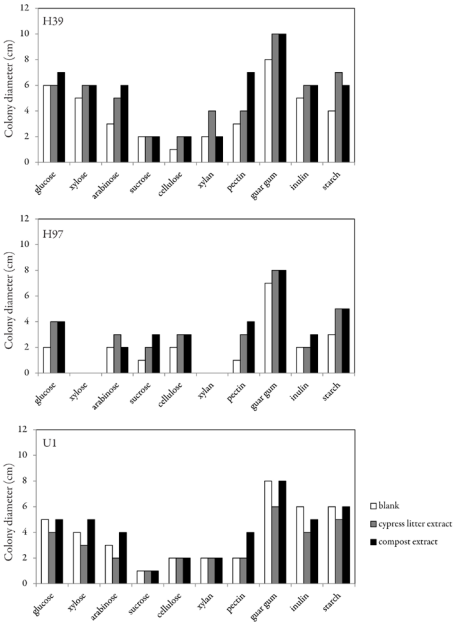

Figure 5 | Growth of *A. bisporus* monokaryons (H39, H97) and dikaryon (U1) on various carbon sources with and without the addition of humic extracts. Growth was quantified by eye independently by two researchers and averaged. Differences between duplicate plates were less than 1 mm.

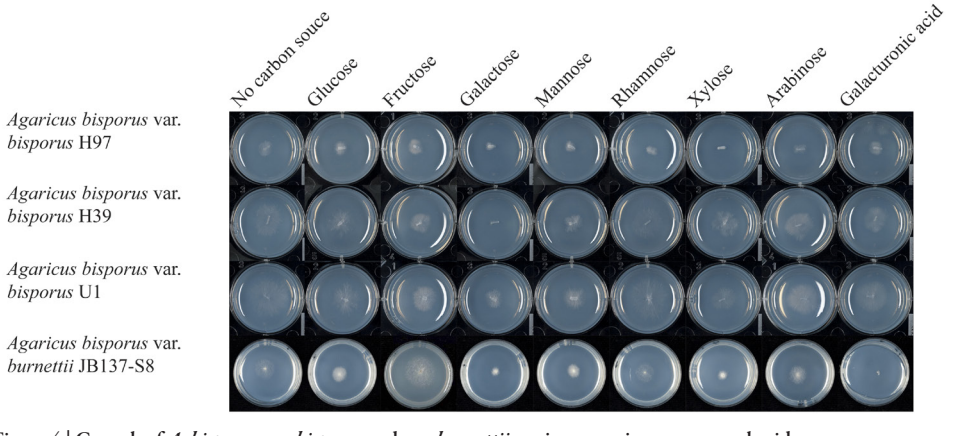

Figure 4 | Growth of *A. bisporus* var. *bisporus* and var. *burnettii* strains on various monosaccharides.

A. bisporus var. *bisporus* H97, H39 and their dikaryon U1, *A. bisporus* var. *burnettii* JB137-s8 and the monokaryotic strain of *C. cinereus* were grown on minimal medium containing 25 mM of monosaccharides and 1% of polysaccharides alone or with 0.1 % of humic extracts. The U1 heterokaryon grew similarly on most monosaccharides and polysaccharides with or without humic extracts, suggesting that the humic extracts do not stimulate or inhibit vegetative mycelial growth under these conditions. On some carbon sources a small reduction in growth was observed in the presence of the cypress litter extract (Figure 5). In contrast, growth of the monokaryons H97 and H39 was stimulated by the humic extracts, but the extent of this depended on the carbon source that was available. *A. bisporus* H97 grew better on glucose with addition of humic extracts. Growth of H39 was more dispersed on xylose, arabinose and glucose with pine soil and compost extracts than media without the extracts (Figure 5). Both H97 and H39 grew better on guar gum, pectin, soluble starch and inulin when the extracts were added. This suggests a synergy between the presence of humic extracts and degradation of the hemicellulose analogue guar gum to stimulate colonization of the substrate. Overall the positive effect of the compost extract was stronger than the Cyprus litter extract, which is likely due to the adaptation of the commercial *A. bisporus* strains to this substrate.

Growth of *C. cinereus* with or without humic extracts did not reveal any significant differences (data not shown), suggesting that the positive effect of the extracts is not a general phenomenon, but a specific trait of compost-inhabiting fungi.

Conclusions

Our genomic and transcriptomic data suggest that *A. bisporus* has the decay machinery to decompose lignocellulosic material; yet to our knowledge, it has not been shown to decompose wood in nature. Although there are similarities in genome composition, *A. bisporus* fits neither brown rot nor white rot classifications. We hypothesized that a humicolous fungus adapted to growth in a humic-rich environment is atypical of classic wood degrading fungi, and transcriptome expression data support this view. The wide repertoire of HTP, β-etherases, MCO, and CYP450 oxidoreductases and their striking up-regulation in mycelium colonizing compost suggest a broad mode of attack on decaying lignin and related metabolites and an adaptation to challenges posed by complex composts. The large gene repertoire of compost induced CAZymes and oxidoreductases, together with high protein degradation and N scavenging abilities are key features of *A. bisporus* adaptation to humic rich ecosystems. This study reveals genetic and enzymatic mechanisms governing adaptation of *A. bisporus* to a humic rich ecological niche created by primary degradation of plant material, demonstrating the critical role such fungi contribute to soil structure and carbon sequestration.

Acknowledgements

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Supplementary data

All supplementary data can be found at:
http://www.cbs.knaw.nl/index.php/pubphysiology/thesis/618-thesis-aleksandrina-patyshakuliyeva
or obtained from the author.

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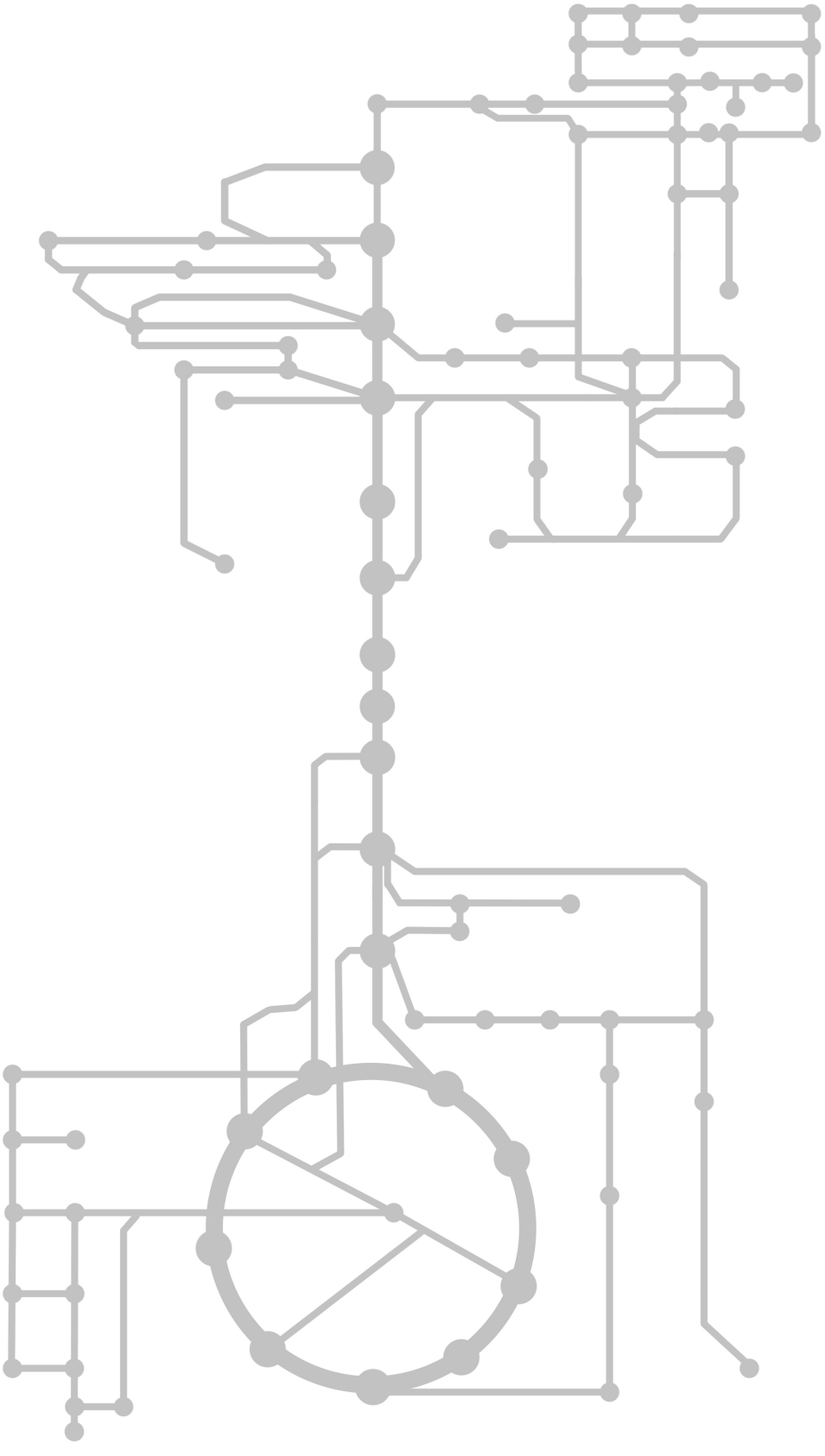

CHAPTER 3

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

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3

AGARICUS BISPORUS IS COMMERCIALLY GROWN ON COMPOST, in which the available carbon sources consist mainly of plant-derived polysaccharides that are built out of various different constituent monosaccharides. The major constituent monosaccharides of these polysaccharides are glucose, xylose, and arabinose, while smaller amounts of galactose, glucuronic acid, rhamnose and mannose are also present.

In this study, genes encoding putative enzymes from carbon metabolism were identified and their expression was studied in mycelium grown compost, casing layer and fruiting bodies 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.

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 & 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 (Tautorus & Townsley, 1984; Fermor et al., 1985).

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 (Lynch, 1993; Lawther et al., 1995; 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 (de Vries & Visser, 2001; Coutinho et al., 2009). 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 (Gerrits, 1969; Fermor et al., 1991; 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 & 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 (Wells et al., 1987; Claydon et al., 1988). Lignin degradation by *A. bisporus* decreases towards the end of the mushroom production cycle (Waksman & Nissen, 1932; Wood & Leatham, 1983; Durrant et al., 1991).

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 (Timell, 1967; Hayashi, 1989; de Vries & Visser, 2001), 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 & 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 & 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 (Hammond, 1981, 1985; Wannet et al., 1999; Beecher et al., 2001). Mannitol functions as an osmolyte, which accumulates to high levels during fruiting body growth while after sporulation the level of mannitol decreases rapidly (Hammond & Nichols, 1976). It might also serve as a post-harvest reserve carbohydrate (Hammond, 1981; Wood, 1985; Beecher et al., 2001). 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; Wells et al., 1987; Wannet et al., 1999).

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.

Materials and Methods

Materials used

Compost, casing layer and fruiting bodies (will be referred to as "tissues") cultures were harvested at the first flush stage of *A. bisporus* strain A15 and were stored at -20 °C. Samples (about 100 g) were collected, freeze dried and milled (<1 mm) (Retsch 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, Merck or Fluka (Busch, Switzerland).

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.

Analytical and spectrometric methods

Neutral carbohydrate composition

Neutral carbohydrate composition of wheat straw, compost and casing layer was analysed according to Englyst (Englyst & Cummings, 1984) using inositol as an internal standard. Samples were treated with 72% (w/w) H_2SO_4 (1h, 30°C) followed by hydrolysis with 1M H_2SO_4 for 3h at 100°C and the constituent sugars released were derivatised and analyzed as their alditol acetates using gas chromatography (GC). The amount of neutral carbohydrates was corrected for mannitol, sorbitol and trehalose.

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 & Asboe-Hansen, 1973) using an autoanalyser (Skalar Analytical BV, Breda, The Netherlands). Glucuronic acid was used as a reference.

Lignin content

Samples of wheat straw, compost and casing layer were analyzed 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-hydrolysed 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 Group GmbH, 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.

Protein content

Nitrogen content of wheat straw, compost and casing layer was analyzed 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).

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.

Chromatographic methods

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, and the mobile phase consisted of (A) 0.1 M NaOH, (B) 1 M NaOAc in 0.1 M NaOH and (C) H_2O. The elution profile was as follows: 0-40 min 100% C; 40.1-45.1 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 0.5 M NaOH at 0.1 ml/min 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, and the mobile phase consisted of (A) 0.1 M NaOH, (B) 1 M NaOAc in 0.1 M NaOH and (C) H_2O. The elution profile was as follows: 0-5 min 100% A, 5-25% 0-30% B, 25.1-30 min 100% B, 30-50 min 100% A.

Water soluble oligosaccharides were separated with a combination of linear gradients from two types of eluents, A: 0.1 M NaOH and B: 1 M NaOAc in 0.1 M NaOH. The elution profile was as following: 0-35 min: 0-38% B, cleaning step 3 min 100% B 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 I 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.

Organic acid analysis

Oxalic acid and citric acid were determined with an Ultimate system (Dionex, Sunnyvale, 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 (Voragen et al., 1986). Elution was performed by using 5 mM H_2SO_4 as eluent at a flow rate of 0.6 ml min^{-1} at 40 °C.

Esterified acetic acid content

Samples of compost and casing layer (20 mg) were saponified with 1 ml of 0.4 M 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 5 mM H_2SO_4 as eluent at a flow rate of 0.6 ml min^{-1} at 40°C. The level of acetic acid substituents was corrected for the free acetic acid in the sample.

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. *burnettii* (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 *Aspergilli*, *P. chrysosporium* or *P. placenta*.

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 pasteurised compost was oven dried for 48 h at 80 °C. Dried compost was boiled in distilled water (7.5 g / l) 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 & 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).

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 randomised 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). 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 3um). 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.

Results

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 (Table S1).

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. 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 (Table S2).

Glycolysis and 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 1, Figure S1 and S2). In contrast, the gluconeogenic gene encoding phosphoenolpyruvate carboxykinase (PEPCK) was 8-fold upregulated in fruiting bodies.

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) (Figure 1, Figure S1 and S2). There is no consistent effect on either the oxidative or the non-oxidative part of the PPP.

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 (Table S2). An exception was the putative L-xylulose reductase encoding gene that had reduced expression levels in compost and casing compared to plate-grown mycelium.

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 2). Expression of genes from the D-galactose Leloir pathway was similar or elevated in all samples compared to plate-grown mycelium (Table S2). In contrast, nearly all genes of the D-galactose oxido-reductive pathway were upregulated in compost and downregulated in fruiting bodies (Table S2). Most genes from the rhamnose and mannose catabolic pathways (Table S1) (Watanabe et al., 2008) were similar or upregulated in compost, casing layer and fruiting bodies, compared to plate-grown mycelium (Table S2).

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 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 (Table S2). 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.

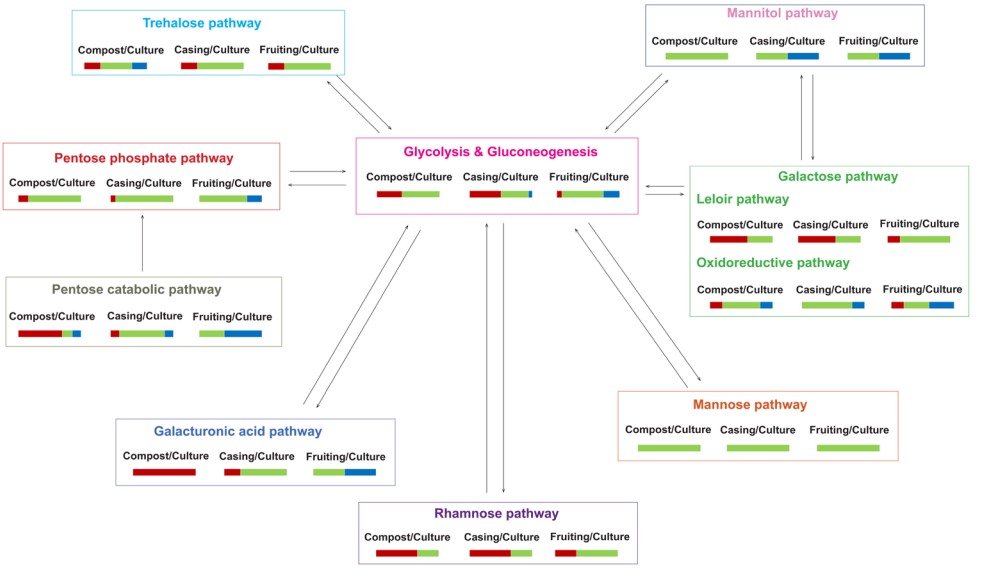

Figure 1 | Schematic representation of the expression of genes of the different carbon metabolic pathways. Bars under the growth stages indicate the percentage of genes that are 2-fold upregulated (red), between 2-fold upregulated and 2-fold downregulated (green), and more than 2-fold downregulated (blue) in the sample compared to culture-grown mycelium.

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.

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 (Table S1), 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*.

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 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.

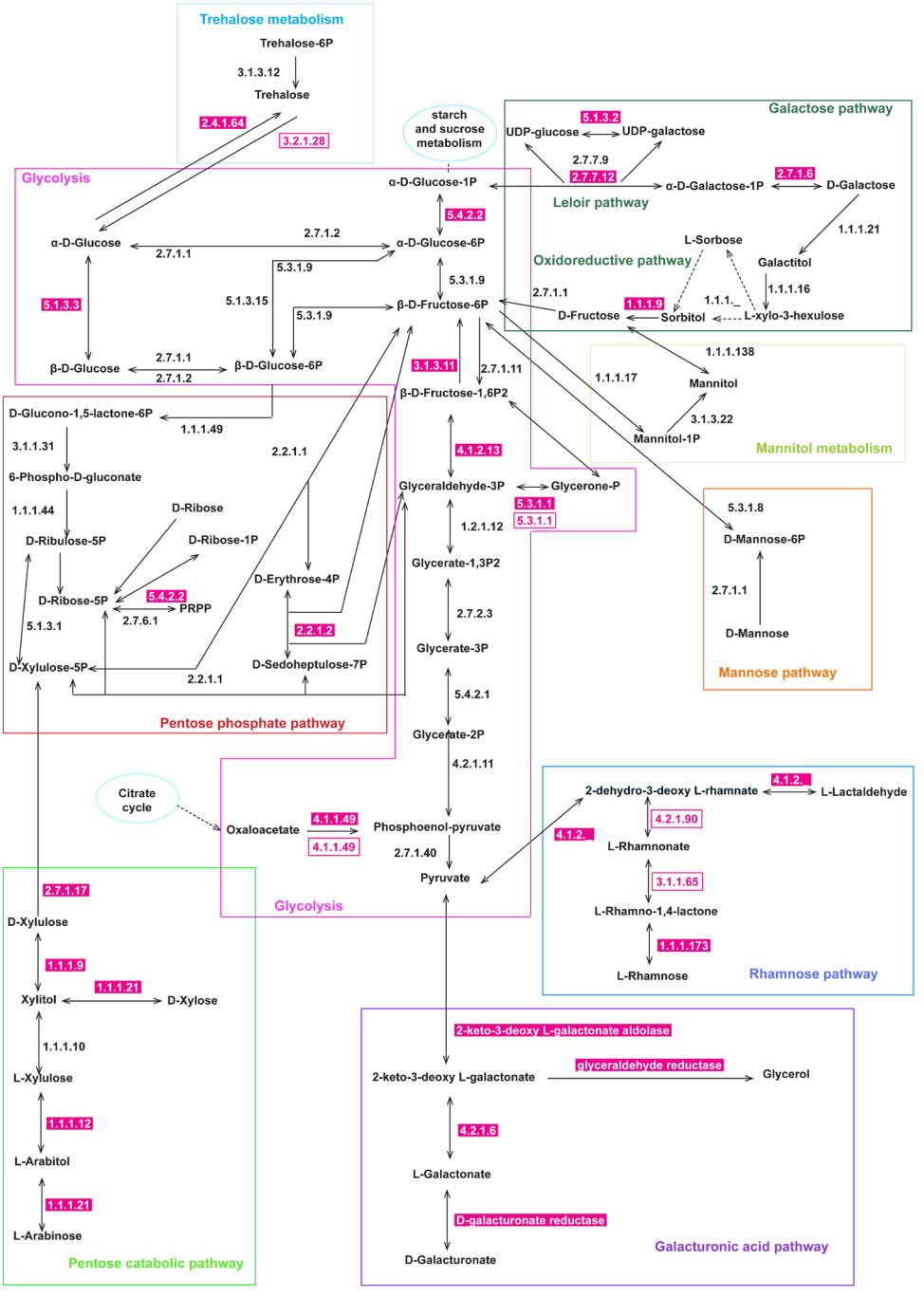

Figure 2 | Map of the central metabolism in *A. bisporus*. Gene products contributing to these pathways are indicated. EC numbers in pink boxes indicate that genes encoding these enzymes are upregulated in compost (white numbers) or fruiting bodies (pink numbers) compared to plate-grown mycelium.

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 tissues. 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 Table S3, 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 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.

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.

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 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 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 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 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 3).

Soluble oligosaccharides were detected in the compost, while none were detected in the casing layer or fruiting bodies (Figure 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 I digest of wheat arabinoxylan in order to identify them (Kormelink et al., 1993). Mainly xylobiose (Figure 3A), xylotriose (Figure 3C), and presumably xylo-oligomers with attached glucuronic acid or its 4-O-methyl ether (Figure 3E) 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 polymerisation and arabinose substituted xylo-oligomers.

Table 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	0.89	4	6.1
Galactose	1.34	3.3	7
Rhamnose	0.8	1.4	2
Glucose	45	47	60
Uronic acids	3.9	8.6	9.2
Acetic acid (mol Ac/100 mol Xyl)	32	12	9

[a]Small part of inorganic material is included.

Table 3 | Concentration (mg/kg) 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

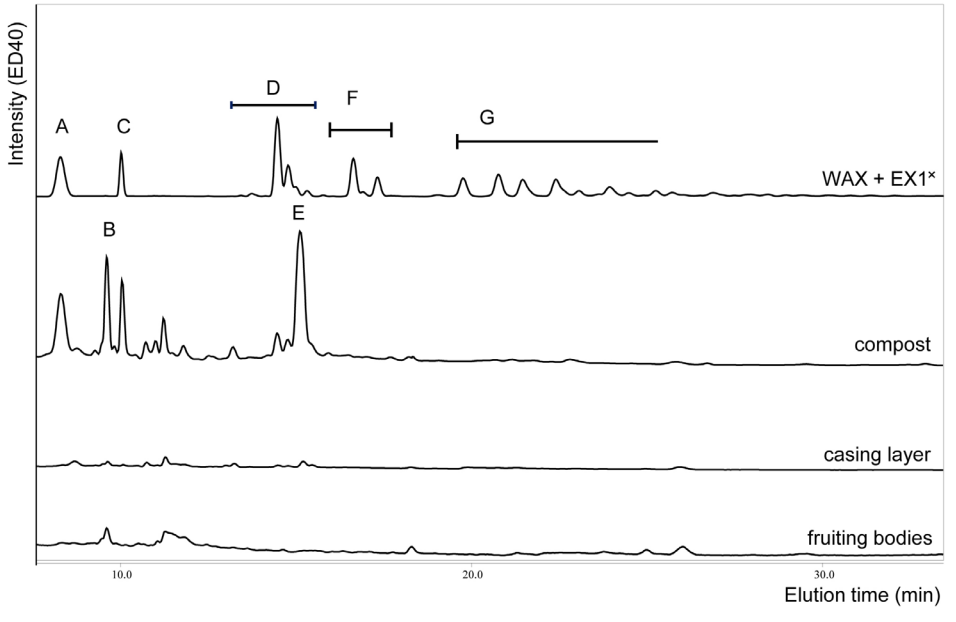

Figure 3 | HPAEC elution patterns of the water soluble fraction of compost, casing layer and fruiting bodies (xylobiose (A), cellobiose (B), xylotriose (C), single substituted xylo-oligomers (D), likely xylo-oligomers with GlcA substituent (E), double substituted xylo-oligomers (F), multiple substituted xylo-oligomers (G). Water extract of fruiting body was diluted 20 times. *WAX + EX1- digest of wheat arabinoxylan and endoxylanase I (Kormelink et al., 1993).

Discussion

In this study, genes encoding carbon metabolic genes were identified in the genome of *A. bisporus* and their expression in mycelium grown compost, casing layer and fruiting bodies was compared to the available carbohydrates and the expression of genes encoding carbohydrate modifying enzymes.

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 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 (van Peij et al., 1998; Hasper et al., 2002). 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 (van Peij et al., 1998; Yuan et al., 2008; Battaglia et al., 2011; Gruben, 2012; Gruben et al., 2012; vanKuyk et al., 2012). 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.

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.

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 in specific tissues. 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 & 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 centre 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 (Figure S3).

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 tissues of *A. bisporus*. This correlates well with the presence of free glucose in all samples, suggesting that hexose catabolism is an important factor in mycelium grown compost, casing layer and fruiting bodies of *A. bisporus*. The PPP has been described as the major route of glucose catabolism in fruiting bodies of *A. bisporus* (Hammond & Nichols, 1976; Hammond, 1977; Hammond & Nichols, 1977) as well as *Lentinula edodes* (Tan & 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* (Schwalb, 1974; Moore & Ewaze, 1976; Chakraborty et al., 2003).

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 & 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 mycelium grown compost, casing layer and fruiting bodies and the expression levels suggest that oxalic acid formation occurs in all tissues. 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 & Coote, 1999), it is tempting to speculate that the accumulation of citric acid in fruiting bodies may also be involved in the defence 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 & Moore, 1995). High expression of isocitrate lyase was also reported in brown-rot fungi, where this enzyme produced succinate and glyoxylate from isocitrate (Munir et al., 2001; Martinez et al., 2009). Progressive downregulation of this gene was observed in the casing layer during the shift from vegetative mycelium to fruiting body (Eastwood et al., 2013).

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 mycorrhiza *L. bicolor* obtains carbon from its symbiotic partner in the form of sucrose, placing a much lower demand on a versatile carbon metabolism.

Conclusions

The data from our study demonstrates that overall there is a clear correlation between expression 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 recognised 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 (Wells et al., 1987; Claydon et al., 1988; Burton et al., 1994).

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 mycelium grown compost, casing layer and fruiting bodies 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

All supplementary data can be found at:
http://www.cbs.knaw.nl/index.php/pubphysiology/thesis/618-thesis-aleksandrina-patyshakuliyeva
or obtained from the author.

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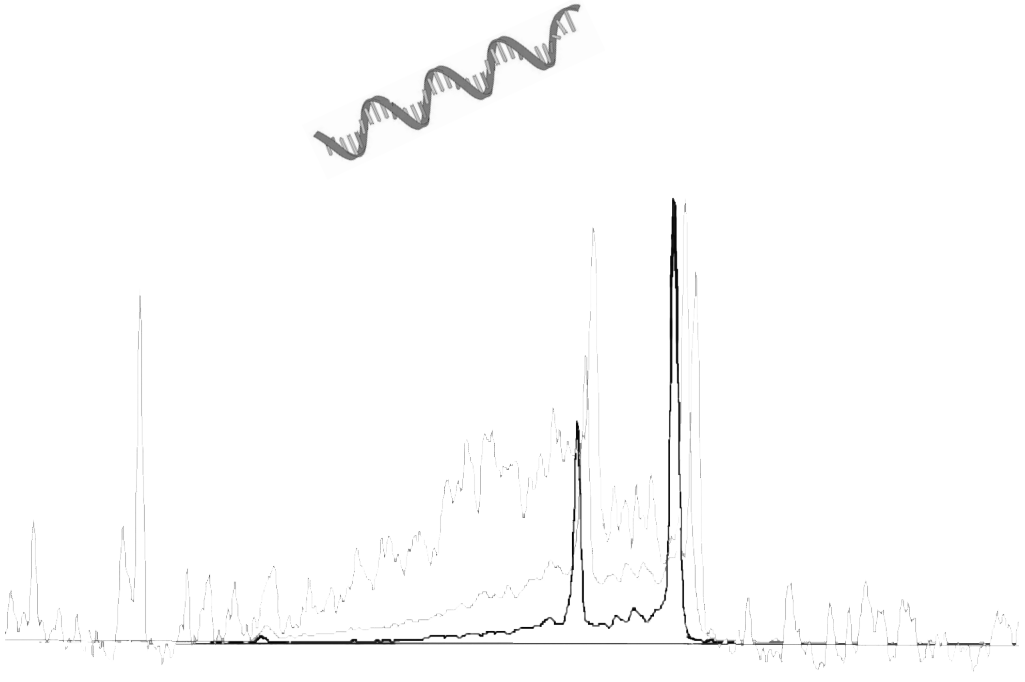

CHAPTER 4

An improved and reproducible protocol for the extraction of high quality fungal RNA from plant biomass substrates

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ISOLATION OF HIGH QUANTITY AND QUALITY RNA is a crucial step in the detection of meaningful gene expression data. Obtaining intact fungal RNA from complex lignocellulosic substrates is often difficult, producing low integrity RNA which perform poorly in downstream applications. In this study we developed an RNA extraction method using CsCl centrifugation procedure, modified from previous reports and adapted for isolation of RNA from plant biomass. This method provided high level of integrity and good quantity of RNA which were suitable for reliable analyses of gene expression and produced consistent and reproducible results.

Introduction

Lignocellulose is the most abundant, renewable and sustainable organic carbon source on earth which makes it an attractive resource for biofuel production as well as a large variety of chemicals and biomaterial (Olsson et al., 2004; Lin & Tanaka, 2006; Himmel & Picataggio, 2009). Lignocellulose is a structural component of plant cell walls which makes up the main fraction of crop residues, forest litter, animal manures or by-products from agriculture, industries but also domestic garbage. Lignocellulose is composed of cellulose, hemicellulose and lignin together with pectin and structural proteins which form a tight and a very complex structure (Martinez et al., 2005; Dashtban et al., 2009).

A diverse set of enzymes is required for complete hydrolysis of lignocellulose, with different activities targeting the variety of chemical bonds in this complex substrate (Dashtban et al., 2009). Filamentous fungi play a key role in degradation of plant cell wall compounds in nature: in soil, compost, forest litter etc. and in the recycling of carbon back into the ecosystem (Lim, 2004; Lundell et al., 2010). They produce a high number and a broad variety of extracellular enzymes that have different catalytic activities and act synergistically (Sánchez, 2009; Chundawat et al., 2011).

Advances in molecular technologies such as proteomics and transcriptomics have enabled the identification of these enzymes or their transcripts and provide insights on how these fungi use their enzymes to efficiently degrade the substrate. However, it remains highly challenging to extract fungal RNA from complex substrates such as compost, soil and forest residues due to the low yields and integrity of RNA, poor reproducibility and co-extraction of humic acids and other organic substances, which can inhibit downstream analyses such as detection and measurement of nucleic acid, PCR amplification, hybridization and RT-PCR (Kreader, 1996; Mendum et al., 1998; Alm et al., 2000; Bachoon et al., 2001; Wang & Fujii, 2011).

For example, extracted RNA from the commercially grown white button mushroom *Agaricus bisporus* cultivated in composted mixture is frequently contaminated with humic substances that are dark in colour, resistant to degradation (Aiken et al., 1985) and interfere with many molecular biological analyses, as even small amounts of humic compounds may affect the detection of gene expression. The main issue with isolating RNA from the wood-decaying white rot basidiomycete *Dichomitus squalens* is high content of endogenous RNases in fungal cells, which makes the extraction of intact RNA a very complicated process.

Here, we describe an efficient method for isolating high-quality RNA from diverse substrates including compost, straw, spruce and microcrystalline cellulose (Avicel). RNA was extracted from different stages of development of *A. bisporus* and from *D. squalens* grown on three different substrates. This method enabled the reproducible extraction of high yield, purity and integrity RNA not only from various substrates but also from different fungal species and developmental stages.

Materials and Methods

Fungal strains, culture conditions, and sample collection

Fungal samples used for RNA extractions in this study came from different sources. *A. bisporus* strain 219 30P (Sylvan, USA) was grown in compost, which is based on wheat straw, horse and chicken manure, gypsum and water, according to commercial practice at the CNC (Coöperatieve Nederlandse Champignonwekersvereniging, Milsbeek, the Netherlands). Mycelium colonized compost samples were harvested at the primordial stage (the first primordia became visible) and at

the first flush of mushroom production. Collected samples were immediately frozen and stored at -20 °C until being processed.

D. squalens FBCC312 was obtained from the Fungal Biotechnology Culture Collection (FBCC), Department of Food and Environmental Sciences, University of Helsinki (email: fbcc@helsinki.fi), and the fungus was maintained on 2% malt agar plates (2% (wt/vol) malt extract (Biokar, France), 2% (wt/vol) agar (Biokar, France). For the fungal inoculum, *D. squalens* was cultivated in 250 ml Erlenmeyer flasks containing 75 ml liquid low-nitrogen aspargine (LN-AS) medium, pH 4.5 (Hatakka & Uusi-Rauva, 1983), with 0.05% (v/v) glycerol (VWR, France) as a carbon source. LN-AS cultures were inoculated with three mycelium-covered plugs (Ø 7 mm) from 2% malt agar plates and incubated stationary at 28°C for 7 days. Solid-state cultures contained 2 g (dry weight) of Norway spruce (*Picea abies*) wood sticks or wheat (*Triticum aestivum*) straw (2 cm pieces in length) on top of the 1% (wt/vol) water agar. Moisture content of the cultures was adjusted to 60% with sterile water. The cultures were inoculated with 2 ml of homogenised *D. squalens* mycelium (Mäkelä et al., 2002) from the LN-AS cultures, and incubated at 28°C for 21 d. Microcrystalline cellulose (Avicel) cultures of *D. squalens* were grown in 250 ml Erlenmeyer flasks that contained 100 ml LN-AS liquid medium supplemented with 1% (wt/vol) Avicel® PH-101 cellulose (Fluka, Ireland) and 0.25 % (w/v) Tween20 (Fluka, Ireland). The Avicel cultures were inoculated with 4 ml of homogenised mycelium from the LN-AS cultures and incubated under agitation (120 rpm) at 28°C for 14 d. The fungal-colonized spruce wood sticks and wheat straw, and the mycelia from the submerged Avicel cultures were snap-frozen in liquid nitrogen and used immediately for RNA extraction.

RNA preparation for CsCl gradient ultracentrifugation

Frozen compost samples (1g of wet weight) and cultures of *D. squalens* (2 g of wet weight) were ground in liquid nitrogen to fine powder by using pre-cooled mortar and pestle. The frozen ground samples were mixed with 10 ml of guanidinium thiocyanate (GIT) extraction buffer (4 M GIT (Amresco, USA); 25 mM sodium citrate (Riedel-de-Haën, Germany), pH 7.0; 0.5 % N-Lauroyl sarcosine (Sigma, USA); 0.1 M β-mercaptoethanol (Sigma, USA)) (Chirgwin et al., 1979), vortexed vigorously (for compost samples) or mixed (for *D. squalens* samples) to ensure that all clumps are dispersed and incubated for 10 min at room temperature. Samples were then centrifuged for 10 min at 4°C and 10000 rpm. The aqueous layer was transferred to another RNAse free tube. All RNA samples extracted from *D. squalens* could be stored at -80°C or directly used for the CsCl gradient centrifugation step. For compost samples the GIT buffer was adjusted to pH 5.0 by using 25 mM sodium citrate (pH 5.0). Compost samples needed additional extraction with an equal volume of chloroform (Sigma, USA) /isoamyl alcohol (Merck, USA) (24:1 v/v), mixed fully by inversion and incubated for 2 min at room temperature. The tubes were centrifuged at 10000 rpm for 15 min at 4°C. The aqueous phase was collected carefully in a new tube and the chloroform/isoamyl alcohol extraction was repeated one more time. After the second chloroform/isoamyl alcohol extraction samples were immediately treated with the CsCl centrifugation procedure (Figure 1).

RNA extraction using a CsCl gradient centrifugation step, quantification and quality analysis of RNA samples

RNA extracts were layered over a 2 ml CsCl solution (5.7 M CsCl (Serva, Germany); 25 mM sodium citrate, pH 5.0) in 13.2 ml polyallomer ultracentrifuge tubes (Beckman-Coulter, Brea, CA, USA) and centrifuged at 33000 rpm for 21 h at 4°C in a Optima L-90K ultracentrifuge, using the SW-41 Ti swinging bucket rotor (Beckman-Coulter, Brea, CA, USA). After centrifugation,

supernatant was carefully removed by pipetting, the tube was inverted and all but the bottom 1 cm was sheared off. The RNA in the clear pellet was rinsed with 100 µl of DEPC-treated water and then dissolved in 50 µl of DEPC-treated water and stored at -80 °C (Figure 1).

Quality and quantity check of all RNA samples were performed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

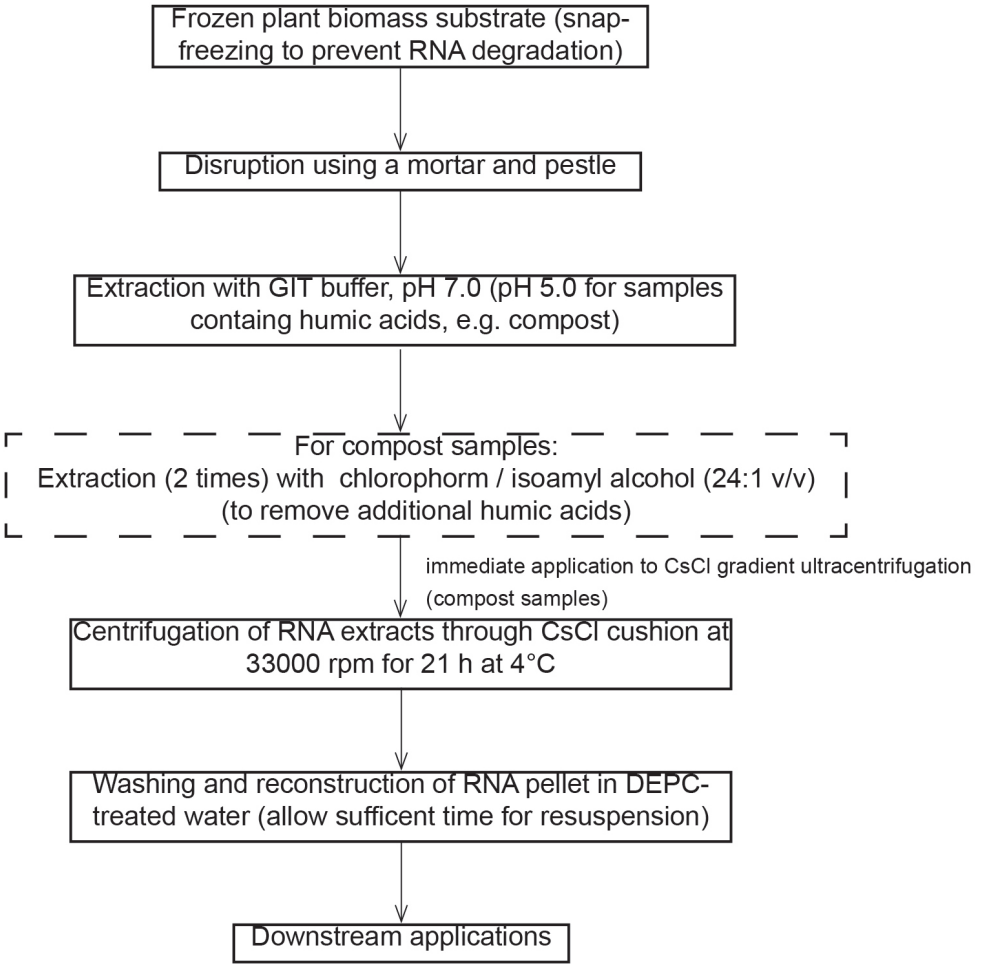

Figure 1 | Flow chart representing the procedure and critical parameters for RNA extraction using CsCl gradient centrifugation.

Quantative RT-PCR (qRT-PCR) for A. bisporus

To assess the performance of the isolated RNA from compost samples, qRT-PCR assay was performed based on the *A. bisporus gpd* gene (glyceraldehyde-3-phosphate dehydrogenase, *A. bisporus* genome Protein ID: 138631). Briefly, 2 µg RNA was converted into cDNA in a 20 µl reaction (ThermoScript™ RT–PCR system, Invitrogen, Carlsbad, USA) according to the instructions of the manufacturer.

The sequences of all primers for qPCR analysis were designed using the Primer Express 3.0 software (Applied Biosystems, Foster City, CA, USA), for the *gpd* gene, the forward

primer was 5'-TCGATCTTGTTGTTCGTCTTGAG-3' and the reverse primer was: 5'-GCGCATGACCTCCTTGATTT-3'. The primers were tested to determine the optimal primer concentrations and efficiency. Combinations of the 50 nM, 300 nM and 900 nM (final concentration) per primer pair were checked and based on the dissociation curve the optimal primer concentration per primer pair was set. For the *gpd* gene, forward/reverse primers concentrations were 900 nM/900 nM with 94 % efficiency.

qPCR was performed using an ABI 7500 fast real-time PCR system (Applied Biosystems, Foster City, CA, USA). 20 µl reactions consisted of 2 µl forward and reverse primers at optimal concentration, 20 ng cDNA sample, 10 µl ABI Fast SYBR Master Mix (Applied Biosystems, Foster City, CA, USA), and water to a final volume of 20 µl. The cycling parameters were 95°C for 20 s, followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. A dissociation curve was generated to verify that a single product was amplified. Control reactions included water only and RNA (i.e. not converted to cDNA to detect residual DNA in the sample). Two biological and three technical replicates were analyzed.

Results and Discussion

The isolation of high-quality RNA and analysis of mRNA transcripts from fungi growing on intricate substrates such as soil, woody and non-woody vegetation and litter is an important step to increase our understanding of the complex processes within fungal communities during growth on these substrates, such as gene regulation of genes encoding lignocellulose-degrading enzymes (Poretsky et al., 2005). However, this has proven a significant challenge while most commonly used methods are often suitable only for specific biomass substrates or specific fungi. Also, reproducibility has often been an issue with existing protocols when used for a wider range of substrates (Mettel et al., 2010; Leite et al., 2012; Wang et al., 2012). In this study, we developed an efficient and reproducible procedure for extracting high-quality RNA from complex substrates that is more widely applicable. As test cases we used two fungi under growth conditions that have proven particularly difficult to obtain high quality RNA from.

The quality and quantity of RNA was significantly improved by CsCl gradient ultracentrifugation procedure

Methods for RNA isolation were performed for different stages of development of *A. bisporus* grown in compost and for the white rot fungus *D. squalens* grown on straw, spruce and microcrystalline cellulose (Avicel). Initial attempts to extract RNA from compost samples had varied success, and several RNA isolation protocols were tested before a reproducible and successful method for each sample was developed. Many standard methods including Trizol reagent and/or guanidine thiocyanate in combination with water-saturated phenol, plant or soil RNA isolation protocols as well as many other commercial kits did not work efficiently or were not reproducible when applied to compost grown mycelium of *A. bisporus* (Table S1, Figure S1) (Chang et al., 1993; Meng et al., 2012; Morin et al., 2012). The main reason for low quality RNA was due to humic substances in compost that interfere with RNA extraction and subsequent downstream applications (Trevors, 1996; England et al., 2001; Sayler et al., 2001; England & Trevors, 2003). Most of the standard procedures produced dark coloured pellets indicating the binding of humic substances to RNA therefore making the pellets very hard to dissolve in water, and resulting in low yields of impure RNA (Figure S1). Column purification of these RNA samples was usually unsuccessful in removing the

humic substances and often leads to poor RNA quantity and integrity or in some cases a complete loss of RNA (Figure S1). The method described here efficiently removed most humic substances and resulted in a good quantity of high-quality RNA (Figure 2).

The best protocol to obtain high purity RNA from compost samples was to use the GIT extraction buffer at pH 5.0 as it was shown that the amounts of co-extracted humic acids in raw extracts prepared under low pH conditions were much lower than those prepared under high pH conditions (Mettel et al., 2010). Including chloroform/isoamyl alcohol clean-up steps into the procedure greatly purified RNA extracts and decreased the presence of humic substances. Further, it was important not to freeze extracted compost samples but to proceed immediately to the CsCl gradient ultracentrifugation step (see Materials and Methods). In contrast, RNA isolated from *D. squalens* in the GIT extraction buffer (pH 7.0) could be stored at -80°C. *D. squalens* produces RNases very fast under stress conditions (Mäkelä MR & Hildén KS, unpublished results) which interfere with RNA isolation. Therefore, the crucial step in RNA extraction from *D. squalens* was preventing RNA degradation by immediately snap-freezing harvested mycelium, grinding under liquid nitrogen and subsequently addition of the GIT extraction buffer. The CsCl ultracentrifugation step is responsible for separation of RNA from endogenous contaminants, proteins, small molecules, coloured aromatic compounds derived from solid lignocellulose and most importantly, humic substances, resulting in the improved RNA integrity (Vares et al., 1995).

Using these modifications high-quality RNA suitable for downstream applications such as qRT-PCR and transcriptomics was obtained (all samples were used in RNAseq analysis for different projects). The quality and quantity of all RNA samples was determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) as it was shown that NanoDrop ND-1000 spectrophotometry (NanoDrop Technologies, Welmington, DE, USA) cannot be used to quantify the RNA content, due to the overlapping absorption spectra of humic acids and RNA (Mettel et al., 2010). CsCl ultracentrifugation resulted in high RNA concentrations, with RNA isolated from compost samples having the highest mean level of RNA (1780 ng/µl) (Figure 2). The CsCl extraction yields RNA with much higher quantity compared to other RNA isolation techniques as this method is based on both chemical and physical extraction which separates biomolecules due to their density resulting in obtaining RNA as a pellet because there is no attainable CsCl concentration at which RNA will bind (Kurland, 1960). Generally pelleting occludes other components of a mixture but since RNA is likely to be the most dense component in this case the pellet will represent highly purified RNA (Kurland, 1960). It is critical to allow enough time for resuspension of this pellet or the yield of RNA will be reduced.

RNA molecules are thermodynamically stable but can be degraded by RNases present in the sample, which can result in short RNA fragments that can interfere with downstream applications (Schroeder et al., 2006). Agilent (Agilent Technologies, Santa Clara, CA, USA) has developed software to calculate an RNA integrity number (RIN), a qualitative assessment of RNA quality (Schroeder et al., 2006). Electropherograms of all extracted RNA show intact 18S rRNA and 28S rRNA with 6 and higher RIN values (Figure 2). The 28S rRNA/18S rRNA ratios for RNA isolated from all compost samples were slightly higher than 2.0, which is normal for intact RNA.

For RNA extracted from spruce, straw and Avicel samples the 28S rRNA/18S rRNA ratio was computed to be close to zero. The 28S rRNA/18S rRNA ratio is calculated automatically and it was observed that by using the biosizing software the ribosomal ratios assessments are compromised by the fact that their calculation is based on area measurements and therefore strongly dependant on definition of start and end points peaks (Imbeaud et al., 2005). Due to this lack of reliability, rRNA ratios may not be used as a gold standard for assessing RNA integrity (coefficient variation (CV) 19-24%) (Imbeaud et al., 2005; Fleige & Pfaffl, 2006; Schroeder et al., 2006). This indicates that

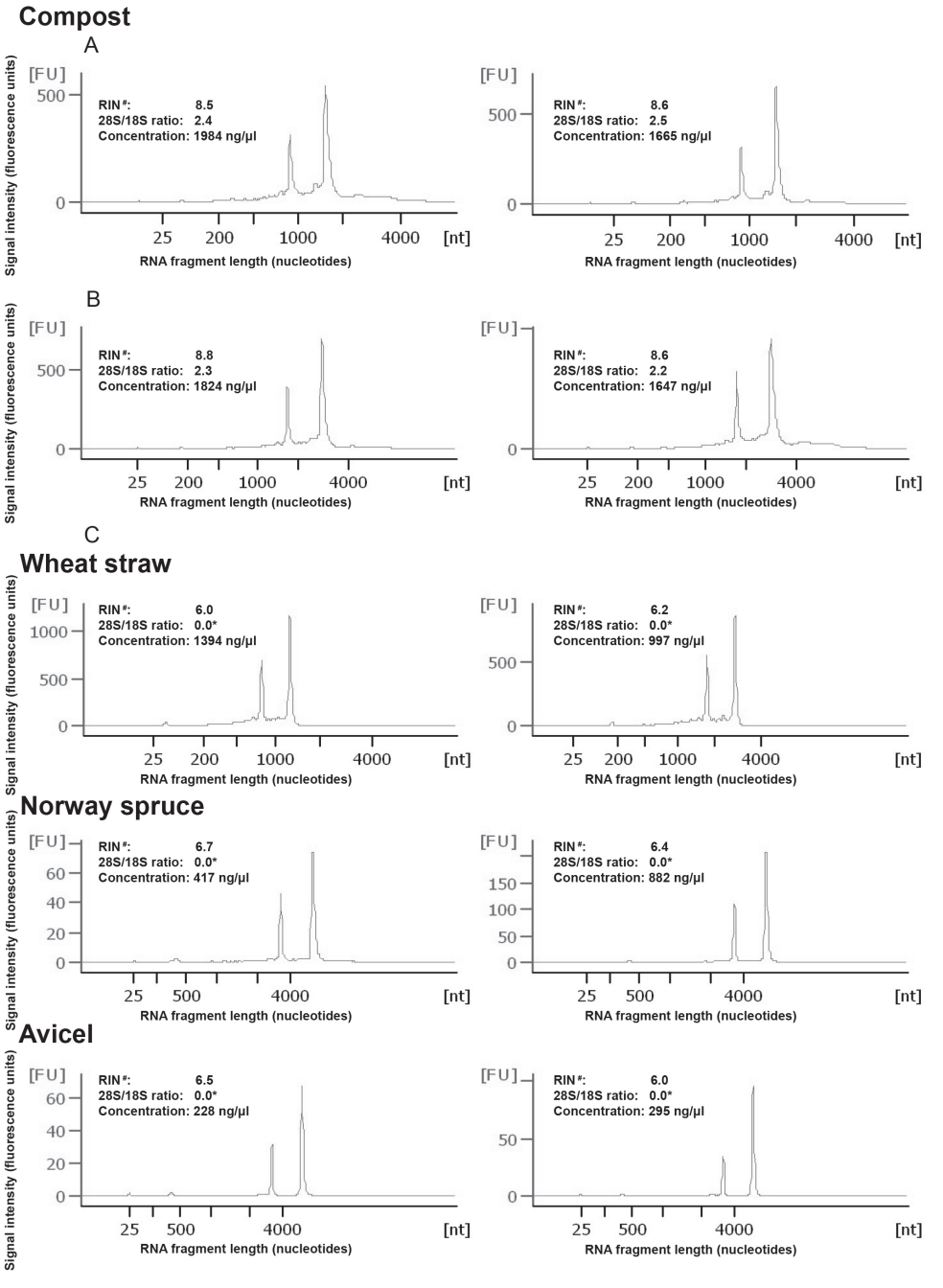

Figure 2 | Chromatograms of microcapillary electrophoresis from RNA samples. A: RNA isolated from *A. bisporus* grown in compost (primordial stage of development). B: RNA isolated from *A. bisporus* grown in compost (first flush). C: RNA isolated from mycelium of *D. squalens* grown on wheat straw, Norway spruce and Avicel. All RNA samples are presented in two biological replicates. #RIN = RNA Integrity Number. *This value is not reliable, see explanation in Results and Discussion.

total RNA with no or low 28S rRNA/18S rRNA ratios is not necessarily of poor quality especially if the electropherogram shows no evidence of degradation (e.g. no increased baseline signal between ribosomal bands or below 18S rRNA band) (Imbeaud et al., 2005).

The CsCl method may provide benefits for downstream applications

RT-qPCR is an important tool for quantification of the changes in gene expression and depends on the use of intact RNA; the presence of humic substances or high levels of DNA in RNA extracts may strongly compromise the results of downstream procedures which are often labour-intensive, time consuming and highly expensive (Fleige et al., 2006). To evaluate whether the RNA obtained by CsCl gradient ultracentrifugation procedure was suitable for downstream applications, RNA isolated from different developmental stages of *A. bisporus* grown in compost was used for qPCR analysis. As expected, *gpd*, a constitutively expressed house-keeping gene, was successfully amplified and Ct values were determined (Figure 3A). Particular attention was paid to verify that DNA was not present in the RNA preparations by performing qPCR on RNA samples without reverse transcriptase reaction. Both negative controls (no-template and no-RT) showed no PCR products (Ct values were not obtained). Moreover, the amplification the *gpd* gene of different cDNA concentrations of primordial stage and first flush of *A. bisporus* showed high efficiency (99.4 % and 101.5 %, respectively) which confirms sufficient quantity of high-quality RNA and indicates that the assay gave reproducible results regardless of the input amount of cDNA (Figure 3B).

In conclusion, this study presents a reliable, routinely reproducible and efficient method for RNA extraction based on CsCl gradient ultracentrifugation, that resulted in high quality RNA from *A. bisporus* (various stages of development) and *D. squalens* grown on diverse complex substrates (compost - *A. bisporus*; straw, spruce and Avicel - *D. squalens*). The extracted RNA had high integrity and quantity and performed excellent in RT-qPCR assay of *A. bisporus*, indicating sustainability and suitability for downstream applications. CsCl ultracentrifugation seems to meet all experimental requirements as a method of choice for isolating RNA free of humic acids, proteins, DNA, polysaccharides, and other components and can easily be adapted for isolating RNA from other plant biomass substrates and other fungi.

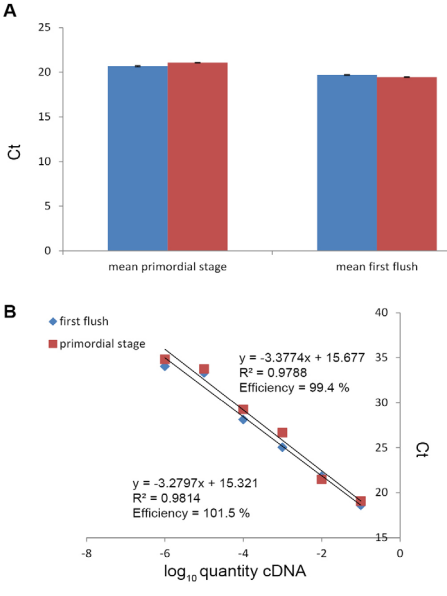

Figure 3 | qRT-PCR analysis of gpd gene of two different stages of development of *A. bisporus* (primordial stage and first flush). The assay was reproducible and consistent, two biological replicates were tested, and standard deviation was calculated with three technical replicates (A). Calibration curves show normalization and efficiency of the assay (B).

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Supplementary data

All supplementary data can be found at:
http://www.cbs.knaw.nl/index.php/pubphysiology/thesis/618-thesis-aleksandrina-patyshakuliyeva
or obtained from the author.

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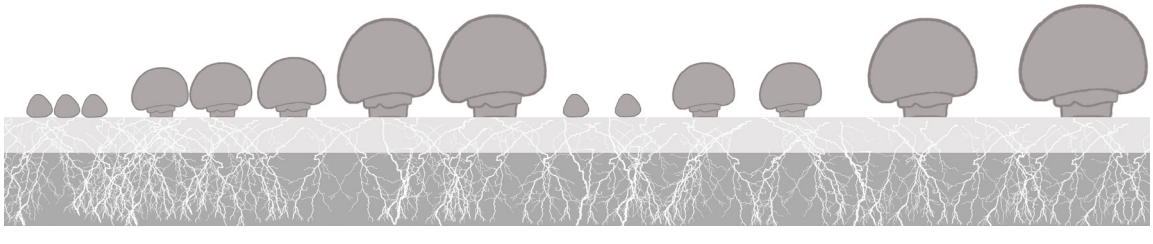

CHAPTER 5

Uncovering the abilities of *Agaricus bisporus* to degrade plant biomass throughout its life cycle

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THE ECONOMICALLY IMPORTANT edible basidiomycete mushroom *Agaricus bisporus* thrives on decaying plant material in forests and grasslands of North America and Europe. It degrades forest litter and contributes to global carbon recycling, depolymerizing (hemi-)cellulose and lignin in plant biomass. Relatively little is known about how *A. bisporus* grows in the controlled environment in commercial production facilities and utilizes its substrate. Using transcriptomics and proteomics, we showed that changes in plant biomass degradation by *A. bisporus* occur throughout its life cycle. Ligninolytic genes were only highly expressed during the spawning stage day 16. In contrast, (hemi-)cellulolytic genes were highly expressed at the first flush while low expression was observed at the second flush. The essential role for many highly expressed plant biomass degrading genes was supported by exo-proteome analysis. Our data also supports a model of sequential lignocellulose degradation by wood decaying fungi proposed in previous studies concluding that lignin is degraded at the initial stage of growth in compost and is not modified after the spawning stage. The observed differences in gene expression involved in (hemi-)cellulose degradation between the first and second flushes could partially explain the reduction in the number of mushrooms during the second flush.

Introduction

Agaricus bisporus, commonly known as the button mushroom, is a saprotrophic secondary decomposing basidiomycete, mainly occupying ecological niches rich in lignocellulose such as leaf and forest litter, and grasslands, and thus has an important role in carbon recycling. In addition, *A. bisporus* is the most widely produced mushroom for human consumption in the world. Commercially it is cultivated on a variety of organic substrates, such as wheat straw, and horse and chicken manure based compost (Gerrits, 1988). Spawn (*A. bisporus* mycelium which is developed on cereal grain) is introduced into this composted mixture and the fungus starts growing vegetatively while colonizing its substrate (Chang & Miles, 1989). During commercial cultivation, the switch from the vegetative to reproductive stage occurs when fully colonized compost is covered with a non-nutritious "casing" layer (mainly peat and lime). Further distribution of mycelial cords in the casing layer and its aggregation forms primordia which continue to develop into mature mushrooms. After a developed crop (flush) is harvested, new flushes appear in a rhythmic mode almost at weekly intervals (Van Griensven, 1988). This controlled environment also provides an ideal system to study the degradation of plant biomass during the different stages of a basidiomycete life cycle.

Despite the ecological and economic importance of button mushrooms, relatively little is known about how it degrades substrate throughout its life cycle. Several studies of the compost composition have shown that it consists mainly of cellulose, hemicellulose and lignin (Gerrits et al., 1967; Iiyama et al., 1994; Jurak et al., 2014) with the major carbohydrate fraction represented by xylosyl and glucosyl residues (Jurak et al., 2014). *A. bisporus* grows well on compost, which correlates with its potential to secrete large amounts of Carbohydrate Active enZymes (CAZy, http://www.cazy.org) (Lombard et al., 2014) to degrade lignocellulose and use released constituents as the carbon source (Gerrits, 1969; Wood et al., 1991; Yague et al., 1997; Hildén et al., 2013).

The complete genome sequence of *A. bisporus* (Morin et al., 2012) facilitated the identification of a full repertoire of genes encoding CAZymes and showed that *A. bisporus* has adapted to the environment where lignocellulose, lignocellulosic-derived material and humic substances are present (Morin et al., 2012). Using a transcriptomics approach of a single time point (the moment when the first flush of mushrooms was produced), recent studies demonstrated a predominant substrate preference for cellulose and xylan based on which genes were expressed during growth of *A. bisporus* on compost (Morin et al., 2012) and the carbohydrate composition of compost (Patyshakuliyeva et al., 2013). Also, there is a difference in the set of CAZyme encoding genes expressed in compost grown mycelium and in fruiting bodies. Genes encoding plant cell wall degrading enzymes were expressed in vegetative mycelium grown in compost and largely absent in fruiting bodies (Patyshakuliyeva et al., 2013). However, genes encoding fungal cell wall modifying enzymes were expressed in both samples but the gene sets were different (Patyshakuliyeva et al., 2013).

Thus far, the above-mentioned studies shed light on the lignocellulose degrading capabilities of *A. bisporus*, its adaptation to humic-rich environment and importance for terrestrial carbon cycling. However, these studies provide mainly snap-shots of the physiology of *A. bisporus* focusing mainly on a transcriptome analysis of only single time point (first flush). Therefore, the study presented here aims to gain a thorough understanding of the carbon nutritive needs of *A. bisporus* and its potential to degrade plant biomass at different stages of its life cycle using the controlled environment of indoor commercial cultivation. This study represents the first temporal transcriptome and secretome analysis of *A. bisporus* in compost as well as composition analysis of the substrate beginning with the vegetative mycelium associated with the spawning stage and

ending with the growth stage when all second flush mushrooms were harvested.

Materials and Methods

Strain and sample collection

A. bisporus commercial heterokaryon strain A15 (Sylvan, USA) was used in this study. The sequenced H97 together with H39 are homokaryons and parental strains of the commercial strain U1. Commercial mushroom strain A15 is a clone of U1 and developed by clonal vegetative propagation, or quasi-clones derived from spores which retain the great majority of the parental genotype (Kerrigan et al., 1993). A15 shares a single basic genotype with U1. Phase I and II mushroom compost, which is based on wheat straw, horse and chicken manure, gypsum and water, were prepared according to commercial practice at CNC-C4C (Milsbeek, the Netherlands). Phase II compost was inoculated with *A. bisporus* spawns (Sylvan, USA) and incubated at 25°C for 16 days. Fully colonized compost was covered with casing layer (peat and lime) according to established commercial practice and incubated until first and second flushes of mushrooms were developed and harvested.

Mycelium colonized compost samples were collected at the different growth stages of *A. bisporus* (Table 1). Compost samples were taken in duplicate for each growth stage. All samples were immediately frozen and stored at -20°C until being processed for transcriptome, secretome, carbohydrate composition analysis and pyrolysis.

Table 1 | Growth stages of *A. bisporus* mycelium colonized compost used in this study.

Compost sample	Description	Days after spawns were introduced into Phase II compost
Spawning stage d16	Compost at the end of the spawning stage	16
Pinning stage	Compost when pinning has clearly started and the first pinheads were visible	30
1st flush	Compost of the 1st flush, pinheads have become harvestable mushrooms	39
After 1st flush	Compost after all 1st flush was harvested	40
2nd flush	Compost of the 2nd flush, pinheads have become harvestable mushrooms	45
After 2nd flush	Compost after all 2nd flush was harvested	48

RNA extraction, cDNA library preparation, and RNA-seq

Total RNA was isolated from the collected compost samples using a CsCl gradient centrifugation method (Patyshakuliyeva et al., 2014). RNA integrity and quantity were checked with the RNA6000 Nano Assay using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). cDNA library preparation and sequencing reactions were conducted in the BGI Tech Solutions Co., Ltd. (Hong Kong). Illumina library preparation, clustering, and sequencing reagents were used throughout the process following the manufacturer's recommendations (http://illumina.com). Briefly, mRNA was purified using poly-T oligonucleotide-attached magnetic beads and then fragmented. The first and second strand cDNA was synthesized and end repaired. Adaptors were ligated after adenylation at the 3' end. After gel purification, cDNA templates were enriched by PCR. cDNA libraries were validated using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and quantified by qPCR. Single-Read samples were sequenced using Illumina HiSeq™ 2000 platform (http://illumina.com). In average 51 bp sequenced reads were constituted, producing approximately 450MB raw yields for each sample.

RNA-seq data analysis and functional annotation

Raw reads were produced from the original image data by base calling. After data filtering the adaptor sequences, highly "N" containing reads (>10% unknown bases) and low quality reads (more than 50% bases with quality value <5%) were removed. After data filtering, in average ~95% clean reads remained in each sample, the average amount of clean reads was 11.84M. Clean reads were then mapped to the genome of *Agaricus bisporus* var *bisporus* (H97) v2.0 (Morin et al., 2012) using SOAPaligner/SOAP2 (Li et al., 2009). No more than 2 mismatches were allowed in the alignment. In average 76.44% total mapped reads to the genome was achieved. The gene expression level was calculated by using RPKM method (Mortazavi et al., 2008). Genes with expression value higher than 300 were considered highly expressed (approximately top 5%, Table S1) and differential expression was identified by CyberT bayesianANOVA algorithm (Kayala & Baldi, 2012) with a cutoff value of fold change >1.5 and p-value (corrected by multiple tests) <0.05. The RNA-seq data has been submitted to GEO (Edgar et al., 2002) with accession number: GSE65800.

Gene sequences were aligned to NCBI non-redundant (Nr) and InterPro database (Mitchell et al., 2015) for functional annotations using BLASTX (Mount, 2007) with cutoff value of 1E-20. Information on Gene Ontology (GO) (Ashburner et al., 2000), Cluster of Orthologous Groups (COG) (Tatusov et al., 2003) and Kyoto Encyclopaedia of Genes and Genome (KEGG) (Kanehisa & Goto, 2000) were downloaded from JGI (http://genome.jgi-psf.org/Agabi_varbisH97_2/) using default settings. Proteins with putative signal peptide were predicted by SignalP 4.0 (Petersen et al., 2011). The annotations were then mapped to the expressed genes accordingly.

Quantitative RT-PCR (qRT-PCR) validation

RNA samples for RNA-seq experiments were also used for qRT-PCR validation of genes with varying transcript abundance. Briefly, 2 μg RNA was converted into cDNA in a 20 μl reaction (ThermoScript™ RT–PCR system, Invitrogen, Carlsbad, USA) according to the instructions of the manufacturer.

The sequences of all primers for qRT-PCR analysis were designed using the Primer Express 3.0 software (Applied Biosystems, Foster City, CA, USA). The primers were tested to determine the optimal primer concentrations and efficiency. Combinations of the 50 nM, 300 nM and 900 nM (final concentration) per primer pair were checked and based on the dissociation curve the optimal primer concentration per primer pair was set. The primer sequences and optimal concentrations of

the tested genes and the reference gene are listed in Table S2.

qRT-PCR was performed using an ABI 7500 fast real-time PCR system (Applied Biosystems, Foster City, CA, USA). 20 µl reactions consisted of 2 µl forward and reverse primers at optimal concentration, 20 ng cDNA sample, 10 µl ABI Fast SYBR Master Mix (Applied Biosystems, Foster City, CA, USA), and water to a final volume of 20 µl. The cycling parameters were 95°C for 20 s, followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. A dissociation curve was generated to verify that a single product was amplified. Transcript levels were normalized against the *gpd* gene (glyceraldehyde-3-phosphate dehydrogenase, *A. bisporus* genome Gene ID: 138631) expression and quantified according to the formula $2^{-(Ct\ gene\ X - Ct\ gpd)}$ (Livak & Schmittgen, 2001). Control reactions included water only and RNA (i.e. not converted to cDNA to detect residual DNA in the sample). Two biological and three technical replicates were analyzed.

Secretome extraction

For secretomic analysis, collected mycelium grown compost (10 g) samples (Table 1) were mixed with distilled water (100 ml) in 250 cm^3 Erlenmeyer flasks. All the flasks were placed in an incubator shaker at 200 rpm for 1 h at 4°C. The samples were then centrifuged at 10000 rpm for 15 min and the supernatant was concentrated 4x using a vacuum concentrator Speedavac (Savant Instruments, Inc., Farmingdale, NY).

Protein preparation and analysis by mass spectrometry

Protein separation and digestion

30 µl of each of the samples were run on a 12% Bis-Tris 1D SDS-PAGE gel (Biorad) for 2-3 cm and stained with colloidal coomassie dye G-250 (Gel Code Blue Stain Reagent, Thermo Scientific). The lane was cut into two bands, which were treated with 6.5 mM dithiothreitol (DTT) for 1 h at 60 °C for reduction and 54 mM iodoacetamide for 30 min for alkylation. The proteins were digested overnight with trypsin (Promega) at 37 °C. The peptides were extracted with 100% acetonitrile (ACN) and dried in a vacuum concentrator (Thermo Scientific, Bremen).

Mass spectrometry: RP-nanoLC-MS/MS

Samples were resuspended in 10% formic acid (FA) / 5% DMSO and 30% of the sample was analyzed using a Proxeon Easy-nLC100 (Thermo Scientific) connected to an Orbitrap Q-Exactive mass spectrometer. Samples were first trapped (Dr Maisch Reprosil C18, 3 um, 2 cm x 100 µm) before being separated on an analytical column (Agilent Poroshell EC-C18, 2.7 µm, 40 cm x 50 µm), using a gradient of 60 min at a column flow of 150 nl min-1. Trapping was performed at 8 µL/min for 10 min in solvent A (0.1 M acetic acid in water) and the gradient was as follows 7- 30% solvent B (0.1 M acetic acid in acetonitrile) in 31 min, 30-100% in 3 min, 100% solvent B for 5 min, and 7% solvent B for 13 min. Nanospray was performed at 1.7 kV using a fused silica capillary that was pulled in-house and coated with gold (o.d. 360 µm; i.d. 20 µm; tip i.d. 10 µm). The mass spectrometers were used in a data-dependent mode, which automatically switched between MS and MS/MS. Full scan MS spectra from m/z 350 – 1500 were acquired at a resolution of 35.000 at m/z 400 after the accumulation to a target value of 3E6. Up to ten most intense precursor ions were selected for fragmentation. HCD fragmentation was performed at normalized collision energy of 25% after the accumulation to a target value of 5e4. MS2 was acquired at a resolution of 17.500 and dynamic exclusion was enabled (exclusion size list 500, exclusion duration 10 s).

Data analysis

Raw files were processed using Proteome Discoverer 1.3 (version 1.3.0.339, Thermo Scientific, Bremen, Germany). Database search was performed using the genome of *A. bisporus* var. *bisporus* (H97) v2.0 (Morin et al., 2012) and Mascot (version 2.4.1, Matrix Science, UK) as the search engine. Carbamidomethylation of cysteines was set as a fixed modification and oxidation of methionine was set as a variable modification. Trypsin was specified as enzyme and up to two miss cleavages were allowed. Data filtering was performed using percolator, resulting in 1% false discovery rate (FDR). Additional filters were; search engine rank 1 peptides and Mascot ion score >20. Raw files corresponding to one sample were merged into one result file. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (Vizcaino et al., 2014) via the PRIDE (Martens et al., 2005) partner repository with the dataset identifier PXD001817.

Pyrolysis and carbohydrate composition analysis of compost

Neutral carbohydrate content and composition was determined in duplicates, as described by Jurak et al. (Jurak et al., 2014). Lignin composition was determined by analytical pyrolysis-GC-MS (Py-GC/MS) method (Jurak, 2015).

Results

Transcriptome of various growth stages of A. bisporus grown in compost under commercial cultivation conditions

High throughput sequencing (RNA-seq) was performed to examine transcriptome of *A. bisporus* grown in compost and the gene expression at different growth stages. The RPKM method (Reads Per Kilobase per Million) (Mortazavi et al., 2008) was used to determine the normalized mRNA abundance. RPKM values were calculated for each of the biological replicates. In this analysis RPKM 300 was taken as the threshold for highly expressed genes. A cutoff of fold change >=1.5 and p-value <0.05 was used to identify differentially expressed genes between all growth stages. The most significant expression difference up to 1000 folds was found when the growth stages were compared to the spawning stage day 16 (Table S3).

Expression of CAZy genes

CAZyme encoding genes represented 3% (376 of 9620 genes) of the total *A. bisporus* genes identified in all growth stages, with genes from the Glycoside Hydrolase families (GHs) representing approximately half (46%) of the total CAZy mRNA (Table S3). The CAZymes encoding genes from the Auxilary Activities (AAs), the Carbohydrate Esterases (CEs) and the Polysaccharide Lyases (PLs) families represented 22%, 9% and 2%, respectively, of the total CAZy mRNA (Table S3).

Among all the growth stages of *A. bisporus* in compost, transcripts predicted to encode cellulose degrading enzymes were generally the most abundant ones (Figure 1 and Table S3). On average, expression of the genes that are involved in degradation of cellulose slightly increased over time from the spawning stage day 16 until the first flush (Table S3). After all the first flush mushrooms were harvested, expression of the genes dropped and then again increased after all the second flush mushrooms were harvested. Exceptions to this profile included one out of six AA9 lytic polysaccharide monooxygenase (Gene ID 196143) encoding genes, which showed the highest

Figure 1 | Visualization of the RNA expression levels, carbohydrate and lignin contents across the growth of *A. bisporus* in compost.

*Sum of arabinosyl, xylosyl, glucuronic acid.

expression during spawning stage day 16 (Figure 1 and Table S3). Highly expressed cellulolytic genes were found during the first flush and during the growth stage when all second flush mushrooms were harvested as compared to the other growth stages. These genes reached an expression level above 300 RPKM and include six AA9 lytic polysaccharide monooxygenases, three GH5_5 and one GH12 endoglucanase, one GH1 β-glucosidase, and one GH6 and one GH7 cellobiohydrolase (Figure 1).

Similar to the expression of cellulolytic genes, transcripts predicted to encode hemicellulose degrading enzymes were most abundant during the first flush and during the growth stage when all second flush mushrooms were harvested and had comparatively low abundance during the other stages of *A. bisporus* growth in compost (Figure 1 and Table S3). Hemicellulolytic genes with RPKM values above 300 are represented by one GH10 and two GH11 endoxylanases, one GH27 α-galactosidase, one GH35 β-galactosidase, one GH5_22 β-xylosidase, one GH51 α-arabinofuranosidase, four GH16 β-1,3-endoglucanses, one GH12 xyloglucan β-1,4-endoglucanase, one GH5_7 endomannanase and one CE1, four CE4 and one CE5 acetylxylan esterases (Figure 1).

RNA-seq analysis revealed that genes encoding CAZymes involved in lignin degradation had their greatest transcript abundance of during spawning stage day 16 and lower abundance in all the other growth stages (Figure 1 and Table S3). Highly expressed ligninolytic genes during the cultivation of *A. bisporus* in compost are two AA1_1 laccases, one AA2 manganese peroxidase, two AA3_2 GMC oxidoreductases (Glucose-Methanol-Choline oxidoreductase), four AA5_1 glyoxal oxidases, and one AA6 1,4-benzoquinone reductase.

RNA-seq expression validation by quantitative RT-PCR (qRT-PCR)

In order to validate the expression profiles obtained by the RNA-seq analysis, twenty CAZyme encoding genes with varying mRNA abundance were tested by quantitative RT-PCR. Expression trends of the selected genes obtained by qRT-PCR confirmed those obtained with RNA-seq data (Figure 2 and Figure S1).

Proteomic analysis of secreted proteins

The time between gene expression and production (and if needed secretion) of the corresponding proteins can differ for individual proteins, and therefore proteomes of the various growth stages were also analyzed. There were 641 proteins identified with 1% false discovery rate (FDR) for all six growth stages together. 168 (26%) of all secreted proteins were identified as CAZymes (Table S4). 96 (57%) of the identified CAZymes were from GH families, while 42 (25%), 17 (10%) and 6 (4%) represent AA, CE and PL families, respectively. Protein abundance was approximated by PSM (Peptide Spectrum Match) values and ranked on this basis.

Several cellulose degrading enzymes were detected in the secretome of *A. bisporus* grown in compost. GH6 and GH7 cellobiohydrolyses as well as GH5_5 endoglucanases were abundantly produced demonstrating high correlation with RNA-seq data (Table 2). But unlike the transcriptome profile which showed a decrease in gene expression at the stage when all the first flush mushrooms were harvested, corresponding CAZymes with cellulolytic activities were still present during that stage (Table 2, Table S3 and S4). These could be the same proteins which were secreted at the earlier first flush stage when corresponding genes were highly expressed. A drop in protein secretion was observed during the second flush followed by an increase at the stage when all the second flush mushrooms were harvested showing a similar trend as was observed for the RNA-seq data.

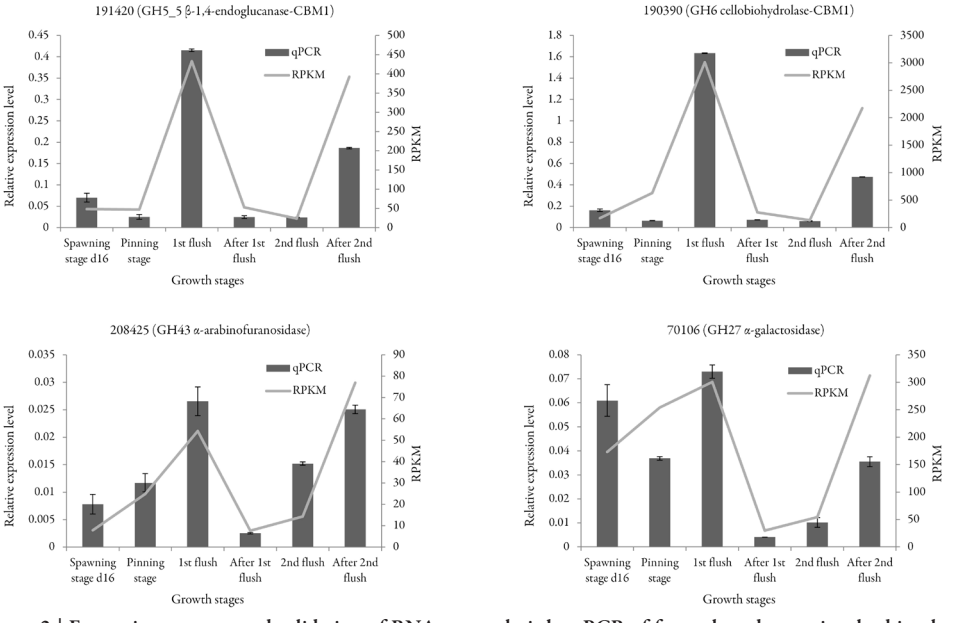

Figure 2 | Expression patterns and validation of RNA-seq analysis by qPCR of four selected genes involved in plant biomass degradation by *A. bisporus* (validation of all twenty genes, see Figure S1). Columns and bars represent the means and standard errors of qPCR. Lines represent RPKM value.

Also several hemicellulose hydrolyzing enzymes were identified and quantified (Table S2). The most abundant secreted proteins were represented by GH10 and GH11 endoxylanases, GH27 α-galactosidase, GH31 α-xylosidase and α-glucosidase, GH35 β-galactosidase and GH51 α-arabinofuranosidase (Table 2). These abundantly produced CAZymes correlate with the RNA-seq analysis of the corresponding genes. A similar pattern of concordance between secretome and transcriptome profiles for hemicellolytic functions was observed regarding differences between various growth stages of *A. bisporus* as was observed for cellulose degradation.

In addition to cellulases and hemicellulases, we detected a number of ligninolytic enzymes with the AA1_1 laccases being the most abundant (Table 2), which were also the most highly expressed ligninolytic genes in the transcriptome data.

Composition analysis of compost samples

Compost samples of the various growth stages of *A. bisporus* were analyzed for cellulose, hemicellulose and lignin content. Changes in carbohydrate composition of compost samples occurred with mushroom growth. Cellulose and hemicellulose content, based on dry matter, decreased from 14.4 and 10.8 (w/w%) in the compost of the spawning stage day 16, to 8.1 and 5.5 (w/w%), respectively, in the compost of the stage when all the second flush mushrooms were harvested (Table 3 and Figure 1). Lignin composition was measured by analytical pyrolysis and S:G (syringol + vinylsyringol units / guaiacol + vinylguaiacol units) ratio was estimated. In contrast to cellulose and hemicellulose composition, the largest changes in lignin content appeared to happen at the spawning stage day 16 compared to composting stages (Phase I and Phase II of compost) (Jurak, 2015) (Table 3 and Figure 1). No major changes occurred in lignin structure (S:G ratio all in a range of 0.43-0.49, no change) after the spawning stage day 16 until the stage when all second flush mushrooms were harvested (Table 3 and Figure 1).

Table 2 | Selected most abundant proteins secreted by *A. bisporus* during growth in compost.

Gene ID	Putative function	# PSM* Spawning stage d16	# PSM* Pinning stage	# PSM* 1st flush	# PSM* After 1st flush	# PSM* 2nd flush	# PSM* After 2nd flush
			Cellulolytic families				
191420	GH5_5 endoglucanase-CBM1	6.5	15	38	87.5	34	32.5
190390	GH6 cellobiohydrolase-CBM1	9	13.5	56	113	37	51
194521	GH7 cellobiohydrolase-CBM1	21.5	42	161	283.5	122	142.5
219902	GH3 β-glucosidase	6.5	67	78.5	82.5	61.5	72.5
			Hemicellulolytic families				
133541	GH10 endoxylanase-CBM1	13.5	23	66	132.5	82	83
196181	GH11 endoxylanase	2.5	10	140	119.5	25	54
70106	GH27 α-galactosidase	16	22.5	69	103	31.5	45
64273	GH31 α-xylosidase	57.5	75	109.5	86	53	67
152299	GH35 β-galactosidase	104	168.5	158.5	178	165	193.5
194576	GH51 α-arabinofuranosidase	81.5	132.5	155.5	132	147.5	167.5
			Ligninolytic families				
139148	AA1_1 laccase	545.5	561	108.5	120.5	390.5	293
146228	AA1_1 laccase	293.5	292.5	56	53	176.5	144.5
221245	AA2 manganese peroxidase	48	53.5	44.5	32.5	34	40
193903	AA5_1 glyoxal oxidase	71.5	54.5	65.5	56	44	51.5

*#PSM Peptide Spectrum Match identified by mass spectrometry RP-nanoLC-MS/MS.

Table 3 | Composition analysis of compost samples of various growth stages of *A. bisporus*.

Growth stage	Cellulose[1] (w/w%)	Hemicellulose*[1] (w/w %)	Lignin (S:G)
Spawning stage d16	14.4 ± 0.1	10.8 ± 0.1	0.49 ± 0.02
Pinning stage	12.9 ± 1.2	7.7 ± 0.1	0.46 ± 0.01
1st flush	10.8 ± 0.6	6.8 ± 0.2	0.43 ± 0.01
After 1st flush	8.8 ± 0.1	6.3 ± 0.1	0.48 ± 0.06
2nd flush	7.6 ± 0.7	5.8 ± 0.3	0.44 ± 0.01
After 2nd flush	8.1 ± 0.2	5.5 ± 0.02	0.46 ± 0.01

*Sum of arabinosyl, xylosyl, glucuronic acid.
[1]Presented as anhydro sugars, based on dry matter.

Central carbohydrate metabolism

Genes associated with central carbohydrate metabolism were identified including glycolysis (27 genes), pentose phosphate pathway (PPP, 14 genes), pentose catabolic pathway (PCP, 4 genes), D-galactose catabolism (12 genes), D-galacturonic acid catabolism (5 genes), L-rhamnose catabolism (2 genes), D-mannose catabolism (4 genes), mannitol metabolism (3 genes), trehalose metabolism (8 genes) and organic acid metabolism (25 genes), and gene expression was analyzed at different growth stages of *A. bisporus* (Table S3).

Genes involved in glycolysis, PPP and PCP were expressed during all growth stages of *A. bisporus* (Table S3). Among the highly expressed genes (RPKM values > 300) glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12); enolase (EC 4.2.1.11) and pyruvate kinase (EC 2.7.1.40) encoding genes were detected, which represent key players of glycolysis, as well as transaldolase (EC 2.2.1.2) and 6-phosphogluconate dehydrogenase (EC 1.1.1.44) encoding genes of the PPP (Table S3), indicating hexose utilization by this organism. Only some pentose catabolic genes were highly expressed, namely those encoding L-arabinose (EC 1.1.1.21) and D-xylulose (EC 1.1.1.9) reductases. In contrast, the D-xylulose kinase (EC 2.7.1.17) encoding gene, which is a key enzyme of pentose catabolism, was expressed at much lower level, suggesting that *A. bisporus* favors hexose over pentose utilization.

In most basidiomycetes, mannitol is formed by direct reduction of fructose through mannitol 2-dehydrogenase using either NAD^+ (EC 1.1.67) or $NADP^+$ (EC 1.1.1.138) as a cofactor (Hult et al., 1980; Voegele et al., 2005). A gene encoding mannitol 2-dehydrogenase (EC 1.1.1.138) was identified in the *A. bisporus* genome and has the highest expression at the first flush while this expression decreases (almost 5 times lower compared to the first flush) at the second flush (Table S3).

Expression of non-CAZy and non-central carbohydrate metabolic genes

To classify the function of the predicted *A. bisporus* genes, the euKaryotic Orthologous Groups (KOG) (Tatusov et al., 2003), Gene Ontology (GO) (Ashburner et al., 2000) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa & Goto, 2000) annotations were performed (Table S5). Out of the total 9620 genes, 6545 were associated with KOG, 4634 with KEGG and 2983 with GO (component, function and process), respectively.

KOG represented genes were assigned to the four main KOG groups (cellular processes and signaling; information storage and processing; metabolism and poorly characterized) (Table S5). 464 KOG genes were highly expressed reaching an RPKM of greater than 300 during various growth stages of *A. bisporus* in compost. Information storage and processing was found to be the major category from the KOG classifications with approximately 24% (98 - 113 genes) of the total highly expressed genes during all the growth stages except spawning stage day 16 where only 14% of these genes were significantly expressed (Figure S2). Most of the upregulated genes in all the growth stages compared to the spawning stage day 16 (10 – 15%) belong to cellular processes and signaling KOG group (Figure S2). These include genes encoding GTP-binding protein (gene ID 184342), 20S proteasome subunit (gene ID 193671) and myosin class I (gene ID 182136) (Morin et al., 2012) (Table S5) and have high similarity to genes with the same function from *Laccaria bicolor* (Martin et al., 2008), *Gloeophyllum trabeum* (Floudas et al., 2012), *Coprinopsis cinerea* (Stajich et al., 2010) and *Ceriporiopsis subvermispora* (Fernandez-Fueyo et al., 2012). The majority of the downregulated genes (3 – 7%) in all the growth stages compared to the spawning stage day 16 represents the category of metabolism (Figure S2). These include genes involved in secondary metabolism: cytochrome P450 genes (genes ID 120936; 189837) (Morin et al., 2012;

Doddapaneni et al., 2013) and a gene encoding sterol C5 desaturase (gene ID 218236) (Morin et al., 2012) which is involved in lipid metabolism (Table S5). These cytochrome P450 genes fall into clans CYP52 (gene ID 120936) which is homologous to the CYP63 family in *Phanerochaete chrysosporium* (Doddapaneni et al., 2005), and to CYP61 (gene ID 189837), which encodes the sterol 22 desaturase in *Saccharomyces cerevisiae* (Skaggs et al., 1996).

Discussion

This study is the first exploration of the transcriptome and proteome of different growth stages of the white button mushroom cultivated in compost, using high throughput RNA-sequencing and mass spectrometry, and is also the most detailed study to date of the progression of plant biomass decay during the life cycle of a basidiomycete fungus. Our integrative analysis of this comprehensive data set demonstrates the capacity of the polysaccharide degradation of *A. bisporus* grown in compost and its ability to utilize this substrate during the cultivation process.

Overall, genes encoding a complete repertoire of cellulolytic and hemicellulolytic (mainly xylanolytic) activities were highly expressed in compost (Figure 1) demonstrating a good correlation with the composition of the substrate (Jurak et al., 2014). Furthermore, a high correlation was observed between expression profiles of the RNA-seq and qRT-PCR data (Figure 2 and Figure S1).

Our results indicate that when *A. bisporus* was cultivated in compost in an indoor, controlled commercial environment, the expression pattern of CAZyme encoding genes and corresponding extracellular protein production changed over time during its life cycle. Figure 1 shows an overview of the major expression changes concerning plant polysaccharide degradation. Interestingly, the transcriptome analysis revealed large differences in expression of genes encoding cellulases and hemicellulases between the first flush when these genes were highly expressed and the second flush when low expression was observed. Such a difference in gene expression might in part explain the difference in yield between two different flushes and could point to a non-synchronicity in gene expression requirement during the second flush under these cultivation conditions. During the first flush yield of the mushrooms was 21 kg/m^2 while the second flush only yielded 8.5 kg/m^2. It is possible that an earlier stimulation of gene expression during the second flush could potentially lead to higher mushroom production.

Ligninolytic genes showed a different expression pattern than the (hemi-) cellulolytic genes. These genes were highly expressed during the spawning stage day 16 and had low expression during all the other stages (Figure 1 and Table S3). This expression pattern corresponds with analytical pyrolysis which showed that the lignin fraction of compost remained practically constant after spawning stage and until the entire second flush was harvested (Figure 1). This could indicate that lignin is not modified after the spawning stage.

Finally, secretome analysis supported the important role for many genes involved in plant biomass degradation, particularly those associated with high transcript levels. However, the correlation between gene expression and protein production changed during life cycle of *A. bisporus*. Variation between transcriptome and proteome was observed after the entire first flush was harvested when gene expression levels decreased while enzymes were still highly abundant. Most likely the proteins were secreted at the first flush stage and remained active in compost. Lack of correlation between transcriptomics and proteomics data can be due to various factors such as different levels of regulation, stability of mRNA, rate of mRNA transcription and protein translation as well as protein stability and the biochemical diversity of proteins (Fournier et al., 2010; Vogel et al., 2011; Vogel & Marcotte, 2012). This could explain the variation in the correlation between the secretion

level of individual proteins and the expression level of their corresponding genes.

Extensive studies of transcriptome and secretome of various wood decaying basidiomycete fungi involved time-dependent degradation or modification of lignocellulosic substrates. For example, wood decay by the white rot fungus *Phanerochaete carnosa* revealed high expression levels of ligninolytic genes (lignin and manganese peroxidases) at early stages of cultivation followed by high expression levels of (hemi-)cellulolytic genes (cellobiohydrolase, xylanase, mannanase, acetyl xylan and glucuronoyl esterases) at later stages of cultivation (MacDonald & Master, 2012). Sequential production of lignocellulose degrading enzymes was also detected during cultivation of another white rot basidiomycete *Ceriporiopsis subvermispora* which showed early production of ligninolytic enzymes (laccase, manganese peroxidase, aryl alcohol oxidase) followed by production of cellulose and hemicellulose main chains cleaving endo-acting enzymes (GH10 endoxylanase, GH5_5 endoglucanase and GH7 cellobiohydrolase) and, in the end of cultivation, a variety of cellulose degrading enzymes along with GH10 endoxylanase were dominant (Hori et al., 2014). The similarity in genome composition of *A. bisporus* with white rot fungi (Morin et al., 2012) is also supported by the expression profile obtained in our study.

In addition to capabilities of *A. bisporus* to degrade plant polysaccharides present in compost during its life cycle, central carbon metabolism including all major pathways as well as storage carbohydrate metabolism were studied at different growth stages of white button mushroom. Carbon catabolic genes were expressed during the entire cultivation process of *A. bisporus* in compost starting from spawning stage day 16 until all the second flush mushrooms were harvested. This active metabolism throughout its life cycle, indicates that *A. bisporus* does not have dormant phases in this method of cultivation. Genes encoding enzymes with essential metabolic role from glycolysis and PPP were highly expressed compared to PCP indicating that *A. bisporus* prefers hexoses over pentoses.

Regarding organic acid metabolism, genes encoding glyoxylate (GLOX) cycle key enzymes (isocitrate lyase and malate synthase) (Munir et al., 2001) were significantly expressed, while genes encoding essential enzymes of tricarboxylic acid cycle (TCA) (isocitrate dehydrogenase, 2-oxoglutarate dehydrogenase and citrate synthase) (Matsuzaki et al., 2008; Kubicek et al., 2011) had low expression (Table S3). This could indicate a higher flux in the GLOX cycle compared to TCA. It has been reported that basidiomycetes have a bicycle TCA/GLOX system and malate can be shuttled from one cycle to the other and be oxidized by malate dehydrogenase to oxaloacetate which further can be converted to oxalate (Munir et al., 2001). The high expression of genes encoding malate dehydrogenase, isocitrate lyase and malate synthase during growth of *A. bisporus* in compost suggests a need for the production of oxalate under these conditions (Table S3). It has been demonstrated that oxalic acid plays a crucial role in initial lignocellulose degradation before enzymatic hydrolysis (Hastrup et al., 2011). Oxalic acid typically accumulates in cultures of brown rot fungi, while its detected in lower amounts in white rot fungi (Dutton et al., 1993; Mäkelä et al., 2002; Hastrup et al., 2012). Moreover, metabolic change at the transition step from vegetative growth to fruiting body formation of brown rot fungus *Fomitopsis palustris* in the GLOX and TCA cycles was observed. Oxalate biosynthesis played an important role in vegetative growth, while glutamate synthesis played a major role in fruiting body formation (Yoon et al., 2002). However, no significant correlation between growth stages of *A. bisporus* and GLOX / TCA cycles were found in our data. Further research is needed to better understand the role of GLOX / TCA cycles in *A. bisporus* throughout its life cycle.

Another interesting feature that was observed refers to mannitol metabolism. It has been shown that mannitol acts as an osmoregulatory compound promoting a flow of water from compost to the fruiting body to maintain turgor and fruiting body development in *A. bisporus*

(Kalberer, 1990; Stoop & Mooibroek, 1998). Additionally, it has been demonstrated that genes involved in mannitol pathway were significantly lower in fruiting bodies than in compost which indicated synthesis of mannitol in the vegetative mycelium and its transport to the fruiting body (Patyshakuliyeva et al., 2013). Mannitol 2-dehydrogenase is a key enzyme for mannitol production in most basidiomycetes (Hult et al., 1980; Voegele et al., 2005). In this study we observed that the gene encoding this enzyme had the highest expression at the first flush while at the second flush the expression level of this gene decreased (Table S3). Referring to the importance of mannitol for fruiting body development low expression of mannitol 2-dehydrogenase gene at the second flush may correspond with lower number of mushrooms harvested at the second flush compared to the first flush. Together with the lower expression of (hemi-) cellulolytic genes in the second flush, this indicates significant differences in the physiology of *A. bisporus* during production of the first and second flush mushrooms.

In conclusion, our integrative analysis of various growth stages of *A. bisporus* cultivated in compost in a controlled environment demonstrates changes in plant biomass degradation throughout its life cycle. Most notable differences in gene expression were detected between first and second flushes, which involved cellulolytic and hemicellulolytic genes as well as the mannitol 2-dehydrogenase encoding gene. This observation could explain the loss in number of mushrooms during second flush.

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Supplementary data

All supplementary data can be found at:
http://www.cbs.knaw.nl/index.php/pubphysiology/thesis/618-thesis-aleksandrina-patyshakuliyeva
or obtained from the author.

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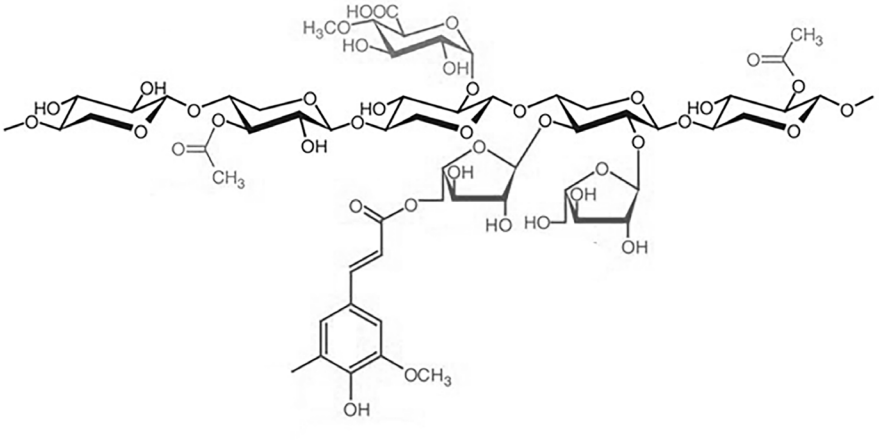
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CHAPTER 6

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

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6

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.

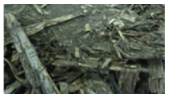
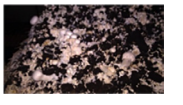
Introduction

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* (Iiyama et al., 1994; Jurak et al., 2014). 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 (Jurak et al., 2014). A casing layer (mixture of peat and lime) is put on top of the mature compost to induce fruiting body formation (Hayes 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 these fractions remain 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 (Jurak et al., 2014). 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 these species. Others have already studied enzyme activities in compost during mycelium growth and fruiting body formation (Wood, 1980; Bonnen et al., 1994; Savoie, 1998). 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 (Patyshakuliyeva et al., 2013). 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 during the growth of *A.bisporus* in compost at different time points and compare these both to the genome information described by Patyshakuliyeva et al., 2013 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 1).

Table 1 | Compost sample codes and description.

Sample	Description	
PII_{end}	Compost just before addition of spawn (*A. bisporus* mycelium which is developed on cereal grain)	
Spawning stage d16	Compost at the end of the spawning stage (16 days after spawns were introduced into Phase II compost)	
Filling stage	Compost after filling of the growth beds with compost of the spawning stage d16 and covered with casing layer (17 days after spawns were introduced into Phase II compost)	
Pinning stage	Compost when pinning has clearly started and the first pinheads were visible (30 days after spawns were introduced into Phase II compost)	
After 1st flush	Compost after all 1st flush was harvested (40 days after spawns were introduced into Phase II compost)	
After 2nd flush	Compost after all 2nd flush was harvested (48 days after spawns were introduced into Phase II compost)	

Materials and Methods

Compost samples

The strain used in this study is the commercial *A. bisporus* strain Sylvan A15, (Sylvan, USA). For a first screening of enzyme activities, compost samples of end of Phase II of the composting process (PIIend) and spawning stage day 16 (PIII-16) were supplied by CNC as described previously (Jurak et al., 2014). For a second, more extensive screening, fresh compost samples of end of Phase II, which is the compost phase just before addition of spawn (*A. bisporus* mycelium which is developed on cereal grain) and of 16 days of mycelium grown compost (spawning stage day 16) were obtained. Further, compost samples obtained during the filling of the beds with spawning stage day 16 compost and covered with casing layer, pinning of *A. bisporus*, when the entire first and second flushes were harvested, were supplied by CNC; all were from the same timeline. Sample codes and description are summarized in Table 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.

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. 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 growth stages.

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.

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 (data not shown).

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 aluminum 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 PIIend and

spawning stage day 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 *Aspergillus awamori* (Gruppen et al., 1992; Kormelink et al., 1993a). Substrates were incubated in water with enzyme extracts from PIIend, spawning stage day 16, filling, pinning, when the entire first and second flushes were harvested. 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 1st flush, and sequentially incubated with pure GH43 AXH-d3 arabinofuranosidase (Van Laere et al., 1999) as described above.

Analytical methods

Neutral carbohydrate and uronic acid content and composition were determined in duplicate, as described in (Jurak et al., 2014).

High Performance Size Exclusion Chromatography (HPSEC) was performed as described in (Jurak et al., 2014). 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 2).

High Performance Anion Exchange Chromatography (HPAEC) was performed as described in (Jurak et al., 2014). Oligosaccharides released from WAX were identified using a profile of WAX degraded by pure, well characterized endo-xylanase was used (Kormelink et al., 1993b). Enzyme digests were diluted 10 times prior to analysis.

Table 2 | First screening of extracellular enzyme activities from the compost in PII_{end} and spawning stage d16 analyzed by HPSEC after 24h 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)

Results and Discussion

Preliminary screening of enzymes from extracts of PIIend and spawning stage day 16

A first screening was performed for two enzyme extracts, PIIend and spawning stage day 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 2.

Wheat arabinoxylan (WAX) was completely degraded by extracts from PIIend and partially by spawning stage day 16. This was expected as xylan is, next to cellulose, the main carbohydrate source in compost used for mushroom growth (Jurak et al., 2014). In addition, cellulose (CMC) was partially degraded by PIIend-extract, whereas not at all by spawning stage day 16-extract. For the pectic substrates, almost no degradation was observed, apart from branched arabinan by spawning stage day 16-extract. Further, partial degradation was observed by both extracts from PIIend and spawning stage day 16 on model galactomannan and xyloglucan. Recently it was shown that the *A. bisporus* genome encodes enzymes targeting all plant polysaccharides (Patyshakuliyeva et al., 2013), 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 spawning stage day 16 (Table 2). However, no activity towards homogalacturonan, rhamnogalacturonan I (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 spawning stage day 16.

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 (Jurak et al., 2014), 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. 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 PIIend, spawning stage day 16, filling, pinning, and when the entire first and second flushes were harvested, 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 PIIend, spawning stage day 16, filling, pinning, when the entire first and second flushes were harvested, 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 (Figure S1).

Xylan degrading activities

The overall hydrolytic enzyme activity tested after incubation for 4h showed similar trends for WAX and birchwood xylan (Figure 1). Enzyme activity on both substrates was lowest during filling and increased over time with the highest activity after the entire first flush was harvested. After that the activity tended to decline for about 20% during the stage when the entire second flush was harvested. 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, after the first 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 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 4 h incubation (Figure 1). The β-xylosidase activity was lowest (0.1 nmol pnp ml^{-1} min^{-1}) in the extract of the filling stage and it increased during pinning stage and had the highest activity in compost collected after the entire first flush of mushrooms was harvested (0.9 nmol pnp ml^{-1} min^{-1}). During the growth of *A. bisporus* in compost (from filling until the stage when the entire second flush was harvested) 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 (Figures 2 and 3). The enzyme extract obtained from compost without mycelium (PIIend) completely degraded polymeric WAX into monomeric and small oligomeric compounds with a molecular mass <12 kDa (Figure 2). Over the period of mushroom growth (spawning stage day 16 to after second flush) two trends could be observed: partial and complete degradation of WAX. Partial degradation was obtained with extracts from spawning stage day 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 (PIIend) to *A. bisporus* (spawning stage day 16 and filling). Complete degradation of high molecular weight WAX was obtained with enzyme extracts from the period of pinning stage until the entire second flush was harvestedindicating an increase in the activity of xylanases. Again, maximum activity was achieved by the after first flush-extract. These results were in line with the results obtained by the reducing end assay (PAHBAH) after 24 h.

Degradation of 4-*O*-methyl-glucuronic acid substituted xylan (birchwood xylan, Figure 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 after first 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 Figure 3. WAX hydrolysed with the PIIend-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 (Table S1). Together with the presence of xylo-oligomers substituted with arabinose this indicated a poor arabinofuranosidase activity in PIIend. The HPAEC chromatogram of WAX incubated with extracts from spawning stage day 16 and filling (Figure 3) showed only low amounts of arabinose,

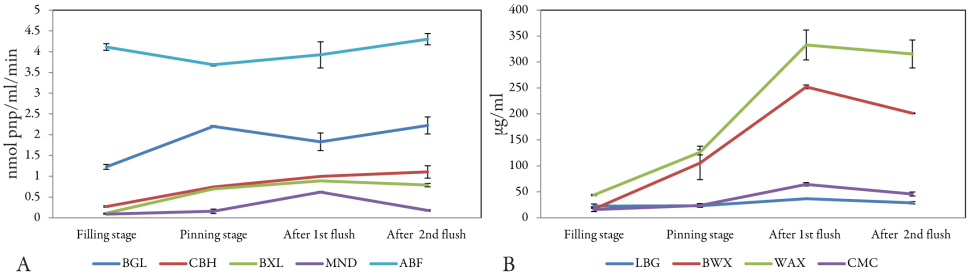


Figure 1 | Relative enzyme activities in water extracts (4h incubation) of various stages of mushroom production.
A: exo-activities (nmol pnp/ml/min). B: total hydrolysis based on reducing sugar determination (µg/ml). BGL= β-glucosidase, CBH= cellobiohydrolase, BXL= β-xylosidase, ABF= α-arabinofuranosidase,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

Table 3 | Total carbohydrate content, carbohydrate molar composition and xylan degree of substitution of compost samples obtained at PIIend, spawning stage d16, filling, pinning, after 1st and after 2nd flushes.

	PII_{end}	Spawning stage d16	Filling	Pinning	After 1st flush	After 2nd flush
Total carbohydrates (% w/w)[a]	26 ± 1	27 ± 1	22 ± 1	23 ± 1	18 ± 1	16 ± 1
Carbohydrate composition (molar %)[b]						
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[c]	15	14	17	16	23	26
GlcA/Xyl[c]	11	13	18	21	31	35

[a]Weight percentage is based on dry matter of composting phases.
[b]As anhydro-sugars; STDEV < 0.5 for all samples.
[c]Ratio mol substituents/100mol of xylosyl residues; abbreviations: Ara, arabinosyl; GlcA, glucuronic acid.

no xylose and very small amounts of xylan-oligomers. This was also in line with the results obtained by HPSEC (Figure 2), which showed mainly large molecular weight xylan remaining.

These results matched with previous research (Jurak et al., 2014) 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 and after the entire first and second flushes were harvested showed much more xylose and arabinose as well as xylobiose and xylotriose than from PIIend, spawning stage day 16 and Filling (Figure 3), being the result from a nearly complete WAX hydrolysis as was also detected on HPSEC (Figure 2).

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 at the stage of the first flush of *A. bisporus*, as published previously (Patyshakuliyeva et al., 2013).

In contrast, double substituted xylo-oligomers remained (Figure 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* (Sorensen et al., 2006) and one by *Bifidobacterium adolescentus* (van den Broek et al., 2005). For *A. bisporus* (Patyshakuliyeva et al., 2013) putative genes encoding enzymes belonging to the family GH43 were found to be up regulated in compost (1st flush) compared to plate grown mycelium, but, it is shown in a phylogeny tree that these GH43 genes are not likely to be active towards the doubly substituted xylan parts (Jurak et al., 2015).

Figure 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 significantly expressed during the growth of *A. bisporus* in compost as analyzed in the same batch of samples as used in this research (Jurak et al., 2015). 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 (Figure 2) in enzyme extracts obtained from various growth stages during the commercial cultivation of *A. bisporus*. 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.

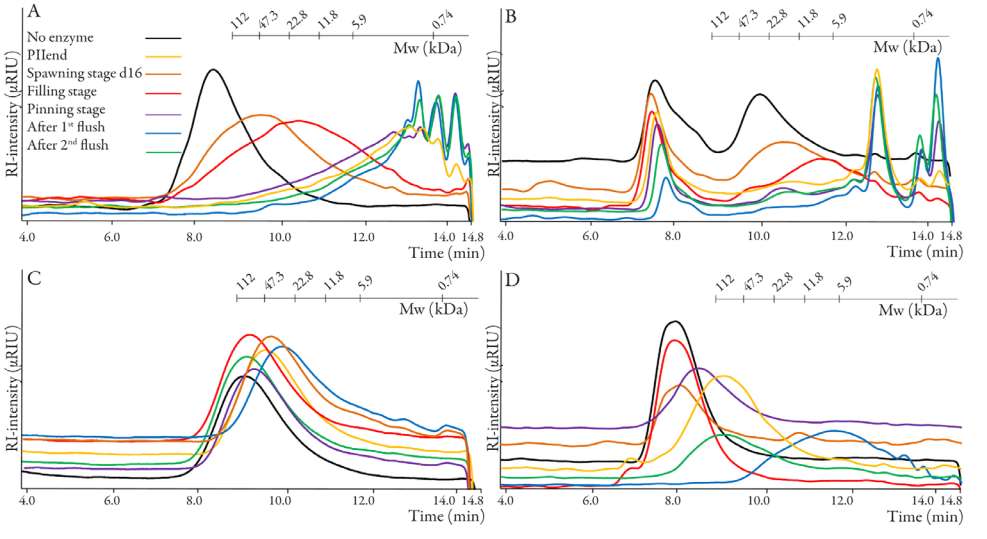


Figure 2 | High performance size exclusion profiles of wheat arabinoxylan (A), birchwood xylan (B), carboxymethyl cellulose (C) and galactomannan (D), after degradation with enzyme extracts obtained from compost of different stages of mushroom growth (24h incubation).

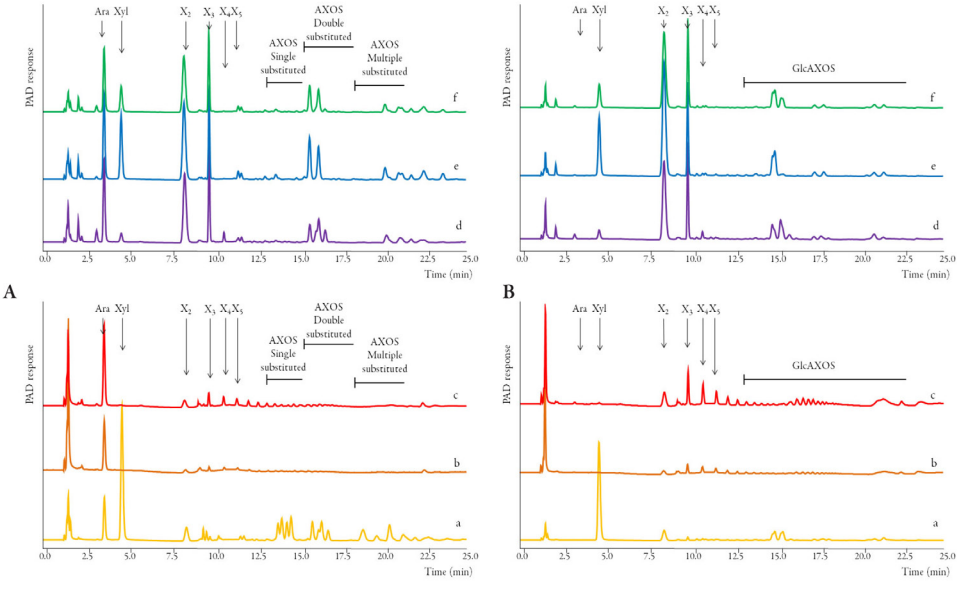


Figure 3 | HPAEC elution profile of WAX (A) and birchwood xylan (B) incubated (24h) with a: PII_{end}, b: spawning stage d16, c: filling stage, d: pinning stage, e: after 1st flush and f: after 2nd flush extracellular enzymes. AXOS = oligomers substituted with arabinose. GlcAXOS = oligomers substituted with 4-O-methyl-glucuronic acid.

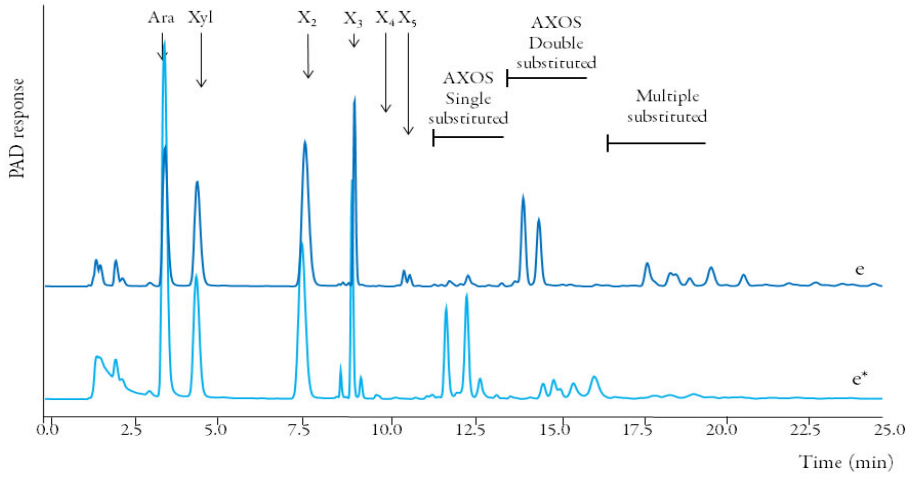


Figure 4 | HPAEC elution profile of WAX incubated (24h) first with compost extract of the stage when the entire 1st flush was harvested (e) and then sequentially with pure GH43 AXH-d3 arabinofuranosidase (e*) (Van Laere et al., 1999). AXOS = oligomers substituted with arabinose.

Cellulose degrading activities

Overall, cellulase activity tested on CMC after 4 h confirmed the activity of cellulases in the compost throughout the fruiting body formation as previously reported by Wood and Goodenough (1977) (Figure 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 during pinning stage (23 μg Glc ml^{-1}). Highest cellulase activity was observed in the compost-extract obtained after the entire first flush of mushrooms (64μg Glc ml^{-1}) and the activity decreased slightly during the stage when the entire second flush was collected (46 μg Glc ml^{-1}).

Cellobiohydrolase activity (4h incubation) was lowest in the extract from the filling stage (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}) during the stage when the entire second flush was harvested (Figure 1). In the extract from filling, β-glucosidase activity was the lowest (1.2 nmol pnp ml^{-1} min^{-1}), while during pinning, activity was much higher (2.2 nmol pnp ml^{-1} min^{-1}) and it decreased again during the stage when the entire first flush was harvested (1.8 nmol pnp ml^{-1} min^{-1}). While during the stage when the entire second flush was harvested, β-glucosidase activity slightly increased and was the same as during the pinning stage.

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 (spawning stage day 16) cellulase activity decreased compared to PIIend. From filling to the stage when the first flush was harvested cellulase activity increased and after the second flush was harvested a slight decrease in activity was detected (Figure 2).

Overall, the trend of CMC hydrolysis by enzyme extracts from the various growth stages observed with HPSEC was in line with the trend observed for the same samples analyzed by PAHBAH assay after 4 (Figure 1) and 24 h (data 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 & 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, 1969). The results of our study confirm this, since we obtained high cellulase and hemicellulase activities during pinning stage, as well as when the entire first and second flushes were harvested. Our data also fit with the previous transcriptomics study of *A. bisporus*, which demonstrated that cellulase encoding genes were upregulated in the compost compared to plate grown mycelium (Patyshakuliyeva et al., 2013). Xylanase activities was higher than most cellulase activities in compost, which confirms a precious 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 compared to plate grown mycelium (Patyshakuliyeva et al., 2013).

Mannan degrading activities

Figure 2 shows HPSEC profiles of galactomannan degraded by enzyme extracts (24 h incubation). For both the exo-activity and the overall enzyme activity (Figure 1) the same trend was observed. In extracts from the filling and pinning stages very low mannanase activity was detected. Nevertheless, after the entire first flush was harvested a remarkable increase in both endo- and exo-activity was

observed. While after the second flush was harvested the overall mannanase activity decreased (Figure 2) and exo-activity decreased for 70% (Figure 1). Overall, this trend was similar to the one observed for cellulases and xylanases.

Compost composition and carbohydrate structures

Table 3 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 (Iiyama et al., 1994; Jurak et al., 2014).

PIIend 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 (Jurak et al., 2014). This indicated that only low amounts of carbohydrates were metabolized from the compost carbohydrates by the microbial population during spawning stage day 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 during spawning stage day 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 (when the entire second flush was harvested) 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 spent compost (when the entire second flush was harvested), a gradual decrease in xylosyl and an increase in arabinosyl and uronic acids, most likely glucuronic acid residues, was observed (Table 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 the stage when the entire second flush was harvested (Table 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 (when the entire second flush was harvested) mainly xylan substituted with glucuronic acid or two arabinosyl residues per xylosyl residue is present.

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. Maximal overall enzymatic activity was observed when the entire first flush of mushrooms was harvested. 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

All supplementary data can be found at:
http://www.cbs.knaw.nl/index.php/pubphysiology/thesis/618-thesis-aleksandrina-patyshakuliyeva
or obtained from the author.

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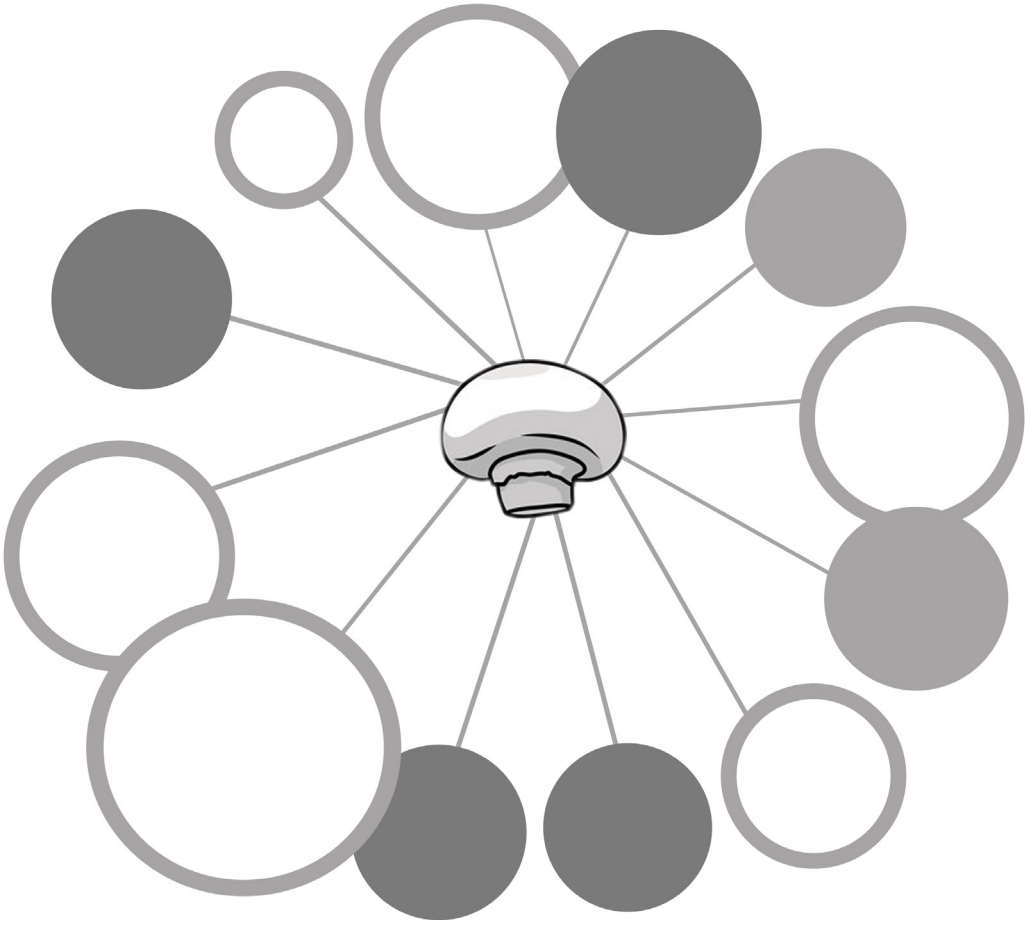

CHAPTER 7

General Discussion

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7

EDIBLE MUSHROOMS have been known for ages in many cultures with respect to their unique culinary values such as flavor and texture profiles, nutritional properties and potential health benefits. The cultivation of edible mushrooms has become a multibillion dollar international industry and is considered as a sustainable alternative to traditional crops. *Agaricus bisporus*, the white button mushroom, is economically the most important mushroom cultivated worldwide. Among all the cultivated mushrooms, *A. bisporus* grows on the most complex substrate. This is known as compost and mainly consists of wheat straw, horse and chicken manure, gypsum and water (Sánchez, 2004). Therefore lignocellulose which is a complex mixture of cellulose, hemicellulose and lignin usually constitutes an important fraction of the total organic matter. During the composting process raw materials are modified to a better decomposed form that is more suitable for *A. bisporus*.

Also of interest is the fact that *A. bisporus* belongs to an ecological niche rich in humus like substrates such as forest litter (and commercial mushroom compost), and plays a significant role in nature by contributing to the global carbon cycle.

The current state of knowledge of the growth and decomposition of the substrate by *A. bisporus* is very limited. The research described in this thesis aimed to explore the abilities of *A. bisporus* to grow in compost and degrade this substrate, as well as to study the molecular mechanisms underlying this phenomenon.

Adaptation to humic-rich environment

The most significant source of carbon in land ecosystems is the plant cell wall, composed primarily of cellulose, hemicellulose, and lignin. Agaricomycetes belong to the fungal phylum Basidiomycota and are capable of using this pool as a carbon and energy sources, acting as wood or litter decomposers. Collectively referred to as white and brown rot fungi, wood decayers use different strategies to decompose plant biomass. Recently, studies have begun to address wood decay mechanisms through the availability of an increasing number of sequenced genomes (Martinez et al., 2009; Eastwood et al., 2011; Floudas et al., 2012; Riley et al., 2014). However, less is known regarding litter decomposition of the forest floor which is also rich in humic substances. Here lies an opportunity to explore novel plant biomass degrading strategies that might not be seen in the wood decomposing fungi, nor in coprophillic fungi such as *Coprinopsis*.

A. bisporus represents a good model organism for the growth in a humic-rich leaf-litter environment. To uncover the fate of the forest litter degradation, the commercial European bisporic (var. *bisporus*) homokaryon H97 and the wild North American tetrasporic (var. *burnettii*) homokaryon, JB137-S8, were sequenced (Morin et al., 2012) (**Chapter 2**). The two genomes var. *bisporus* and var. *burnettii* have sizes 30.2 and 32.6 Mb with 10,438 and 11,289 protein coding genes estimated respectively (Figure 1). The degree of synteny between these two *A. bisporus* genomes was evaluated highlighting a number of local inversions and translocations. These chromosomal rearrangements possibly contribute to the relatively poor rate of recombination and low viability of haploid meiotic spores from crosses between the two varieties (Foulongne-Oriol et al., 2010).

Overall, the Carbohydrate Active enZymes (CAZymes) repertoire (188 Glycoside Hydrolases (GH), 59 Polysaccharide Lyases (PL) and 10 Carbohydrate Esterases (CE)) encoded by the *A. bisporus* genome is close to that of wood decaying fungi, and notably different from phylogenetically related fungi such as the coprophilous *Coprinopsis cinerea* and the mycorrhizal symbiont *Laccaria bicolor*. These differences are limited to a few families, but these distinctions might have consequences for degradation of plant cell wall polysaccharides. For example, the *A. bisporus* genome contains genes encoding GH6 and GH7 families, which represent an important group typically featuring cellobiohydrolases. In contrast, those families were not identified in *L. bicolor* or in brown rots (except Boletales), while they were identified in all white rot fungi. In addition to the distinct repertoire of CAZymes, analysis of the oxidative ligninolytic machinery of *A. bisporus* revealed only two manganese peroxidases (MnPs) from all heme-including peroxidases, several laccases (12) and a large set (24) of heme-thiolate peroxidases (HTPs) which is complimented by a number (9) of H_2O_2-producing enzymes such as copper radical oxidases (CROs) and glyoxal oxidases (GLXs). *A. bisporus* has the largest number of HTPs, which are considered a hybrid of peroxidases and monooxygenases (Hofrichter et al., 2010), compared to other fungi involved in lignocellulose degradation. Relative to white rots, the reduced number of heme-including peroxidases may reflect a different method of lignin decomposition and its accessibility in compost compared to woody substrates. Moreover, the high number of HTPs could compensate for the almost absence of heme-including peroxidases in *A. bisporus*.

In **Chapter 2** gene expression during the first flush mushrooms of the compost, casing layer and fruiting bodies compared to mycelium grown on agar medium was profiled. 51% of the genes encoding GHs, PLs and CEs were upregulated in compost, while only 17% of these genes were upregulated in casing layer and in fruiting bodies (Figure 1). Furthermore, expanded number of genes involved in degradation of lignin derivatives such as HTPs, GLXs and β-etherases were also upregulated in compost relative to defined media.

This unique repertoire of genes and its expression pattern may enhance survival in humic-rich

and partially degraded organic matter. Genome composition together with transcriptome analysis does not easily assign *A. bisporus* to either white rot or brown rot life style. Therefore this analysis uncovered a new "humicolous" group containing species such as *A. bisporus* which are adapted to grow in a humic-rich environment (Figure 1) (Morin et al., 2012).

Unraveling different gene expression pattern with respect to CAZymes and carbon utilization in compost, casing layer and fruiting bodies

The whole genome sequence and the transcriptome analysis of *A. bisporus* were the first to be available from the large family of Agaricaceae, and provide a solid foundation for understanding its particular nutrition mode and ecological adaptation (Chapter 2) (Morin et al., 2012). To uncover the fate of the carbon fluxes in *A. bisporus* and to investigate the changes in compost and casing layer mycelium and in fruiting bodies of the first flush mushrooms, in **Chapter 3** genes involved in central carbon metabolism were identified and their expression was analysed. This analysis suggests a specific response to the presence of carbon, its further utilization by *A. bisporus* and its flow from mycelial network in compost into the fruiting bodies. In particular, only genes involved in hexose catabolism were expressed in fruiting bodies while carbon catabolic genes of most pathways of central carbon metabolism were expressed in compost grown mycelium (Figure 1). These results show that a much larger variety of monosaccharides can be used by *A. bisporus* compost colonized mycelium, while only hexoses are transferred to the fruiting bodies from the vegetative mycelium. Moreover, the detected levels of free monosaccharides in compost and fruiting bodies correlated with the expression of carbon catabolic genes. This analysis provides a new understanding of the pathways that allow *A. bisporus* to obtain carbon as an energy source and to maintain fruiting body development despite reduced expression of multiple genes involved in carbon utilization in fruiting bodies.

In addition to carbon utilization, a detailed analysis of the expression of the genes encoding plant and fungal polysaccharide degrading / modifying enzymes was performed in **Chapter 3** to further evaluate degradation of the substrate by *A. bisporus*. Analysis of this data showed that in the compost the main focus was on degrading the plant biomass as a broad scope of genes was expressed, related to different polysaccharides. Approximately 64% and 90% of cellulolytic and xylanolytic genes respectively were expressed in compost (Figure 1). Interestingly, genes involved in utilization of pentoses were highly upregulated in compost, supporting a good correlation between release and conversion of these sugars by *A. bisporus* grown in compost.

While *A. bisporus* compost colonized mycelium at the first flush had elevated levels of plant biomass degrading genes, the casing layer appeared to be an intermediate phase in which plant biomass degradation still took place, but also genes encoding fungal cell wall modifying enzymes such as GH18 chitinases were slightly upregulated. It has been established that the casing layer is a nutritionally poor substrate (Hayes, 1981), and the *A. bisporus* mycelial network in this layer allows symplastic movement of carbohydrates from the mycelium in compost into the developing fruiting bodies.

Finally, in the fruiting bodies, the main emphasis was on modification of fungal polysaccharides. 30% from all genes encoding fungal cell wall modifying enzymes were expressed in the first flush fruiting bodies (Figure 1). However, some of these genes were also expressed in casing layer and compost confirming that synthesis and modification of the cell wall occurred during the

entire growth of *A. bisporus*. Although, various set of cell wall modifying genes were expressed implying that the morphology of compost, casing layer and fruiting bodies is different.

Overall, expression of plant polysaccharide degradation related genes had a good correlation with the carbohydrate composition present in the first flush compost, casing layer and fruiting bodies. Taken together, identification of the genes of the central carbon metabolism and CAZymes as well as its expression analysis demonstrates that the release of the carbohydrates from the compost, its utilization and metabolism are highly differentiated in *A. bisporus*.

Novel insights into plant biomass degrading abilities of *A. bisporus* during its growth in compost

The white button mushroom *A. bisporus* is a major horticultural crop produced worldwide which is commercially cultivated on lignocellulosic biomass from agricultural waste residues. However, the important questions as to how the fungus degrades its substrate throughout its life cycle has remained unanswered. So far only little is known about the changes in plant biomass degradation during the growth of *A. bisporus* in compost. On one hand, *A. bisporus* produces a large range of enzymes to degrade lignocellulose (Wood & Thurston, 1991; Bonnen et al., 1994; Savoie, 1998). Moreover, the genome sequence of *A. bisporus* identified a wide set of genes encoding plant cell wall CAZymes (Chapter 2) (Morin et al., 2012) and detailed analysis of the expression of these genes was performed in compost, casing layer and fruiting bodies of the first flush (Chapter 3) (Patyshakuliyeva et al., 2013). On the other hand, 17% of the original compost polysaccharides remains unutilized at the end of harvesting of the last flush (Iiyama et al., 1994). Consequently, Chapter 5 and 6 were set out to address this problem and to look inside this black box using in-depth integrative analysis of transcript expression and protein secretion as well as carbohydrate degrading enzyme assays data from *A. bisporus* mycelium grown compost at different growth stages (including spawning, pinning, the first flush, the second flush stages and when the entire first and second flushes were harvested stages).

Transcriptome analysis required high quality and quantity of RNA. It has been pointed out that the major challenge facing gene expression analyses in complex substrates (compost, soil and forest residues) today is the development of a reproducible RNA extraction method which would produce high integrity RNA suitable for all downstream applications (Mendum et al., 1998; Alm et al., 2000; Bachoon et al., 2001). The methodology based on the CsCl gradient ultracentrifugation developed in **Chapter 4** allowed obtaining high levels of quality and quantity of RNA not only from different growth stages of *A. bisporus* mycelium grown compost but also from the white rot fungus *Dichomitus squalens* which was grown on straw, spruce and microcrystalline cellulose (Avicel).

The main issue with extracting RNA from compost is the high content of humic substances in composted mixture, which makes isolation of intact RNA a very complicated process (Aiken et al., 1985). Extracting RNA from compost samples using guanidinium thiocyanate (GIT) extraction buffer at pH 5.0 as well as extra chlorophorm/isoamyl alcohol clean-ups, lowered co-extraction of humic acids and therefore increased RNA integrity. Previously it has been also demonstrated that the preparation of RNA extracts under lower pH conditions decreased the amount of co-extracted humic acids (Mettel et al., 2010). Furthermore, the resulting RNA extracts had to be proceeded immediately to the CsCl gradient ultracentrifugation. The CsCl gradient ultracentrifugation is a crucial step in separating RNA from various endogenous contaminants including humic substances and is suitable for use in any procedure requiring highly purified RNA from environmental samples.

The RNA isolation protocol described in Chapter 4 (Patyshakuliyeva et al., 2014) was used to extract RNA from *A. bisporus* (various growth stages) grown in compost and enabled transcriptome analysis of these samples which is one of the main focuses of **Chapter 5**. This Chapter represents, to our knowledge, the first study in which transcriptome and proteome data for different growth stages of *A. bisporus* in compost starting from spawning stage until the stage when all the second flush mushrooms were harvested were analysed in conjunction with data on composition analysis of these compost samples.

First, genes encoding lignocellulolytic CAZymes and their expression patterns were examined. Transcripts predicted to encode cellulose and hemicellulose (mostly xylan) degrading enzymes were most abundant in compost which correlates well with the transcript profile of the mycelium grown compost studied in Chapters 2 and 3 (Morin et al., 2012; Patyshakuliyeva et al., 2013). Also this gene expression pattern is consistent with the composition of the compost (Iiyama et al., 1994; Jurak et al., 2014). Furthermore, this analysis showed changes in gene expression during the cultivation of *A. bisporus* in compost over time. Cellulolytic and hemicellulolytic genes were highly expressed during the first flush and during the growth stage when all second flush mushrooms were harvested and had comparatively low abundance during the other stages of *A. bisporus* growth in compost. Ligninolytic genes had greatest transcript abundance during spawning stage day 16 and lower abundance in all the other growth stages which agrees well with data from analytical pyrolysis showing the most changes in lignin content at the spawning stage day 16 compared to Phase I and II composting stages (Figure 1) (Jurak et al., 2015). In this Chapter it was shown that the lignin composition remained unmodified after the spawning stage day 16 until the stage when the second flush was completely harvested. Most importantly, this transcriptome analysis demonstrated significant expression differences in genes encoding cellulose and hemicellulose degrading enzymes between first and second flushes which could partly explain a big reduction (the first flush – 21 kg/m^2; the second flush – 8.5 kg/m^2) in mushroom production during the second flush (Figure 1).

To further evaluate abilities of *A. bisporus* to degrade the substrate during its life cycle, secreted proteins were determined in the same compost samples which were used for transcriptome analysis. Overall, produced proteins associated with lignocellulose degradation are in agreement with the abundance of transcript levels performing a good correlation between different data sets. However, when the transcriptome profile showed decrease in gene expression at the stage when all the first flush mushrooms were harvested, the corresponding CAZymes with cellulolytic and hemicellulolytic activities were still present during that stage. These could be the same proteins which were already secreted at the earlier first flush stage when corresponding genes were highly expressed.

Several studies reported hemicellulolytic abilities of *A. bisporus* highlighting isolated genes and activities of their corresponding enzymes, cellobiohydrolases GH6, GH7 and endoxylanases GH10, in compost (Raguz et al., 1992; Chow et al., 1994; Yague et al., 1997; De Groot et al., 1998). Analysis of these cellulase and endoxylanase activities showed their presence throughout growth of *A. bisporus* in compost with increased activities starting from the pinning stage and reaching the highest level of activities at the postharvest stage after the first flush mushrooms were harvested (Wood & Goodenough, 1977; Claydon et al., 1988; Whiteford et al., 2000). These observations are also compatible with proteomics analysis of secreted GH6, GH7 and GH10 CAZymes families which had the highest abundance at the stage when the entire first flush was harvested.

Recent transcriptome and secretome investigations of white rot fungi such as *Phanerochaete carnosa* and *Ceriporiopsis subvermispora* also demonstrated that degradation and modification of lignocellulosic substrates which was time-dependant (MacDonald & Master, 2012; Hori et al., 2014). These studies observed sequential lignocellulolytic gene expression and secretion of

associated proteins revealing high expression/production of ligninolytic genes/proteins at early stages of cultivation followed by high expression/production of (hemi-) cellulolytic genes/proteins at later stages. The combined analysis of the *A. bisporus* genome (Chapter 2) (Morin et al., 2012) and expression dataset from this Chapter enabled to distinguish similarity between white rot fungi and litter decomposing fungus *A. bisporus*.

To gain insight into the carbon nutritive needs of the fungus, in Chapter 5 genes involved in central metabolism were also studied during the growth of *A. bisporus* in compost. These genes were expressed throughout the entire life cycle if *A. bisporus* indicating an active metabolic process without dormant stages. In addition genes involved in hexose catabolism which encode enzymes with significant metabolic function were higher expressed compared to pentose catabolic genes. This could suggest preference for hexoses over pentoses during the growth of *A. bisporus* in compost. Interestingly, a gene encoding mannitol 2-dehydrogenase which is an essential enzyme for mannitol metabolism and for the fruiting body development, respectively, was highly expressed at the first flush and lowly expressed at the second flush (Figure 1). By integrating this data to the expression pattern of (hemi-) cellulolytic genes, the loss in the yield during the harvesting of the second flush could be in part explained.

Another approach to investigate abilities of *A. bisporus* to degrade the substrate during its life cycle was detection of enzymatic activities in compost (**Chapter 6**). Comprehensive enzyme activity screening was assessed at the stages of the end of the Phase II composting process, spawning stage day 16, at the stage when colonized compost was covered with casing layer, at the pinning stage and at the stages when the entire first and second flushes of mushrooms were harvested. Based on the composition of the compost (Jurak et al., 2014), cellulose and xylan degrading enzymes activities were selected for screening. Overall, cellulolytic and xylanolytic activities were detected throughout the growth of *A. bisporus* in compost which correlates well with previous studies regarding these enzymatic activities (Wood & Goodenough, 1977; Claydon et al., 1988; Whiteford et al., 2000) as well as with cellulolytic and xylonolytic gene expression and protein secretion described in Chapter 5. Moreover, most of the measured carbohydrate degrading enzyme activities had their highest levels at the stage when all the first flush was harvested confirming the pattern observed for secreted proteins showed in Chapter 5. This is also supported by several observations related to the release of cellulose and xylan degrading enzymes during the growth of *A. bisporus* in compost over time (Wood & Goodenough, 1977; Claydon et al., 1988; Whiteford et al., 2000).

In-depth analysis of enzyme activities during the growth of *A. bisporus* in compost using High Performance Size Exclusion Chromatography (HPSEC) and High Performance Anion Exchange Chromatography (HPAEC) revealed absence of arabinofuranosidase activity which releases arabinosyl units from doubly substituted xylan as well as the lack of α-glucuronidase activity. Consequently, arabinosyl and α-glucuronic acid substituted xylan accumulated in compost during the development of *A. bisporus* (Figure 1). Previously it has been also demonstrated that during the spawning stage of mycelium growth xylan was only partially degraded (Jurak et al., 2014). Some arabinofuranosidases from CAZy family GH43 are able to remove arabinosyl units from doubly substituted xylooligomers (van den Broek et al., 2005; Sorensen et al., 2006). Four genes encoding GH43 family and two genes encoding GH115 α-glucuronidases were identified in *A. bisporus* genome (Chapter 2) (Morin et al., 2012), but transcriptome analysis revealed no or low expression of these genes (Chapter 5). Two of the secreted GH43 and two of GH115 proteins were identified in proteomics analysis with very low abundance (Chapter 5). Taken together Chapter 5 and 6 data sets, substantial evidence implicates improvement of commercial mushroom production by either stimulation of gene expression during the second flush which could potentially lead to higher mushroom production or by adding lacking xylan de-branching enzymes to the compost

as well as by developing new commercial *A. bisporus* strain capable of degrading substituted xylan.

The potential for improving mushroom production

The observations in this thesis provide functional models that may explain the plant biomass degrading abilities of *A. bisporus*. Definitive mechanisms remain uncertain, but these investigations identify a set of potential important genes as well as secreted enzymes, including those which are missing and potentially significant for the substrate degradation. More detailed functional analysis could offer novel insights and improvements into degrading abilities of *A. bisporus* which may be a contributing factor for the mushroom industry.

The sequenced *A. bisporus* genome (Chapter 2) (Morin et al., 2012) has become a highly valuable resource for performing genomic and metabolic comparisons among fungi (Floudas et al., 2015; Kohler et al., 2015). Beyond its interest for fundamental knowledge, the release of the *A. bisporus* genome sequence opens a new era for breeding applications. All the cultivars used by mushroom industry all over the world belong to *A. bisporus* var. *bisporus* which has a predominantly pseudohomothallic life style. The additionally sequenced genome of *A. bisporus* var. *burnettii* (Chapter 2) (Morin et al., 2012) which is different in its ecology, mode of reproduction (mainly heterothallic) as well as morphological features, and further rigorous comparative genomics analysis can provide a better understanding of the ecological adaptation of this variety to its desert habitat (Callac et al., 1993; Kerrigan et al., 1994). In addition, *A. bisporus* var. *burnettii* is able to produce high yields of mushrooms at various ranges of temperatures (17 °C, 25 °C and 30°C) (Largeteau et al., 2011; Navarro & Savoie, 2015), while *A. bisporus* var. *bisporus* develops fruiting bodies with less productivity and less early fruiting at 25 °C compared with 17 °C (Largeteau et al., 2011). Identification of several candidate genes combined with QTL (Quantitative Trait Loci) mapping and transcript analyses have shed light on understanding the abilities of *A. bisporus* var. *burnettii* to produce mushrooms at 25 °C (Foulongne-Oriol et al., 2014). This analysis combined with the following detailed investigation of the genomes can contribute to designing breeding schemes between two varieties and lead to improved mushroom production. However, the difference in the quality of the two genome sequences (nearly complete *A. bisporus* var. *bisporus* assembly and the fragmented one of var. *burnettii*) should be taken into account for the further research. To achieve not only better foundations for breeding programmes but also to get more information on the different reproductive styles within *A. bisporus*, the sequencing of the genome of the third variety, *A. bisporus* var. *eurotetrasporus*, which represents a true homothallic life style should be considered.

The expression and proteome profiles, broad assays of carbohydrate degrading enzymes of various growth stages of *A. bisporus* cultivated in compost in conjunction with the substrate composition and carbohydrate structure analysis (Chapter 3, 4, 5 and 6) can provide a valuable resource for further studies which could improve substrate utilization leading to higher mushroom production. Addition of the third and maybe even fourth flushes of mushroom for transcriptome analysis can reveal if gene expression of those flushes will follow the same pattern of highly expressed (hemi-) cellulolytic genes of the first flush or low expression of these genes of the second flush. Also investigating the expression levels of each day during the harvesting of mushroom flushes (usually mushrooms of every flush are harvested within few days) can direct mushroom industry at which particular stage stimulation of gene expression can be applied to promote higher mushroom production. Remarkably, the lack of arabinofuranosidase and α-glucuronidase activities was observed in compost (Chapter 6). Thus, it will be important to find alternatives for employment of these enzymes to the compost which could give access to the easily available carbon source and satisfy

the nutritive needs of *A. bisporus*. Future research should also address collecting and exploring new wild type strains which could have the ability to degrade substituted xylan. We had an opportunity to look at the progeny from two *A. bisporus* var. *bisporus* homokaryons regarding carbon substrate preference by growing these strains on various mono-, polysaccharides and complex substrates (results not shown). Most differences observed between these homokaryons were related to xylan degradation. The heterokaryon and one of the homokaryons grew much better on pentoses and xylan compared to the other parental strain. This is undoubtedly significant evidence that in-depth research of various *A. bisporus* strains could provide valuable insights for the development of a new commercial strain with better substrate degrading and further carbohydrate utilization abilities. All these small improvements may result in a substantial increase of the substrate conversion leading to higher yield of this tasty mushroom product.

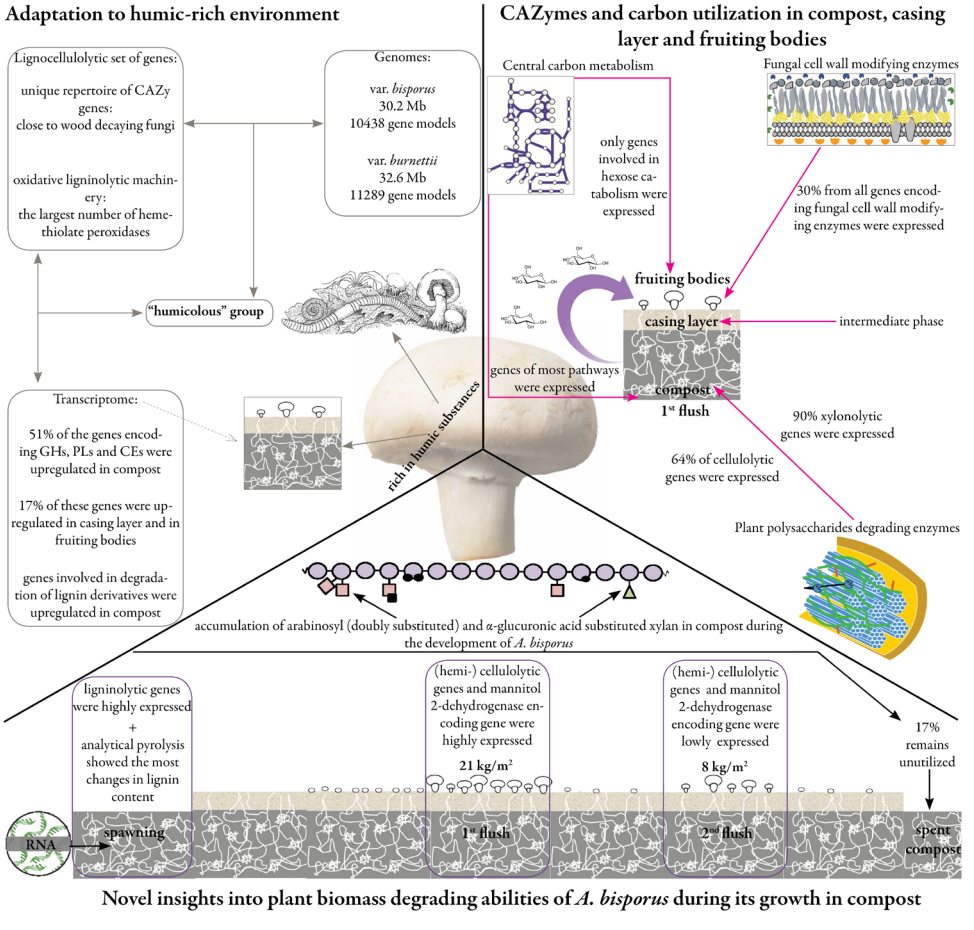

Figure 1 | Summary of the research described in this thesis. Plant biomass utilization by *A. bisporus*.

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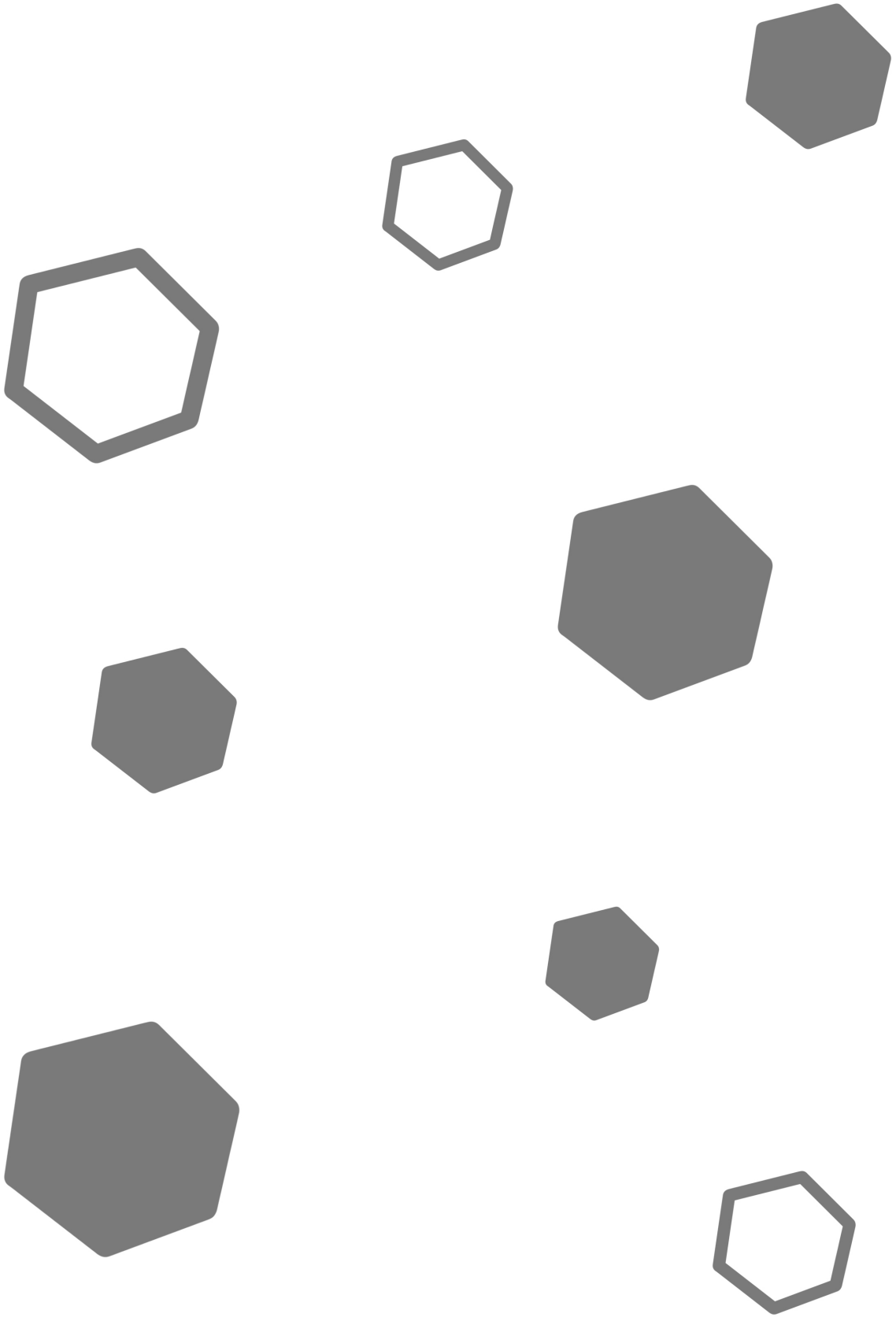

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Acknowledgements

Unraveling the mystery of commercial cultivation of *Agaricus bisporus*: plant biomass utilization and its effect on mushroom production

Summary

MUSHROOM FORMING FUNGI, Agaricomycetes, are the most familiar and conspicuous of all the Fungi, because they mostly produce a great variety of large fruiting bodies. Agaricomycetes represent a diverse class of the phylum Basidiomycota (Basidiomycetes) with 21000 described species that have important ecological roles in nature. Among this class of fungi, there are species that are saprotrophs (decompose organic matter for their nutrition), plant pathogens, and mutualistic symbionts with the roots of various plant (ectomycorrhizae).

Mushrooms are not only important components of ecosystems but also have a strong impact on people's daily lives. They play an essential role in agriculture, medicine, food industry, textiles, bioremediations and many other industrial fields. The majority of edible mushrooms are Agaricomycetes. Most of the wild-grown edible mushrooms are ectomycorrhizae, such as porcini (*Boletus edulis*), chanterelle (*Cantharellus cibarius*), and matsutake (*Tricholoma matsutake*). The most popular cultivated edible Agaricomycetes are button mushrooms or champignons (*Agaricus bisporus*), shiitake (*Lentinula edodes*) and oyster mushrooms (*Pleurotus ostreatus*).

A. bisporus is the most commonly cultivated, consumed and recognized mushroom worldwide, and an important component of the human diet. The first successful cultivation of the white button mushroom was recorded in France in the seventeenth century. Today, China, the United States and The Netherlands are the largest growers of cultivated white button mushrooms. It grows on complex substrates (compost, humic soil) to which it is highly specialized. Commercially *A. bisporus* grows on compost which consists of animal manure (usually horse and chicken), wheat straw, gypsum, water and different additives. Mushroom production involves several sequential steps which include composting stage (Phase I and II: when all the compost ingredients are mixed, heated, conditioned and pasteurized), spawning stage (broadcasting of spawns (cereal grains colonized by the mushroom mycelium) and fully colonization of compost by the mushroom mycelium), casing layer stage (an overlay of peat and lime applied to colonized compost), pinning stage (primordia or "pins" are knots of mycelium that develop into mushrooms), and the stage of harvestable mushrooms which grow in repeating cycles called "flushes".

Wheat straw is the main component of the mushroom compost. This plant material comprises three main types of carbon-based polymers – cellulose, hemicellulose and lignin – collectively called lignocellulosic biomass. Cellulose is a polymer of glucose, while hemicelluloses are polymers that incorporate a range of different sugars. Lignin is a complex mixture of polymers made from ring-shaped phenolic groups with carbon-based structures. To obtain all needed nutrients for growth *A. bisporus* secretes various types of enzymes that attack and take apart the recalcitrant lignocellulosic cell walls of the plant material to liberate the sugars.

Growth of *A. bisporus* is one of the few biotechnological processes in which all components of lignocellulose are converted into a new product. Utilization of the substrate is not optimal and a significant amount of the substrate remains unused at the end of mushroom production. Understanding of the process of substrate degradation has been conspicuously lacking despite the prime importance of *A. bisporus* to the multibillion dollar mushroom industry. Therefore, there is an enormous interest in improving substrate conversion for mushroom production. To accomplish this, we need a better understanding of the reactions involved in biomass degradation by *A. bisporus* during its commercial cultivation. This PhD thesis provides new insights into this mystery.

In an effort to determine whether *A. bisporus* has genes involved in production of

lignocellulose degrading enzymes, the genome sequence of *A. bisporus* was elucidated in **Chapter 2**. A full repertoire of these genes was identified, demonstrating similarity to that of wood degrading fungi. The *A. bisporus* genome contains a striking expansion of genes (relative to other lignocellulose degrading fungi) that enhance degradation of lignin, such as heme-thiolate peroxidases and β-etherases. This suggests a broad attack on breaking down lignin and related metabolites found in humic-rich environments, such as compost. Genes encoding heme-thiolate peroxidases and β-etherases together with other genes encoding lignin degrading enzymes (manganese peroxidases, multicopper radical oxidases and cytochrome P450s) are expressed during growth in compost. This analysis revealed a new classification, "humicolous", for species such as *A. bisporus* adapted to grow in humic-rich, partially decomposed environment.

Chapter 3 addresses identification of the carbon metabolic genes to understand the carbon nutritive needs of the fungus. Expression of these genes reveals a highly specialized metabolism aimed at hexose sugars in the fruiting body, while in the compost-grown mycelium a much more diverse metabolism is active. Furthermore, Chapter 3 also demonstrates the essential difference between the compost-, casing layer-grown mycelium, and fruiting bodies of *A. bisporus*. Compost-grown mycelium is mainly focused on degrading the plant biomass while fruiting bodies are emphasised on modification of the *A. bisporus* cell wall. The casing layer is an intermediate phase in which both plant biomass decomposition and modifications of fungal polysaccharides take place.

To further evaluate the degradation of the substrate by *A. bisporus*, the fungus was commercially cultivated and compost samples were taken at selected time points (spawning stage, pinning stage, the first flush, the second flush stages and when entire first and second flushes were harvested). Transcriptome (gene expression), secretome (protein production), substrate composition and enzymatic activities were analyzed throughout the growth of *A. bisporus* in compost. Analysis of the transcriptome required isolation of high quality and quantity of RNA. Extraction of RNA from complex plant biomass based materials with high content of humic substances such as compost is the greatest challenge. In **Chapter 4** several protocols have been tested to isolate pure and sufficient amounts of RNA from compost in a reproducible way. An RNA extraction protocol was investigated using the CsCl ultracentrifugation method. This method showed to be successful providing high level of integrity and good quantity of RNA. Extracted RNA was suitable for reliable analyses of gene expression and produced consistent and reproducible results. The method is also suitable for other plant biomass based samples.

Chapter 5 demonstrates how *A. bisporus* degrades its substrate during the commercial cultivation process. In this case, genes that allow *A. bisporus* to produce the correct mixture of lignocellulose degrading enzymes were determined and compared with related secreted proteins and substrate composition. Such a broad comparison offers a wealth of information. The combined data reveals that lignin is mainly degraded at the beginning of the cultivation process (spawning stage) and stays not modified from the pinning stage until the end of the cultivation. This complements evidence for the sequential lignocellulose degradation by wood degrading fungi.While major differences in cellulose and hemicellulose degradation found between first and second flushes of mushrooms. These genes are highly expressed at the first flush while low expression is observed at the second flush of harvested mushrooms. This finding could answer the long-standing question regarding the differences in the mushroom yield between flushes (first flush of mushrooms has much higher yield compared to following flushes). In addition to lignocellulose degradation, carbon catabolism was analyzed during the commercial growth of *A. bisporus* uncovering preferences of hexose sugars over pentoses by this fungus.

It is also crucial to determine enzyme activities in compost during the commercial cultivation. **Chapter 6** shows that enzymes which break apart cellulose and hemicellulose are present throughout

the growth of *A. bisporus* in compost. Another puzzling aspect of substrate utilization by *A. bisporus* is the unutilized part of the compost at the end of the commercial cultivation. Hemicellulose xylan substituted with arabinosyl and glucuronic acid remains not being degraded by the end of the mushroom cultivation.

This work is significant as the first report on the entire commercial cultivation process of *A. bisporus* and substrate degradation with a clear genetic background. The combination of genomic, transcriptomic, proteomic data together with enzymatic activities and substrate composition analyses unravels the mystery of the growth of *A. bisporus* in compost and opens new avenues for degradation of lignocellulose by this fungus, providing potentials for improving mushroom production. Findings of this PhD research are a treasure trove for researchers and mushroom industry. But it is just a beginning.

Het mysterie van de commerciële teelt van *Agaricus bisporus* ontrafelen: gebruik van plantaardige biomassa en het effect daarvan op de teelt van champignons

Nederlandse Samenvatting

VAN ALLE SCHIMMELS ZIJN DE PADDENSTOEL VORMENDE SCHIMMELS, de agaricomyceten, het meest bekend en opvallend, omdat zij een grote verscheidenheid aan vruchtlichamen produceren. Agaricomycetes spelen een belangrijke ecologische rol in de natuur en vertegenwoordigen met 21.000 beschreven soorten een klasse van de phylum Basidiomycota (Basidiomyceten) met een grote diversiteit. Verschillende agaricomyceten soorten zijn saprotroof (voeden zich uitsluitend met 'dode' organische materie), planten pathogeen of leven als symbiont met de wortels van verschillende planten (ectomycorrhizae).

Paddenstoelen zijn niet alleen ecologisch belangrijk, maar hebben ook een grote invloed op het dagelijks leven. Zij spelen namelijk een essentiële rol in de landbouw, de geneeskunde, de voedingsindustrie, de textielindustrie, bioremediatie en vele andere industriële sectoren. De meerderheid van de eetbare paddenstoelen behoren tot de agaricomyceten. De meeste van de in de natuur voorkomende eetbare paddenstoelen zijn ectomycorrhizae, zoals eekhoorntjesbrood (*Boletus edulis*), cantharellen (*Trechter cibarius*) en matsutake (*Tricholoma matsutake*). De meest populaire geteelde eetbare agaricomycetes zijn de champignon (*Agaricus bisporus*), shiitake (*Lentinula edodes*) en oesterzwammen (*Pleurotus ostreatus*).

A. bisporus is de meest geteelde, geconsumeerde en bekendste paddenstoel in de wereld en daarbij is *A. bisporus* ook nog een gezonde voedingsbron voor mensen. De champignon werd voor het eerst succesvol gecultiveerd in Frankrijk in de zeventiende eeuw. Tegenwoordig zijn China, de Verenigde Staten en Nederland de grootste telers van de champignon. Champignons zijn gespecialiseerd in het groeien op complexe substraten (compost en humus) en in de afbraak hiervan. *A. bisporus* wordt commercieel geteeld op compost. Deze compost bestaat uit dierlijke mest (meestal paarden en kippenmest), tarwe-stro, gips, water en verschillende toevoegingen. De teelt van champignon bestaat uit verschillende opeenvolgende stappen. Het begint met een compostering proces bestaande uit fase I en II. Hierin worden alle compost ingrediënten gemengd, verwarmd, geconditioneerd en gepasteuriseerd. Hierna komt de doorgroei (spawning) periode, waarin het broed (spawn), dat zijn graankorrels gekoloniseerd door schimmel mycelium, door de compost wordt gemixt. Vanuit het broed wordt de compost volledig gekoloniseerd met mycelium. De broed periode wordt gevolgd door de dekaarde (casing layer) periode. Dit is de periode waarbij een laag van turf en kalk wordt aangebracht op de met schimmel gekoloniseerde compost. Tijdens de volgende periode worden de primordia of "knoppen" (pins) gevormd, daarom wordt dit het de knopvorming (pinning) periode genoemd. De mycelium knoppen ontwikkelen zich tot champignons/vruchtlichamen. In de oogst periode worden de volgroeide champignons geoogst. Dit gebeurd in herhalende cycli genaamd "vluchten".

Tarwe stro is de belangrijkste component van de compost waarop champignons worden gegroeid. Dit planten materiaal bestaat uit drie hoofdtypen van koolstof gebaseerde polymeren: Cellulose, hemicellulose en lignine. Deze drie typen worden samen de 'lignocellulosic biomass' genoemd. Cellulose is een polymeer van alleen glucose, terwijl hemicellulose een polymeer is van verschillende suikers. Lignine is een complex mix van polymeren van ringvormige fenolische groepen met op koolstof gebaseerde structuren. Om de benodigde voedingsstoffen te verkrijgen, scheidt *A. bisporus* verschillende typen enzymen uit. Deze enzymen kunnen de 'recalcitrante' lignocellulosische celwanden, waaruit het plantenmateriaal bestaat, afbreken en zo de benodigde suikers vrij maken.

De groei van *A. bisporus* is een van de weinige biotechnologische processen waarin alle onderdelen van lignocellulose worden omgezet in een nieuw product. De conversie van het substraat is niet optimaal, en daardoor blijft er aan het eind van de champignon productie een significante hoeveelheid substraat over. Ondanks het enorme belang van *A. bisporus* in de miljarden dollar paddenstoelen industrie, ontbreekt het de kennis over substraat afbraak door *A. bisporus*. Daarom zijn champignon telers zeer geïnteresseerd in het verbeteren van de substraat omzetting. Hiervoor moet er meer kennis worden verzameld over planten biomassa afbraak door commercieel geteelde *A. bisporus*. Dit proefschrift geeft nieuwe inzichten in dit mysterie.

In **Hoofdstuk 2** is de genoom sequentie van *A. bisporus* bestudeerd met het doel om alle genen te identificeren die betrokken zijn bij de productie van lignocellulose afbrekende enzymen. Uit de genoom sequentie blijkt dat het repertoire van genen die betrokken zijn bij lignocellulose afbraak overeenkomt met het repertoire van hout afbrekende schimmels. Het *A. bisporus* genoom bevat een opvallende hoeveelheid genen (ten opzichte van andere lignocellulose afbrekende schimmels) die de afbraak van lignine versnellen, zoals heem-thiolaat peroxidasen en β-etherasen. Dit suggereert dat lignine en verwante metabolieten in humus-rijke omgevingen zoals compost wordt afgebroken door verschillende mechanismes. Genen die coderen voor lignine afbrekende (co)enzymen (heem-thiolaat peroxidasen, β-etherasen, mangaan peroxidasen, oxidasen multikoper radicalen en cytochroom P450's) worden tijdens de groei van *A. bisporus* in compost tot expressie gebracht. De genoom analyse onthulde een nieuwe classificatie voor schimmels die zich hebben aangepast aan humus rijke, deels afgebroken omgeving, zoals *A. bisporus*. Deze klasse schimmels zijn 'humicoulous' genoemd.

In **Hoofdstuk 3** worden de koolstof metabolisme genen geïdentificeerd die betrokken zijn bij nutriënten behoefte van *A. bisporus*. Expressie van deze genen laat een zeer gespecialiseerde stofwisseling zien. Hierbij is het metabolisme in de paddenstoel voornamelijk gericht op de omzetten van hexose suikers, terwijl het mycelium dat in de compost groeit een veel gevariëerder metabolisme heeft. Hoofdstuk 3 toont ook een significant verschil in metabolisme tussen het mycelium groeiend in compost of in de casing laag en het metabolisme in vruchtlichamen van *A. bisporus*. Mycelium dat in compost groeit is vooral gericht op het afbreken van de plantaardige biomassa, terwijl het metabolisme van de vruchtlichamen zich voornamelijk richt op het modificeren van de *A. bisporus* celwand. Het mycelium dat in de casing laag groeit laat een tussenvorm zien. Het metabolisme in deze tussenvorm is op zowel de afbraak van plantaardige biomassa als op het modificeren van de polysacchariden van de schimmel celwand gericht.

De substraat afbraak door *A. bisporus* werd verder geëvalueerd door van de commercieel geteelde champignons compost monsters te nemen op verschillende tijdstippen (doorgroei periode, knopvorming periode, de eerste vlucht, de tweede vlucht). Gedurende de groei van *A. bisporus* werden het transcriptoom (gen expressie), het secretoom (eiwit productie), evenals de substraat samenstelling en de enzymatische activiteiten in de compost geanalyseerd. De analyse van het transcriptoom vereiste RNA van hoge kwaliteit en kwantiteit. Extractie van zuiver RNA uit complexe plantaardige biomassa dat een hoog gehalte aan humus stoffen heeft, zoals compost, is een grote uitdaging. In **Hoofdstuk 4** zijn verschillende protocollen getest om zuiver en een voldoende hoeveelheid van RNA uit compost te isoleren. Een onderzocht RNA-extractie protocol maakt gebruik van de CsCl ultracentrifuge. Deze methode bleek uitermate succesvol. Het geïsoleerde RNA was van hoge kwaliteit en kwantiteit. Het geëxtraheerde RNA was geschikt voor analyse van gen expressie en gaf betrouwbare en reproduceerbare resultaten. De methode bleek ook geschikt te zijn voor RNA isolatie uit andere plantaardige biomassa's.

Hoofdstuk 5 laat zien hoe *A. bisporus* het substraat afbreekt tijdens de commerciële teelt. In deze studie werden de genen waarmee *A. bisporus* de juiste mix lignocellulose afbrekende

enzymen bepaald, vergeleken met verwante gesecreteerde eiwitten en substraatcompositie. Zo'n brede vergelijking biedt een schat aan informatie. Uit deze gecombineerde data blijkt dat lignine voornamelijk wordt afgebroken in het begin van het groei proces (doorgroei periode). Vanaf de knopvorming periode tot het einde van de cultivatie blijft de lignine samenstelling ongewijzigd. Dit is in overeenstemming met het bewijs voor de sequentiële lignocellulose afbraak door hout afbrekende schimmels. Ook zijn er grote verschillen in cellulose en hemicellulose afbraak tussen de eerste en tweede vlucht van champignons. De genen betrokken bij de afbraak van cellulose en hemicellulose komen hoog tot expressie tijdens de eerste vlucht, terwijl een lage expressie wordt waargenomen tijdens de tweede vlucht. Dit resultaat kan de vraag over de verschillen in de paddenstoel opbrengst tussen de vluchten beantwoorden (de oogst van de eerste vlucht heeft een veel hogere opbrengst in vergelijking met volgende vluchten). Naast lignocellulose afbraak werd het koolstof katabolisme geanalyseerd tijdens de commerciële teelt van *A. bisporus*. Deze analyse laat zien dat *A. bisporus* een voorkeur voor hexose suikers heeft ten opzichte van pentose suikers.

Om de compost afbraak van *A. bisporus* te begrijpen is het ook van cruciaal belang om de enzym activiteiten in compost te bepalen tijdens de commerciële teelt. **Hoofdstuk 6** toont aan dat de cellulose en hemicellulose afbrekende enzymen gedurende de gehele groei van *A. bisporus* in de compost aanwezig zijn. Een ander onbekend aspect van het substraat gebruik door *A. bisporus* is dat na afloop van de commerciële teelt een deel van de compost nog niet is verbruikt. Aan het eind van de teelt blijft er nog een met name het hemicellulose xylaan vertakt met arabinose en glucuronzuur over.

Dit werk is het eerste verslag over het gehele commerciële teelt proces van *A. bisporus* met de focus op substraat afbraak en het genetische aspect dat hieraan ten grondslag ligt. De combineerde analyse van genomische, transcriptomische, en proteomische data samen met de enzymatische activiteiten en de substraat samenstelling ontrafelt het mysterie van de groei van *A. bisporus* in compost. Deze kennis opent nieuwe mogelijkheden voor het verbeteren van de afbraak van lignocellulose door deze schimmel en hiermee mogelijk een efficiëntere productie van champignons. Bevindingen in dit promotieonderzoek geeft een nieuwe kijk op de commerciële teelt van *A. bisporus.* Maar het is slechts een begin.

Раскрытие тайны промышленного культивирования *Agaricus bisporus*: использование растительной биомассы и ее влияние на продуктивность шампиньонов

Краткое изложение на русском языке

ГРИБЫ АГАРИКОМИЦЕТЫ (лат. Agaricomycetes), представляют собой наиболее известный и легко узнаваемый класс царства Грибов, обладающих большим разнообразием крупных плодовых тел. Класс Агарикомицеты широко распространен и разнообразен. Этот класс включен в отдел Базидиомикота (Базидиомицеты) и на сегодняшний день представлен 21000 описанных видов, играющих важную роль в биологическом круговороте веществ в природе. Грибы этого класса можно разделить на несколько экологических групп: сапротрофы (живущие за счет разложения органического вещества), растительные патогены, и мутуалистические симбионты, образующие взаимовыгодные отношения с корнями различных растений (эктомикориза).

Грибы являются не только важным компонентом экосистемы, но также оказывают большое влияние на повседневную жизнь людей. Они играют важную роль в сельском хозяйстве, медицине, пищевой и текстильной промышленности, биоремедиации и многих других промышленных областях. Большинство съедобных грибов относятся к классу Агарикомицеты. Большая часть дикорастущих съедобных грибов представлена эктомикоризой, например белый гриб (*Boletus edulis*), лисички (*Cantharellus cibarius*), и мацутакэ (*Tricholoma matsutake*). Самыми популярными культивируемыми агарикомицетами являются шампиньоны (*Agaricus bisporus*), шиитаке (*Lentinula edodes*) и вешенки (*Pleurotus ostreatus*).

A. bisporus (шампиньон двуспоровый) – наиболее часто культивируемый, потребляемый и известный гриб во всем мире, который также является важным компонентом рациона человека. Первое успешное культивирование шампиньонов было зарегистрировано во Франции в семнадцатом веке. На сегодняшний день, Китай, Соединенные Штаты и Нидерланды являются крупнейшими производителями шампиньонов. Этот гриб растет на сложных субстратах (компост, почва богатая гуминовыми веществами), к которым он хорошо приспособлен. В промышленности, *A. bisporus* растет на компосте, который состоит из навоза (как правило, лошадиного и куриного), пшеничной соломы, гипса, воды и различных добавок. Технология промышленного выращивания шампиньонов включает в себя несколько стадий. Культивирование начинается с подготовки компоста, включающей в себя две фазы (Фаза I и Фаза II). Во время этого процесса все ингредиенты компоста смешивают, нагревают, кондиционируют и пастеризуют. На стадии посева и разрастания готовый компост засевается мицелием шампиньона, представляющим собой зерна злаков, покрытые мицелием *A. bisporus*. Компост равномерно перемешивают с посевным мицелием, который далее разрастается в компосте. После этого наступает следующая стадия - нанесение покровной смеси. Этой смесью, состоящей из торфа и известняка, покрывают заросший мицелием компост. На этой стадии мицелий шампиньона продолжает разрастаться, но уже в покровном слое. В результате мицелиальные гифы соединяются и образуют сначала «звездочки», потом узелки, и из них уже образуются примордии или булавочные головки (стадия формирования примордий). В течение некоторого времени примордии развиваются в плодовое тело шампиньонов. Плодовые тела шампиньонов образуются несколькими волнами плодоношения, первая из которых приносит наибольший урожай (стадия плодообразования и сбора грибов).

Пшеничная солома является основным компонентом компоста для промышленного

производства шампиньонов. Этот растительный материал состоит из трех основных видов полимеров на основе углерода - целлюлозы, гемицеллюлозы и лигнина. Комплексно эти полимеры называются лигноцеллюлозной биомассой. Целлюлоза – это полимер глюкозы, в то время как гемицеллюлоза представляет собой полимеры, которые включают в себя целый ряд различных сахаров. Лигнин - сложная смесь полимеров, состоящих из кольцеобразных фенольных групп с углеродной основой. *A. bisporus* выделяет различные типы ферментов, атакующие и разлагающие лигноцеллюлозные клеточные стенки растительного материала, и высвобождающие различные сахара. Эти сахара являются необходимыми питательными веществами для роста и развития шампиньона.

Рост *A. bisporus* в компосте является одним из нескольких биотехнологических процессов, когда все компоненты лигноцеллюлозы преобразуются в новый продукт. Тем не менее, утилизация субстрата не оптимальна и значительное его количество остается неиспользованным в конце процесса производства шампиньонов. Несмотря на первостепенное значение *A. bisporus* для многомиллиардной промышленности шампиньонов, процесс разложения и утилизации субстрата плохо изучен. В связи с этим, в грибной промышленности появился огромный интерес к улучшению конверсии субстрата для производства шампиньонов. Для достижения более продуктивного производства шампиньонов, необходимо понять и объяснить реакции участвующие в деградации растительной биомассы во время промышленного культивирования *A. bisporus*. Данная докторская диссертация раскрывает новые аспекты этого загадочного процесса.

Для того чтобы определить, имеет ли *A. bisporus* гены, кодирующие выработку ферментов принимающих участие в процессе разложения лигноцеллюлозы, геном *A. bisporus* был секвенирован и описан во **2-ой Главе**. Полный арсенал этих генов был выявлен, и проявил сходство с набором генов, которым обладают грибы разлагающие древесину. Геном *A. bisporus* содержит поразительное количество генов (относительно геномов других грибов разлагающих лигноцеллюлозу), которые усиливают разложение лигнина, такие как гем-тиолят пероксидазы и β-эстеразы. Эти данные предполагают многостороннее разложение лигнина, а также наличие связанных с ним метаболитов, присутствующих в средах богатых гумином, таких как компост. Гены кодирующие гем-тиолят пероксидазу и β-эстеразу, вместе с другими генами кодирующими выработку ферментов, разрушающих лигнин (пероксидазы марганца, голубые медьсодержащие оксидазы и цитохром P450), экспрессированы во время роста *A. bisporus* в компосте. Анализ, представленный во 2-ой Главе, также выявил новую классификацию, «humicolous», для таких видов, как *A. bisporus*, которые адаптированы к росту в частично разложившихся средах богатых гумином.

3-я Глава направлена на идентификацию генов принимающих участие в метаболизме углеводов для выявления питательных потребностей шампиньона. Исследование экспрессии этих генов выявило что *A. bisporus* обладает высокоспециализированным метаболизмом. Плодовые тела *A. bisporus* нацелены на метаболизм гексоз. В то время как мицелий, находящийся в компосте обладает гораздо более разнообразным метаболизмом. Кроме того, 3-я Глава также демонстрирует существенную разницу между мицелием присутствующим в компосте, в покровном слое и плодовыми телами *A. bisporus*. Мицелий, находящийся в компосте в основном сосредоточен на разложение растительной биомассы, в то время как плодовые тела направлений на модификацию клеточной стенки *A. bisporus*. Между тем покровный слой является промежуточной фазой, в которой происходит как разложение растительной биомассы, так и модификация полисахаридов клеточной стенки шампиньона.

Для дальнейшего исследования как *A. bisporus* разлагает субстрат, этот гриб культивировался в промышленных условиях и образцы компоста были собраны на

определенных стадиях развития гриба (стадия посева и разрастания мицелия; стадия сформированных примордий; стадия первой и второй волны выращенных шампиньонов; а также стадия, когда полностью грибы первой и второй волны были собраны). Транскриптом (экспрессия генов), секретом (совокупность секретируемых белков), состав субстрата, а также ферментативные активности были анализированы в процессе роста *A. bisporus* в компосте. Для проведения анализа транскриптома необходима РНК высокого качества и в большом количестве. Выделение РНК из комплексных субстратов растительной биомассы с высоким содержанием гуминовых веществ (например, компост) является сложной задачей. В **4-ой Главе** несколько методов выделения РНК из компоста были протестированы, для того чтобы получить беспримесную РНК в достаточном количестве. Один из методов изоляции РНК был исследован с помощью техники градиентного CsCl ультрацентрифугирования. Этот метод оказался высокоэффективным и обеспечил РНК высокого уровня целостности и в большом количестве. Выделенная этим методом РНК из компоста соответствовала требованиям необходимым для анализа экспрессии генов. Этот метод был протестирован и на других образцах основанных на растительной биомассе и также показал высоко воспроизводимые результаты.

5-ая Глава раскрывает как *A. bisporus* разлагает субстрат в процессе коммерческого культивирования. В этом случае гены, которые необходимы *A. bisporus* для выработки определенной смеси ферментов разлагающих лигноцеллюлозу, были выявлены и сопоставлены с соответствующими секретируемыми белками и с составом субстрата. Такой обширных анализ данных и их сопоставление дает огромное количество информации. Исследование этих комбинированных данных обнаружило, что разложение лигнина в основном происходит в начале процесса культивирования (на стадии посева и разрастания мицелия). В то время как с начала стадии формирования примордий и до конца всего процесса культивирования лигнин остается не измененным. Это дополняет доказательства о последовательном разложении лигноцеллюлозы древо-разлагающими грибами. В то же время основные различия в разложении целлюлозы и гемицеллюлозы были обнаружены между первой и второй волнами сбора грибов. Высокая экспрессия генов, принимающих участие в этих процессах, была выявлена в первой волне сбора шампиньонов. А низкая экспрессия этих генов наблюдалась во время второй волны сбора грибов. Это открытие может привести к ответу на давно существующий вопрос о различиях в количестве собранных шампиньонов между волнами сбора (первая волна сбора грибов имеет гораздо более высокую урожайность по сравнению с последующими волнами сбора). В дополнение к исследованию процесса разложения лигноцеллюлозы, катаболизм углеводов был анализирован в процессе коммерческого роста *A. bisporus*. Этот анализ выявил предпочтения шампиньонов в отношении гексозных сахаров вместо пентоз.

Также большое значение имеет определение активности ферментов в компосте в процессе коммерческого культивирования грибов. В **6-ой Главе** было определено, что ферменты, разрушающие целлюлозу и гемицеллюлозу, присутствуют в процессе всего роста *A. bisporus* в компосте. Еще один важный вопрос об утилизации субстрата шампиньоном *A. bisporus* остается нераскрытым - это оставшаяся неиспользованной часть компоста в конце промышленного выращивания гриба. В данной исследовательской работе было выявлено, что гемицеллюлоза - ксилан с ответвлениями арабинозы и глюкуроновой кислоты остается не разложенной в конце культивирования шампиньонов.

Настоящая докторская работа является первым важным отчетом о полном коммерческом процессе культивирования шампиньонов, разложении и утилизации субстрата, подтвержденным генетическими данными. Комбинирование данных генома, транскриптома,

протеома вместе с анализом ферментативной активности и составом субстрата раскрывает тайну промышленного культивирования *A. bisporus* в компосте и открывает новые пути в понимании процесса разложения лигноцеллюлозы этим грибом, а также предоставляет возможности для улучшения производства этих грибов. Результаты этого научного исследования являются кладом для исследователей и грибной промышленности. Но это только начало.

Curriculum Vitae

ALEKSANDRINA PATYSHAKULIYEVA was born on October 16th in Balkanabat, Turkmenistan. She followed her secondary education in specialized school 64, Ashgabat, Turkmenistan, and graduated in 2004 with an Honors Certificate of Secondary Education. In October of the same year she began her Pre-University study in Medicine and Biology at Belarusian State University, Minsk, Belarus, and received her Honors Graduation Certificate in 2005. She continued her higher education at the Belarusian State University, where she obtained both her Bachelor's and Master's degrees in Biology (Biotechnology), and was also qualified as Lecturer of Biology in 2010. As part of this higher education, she did her Bachelor's and Master's internships under the supervision of Assoc. Prof. Alina M. Khodosovskaya at the Department of Molecular Biology of the Belarusian State University. During her main Master research training, she investigated molecular differentiation of the Belarusian *Phytophthora infestans* strains according to their belonging to certain haplotypes of mitochondrial DNA.

In November 2010 Aleksandrina started her PhD in Fungal Physiology in conjunction with Utrecht University, The Netherlands, and CBS-KNAW Fungal Biodiversity Centre, The Netherlands, under the supervision of Prof. Dr. ir. Ronald P. de Vries. The results of her research in this period are described in this thesis, entitled "Unraveling the mystery of commercial cultivation of *Agaricus bisporus*: plant biomass utilization and its effect on mushroom production".

List of Publications

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.
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Abstracts and Presentations

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. 17th International Congress of the Hungarian Society for Microbiology, July 8-10, 2015, Budapest, Hungary (**Oral Presentation**)

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., Ronald P. de Vries The 28th Fungal Genetics Conference, March 17-22, 2015, Pacific Grove, California, USA (Poster Presentation)

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