Fungal treatment of lignocellulosic biomass

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Thesis

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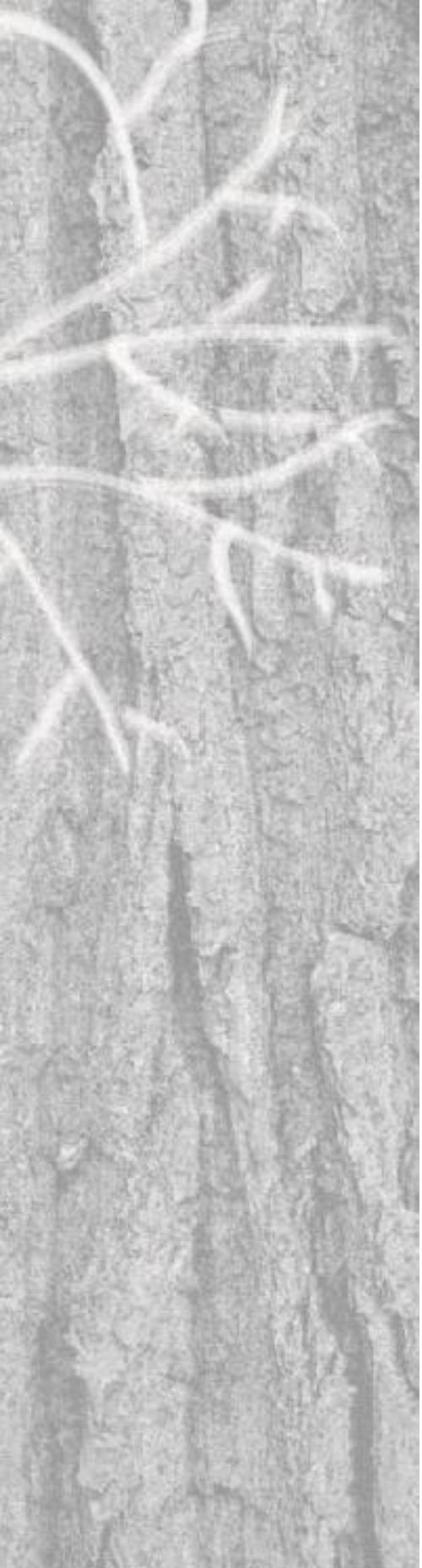

CHAPTER 1

General introduction

1.1 Competition between food, feed and fuel

The world population is expected to grow to 9 billion people in 2050 (FAO, 2009). This increase in world population means an increasing demand for food and energy. Not only the world population but also the standard of living is expected to increase in many countries. This increase is higher for developing countries than for developed countries, resulting in a more equal per capita income all over the world (FAO, 2009). Yotopoulos (1985) describes an increasing demand for grains when income increases. Of the global grain production about 40% is fed to livestock and in well-developed countries this is even up to 70% (Lundqvist et al., 2008). As such, more grains will be used to produce animal products as the annual income and prosperity is increasing (Yotopoulos, 1985; Banerjee, 2011), thereby increasing demand grain demand.

Since fossil fuels are expected to become scarce, alternatives are being developed. One of the alternatives is the production of bioethanol. The EU countries have agreed to achieve as a target 27% of the total energy to be renewable in 2030 (European Commission, 2014). Currently in the U.S.A. and Brazil, bioethanol is being produced on a large scale. This bioethanol production is mainly based on grains, sugarcane, corn and soy, products that are also used in food and feed.

In order to meet the increasing demand for food, feed and fuel, more arable land is needed. Up to now, the amount of arable land is increasing, but not to the same extent as the demand for agricultural products (FAO, 2009). Improving the efficiency of land use will increase production and is one of the many ways to meet the increasing demand. Another option is the utilization of products that are now considered as waste. For example, with the production of wheat, also a substantial amount of straw (1.3 kg straw per kg wheat (Talebnia et al., 2010)) is produced which is regarded as waste. By using the whole plant, food production can be combined with feed and fuel production on the same plot of land. Using the whole plant for feed production will not only lead to a more efficient land use, but also to a reduction of agricultural waste.

1.2 Lignocellulosic biomass

Those parts of the plants that are regarded as waste often contain a relatively high concentration of plant cell walls (~ 80%). Plant cell walls consist of a lignocellulosic complex in which lignin, hemicellulose and cellulose are tightly bound to each other via covalent and non-covalent bonds (Jeffries, 1994). Cellulose is an unbranched chain of (1,4)-linked β-D-glucan, which forms crystalline microfibrils (Cosgrove, 2005). The alignment of these microfibrils provides structure to the plant cell walls. Hemicellulose is found mainly in three forms, xylan, xyloglucan and mannan. Wheat straw contains mainly xylan that has a backbone of β-1,4 linked D-xylose residues, which can be acetylated and/or substituted with α-1,2- or α-1,3-linked L-arabinose (arabinoxylan) and/or α-1,2-linked D-glucuronic acid (glucuronoxylan) (Cosgrove, 2005). Hemicellulose is bound to cellulose to give more structure to plant cell walls. In young plants, cell walls consist of only cellulose and hemicellulose. Upon growth of the plant, the cells elongate by taking up water and become thinner and weaker. To prevent

collapsing of the structure, elongated cells are consolidated by integration of new polymers such as lignin (Cosgrove, 2005). Lignin is thus very important for plants, as it provides rigidity and protects the plants against microbial attack. These characteristics are different for each plant species, as a tree is more rigid than grass for example. Consequently, the plant cell wall composition differs among plant species. Lignin is a highly complex polymer consisting of syringyl (S), guaiacyl (G) and p-hydroxyphenyl (H) building blocks (Buranov and Mazza, 2008). The ratio between these building blocks differs between plant species, and may even differ within plant species (Lapierre et al., 1995) and within a plant. Consequently, lignocellulose has a unique composition in each plant species.

1.3 Lignocellulosic biomass in ruminant nutrition

From an energy yielding point of view, lignocellulose in feed production will be most interesting as ruminant feed ingredient. Ruminants have a digestive system adapted to a plant based diet. An important part of a ruminant diet is plant cell walls, often referred to as fiber (Van Soest, 1978). The nutrient requirements of dairy cattle (NRC, 2001) recommend at least 19% (neutral detergent) fiber originating from forage in the diet of dairy cows. Replacement of part of this forage by lignocellulosic biomass originating from agricultural waste may require less arable land needed for feed production.

Generally, cellulose and hemicellulose in plant cell walls can be degraded in the rumen by microbes such as *Fibrobacter succinogenes*, *Ruminococcus flavefaciens* and *Ruminococcus albus* (Fukuma et al., 2015; Krause et al., 2003). Although *F. succinogenes* are thought to be the most important fibrolytic organisms, it performs better in co-culture with other (non-fibrolytic) microorganisms (Fukuma et al., 2015). Not only bacteria, but also the anaerobic fungi in the rumen can degrade plant cell wall polysaccharides. It is known that they can solubilize even up to 34% of the lignin in monocultures (Krause et al., 2003; McSweeney et al., 1994). *In vivo*, these anaerobic fungi will grow too slowly to efficiently digest fibers in the rumen. The involvement of different organisms suggests that fiber digestion in the rumen is a complex system. Fiber digestion in the rumen is not yet fully understood, because not all interactions are known and not all rumen microbes have been cultured or identified. Nevertheless, it has been clearly demonstrated that lignin is negatively correlated to the digestibility of a feedstuff (Arora and Sharma, 2009; Krause et al., 2003; Tuyen et al., 2012). The negative influence of lignin is caused by the tight linkages with carbohydrates in plant cell walls. Lignin, therefore, blocks the cellulose and hemicellulose making it inaccessible to the microbiota in the rumen. As such, lignin removal increases the accessibility of carbohydrates for rumen microbes.

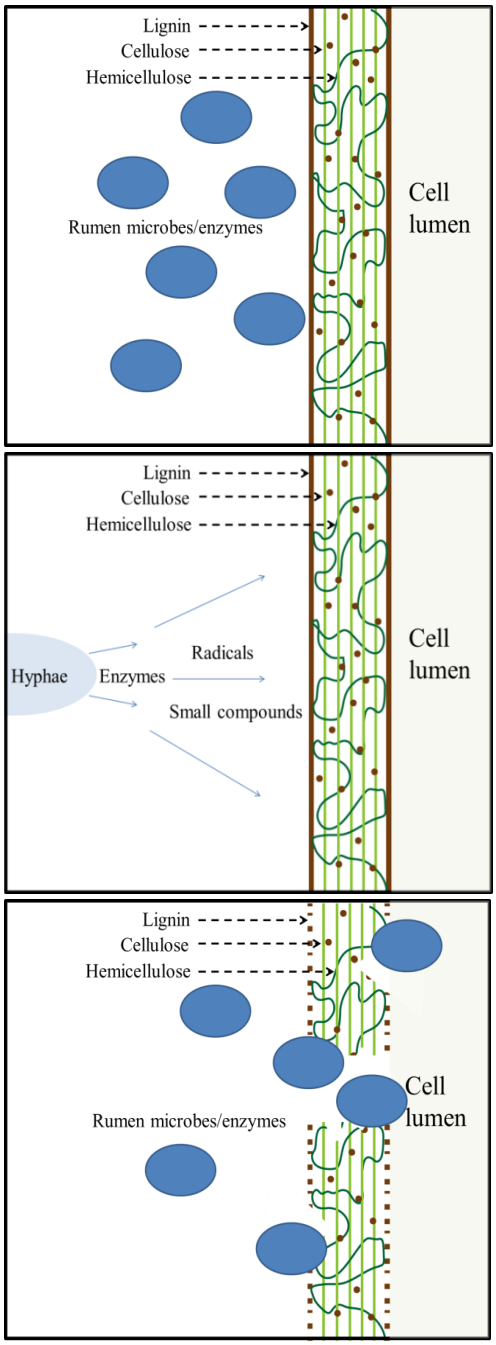

Figure 1.1 Schematic overview of the principle of fungal pre-treatment. Modified from Isroi et al. (2011).

1.4 Lignocellulosic biomass in bioethanol production

Bioethanol production is currently based on feedstock that is rich in starch, the so called first generation biofuels. In the human diet, starch is an important ingredient and to reduce competition between food and fuel, current research focusses on the development of second generation biofuels based on lignocellulosic feedstock (Banerjee, 2011). Enzymatic saccharification of carbohydrates in lignocellulosic material yields sugar monomers such as glucose and xylose. During anaerobic fermentation six carbon sugar monomers can be converted to ethanol by yeast. Some yeast strains also can convert five carbon sugar monomers to ethanol (Hendriks and Zeeman, 2009).
Enzymatic saccharification is the key step determining the efficiency of the bioethanol production process. The yield of bioethanol will increase when more carbohydrates are converted to sugar monomers. The conversion to monomers depends on the accessibility of the carbohydrates. In lignocellulose, the lignin is blocking the accessibility of the carbohydrates, leading to a lower yield of monomers for bioethanol production (Mosier et al., 2005). To increase the yield, lignin removal is essential.

1.5 Fungal pre-treatment

Lignin removal is the key to increase the utilization of lignocellulosic biomass. In nature, lignin is degraded by fungi with some white rot fungi known to be highly selective lignin degraders during the early stages of vegetative growth (Palmer and Evans, 1983; Pandey and

Pitman, 2003). Lignin, along with some hemicellulose, can be degraded up to 60%, with most of the cellulose left intact (Tuyen et al., 2012; 2013). It is thought that the removal of lignin and partial digestion of the carbohydrates by white rot fungi during vegetative growth facilitates the uptake of carbohydrates during the rapid production of fruiting bodies. This is supported by the observations of a switch from lignin degradation to an exclusive carbohydrate degradation when fruiting bodies are formed (Ohga et al., 2000).
The selective lignin degrading properties of white rot fungi can be used in a biological, fungal pre-treatment. The principle of fungal pre-treatment of lignocellulosic biomass is shown in Figure 1.1. The intact cell wall contains lignin that is tightly bound to hemicellulose and cellulose. Due to the linkages, lignin forms a (physical) barrier for rumen microbes/enzymes to reach the carbohydrates. During the fungal pre-treatment, the lignocellulosic biomass is colonized by fungal hyphae.
Upon colonization, the fungi produce ligninolytic enzymes to degrade lignin. Although Figure 1.1 suggest a physical appearance of the hyphae in the plant cell wall, both the hyphae and enzymes are too large to enter the cell walls. To reach the lignocellulose from a distance, the fungal ligninolytic enzyme system produces small radical components that can diffuse into the cell wall (Martínez et al., 2005). The radicals break down lignin in the dense lignocellulosic structure. Once the cell wall structure has opened up, direct enzymatic lignin degradation takes place. At a certain moment when sufficient cellulose is accessible and fruit body production starts, it is expected that the fungus switches from a lignin degrading to a cellulose degrading mechanism. The fungal treatment should be stopped just before this switch, since in theory, this is the moment where most of the lignin is degraded and maximum accessibility of cellulose (and hemicellulose) is obtained. Consequently, the accessibility of the cell wall carbohydrates is also maximum for rumen microbes and enzymes in bioethanol production. In theory, a fungal pre-treatment has great potential to increase cellulose accessibility. It would provide a cheap and safe alternative for current physical and chemical pre-treatments.

1.6 Aims and thesis outline

The possibilities of fungal pre-treatment to increase the utilization of lignocellulosic biomass are investigated in this thesis. This thesis starts with an overview of the scientific literature on fungal pre-treatment of lignocellulosic biomass to increase rumen degradability to set the current state-of-the-art.
The first aim in this thesis was to corroborate the scientific literature. Based on the state-of-the-art described in Chapter 2, four sources of lignocellulosic biomass were chosen for a pre-treatment with four different edible fungi (Chapter 3). A pre-treatment was performed with *Ganoderma lucidum*, *Lentinula edodes*, *Pleurotus eryngii* and *Pleurotus ostreatus* grown on wheat straw, wood chips, miscanthus and rice straw. Here, the potential of a fungal pre-treatment to increase rumen degradability was confirmed and *L. edodes* treatments of wheat straw and wood chips were the most promising combinations.

The second aim in this thesis was a detailed investigation of the changes in the biomass. In Chapter 4, pyrolysis gas chromatography/mass spectrometry (Py-GC/MS) was used to analyze changes in lignin of wheat straw and wood chips upon an *L. edodes* treatment. Although not tested in Chapter 3, the literature overview (Chapter 2) describes the potential of *Ceriporiopsis subvermispora* for fungal pre-treatment. To demonstrate the potential of a *C. subvermispora* pre-treatment, it was compared to an *L. edodes* pre-treatment in Chapter 5. The lignin degrading strategies by *C. subvermispora* and *L. edodes* are studied by a detailed characterization of the fungal treated substrates.

The third aim in this thesis was to investigate options to reduce the incubation time to make fungal pre-treatment a viable technology. The fungal pre-treatment described in Chapters 3 to 5 requires an incubation time of up to 12 weeks. This incubation time is too long to make fungal pre-treatment competitive with current pre-treatments of lignocellulosic biomass. Chapters 6 and 7 describe optimization strategies of the fungal pre-treatment. Optimization is defined as a more selective lignin degradation without carbohydrate degradation in a shorter time. The optimization strategy described in Chapter 6 is the addition of compounds that stimulate growth or enzyme production and activity. In Chapter 7 the effect of particle size of the biomass and the amount of fungus added, was studied.

Finally, Chapter 8 integrates the results of the previous chapters and discusses them with respect to feed and fuel applications.

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CHAPTER 2

Fungal treated lignocellulosic biomass as ruminant feed ingredient: a review

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Abstract

In ruminant nutrition, there is an increasing interest for ingredients that do not compete with human nutrition. Ruminants are specialists in digesting carbohydrates in plant cell walls; therefore lignocellulosic biomass has potential in ruminant nutrition. The presence of lignin in biomass, however, limits the effective utilization of cellulose and hemicellulose. Currently, most often chemical and/or physical treatments are used to degrade lignin. White rot fungi are selective lignin degraders and can be a potential alternative to current methods which involve potentially toxic chemicals and expensive equipment. This review provides an overview of research conducted to date on fungal pre-treatment of lignocellulosic biomass for ruminant feeds. White rot fungi colonize lignocellulosic biomass, and during colonization produce enzymes, radicals and other small compounds to breakdown lignin. The mechanisms on how these fungi degrade lignin are not fully understood, but fungal strain, the origin of lignocellulose and culture conditions have a major effect on the process. *Ceriporiopsis subvermispora* and *Pleurotus eryngii* are the most effective fungi to improve the nutritional value of biomass for ruminant nutrition. However, conclusions on the effectiveness of fungal delignification are difficult to draw due to a lack of standardized culture conditions and information on fungal strains used. Methods of analysis between studies are not uniform for both chemical analysis and *in vitro* degradation measurements. *In vivo* studies are limited in number and mostly describing digestibility after mushroom production, when the fungus has degraded cellulose to derive energy for fruit body development. Optimization of fungal pre-treatment is required to shorten the process of delignification and make it more selective for lignin. In this respect, future research should focus on optimization of culture conditions and gene expression to obtain a better understanding of the mechanisms involved and to allow the development of superior fungal strains to degrade lignin in biomass.

2.1 Introduction

The human population is increasing and consumption patterns are changing towards animal based products such as meat and milk, increasing the demand for animal feed (Boland et al., 2013). To ensure food security, future feed ingredients should not compete with use in human nutrition. Lignocellulosic biomass, consisting of plant cell walls containing lignin, cellulose and hemicellulose, is one of the most abundant waste streams in the world. Cellulose and hemicellulose are polysaccharides which, when converted to sugars, can be used as an energy source. The presence of lignin in biomass forms a major obstacle for the effective utilization of cellulose and hemicellulose by rumen microbes and presently, chemical and/or physical treatments are used to degrade lignin (Chaturvedi and Verma, 2013). There is an increasing interest in alternative treatments to the use of chemicals or expensive physical treatments such as the utilization of white rot fungi to degrade lignin in biomass as indicated by the growing number of scientific papers on this subject. Although white rot fungi have a large potential in selectively removing lignin in biomass, this method requires further optimization to be competitive to conventional treatments such as urea or other alkali treatments. This review intends to provide an overview of research conducted to date using white rot fungi and also intends to illustrate that it is difficult to draw general conclusions from data in the literature on how to improve this type of biological pre-treatment. This is in part due to the large variation in methods used or lack in standardization of methods and, sometimes, to the absence of important details such as strain identity. Although the same principle of lignin removal counts for biofuel production, this review focuses on ruminant nutrition. This overview identifies the main steps in the fungal pre-treatment that need to be standardized and further optimized to make this method a competitive alternative for more conventional approaches.

2.2 Plant cell walls and lignin

Lignocellulosic biomass can be defined as plant materials with a high content of lignified plant cell walls which contain, besides lignin, also hemicellulose and cellulose. Further, only small amounts of ash, proteins and pectins are present in lignocellulosic biomass (Grabber, 2005). Lignin content and composition differ between species and changes during plant maturation (Susmel and Stefanon, 1993; Grabber, 2005) when more lignified secondary cell walls are developed (Grabber, 2005). Cellulolytic microbes in the rumen can degrade most of the carbohydrates present in plants, and therefore roughage high in plant cell walls can be used as a main feed ingredient for ruminants. Cellulose, and also hemicellulose, in plant cell walls is limitedly accessible to the rumen microbes, because of the direct (covalent) or indirect (ester or ether) linkage to lignin (Susmel and Stefanon, 1993; Jeffries, 1994; Jalč, 2002; Vanholme et al., 2010; Ding et al., 2012). Cellulose is present in either a crystalline or amorphous form with the former being more difficult to degrade by enzymes (Dashtban et al., 2009). Although the term lignin describes a large group of aromatic polymers, in general, lignin consists of 3 building blocks namely p-coumaryl alcohol(p-hydroxyphenyl propanol), coniferyl

alcohol (guaiacyl propanol) and sinapyl alcohol (syringyl propanol) (Jeffries, 1994; Vanholme et al., 2010). Lignin plays an important role in providing strength, protection against pathogens, improving water conduction and preventing degradation of structural polysaccharides by hydrolytic enzymes (Grabber, 2005). Lignin can only be degraded aerobically and is not degraded in the anaerobic condition in the rumen. As such, the amount of dietary lignin is, therefore, negatively correlated to dry matter digestibility (Arora and Sharma, 2009) and as such is used as an indigestibility marker (Van Soest, 1993). The structure of lignin is as important as the type and ratios of its constituent building blocks in terms of the degradability by wood decay fungi. Highly branched lignin is less degradable than more linear lignin (Grabber, 2005) and syringyl-rich lignin, for example, renders poplar more resistant to degradation by wood decay fungi (Skyba et al., 2013).

Plant residues with a high lignin content, although rich in cellulose and hemicellulose, are often regarded as organic) waste. In the European Union, a total of ~153 M dry tons of agricultural residues are produced originating from, for example, wheat, barley, rye, oats, maize and forests (Diamantidis and Koukios, 2000; Ericsson and Nilsson, 2006; Scarlat et al., 2010). Municipal waste, approximately 10% of the total waste, has a variable composition and can contain amongst others paper and paper products, food and garden waste (Eurostat, 2009; Blumenthal, 2011). Organic waste is now mainly used in biogas and energy production, soil improvement, as bedding in animal husbandry, mushroom production and disposed of in landfills (Scarlat et al., 2010; Blumenthal, 2011). Another source of lignocellulose includes energy crops (e.g. miscanthus) specially bred for biofuels which contain a high carbohydrate content (McKendry, 2002).

2.3 Chemical and/or physical methods to degrade lignin

Current methods to increase the accessibility of cellulose and hemicellulose in lignocellulosic biomass include physical, physicochemical and chemical treatments. The effectiveness of these treatments has been reviewed by several authors. Therefore, only a short summary including advantages and disadvantages will be provided here. For more in-depth discussions on this topic, see articles by Hendriks and Zeeman (2009), Agbor et al. (2011), Sarkar et al. (2012), Chaturvedi and Verma (2013) and Sarnklong et al. (2010).

Physical treatments of lignocellulosic biomass to improve digestibility, like grinding, soaking or pelleting, can result in a lower cellulose crystallinity resulting in cellulose being better degradable by enzymes (Hendriks and Zeeman, 2009; Sarnklong et al., 2010; Agbor et al., 2011; Sarkar et al., 2012). These physical treatments have a limited feasibility to be applied on farm, because machines or industrial processes are required (Sarnklong et al., 2010). However, often reduction of particle size is needed for successful processing (Mosier et al., 2005).

Chemical treatments with acids (e.g. sulphuric or nitric acids) or alkalis (e.g. sodium hydroxide or potassium hydroxide) result in a lower cellulose crystallinity and high

sugar yields (Hendriks and Zeeman, 2009; Agbor et al., 2011; Sarkar et al., 2012). Although sodium hydroxide is used for pre-treatment to improve digestibility of rice straw, it is an expensive method which can cause environmental pollution (Sarnklong et al., 2010; Chaturvedi and Verma, 2013). Urea treatment is a relatively cheap and safe method, which in addition adds nitrogen, increasing the nutritive value for ruminants. However, urea treatment increases *in vitro* dry matter degradability of rice straw only by 10–17%, which can be considered to only be a minor improvement (Sarnklong et al., 2010). Although not yet reported for ruminant feed ingredients, another strategy is to treat the biomass with oxidative chemicals such as hydrogen peroxide or acetic acid. However, these chemicals are not selective and will cause losses of cellulose and hemicellulose (Hendriks and Zeeman, 2009).

Physicochemical treatments combine different methods, such as heat, moisture, pressure and chemicals to improve cellulose accessibility. Disadvantages are the high energy demands, the need for special equipment, and production of toxic waste which can limit further downstream processing (Hendriks and Zeeman, 2009; Agbor et al., 2011; Sarkar et al., 2012).

Fibrolytic enzymes from fungi or bacteria have been added to lignocellulosic biomass in order to improve the accessibility of cellulose and hemicellulose. Addition of fibrolytic enzymes from different white rot fungi to wheat straw did result in a higher *in vitro* neutral detergent fiber (NDF) rumen degradability of 13% (Rodrigues et al., 2008).

Biological pre-treatments using fungi are low energy and safe alternatives to other treatments (Akhtar et al., 1992; Howell et al., 2009; Rasmussen et al., 2010; Giles et al., 2011). Fungal treatment resulted in up to 38% energy savings compared to an untreated control in biopulping process (Akhtar et al., 1998). Although fungal treatment is regarded as a cheap alternative pre-treatment of lignocellulosic biomass, a thorough cost analysis including energy savings, reduction in use of chemical agents, savings on equipment, throughput and environmental consequences should be conducted (Tian et al., 2012). In the latter calculation, loss of dry matter in fungal treatments should also be taken into account because fungi consume carbohydrates. The time required for fungal delignification and the difficulties for up-scaling make the process not yet feasible (Chaturvedi and Verma, 2013). In an on-farm application of fungal treatment, time may be less of an issue, while changes in temperature due to environmental conditions can be a problem (Wan and Li, 2010). This review will show that these limitations can be overcome and that fungi can be used as a potential pre-treatment of lignocellulosic biomass to produce ruminant feed.

None of the currently available methods results in 100% conversion of biomass into available sugars, and there always is a loss of biomass involved with the various treatments, thereby, increasing costs (Chaturvedi and Verma, 2013). Although 100% conversion is unrealistic, methods to improve biomass use should be optimized.

Table 2.1 Fungal species and substrate used to degrade lignin in biomass for animal nutrition.

Bjerkandera adusta – oil palm[18], wheat[26]	*Lentinula edodes* –cedar[16], corn[27], oil palm[11, 18, 27], rice[27], sugarcane[27], wheat[26]	*Pleurotus cornucopiae* – wheat[25]
Ceriporiopsis subvermispora – bamboo[17], cedar[16], corn[27], oil palm[11, 18, 27], rice[20, 27], sugarcane[27], wheat[26]	*Lyophyllum ulmarium* – wheat[13]	*Pleurotus florida* – water hyacinth[15]
Corprinus fimetarius – rice[19]	*Phanerochaete chrysosporium* – birch[6], oil palm[18], rice[14, 20], spruce[6], wheat[2, 26, 28]	*Pleurotus ostreatus* – cedar[16], cocoa[3], corn[27], oil palm[18, 27], rice[27], sugarcane[27], wheat[1, 7, 10, 13, 21, 23, 24, 25, 26, 28]
Cyathus stercoreus – wheat[2]	*Phellinus laevigatus* – wheat[13]	*Pleurotus sajor-caju* – birch[6], rice[8], spruce[6], wheat[8, 9, 25]
Daedalea guercina – wheat[13]	*Phlebia brevispora* –oil palm[11, 18], rice[20], wheat[4, 5, 26]	*Pleurotus sapidus* – wheat[25]
Dichomitus squalens – cedar[16], wheat[2]	*Phlebia fascicularia* – rice[20], wheat[4]	*Polyporus brumalis* – wheat[13]
Ganoderma lucidum – oil palm[11, 18], wheat[22, 26]	*Phlebia floridensis* –rice[20], wheat[4]	*Polyporus ciliates* – wheat[12, 13]
Hericium clathroides – wheat[13]	*Phlebia radiata* –rice[20], wheat[4]	*Schizophyllum commune* – oil palm[18], wheat[26]
Inonotus andersonii – wheat[13]	*Pholiota nameko* – cedar[16]	*Trametes gibbosa* – wheat[13]
Inonotus dryophylus – wheat[13]	*Pleurotus citrinopileatus* – water hyacinth[15]	*Trametes versicolor* – birch[6], oil palm[18], spruce[6], wheat[21, 26, 28]
Inonotus obliquus – wheat[13]	*Pleurotus eryngii* –corn[27], oil palm[11, 18, 27], rice[27], sugarcane[27], wheat[26, 28]	*Volvariella volvacea* – wheat[26]
Laccaria amethystine – wheat[28]		
Lentinus tigrinus – wheat[12, 13]		

References: 1 Adamovic et al. (1998), 2 Agosin et al. (1986), 3 Alemawor et al. (2009), 4 Arora and Sharma (2009), 5 Arora and Sharma, 2011, 6 Asiegbu et al. (1996), 7 Bakshi et al. (1985), 8 Bisaria et al. (1997), 9 Calzada et al. (1987), 10 Fazaeli et al. (2006), 11 Hassim et al. (2012), 12 Jalč et al. (1994), 13 Jalč et al. (1998), 14 Liang et al. (2010), 15 Mukherjee and Nandi (2004), 16 Okano et al. (2005), 17 Okano et al. (2009), 18 Rahman et al. (2011), 19 Rai et al. (1989), 20 Sharma and Arora (2010a), 21 Shrivastava et al. (2011), 22 Shrivastava et al. (2012), 23 Streeter et al. (1982), 24 Tripathi and Yadav (1992), 25 Tsang et al. (1987), 26 Tuyen et al. (2012), 27 Tuyen et al. (2013), 28 Valmaseda et al. (1991a).

2.4 Fungal pre-treatments

Wood decay fungi have a unique mechanism to degrade lignin, and can thus be used as a potential pre-treatment to upgrade or improve lignocellulosic biomass for use as a ruminant feed ingredient. However, biological pre-treatment of biomass has been considered to be difficult to carry out on an industrial scale (Chaturvedi and Verma, 2013) because the method requires sterile conditions, a major portion of cellulose and hemicellulose are consumed by fungi and that the process takes relatively long. This review will illustrate that some of these disadvantages have already been solved and others can be overcome, for example by the use of selective (improved) fungal strains and optimized cultivation conditions. In addition, the mushroom industry has expertise and equipment for collection and mixing large amounts of organic waste (Noble and Gaze, 1996) and for inoculation with fungi even under nonsterile conditions using spawn technology. Adapting these systems offers opportunities to make fungal treatment of lignocellulosic biomass a competitive alternative for the presently used chemical/physical methods.

Table 2.2 Fungal strain, culture conditions and method used to determine lignin in studies describing *P. ostreatus* treatment of wheat straw.

Fungal strain	Temperature/time	Inoculum	Lignin method	Reference
Unknown	24°C/28 days	2% mycelium (on medium based on wheat grain) of substrate fresh weight	Acid detergent lignin	Adamovic et al. (1998)
P. ostreatus (JacQ:Fr) Kumm. 476	28°C/30 days	100 ml water containing mycelium in 300 g wet straw	Acid detergent lignin	Jalč et al. (1998)
P. ostreatus CBS 411.71	28°C/21 days	2 ml inoculum in 2 g dry straw	Klason lignin	Salvachúa et al. (2011)
P. ostreatus (F6) (Jacquin ex Fr.) Kammer	30°C/30 days	0.5% w/w fungal dry mass/straw	Acid detergent lignin	Shrivastava et al. (2011)
P. ostreatus strain MES 03449	24°C/49 days	5 g grain spawn in 160 g wet straw	Acid detergent lignin	Tuyen et al. (2012)

2.4.1 Principle of fungal pre-treatment

Wood decaying fungi can generally be divided into three groups, i.e. white rot fungi that selectively degrade lignin, especially during the early phase of colonization followed by a fruiting stage in which polysaccharides are degraded, brown rot fungi that degrade polysaccharides and modify lignin, and soft rot fungi that degrade lignin and polysaccharides simultaneously (Jalč, 2002). The selective degradation of lignin by white rot fungi makes these organisms highly useful for pre-treatment of lignocellulosic biomass (Palmer and Evans, 1983), especially when the treatment is terminated before the fungus starts to degrade cellulose for fruit body production. It is during this latter stage that fungi require relatively large amounts of hemicellulose and cellulose as a carbon and energy source to produce fruit bodies. The mycelium of the white rot fungi colonizes a substrate and lignin is enzymatically degraded to reach the polysaccharides (Kirk and Farrell, 1987; Hammel, 1997). During growth of the fungi, nitrogen from the lignocellulosic biomass is incorporated into fungal proteins and as a result of the degradation of other nutrients, an enrichment of nitrogen, and thus crude protein can be found.

2.4.2 The complex toolbox of fungi for lignin degradation

Fungal delignification is a complex, aerobic process. Several heme containing peroxidases are involved, like the “classic” fungal lignin peroxidase (LiP), manganese peroxidase (MnP) and versatile peroxidase (VP) (Kirk and Farrell, 1987), and the recently described novel types of heme-dependent peroxidases, aromatic peroxygenases (APOs), and dye-decolorizing peroxidases (DyPs) (Hofrichter et al., 2010). Laccase is another important enzyme involved in lignin degradation. The above-mentioned peroxidases use hydrogen peroxide as the electron acceptor to catalyze a number of

oxidative reactions. Fungi contain several enzymes to produce hydrogen peroxide, like arylalcoholoxidase, (methyl)glyoxal oxidase, glucose-1-oxidase, glucose-2-oxidase and cellobiose:quinone oxidoreductase (Kirk and Farrell, 1987; Reid, 1995; Hammel, 1997; Leonowicz et al., 1999; Isroi et al., 2011). The latter enzyme is also involved in cellulose degradation during depolymerization of lignin by laccase, indicating a relationship between lignin and cellulose depolymerization (Leonowicz et al., 1999). Genes encoding for LiP, MnP, laccase and glyoxal oxidase were first described in *Phanerochaete chrysosporium* (Cullen, 1997). Several hundreds of genes have now been described for laccase, and over 65 different genes have been sequenced for LiP and MnP (Janusz et al., 2013). The availability of whole genome sequences of a large number of fungi has even allowed the estimation of the origin of lignin degradation and suggests that this may have coincided with the sharp decrease in the rate of organic carbon burial around the end of the carboniferous period (Floudas et al., 2012).

Lignin has a highly dense structure that does not allow penetration by enzymes. The radicals produced by peroxidases, however, are sufficiently small to penetrate the dense lignin structure and lignin can thus be mineralized at a distance from the fungal cell (Hammel, 1997; Tian et al., 2012). The involvement of radicals in the process indicates that degradation is not a highly specific process. To protect themselves against the radicals, the fungi produce superoxide dismutase, which catalyzes reactions to convert superoxide anion radicals into oxygen and hydrogen peroxide (Leonowicz et al., 1999). Lignolytic enzyme activities are not always correlated with lignin degradation, demonstrating that the function of the lignolytic enzyme complex is not yet fully understood (Valmaseda et al., 1991b; Sarnklong et al., 2010; Isroi et al., 2011; Shrivastava et al., 2011). The complexity and the large number of enzymes and radical components involved in lignin degradation indicate that this can only be performed optimally in a fungal system. This is underlined by the minimal increase in *in vitro* degradability of wheat straw after incubation of straw with extracts of different white rot fungi (Rodrigues et al., 2008).

2.4.3 Fungal treated biomass as feed ingredient

Fungal pre-treated lignocellulosic biomass can be used for a number of different purposes besides as an animal feed ingredient, such as bioethanol and methane production and the production of fine chemicals. Each purpose requires a different source of lignocellulosic biomass, but each purpose requires degradation of lignin without, or minimal, loss of carbohydrates. Fungal treatment for biofuel production has recently been reviewed by Wan and Li (2012). This review intends to discuss mainly fungus–substrate combinations tested for animal nutrition. Nevertheless, nutritionists can learn from lignin and cellulose degradation data in biofuel studies.

The potential feed ingredients for ruminant nutrition studied include residues of wheat, rice, corn, oil palm, cocoa, bamboo, sugarcane, water hyacinth, cedar, birch and spruce (Table 2.1). Approximately 35 different fungal species have been studied to improve the use of these lignocellulosic substrates for ruminant nutrition (Table 2.1). Substrates are

often chosen based on potential nutritional value, but the choice is also influenced by geographical availability. The fungus–substrate combinations show a large variation in the extent of delignification and accessibility of cellulose and also hemicellulose.

2.4.4 Variations between fungal treatments

Since each type of organic biomass has a unique cell wall composition, the best way to compare effectiveness of the pre-treatment is to evaluate the effect of different fungi grown on the same substrate. Wheat straw is one of the most widely studied lignocellulosic biomasses as substrate in fungal pre-treatment for ruminant nutrition (Table 2.1), but also for other purposes like biofuel. For each fungal species studied on wheat straw, the lignin and cellulose degradation of wheat straw as a percentage of lignin and cellulose in the dry matter compared to untreated wheat straw (each data point represents one fungal species per study) are plotted in Figure 2.1. Fungal species that show a high selectivity in degradation of lignin with only a minor use on cellulose (>20% lignin and <5% cellulose degraded) are *Ceriporiopsis subvermispora*, *Lentinula edodes*, *Hericium clathroides*, *Pleurotus ostreatus*, and *Pleurotus eryngii*, representing only 5 out of the 41 species represented in Figure 2.1, indicating that these species would be the best candidates for selectively removing lignin from wheat straw.

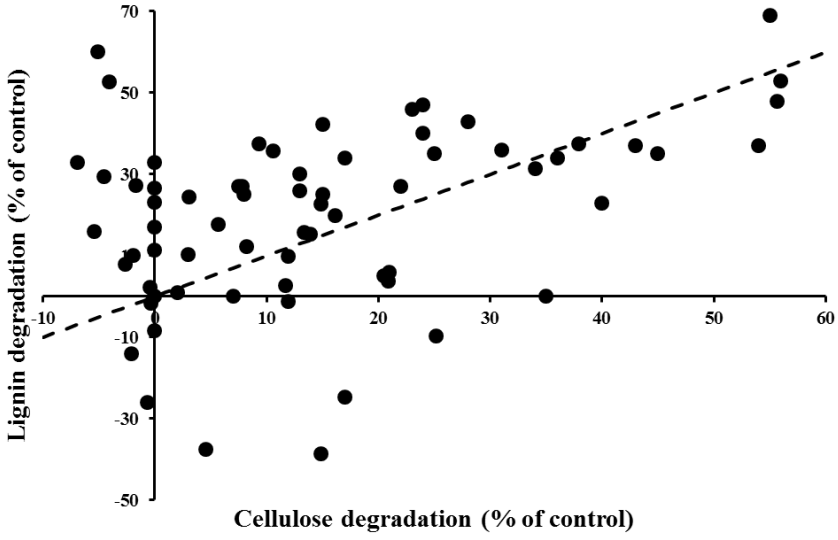

Figure 2.1 Lignin and cellulose degradation in fungal treated wheat straw compared to untreated wheat straw (control). Each data point represents one fungal strain per literature source. Dashed line: line of unity.
From: Adamovic et al. (1998), Bisaria et al. (1997), Fazaeli et al. (2006), Gaitán-Hernández et al. (2011), Jalč et al. (1994), Jalč et al. (1998), Kluczek-Turpeinen et al. (2007), Salvachúa et al. (2011), Shrivastava et al. (2011), Shrivastava et al. (2012), Streeter et al. (1982), Tuyen et al. (2012) and Valmaseda et al. (1991a).

The large variation in lignin and cellulose degradation indicates that most studies are difficult to compare. The latter is illustrated in Figure 2.2 where wheat straw treatments are compared where only *P. ostreatus* is used. Although all five studies, except Shrivastava et al. (2011), found more lignin than cellulose degradation by *P. ostreatus* on wheat straw, results vary from very selective lignin degradation (Adamovic et al., 1998; Tuyen et al., 2012) to cellulose degradation comparable to lignin (Jalč et al., 1998; Salvachúa et al., 2011). The incubation conditions (time and temperature), length of incubation and strains used vary considerably and may explain the differences found between these studies (Table 2.2). It should be mentioned that in addition to cellulose and lignin, also hemicellulose is degraded. From the studies reported in Figure 2.1, there was a significant ($P < 0.05$) but weak correlation (R below 0.6) between the degradation of the three compounds (lignin, cellulose and hemicellulose) indicating that more factors than these are of importance. Rahman et al. (2011) and Hassim et al. (2012) both describe a fungal treatment of oil palm fronds using *Phlebia brevispora*. While *P. brevispora* improved the apparent rumen degradable carbohydrate contents by 100%, as measured by the volatile fatty acid production *in vitro* in rumen fluid of lambs in the Rahman et al. (2011) study, Hassim et al. (2012) observed no effect. Differences between substrate batches or animals used for the *in vitro* assay might explain these differences.

2.4.5 The most effective fungus for fungal treatment

Ranking fungi tested on the same substrate within one study provides a sound comparison of the effectiveness of individual fungi as a pre-treatment. It can be concluded from studies comparing fungi grown on wheat straw that *P. brevispora*, *Trametes versicolor*, *C. subvermispora* and *Polyporus ciliates* can improve the dry matter and organic matter digestibility (Valmaseda et al., 1991a; Jalč et al., 1994; Arora et al., 2011; Tuyen et al., 2012; Tuyen et al., 2013). Lignin degradation explained approximately 80% of the weight loss caused by *P. eryngii* treatment of wheat straw for two months (Valmaseda et al., 1990).

C. subvermispora and *P. eryngii* perform the best across substrates, resulting in the most structural changes, or *in vitro* rumen degradability of organic matter (Valmaseda et al., 1990; del Río et al., 2001; Okano et al., 2005; Hassim et al., 2012; Tuyen et al., 2012; Tuyen et al., 2013). Gene expression patterns confirm the selectivity of *C. subvermispora* for lignin degradation with an extension of the known mnp gene family found in this fungus with additional new types of peroxidases which are all highly expressed on lignocellulosic substrates. In addition, the number and expression level of glycohydrolase genes of *C. subvermispora* was lower than that of *P. chrysosporium*, a less selective fungus (Fernandez-Fueyo et al., 2012).

The use of a combination of different fungi seems to have additional beneficial effects compared to the use of monocultures. Co-cultures of *Pleurotus sajor-caju*, *T. versicolor* and *P. chrysosporium* resulted in degradation of 16% lignin in spruce sawdust, while monocultures resulted in a delignification of 0 to 5% (Asiegbu et al., 1996). Also co-

cultures of either *C. subvermispora* or *Physisporinus rivolus* cultured with *P. ostreatus* gave an increased loss of lignin in aspen wood (Chi et al., 2007) compared to incubation with monocultures. It should be noted, however, that the extent to which each fungus contributes in co-culture studies is not always clear, leaving the possibility that mainly one culture is left when the substrate was analyzed.

In general, it can be concluded that fungal treatments described in the literature were mainly investigated in small studies, concerning single substrates or fungal species. Because each study uses different culture conditions, comparisons can only be made within studies. Nevertheless, a number of studies showed the beneficial effects of fungi on the bioavailability of cellulose and hemicellulose. To make fungal treatment of biomass competitive to present-day methods used in ruminant nutrition, however, a better understanding of the process is needed to increase the effectiveness.

2.5 Important factors for fungal treatment

Although fungal pre-treatments of lignocellulosic material are environmentally and economically friendly, it is a relatively time consuming process in which also carbohydrates are used for the metabolism of the fungus compared to other treatments and a special bioreactor should be designed to create aerobic and aseptic conditions (Flachowsky et al., 1999; Okano et al., 2005; Ramirez-Bribiesca et al., 2011; Zhu et al., 2011; Sarkar et al., 2012; Tian et al., 2012; Tuyen et al., 2012). However, the large scale on which biomass is inoculated and colonized in tunnels by the button mushroom

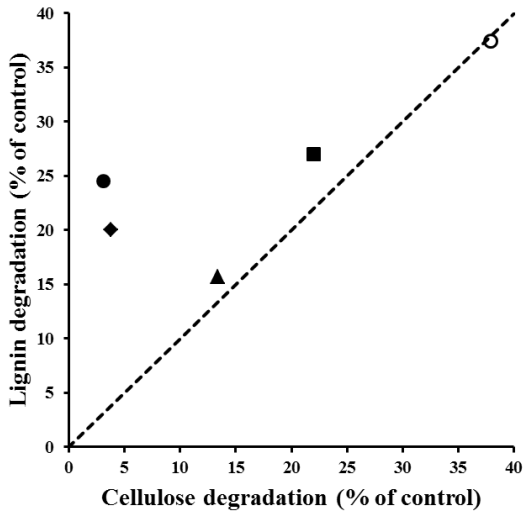

Figure 2.2 Lignin and cellulose degradation in wheat straw by *P. ostreatus* plotted as % decrease of dry matter compared to untreated wheat straw (control). Dashed line: line of unity. From: ♦ Adamovic et al. (1998), ▲ Jalč et al. (1998), ■ Salvachúa et al. (2011), ○ Shrivastava et al. (2011) and ● Tuyen et al. (2012).

industry offers potential to adapt this system for pre-treatment with fungi on a large scale. Optimization of the process by selecting the most effective strain, the right substrate and culture conditions can reduce treatment time and carbohydrate losses.

2.5.1 Fungal strain

Differences in lignin degradation between fungal species are observed (see section 2.4.2), but within a species also differences between different strains can be observed. Only a few studies have compared different strains of the same fungal species. Two different strains of *P. ostreatus*, grown on sugarcane bagasse produced different amounts of the enzymes laccase (up to a fourfold difference), xylanase (up to a threefold difference) and carboxy methyl cellulase (up to a threefold difference) (Membrillo et al., 2008). Both strains also responded differently to different culture conditions, like particle size and additives such as manganese (Membrillo et al., 2008). Stajic et al. (2006) also found different laccase and peroxidase production by two different *P. ostreatus* strains grown on grapevine sawdust. Three different strains of *L. edodes* produced different enzymes during each phase of the mushroom production process (Cavallazzi et al., 2004). Differences between strains with respect to lignin degradation can also be used to improve strain performance by breeding (del Vecchio et al., 2012). Publicly available and private culture collections have large numbers of strains varying in genotype of many white rot fungi. This offers the opportunity to directly screen for superior strains or improve strains as pre-treatment for production of feed ingredients by breeding.

2.5.2 Substrate

One of the factors that hamper an accurate comparison between different pre-treatment experiments is the variation in substrate constitution. Lignocellulosic biomass from the same origin has not always the same composition. Labuschagne et al. (2000) demonstrated this by comparing the composition of wheat straw obtained from 15 different cultivars, grown on the same field and during the same period. Although the conditions may not have been optimal for all cultivars, the latter study shows that the reducing sugar content between varieties can differ considerably, i.e. between 13.1 and 40.7 mg/g straw. Mushroom yield of *P. ostreatus* varied between 123 and 262 kg mushrooms per ton of substrate on different batches of wheat straw (Labuschagne et al., 2000). Lignin degradation by fungi also depends on the batch of wheat straw as demonstrated by Arora and Sharma (2009) who showed that *Phlebia floridensis* degrades lignin in wheat straw from the north eastern zone of India more efficiently and selectively compared to wheat straw from other regions (and climates). Timing and kinetics of laccase production of three different fungi changed when wheat straw from a different origin was used (Arora and Sharma, 2009). Cell wall composition also varied between different cultivars of other crops, such as miscanthus and switch grass (Van Hulle et al., 2010).

Lignin composition is as important as content. Guaiacyl–syringyl units are more difficult to degrade than other units, and guaiacyl-units degrade later than syringyl-units, which have a lower redox potential and lower condensation degree than guaiacyl-units (Terrón et al., 1995; Burlat et al., 1998; Martínez et al., 2001). Lignin composition of various substrates differs considerably. For example, spruce contains a relative distribution of p-hydroxyphenyl:guaiacyl:syringyl of 2:98:0, while the relative distribution in birch is 0:24:76 (p-hydroxyphenyl was not determined) and in wheat straw 5:49:46 (Lapierre, 1993). These variations can have a major effect on the extent of degradation by fungi (Obst et al., 1994; Skyba et al., 2013). Linkages between lignin and the carbohydrates do have an effect on efficient lignin degradation by fungi. Applying a chemical treatment before fungal treatment might already break down the more difficult degradable parts of a substrate. The latter, however, increases the risk that the fungus reproduces first on the easily degradable carbohydrates before degrading the lignin (Reid, 1989).
In ruminant nutrition, lignin is negatively correlated to dry matter and especially fiber digestibility, with the lignin structure determining to which extent digestibility is affected (Jung and Deetz, 1993). The latter is likely due to different linkages between lignin and carbohydrates. Ether linkages for example cannot be broken down under anaerobic conditions (Jung and Allen, 1995). The presence of such linkages will decrease digestibility in the anaerobic conditions of the rumen of animals.

2.5.3 Culture conditions

Biological pre-treatments with fungi are mostly performed as a solid state fermentation, meaning that a moistened substrate is used instead of a fluid mixture (Reid, 1989). Advantages of solid state fermentation over submerged fermentation are the requirement for smaller, simpler and cheaper fermenters, the production of less effluent, and better aeration of the substrate (Reid, 1989; Zhu et al., 2011). Solid state fermentation is a natural environment for lignin degrading fungi and a less attractive environment for competing bacteria (Reid, 1989). Solid state fermentation is influenced by sterilization, inoculum, degree of aeration, particle size, and culture conditions, such as moisture content, pH, temperature and time (Reid, 1989). The importance of these factors in fungal pre-treatment of lignocellulosic biomass has recently been reviewed by Wan and Li (2012).
Factors associated with the substrate that can influence lignin degradation by fungi are sterilization and particle size. Sterilization by autoclaving can increase lignin and cellulose content of wheat straw (twice autoclaving for 1 h) (Tuyen et al., 2012). However, this is not observed for all substrates and all sterilization conditions (Makkar and Singh, 1992; Tuyen et al., 2013). Reducing particle size is regarded as a physical pre-treatment during which cellulose crystallinity is reduced (Agbor et al., 2011). Adjusting particle size for better fungal treatment actually means that a physical process is combined with fungal treatment. In ruminant nutrition, particle size should be balanced between surface available for the rumen microbes and the rumen residence time. In practice this means that fibrous materials ground too fine, reduce fiber digestion

(Tamminga, 1993). Not only particle size, but also shape is important for rumen digestion (Kammes and Allen, 2012). In fungal treatment, it should be noted that between smaller particles, which are easier to colonize, there is less space for oxygen (Reid, 1989). Aeration is also influenced by the moisture content; if too much water is present, less space for oxygen is available.

The time required for colonization of the substrate is influenced by the amount and type of inoculum. Spores are the smallest possible inoculum unit. Vegetative spores are preferred because they have the same genotype as the strain that produces these spores, whereas generative spores are offspring and principally have an altered genotype and potentially different phenotypic characteristics (Royse, 1997; Saxena et al., 2001). Generative spores might lead to inconsequent performance of the fungal strain. A type of inoculum that is used on a large scale by the mushroom industry is spawn (mycelium-coated grains) (Tripathi and Yadav, 1992). Mycelium-coated grains are larger than spores resulting in less inoculation points in the same volume inoculum added. The simplest method of inoculation is the use of a small part of the fungal treated substrate as an inoculum for the next batch of substrate (Reid, 1989). However, transferring colonized substrate too often can result in degeneration of the fungus due to a decreased stability of the genome. The quantity of inoculum is also important, i.e. a larger amount of inoculum leads to a shorter time needed to fully colonize the substrate. More inoculum does not necessarily mean more enzyme production by the fungi, meaning that lignin degradation may not be increased when more inoculum is added (Mehboob et al., 2011).

Nitrogen, such as peptone, NH_4Cl, malt extract, and carbon sources, such as cane molasses or glucose, added to the culture can induce faster colonization (Levin et al., 2008; Sharma and Arora, 2010b; Mehboob et al., 2011; Wan and Li, 2011). In nature, lignin degradation occurs under nitrogen deficient conditions; therefore, addition of nitrogen to the fungal culture can repress the ligninolytic effect of some fungal species (Kirk and Farrell, 1987; Reid, 1989).

In ruminant nutrition, nitrogen is important for nutritional reasons. Adding protein (8.5% in the form of soybeans) to fibrous materials increased rumen dry matter, cellulose, hemicellulose and energy digestibility (Males, 1987). Furthermore, a low crude protein content is often regarded as one of the major limitations of feeding straw to ruminants, and nitrogen addition can provide additional nutritional value to lignocellulosic biomass (Flachowsky et al., 1999). Because nitrogen is important in ruminant nutrition, the addition of nitrogen to lignocellulosic biomass should be considered in fungal treatment. In case nitrogen has a negative effect on fungal treatment, addition of nitrogen at a later stage, before feeding, could be an option. Increasing nitrogen content by performing a urea treatment before culturing of *P. ostreatus* on wheat straw could lead to more lignin degradation and a higher *in vitro* dry matter digestibility (Tripathi and Yadav, 1992).

Copper, manganese, linoleic acid, dirhamnolipid and veratryl alcohol addition can enhance the production of peroxidases by fungi (Kerem and Hadar, 1995; Palmieri et al., 2000; Scheel et al., 2000; Alemawor et al., 2009; Cunha et al., 2010; Liang et al., 2010;

Salame et al., 2010; Sharma and Arora, 2010b; Gassara et al., 2011; Zhu et al., 2011; Wan and Li, 2012). These additives, when used in the pre-treatment of lignocellulosic biomass with fungi, will be subsequently present in the feed and, therefore, effects on ruminants should be considered. Manganese can stimulate *in vitro* cellulose digestion in rumen fluid, when no trace elements are added to the incubation mixture (Hidiroglou, 1979). Low concentrations (<5%) of linoleic acid can be fed to ruminants without affecting rumen fermentation with higher concentrations resulting in inhibition of microbial synthesis and biohydrogenation (Jalč et al., 2007; Szumacher-Strabel et al., 2009). Dirhamnolipids, a biosurfactant, can increase rumen digestibility by interaction between enzyme and substrate and increase endogenous enzyme release in the rumen (Liu et al., 2013).

2.5.4 Mushroom production

Wood decaying fungi make polysaccharides more accessible by degrading lignin to prepare the substrate for production of fruit bodies. Cultures of *P. ostreatus* grown on wheat straw used 20% cellulose, 32.3% hemicellulose and 37.4% lignin after 41 days when not allowed to fructify. The same strain producing mushroom at the end of the cycle degraded 62.1% cellulose, 60.8% hemicellulose and 41.8% lignin of the wheat straw (Ginterová and Lazarová, 1987). The latter shows that the fungus is preparing the substrate for mushroom production. During mushroom production, cellulose is degraded and, therefore, unavailable for rumen microbes, resulting in a lower *in vitro* dry matter digestibility (Mukherjee and Nandi, 2004). *In vivo*, sheep had a lower intake of *P. ostreatus* treated wheat straw after mushroom production than *P. ostreatus* treated straw before mushroom production (Fazaeli et al., 2006). Mushroom production can yield two high value products, the mushrooms for human consumption and the spent substrate as feed ingredient. However, producing mushrooms and obtaining a more digestible delignified substrate does not appear to be easy to combine (Tsang et al., 1987).
For successful fungal treatment, in order to obtain optimal value in animal nutrition, the process should be terminated before fruit bodies are formed, meaning that timing is very important in fungal treatment of lignocellulosic biomass.

2.6 Ways to analyze effect of fungal treatments on feed composition

In animal nutrition, the Van Soest method (Van Soest et al., 1991) is commonly used in which the insoluble fiber, called neutral detergent fiber (NDF), is estimated. This insoluble fiber is assumed to consist of hemicellulose, cellulose and lignin. Acid detergent fiber (ADF) contains acid insoluble cellulose and lignin. Acid detergent lignin (ADL) in the Van Soest method is determined by adding 72% sulphuric acid to the ADF fraction to dissolve cellulose with the remaining fraction being considered to consist of lignin (Van Soest et al., 1991). In the literature, often Klason lignin is determined for which 72% sulphuric acid is applied directly to the organic matter. Although ADL and Klason lignin are comparable methods, a 4% difference between these methods was found on cotton stalks treated with *P. ostreatus* (Kerem and Hadar, 1995; Brinkmann et

al., 2002; Hatfield and Fukushima, 2005). Another comparable method to estimate lignin content is permanganate oxidation of the ADF fraction. However, noncellulosic polysaccharides present in the ADF fraction could interfere, resulting in an overestimation of lignin (Hatfield and Fukushima, 2005). Pyrolysis gas chromatography–mass spectrometry (GC–MS) can discriminate between different building blocks of lignin and showed different degradation products after treating eucalyptus wood with either *Bjerkandera adusta*, *C. subvermispora*, *Coniophora puteana*, *Crepidotus variabilis*, *Melanotus hypatochrous*, *P. chrysosporium*, *Phlebia radiata*, *Pleurotus pulmonarius*, *Trametes trogii* (del Río et al., 2001). Pyrolysis GC–MS is a powerful analytical technique, although the equipment is relatively expensive (Martínez et al., 2001). Although different methods have been used, the quantification of lignin remains difficult as there is no standard as reference since lignin can be a group of aromatic polymers (Hatfield and Fukushima, 2005). Effects of fungal treatment, therefore, should be considered within studies where fungal treated material is compared to untreated lignocellulose. Absolute numbers can provide more information on losses instead of contents. Lignin may not necessarily be degraded but can be modified during the fungal treatment and thus having an effect on the bioavailability of cellulose and hemicellulose. This effect can easily be missed as has been shown in lignin analysis in tulip poplar wood chips treated with *C. subvermispora* followed by treatment with *Postia placenta* (Giles et al., 2011; Ding et al., 2012). In ruminant nutrition, *in vitro* methods are often used to determine the degradability by rumen microbes. In many studies, the *in vitro* gas production technique (Cone et al., 1996) is used to determine the degree and rate of gas production, which has a linear relationship with the organic matter fermentation in rumen fluid. Within gas production measurement techniques, different methods are used to quantify digestibility, i.e. using only gas production or the reduction in substrate. Factors that affect these methods are the donor animal, temperature, pH and the type of buffers used (Cone et al., 1996). Kinetics of gas production by rumen microbes provides important information; therefore systems which measure continuously are preferred (Cone et al., 1997). Another method often used is a two-phasic method, in which incubation in rumen fluid is followed by a pepsin treatment. After incubation, the weight loss of the solid residue, as a measure of the content digested, i.e. degraded into soluble compounds, is determined (Tilley and Terry, 1963). Results of rumen degradation analysis should always be interpreted in relation to the method used (Getachew et al., 1998). Results of *in vitro* studies on fungal treated material should be compared with an untreated control and a highly digestible feed ingredient, to provide additional insight into the possibilities to use *in vivo*. *In vitro* degradability results cannot be directly related to *in vivo* digestibility, because feed intake and palatability of fungal treated material are not taken into account.

2.7 Fungi in ruminant nutrition

2.7.1 Microorganisms as feed additive

Microbial biomass can be used as an alternative source for protein. Fungi can have additional value because they may contain some essential amino acids, although sulfur-containing amino acids are often deficient in fungi (Kuhad et al., 1997). Nevertheless, mycelium of *P. ostreatus* does contain cysteine (13.4 mg/g dry mycelium) and methionine (5.9 mg/g dry mycelium) (Hadar and Cohen-Arazi, 1986). The crude protein (N × 6.25) content in *P. ostreatus* mycelium (when grown on synthetic medium) was 25.7% of the dry matter, which only partly represents protein as chitin present in fungal cells also contains nitrogen (Manu-Tawiah and Martin, 1987). Chitin, formed from polymeric N-acetyl glucosamine, together with β-glucan, polymer consisting of glucose, constitutes fungal fiber (Sadler, 2003). β-Glucans could be an additional source of glucose in the rumen, or replace part of the carbohydrates, hemicellulose and cellulose, that are degraded by the fungus. There are studies indicating that pure chitin can be degraded by some microbes in the rumen (Fadel El-Seed et al., 2003; Miltko et al., 2010). Important to note when considering nutritional values of fungal biomass is that it is highly dependent on the growth stage of the fungus (Manu-Tawiah and Martin, 1987). Mushrooms of *P. ostreatus* contain approximately 10% more nitrogen (as percentage of dry weight) than mycelium of the same fungus (Manu-Tawiah and Martin, 1987).

Using microorganisms as feed additives is not a new concept, since yeasts are being used to stimulate digestion. Active yeast as feed additive could promote the development of the rumen in young animals by stimulating the development of the microbial population, stabilize rumen pH and increase fiber digestion by influencing cellulolytic microbes (Chaucheyras-Durand et al., 2008). Yeast fed as feed additive to cows may increase metabolizable energy of maize silage (Guedes et al., 2008).

2.7.2 *In vitro* digestibility of fungal treated biomass

In vitro studies show that fungal treated material has potential to be used as a feed ingredient. A number of fungal-substrate combinations have been tested using *in vitro* methods, including weight loss methods as described by Tilley and Terry (1963) and gas production measurements as described by Cone et al. (1996), using either rumen fluid or fecal inoculum from different ruminant species, e.g. cows, sheep or goats. These fungus–substrate combinations improved *in vitro* degradability including *C. subvermispora*, *Daedalea quercina*, *Ganoderma* sp. Rckk02, *H. clathroides*, *Inonotus andersonii*, *Inonotus dryphilus*, *Inonotus obliquus*, *Laccaria amethystine*, *L. edodes*, *P. brevispora*, *P. chrysosporium*, *P. ciliatus*, *P. eryngii*, *Phellinus laevigatus*, *P. sajor-caju*, *P. ostreatus*, *Trametes gibbosa*, *T. versicolor* on wheat straw (Streeter et al., 1982; Valmaseda et al., 1991a; Tripathi and Yadav, 1992; Jalč et al., 1994; Bisaria et al., 1997; Jalč et al., 1998; Fazaeli et al., 2006; Arora and Sharma, 2009; 2011; Shrivastava et al., 2011; Shrivastava et al., 2012; Tuyen et al., 2012), *P. chrysosporium*, *P. sajor-caju*, *T. versicolor* on spruce wood (Asiegbu et al., 1996), *C. subvermispora*, *L. edodes*, *P. brevispora*, *P. eryngii*, *P. ostreatus* on oil palm fronds (Rahman et al., 2011; Hassim et

al., 2012; Tuyen et al., 2013), *Pleurotus citrinopileatus*, *Pleurotus florida* on water hyacinth (Mukherjee and Nandi, 2004), *C. subvermispora*, *Dichomitus squalens*, *L. edodes*, *Pholiota nameko*, *P. ostreatus* on red cedar wood (Okano et al., 2005), *C. subvermispora*, *P. brevispora*, *P. chrysosporium*, *P. eryngii*, *Phlebia fascicularia*, *P. floridensis*, *P. radiate* on rice straw (Sharma and Arora, 2010a; Tuyen et al., 2013), *C. subvermispora*, and *L. edodes* on sugarcane bagasse (Tuyen et al., 2013). *In vitro* fermentation properties of wheat straw were increased to the levels of hay after treatment with *P. ciliatus* (Jalč et al., 1994). An improvement of *in vitro* degradability not always necessarily results in a high value feed as shown by Okano et al. (2005), who found an increase in degradability of cedar sapwood chips after treatment with *C. subvermispora* and *L. edodes*, but degradability was still lower than that of rice straw. An increased *in sacco* degradation of wheat straw was found after treatment with *D. squalens* and *Cyathus stercoreus* compared to untreated wheat straw (Agosin et al., 1986).

2.7.3 *In vivo* digestibility of fungal treated biomass

Only a few papers describe *in vivo* studies in which fungal treated material is fed to ruminants. An issue that may rise is palatability, i.e. whether animals will accept fungal treated material as feed. Generally, a high feed intake is essential for production animals, and intake is higher of better digestible feeds or feed ingredients. After mushroom production of *P. sajor-caju* on wheat straw, the substrate was fed to male lambs resulting in increased fat and cellulose digestibility, without affecting feed intake (Calzada et al., 1987). Feeding solely fungal (*Coprinus fimetarius*) treated rice straw resulted in a higher dry matter intake and cell wall digestibility by goats compared to urea treated rice straw (Rai et al., 1989). Goats seem to accept *Ganoderma* sp. treated wheat straw, with additional groundnut cake to meet crude protein requirements. A higher digestibility of dry matter, organic matter, crude protein, hemicellulose and cellulose was observed when goats were fed fungal treated material (Shrivastava et al., 2012). Cows seem to be more selective, since a maximum of 17% wheat straw after mushroom production by *P. ostreatus* could be fed (Adamovic et al., 1998). Animal species can have a large influence on the effect measured, since fiber intake is higher in cattle than in sheep and goats, and fiber digestion is higher in cattle and goats than in sheep (Galyean and Goetsch, 1993). Mushroom production by *Pleurotus* sp. lowered dry matter digestibility of wheat straw in buffaloes (Bakshi et al., 1985). Most *in vivo* studies include mushroom production in their fungal treatment, which is assumed to result in a lower cellulose content (see section 2.5.4). Mushroom production can have an effect on voluntary intake by sheep, since before mushroom production intake was higher than after mushroom production (Fazaeli et al., 2006). Using fungi that produce edible mushrooms has the advantage that also the mycelium is generally regarded as save (GRAS status). One of the most effective and selective lignin degraders, *C. subvermispora*, is not used for mushroom production and it is, therefore, not known if this fungus can be consumed safely by animals. One *in vivo* study has been conducted using *C. subvermispora* treated bamboo fed to sheep where the material was included in a diet with alfalfa hay, wheat

bran and soybeanmeal. The fungal treated bamboo resulted in a higher organic matter, NDF and ADF digestion than untreated bamboo. To ensure that the animals accepted the material, it was ground to 2 mm (Okano et al., 2009). This means that this fungus may not be harmful for sheep in the short term when fed as part of a compound diet. However, long term studies should be conducted to ensure that the feeding of *C. subvermispora* to animals is safe. Furthermore, the studies presenting *in vivo* data are limited to digestion data. Additional information is required on the effect of feeding fungal treated lignocellulosic biomass on the quality and safety of animal products for human consumption such as milk and meat.

2.8 Future work

Optimization based on knowledge of mechanisms involved in lignin degradation. For optimization of a fungal pre-treatment of lignocellulosic biomass, culture conditions play an important role. Since substrates differ in cell wall composition, they may require different culture conditions for optimal performance of the fungi. Comparison of studies not only requires accurate documentation and reporting of culture conditions (as discussed in section 2.4.3), but also standardized analysis of fungal treated material.

Comparing lignin and cellulose degradation under optimal and suboptimal conditions for a fungal treatment will provide more insight into the underlying mechanisms. Enzyme and smaller reactive oxidative compound, such as organic acids or mediators, production is important in lignin degradation and characterization of these enzymes and oxidative compounds during fungal delignification can provide valuable information (Dashtban et al., 2009; Tian et al., 2012). Previous studies have shown that there is a clear negative correlation between the analyzed lignin content of substrates and the positive effect fungi have on digestibility (Tuyen et al., 2013). However, also composition of lignin is important for fungal degradation as discussed in section 2.5.2. Changes in lignin composition and content during fungal treatment can be used to predict the effectiveness of a specific fungus–substrate combination. Chemical changes have been found in aromatic rings and conjugated ester bonds in lignin of corn stover after treatment with *Irpex lacteus* (Yang et al., 2010). Degradation of the building blocks of lignin (syringyl, guaiacyl or p-hydroxyphenyl units, see section 2.2) provides different products after the fungal treatment of wheat straw (Martínez et al., 2001). Different peak patterns, when pyrolysis GC–MS was used, were found in spruce treated with *T. versicolor* compared to untreated sound wood (Sáiz-Jiménez and De Leeuw, 1984). Degradation patterns of plant cell walls should also be studied using microscopy to determine which part of the cell is degraded first. Unraveling the mechanisms of fungal delignification, however, remains difficult, as both enzymes and degradation products can be bound to cells, leading to an underestimation (Calvo et al., 1995; Valásková and Baldrian, 2006). Measurement of gene expression of fungi grown under optimized conditions, compared to suboptimal conditions, can provide additional information on the mechanisms of action of lignin degradation. Fungi selected for efficiency and extent of lignin

degradation can be used to map genes involved and this information can be used for breeding superior strains.
Optimal culture conditions found in laboratory scale experiments are not necessarily optimal under more practical, larger scale treatments. Challenges to treat lignocellulosic biomass with fungi on a larger scale are the removal of heat and controllable conditions as reviewed by Reid (1989) and Zadrazil et al. (1996). In the mushroom production industry, solid state fermentation is practiced on a large scale, and may provide a model for fungal pre-treatments (Sánchez, 2004). Once the optimal fungal pre-treatment has been developed, the effects of fungal treated feed ingredients on animal production should be studied including production efficiency, animal health and welfare, animal product quality and the environmental impact.

2.8.1 Fungal strain improvement

Classic breeding or breeding by molecular techniques can be used to improve the lignin degradation capacity of fungi. Classic breeding of fungi involves crossbreeding different strains using compatible monokaryons (Ramírez et al., 2010). Del Vecchio et al. (2012) showed that laccase production by *P. ostreatus* could be improved 5 fold by classic breeding efforts.
Molecular techniques have been described to improve the MnP production of *P. ostreatus* by homologous recombinant gene expression of mnp genes (Irie et al., 2001; Tsukihara et al., 2006). Introducing mnp cDNA of *P. ostreatus* in *Coprinus cinereus* combined the high MnP production of *P. ostreatus* and the fast growth of *C. cinereus*, resulting in a higher lignin degradation after 16 days (Ogawa et al., 1998). Cellulose degradation could be lowered and selectivity could be improved by developing a cellulase-deficient *P. ostreatus* strain (Chalamcherla et al., 2010). A cellobiose dehydrogenase deficient *T. versicolor* strain degraded less carbohydrates, but this did not result in an improved *in vitro* rumen degradability (Ramirez-Bribiesca et al., 2011). Breeding of lignin degrading fungi has mainly focused on genes encoding for lignin degrading enzymes, but it is important to note that not all genes present are expressed. For example, both *C. subvermispora* and *Phanerochaete sordida* have LiP genes, but do not produce LiP (Rajakumar et al., 1996). Enzyme production is not always related to lignin degradation as discussed in section 2.4.2, therefore, breeding based on lignin degrading characteristics is advisable.
Expression of genes can be influenced by environmental factors. Laccase production is multifactorially regulated, as shown by downregulation of 38% of the laccase gene expression in *P. ostreatus* in solid state fermentation, compared with submerged fermentation (Castanera et al., 2012; del Vecchio et al., 2012). Substrate influences the type of isozymes produced by *C. subvermispora* (Salame et al., 2010). Mainly nitrogen and carbon are important in the expression of genes involved in lignin degradation (Conesa et al., 2002; Janusz et al., 2013). In selective lignin degradation, optimal culture conditions are as important as genotype of the fungus.

2.9 Conclusions

Lignin in plant cell walls is blocking cellulose and hemicellulose, so that these carbohydrates are less accessible for rumen microbes. Delignifying white rot fungi are capable of increasing the bioavailability of cellulose and hemicellulose, and can thus present a cheap and environmental friendly alternative for commonly used chemical extraction methods. Selectively delignifying fungi colonize the substrate where after they start producing enzymes and radicals to break down the lignin structures, making cellulose and hemicellulose readily available to produce fruit bodies. Lignin degradation is a highly complex process, involving enzymes, mainly peroxidases, radicals and other small compounds, such as chelates, and not yet fully understood.

Important factors in the fungal treatment are the fungal strain chosen, the substrate studied, time of incubation and culture conditions. In the literature, details on fungal strain and culture conditions are often missing, making it difficult to compare and reproduce studies. Nevertheless, many different fungus–substrate combinations result in higher *in vitro* degradability in rumen fluid. In general, *C. subvermispora* and *P. eryngii* perform the best regarding structural changes and improvement *in vitro* rumen degradability of organic matter on different substrates. Increased *in vivo* dry matter, organic matter, crude protein and fiber digestibility by sheep and goats is found after fungal treatment of lignocellulosic biomass. Important in ruminant nutrition is safety of the fungus used and more information is required on the safe use of fungal treated biomass as feed ingredients for animals as well as subsequent safety of the human consumable products in long term *in vivo* studies.

Disadvantages of fungal treatment are the long incubation period needed and use of carbohydrates by the fungi. To use the fungal pre-treatment for ruminant nutrition, optimization is essential. Detailed description on procedures of different fungal pre-treatment methods should be described clearly and effects of the treatment on substrate composition and feeding value should be assessed by standardized and well described methods. Studies in the literature should clearly describe fungal strains and culture conditions used, making reproduction, comparison, and thus optimization possible. Knowledge on lignin degradation mechanisms should be obtained by comparing gene expressions of fungi grown under optimized and suboptimal conditions. Knowledge on working mechanisms at a gene level can also be used to breed superior fungal strains, which could provide tools to improve the degrade lignin more selectively during a shorter period of time.

Important factors in lignocellulosic digestibility should be studied at a rumen level. Furthermore, until now only small laboratory scale experiments have been performed concerning single substrates or fungal species. More elaborative experiments in practice should be conducted to develop a competitive alternative to conventional pre-treatments of lignocellulosic biomass for ruminant nutrition.

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CHAPTER 3

Fungal treatment of lignocellulosic biomass: importance of fungal species, colonization and time on chemical composition and *in vitro* rumen degradability

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Abstract

The aim of this study is to evaluate fungal treatments to improve *in vitro* rumen degradability of lignocellulosic biomass. In this study four selective lignin degrading fungi, *Ganoderma lucidum*, *Lentinula edodes*, *Pleurotus eryngii* and *Pleurotus ostreatus*, were used to pre-treat lignocellulosic biomass and to make the carbohydrates in the lignocellulose available for rumen microbes. Wheat straw, miscanthus, wood chips and rice straw were used as models for lignocellulosic biomass. Samples obtained after 12 weeks of incubation were assessed for fungal growth, *in vitro* gas production (IVGP) as measure of rumen fermentation capacity and fiber composition. Most effects on IVGP and lignin degradation were found after 12 weeks of fungal treatment. Twelve weeks of treatment with *L. edodes* improved the IVGP of wheat straw by 58.5 ml/g OM (23.1% increase), of miscanthus by 80.5 ml/g OM (43.7% increase) and of wood chips by 123.4 ml/g OM (229% increase). All fungi improved the IVGP of wood chips. Although all fungi grew on rice straw, the IVGP was not improved. All four fungal species caused increased cellulose concentration of wheat straw, miscanthus and wood chips. However, when expressed as absolute amounts, cellulose degradation occurred. In wheat straw and miscanthus, lignin was degraded best by *L. edodes*. Improvement of IVGP by *L. edodes* was correlated to lignin degradation. Lignin degradation and dry matter loss were correlated to the mycelium growth of *L. edodes*. These correlations differed between substrates and fungi. Fungal growth was not always a good predictor for the performance of the fungus. Here, *L. edodes* performed best, regarding IVGP and selective lignin degradation.

3.1 Introduction

Cellulose is one of the most abundant polysaccharides present in the world (Buranov and Mazza, 2008) and an important substrate for rumen microbes thus important in ruminant nutrition. Within plant cell walls, cellulose is interwoven with hemicellulose and lignin, a recalcitrant polymer providing rigidity and protection to plants (Vanholme et al., 2010). Lignin cannot be degraded through anaerobic fermentation in the rumen and has been shown to be negatively related to digestibility in ruminant animals (Arora and Sharma, 2009). Agricultural by-products, like wheat and rice straw or municipal waste, like wood chips from pruning, are often rich in carbohydrates in the form of cellulose and hemicellulose. However, these by-products cannot be used optimally by ruminants because of the effect of lignin (Lapierre, 1993; Buranov and Mazza, 2008). Also in crops like miscanthus which are specially bred for a high carbohydrate content, cellulose has a limited accessibility due to the relatively high lignin content (Van Hulle et al., 2010). Several methods to degrade or modify lignin to improve cellulose and hemicellulose accessibility exist, and presently mainly acid and alkaline chemicals are used (Howard et al., 2004; Sarnklong et al., 2010). The use of chemicals is not only costly but also a potential pollutant to the environment (Dashtban et al., 2009). Biological degradation of lignin by fungal lignolytic enzymes can offer a viable and environmentally friendly alternative. Especially white-rot fungi are specialists in degrading lignin and some are highly selective, i.e. cellulose is hardly degraded (Kirk and Farrell, 1987). Fungi degrade organic matter by excreting enzymes and since these enzymes have a very limited range of diffusion, the extent of colonization is relevant for proper lignin degradation and is the first measure for a successful treatment (Kirk and Farrell, 1987). Ergosterol, a component of cell membranes specific for fungi, can be used to estimate fungal biomass. During colonization, fungi secrete a complex mixture of compounds that can degrade the lignocellulose complex. Especially compounds involved in oxidative degradation or generation of radicals support lignin degradation (Wong, 2009). Each fungal species has a specific strategy, and shows different selectivity to degrade lignin. A number of macro fungi degrade lignin and hemicellulose during vegetative growth and cellulose is mainly degraded during fruiting body production. This vegetative phase is thus highly suitable to improve accessibility of cellulose. The time required for this treatment is another important factor. In the study reported here, fungal treatment was followed at different duration of treatment to determine whether lignin degradation shifts to cellulose degradation after a prolonged treatment. To study the improvement in cellulose accessibility by fungal lignin degradation, a model for ruminant digestion was used. The microbes in the rumen are specialists in degrading cellulose in plant cell walls, but are unable to degrade lignin in the anaerobic rumen environment. When more carbohydrates are available, rumen microbes will degrade more substrate resulting in an increased production of volatile fatty acids. This increased accessibility of cellulose to rumen microbes can be simulated by the routinely used *in vitro* gas production technique (Cone et al., 1996). Based on previous research, four edible fungi were selected namely, *Ganoderma lucidum*, *Lentinula edodes*, *Pleurotus eryngii*, and *Pleurotus ostreatus*

(Tuyen et al., 2012) as these were shown to act as specific lignin degraders and improve *in vitro* degradability of lignocellulosics in rumen fluid (Tuyen et al., 2012). In the present study, the differences between four fungal species on lignin degradation in wheat straw, rice straw, miscanthus and wood chips originating from municipal waste were examined. Also the effects of colonization rate and duration of treatment on lignin degradation by these fungi was studied. Chemical changes and cellulose accessibility as measured by the *in vitro* gas production technique were compared and related to the fungal biomass formed.

3.2 Material and methods

3.2.1 Fungal strains and spawn preparation

The fungal strains used in this study (*G. lucidum* (strain MES 02102), *L. edodes* (strain MES 11910), *P. eryngii* (strain MES 03757) and *P. ostreatus* (strain MES 03449)) were preserved in liquid nitrogen at the Department of Plant Breeding (Wageningen UR, Wageningen, The Netherlands). Initial cultures of these fungi were developed on malt extract agar plates at 24°C until mycelium covered most of the plate surface. Pieces of colonized agar culture (~1 cm^2) were added to sterilized sorghum grains and incubated at 24°C until all grains were colonized by mycelium. This spawn was kept at 4°C until further use.

3.2.2 Substrate preparation

The substrates used were wheat straw, miscanthus (*Miscanthus giganteus*), rice straw (obtained from Indonesia but, otherwise, from an unknown source), and wood chips consisting of a mixture of wood species. All substrates were chopped into pieces of approximately 3 cm. Rice straw already consisted of smaller particles of sizes between 2 and 3 cm. Samples were taken from each substrate to serve as a control before autoclaving. Nets, with pores large enough to allow water to enter without substrate leaving the nets, were used to submerge the substrate in tap water for 3 d to allow moisture to fully penetrate the material. Excess water was drained (final dry matter content was 210 g/kg for wheat straw, 290 g/kg for miscanthus, 270 g/kg for rice straw and 340 g/kg for wood chips) before the substrates were autoclaved twice. The substrate was transferred to autoclavable bags for the first sterilization at 121°C for 1 h. After cooling, the material was weighed (approximately 80–90 g dry matter) into 1.2 l polypropylene containers and covered with a lid containing a filter (model TP1200+TPD1200 XXL Combiness, Nazareth, Belgium). Each container was autoclaved at 121°C for 1 hour to kill the germinated spores left after the first autoclaving step. After cooling, the containers with the sterile substrates were stored at room temperature until further use.

3.2.3 Substrate inoculation

To each container, approximately 8–10 g of spawn was added aseptically and mixed to distribute the spawn equally over the substrate. Each container with sample was incubated for either 0, 2, 4, 8 or 12 weeks in an air-conditioned chamber at 24°C with a relative humidity of 75%. Each treatment was done in triplicate. After incubation, the containers were weighed and approximately 15% of the treated substrate including mycelium was air-dried at 70°C until constant weight for chemical analysis and *in vitro* gas production measurement. The other part was freeze dried and stored at room temperature until ergosterol determination. After drying the material was weighed again for dry matter (DM) loss determination. The dried wheat straw, miscanthus and rice straw were ground over a 1 mm sieve using a Peppink 100 AN cross beater mill (Peppink, Deventer, The Netherlands). The dried wood chips were finely ground over a 1 mm sieve using a Retch ZM100 centrifugal mill (Retsch, Haan, Germany) to obtain a homogenous sample.

3.2.4. Chemical analysis

Fiber analysis was performed according to the method of Van Soest et al. (1991). Neutral detergent fiber was determined with heat stable amylase, but without addition of sodium sulphite. All fiber fractions are expressed excluding residual ash. The hemicellulose concentration was calculated as the difference between the neutral detergent fiber (NDF) and the acid detergent fiber (ADF). Cellulose was calculated as the difference between ADF and acid detergent lignin (ADL). For DM determination, air-dried material was dried at 103°C for 4 hours (ISO 6496). Ash content was determined by combustion for 3 hours at 550°C in a muffle furnace (ISO 5984). Absolute numbers were calculated using data on the remaining air dry matter after fungal incubation corrected for DM content in dried material as determined by oven drying at 103°C.

3.2.5 *In vitro* gas production (IVGP) technique

In vitro gas production was performed according to the procedure described by Cone et al. (1996). In summary, rumen fluid of fistulated non-lactating cows fed a grass silage based diet was mixed with an buffer solution under anaerobic conditions. Air dried samples (500 mg) were incubated in 60 ml buffered rumen fluid for 72 hours at 39°C. The gas production was recorded automatically as described by Cone et al. (1996) and related to the organic matter (OM) content of the samples.

3.2.6 Ergosterol determination

Ergosterol determination was based on Niemenmaa et al. (2008). Briefly, ground, freeze dried material (200 mg) was saponified with 3 ml 10% KOH/methanol for 1 hour at 80°C. Ergosterol was extracted by adding 1 ml water and 2 ml hexane, with the hexane phase being collected after shaking and centrifuging for 10 minutes at 4000 rpm. This step was repeated once more before both hexane phases were pooled. Hexane was

evaporated under vacuum and the remaining ergosterol dissolved in methanol. The recovery of the internal standard 7-dehydrocholesterol (9.6 µg added) was used to correct ergosterol concentrations for extraction efficiency (Sigma Aldrich, St. Louis, Missouri, USA) (Niemenmaa et al., 2008). Ergosterol was analyzed using an high performance liquid chromatography (HPLC) fitted with a reversed phase C18 column (250 × 4.6 mm, Phenomex aqua 5 µm). The liquid phase was 90% methanol and 10% (1:1) 2-propanol/hexane. Areas under the peak were corrected for the extraction efficiency based on the internal standard using Empower 2 software (Waters Corporation, Milford, Massachusetts, USA). To convert the amount of ergosterol measured into dry weight of mycelium, mycelium of each fungus was grown on malt extract agar with cellophane and freeze dried. Known amounts of dry mycelium were subjected to ergosterol extraction. For each fungus, the amount of ergosterol per mg mycelium was calculated and used to express the data in mg mycelium per g substrate.

3.2.7 Statistical analysis

IVGP data of the fungal treatment of each substrate were subject to generalized linear model (GLM) analysis in SAS 9.2. The following model was used:

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

in which Y_{ij} is the observation j in treatment i; μ is the overall mean; α_i is the fixed effect of fungal treatment i; β_j is the fixed effect of time j; ω_{ij} is the random error. Ergosterol, chemical composition and IVGP data of the fungal treatment at each duration of treatment compared to the uninoculated, autoclaved control of each substrate were subject to generalized linear model (GLM) analysis in SAS 9.2. Post-hoc multiple comparison with Tukey's significant test at a level of $\alpha = 0.05$ was performed to determine the significance between the treatments. The following model was used:

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

in which Y_{ij} is the observation j in treatment i; μ is the overall mean; α_i is the fixed effect of treatment i; ω_{ij} is the random error. Correlations between IVGP and ADL, mycelium growth and ADL, and mycelium growth and IVGP were performed by correlation analysis in SAS 9.2. The correlations are provided as R^2: square of Pearson correlation coefficient R.

3.3 Results

3.3.1 Growth of fungi

All fungi used in the present study were able to grow on all substrates. After 2 weeks, mycelium growth was still low for *G. lucidum* on wood chips, *L. edodes* on miscanthus, rice straw and wood chips, and *P. ostreatus* on miscanthus and wood chips (Figure 3.1). After 4 weeks, significant (P = 0.0147) growth of only *G. lucidum* on wheat straw was

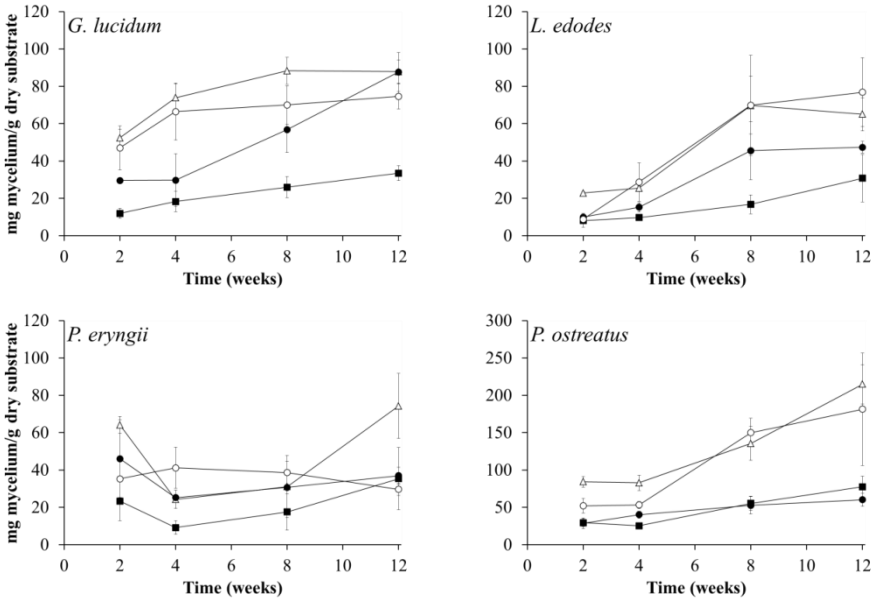

Figure 3.1 Mycelium concentration of *Ganoderma lucidum*, *Lentinula edodes*, *Pleurotus eryngii* and *Pleurotus ostreatus* grown for 0, 2, 4, 8 and 12 weeks on (Δ) wheat straw, (■) miscanthus, (○) wood chips, (●) rice straw. Error bars indicate standard deviations, n=3.

observed (Figure 3.1). In some fungal treated substrates, very limited or no growth was visually observed, especially for *P. eryngii*, explaining the low amount of mycelium measured for this species after 2 and 4 weeks (Figure 3.1). For each time point, a different container was used to measure ergosterol concentration. With very low or no growth, a variation between containers might erroneously suggest a decrease in fungal biomass as is seen in Figure 3.1. In the period from 4 to 8 weeks of incubation, a significant increase of mycelium of *L. edodes* was seen on wheat straw ($P = 0.0213$), miscanthus ($P = 0.0075$) and rice straw ($P = 0.02$) (Figure 3.1). In the same period, also a significant increase of mycelium of *P. ostreatus* was found on wheat straw ($P = 0.0333$) and wood chips ($P = 0.0235$) (Figure 3.1). A significant increase in ergosterol concentration for *G. lucidum* on wood chips was only found in the period between 2 and 8 weeks of incubation (Figure 3.1). *G. lucidum* showed a significant ($P = 0.0319$) ($P < 0.05$) growth only on miscanthus after 12 weeks (Figure 3.1). A plateau in mycelium concentration was reached after 8 weeks of incubation of *G. lucidum* on wheat straw and wood chips, *P. ostreatus* on all substrates except wheat straw and *L. edodes* on all substrates ($P < 0.05$) (Figure 3.1). For *P. ostreatus*, mycelium concentration was highest on wheat straw, while *L. edodes* grew best on rice straw (Figure 3.1). *G. lucidum* produced equal amounts of mycelium on wheat straw and miscanthus, but wheat straw was colonized quicker than miscanthus.

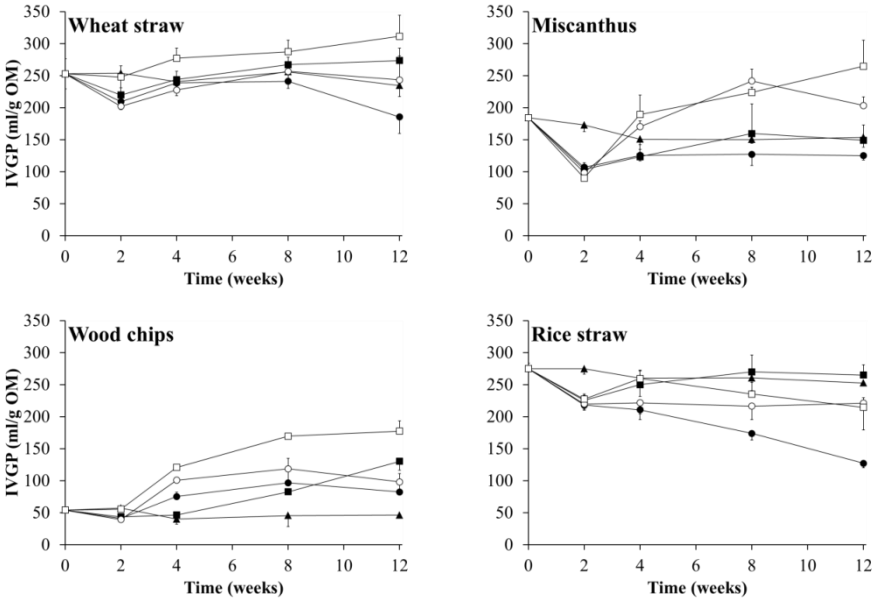

Figure 3.2 *In vitro* gas production after 72h (IVGP) of incubation in rumen fluid of substrates treated with four different fungi for 0, 2, 4, 8 and 12 weeks ((▲) untreated autoclaved material, (●) *Pleurotus ostreatus*, (■) *Pleurotus eryngii*, (○) *Ganoderma lucidum*, (□) *Lentinula edodes*). Error bars indicate standard deviations, n=3.

3.3.2 IVGP

Untreated, autoclaved wheat straw showed an IVGP of 252.8 ml/g OM, IVGP of untreated autoclaved miscanthus, wood chips and rice straw was 184.2, 54.0, and 274.9 ml/g OM, respectively. There was a significant ($P < 0.01$) interaction between fungus and time for all substrates, indicating that the different fungi had different effects over time. While none of the fungi increased IVGP of wheat straw and wood chips during 4 weeks of treatment, IVGP of miscanthus was improved between 2 and 4 weeks treatment with *G. lucidum* ($P = 0.0018$) and *L. edodes* ($P = 0.0111$) (Figure 3.2). All fungal species tested here increased ($P < 0.05$) IVGP of wood chips after 8 weeks. Eight weeks of *G. lucidum* treatment of miscanthus resulted in a maximum in IVGP (Figure 3.2). Twelve weeks of *L. edodes* treatment resulted in an increase in IVGP of 58.5 ml/g OM (23.1%) for wheat straw ($P < 0.05$), 80.5 ml/g OM (43.7%) for miscanthus ($P = 0.0196$) and 123.4 ml/g OM (228.8%) for wood chips ($P < 0.0001$) compared to untreated, autoclaved material (Figure 3.2). Furthermore, 12 weeks of treatment of wood chips with *P. eryngii* increased ($P < 0.0001$) IVGP by 76.1 g/ml OM (141.1% increase) (Figure 3.2). The IVGP of rice straw was not improved by fungal treatment (Figure 3.2).

3.3.3 Chemical composition

L. edodes treatment resulted in a significant improvement in IVGP of wheat straw and even in the greatest improvement in IVGP of miscanthus and wood chips as shown in Figure 3.2. Therefore, chemical composition of *L. edodes* treated material was measured

after 2, 4, 8 and 12 weeks (Table 3.2), while for the other fungi chemical composition was measured only after 12 weeks (Table 3.1). Furthermore, as no improvement in IVGP of rice straw compared to the untreated autoclaved control was observed, no chemical analyses of the material were done. *L. edodes* caused a decrease ($P < 0.05$) in hemicellulose and ADL concentration of wheat straw from 2 weeks on. Cellulose concentration was increased ($P < 0.05$) in miscanthus from 2 weeks on (Table 3.2). Treatment of wheat straw with *L. edodes* for 8 weeks or longer resulted in an increased cellulose concentration (Table 3.2). *L. edodes* did decrease ($P < 0.0001$) ADL concentration of not only wheat straw, but also of wood chips and miscanthus during 8 weeks of treatment (Table 3.2). However, the absolute amount of ADL in miscanthus started decreasing from 2 weeks on, as did the absolute amount of hemicellulose ($P < 0.01$) (Table 3.2). After 8 weeks of treatment, cellulose in miscanthus started to be degraded ($P < 0.01$) by *L. edodes* (Table 3.2). Although all fungi decreased the ADL and hemicellulose concentration of wheat straw and did not decrease the cellulose concentration, the IVGP of wheat straw was only increased after 12 weeks of treatment with *L. edodes*. This fungus caused a higher loss of ADL (65.6 g/kg DM) in wheat straw compared to the other fungi, but also a high loss of hemicellulose of 172.9 g/kg DM (Table 3.1). *L. edodes* also caused the highest loss (164.4 g/kg DM) of hemicellulose in miscanthus. Besides *L. edodes*, *G. lucidum* degraded ($P < 0.0001$) ADL in miscanthus, and *P. eryngii* ($P = 0.0072$) and *P. ostreatus* ($P = 0.0017$) caused a decreased ADL concentration in wood chips after 12 weeks treatment (Table 3.2). *L. edodes* decreased ($P < 0.0001$) the hemicellulose concentration most in miscanthus and was the only fungus which degraded ($P < 0.05$) hemicellulose at the same rate compared to other components in wood chips (Table 3.1). After 12 weeks of colonization, all fungi increased the cellulose concentration in wheat straw and miscanthus ($P < 0.05$) (Table 3.1). It is important to note that cellulose in miscanthus was degraded by all fungi except *P. eryngii* varying from 3 up to7.5 g (which corresponds to 7 up to 20%) of the absolute amount of cellulose present in the original substrate. The absolute amount of cellulose in wheat straw, decreased only after treatment with *P. ostreatus* (17.2 g) for 12 weeks (Table 3.1). The latter is in line with the highest DM loss in wheat straw caused by this fungus. The fact that *G. lucidum* degraded cellulose after 12 weeks treatment of miscanthus suggests that in the current experiment, 12 weeks of *G. lucidum* treatment of miscanthus is too long to result in maximum rumen fermentation. None of the fungi degraded cellulose of wood chips significantly, both when expressed as concentration or absolute amounts, whereas the absolute amount of ADL was decreased ($P < 0.05$) by *L. edodes* and *P. ostreatus* treatment for 12 weeks (Table 3.2). Throughout the fungal treatment, substrates are enriched in terms of ash and crude protein concentration (Tables 3.1 and 3.2).

Table 3.1 Chemical composition of wheat straw, miscanthus and wood chips before (untreated, autoclaved control) and after 12 weeks of treatment with four different fungi.

		Concentration (g/kg DM)					Absolute amount (g)					DM loss
Substrate	Treatment	HC	Cell	ADL	Ash	CP	HC	Cell	ADL	Ash	CP	(%)
Wheat straw	Control	260.1^{a}	479.6^{a}	81.1^{a}	24.1^{a}	26.6^{a}	24.2^{a}	44.6^{a}	7.5^{a}	2.2	2.5	0^{a}
	G. lucidum	185.2^{b}	525.8^{bc}	56.7^{b}	27.7^{ab}	31.3^{a}	14.4^{b}	40.9^{a}	4.4^{b}	2.2	2.4	16^{b}
	L. edodes	87.2^{c}	544.2^{b}	15.5^{c}	36.4^{c}	42.6^{b}	5.7^{c}	35.6^{a}	1.0^{c}	2.4	2.8	30^{b}
	P. eryngii	171.3^{bd}	507.4^{c}	43.9^{d}	31.2^{b}	34.7^{c}	13.7^{b}	40.6^{a}	3.5^{d}	2.5	2.8	14^{ab}
	P. ostreatus	165.5^{d}	477.6^{d}	46.7^{d}	44.9^{d}	51.8^{d}	8.3^{c}	24.0^{b}	2.4^{e}	2.3	2.6	46^{c}
	SEM	6.97	7.29	0.85	0.91	0.61	0.85	1.71	0.13	0.12	0.10	0.03
	P-value	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.41	0.09	<0.01
Miscanthus	Control	285.2^{a}	471.2^{a}	101.4^{a}	9.4^{a}	19.1^{a}	22.5^{a}	37.2^{a}	8.0^{a}	0.7^{a}	1.5	0^{a}
	G. lucidum	194.2^{b}	524.0^{b}	72.3^{b}	12.3^{b}	22.0^{b}	12.7^{b}	34.2^{b}	4.7^{b}	0.8^{b}	1.4	17^{b}
	L. edodes	120.8^{c}	582.3^{c}	36.8^{c}	14.4^{c}	27.4^{c}	6.2^{c}	29.7^{c}	1.9^{c}	0.7^{ab}	1.4	35^{c}
	P. eryngii	253.4^{d}	508.3^{b}	96.2^{a}	10.4^{a}	19.1^{a}	19.5^{d}	39.2^{d}	7.4^{a}	0.8^{b}	1.5	3^{a}
	P. ostreatus	239.2^{d}	516.9^{b}	96.3^{a}	13.0^{b}	23.1^{b}	14.6^{b}	31.6^{c}	5.9^{d}	0.8^{b}	1.4	23^{d}
	SEM	5.28	2.81	1.31	0.24	0.47	0.47	0.41	0.12	0.01	0.03	0.02
	P-value	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.08	<0.01
Wood chips	Control	198.2^{a}	453.0	198.2^{a}	96.6^{a}	29.8^{a}	11.0^{ab}	35.3	15.4^{a}	7.5	2.3	0^{a}
	G. lucidum	151.3^{ab}	448.4	161.6^{ab}	96.8^{ab}	29.5^{ab}	11.3^{ab}	33.7	12.2^{ab}	7.3	2.2	4^{ab}
	L. edodes	105.6^{c}	442.9	107.0^{c}	131.3^{b}	40.1^{c}	6.8^{b}	28.5	6.9^{c}	8.5	2.6	17^{c}
	P. eryngii	164.6^{abd}	465.0	146.9^{b}	103.6^{ab}	33.2^{abcd}	15.0^{a}	41.9	13.1^{ab}	9.7	3.0	3^{ab}
	P. ostreatus	143.0^{abcd}	421.3	135.2^{bc}	128.3^{ab}	40.9^{cd}	8.9^{ab}	26.2	8.4^{bc}	7.8	2.5	21^{c}
	SEM	9.06	11.19	7.96	11.27	1.87	1.58	3.37	1.04	1.44	0.22	0.03
	P-value	<0.01	0.26	<0.01	0.04	<0.01	0.02	0.06	<0.01	0.49	0.19	<0.01

Values with different superscripts within column are significantly (P<0.05) different. ADL, acid detergent lignin; HC, hemicellulose; Cell, cellulose; CP, crude protein; DM loss, dry matter loss.

Table 3.2 Chemical composition of substrates measured before (untreated, autoclaved control) and after treatment with *L. edodes* for 2, 4, 8 and 12 weeks.

Substrate	Duration of treatment (weeks)	Concentration (g/kg DM)					Absolute amount (g)					DM Loss (%)
		HC	Cell	ADL	Ash	CP	HC	Cell	ADL	Ash	CP	
Wheat straw	Control	260.1^{a}	479.6^{bc}	81.1^{a}	24.1^{c}	26.6^{d}	24.2^{a}	44.6^{a}	7.5^{a}	2.2	2.5	0^{c}
	2	202.7^{b}	461.2^{c}	74.6^{b}	28.6^{b}	30.8^{c}	17.4^{b}	39.5^{ab}	6.4^{b}	2.5	2.6	8^{c}
	4	148.1^{c}	496.3^{b}	57.0^{c}	30.1^{b}	32.9^{c}	12.2^{c}	41.0^{ab}	4.7^{c}	2.5	2.7	11^{bc}
	8	94.6^{d}	537.3^{a}	33.2^{d}	34.4^{a}	37.5^{b}	6.9^{d}	39.1^{ab}	2.4^{d}	2.5	2.7	22^{ab}
	12	87.2^{d}	544.2^{a}	15.5^{e}	36.4^{a}	42.6^{a}	5.7^{d}	35.6^{b}	1.0^{e}	2.4	2.8	30^{a}
	SEM	6.63	6.81	1.28	0.61	0.53	0.70	1.85	0.17	0.09	0.11	0.03
	P-value	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.07	<0.01	0.24	0.39	<0.01
Miscanthus	Control	285.2^{a}	464.7^{d}	101.4^{b}	9.4^{c}	19.1^{c}	22.5^{a}	37.2^{a}	8.0^{a}	0.7^{b}	1.5	0^{c}
	2	241.3^{b}	442.4^{e}	139.4^{a}	9.9^{c}	19.8^{c}	18.7^{b}	34.3^{ab}	10.8^{b}	0.8^{a}	1.5	2^{c}
	4	185.6^{c}	501.1^{c}	97.5^{b}	10.6^{c}	21.3^{c}	12.6^{c}	34.1^{ab}	6.6^{c}	0.7^{b}	1.4	14^{bc}
	8	149.1^{d}	549.7^{b}	73.6^{c}	12.1^{b}	24.6^{b}	8.8^{d}	32.2^{bc}	4.3^{d}	0.7^{b}	1.4	26^{ab}
	12	120.8^{e}	575.8^{a}	36.8^{d}	14.4^{a}	27.4^{a}	6.2^{e}	29.7^{c}	1.9^{e}	0.7^{ab}	1.4	35^{a}
	SEM	4.76	3.43	1.87	0.29	0.48	0.50	0.75	0.17	0.01	0.03	0.03
	P-value	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.05	<0.01
Wood chips	Control	140.8^{a}	445.5^{c}	198.2^{a}	96.6	29.8^{c}	11.0^{a}	35.3	15.4^{a}	7.5	2.3	0
	2	96.3^{ab}	486.2^{b}	187.3^{ab}	105.5	30.3^{bc}	7.8^{ab}	39.2	15.1^{a}	8.5	2.4	0
	4	103.6^{ab}	497.4^{ab}	163.8^{b}	86.6	32.8^{bc}	6.8^{ab}	31.1	10.1^{b}	5.4	2.1	20
	8	79.9^{b}	520.6^{a}	126.6^{c}	104.8	35.4^{ab}	5.6^{b}	36.1	8.8^{b}	7.2	2.5	11
	12	105.6^{ab}	436.5^{c}	107.0^{c}	131.3	40.1^{a}	6.8^{ab}	28.5	6.9^{b}	8.5	2.6	17
	SEM	9.80	6.69	5.48	10.33	1.14	1.04	2.61	0.83	0.80	0.18	0.07
	P-value	0.02	<0.01	<0.01	0.10	<0.01	0.04	0.10	<0.01	0.11	0.44	0.16

Values with different superscripts within column are significantly (P<0.05) different. ADL, acid detergent lignin; HC, hemicellulose; Cell, cellulose; CP, crude protein; DM loss, dry matter loss.

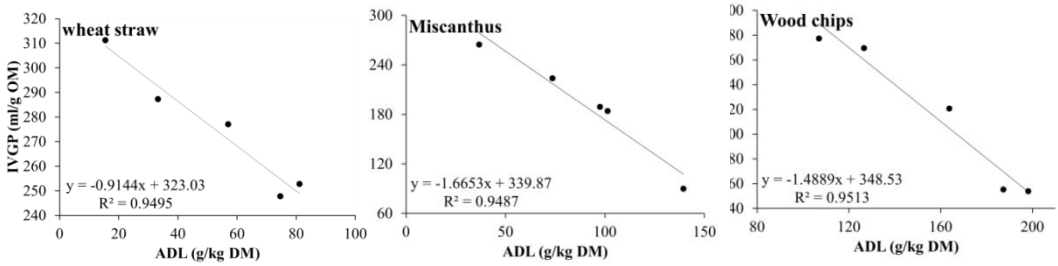

Figure 3.3 Relationship between *in vitro* gas production after 72h (IVGP) and acid detergent lignin (ADL) for *Lentinula edodes* grown on wheat straw, miscanthus and wood chips.

3.3.4 Correlations

A strong correlation was found for all substrates, except rice straw, between changes in ADL concentration by *L. edodes* and changes in IVGP (wheat straw: $R^2 = -0.95$ ($P < 0.05$), miscanthus: $R^2 = -0.95$ ($P < 0.05$), wood chips: $R^2 = -0.95$ ($P < 0.05$)) (Figure 3.3). The growth of *L. edodes* as measured by the ergosterol concentration was correlated to a decrease in ADL concentration for wheat straw: $R^2 = -0.88$ ($P < 0.05$) and wood chips: $R^2 = -0.90$ ($P < 0.05$) (Figure 3.4). Although a R^2 of −0.65 was found for miscanthus, this correlation was not significant. DM loss and mycelium growth (*L. edodes*) was strongly correlated in wheat straw ($R^2 = 0.91$ ($P < 0.05$)) and miscanthus ($R^2 = 0.93$ ($P < 0.05$)) (Figure 3.5). *L. edodes* caused a maximum DM loss of wood chips. This DM loss did not change significantly after 4 weeks of incubation, which can be explained by a large variation between replicates. Degradation of ADL in wood chips by *L. edodes* continued after 4 weeks, however, this was not seen in the DM loss data. The efficiency of the mycelium, thus the ADL degradation per g mycelium, differed between substrates. On wheat straw, each g of *L. edodes* mycelium was responsible for 1.14 g of ADL degradation, while for miscanthus and wood chips, *L. edodes* degraded 0.84 and 3.1 g ADL respectively. Fungal growth is not always related to the effectiveness of the fungal treatment, as is demonstrated by the fact that there was no correlation between the mycelium growth and the increase in IVGP for rice straw. The latter may be due to the lower ADL concentration (33.9 g/kg DM) compared to the other substrates. Mycelium growth may be a predictor of lignin degradation, but this should be compared within substrates and not between substrates.

3.4 Discussion

3.4.1 Growth of fungi

A prerequisite for an effective fungal treatment of lignocellulosic material is a good colonization by mycelium. Mycelium content can be estimated by measuring ergosterol, a sterol present in fungi and protozoa (Seitz et al., 1977; Mille-Lindblom et al., 2004). Although ergosterol content of mycelium is dependent on the different growth stages,

such as lag, exponential and stationary phase (Gao et al., 1993), we assumed that the ergosterol content of mycelium grown on artificial medium is comparable to mycelium grown on the substrates investigated. As each fungus has a unique ergosterol content, it was assessed in each fungal species. After growth on malt extract agar, *G. lucidum*, *L. edodes*, *P. eryngii* and *P. ostreatus* contained 2.4, 2.3, 1.7 and 1.9 µg ergosterol/mg dried mycelium, respectively. Ergosterol content of *L. edodes* found here (2.3(±0.47) µg ergosterol/mg mycelium) was lower than described by Silva et al. (2005) who found 4.1 µg ergosterol/mg fungal biomass. The different ergosterol content can be due to different culture methods, since Silva et al. (2005) cultured *L. edodes* in a liquid culture. The amounts of ergosterol during fungal treatment found in the current study are comparable to literature. In the present study, 417 µg ergosterol of *P. ostreatus* per g wheat straw was found after 12 weeks, which is comparable to ergosterol concentrations (500 µg/g) described for the same fungus-substrate combination by Robertson et al. (2008). Okeke et al. (1994) described ergosterol concentrations of oak sawdust logs after 14 weeks *L. edodes* cultivation between 80.0 and 209.0 µg ergosterol/g substrate depending on the *L. edodes* strain used. In the current study 81.7 µg ergosterol/g wood chips was found after 12 weeks *L. edodes* treatment, which is within the range of Okeke et al. (1994). Growth of *P. eryngii* shows large variations on sawdust of different origin, therefore ergosterol concentrations of the wood chips originating from a mixture of species in the current study cannot be compared to literature (Ohga, 2000). The immersion and subsequently draining of the substrates before sterilization and inoculation will remove most of the water soluble compounds. Water extracts can consist of free sugars, alditols, organic acids, and inorganic acids (Chen et al., 2007; Chen et al., 2010). As a result, the easy accessible sugars are removed, forcing the fungi to degrade the more difficult lignocellulosic structures. The formation of fungal biomass is related to the degree of colonization thus the fraction of the substrate that is exposed to the extracellular enzymes of the fungi. The ratio between ligninolytic enzymes and cellulolytic enzymes can be an indication for the efficiency of the fungal treatment (Fernandez-Fueyo et al., 2012) although ligninolytic enzyme production is not always directly related to lignin degradation (Shrivastava et al., 2011).

3.4.2. IVGP

IVGP was used to determine effectiveness of fungal treatment. Most fungal substrate combinations showed a lower IVGP after 2 weeks of incubation. This drop in *in vitro* degradability was also described by Zadrazil (1977), who found that after 20 days the fungal treated wheat straw was less degradable than the initial straw due to consumption of easy accessible nutrients by the fungus. Fungal treatment of wood chips did not result in a drop in IVGP, suggesting wood chips contain less easy accessible nutrients for the fungi. The initial IVGP of wheat straw (252.8 ml/g OM) was higher than found by Tuyen et al. (2012) (200 ml/g OM). Differences might due to the use of different batches or to the use of different cultivars, which are characteristics that have an effect on fungal treatment ((Labuschagne et al., 2000; Arora and Sharma, 2009)). This is again confirmed

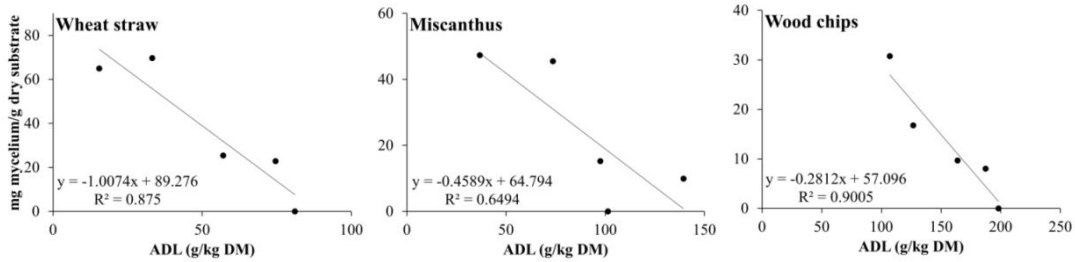

Figure 3.4 Relationship between substrate mycelium concentration and acid detergent lignin (ADL) for *Lentinula edodes* grown on wheat straw, miscanthus and wood chips.

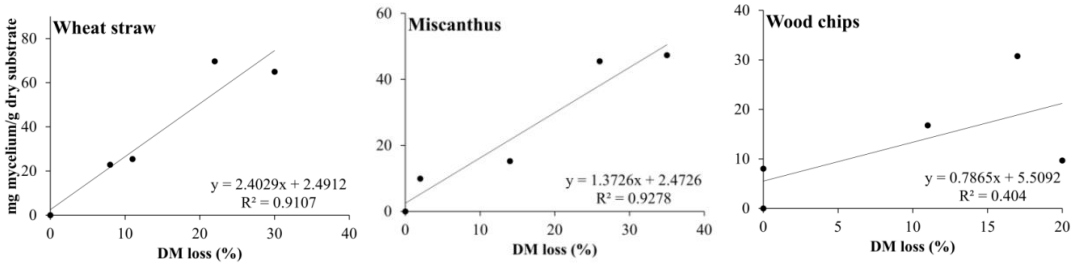

Figure 3.5 Relationship between substrate dry matter (DM) loss and mycelium concentration for *Lentinula edodes* grown on wheat straw, miscanthus and wood chips.

in the present study, where no improvement was found in IVGP of wheat straw during the first 8 weeks of treatment, while Tuyen et al. (2012) found an increase in IVGP after 7 weeks of treatment with *L. edodes*, *P. eryngii* and *P. ostreatus*, using a similar spawn:substrate ratio. The use of different batches, cultivars and growth conditions of rice straw could explain differences in effectiveness of fungal treatment. Tuyen et al. (2013) found an increase in IVGP of fungal treated rice straw using *Ceriporiopsis subvermispora*, but not with *L. edodes*, *P. eryngii* or *P. ostreatus*. In other studies, an increased *in vitro* degradability of rice straw was found after 30 days of treatment with *Cyathus stercoreus* (Karunanandaa et al., 1992) or after 20 days of treatment with *Pleurotus sajor-caju* (Bisaria et al., 1997). Future studies on fungal treatment of rice straw should use different fungal species than those used in the present study. Each wood type contains a different concentration and composition of lignin (Lapierre, 1993), resulting in different effects of fungal treatments. Since here a mixture of different wood types was used, the results of the wood chips presented in the current study are difficult to compare to data in the literature. Okano et al. (2005) reported an increase in *in vitro* degradability after 8 weeks of fungal treatment of cedar wood with *L. edodes* and *C. subvermispora*. However, the study concluded that although the *in vitro* degradability of cedar wood increased compared to the untreated control, it was still lower than rice straw. In the present study, fungal treatment of wood chips resulted in a relatively large increase of 123.4 ml/g OM (228.8%) in IVGP when treated with *L. edodes*. However,

the total IVGP after fungal treatment is lower than IVGP of the other substrates without treatment (Figure 3.2). This is likely due to the high amount of lignin in wood compared to the other substrates, i.e. the lignin concentration of wood after 12 weeks of treatment is similar or higher than lignin concentration of the other substrates without treatment. This suggests that wood chips might not be suitable as an animal feed ingredient; however it is a good model substrate for bioethanol production and to study lignin degradation by fungi. The current results of miscanthus are also difficult to compare to literature, because fungal treatment of *Miscanthus giganteus* has not been described previously. Osono (2010) described a 12 weeks treatment of *Miscanthus sinensis* with *Trametes* versicolor, and showing some lignin degradation.

3.4.3 Chemical composition

The chemical composition of the substrates was determined using the method by Van Soest et al. (1991). In the current study hemicellulose is defined as the difference between NDF and ADF and cellulose as the difference between ADF and ADL. However, care has to be taken when using these definitions. NDF is known to contain some proteins, ADF contains some pectins and hemicelluloses, and ADL does not include the acid soluble lignin. As a result, hemicellulose and cellulose are overestimated and lignin is underestimated (Jung, 1997; Godin et al., 2014). Also the pectin, hemicellulose and lignin may have different properties in different plant species (Jung, 1997). Nonetheless, acid soluble lignin is better digestible than ADL, which is negatively correlated with digestibility (Lowry et al., 1994; Jung et al., 1997). ADL may not be considered as chemically defined lignin, but it defines the indigestible part of the cell wall in terms of fermentability (Lowry et al., 1994). The pitfalls of the analysis method used in the current study should be considered during interpretation of the results. Comparisons within substrates are valid, since they are based on the same analytical method. Chemical composition of the substrates on each duration of treatment was expressed as a percentage of the DM (Tables 3.1 and 3.2). This is the most common manner to present data of fungal treated biomass (Zadrazil, 1977; Karunanandaa et al., 1992; Bisaria et al., 1997; Jalč et al., 1998; Okano et al., 2005; Arora and Sharma, 2009; Osono, 2010; Salvachúa et al., 2011; Shrivastava et al., 2011; Tuyen et al., 2012; Tuyen et al., 2013). In this way, enrichment in or reduction of components due to the preference of fungi for degradation of certain components can be determined. In addition, nutrient concentration data are valuable information in animal nutrition to ascertain the nutritional value of a feed ingredient. However, for a better understanding of the processes occurring during fungal treatment, and to see the actual components which have been degraded, absolute values using DM loss data are more informative. The importance of absolute values can be demonstrated by the changes observed in cellulose. The absolute amount of cellulose cannot increase upon fungal treatment, as demonstrated in absolute values. Nevertheless, the concentration of cellulose increased due to degradation of other components in the substrates. The degradation of components that contributes most to the DM loss can be calculated by adding absolute

values up in a mass balance. The absolute amounts of crude protein and ash, however, do not change, since fungi are not able to fixate nitrogen from the air (Millbank, 1969) and minerals remain within the substrate. The only exception is a change ($P < 0.05$) in absolute amounts of ash in miscanthus and the cause for this change is not clear. Expression of results in absolute amounts indicated that lignin degradation by these fungi in wheat straw and miscanthus is not as selective as the data on concentrations suggest, meaning that not only lignin but also cellulose is degraded by the fungi. Salvachua et al. (2011), Jalč et al. (1998) and Shrivastava et al. (2011) described the simultaneous degradation of lignin and cellulose on wheat straw by *P. ostreatus*. Both in the present study and the study of Tuyen et al. (2012), *P. ostreatus* was found to be a selective lignin degrader on wheat straw. Again the differences may be due to different batches or cultivar of wheat straw, fungal strains, duration of treatment, inoculum type and inoculum:substrate ratios, but also ways of expressing data. The absolute amount of ADL and ash in miscanthus increased after 2 weeks treatment with *L. edodes* (Table 3.2). It is unlikely that *L. edodes* produces compounds that contribute to the ADL and ash fractions. It is unclear why the ADL absolute amount in increasing after 2 weeks, but it might be due to an incorrect measurement of the dry matter content of that particular fraction. The decrease in ADL concentration of wheat straw was 47.9 g/kg DM (which corresponds with 59%) after 8 weeks, which is higher than observed (47%) by Tuyen et al. (2012) after 7 weeks of treatment. This does not correspond to the results of the IVGP after 8 weeks of *L. edodes* treatment, since only an increase of 35 ml/g OM was found, while Tuyen et al. (2012) found an increase of approximately 70 ml/g OM compared to the control. The difference might be explained by the larger amount of hemicellulose degraded in the current study compared to Tuyen et al. (2012). In contrast, literature states that cellulose is better digestible than hemicellulose, explaining the highest IVGP in the sample with the lowest hemicellulose concentration in the *L. edodes* treated material (Sullivan, 1966; Keys et al., 1969). In the current study, the correlations found between ADL and IVGP indicates that lignin (here measured as ADL) is limiting the access of rumen microbes to degrade the substrates. A negative correlation between the concentration of lignin and dry matter digestibility has been described before by Arora and Sharma (2009). The way lignin concentration is measured does not allow the assessment to what extent linkages between lignin and carbohydrates are affected by fungi or how lignin is modified without changing its solubility. Both processes might also have an effect on the accessibility of carbohydrates (Rollin et al., 2011).

3.5 Conclusions

L. edodes was the most selective lignin degrading fungus of the four tested and showed the greatest improvement in IVGP and chemical changes on wheat straw, miscanthus and wood chips. Although nutrient concentration data indicated an increased cellulose concentration, mass balance measurements showed degradation of cellulose. All fungi grew on rice straw, but did not improve IVGP. Twelve weeks were required for the greatest IVGP improvement of wheat straw, miscanthus and wood chips. *L. edodes*

growth was positively correlated to lignin degradation and DM loss, although this correlation differed per substrate. Lignin degradation by *L. edodes* was correlated to increasing IVGP.

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CHAPTER 4

Preferential lignin degradation in wheat straw and wood chips by the white-rot fungus *Lentinula edodes* and its influence on *in vitro* rumen degradability

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Submitted

Abstract

The present work investigated the influence of the lignin content and composition in fungal treatment to improve rumen degradability. Wheat straw and wood chips, differing in lignin composition, were treated with *Lentinula edodes* for 0, 2, 4, 8 and 12 weeks and analyzed for lignocellulose composition using pyrolysis-gas chromatography-mass spectrometry and detergent fiber analysis. *L. edodes* preferentially degraded lignin in wheat straw and wood chips, leaving a substrate enriched in cellulose. More syringyl (S)-units than guaiacyl (G)-units were degraded in lignin, resulting in a decreased S/G ratio. A decreasing S/G ratio (wheat straw: r=-0.72, wood chips: r=-0.75) and selective lignin degradation (wheat straw: r=-0.69, wood chips: r=-0.88) were correlated with *in vitro* gas production (IVGP), a good indicator for rumen degradability. Effects on IVGP were similar for wheat straw and wood chips indicating that lignin content and 3D-structure of cell walls influence *in vitro* rumen degradability more than lignin composition.

4.1 Introduction

Carbohydrates in plant cell walls can be an important source of nutrients for ruminants. However, these carbohydrates are bound to lignin, which can be degraded only under aerobic conditions by fungi and some bacteria (Ahmad et al., 2010), and as such cannot be broken down in the low oxygen environment of the rumen. Currently, several chemical and physical pre-treatments are used to make the carbohydrates in lignocellulosic substrates more available for degradation in the rumen (Sarnklong et al., 2010; van Kuijk et al., 2015). Biological treatments using fungi that selectively degrade lignin may be more cost effective and less harmful for animals and the environment compared to current pre-treatment methods. Studies reported in the scientific literature describe treatments by different fungi to pre-treat various substrates suitable as ruminant feed ingredient (van Kuijk et al., 2015). Among them, the white rot fungus *Lentinula edodes* was found to be highly promising due to its selective lignin degradation pattern (Tuyen et al., 2012; 2013). In addition, this fungus has a 'generally regarded as safe' (GRAS) status and is, therefore, a potentially suitable fungus for fungal pre-treatment of feed ingredient (Tuyen et al., 2012). However, due to the GRAS status of the fungus and its edible mushrooms, hitherto the majority of research has focused on mushroom production (Permana et al., 2000; Gaitán-Hernández et al., 2006; Lin et al., 2015).

Scientific studies describing *L. edodes* treatment of lignocellulosic biomass to increase rumen degradability often report changes in lignin content as measured by the Van Soest acid detergent lignin (ADL) methodology (Van Soest et al., 1991; Tuyen et al., 2012; 2013; van Kuijk et al., 2015). However, ADL does not represent the total lignin content, since acid soluble lignin is not included in the ADL fraction leading to an underestimation (Jung, 1997; Godin et al., 2014). Lignin is a complex aromatic polymer produced by the oxidative coupling of three main monolignols, sinapyl, coniferyl and *p*-coumaryl alcohols, differing in their degree of methoxylation (Ralph et al., 2004). When incorporated into the lignin polymer, these monolignols give rise to the syringyl (S), guaiacyl (G) and *p*-hydroxyphenyl (H) units, respectively, generating a variety of structures and linkages within the polymer, including β–*O*–4′ alkyl-aryl ethers, phenylcoumarans and resinols, amongst others. Lignin composition is different for each plant species, for example, lignin in grasses and herbaceous plants consists of S-, G-, and H-units, whereas softwoods present mainly G-lignin units and hardwoods present S- and G-units in different proportions (Vanholme et al., 2010). Lignin composition can be measured using pyrolysis coupled to gas chromatography-mass spectrometry (Py-GC/MS) (del Río et al., 2001). During pyrolysis the plant material is heated at high temperatures (usually around 500-700 ºC) in an oxygen-free environment to break down the macromolecular components of plant cell walls to smaller compounds, which are subsequently analyzed in a GC/MS system. As such, Py-GC/MS is a useful tool to monitor the extent of fungal degradation of lignocellulosic constituents, which cannot be straight-forwardly detected with the standard gravimetric methods, such as the detergent fiber method (Faix et al., 1991; del Río et al., 2001; del Río et al., 2002).

In this study it is hypothesized that in addition to the total lignin content, the lignin composition (ratios between S-, G-, and H-units) and the 3D-structure formed by lignin and carbohydrates in plant cell walls also determine the efficiency of the fungal treatment to improve rumen degradability. In this paper, two substrates differing in lignin composition (wheat straw and wood chips) were treated with *L. edodes* and analyzed for cell wall components using the detergent fiber method, and lignin composition using Py-GC/MS. Rumen degradability of treated substrates was measured by the *in vitro* gas production technique.

4.2 Material and methods

4.2.1 Fungal strains and spawn preparation

Lentinula edodes (strain MES 11910) was cultured on malt extract agar plates at 24 °C until most of the plate surface was covered with mycelium. Pieces of colonized agar culture (~1 cm^2) were added to sterilized sorghum grains and incubated at 24 °C until all grains were colonized by mycelium. This spawn was kept at 4 °C until further use.

4.2.2 Substrate preparation

The substrates used were wheat straw and municipal trimmings consisting of a mixture of chips from different wood species. Both substrates were chopped into pieces of approximately 3 cm length and submerged in tap water for 3 d at room temperature to allow moisture to fully penetrate. After removal of excess water, substrates were autoclaved twice with the first sterilization performed in autoclavable bags at 121 °C for 1 h. After cooling, the material was weighed into 1.2 l polypropylene containers fitted with a cover containing a filter allowing gas exchange, but preventing contamination (model TP1200+TPD1200 XXL Combiness, Nazareth, Belgium). Each container was filled with approximately 80-90 g dry matter of wheat straw or wood chips before being autoclaved a second time at 121 °C for 1 hour to kill the remaining germinated spores in the substrates. After cooling, the sterile substrates were kept in the container at room temperature until further use. Three of these autoclaved containers were used as an uninoculated control (0 weeks treatment).

4.2.3 Substrate inoculation

To each remaining containers, approximately 8-10 g of spawn (sorghum grains colonized with a pure culture of *L. edodes*) was added and mixed to distribute the spawn equally over the substrate. Both handlings were performed aseptically. Each container was then incubated for 2, 4, 8 or 12 weeks at 24 °C and 70% relative humidity in a climate controlled chamber. All conditions were tested in triplicate.

After incubation, the substrate was air-dried at 70 °C until constant weight. The dried wheat straw was ground with a Peppink 100 AN cross beater mill (Peppink, Deventer, The Netherlands) over a 1 mm sieve. The dried wood chips were first coarsely ground over a 1 mm sieve using a Retch SM2000 cutting mill (Retch, Haan, Germany) before

being ground over a 1 mm sieve using a Retch ZM 100 centrifugal mill (Retch, Haan, Germany). Samples were stored at 4 ºC until chemical analyses.

4.2.4 Fiber analysis

Samples were analyzed according to the Van Soest method (Van Soest et al., 1991). The hemicellulose content was calculated as the difference between the neutral detergent fiber (NDF) and the acid detergent fiber (ADF). The lignin content was determined as the 'acid detergent lignin' (ADL) content, that was defined as the part of the cell wall that is not soluble in acid detergent reagent and 72% sulphuric acid. The cellulose content was calculated as the difference between ADF and ADL. For dry matter (DM) determination, air-dried material was dried at 103 °C for 4 h. Ash content was determined by combustion for 3 hours at 550 °C in a muffle furnace. The data for three replicate samples were averaged and expressed as g kg^{-1} DM.

4.2.5 Pyrolysis-GC/MS

Pyrolysis-GC/MS (approximately 1 mg) was performed with a 3030 micro-furnace pyrolyzer (Frontier Laboratories Ltd.) connected to an Agilent 7820A GC using a DB-1701 fused-silica capillary column (60 m x 0.25 mm, 0.25 μm film thickness) and an Agilent 5975 mass selective detector (EI at 70 eV). The pyrolysis was performed at 500 ºC. The oven temperature of the gas chromatograph was programmed from 100 ºC (4 minutes) to 280 ºC (2 minutes) at 3 ºC $minute^{-1}$. Helium was the carrier gas (1 ml $minute^{-1}$). The compounds were identified by comparing their mass spectra with those of the Wiley and NIST libraries and those reported in literature (Faix et al., 1990; Ralph and Hatfield, 1991). Peak areas corrected for molecular weight were calculated for the carbohydrate and lignin-degradation products, the summed areas were normalized, and the data for three replicate samples were averaged and expressed as percentages.

4.2.6 *In vitro* gas production technique

The *in vitro* gas production (IVGP) technique was performed according to the procedure previously described (Cone et al., 1996). In short, rumen fluid of 2 fistulated non-lactating cows fed a grass silage based diet was mixed with a buffer solution under anaerobic conditions. Air dried samples (500 mg) were incubated in 60 ml buffered rumen fluid (final dilution 3 times) for 72 hours at 39 °C. The gas production was automatically recorded as previously described (Cone et al., 1996), and the data for three replicate samples were averaged and expressed as ml gas g^{-1} organic matter (OM).

4.2.7 Statistical analysis

A generalized linear model (GLM) analysis in SAS 9.3 was used to compare fiber composition and IVGP of the fungal treatment at each incubation time to the autoclaved, uninoculated control of each substrate. The following model was used:

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

in which Y_{ij} is the observation *j* in treatment *i*; μ is the overall mean; α_i is the fixed effect of treatment *i*; ω_{ij} is the random error. Post-hoc multiple comparison with Tukey's significant test at a level of $\alpha = 0.05$ was performed to determine the significance between the treatments.

Regression analysis between IVGP and fiber composition of fungal treated substrates was analyzed in SAS 9.3.

The correlation between IVGP and pyrolysis GC/MS data was analyzed in SAS 9.3. The correlations are provided as the Pearson correlation coefficient (r).

4.3 Results

4.3.1 Composition of wheat straw and wood chips during fungal treatment

The ADL, hemicellulose and cellulose content of wheat straw and wood chips before and after *L. edodes* treatment for 2, 4, 8 and 12 weeks is shown in Table 4.1. Untreated wheat straw had a lower ADL, higher hemicellulose and similar cellulose content compared to untreated wood chips. Upon *L. edodes* treatment, the content of ADL and hemicellulose of wheat straw decreased ($P<0.05$), while the cellulose content increased

Table 4.1 Chemical composition of autoclaved wheat straw and wood chips before and after treatment with *L. edodes* for 2, 4, 8 and 12 weeks.

Substrate	Treatment time (weeks)	Contents (g/kg DM)			Amounts (g)			DM loss (%)	IVGP (ml/g OM)
		ADL	HC	Cell	ADL	HC	Cell		
Wheat straw	0	81.1^{a}	260.1^{a}	479.6^{bc}	7.5^{a}	24.2^{a}	44.6	0^{c}	252.8^{ab}
	2	74.6^{b}	202.7^{b}	461.2^{c}	6.4^{b}	17.4^{b}	39.5	8^{c}	247.8^{b}
	4	57.0^{c}	148.1^{c}	496.3^{b}	4.7^{c}	12.2^{c}	41.0	11^{bc}	277.0^{ab}
	8	33.2^{d}	94.6^{d}	537.3^{a}	2.4^{d}	6.9^{d}	39.1	22^{b}	287.3^{ab}
	12	15.5^{e}	87.2^{d}	544.2^{a}	1.0^{e}	5.7^{d}	35.6	30^{a}	311.2^{a}
	RMSE	2.22	11.48	11.80	0.29	1.22	3.21	0.05	22.40
	P-value	<0.01	<0.01	<0.01	<0.01	<0.01	0.07	<0.01	0.03
Wood chips	0	198.2^{a}	140.8^{a}	445.5^{c}	15.4^{a}	11.0^{a}	35.3	0	54.0^{c}
	2	187.3^{ab}	96.3^{ab}	486.2^{b}	15.1^{a}	7.8^{ab}	39.2	0	55.4^{c}
	4	163.8^{b}	103.6^{ab}	497.4^{ab}	10.1^{b}	6.8^{ab}	31.1	20	120.7^{b}
	8	126.6^{c}	79.9^{b}	520.6^{a}	8.8^{b}	5.6^{b}	36.1	11	169.6^{a}
	12	107.0^{c}	105.6^{ab}	436.5^{c}	6.9^{b}	6.8^{ab}	28.5	17	177.4^{a}
	RMSE	9.49	16.97	11.59	1.44	1.81	4.53	1.2	16.12
	P-value	<0.01	0.02	<0.01	<0.01	0.04	0.10	0.16	<0.01

Values with different superscripts within column are significantly ($P<0.05$) different. ADL, acid detergent lignin; HC, hemicellulose; Cell, cellulose; DM loss, dry matter loss; IVGP, *in vitro* gas production; RMSE, root-mean-square error.

(P<0.05) compared to the untreated control. Expressed in absolute amounts, ADL decreased (P<0.05) up to 87%, hemicellulose (P<0.05) up to 77% and cellulose was degraded up to 20%, but this was not significant (P=0.07) (Table 4.1). The amount of dry matter significantly decreased by 30% after 12 weeks. Fungal treatment of wood chips also resulted in a decrease (P<0.05) in the ADL content during the 12 weeks of incubation. No significant decrease in hemicellulose and cellulose content was observed after 12 weeks incubation compared to the autoclaved control. The same applied to the absolute amounts of each compound, only ADL decreased significantly (P<0.05) over time during *L. edodes* treatment of wood chips compared to the uninoculated control.

4.3.2 *In vitro* gas production (IVGP) of fungal treated samples

IVGP of wheat straw increased (P=0.058) from 252.8 ml g^{-1} OM in the untreated control sample (0 weeks treatment) to 311.2 ml g^{-1} OM after 12 weeks of *L. edodes* treatment, a 23% increase (Table 4.1). For fungal treated wood chips, the IVGP of the uninoculated control (0 weeks treatment) was 54.0 ml g^{-1} OM and a significant increase (P<0.05) was already seen after 4 weeks treatment, and continued to increase during the 12 weeks treatment up to a value of 177.4 ml g^{-1} OM, representing a nearly 230% increase compared to the untreated sample (Table 4.1).

Regression analysis between IVGP (ml g OM^{-1}) and cell wall composition (g kg^{-1}) yielded the following equation:

$$IVGP = 0.26 \times hemicellulose + 0.33 \times cellulose - 1.34 \times ADL + 135.86$$

This equation indicates that changes in ADL have more effect on IVGP than hemicellulose and cellulose. The large influence of ADL on IVGP is the reason to study lignin in more detail.

4.3.3 Py-GC/MS analyses of uninoculated and fungal treated substrates

The pyrograms of wheat straw and wood chips during fungal treatment with *L. edodes* are shown in Figures 4.1 and 4.2. The identities and relative abundances (mean average of three replicates) of the compounds released are provided in Table 4.2 (wheat straw) and Table 4.3 (wood chips). The pyrolysis of untreated wheat straw (Figure 4.1a) and wood chips (Figure 4.2a) released a similar set of compounds derived from the carbohydrate and lignin moieties, although in different proportions (Table 4.2 and 4.3).

The relative abundances of lignin-derived phenols decreased with incubation time of wheat straw and wood chips with *L. edodes*. In the case of wheat straw degraded by *L. edodes* (Figure 4.1, Table 4.2), the percentage of compounds released that were derived from carbohydrates upon Py-GC/MS varied from 59.6% in the control sample (0 weeks

Table 4.2 Identities and relative abundance (mean average of three replicates) of the compounds released upon pyrolysis GC/MS of autoclaved wheat straw before and after treatment with *L. edodes* for 2, 4, 8 and 12 weeks.

Label	Compound	Origin[a]	Time (weeks)				
			0	2	4	8	12
1	(*2H*)-furan-3-one	C	3.4	4.1	4.4	4.9	5.2
2	Propanal	C	6.1	5.4	4.4	4.7	6.7
3	Furfural	C	6.0	10.1	10.4	11.2	9.7
4	2,3-dihydro-5-methylfuran-2-one	C	6.0	5.4	5.1	5.4	7.0
5	(*5H*)-furan-2-one	C	4.6	3.8	3.9	3.8	4.9
6	4-hydroxy-5,6-dihydro-(*2H*)-pyran-2-one	C	7.9	10.9	10.8	9.1	6.5
7	2-hydroxy-3-methyl-2-cyclopenten-1-one	C	3.4	3.0	3.8	5.4	5.9
8	Phenol	LH	1.1	0.9	1.0	1.1	1.0
9	Guaiacol	LG	3.4	2.5	2.4	2.2	1.6
10	3-hydroxy-2-methyl-(*4H*)-pyran-4-one	C	1.0	1.1	1.7	2.5	2.7
11	4-hydroxymethyl-1,4-butyrolactone	C	2.9	3.1	2.7	2.8	3.9
12	4-methylguaiacol	LG	1.3	1.0	0.7	0.5	0.3
13	4-ethylguaiacol	LG	0.4	0.3	0.2	0.1	0.1
14	5-hydroxymethyl-2-tetrahydrofuraldehyde-3-one	C	1.7	1.5	2.4	2.8	3.0
15	1,4-anhydroarabinofuranose	C	1.7	1.7	1.9	1.6	1.3
16	4-vinylphenol	LH/PCA	9.3	6.3	3.1	2.2	0.8
17	4-vinylguaiacol	LG/FA	8.3	5.3	2.9	2.1	0.8
18	Eugenol	LG	0.4	0.2	0.2	0.1	0.0
19	5-hydroxymethyl-2-furfuraldehyde	C	1.7	3.3	3.6	3.2	4.7
20	Syringol	LS	2.9	2.1	1.7	0.8	0.5
21	*cis*-isoeugenol	LG	0.2	0.1	0.1	0.1	0.0
22	1,4-dideoxy-D-glycerohex-1-enopyranos-3-ulose	C	0.8	1.0	1.4	2.0	1.7
23	*trans*-isoeugenol	LG	1.5	0.9	0.6	0.4	0.2
24	1,4-anhydroxylofuranose	C	2.4	2.1	3.5	2.8	2.4
25	4-methylsyringol	LS	1.4	1.2	0.7	0.2	0.1
26	Vanillin	LG	1.8	1.0	1.0	0.6	0.3
27	4-ethylsyringol	LS	0.2	0.1	0.1	0.0	0.0
28	vanillic acid methyl ester	LG	0.1	0.3	0.2	0.1	0.1
29	acetovanillone	LG	0.4	0.5	0.5	0.5	0.3
30	4-vinylsyringol	LS	2.2	1.2	0.7	0.3	0.2
31	guaiacylacetone	LS	0.4	0.3	0.2	0.1	0.1
32	4-allyl-syringol	LS	0.6	0.3	0.1	0.1	0.0
33	propiovanillone	LG	0.1	0.1	0.1	0.1	0.0
34	*cis*-4-propenylsyringol	LS	0.4	0.3	0.1	0.1	0.0
35	*trans*-4-propenylsyringol	LS	2.0	1.5	0.4	0.3	0.1
36	levoglucosane	C	10.0	15.6	21.6	25.1	27.3
37	syringaldehyde	LS	0.9	0.5	0.4	0.2	0.1
38	syringic acid methyl ester	LS	0.1	0.2	0.2	0.1	0.1
39	acetosyringone	LS	0.6	0.6	0.4	0.2	0.1
40	syringylacetone	LS	0.3	0.3	0.2	0.1	0.0
41	propiosyringone	LS	0.1	0.1	0.0	0.0	0.0
	% Lignin		40.4	27.9	18.1	12.6	7.1
	% Carbohydrates		59.6	72.1	81.9	87.4	92.9
	Lignin/Carbohydrate ratio		0.7	0.4	0.2	0.1	0.1
	H		10	7	4	3	2
	G		18	12	9	7	4
	S		12	8	5	3	1
	Syringyl/Guaiacyl ratio		0.7	0.7	0.5	0.4	0.4
	$(Syringyl/Guaiacyl)_{except\ vinyl}$ ratio[b]		1.0	1.0	0.7	0.5	0.4
	Ph-C0-2/Ph-C3[c]		5.7	6.0	8.2	9.5	10.9
	% Cα-oxidized lignin		10.2	11.5	15.2	15.2	16.4
	% Cα-oxidized G-units		5.9	6.5	9.4	10.4	11.3
	% Cα-oxidized S-units		4.4	4.9	5.9	4.8	5.1

[a]C, carbohydrate-derived compounds; LH, *p*-hydroxycinnamyl lignin-derived compounds; LG, guaiacyl-lignin derived compounds; S, syringyl-lignin derived compounds; PCA, *p*-coumarates; FA: ferulates. [b]All G- and S-derived peaks were used for the estimation of the S/G ratio, except 4-vinylguaiacol (which also arises from ferulates), and the analogous 4-vinylsyringol.[c]Ratio of lignin-derived phenols with none, 1 and 2 carbons in the side-chain to lignin-derived phenols with 3 carbons in the side-chain.

Table 4.3 Identities and relative abundance (mean average of three replicates) of the compounds released upon pyrolysis GC/MS of autoclaved wood chips before and after treatment with *L. edodes* for 2, 4, 8 and 12 weeks.

Label	Compound	Origin[a]	Time (weeks)				
			0	2	4	8	12
1	(*2H*)-furan-3-one	C	2.7	3.2	3.4	3.9	4.0
2	propanal	C	2.5	2.1	2.2	2.2	2.2
3	Furfural	C	6.1	8.3	8.6	8.8	9.2
4	2,3-dihydro-5-methylfuran-2-one	C	4.0	3.4	3.5	4.1	3.8
5	(*5H*)-furan-2-one	C	2.6	2.7	2.5	2.7	2.4
6	4-hydroxy-5,6-dihydro-(*2H*)-pyran-2-one	C	5.5	7.8	7.3	7.1	7.4
7	2-hydroxy-3-methyl-2-cyclopenten-1-one	C	4.3	3.3	3.7	4.3	3.9
8	Phenol	LH	1.8	1.8	1.8	1.6	1.5
9	Guaiacol	LG	4.2	3.9	4.3	4.5	4.9
10	3-hydroxy-2-methyl-(*4H*)-pyran-4-one	C	1.3	1.0	1.1	1.4	1.4
11	4-hydroxymethyl-1,4-butyrolactone	C	1.1	1.0	1.0	0.7	0.8
12	4-methylguaiacol	LG	4.9	3.6	3.3	2.3	2.3
13	4-ethylguaiacol	LG	0.8	0.4	0.4	0.4	0.2
14	5-hydroxymethyl-2-tetrahydrofuraldehyde-3-one	C	1.7	1.8	2.1	2.6	2.9
15	1,4-anhydroarabinofuranose	C	0.9	0.8	0.7	0.8	0.7
16	4-vinylphenol	LH	1.0	1.0	0.7	0.7	0.6
17	4-vinylguaiacol	LG	5.8	4.9	4.4	3.2	2.5
18	Eugenol	LG	1.3	0.9	0.8	0.6	0.5
19	5-hydroxymethyl-2-furfuraldehyde	C	1.6	2.5	2.4	2.7	3.0
20	Syringol	LS	3.8	3.3	2.8	2.4	1.9
21	*cis*-isoeugenol	LG	0.9	0.6	0.5	0.3	0.2
22	1,4-dideoxy-D-glycerohex-1-enopyranos-3-ulose	C	0.4	1.3	1.3	1.3	1.6
23	*trans*-isoeugenol	LG	4.4	2.7	2.3	1.6	1.3
24	1,4-anhydroxylofuranose	C	0.8	1.2	1.1	1.3	1.4
25	4-methylsyringol	LS	3.2	2.6	1.9	1.4	1.0
26	Vanillin	LG	1.9	2.0	1.7	1.4	1.5
27	4-ethylsyringol	LS	0.6	0.4	0.3	0.2	0.1
28	vanillic acid methyl ester	LG	0.3	0.5	0.6	0.7	0.8
29	acetovanillone	LG	1.0	1.0	1.0	0.9	0.9
30	4-vinylsyringol	LS	4.4	3.5	2.4	1.8	1.3
31	guaiacylacetone	LS	0.7	0.6	0.6	0.4	0.4
32	4-allyl-syringol	LS	1.3	0.9	0.6	0.5	0.3
33	propiovanillone	LG	0.2	0.2	0.6	0.5	0.3
34	*cis*-4-propenylsyringol	LS	0.8	0.7	0.5	0.3	0.6
35	*trans*-4-propenylsyringol	LS	5.2	3.6	2.5	1.9	1.6
36	levoglucosane	C	12.6	17.0	22.2	26.3	28.5
37	syringaldehyde	LS	1.8	1.7	1.1	0.8	0.7
38	syringic acid methyl ester	LS	0.1	0.2	0.2	0.3	0.3
39	acetosyringone	LS	0.9	0.9	0.7	0.6	0.5
40	syringylacetone	LS	0.6	0.7	0.6	0.5	0.4
41	propiosyringone	LS	0.3	0.2	0.2	0.1	0.1
	% Lignin		52.1	42.7	36.6	29.9	26.9
	% Carbohydrates		47.9	57.3	63.4	70.1	73.1
	Lignin/Carbohydrate ratio		1.1	0.7	0.6	0.4	0.4
	H		3	3	3	2	2
	G		26	21	20	17	16
	S		23	19	14	11	9
	Syringyl/Guaiacyl ratio		0.9	0.9	0.7	0.7	0.6
	Ph-C0-2/Ph-C3[b]		2.3	2.8	3.1	3.5	3.6
	% Cα-oxidized lignin		12.3	15.7	16.5	17.4	19.2
	% Cα-oxidized G-units		6.4	8.5	10.7	11.3	13.0
	% Cα-oxidized S-units		5.9	7.2	5.8	6.1	6.2

[a]C, carbohydrate-derived compounds; LH, *p*-hydroxycinnamyl lignin-derived compounds; LG, guaiacyl-lignin derived compounds; S, syringyl-lignin derived compounds. [b] Ratio of lignin-derived phenols with none, 1 and 2 carbons in the side-chain to lignin-derived phenols with 3 carbons in the side-chain.

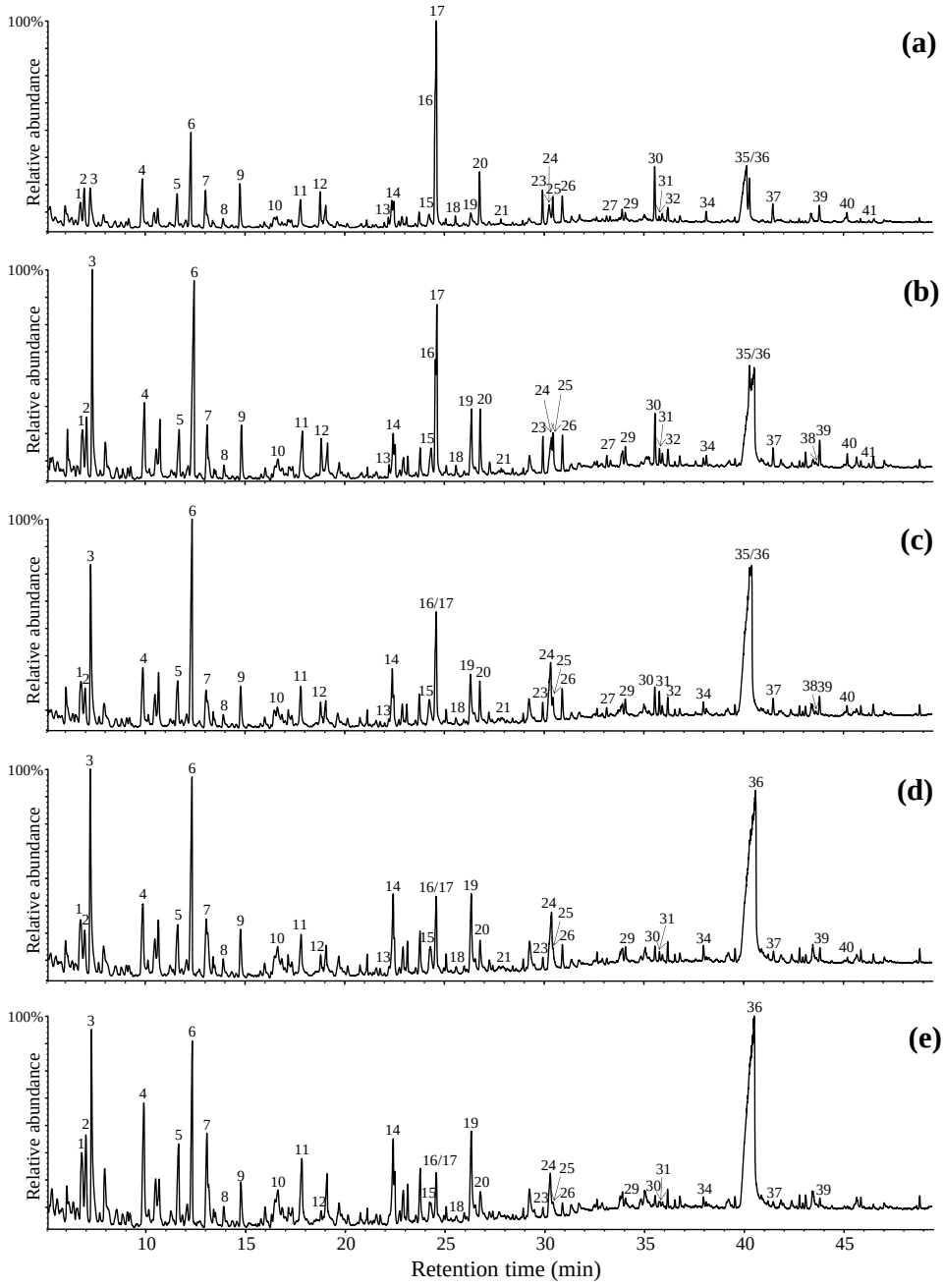

Figure 4.1 Py-GC/MS chromatograms of wheat straw degraded with the fungus *Lentinula edodes*. (a) untreated wheat straw control (0-weeks incubation); (b) wheat straw degraded for 2 weeks; (c) 4 weeks; (d) 8 weeks; (e) 12 weeks. The identities and relative abundances of the compounds represented by the numbered peaks are listed in Table 4.2.

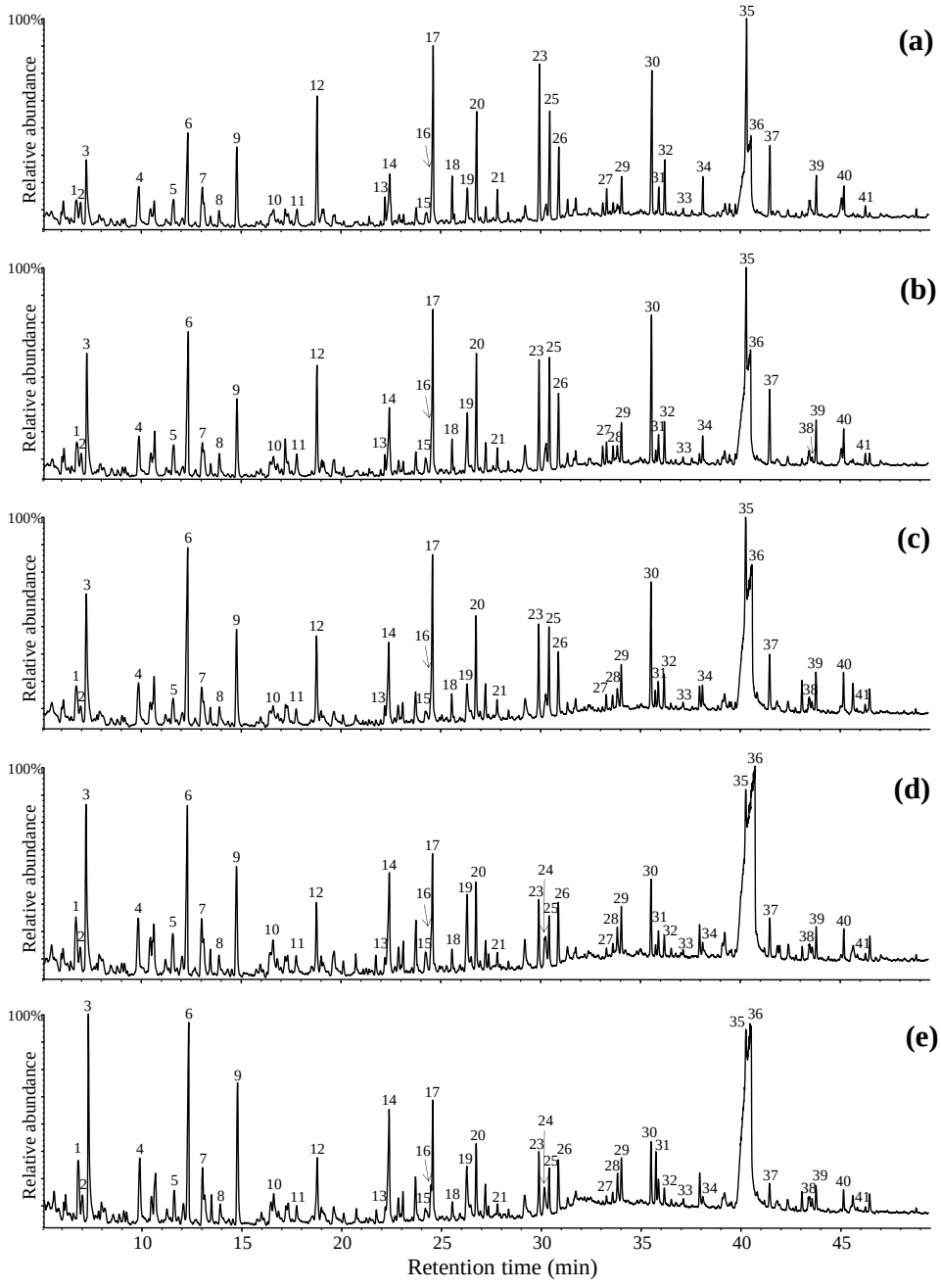

Figure 4.2 Py-GC/MS chromatograms of wood chips degraded with the fungus *Lentinula edodes*. (a) untreated wood chips (0-weeks incubation); (b) wood chips degraded for 2 weeks; (c) 4 weeks; (d) 8 weeks; (e) 12 weeks. The identities and relative abundances of the compounds represented by the numbered peaks are listed in Table 4.3.

incubation time) to 92.9% after 12 weeks of incubation, while the lignin-derived phenols (H+G+S) varied from 40.4% in the control sample to only 7.1% after 12 weeks of incubation. In the case of wood chips incubated with *L. edodes* (Figure 4.2, Table 4.3), the percentage of carbohydrate-derived compounds released upon Py-GC/MS varied from 47.9% in the control sample (0 weeks incubation time) to 73.1% after 12 weeks of incubation, while the lignin-derived phenols varied from 52.1% in the control sample to 26.9% after 12 weeks of incubation. The lignin/carbohydrate (L/C) ratio estimated upon Py-GC/MS decreased from 0.7 in the wheat straw control sample (0 weeks incubation time) to 0.1 in the wheat straw degraded for 12 weeks (Table 4.2), and from 1.1 in the wood chips control sample (0 weeks incubation time) to 0.4 for the wood chips degraded for 12 weeks (Table 4.3).

Among the lignin-derived phenols, the pyrograms of wheat straw show compounds derived from *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) lignin units, whereas lignin in wood chips contained mainly G- and S-units (Tables 4.2 and 4.3). For wheat straw, the ratio between S- and G-units (S/G ratio) gradually decreases with incubation time from 0.7 in the control sample (0 weeks incubation time) to a final value of 0.4 after 12 weeks (Table 4.2). However, 4-vinyl-guaiacol (compound **17**) may also arise from ferulates on arabinoxylans and, therefore, the lignin S/G ratio may be underestimated (del Río et al., 2012a). Hence, a more accurate S/G ratio of the lignin in wheat straw was obtained by ignoring 4-vinylguaiacol (compound **17**) (and the analogous 4-vinylsyringol, compound **30**). This S/G ratio estimated a value of 1.0 in the untreated wheat straw and shows a continuous decrease until a value of 0.4 after 12 weeks of incubation (Table 4.2). Likewise, in the case of wood chips, a decrease of the lignin S/G ratio was also observed during fungal incubation time. The S/G ratio estimated by Py–GC/MS for the untreated wood chips sample was 0.9, and decreased from 4 weeks on steadily during incubation down to a value of 0.6 in the wood chips incubated for 12 weeks (Table 4.3). The S/G ratio in wood chips was not corrected for 4-vinylguaiacol and 4-vinylsyringol, since ferulates are abundant in grasses, but not important in wood (del Río et al., 2012a).

The Ph-C0-2/Ph-C3 ratio represents the ratio between lignin units in which side chain degradation occurred (Ph-C0-2) and the original lignin units (Ph-C3). The Ph-C0-2/Ph-C3 ratio in wheat straw increased from 5.7 in the untreated sample up to 10.9 in the 12-weeks treated sample. In wood chips the Ph-C0-2/Ph-C3 ratio increased from 2.3 in the untreated sample up to 3.6 in the 12-weeks treated sample. As seen in Figure 4.3, Ph-C3 compounds originating from both S- and G-units decreased at a similar rate while Ph-C0-2 compounds originating from S-units (compounds **20**, **25**, **27**, **30**, **37**, **38** and **39**) are degraded faster than Ph-C0-2 compounds originating from G-units (compounds **9**, **12**, **13**, **16**, **26**, **28** and **29**). As a result, more Ph-C0-2 compounds originating from G-units than from S-units were found in both the fungal degraded wheat straw and wood chips, in accordance with the fact that *L. edodes* degraded more S-units than G-units.

In addition, some Cα-oxidized phenolic compounds such as aromatic aldehydes, acids and ketones were found during pyrolysis of wheat straw and wood chip samples treated

with *L. edodes* (Table 4.2 and 4.3). Among them, the relative abundance of lignin-derived compounds oxidized at the α-carbon, clearly increase after fungal treatment of wheat straw and wood chips with *L. edodes* (Tables 4.2 and 4.3). In the case of wheat straw treated with *L. edodes*, the percentage of the Cα-oxidized compounds increases continuously during fungal incubation from 10.2% in the control sample (0 weeks incubation) up to 16.4% in the wheat straw sample after 12 weeks of fungal incubation. Similarly, in the case of the wood chips treated with *L. edodes*, the percentage of Cα-oxidized compounds also increases during fungal incubation from 12.3% in the control sample (0 weeks incubation) up to 19.2% after 12 weeks of fungal degradation. Interestingly, more Cα-oxidized lignin compounds were found originating from G-lignin units (compounds **26**, **28**, **29** and **33**) than from S-lignin units (compounds **37**, **38**, **39** and **41**) in both wheat straw and wood chips, as reflected in Figure 4.4.

4.3.4 Correlations between IVGP and composition of the substrates

IVGP showed a linear relationship to changes in cell wall composition (ADL to carbohydrate (hemicellulose + cellulose) ratio) as determined by the detergent fiber analysis (Figure 4.5). This relation was similar for both wheat straw and wood chips.

The increase in IVGP of fungal treated wheat straw was negatively correlated with the L/C ratio (r=-0.69, P<0.01) determined by Py-GC/MS (Figure 4.6). S/G ratio was also negatively correlated with IVGP (r=-0.72, P<0.01), while a positive correlation was found between IVGP and the percentage of Cα-oxidized lignin compounds (r=0.77, P<0.01) and the Ph-C0-2/Ph-C3 ratio (r=0.51, P=0.05) determined by Py-GC/MS (Figure 4.6). Similar to wheat straw, IVGP of fungal treated wood chips was also negatively correlated to the L/C ratio (r=-0.88, P<0.01) and S/G ratio (r=-0.75, P<0.01) (Figure 4.6), while a positive correlation between IVGP of wood chips and Ph-C0-2/Ph-C3 ratio (r=0.77, P<0.01) and %Cα-oxidized lignin (r=0.62, P=0.01) was found (Figure 4.6).

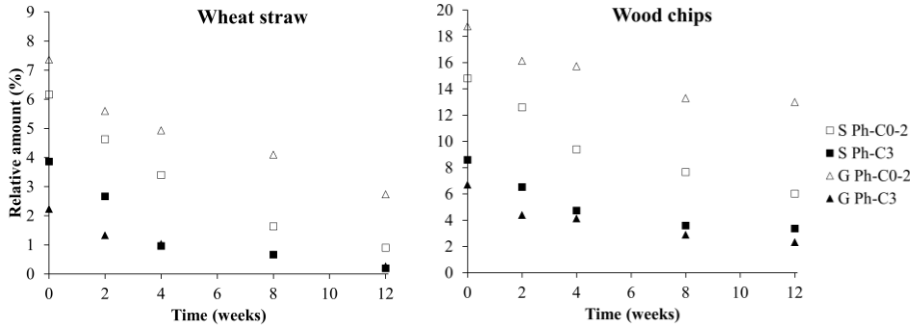

Figure 4.3 Relative amounts of Ph-C3 and Ph-C0-2 compounds originating from S- and G-units present in wheat straw and wood chips during *Lentinula edodes* treatment. □ Ph-C0-2 compounds originating from S-units ■ Ph-C3 compounds originating from S-units Δ Ph-C0-2 compounds originating from G-units ▲ Ph-C3 compounds originating from G-units.

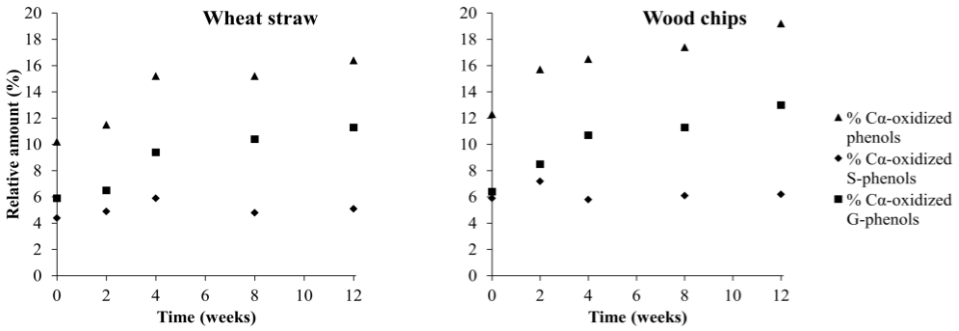

Figure 4.4 Percentage Cα-oxidized lignin units originating from S- and G-units present in wheat straw and wood chips during *Lentinula edodes* treatment. ▲total Cα-oxidized phenols □ Cα-oxidized products originating from S-units ■ Cα-oxidized products originating from G-units.

4.4 Discussion

Regression analysis showed that changes in ADL content influence changes in IVGP most. This is in line with a previous paper (Tuyen et al., 2012), that found that ADL content and IVGP were negatively correlated. ADL represents the undegradable fraction that is defined as lignin (Van Soest et al., 1991). However, it does not include acid soluble lignin, and due to a filtration step, it might not include smaller fragments of lignin (such as degradation products) (Jung, 1997; Godin et al., 2014). The detergent fiber analysis method used in

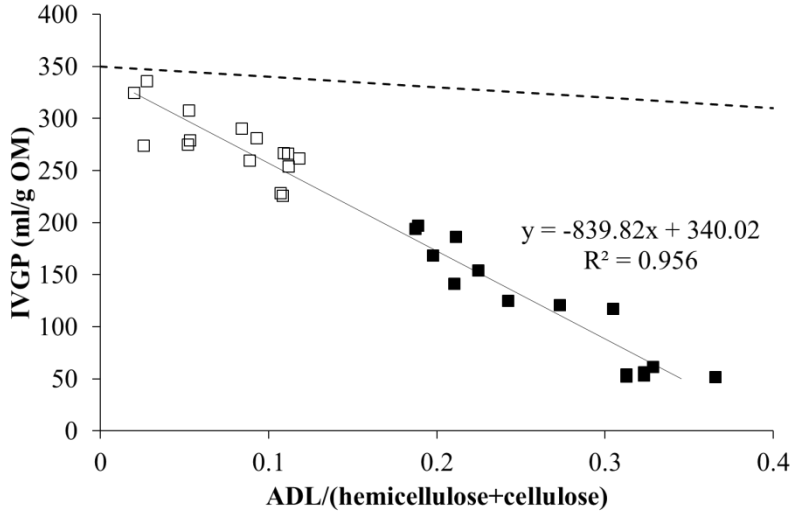

Figure 4.5 Relation between *in vitro* gas production (IVGP) and the lignin to carbohydrates ratio (lignin/hemicellulose+ cellulose) as determined by the detergent fiber analysis. □ wheat straw ■ wood chips, dashed line: potential maximum IVGP.

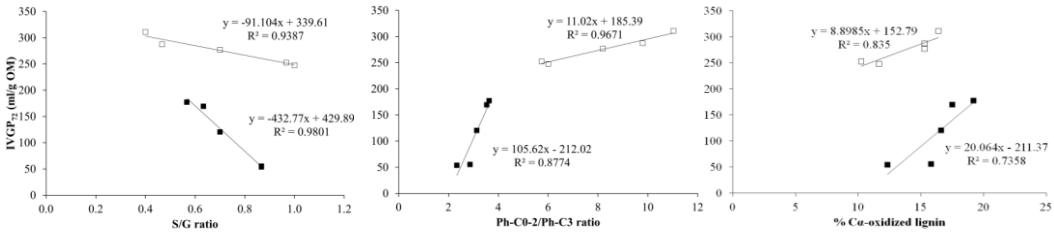

Figure 4.6 Correlations between *in vitro* gas production (IVGP) and lignin/carbohydrate ratio, S/G ratio, Ph-C0-2/Ph-C3 ratio and %Cα-oxidized lignin. □ wheat straw ■ wood chips.

the current study used filter bags with pore size of 25 micron, meaning that smaller, unbound compounds will be lost during analysis. To determine the effect of lignin composition, lignin was studied in more detail using Py-GC/MS. Preferential lignin mainly derived from cellulose indicating a relative enrichment in cellulose in both substrates, although more pronounced in wheat straw. These results confirm preferential lignin degradation found by *L. edodes* in beech and wheat straw (Faix et al., 1991; Tuyen et al., 2012). The higher lignin degradation found mainly derived from cellulose indicating a relative enrichment in cellulose in both substrates, although more pronounced in wheat straw. These results confirm preferential lignin degradation found by *L. edodes* in beech and wheat straw (Faix et al., 1991; Tuyen et al., 2012). The higher lignin degradation found in wheat straw compared to wood chips is likely due to the higher lignin content in wood (L/C ratio of 0.3) compared to wheat straw (L/C ratio of 0.1) and possibly to physical differences in the two types of substrate (such as density of tissues and the surface to content ratio). It must be noted, however, that the observed L/C ratios do not reflect the real content of each moiety since pyrolysis is known to be more sensitive for lignin as the cellulose is significantly underestimated due to intense charring and extensive degradation to non-chromatographed products (Ralph and Hatfield, 1991). However, the L/C ratios observed upon pyrolysis can still be used for comparison of the relative amounts of individual moieties of lignocellulose in the analyzed samples and visualize the direction in which compound concentrations changes. The preferential lignin degradation is confirmed by the increasing occurrence of side chain degradation products (Ph-C0-2) and Cα-oxidized lignin. Interestingly, Ph-C0-2 compounds were also detected in the untreated, autoclaved control. These compounds are generated during the pyrolysis of condensed lignin structures. Since G-units form more condensed structures, more Ph-C0-2 compounds originating from G-lignin were found (Figure 4.3).

The Py-GC/MS data showed a change in lignin composition by *L. edodes*. Two different phases in lignin composition changes by *L. edodes* can be defined. The first phase is characterized by radical attack of lignin, since lignin degrading enzymes cannot enter the intact cell walls during the initial phase of fungal delignification (Martínez et al., 2005). These radicals are likely less specific resulting in a simultaneous degradation of S- and

G-units during the first 2 to 4 weeks of *L. edodes* treatment. Cα-Cβ oxidative cleavage on the other hand does occur under the influence of both the radicals and direct enzymatic degradation (Martínez et al., 2005), resulting in an increased percentage of Cα-oxidized lignin within the first 2 weeks of *L. edodes* treatment as was also shown for other fungal treated lignocellulosic samples (del Río et al., 2001; del Río et al., 2002).

During the second phase, the final stages of the fungal treatment, lignin degradation shifts from radical degradation toward enzymatic degradation (Faix et al., 1991). With enzymatic degradation preferential S- over G-unit degradation starts after 4 weeks of *L. edodes* treatment. In wood chips, this preferential S-unit degradation is accompanied by a significant increase in IVGP. In the calculation of the S/G ratio for wheat straw, 4-vinylguaiacol (compound **17**) and 4-vinylsyringol (compound **30**) were excluded. The presence of these compounds is mostly due to the presence of *p*-coumarates and ferulates, which decarboxylate efficiently under pyrolytic conditions producing these vinyl compounds, as previously shown (del Río et al., 2012b). The decrease of 4-vinylguaiacol (compound **17**) in wheat straw suggests a degradation of ferulates or the arabinoxylans to which ferulates are bound. This result is in accordance with the hemicellulose degradation found by the detergent fiber method. The increase in the relative amounts of partly degraded G-units at a later stage of fungal degradation might indicate that S-units are degraded further and are not detected anymore, which is in accordance with the preferential S-unit degradation by *L. edodes*.

The higher biodegradability of the S-lignin compared to the G-lignin has already been shown in several other studies showing the decrease of the S/G ratio during degradation of other lignocellulosic substrates by different white-rot fungi (Faix et al., 1991; Valmaseda et al., 1991; del Río et al., 2001; del Río et al., 2002; Vane et al., 2003; Choi et al., 2006). S-lignin units are more prone to enzymatic fungal degradation because they have a higher predominance of β-*O*-4 ether linkages compared to G-units that are more recalcitrant to fungal attack due to the formation of condensed linkages (del Río et al., 2002). However, it has recently been indicated that S-rich transgenic poplar woods exhibited improved resistance to fungal degradation (Skyba et al., 2013), which suggests that besides composition of lignin, the 3D-structure of cell walls is important in degradation.

Here, the S/G ratio is correlated to IVGP (Figure 4.6), suggesting that lignin composition has an influence on rumen degradability. Changes in S/G ratio of wood chips have a larger influence on rumen degradability than changes in S/G ratio of wheat straw. A similar trend for Ph-C0-2/Ph-C3 ratio was found in wood chips and wheat straw (Figure 4.6). In the current study, with the degradation of lignin, also its structure is changing, lignin composition and content. The relatively large effect of changes in S/G ratio and Ph-C0-2/Ph-C3 ratio are related to the high lignin content of wood chips. Small changes in lignin will have a relatively large effect on accessibility of rumen microbes. However, the high linear correlation between the lignin/carbohydrate ratio and IVGP (Figure 4.5), where both substrates show an identical trend indicates that lignin content is more important than lignin composition does not have an effect on IVGP. The latter is in line

with previous works that reported no effect of lignin composition on the degradation of polysaccharides in maize cell walls by enzymes of *Trichoderma reesei* and *Aspergillus niger* or rumen microbes (Grabber et al., 1997). It is important to note that lignin degradation started during the first 2 to 4 weeks, while both S/G ratio and IVGP did not change. This suggests that lignin structure, i.e. the binding to carbohydrates, rather than lignin composition is important for rumen degradability. Access of rumen microbes to the fermentable carbohydrates is blocked by the presence of lignin bound to the carbohydrates. This is demonstrated by the theoretical potential maximum IVGP if all ADL would be removed (dashed line in Figure 4.5). The maximum IVGP will be approximately 350 ml g^{-1} OM, since removal of total ADL will result in pure carbohydrates. The maximum IVGP decreases slightly, since ADL is diluting the carbohydrates, i.e. at a higher ADL/(hemicelluloses + cellulose) ratio a lower amount of carbohydrates are present (Figure 4.6). This suggests that both the content of lignin and composition of lignin (S/G ratio) are not influencing IVGP during the first 2 to 4 weeks. Probably the linkages between lignin and carbohydrates (Grabber et al., 2009) and the 3D-structure of lignin block the rumen microbes. If this is true, removal of the linkages between lignin and carbohydrates would result in a theoretical IVGP of approximately 350 ml/g OM (dashed line in Figure 4.5). This value would decrease with an increasing lignin/carbohydrate ratio, because of the diluting effect of lignin. This theoretical IVGP is only true under the assumption that lignin is not toxic for rumen microbes.

Diverse and complex products are generated during degradation of lignin by *L. edodes*. Phenolic compounds originating from lignin such as cinnamic acid and vanillin are described to inhibit cellulose degradation by rumen microbes (Varel and Jung, 1986). However, the concurrent increase of IVGP with the increasing amounts of lignin degradation products indicates that these products do not inhibit rumen microbes, at least not enough to overcome the positive effects of lignin degradation.

The wood chips used in the current study originated from municipal trimmings consisting of a mixture of different wood species. This makes a more detailed comparison between the results found here and in the literature difficult.

In this study, changes in lignin composition were a direct result of lignin degradation since it was mainly related to the mechanisms of fungal degradation and less to substrate properties. Changes in lignin had a similar effect on IVGP of wheat straw and wood chips. It is concluded that lignin content and the 3D-structure of cell walls have a larger influence on *in vitro* rumen degradability than changes in lignin composition.

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CHAPTER 5

Characterization of wheat straw and oak wood chips treated with the white rot fungi *Ceriporiopsis subvermispora* and *Lentinula edodes*

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Abstract

Wheat straw and oak wood chips were incubated with *Ceriporiopsis subvermispora* and *Lentinula edodes* for 8 weeks. Every week, fungal treated substrates were characterized by measuring fungal biomass, measuring changes in chemical composition, visualization of treated material and investigating effects on *in vitro* rumen degradability and enzymatic saccharification.

Fungal biomass, estimated by the ergosterol content, showed *L. edodes* to continuously grow on both wheat straw and oak wood chips during 8 weeks. *C. subvermispora* colonized both substrates during the first week, stopped growing on oak wood chips, and resumed growth after 6 weeks on wheat straw. Detergent fiber analysis and pyrolysis coupled to gas chromatography/mass spectrometry (Py-GC/MS) showed a selective lignin degradation in wheat straw, although also some carbohydrates were degraded. *L. edodes* continuously degraded lignin and hemicellulose in wheat straw while *C. subvermispora* degraded lignin and hemicellulose only during the first 5 weeks of treatment after which cellulose degradation started. In contrast to *L. edodes*, *C. subvermispora* produced alkylitaconic acids during the treatment of wheat straw. The increase in the production of alkylitaconic acids paralleled the degradation and modification of lignin indicating the importance of these compounds in delignification. Both fungi selectively degraded lignin in wood chips. After 4 weeks of treatment, no significant changes in chemical composition were detected.

Light microscopy visualized a dense structure of oak wood chips which was difficult to penetrate by the fungi, explaining the relative lower lignin degradation compared to wheat straw measured by chemical analysis. Most of the structures in wheat straw disappeared during the fungal treatment. The result of all these changes was an increase in substrate utilization due to an increased *in vitro* rumen degradability of wheat straw and oak wood chips. In addition, more glucose and xylose were released after enzymatic saccharification of wheat straw after the fungal treatment compared to untreated material.

5.1 Introduction

Cellulose in lignified plant cell walls can be a source of energy in applications such as animal nutrition and biofuel production. However, the utilization of cellulose in lignocellulosic biomass cannot directly be used for these purposes because of the presence of lignin. This recalcitrant polymer is difficult to degrade and several chemical and/or physical methods have been developed to selectively remove lignin (Agbor et al., 2011). Fungal treatment of lignocellulosic biomass can be a relatively inexpensive and environmental friendly technology to decrease lignin contents and to increase the accessibility of cellulose (Sarnklong et al., 2010; van Kuijk et al., 2015a). In particular, the white-rot fungi *Ceriporiopsis subvermispora* and *Lentinula edodes* have proven to selectively degrade lignin, leaving a substrate enriched in cellulose after 6 to 12 weeks of treatment (Okano et al., 2005; Tuyen et al., 2012, 2013; van Kuijk et al., 2015b). The fungal treated biomass showed increased *in vitro* rumen degradability, demonstrating that the cellulose becomes available for rumen microbes. Most studies investigating the fungal treatment of lignocellulose focus on changes in the end product and the consequences for further downstream processing (Okano et al., 2005; Tuyen et al., 2012; 2013; van Kuijk et al., 2015a; 2015b). The analyses of the intermediate products formed during the treatment might help to understand how fungi degrade lignocellulose and this knowledge might be used to further improve the technology. To this end, the changes in lignocellulose were assessed during 8 weeks incubation with two different fungal species grown on two different substrates. Wheat straw was used as a reference substrate and oak wood chips as a substrate high in lignin. Changes in chemical composition/content were studied using the detergent fiber method (Van Soest et al., 1991) and structural changes in especially lignin were studied using pyrolysis coupled to gas chromatography and mass spectrometry (Py-GC/MS). Light microscopy was used to visualize tissue integrity, degradation of lignin and changes in the availability of cellulose. Furthermore, the growth of the fungus was studied, both microscopically and by measuring ergosterol contents.

5.2 Material and methods

5.2.1 Fungal strains and spawn preparation

Ceriporiopsis subvermispora (strain MES 13094) and *Lentinula edodes* (strain MES 11910) were preserved in liquid nitrogen at Wageningen UR Plant Breeding. Initial culturing of the fungi was done on malt extract agar plates (pH ≈ 5.5) at 24 °C until mycelium was covering most of the plate surface. Spawn was prepared by adding pieces of colonized agar culture to sterilized sorghum grains followed by incubation at 24 °C until all grains were colonized by mycelium. The resulting spawn was kept at 4 °C until further use.

5.2.2 Substrate preparation

Wheat straw (particles of ~ 3 x 0.3 cm length x diameter) and oak wood chips (particles of ~ 2 x 0.5 cm length x thickness) were used as substrates. An excess of water was

added to the substrates and left for 3 days to allow the water to fully penetrate the material. After removal of excess of water by draining, 50 g (on dry matter basis) of wheat straw and 100 g (on dry matter basis) of oak wood chips were weighed into 1.2 l polypropylene containers with a filter cover (model TP1200+TPD1200 XXL Combiness, Nazareth, Belgium). Two containers of wheat straw represented one sample. The material was sterilized by autoclaving for 1 hour at 121 °C, and the containers with sterilized substrate were kept at 20 °C until use. A sample of the autoclaved material was collected (untreated control).

5.2.3 Inoculation of substrate

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

5.2.4 Sampling

Samples were taken every week during a period of 8 weeks. Approximately 90% of the substrate was air-dried at 70 °C for chemical analysis, *in vitro* gas production technique measurement and enzymatic saccharification. The remaining (~ 10%) part was freeze-dried and used to determine ergosterol content and used for Py-GC/MS analyses. For microscopy, samples (fresh) of each treatment were taken only before and after 8 weeks of incubation.

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

5.2.5 Ergosterol

Ergosterol was determined as described by Niemenmaa et al. (2008). In brief ground, freeze dried material (200 mg) was saponified with 3 ml 10% KOH/methanol for 1 hour at 80 °C. Ergosterol was extracted by adding 1 ml water and 2 ml hexane, and the hexane phase was collected in glass tubes after shaking and centrifuging for 10 minutes at 4000 rpm. This step was repeated for optimal extraction and both hexane phases were mixed. Hexane was evaporated under vacuum and ergosterol was dissolved in methanol. The extraction efficiency was calculated on the basis of recovery of the internal standard cholecalciferol (vitamin D3) (9.6 µg added) (Sigma Aldrich, St. Louis, Missouri, USA).

Ergosterol was analyzed using an HPLC fitted with a reversed phase C18 column (250 x 4.6 mm, Phenomex aqua 5 µm, Torrance, California, USA). The liquid phase was 90% methanol and 10% 2-propanol/hexane (1:1, v/v). Areas under the peak were corrected

for the extraction efficiency based on the internal standard using Empower 2 software (Waters Corporation, Milford, Massachusetts, USA).
To calculate a conversion rate of the amount of ergosterol measured to dry weight of mycelium, mycelium of each fungus was grown on malt extract agar with cellophane and freeze dried. Known amounts of dry mycelium were subjected to ergosterol extraction. For each fungus, the amount of ergosterol per mg mycelium was calculated.

5.2.6 Light microscopy

The fresh samples were fixed in a mixture of 3% (v/v) paraformaldehyde, 0.1% (v/v) glutaraldehyde, 0.02% Triton™ X-100 Surfact-Amps™ (ThermoFisher, Bleiswijk, the Netherlands) dissolved in 50 mM PIPES buffer (pH 6.9). Fixed samples were dehydrated in a series of ethanol concentrations until full dehydration was reached in 100% ethanol. Dehydrated samples were stepwise infiltrated with Technovit 7100 (Heraeus Kulzer Benelux, Haarlem, the Netherlands). Polymerization of the Technovit 7100 monomers was done for at least 1 hour at 37 ºC. Resin embedded samples were cut at 5 µm with using a Microm (Adamas Instr, Rhenen, the Netherlands) rotary microtome. The collected sections were stretched on water surface and baked to glass slides at 80 ºC for at least 10 minutes.
Staining of the sections for lignin and cellulose was based on the procedure described by Srebotnik and Messner (1994). In brief, sections were stained for 1 minute in 1% Saffranin O (S2255, Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands) dissolved in 15% ethanol. Excess of dye was removed by washing for 1 minute in water followed by two times 5 minutes in 30% ethanol and 5 minutes in 15% ethanol. After another minute in water to remove the ethanol, sections were stained for 3 minutes in 1% Astra blue (Marker Gene Technologies Inc., Eugene, Oregon, USA) dissolved in 5% ethanol. Excess of dye was removed by washing four times 3 minutes in water.
Fungal hyphae were discriminated in sections of treated samples with 1% Toluidine Blue in 1% sodium tetra borate. Sections were stained during 5 minutes in toluidine blue and excess of dye was removed during 30 minutes in water.

5.2.7 Chemical analysis

Detergent fiber analysis was performed on air-dried, ground material according to the method described by Van Soest et al. (1991) using Ankom fiber analyser 2000 (ANKOM Technology, Macedon, New York, USA). Acid detergent fiber (ADF) was subtracted from neutral detergent fiber (NDF) to calculate the hemicellulose content. Acid detergent lignin (ADL) was subtracted from ADF to calculate the cellulose content. For dry matter determination air-dried material was dried at 103 °C until constant weight. Ash content was determined after combustion for 3 hours at 550 °C in a muffle furnace. Dry matter loss data were used to calculate absolute amounts of nutrients.

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

Py-GC/MS of samples (3.2 mg) were performed with a 3030 micro-furnace pyrolyzer (Frontier Laboratories Ltd., Fukushima, Japan) connected to an Agilent 7820A GC using a DB-1701 fused-silica capillary column (60 m x 0.25 mm, 0.25 μm film thickness) and an Agilent 5975 mass selective detector (EI at 70 eV) (Agilent Technologies, Santa Clara, California, USA). The pyrolysis was performed at 500 ºC. The oven temperature of the gas chromatograph was programmed from 100 ºC (4 minutes) to 280 ºC (8 minutes) at 3 ºC minutes^{-1}. Helium was the carrier gas (1 ml minute^{-1}). The compounds were identified by comparing their mass spectra with those of the Wiley and NIST libraries and those reported in the literature (del Río et al., 2002a; Faix et al., 1990; Ralph and Hatfield, 1991). Peak molar areas were calculated for the carbohydrate and lignin-degradation products, the summed areas were normalized, and the data for three replicate samples were averaged and expressed as percentages. The samples of wheat straw were measured in duplicate, whereas only one sample per treatment was measured for wood chips. For this reason no statistical analysis could be done on the results of wood chips, however numerical trends will be discussed.

5.2.9 *In vitro* gas production (IVGP)

Determination of IVGP was performed according to the procedure described by Cone et al. (1996). Rumen fluid of rumen fistulated non-lactating cows fed a grass silage based diet was collected. To each 60 ml buffered rumen fluid 500 mg air dried sample was added. During 72 hours of incubation at 39 °C the amount of gas produced by anaerobic fermentation were measured. Total gas production was related to organic matter content of the samples.

5.2.10 Enzymatic saccharification

Enzymatic saccharification was tested for wheat straw treated with *C. subvermispora* or *L. edodes* for 8 weeks. Two g of wheat straw (air-dried, ground to 1 mm) were mixed with 19 ml sodium citrate buffer (50 mM, pH 5.3) and 1 ml of enzymes, i.e. mixture of mainly cellulases (CMAX3, Dyadic Nederland BV, Wageningen, The Netherlands). The mixtures were incubated at 50 °C in a rotary shaker (600 rpm). Samples of the supernatant were taken after 0, 4, 8, 12, 24, 48 and 72 hours of incubation to measure the amount of released glucose and xylose. The amount of released glucose was measured using a D-glucose kit (D-glucose assay kit (GOPOD format), Megazyme, Bray, Ireland) and expressed as mg glucose per g of wheat straw. The amount of released xylose was measured using a D-xylose kit (D-xylose assay kit, Megazyme, Bray, Ireland) and expressed as mg xylose per g of hemicellulose present in the wheat straw as determined by the detergent fiber analysis.

5.2.11 Statistical analysis

The results of detergent fiber analysis, Py-GC/MS, IVGP and enzymatic saccharification at different incubation times of the fungal treatment of each substrate were compared

Table 5.1 Ergosterol content and chemical composition of wheat straw before (0 weeks) and after treatment with *C. subvermispora* and *L. edodes*, expressed in g/kg DM and in absolute amounts (g).

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

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

Table 5.2 Ergosterol content and chemical composition of wood chips before (0 weeks) and after treatment with *C. subvermispora* and *L. edodes*, expressed in g/kg DM and in absolute amounts (g).

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

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

using the generalized linear model (GLM) analysis in SAS software version 9.3 (SAS Institute Inc., Cary, North Carolina, USA). Post-hoc multiple comparison with Tukey's significant test at a level of $\alpha = 0.05$ was performed to determine the significance of differences between the treatments. The following model was used:

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

in which Y_{ij} is the observation j at incubation time i; μ is the overall mean; α_i is the fixed effect of incubation time i; ω_{ij} is the random error.
Correlations between IVGP, enzymatic saccharification and ADL were subjected to correlation analysis using SAS software version 9.3. Correlations are provided as the Pearson correlation coefficient r.

5.3 Results

5.3.1 Fungal growth

The changes in ergosterol content in the substrate during incubation were measured as an indication for fungal growth (Table 5.1). The ergosterol content of both in wheat straw and oak wood chips, showed a steady increase during 8 weeks of *L. edodes* treatment indicating a continuous growth of this fungus during the whole incubation period. The ergosterol content of the *C. subvermispora* treated substrates showed a different pattern. Ergosterol content in both wheat straw and oak wood chips increased in the first week of treatment. In the next 7 weeks, the ergosterol content in wood chips did not change significantly. In wheat straw, however, the ergosterol content increased again after 6 weeks of incubation.
Visual, macroscopic, observations confirmed the growth patterns by *C. subvermispora* and *L. edodes* on both wheat straw and wood chips (data not shown).

5.3.2 Microscopy

Sections of untreated and fungal treated wheat straw and oak wood chips were stained for lignin (Safranin O) and accessibility of cellulose in absence of lignin (Astra Blue) (Srebotnik and Messner, 1994), or for visualization of fungal hyphae (Toluidine Blue). The most representative pictures of the stained sections are shown in Figure 5.1 (wheat straw) and Figure 5.2 (wood chips).
Safranin O and Astra Blue staining of untreated wheat straw showed an intact structure with few intercellular spaces and mainly red stained walls (Figure 5.1a). The thick walls in the xylem vessels and vascular bundle sheets reinforced with sclerenchyma are predominantly stained. After *C. subvermispora* treatment most of the structure disappeared (Figure 5.1c). *C. subvermispora* degraded most of the thin walled parenchymatic cells and only some remnants of lignified sclerenchyma are still visible (Figure 5.1c). Safranin O and Astra Blue staining showed the presence of accessible cellulose and some remaining lignin. Toluidine Blue staining revealed *C. subvermispora*

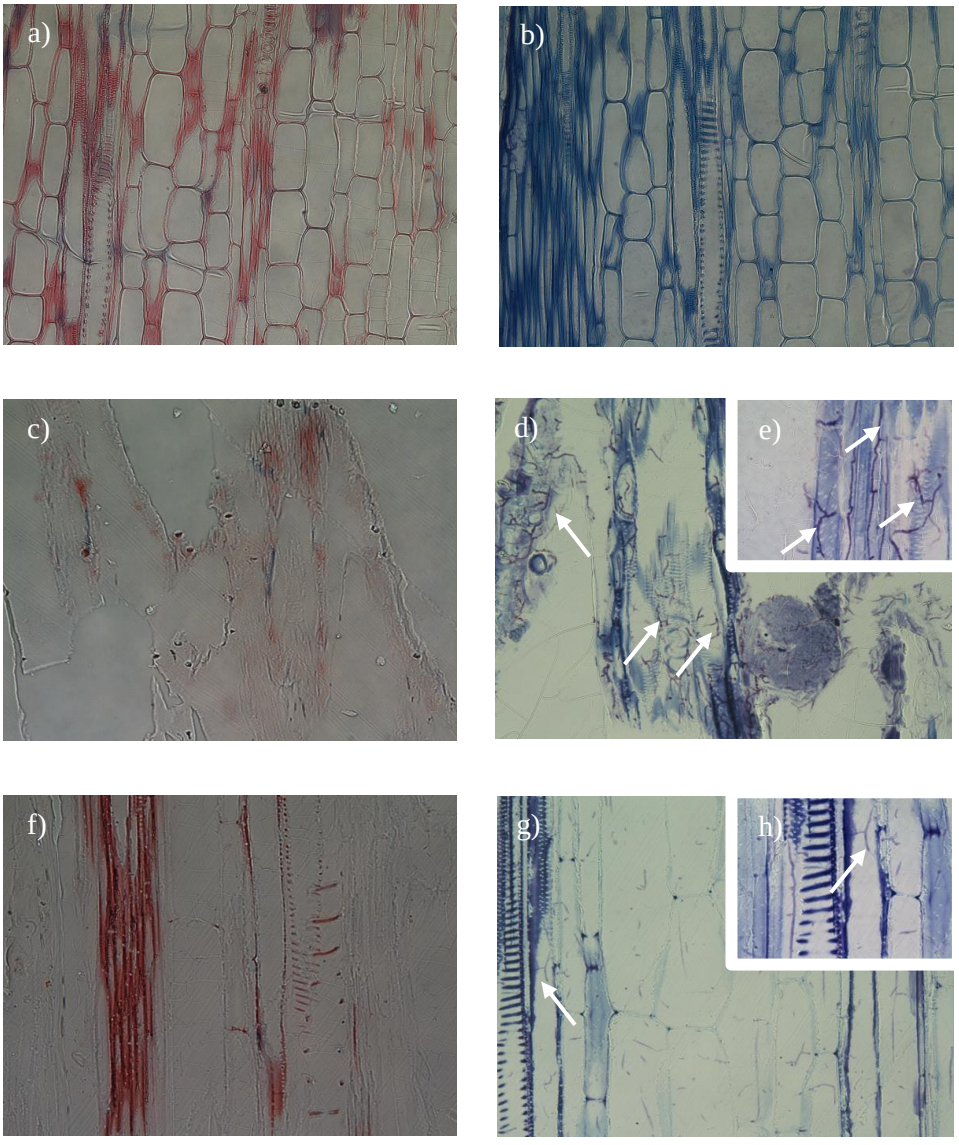

Figure 5.1 Light microscopy of untreated and fungal treated wheat straw. a) autoclaved, uninoculated wheat straw stained with saffranin O and Astra Blue, b) autoclaved, uninoculated wheat straw stained with toluidine blue, c) *C. subvermispora* treated wheat straw for 8 weeks stained with saffranin O and Astra Blue, d) and e) *C. subvermispora* treated wheat straw for 8 weeks stained with toluidine blue, f) *L. edodes* treated wheat straw stained with saffranin O and Astra Blue, g) and h) *L. edodes* treated wheat straw for 8 weeks stained with toluidine blue. Arrows indicate fungal hyphae.

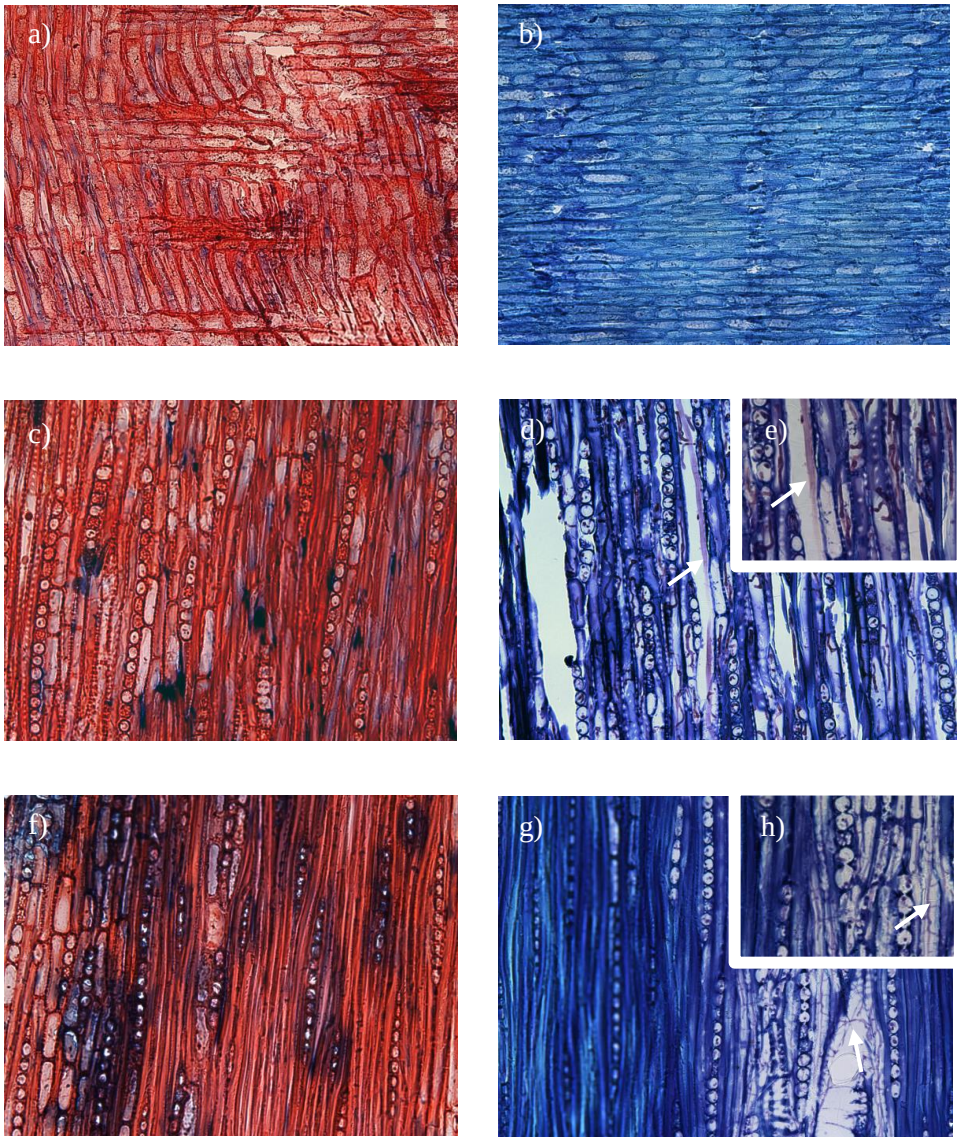

Figure 5.2 Light microscopy of untreated and fungal treated oak wood chips (20x magnification). a) autoclaved, uninoculated oak wood chips stained with saffranin O and Astra Blue, b) autoclaved, uninoculated oak wood chips stained with toluidine blue, c) *C. subvermispora* treated oak wood chips for 8 weeks stained with saffranin O and Astra Blue, d) and e) *C. subvermispora* treated oak wood chips for 8 weeks stained with toluidine blue, f) *L. edodes* treated oak wood chips stained with saffranin O and Astra Blue, g) and h) *L. edodes* treated oak wood chips for 8 weeks stained with toluidine blue. Arrows indicate fungal hyphae.

hyphae (arrows) to be omnipresent in wheat straw after 8 weeks of treatment (Figure 5.1d and 5.1e). Safranin O and Astra Blue staining showed that after 8 weeks of *L. edodes* treatment a clearly visible structure was left, which was stained red for lignin (Figure 5.1f). Toluidine Blue staining showed an overall presence of *L. edodes* hyphae (arrows) throughout the remaining structures (Figure 5.1g and 5.1h).

Sections of untreated oak wood chips showed a dense structure consisting of xylem vessels (Figure 5.2a (Safranin O and Astra blue staining) and 5.2b (Toluidine Blue staining)). This dense structure was still present after 8 weeks of *C. subvermispora* or *L. edodes* treatment. Safranin O – Astra Blue stained fungal treated oak wood chips still showed large parts containing lignin (Figure 5.2c and 5.2f). In contrast to untreated oak wood chips, the vascular bundles stained blue, showing more cellulose became accessible for Astra Blue staining due to the degradation of lignin (Figure 5.2c and 5.2f). Toluidine Blue staining showed the presence of fungal hyphae that had invaded the (physically accessible) structures (Figure 5.2d and 5.2g). The fungal hyphae were not omnipresent as found in wheat straw, which was most clear in *L. edodes* treated oak wood chips. In Figure 5.2g, hyphae of *L. edodes* were only visible in the part of the sample showing wider vessel diameters, whereas the dense vessels were still fully intact.

5.3.3 Chemical composition of fungal treated material

5.3.3.1 Detergent fiber analysis

Table 5.1 and 5.2 show the changes in the composition of wheat straw and oak wood chips during the fungal treatment, using the detergent fiber analysis method (Van Soest et al., 1991). *C. subvermispora* incubation of wheat straw led to a decrease of dry matter of 29% after 8 weeks. The detergent fiber composition of wheat straw changed significantly only during the first 5 weeks of treatment with a continuous decrease in ADL and hemicellulose concentration whereas the cellulose concentration did not change significantly. Despite the fact that the content of fibers did not change significantly between 5 and 7 weeks, the dry matter loss in wheat straw increased continuously upon *C. subvermispora* treatment. Expressed as absolute amounts, also a steady degradation of ADL and hemicellulose was observed. For cellulose, however, the absolute amount in wheat straw was only changing significantly in the first 3 weeks of incubation with *C. subvermispora* (Table 5.1).

The 8 weeks of treatment of wheat straw by *L. edodes* led to a loss of 26% dry matter, a decreased ADL and hemicellulose content and an increased cellulose content (Table 5.1). Absolute amounts confirmed the ADL and hemicellulose degradation, but also showed that *L. edodes* degraded ($P<0.05$) part of the cellulose in wheat straw (Table 5.1).

In oak wood chips, *C. subvermispora* degraded 25% of the dry matter in 8 weeks. Hemicellulose and ADL contents changed only significantly during the first 3 weeks of treatment (Table 5.2), which corresponded with the obtained dry matter loss. From 4 weeks onwards no changes in the content of the main components (ADL, hemicellulose and cellulose) were observed in oak wood chips. *C. subvermispora* did not change the

cellulose content significantly in oak wood chips during the 8 weeks of treatment. Nevertheless, absolute amounts showed that oak wood chips treated for 4 weeks or longer with *C. subvermispora* contained a lower amount of cellulose compared to untreated biomass, resulting in a total degradation of 5.6 g cellulose after 8 weeks of treatment (Table 5.2).

L. edodes caused a dry matter loss in oak wood chips of up to 36%. The composition of the oak wood chips did not change significantly after 5 weeks of *L. edodes* treatment. At the end of 8 weeks of *L. edodes* treatment, the oak wood chips showed a lower ($P<0.05$) ADL and hemicellulose and a higher ($P<0.05$) cellulose content than the control before treatment (Table 5.2). Absolute amounts of detergent fiber fractions showed a degradation ($P<0.05$) of all compounds including cellulose by *L. edodes* in oak wood chips (Table 5.2).

5.3.3.2 Py-GC/MS

The composition of the fungal treated samples was analyzed by Py-GC/MS, as shown in Figure S5.1 and S5.2 for wheat straw and Figure S5.3 and S5.4 for wood chips. The identities and relative abundances of the compounds released by Py-GC/MS are shown in Tables S5.1 and S5.2 for wheat straw and Tables S5.3 and S5.4 for wood chips. The most important results obtained by Py-GC/MS of untreated and fungal treated wheat straw and oak wood chips are shown in Table 5.3.

In the case of wheat straw, the lignin to carbohydrate (L/C) ratio estimated upon Py-GC/MS varied from 2.2 in the untreated material to 0.4 and 0.5 after 8 weeks of treatment with *C. subvermispora* and *L. edodes*, respectively (Table 5.3). In wood chips, a decrease in L/C ratio was found from 1.6 in the untreated material to 0.7 and 0.9 after 8 weeks of treatment with *C. subvermispora* and *L. edodes*, respectively (Table 5.3). The main change observed in the carbohydrate fraction of both wheat straw and wood chips was the increase in the relative content of levoglucosane (peak **38**), a compound originating from cellulose.

Analysis of the lignin-derived compounds indicated that the syringyl (S) units were preferentially degraded over guaiacyl (G) units, as observed by the decrease of the S/G ratio in wheat straw from 0.7 in the untreated material to 0.3 and 0.4 after 8 weeks of treatment with *C. subvermispora* and *L. edodes*, respectively (Table 5.3). The S/G ratio in wood chips decreased from 1.1 to 0.5 and 0.6 after 8 weeks of treatment with *C. subvermispora* and *L. edodes*, respectively (Table 5.3). Moreover, the treatment with *C. subvermispora* and *L. edodes* resulted in the formation of intermediate degradation products of lignin, as shown by the increased ratio of phenolic compounds bearing 0 to 2 C-atoms (Ph-C0-C2) and the intact phenolic compounds bearing 3 C-atoms (Ph-C3) in the side chain (PhC0-2/Ph-C3 ratio). Although in both wheat straw and wood chips the Ph-C0-2/Ph-C3 ratio increased, this effect was more evident in wheat straw. In wheat straw, the side chains were not only degraded, but also the fungi oxidized the Cα-atom, as shown by the increasing percentage in Cα-oxidized lignin compounds upon fungal

Table 5.3 Composition of lignocellulose in wheat straw and wheat straw before and after treatment with *C. subvermispora* or *L. edodes* as determined by Py-GC/MS.

Substrate	Time (weeks)	*C. subvermispora*					*L. edodes*			
		L/C Ratio	S/G ratio	Ph-C0-2 /Ph-C3 Ratio	Cα-oxidized lignin (%)	Alkylitaconic acids (%)	L/C ratio	S/G ratio	Ph-C0-2 /Ph-C3 ratio	Cα-oxidized lignin (%)
Wheat straw	control	2.2^{a}	0.7^{a}	7.6^{c}	4.4^{e}	0	2.2^{a}	0.7^{a}	7.6^{d}	4.4^{d}
	1	1.9^{a}	0.7^{a}	7.3^{c}	4.8^{de}	0	1.9^{a}	0.7^{a}	7.8^{d}	4.5^{d}
	2	1.1^{b}	0.8^{a}	8.7^{bc}	7.6^{cd}	0.2	1.4^{b}	0.7^{a}	8.4^{cd}	6.3^{cd}
	3	0.9^{b}	0.6^{a}	10.7^{abc}	8.3^{bc}	0.5	1.0^{c}	0.6^{a}	9.5^{bcd}	7.4^{bc}
	4	0.5^{c}	0.5^{b}	13.0^{a}	10.7^{ab}	1.3	0.9^{cd}	0.6^{a}	10.0^{bc}	8.7^{abc}
	5	0.5^{c}	0.4^{bc}	11.9^{ab}	12.1^{a}	1.8	0.9^{cd}	0.5^{b}	10.3^{ab}	9.2^{ab}
	6	0.4^{c}	0.4^{bc}	12.3^{ab}	12.4^{a}	2.9	0.7^{de}	0.5^{bc}	10.6^{ab}	9.4^{ab}
	7	0.4^{c}	0.4^{bc}	13.3^{a}	12.3^{a}	3.3	0.7^{de}	0.4^{cd}	10.0^{bc}	10.1^{a}
	8	0.4^{c}	0.3^{c}	12.3^{a}	10.1^{abc}	4.8	0.5^{e}	0.4^{d}	12.1^{a}	9.0^{ab}
Wood chips	control	1.6	1.1	3.6	4.2	0	1.6	1.1	3.6	4.2
	1	1.4	1.0	4.6	5.7	0.1	1.4	0.8	4.4	3.1
	2	1.3	0.9	4.3	4.3	0.3	1.4	0.8	5.8	4.9
	3	0.8	0.5	7.5	3.4	1.1	1.2	0.6	6.2	3.3
	4	0.8	0.6	8.0	4.3	1.2	1.1	0.7	6.2	3.7
	5	0.8	0.6	8.0	3.6	2.8	1.2	0.5	7.3	3.1
	6	0.9	0.5	7.7	3.7	2.5	1.0	0.6	7.4	3.0
	7	0.8	0.5	7.6	4.2	1.6	1.0	0.5	8.7	3.1
	8	0.7	0.5	8.8	4.8	3.0	0.9	0.6	7.9	2.8

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

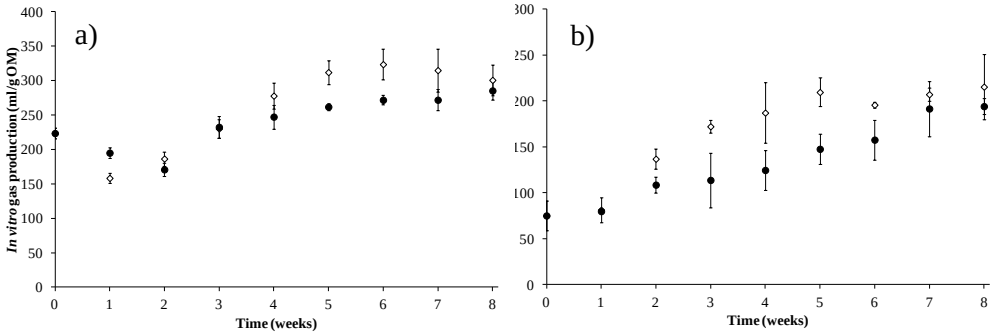

Figure 5.3 *In vitro* gas production of *C. subvermispora* and *L. edodes* treated wheat straw and oak wood chips. a) wheat straw, b) oak wood chips. ◊ *C. subvermispora* ● *L. edodes*. Error bars represent standard deviations (n=3).

treatment. The percentage of Cα-oxidized lignin did not change during fungal treatment of wood chips (Table 5.3). In wheat straw, both the side chain degradation and modification were highly related to each other (*C. subvermispora*: r=0.90, P<0.0001; *L. edodes*: r=0.79, P<0.001). In the case of *C. subvermispora*, the major changes in the lignin polymer occurred during the first 5 weeks of treatment, while no significant changes in L/C ratio, S/G ratio, Ph-C0-2/Ph-C3 ratio and percentage of Cα-oxidized lignin occurred after 5 weeks of treatment of wheat straw. This result corresponds with the stabilizing ADL, hemicellulose and cellulose contents after 5 weeks of treatment according to the detergent fiber analysis. Upon *L. edodes* treatment a gradual decrease (P<0.05) in L/C ratio and a gradual increase (P<0.05) in the Ph-C0-2/Ph-C3 ratio was found during the 8 weeks of treatment. However, during the first 4 weeks of *L. edodes* treatment of wheat straw, the S- and G-lignin units were degraded simultaneously and also Cα-oxidized lignin compounds were formed. After 5 weeks, a preferential S-unit degradation started, but the production of Cα oxidized lignin compounds did not increase anymore. In wood chips, the main changes in L/C ratio, S/G ratio and Ph-C0-2/Ph-C3 ratio were found after 4 weeks of *C. subvermispora* and *L. edodes* treatment.

Three alkylitaconic acids were identified in both substrates treated with *C. subvermispora* including, tetradecylitaconic acid (peak **43**) and *cis*-7-hexadecanylitaconic acid (peak **44**) which increased after 1 week in treated wood chips and 2 weeks in treated wheat straw. Hexadecylitaconic acid (peak **45**) increased after 2 weeks in treated wood chips and 3 weeks in treated wheat straw, also increasing in time during the incubation period (Figure S5.1 and S5.3). The ratio between the different alkylitaconic acids did not change from 3 weeks up to the end of the incubation period. An increasing amount of alkylitaconic acids were produced (Table 5.3), composed of 23% tetradecylitaconic acid (peak **43**), 65% *cis*-7-hexadecanylitaconic acid (peak **44**) and 12% hexadecylitaconic acid (peak **45**) after 3 weeks until the end of *C. subvermispora* treatment of wheat straw (Figure S5.1).

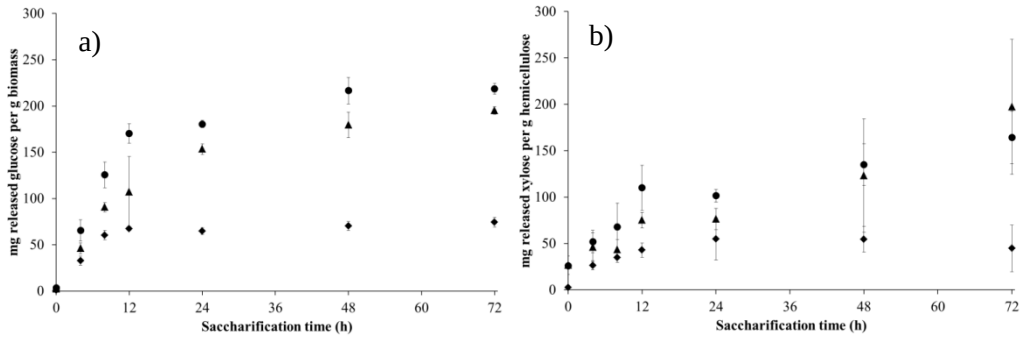

Figure 5.4 Enzymatic saccharification of untreated, *C. subvermispora* and *L. edodes* treated wheat straw. a) glucose, b) xylose. ♦ untreated wheat straw, ▲ wheat straw treated for 8 weeks with *C. subvermispora*, ● wheat straw treated for 8 weeks with *L. edodes*. Error bars represent standard deviations (n=3).

In wood chips the production of alkylitaconic acids stabilized from 5 weeks of *C. subvermispora* treatment (Figure S5.3). Alkylitaconic acids were not detected in wheat straw (Figure S5.2) or wood chips (Figure S5.4) treated with *L. edodes*.

5.3.4 *In vitro* rumen degradability

Total IVGP started at 223.4 ml/g OM for the untreated wheat straw and increased 34.6% to 300.7 ml/g OM after 8 weeks of *C. subvermispora* treatment and 27.7% to 285.3 ml/g OM after 8 weeks of *L. edodes* treatment (Figure 5.3a). During the first 4 to 5 weeks, the IVGP of wheat straw increased ($P<0.05$) to remain similar after this period during both the *C. subvermispora* and *L. edodes* treatment (Figure 5.3a).

The total IVGP started at 75.0 ml/g OM for the untreated oak wood chips and increased 187.2% to 215.4 ml/g OM after 8 weeks of *C. subvermispora* treatment and 158.8% to 194.1 ml/g OM after 8 weeks of *L. edodes* treatment (Figure 5.3b). The IVGP of oak wood chips increased ($P<0.05$) during the first 3 weeks of *C. subvermispora* treatment and during the first 4 weeks of *L. edodes* treatments, after which the IVGP did not change significantly anymore (Figure 5.3b).

5.3.5 Enzymatic saccharification

As a proof of principle, the enzymatic saccharification was only measured for wheat straw. Enzymatic saccharification with a mixture of cellulases (CM) for 72 hours released most ($P<0.05$) glucose in *L. edodes* treated wheat straw compared to *C. subvermispora* treated wheat straw (Figure 5.4a). More ($P<0.05$) glucose was released from fungal treated wheat straw compared to untreated wheat straw (Figure 5.4a).

The amounts of xylose released per g of biomass were not significantly different between untreated and fungal treated materials after 72 hours enzymatic saccharification (data not shown). Due to hemicellulose degradation, fungal treated wheat straw contained less hemicellulose per g of biomass (see Table 5.1). As less hemicellulose was

present, less xylan could be enzymatically converted to xylose. Therefore, xylose release is expressed as mg xylose per g of hemicellulose. The hemicellulose left in the wheat straw was better accessible for enzymatic saccharification by CM, since more ($P<0.05$) xylose per g hemicellulose was released from fungal treated material compared to untreated wheat straw after 72 hours of enzymatic saccharification (Figure 5.4b).

5.3.6 Correlations between IVGP and enzymatic saccharification

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

5.4 Discussion

Fungal degradation of wheat straw can be divided into two phases. The first phase can be defined by the degradation of easily accessible components such as starch, pectin and easily accessible hemicellulose. Wheat straw contains, for example 3-4 % starch (Bart et al., 2013). The degradation of these easily accessible compounds might not be hindered by the presence of lignin and thus also easily accessible by the ruminant microflora. Removal of these compounds will thus lead to a decrease in IVGP compared to the untreated material as seen for wheat straw treated for 1 week with *C. subvermispora* or 2 weeks with *L. edodes*. During the first phase usually no significant lignin degradation occurs as shown in the fiber analysis (no decrease in ADL). However, the Py-GC/MS showed a significant decrease of the L/C ratio in fungal treated already during the first 2 weeks. In the first weeks, lignin degradation/modification in wheat straw started by oxidation of the Cα of lignin by *C. subvermispora*, which is indicated by the increase in acetovanillone (peak **30**).

The second phase can be defined as the delignification phase resulting in an increased IVGP. Significant delignification of wheat straw, as measured by the detergent fiber method, starts after 3 weeks of treatment for both fungi. This process, accompanied by a decrease in L/C ratio as measured by Py-GC/MS, continues until 5 weeks of *C. subvermispora* treatment and 8 weeks of *L. edodes* treatment. The continuous lignin degradation by *L. edodes* resulting in a continuous increase in IVGP of wheat straw was also described by Tuyen et al. (2012). Delignification starts by simultaneous degradation of S- and G-lignin units in wheat straw. However, a preferential degradation of S- over G-lignin units occurred after 4 weeks of *C. subvermispora* treatment and after 5 weeks of *L. edodes* treatment, as observed by the decrease in the S/G ratio from that point

onwards. This is in line with the observation that G-units are more recalcitrant towards fungal attack because of a high condensation degree (del Río et al., 2002b). Preferential S-unit degradation shows that the fungi degrade mainly β-*O*-4-ether-linkages, the predominant linkages in S-units. The formation of degradation products, i.e. increase in side chain degradation represented by the Ph-C0-2/Ph-C3 ratio, indicates that also Cα-Cβ oxidative cleavage of lignin side chains took place during the fungal treatment from 4 weeks onwards. The increase in Ph-C0-2/Ph-C3 ratio is mainly determined by the decrease in Ph-C3 products such as eugenol (peak **21**), *cis*-isoeugenol (peak **24**), *trans*-isoeugenol (peak **25**), 4-allyl-syringol (peak **33**), *cis*-propenylsyringol (peak **35**) and *trans*-propenylsyringol (peak **36**). The fact that Ph-C0-2 products decrease in time suggests that upon fungal treatment these products are further metabolized. The negative correlation between S/G ratio on one hand and IVGP, glucose and xylose release on the other hand suggests that G-units are also more recalcitrant toward degradation by the fungi. However, in the current study, the changes in lignin composition are a direct result of lignin degradation. The real effect of changes in lignin composition on further processing cannot be separated from the effect of changes in lignin content.

Delignification is accompanied by degradation of hemicellulose by both fungi. Hemicellulose is needed as an energy source for the fungi to grow and to produce enzymes. The ergosterol content increase in wheat straw throughout the *L. edodes* treatment indicates that this fungus might use hemicellulose as source of energy. The hemicellulose content of *C. subvermispora* treated wheat straw continuously decreased, while the ergosterol content only significantly increased after 6 weeks of treatment. Nevertheless, the hemicellulose content correlated significantly with the ergosterol content (r=-0.73) during the *C. subvermispora* treatment of wheat straw. An explanation for this correlation can be that this fungus produces xylanases and degrades/solubilizes the main structures of hemicellulose, but not to xylose (Brasil de Souza-Cruz et al., 2004; Guerra et al., 2003). Solubilized hemicellulose will dissolve in neutral detergent reagent and thus not be measured in the detergent fiber analysis as hemicellulose, i.e. the hemicellulose degradation is overestimated. Similarly, *A. bisporus* lacks also enzymes to remove arabinosyl residues from doubly substituted xylose (Jurak et al., 2015), which results in more solubilized hemicellulose. Consequently, double substituted xylose cannot be used as an energy source by the fungus to grow. Degradation products of hemicellulose are not included in the NDF minus ADF fraction. An extensive carbohydrate analysis should be done to ensure hemicellulose is degraded and to see whether monosaccharides originating from hemicellulose are used by the fungus. It is not yet understood why *C. subvermispora* degrades hemicellulose without using it for growth. Possibly hemicellulose degradation cannot be avoided in the process of lignin degradation due to the tight (ether) bonds between the two components (Buranov and Mazza, 2008). Hemicellulose is described to hamper the enzymatic degradation of cellulose, therefore, hemicellulose degradation might be required to increase cellulose accessibility (Meng and Ragauskas, 2014). Cellulose accessibility in wheat straw increased after 4 weeks of *C. subvermispora* treatment and after 5 weeks of *L. edodes*

treatment, as indicated by the increased IVGP. On the other hand, the absolute amount of cellulose decreased after 3 weeks of *C. subvermispora* treatment and 5 weeks of *L. edodes* treatment. This means that although cellulose was degraded, the remaining cellulose after fungal treatment was better accessible for rumen microbes and enzymes than before treatment. The current study shows a decrease in the cellulose content. However, literature shows that *C. subvermispora* has less genes for cellulases compared to other white rot fungi (Fernandez-Fueyo et al., 2012). As a result *C. subvermispora* has an incomplete cellulose degrading system (Guerra et al., 2003; Tanaka et al., 2009). This may indicate that the ADF minus ADL fraction does not represent the whole cellulose content. Similarly, underestimation of the carbohydrate fraction is also inherent to the Py-GC/MS method. Also, both the detergent fiber analysis and Py-GC/MS are generally calculated through ratios. This means that a more detailed, quantitative carbohydrate analysis of fungal treated material is recommended.

The Py-GC/MS data show the production of a series of alkylitaconic acids by *C. subvermispora*, despite the fact that ergosterol data do not show growth. The termination of production of secondary metabolites after active growth, as observed for *C. subvermispora*, has also been observed in other fungi (Calvo et al., 2002). Alkylitaconic acids were not found in the *L. edodes* treated wheat straw. The production of alkylitaconic acids started after 2 weeks of *C. subvermispora* treatment of wheat straw, similar as described for treatment of eucalyptus (del Río et al., 2002a; Gutiérrez et al., 2002). It is interesting to note that alkylitaconic acids are produced continuously throughout the treatment period, contrary to what occurs with ergosterol.

Alkylitaconic acids are involved in lignin degradation through a mechanism of lipid peroxidation by manganese peroxidase (Gutiérrez et al., 2002; Nishimura et al., 2012). Another theory states alkylitaconic acids suppress hydroxyl radicals that are released upon lignin degradation. Suppression prevents the hydroxyl radicals to attack cellulose (Rahmawati et al., 2005). The cellulose content did not significantly decrease during the first 5 weeks of *C. subvermispora* treatment, simultaneous to the increased production of alkylitaconic acids. Nevertheless, the cellulose content was significantly lower after 8 weeks of *C. subvermispora* treatment compared to the control, while the production of alkylitaconic acids continued. The chemical changes in wheat straw may suggest that alkylitaconic acids are both involved in lipid peroxidation and preventing cellulose degradation. This is the first time that the production of alkylitaconic acids has been studied during fungal growth and fungal delignification of lignocellulose. The ergosterol measurements indicate that *C. subvermispora* grows mainly in the first week, stops growing in the next 4 weeks and might resume growth in the last 3 weeks. Unfortunately, the ergosterol measurements were insufficiently accurate in the last weeks which might be due to the formation of some clusters of dense tissue causing variation in biomass in the substrate that can have effect on the accuracy of measuring ergosterol. Nevertheless, the measurements show that the production of alkylitaconic acids and the degradation of lignin, measured as Cα-oxidation and Ph-C0-2/Ph-C3 ratio, both increase independent of the fungal growth.

Preferential lignin degradation by the fungi was also confirmed by microscopy using a combination of Safranin O and Astra Blue staining based on the method of Srebotnik and Messner (1994). Astra blue only stains cellulose in the absence of lignin and is an indirect measure for selective lignin degradation (Srebotnik and Messner, 1994). This indicates that cell walls consisted of a high lignin matrix where cellulose is mostly masked and not accessible. The degradation of plant cell walls from within the cells is often seen in fungi that selectively degrade lignin (Schwarze, 2007). In the current study Safranin O and Astra Blue staining did not stain fungal hyphae. Toluidine Blue showed the presence of fungal hyphae in treated wheat straw and oak wood chips. The presence of subcellular details in hyphae shows that fixation and embedding of the material was done properly. However, the presence of Technovit 7100 may prevent staining of fungal hyphae by Safranin O and Astra Blue.

Enzymatic saccharification was conducted on wheat straw after 8 weeks of fungal treatment. As shown by the chemical analysis and the IVGP measurements, *C. subvermispora* reached an optimal IVGP and lignin degradation after 5 weeks of treatment, after which the fungus started to degrade cellulose. As a result, less cellulose was available for enzymatic saccharification after 8 weeks of treatment, resulting in a lower glucose release upon CM incubation than in *L. edodes* treated wheat straw. Interestingly, the mixture of cellulases contained some xylanase activity, since xylose was released after CM incubation. Here the terms glucose and xylose release are used, while in other studies the term sugar yield is used. Sugar yield is the amount of released sugar from the total amount of sugar present in the biomass (Wan and Li, 2011). In the current study, the initial amount of glucan and xylan in wheat straw was not measured, therefore, sugar yields could not be calculated. In the scientific literature, only a 2 - 25% increase in glucose yield and a 10% increase in xylose yield were reported in wheat straw after 35 days of *C. subvermispora* treatment (Cianchetta et al., 2014; Wan and Li, 2011). In the current study, *C. subvermispora* treatment resulted in 163% more glucose and 341% more xylose compared to untreated wheat straw, indicating that *C. subvermispora* made relatively more cellulose accessible for enzymes compared to untreated wheat straw. However, comparison with the scientific literature is difficult, since in the current study different enzymes and batches of wheat straw were used and sugar yields cannot be calculated.

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

Unlike wheat straw, oak wood chips probably did not contain easily accessible nutrients, since the IVGP did not decrease in the first week. In addition, ADL degradation by *L. edodes* and *C. subvermispora* started already after 2 weeks of treatment and the L/C ratio numerically decreased already after 1 week of fungal treatment. Also, hemicellulose degradation only started after 2 weeks of treatment, while in wheat straw it started in the

first week. This suggests that hemicellulose is less accessible in oak wood chips. The lower accessibility in oak wood chips can be explained by incomplete fungal colonization due to the dense structure of oak wood chips as observed by microscopy. Colonization, and delignification, not only requires physical space for the fungus to grow, but also oxygen within the tissue. The fact that the percentage of Cα-oxidized lignin in wood chips does not clearly increase upon fungal treatment, suggests that the availability of oxygen is the limiting factor. To increase the availability of oxygen, the wood structure has first to be degraded to allow oxygen to enter before further degradation and growth can occur. This stepwise "delignification and colonization" requires a longer treatment time for biomass with a dense structure like oak wood. A strategy to decrease treatment time is to increase the surface to volume ratio of dense biomass to allow for more entry points for fungi.

The second phase in the fungal treatment of oak wood chips was characterized by little changes in composition of the substrate after 4 weeks of treatment. Ergosterol data showed that *C. subvermispora* only grew during the first week of colonization. On oak wood chips, in contrast to wheat straw, no further growth of the fungus was observed. Similarly, Messner et al. (1998) showed a plateau in ergosterol development during *C. subvermispora* treatment of oak wood chips. The fungal growth stopped between 6 and 14 days to continue again afterwards (Messner et al., 1998). These authors confirmed this observation by the temperature development, as the temperature did not change during the plateau period in ergosterol. Messner et al. (1998) suggested that lignin degradation should take place before carbohydrates can be degraded by the fungus. Lignin is degraded by *C. subvermispora* without growing. The production of alkylitaconic acids shows that the fungus is producing secondary metabolites without active growth. The fungus cannot grow until the carbohydrates are accessible, meaning that lignin should be degraded first. Messner et al. (1998) described manganese peroxidase activity to be high during the plateau in the ergosterol data. This indicates that lignin degradation is the first step in the degradation process by *C. subvermispora*. However, in the current study *C. subvermispora* degraded lignin and hemicellulose without changing the cellulose content in both wheat straw and oak wood chips during the plateau period in ergosterol.

5.5 Conclusions

The white rot fungi *C. subvermispora* and *L. edodes* preferentially degrade lignin without changing the cellulose content during growth on wheat straw and oak wood chips. Most changes occurred during the first 4 weeks of fungal treatment, and in general terms, *C. subvermispora* degraded more lignin than *L. edodes*. Both fungi have a different strategy in degrading the lignocellulosic materials. *L. edodes* continuously grows and degrades lignin during the growth, while *C. subvermispora* colonizes the material predominantly during the first week and degrades lignin and hemicellulose without growing. The density of biomass limits the growth of fungi. As a result of the

selective lignin degradation, the IVGP and the sugars released upon enzymatic saccharification increases.

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Supplementary data

Table S5.1 Identities and relative abundances of compounds found with Py-GC/MS in wheat straw treated with *C. subvermispora*.

	Compound	Origin	T=0	T=1	T=2	T=3	T=4	T=5	T=6	T=7	T=8
1	(2H)-furan-3-one	C	1.8^{e}	2.2^{de}	2.9^{cde}	3.2^{cd}	4.1^{bc}	4.6^{ab}	4.7^{ab}	4.8^{ab}	5.7^{a}
2	Propanal	C	7.4	8.0	8.3	7.9	7.7	8.3	8.0	9.2	9.0
3	Furfural	C	2.5^{d}	3.3^{cd}	8.8^{ab}	11.3^{b}	18.2^{a}	21.5^{a}	19.9^{a}	18.7^{a}	18.0^{a}
4	2-acetylfuran	C	0.5	0.4	0.5	0.5	0.7	0.6	0.6	0.9	0.5
5	2,3 dihydro-5-methylfuran-2-one	C	5.2	5.8	7.1	5.7	5.9	6.4	6.5	7.0	8.6
6	5-methyl-2-furfuraldehyde	C	0.5^{d}	0.6^{d}	0.8^{cd}	0.8^{bcd}	1.2^{abc}	1.2^{abc}	1.5^{a}	1.5^{a}	1.4^{ab}
7	(5H)-furan-2-one	C	2.3^{e}	2.5^{de}	2.9^{dec}	3.3^{bcd}	3.6^{abc}	3.4^{abcd}	3.9^{ab}	4.2^{ab}	4.3^{a}
8	4-hydroxy-5,6-dihydro-(2H)-pyran-2-one	C	2.1^{b}	2.3^{b}	5.3^{ab}	7.2^{ab}	10.2^{a}	7.7^{ab}	9.4^{a}	8.4^{a}	4.6^{ab}
9	2,3-dimethylcyclopenten-1-one	C	0.2^{b}	0.2^{b}	0.3^{ab}	0.3^{ab}	0.3^{ab}	0.3^{ab}	0.3^{ab}	0.3^{ab}	0.4^{a}
10	2-hydroxy-3-methyl-2-cyclopenten-1-one	C	5.4	4.9	4.3	4.8	4.8	5.0	5.4	5.5	6.4
11	Phenol	LH	1.3	1.5	1.4	1.2	1.3	1.3	1.1	1.0	2.0
12	Guaiacol	LG	9.5	9.1	9.1	9.7	10.0	10.6	10.1	9.9	12.2
13	3-hydroxy-2-methyl-(4H)-pyran-4-one	C	0.6	0.8	0.9	0.9	1.0	1.3	1.3	1.4	1.9
14	4-hydroxymethyl-1,4-butyrolactone	C	0.5^{b}	0.8^{ab}	1.2^{a}	1.2^{a}	1.2^{a}	1.3^{a}	1.1^{ab}	1.3^{a}	1.2^{ab}
15	4-methylguaiacol	LG	3.9^{a}	3.7^{a}	3.0^{ab}	2.5^{bc}	1.7^{cd}	1.3^{d}	1.0^{d}	0.8^{d}	1.0^{d}
16	4-ethylphenol	LH	0.3^{ab}	0.3^{a}	0.2^{b}	0.2^{b}	0.1^{c}	0.1^{c}	0.1^{c}	0.1^{c}	0.1^{c}
17	4-ethylguaiacol	LG	2.1^{a}	1.8^{a}	1.6^{ab}	1.4^{abc}	0.8^{bcd}	0.7^{cd}	0.6^{cd}	0.5^{d}	0.3^{d}
18	5-hydroxymethyl-2-tetrahydrofuraldehyde-3-one	C	0.0^{b}	0.0^{b}	0.0^{b}	0.0^{b}	0.0^{b}	0.1^{ab}	0.3^{a}	0.3^{ab}	0.3^{a}
19	4-vinylguaiacol	LG	20.8^{a}	18.3^{a}	12.1^{b}	11.5^{b}	6.3^{c}	4.5^{cd}	4.6^{cd}	4.5^{cd}	1.6^{d}
20	4-vinylphenol	LH	7.2^{a}	7.1^{a}	3.8^{b}	3.3^{bc}	1.9^{cd}	1.5^{d}	1.5^{d}	1.2^{d}	0.8^{d}
21	Eugenol	LG	0.7^{a}	0.6^{b}	0.4^{c}	0.3^{c}	0.2^{d}	0.1^{de}	0.1^{de}	0.1^{de}	0.1^{e}
22	Syringol	LS	7.1^{a}	7.2^{a}	8.2^{a}	7.4^{a}	5.4^{b}	4.9^{bc}	4.1^{bc}	4.1^{bc}	3.9^{c}
23	5-hydroxymethyl-2-furfuraldehyde	C	0.5^{b}	0.7^{b}	0.9^{b}	1.3^{b}	1.6^{b}	1.7^{b}	1.8^{b}	2.0^{ab}	3.4^{a}
24	*cis*-isoeugenol	LG	0.5^{a}	0.4^{b}	0.3^{c}	0.2^{c}	0.2^{d}	0.1^{de}	0.1^{de}	0.1^{de}	0.1^{e}
25	*trans*-isoeugenol	LG	2.7^{a}	2.6^{a}	1.7^{b}	1.3^{bc}	0.9^{cd}	0.6^{d}	0.6^{d}	0.6^{d}	0.4^{d}
26	4-methylsyringol	LS	2.1^{a}	2.0^{a}	1.8^{ab}	1.3^{bc}	0.7^{cd}	0.5^{d}	0.5^{d}	0.5^{d}	0.3^{d}
27	Vanillin	LG	1.5	1.3	1.5	1.6	1.6	1.3	1.3	1.1	0.7
28	4-ethylsyringol	LS	0.7^{a}	0.6^{ab}	0.5^{abc}	0.4^{abc}	0.2^{abc}	0.1^{c}	0.2^{bc}	0.4^{abc}	0.0^{c}
29	vanillic acid methyl ester	LG	0.0^{c}	0.0^{c}	0.1^{bc}	0.3^{abc}	0.4^{a}	0.4^{ab}	0.5^{a}	0.6^{a}	0.4^{ab}
30	Acetovanillone	LG	0.2^{c}	0.4^{bc}	0.7^{ab}	0.7^{a}	0.6^{ab}	0.6^{ab}	0.5^{abc}	0.7^{ab}	0.4^{abc}
31	4-vinylsyringol	LS	2.6^{a}	2.6^{a}	1.7^{ab}	1.1^{bc}	0.5^{c}	0.3^{c}	0.3^{c}	0.2^{c}	0.4^{c}
32	Guaiacylacetone	LG	0.6	0.7	0.6	0.5	0.4	0.4	0.3	0.3	0.4
33	4-allyl-syringol	LS	0.4^{a}	0.4^{a}	0.3^{b}	0.2^{bc}	0.1^{cd}	0.0^{d}	0.1^{d}	0.0^{d}	0.0^{d}
34	Propiovanillone	LG	0.0^{b}	0.0^{b}	0.0^{b}	0.0^{b}	0.0^{b}	0.4^{a}	0.4^{a}	0.4^{a}	0.5^{a}
35	*cis*-propenylsyringol	LS	0.3^{a}	0.3^{a}	0.2^{b}	0.2^{bc}	0.1^{cd}	0.0^{d}	0.1^{d}	0.0^{d}	0.0^{d}
36	*trans*-propenylsyringol	LS	2.3^{a}	2.4^{a}	1.4^{b}	0.9^{bc}	0.4^{cd}	0.2^{d}	0.2^{d}	0.2^{d}	0.2^{d}
37	Syringaldehyde	LS	0.4^{ab}	0.3^{abc}	0.4^{a}	0.3^{abcd}	0.2^{bcde}	0.2^{cde}	0.1^{e}	0.1^{e}	0.1^{de}
38	Levoglucosane	C	2.3^{d}	2.3^{d}	3.1^{cd}	3.4^{cd}	4.5^{bcd}	5.1^{abc}	6.1^{ab}	6.3^{ab}	7.2^{a}
39	syringic acid methyl ester	LS	0.1^{c}	0.1^{c}	0.2^{a}	0.2^{ab}	0.1^{abc}	0.2^{abc}	0.1^{abc}	0.1^{bc}	0.1^{bc}
40	Acetosyringone	LS	0.8^{abcd}	0.9^{ab}	1.1^{a}	0.9^{abc}	0.7^{bcde}	0.6^{bcde}	0.5^{cde}	0.5^{de}	0.5^{e}
41	Syringylacetone	LS	0.3^{ab}	0.4^{a}	0.5^{ab}	0.3^{ab}	0.3^{ab}	0.3^{ab}	0.2^{ab}	0.2^{b}	0.2^{ab}
42	Propiosyringone	LS	0.1	0.1	0.1	0.1	0.1	0.2	0.2	0.1	0.1

Values with different superscripts within row are significantly ($P<0.05$) different. [1]C, carbohydrate-derived compounds; LH, *p*-hydroxycinnamyl lignin-derived compounds; LG, guaiacyl-lignin derived compounds; S, syringyl-lignin derived compounds.

Table S5.2 Identities and relative abundances of compounds found with Py-GC/MS in wheat straw treated with *L. edodes*.

	Compound	Origin[1]	T=0	T=1	T=2	T=3	T=4	T=5	T=6	T=7	T=8
1	(2H)-furan-3-one	C	1.8^{c}	2.1^{c}	2.6^{bc}	3.3^{ab}	3.3^{ab}	3.3^{ab}	3.7^{a}	3.6^{ab}	4.2^{a}
2	Propanal	C	7.4^{c}	8.3^{bc}	8.5^{bc}	8.8^{bc}	10.1^{ab}	9.1^{bc}	9.3^{bc}	9.2^{bc}	12.0^{a}
3	Furfural	C	2.5^{d}	3.2^{d}	5.5^{c}	7.6^{abc}	6.9^{bc}	7.5^{bc}	8.5^{ab}	9.7^{a}	7.2^{bc}
4	2-acetylfuran	C	0.5	0.3	0.5	0.7	0.5	0.8	0.9	0.8	1.3
5	2,3 dihydro-5-methylfuran-2-one	C	5.2^{c}	5.2^{c}	5.6^{c}	6.5^{bc}	10.2^{a}	9.5^{ab}	10.2^{a}	9.5^{ab}	12.1^{a}
6	5-methyl-2-furfuraldehyde	C	0.5^{b}	0.5^{b}	0.5^{b}	0.9^{ab}	1.1^{a}	0.9^{ab}	1.2^{a}	1.1^{a}	1.2^{a}
7	(5H)-furan-2-one	C	2.3^{d}	2.6^{cd}	2.8^{bcd}	3.4^{bcd}	3.4^{bcd}	3.6^{bc}	4.0^{ab}	3.8^{ab}	4.9^{a}
8	4-hydroxy-5,6-dihydro-(2H)-pyran-2-one	C	2.1	2.6	4.0	5.1	3.2	4.3	4.9	4.6	3.0
9	2,3-dimethylcyclopenten-1-one	C	0.2	0.3	0.3	0.3	0.6	0.4	0.3	0.3	0.6
10	2-hydroxy-3-methyl-2-cyclopenten-1-one	C	5.4^{cde}	4.8^{e}	5.0^{de}	5.7^{cde}	6.1^{bcde}	6.9^{bc}	6.7^{bcd}	7.6^{b}	9.8^{a}
11	Phenol	LH	1.3	1.3	1.3	1.3	1.0	1.1	1.1	1.0	1.9
12	Guaiacol	LG	9.5^{abc}	8.8^{c}	9.1^{bc}	9.6^{abc}	10.6^{abc}	10.9^{ab}	10.8^{ab}	10.7^{abc}	11.4^{a}
13	3-hydroxy-2-methyl-(4H)-pyran-4-one	C	0.6	0.5	0.7	0.9	1.1	1.0	1.4	1.2	1.5
14	4-hydroxymethyl-1,4-butyrolactone	C	0.5^{ab}	0.8^{ab}	1.0^{ab}	1.1^{a}	0.9^{ab}	0.9^{ab}	1.0^{ab}	0.7^{ab}	0.3^{a}
15	4-methylguaiacol	LG	3.9^{a}	3.7^{ab}	3.5^{ab}	3.0^{bc}	2.6^{c}	2.6^{c}	2.4^{cd}	2.4^{cd}	1.6^{d}
16	4-ethylphenol	LH	0.3^{ab}	0.3^{a}	0.3^{a}	0.2^{abc}	0.2^{bc}	0.2^{abc}	0.2^{cd}	0.2^{cd}	0.1^{d}
17	4-ethylguaiacol	LG	2.1^{a}	1.8^{ab}	1.9^{ab}	1.6^{ab}	1.4^{abc}	1.4^{abc}	1.2^{bc}	1.4^{abc}	0.8^{c}
18	5-hydroxymethyl-2-tetrahydrofuraldehyde-3-one	C	0.0^{b}	0.0^{b}	0.0^{b}	0.0^{b}	0.0^{b}	0.4^{ab}	0.6^{ab}	0.4^{b}	1.1^{a}
19	4-vinylguaiacol	LG	20.8^{a}	19.5^{ab}	15.8^{bc}	12.3^{cd}	9.8^{de}	10.0^{d}	8.6^{de}	8.5^{de}	5.8^{e}
20	4-vinylphenol	LH	7.2^{a}	8.1^{a}	5.3^{b}	3.9^{c}	3.1^{cd}	3.0^{cd}	2.6^{d}	2.5^{de}	1.6^{e}
21	Eugenol	LG	0.7^{a}	0.6^{a}	0.5^{b}	0.4^{c}	0.3^{cd}	0.3^{cd}	0.2^{de}	0.3^{cd}	0.2^{e}
22	Syringol	LS	7.1^{ab}	6.7^{b}	7.3^{a}	7.2^{a}	7.3^{a}	6.7^{b}	5.7^{c}	5.1^{d}	4.4^{e}
23	5-hydroxymethyl-2-furfuraldehyde	C	0.5	0.7	1.0	1.3	0.9	1.0	0.9	1.2	1.3
24	*cis*-isoeugenol	LG	0.5^{a}	0.5^{a}	0.4^{b}	0.3^{bc}	0.2^{d}	0.3^{cd}	0.2^{de}	0.3^{cd}	0.2^{e}
25	*trans*-isoeugenol	LG	2.7^{a}	2.4^{ab}	2.1^{bc}	1.7^{cd}	1.4^{de}	1.4^{de}	1.2^{ef}	1.4^{de}	0.9^{f}
26	4-methylsyringol	LS	2.1^{a}	1.9^{a}	1.9^{ab}	1.5^{abc}	1.2^{bcd}	1.1^{cd}	0.9^{cd}	1.1^{cd}	0.6^{d}
27	Vanillin	LG	1.5	1.4	1.4	1.5	1.4	1.7	1.4	1.5	1.2
28	4-ethylsyringol	LS	0.7^{a}	0.6^{abc}	0.6^{ab}	0.4^{abcd}	0.3^{cde}	0.3^{bcde}	0.3^{de}	0.3^{de}	0.2^{e}
29	vanillic acid methyl ester	LG	0.0	0.0	0.2	0.1	0.2	0.3	0.3	0.6	0.5
30	Acetovanillone	LG	0.2^{c}	0.2^{c}	0.6^{ab}	0.7^{a}	0.7^{a}	0.7^{a}	0.7^{a}	0.6^{a}	0.3^{bc}
31	4-vinylsyringol	LS	2.6^{a}	2.7^{a}	2.0^{ab}	1.3^{bc}	1.1^{cd}	0.8^{cd}	0.6^{cd}	0.5^{d}	0.5^{d}
32	Guaiacylacetone	LG	0.6	0.6	0.6	0.5	0.5	0.5	0.4	0.4	0.4
33	4-allyl-syringol	LS	0.4^{a}	0.4^{a}	0.3^{b}	0.2^{c}	0.2^{cd}	0.1^{d}	0.1^{de}	0.1^{d}	0.1^{e}
34	Propiovanillone	LG	0.0^{b}	0.0^{b}	0.0^{b}	0.0^{b}	0.2^{a}	0.2^{a}	0.3^{a}	0.3^{a}	0.2^{a}
35	*cis*-propenylsyringol	LS	0.3^{a}	0.3^{a}	0.3^{ab}	0.2^{bc}	0.1^{bcd}	0.2^{bcde}	0.1^{cd}	0.1^{cd}	0.1^{d}
36	*trans*-propenylsyringol	LS	2.3^{a}	2.2^{a}	1.6^{b}	1.1^{c}	0.9^{cd}	0.7^{d}	0.6^{de}	0.6^{de}	0.4^{e}
37	Syringaldehyde	LS	0.4^{a}	0.4^{a}	0.3^{ab}	0.3^{ab}	0.2^{ab}	0.2^{ab}	0.1^{ab}	0.2^{ab}	0.0^{b}
38	Levoglucosane	C	2.3^{b}	2.4^{b}	3.3^{ab}	3.9^{ab}	4.7^{ab}	4.1^{ab}	5.1^{a}	5.4^{a}	5.5^{a}
39	syringic acid methyl ester	LS	0.1^{bc}	0.1^{c}	0.1^{abc}	0.2^{abc}	0.2^{a}	0.2^{a}	0.2^{a}	0.2^{ab}	0.1^{abc}
40	Acetosyringone	LS	0.8^{abc}	0.7^{bc}	1.0^{a}	1.0^{ab}	1.0^{a}	0.9^{ab}	0.8^{abc}	0.7^{bc}	0.6^{c}
41	Syringylacetone	LS	0.3^{ab}	0.3^{ab}	0.3^{ab}	0.3^{ab}	0.4^{a}	0.3^{ab}	0.3^{ab}	0.2^{b}	0.2^{b}
42	Propiosyringone	LS	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

Values with different superscripts within row are significantly ($P<0.05$) different. [1]C, carbohydrate-derived compounds; LH, *p*-hydroxycinnamyl lignin-derived compounds; LG, guaiacyl-lignin derived compounds; S, syringyl-lignin derived compounds.

Table S5.3 Identities and relative abundances of compounds found with Py-GC/MS in wood chips treated with *C. subvermispora*.

	Compound	Origin[1]	T=0	T=1	T=2	T=3	T=4	T=5	T=6	T=7	T=8
1	(2H)-furan-3-one	C	2.0	2.4	2.3	2.6	2.8	2.4	2.4	2.7	2.8
2	Propanal	C	1.4	1.8	1.1	1.1	1.8	2.1	1.4	1.4	1.9
3	Furfural	C	4.1	8.9	8.9	9.3	9.2	8.3	9.3	9.1	8.6
4	2-acetylfuran	C	0.3	0.2	0.3	0.3	0.4	0.4	0.4	0.4	0.4
5	2,3 dihydro-5-methylfuran-2-one	C	7.1	5.4	6.0	6.8	7.8	7.9	7.4	7.4	8.3
6	5-methyl-2-furfuraldehyde	C	1.2	1.7	2.0	2.5	2.3	2.4	3.2	3.3	2.7
7	(5H)-furan-2-one	C	2.1	2.2	2.2	2.2	3.1	2.8	2.6	2.4	2.9
8	4-hydroxy-5,6-dihydro-(2H)-pyran-2-one	C	3.0	5.3	4.1	4.9	6.7	5.4	5.8	5.4	6.5
9	2,3-dimethylcyclopenten-1-one	C	0.2	0.2	0.2	0.2	0.3	0.3	0.3	0.2	0.3
10	2-hydroxy-3-methyl-2-cyclopenten-1-one	C	6.4	5.3	6.4	7.0	8.1	7.7	8.2	7.8	8.8
11	Phenol	LH	0.4	0.4	0.3	0.3	0.3	0.3	0.7	0.7	0.6
12	Guaiacol	LG	5.5	8.0	8.1	11.6	13.3	12.9	14.6	13.1	13.6
13	3-hydroxy-2-methyl-(4H)-pyran-4-one	C	0.9	0.4	1.4	2.0	2.3	2.3	2.3	2.3	2.6
14	4-hydroxymethyl-1,4-butyrolactone	C	0.2	0.2	0.0	0.0	0.1	0.0	0.0	0.0	0.0
15	4-methylguaiacol	LG	5.7	5.6	5.4	5.3	3.4	4.5	3.9	4.2	3.4
16	4-ethylphenol	LH	0.8	0.8	1.2	0.2	1.0	0.6	0.3	0.3	0.4
17	4-ethylguaiacol	LG	2.0	1.8	2.6	1.4	1.1	1.3	1.4	1.4	1.2
18	5-hydroxymethyl-2-tetrahydrofuraldehyde-3-one	C	1.4	0.7	0.6	1.5	1.0	0.8	1.1	1.1	0.8
19	4-vinylguaiacol	LG	9.0	7.7	7.6	5.9	5.1	5.3	5.7	6.1	5.1
20	4-vinylphenol	LH	0.2	0.2	0.1	0.8	0.4	1.0	0.6	0.4	0.3
21	Eugenol	LG	0.9	0.7	0.8	0.5	0.5	0.4	0.6	0.5	0.4
22	Syringol	LS	10.2	11.0	9.3	7.8	9.4	8.4	8.3	8.2	8.5
23	5-hydroxymethyl-2-furfuraldehyde	C	0.8	0.9	0.3	2.1	2.8	3.1	2.1	3.0	3.5
24	*cis*-isoeugenol	LG	0.7	0.6	0.6	0.4	0.3	0.3	0.4	0.4	0.3
25	trans-isoeugenol	LG	3.4	2.6	2.6	1.6	1.3	1.2	1.5	1.5	1.2
26	4-methylsyringol	LS	6.5	5.5	5.1	2.5	2.2	2.2	2.3	2.1	1.8
27	Vanillin	LG	0.6	0.8	0.7	0.4	0.6	0.5	0.6	0.6	0.6
28	4-ethylsyringol	LS	1.4	0.8	1.0	0.4	0.4	0.4	0.4	0.5	0.3
29	vanillic acid methyl ester	LG	0.0	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.3
30	Acetovanillone	LG	0.5	0.5	0.3	0.3	0.3	0.3	0.4	0.4	0.4
31	Vinylsyringol	LS	4.1	2.8	2.9	1.2	1.2	1.3	1.3	1.0	1.1
32	Guaiacylacetone	LG	0.3	0.4	0.3	0.3	0.3	0.2	0.3	0.4	0.2
33	4-allyl-syringol	LS	1.1	0.7	0.7	0.3	0.3	0.3	0.3	0.3	0.3
34	Propiovanillone	LG	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
35	*cis*-propenylsyringol	LS	0.7	0.5	0.6	0.2	0.2	0.3	0.3	0.3	0.2
36	*trans*-propenylsyringol	LS	5.1	3.7	3.8	1.3	1.4	1.5	1.4	1.3	1.3
37	Syringaldehyde	LS	0.6	0.6	0.7	0.3	0.4	0.2	0.2	0.2	0.2
38	Levoglucosane	C	7.7	6.5	7.8	13.5	6.9	9.5	7.1	8.9	7.5
39	syringic acid methyl ester	LS	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
40	Acetosyringone	LS	0.7	1.2	0.5	0.3	0.3	0.4	0.3	0.4	0.5
41	Syringylacetone	LS	0.7	0.7	0.8	0.4	0.4	0.6	0.4	0.4	0.4
42	Propiosyringone	LS	0.2	0.1	0.2	0.1	0.1	0.1	0.1	0.1	0.1

[1]C, carbohydrate-derived compounds; LH, *p*-hydroxycinnamyl lignin-derived compounds; LG, guaiacyl-lignin derived compounds; S, syringyl-lignin derived compounds.

Table S5.4 Identities and relative abundances of compounds found with Py-GC/MS in wood chips treated with *L. edodes*.

	Compound	Origin[1]	T=0	T=1	T=2	T=3	T=4	T=5	T=6	T=7	T=8
1	(2H)-furan-3-one	C	2.0	1.6	2.0	2.0	1.9	1.8	2.1	2.1	4.7
2	Propanal	C	1.4	1.4	0.9	1.5	2.4	1.9	1.5	2.4	2.9
3	Furfural	C	4.1	6.8	6.0	7.2	7.3	6.8	7.8	7.5	12.3
4	2-acetylfuran	C	0.3	0.4	0.4	0.5	0.6	0.5	0.5	0.6	0.4
5	2,3 dihydro-5-methylfuran-2-one	C	7.1	6.3	6.5	6.6	7.8	7.2	7.7	8.2	6.8
6	5-methyl-2-furfuraldehyde	C	1.2	2.0	2.1	1.8	2.3	2.0	2.2	2.6	1.7
7	(5H)-furan-2-one	C	2.1	1.7	1.9	2.0	3.4	2.2	2.4	2.3	2.5
8	4-hydroxy-5,6-dihydro-(2H)-pyran-2-one	C	3.0	4.3	4.8	5.6	4.2	4.9	5.4	4.2	4.2
9	2,3-dimethylcyclopenten-1-one	C	0.2	0.2	0.2	0.3	0.4	0.3	0.4	0.3	0.5
10	2-hydroxy-3-methyl-2-cyclopenten-1-one	C	6.4	6.7	7.4	7.9	8.5	8.7	9.4	9.7	9.7
11	Phenol	LH	0.4	0.3	0.2	0.3	0.2	0.4	0.5	0.5	0.4
12	Guaiacol	LG	5.5	8.0	9.4	11.7	11.7	14.7	15.9	15.9	13.8
13	3-hydroxy-2-methyl-(4H)-pyran-4-one	C	0.9	1.6	1.8	2.0	2.5	2.7	3.2	3.2	2.6
14	4-hydroxymethyl-1,4-butyrolactone	C	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
15	4-methylguaiacol	LG	5.7	7.7	6.5	7.8	4.9	6.6	4.2	5.1	3.7
16	4-ethylphenol	LH	0.8	1.2	0.3	0.2	0.3	0.3	0.5	0.4	0.5
17	4-ethylguaiacol	LG	2.0	2.2	1.7	2.6	1.7	1.6	1.3	1.3	1.4
18	5-hydroxymethyl-2-tetrahydrofuraldehyde-3-one	C	1.4	0.5	0.7	0.8	1.0	1.7	1.1	1.3	0.7
19	4-vinylguaiacol	LG	9.0	7.8	7.7	6.9	7.4	7.3	6.9	6.9	6.6
20	4-vinylphenol	LH	0.2	0.1	0.2	0.4	0.4	0.3	0.0	0.0	0.1
21	Eugenol	LG	0.9	1.0	0.7	1.0	0.9	0.9	0.8	0.7	0.7
22	Syringol	LS	10.2	9.6	12.0	10.6	10.9	9.6	9.9	10.2	10.4
23	5-hydroxymethyl-2-furfuraldehyde	C	0.8	0.5	0.6	0.5	0.5	0.8	0.5	0.4	0.5
24	*cis*-isoeugenol	LG	0.7	0.7	0.6	0.6	0.5	0.5	0.4	0.4	0.4
25	trans-isoeugenol	LG	3.4	2.7	2.7	2.2	2.0	1.9	1.7	1.7	1.8
26	4-methylsyringol	LS	6.5	5.6	5.9	3.0	3.1	2.7	2.3	2.1	2.1
27	Vanillin	LG	0.6	0.3	0.9	0.3	0.4	0.4	0.3	0.2	0.3
28	4-ethylsyringol	LS	1.4	1.0	0.9	0.6	0.6	0.5	0.7	0.4	0.3
29	vanillic acid methyl ester	LG	0.0	0.0	0.0	0.1	0.2	0.2	0.2	0.3	0.2
30	Acetovanillone	LG	0.5	0.3	0.4	0.2	0.3	0.3	0.3	0.4	0.2
31	Vinylsyringol	LS	4.1	2.9	2.3	1.6	1.8	1.6	1.3	1.1	1.1
32	Guaiacylacetone	LG	0.3	0.4	0.5	0.3	0.2	0.2	0.2	0.2	0.2
33	4-allyl-syringol	LS	1.1	0.7	0.6	0.4	0.4	0.3	0.4	0.3	0.3
34	Propiovanillone	LG	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
35	*cis*-propenylsyringol	LS	0.7	0.6	0.4	0.3	0.4	0.3	0.3	0.2	0.2
36	*trans*-propenylsyringol	LS	5.1	3.8	2.2	2.0	2.0	1.7	1.7	1.1	1.1
37	Syringaldehyde	LS	0.6	0.5	0.5	0.5	0.4	0.3	0.3	0.3	0.2
38	Levoglucosane	C	7.7	7.4	6.5	6.1	5.2	4.8	4.7	4.8	3.9
39	syringic acid methyl ester	LS	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
40	Acetosyringone	LS	0.7	0.5	0.9	0.5	0.4	0.4	0.3	0.3	0.3
41	Syringylacetone	LS	0.7	0.7	0.6	0.7	0.6	0.5	0.5	0.5	0.5
42	Propiosyringone	LS	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1

[1]C, carbohydrate-derived compounds; LH, *p*-hydroxycinnamyl lignin-derived compounds; LG, guaiacyl-lignin derived compounds; S, syringyl-lignin derived compounds.

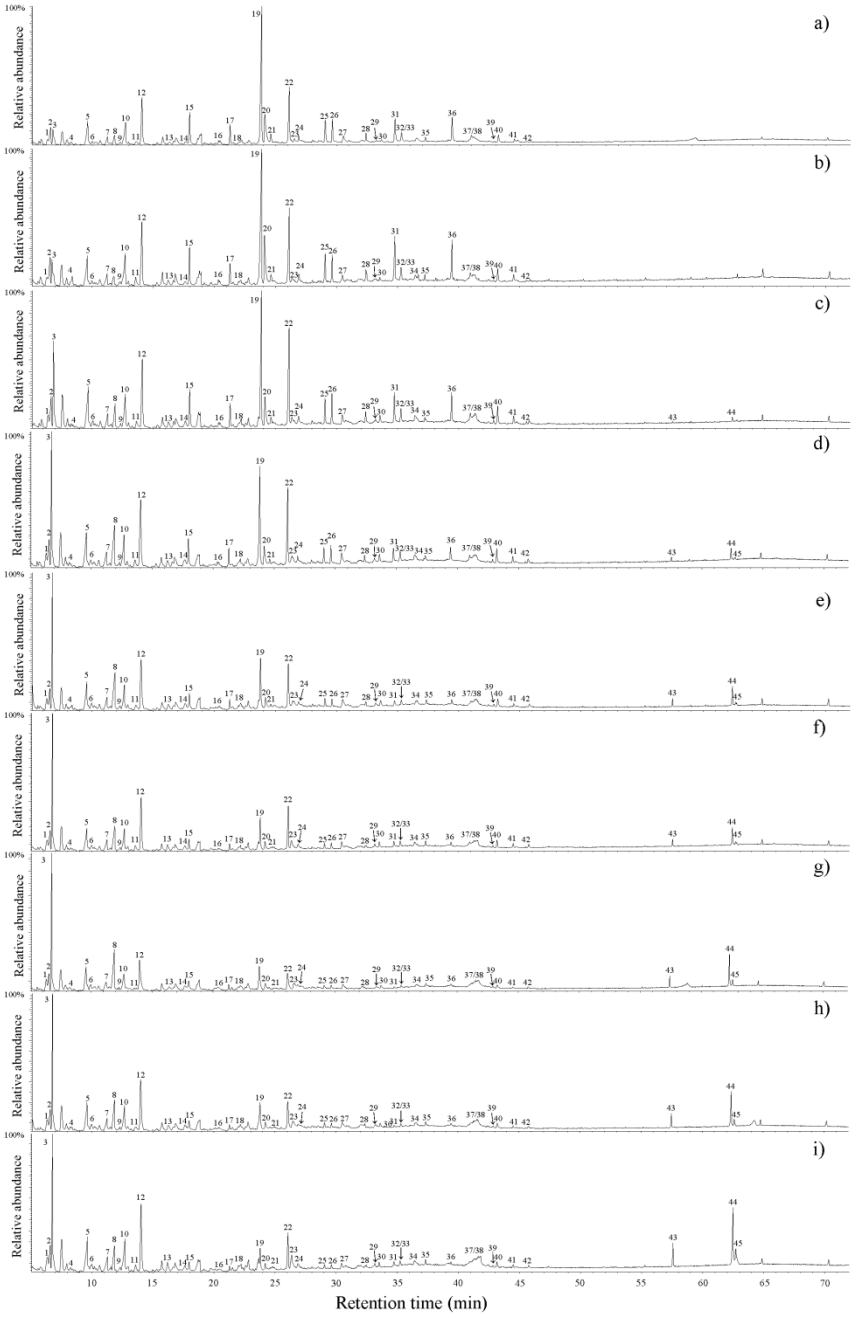

Figure S5.1 Pyrograms of wheat straw treated with *C. subvermispora* for different incubation times: a) 0 weeks, control (autoclaved, untreated wheat straw), b) 1 week, c) 2 weeks, d) 3 weeks, e) 4 weeks, f) 5 weeks, g) 6 weeks, h) 7 weeks, i) 8 weeks.

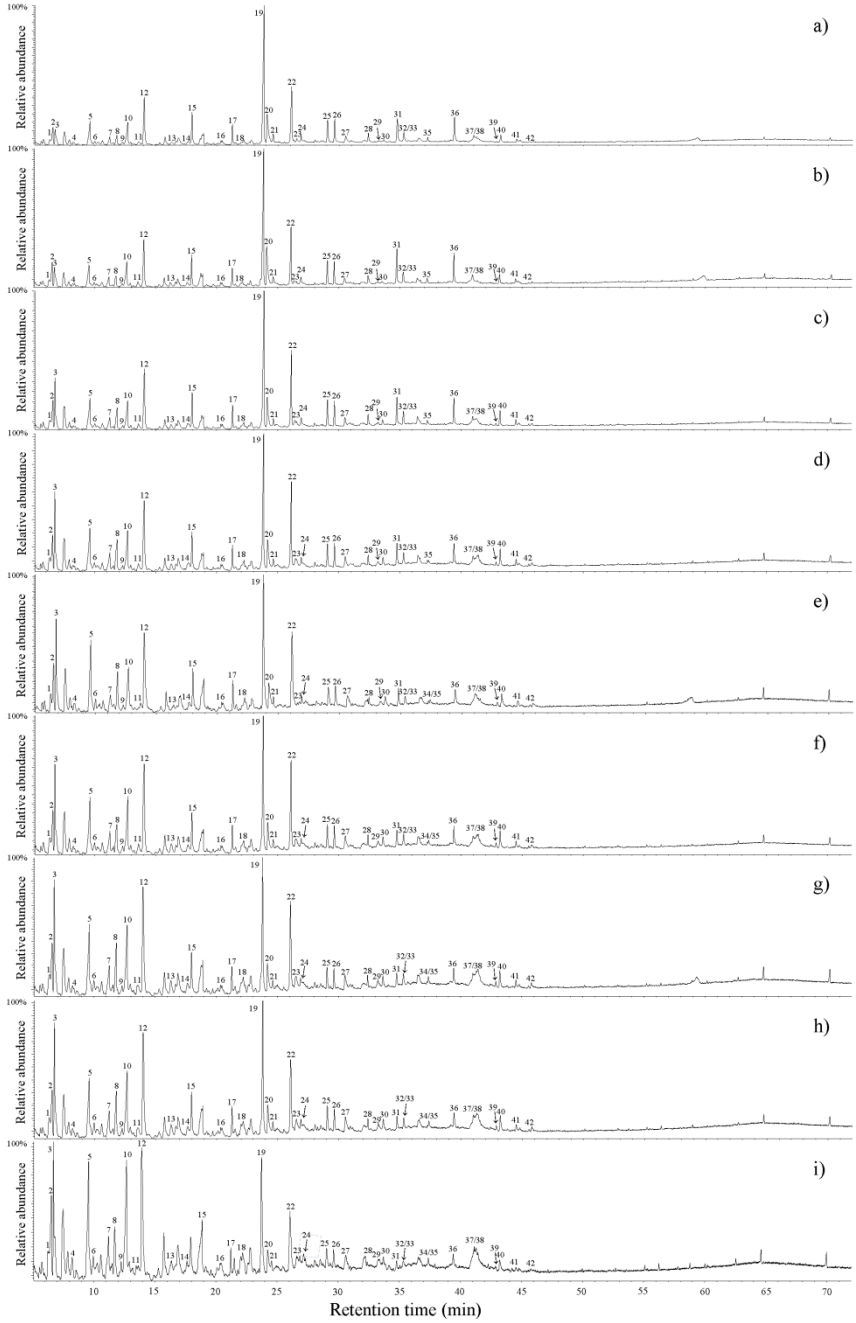

Figure S5.2 Pyrograms of wheat straw treated with *L. edodes* for different incubation times: a) 0 weeks, control (autoclaved, untreated wheat straw), b) 1 week, c) 2 weeks, d) 3 weeks, e) 4 weeks, f) 5 weeks, g) 6 weeks, h) 7 weeks, i) 8 weeks.

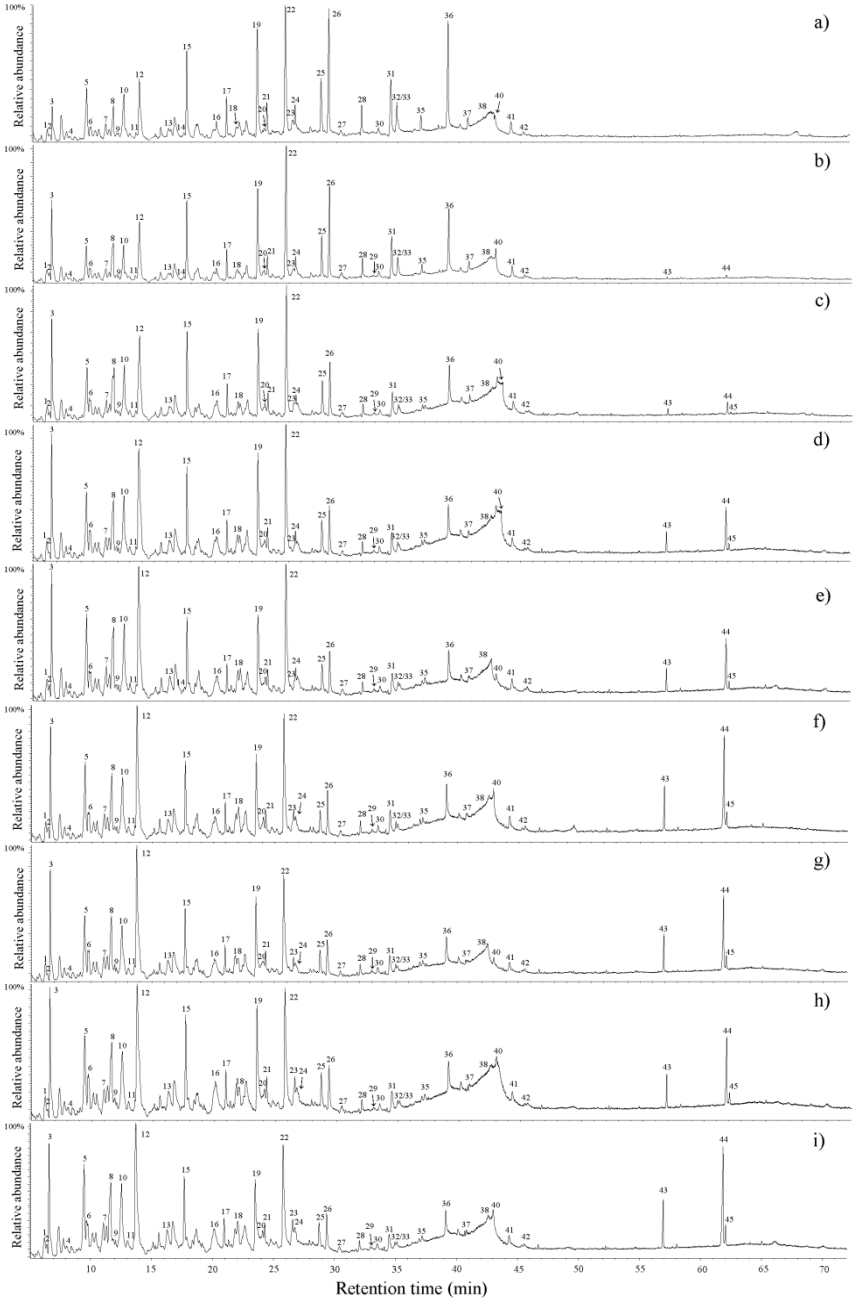

Figure S5.3 Pyrograms of wood chips treated with *C. subvermispora* for different incubation times: a) 0 weeks, control (autoclaved, untreated wheat straw), b) 1 week, c) 2 weeks, d) 3 weeks, e) 4 weeks, f) 5 weeks, g) 6 weeks, h) 7 weeks, i) 8 weeks.

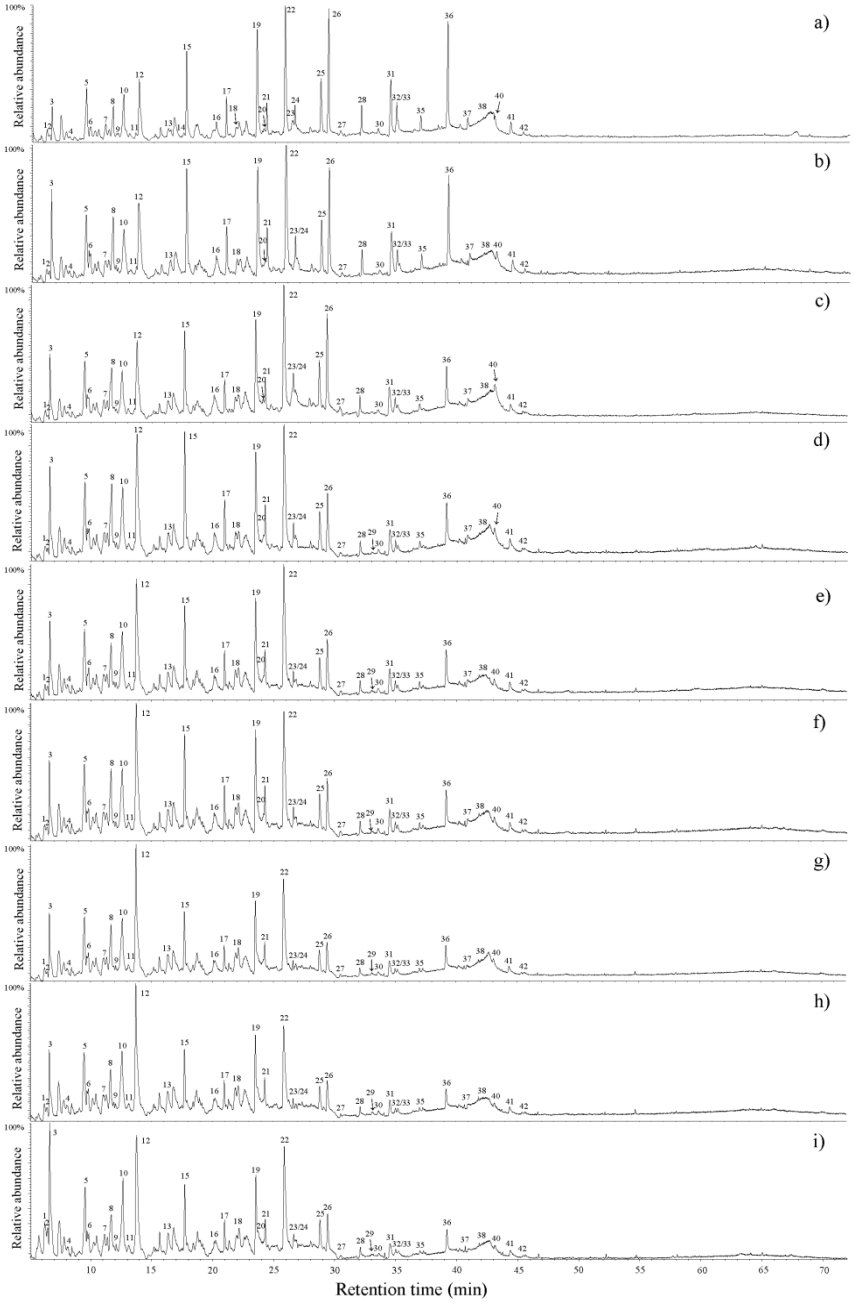

Figure S5.4 Pyrograms of wood chips treated with *L. edodes* for different incubation times: a) 0 weeks, control (autoclaved, untreated wheat straw), b) 1 week, c) 2 weeks, d) 3 weeks, e) 4 weeks, f) 5 weeks, g) 6 weeks, h) 7 weeks, i) 8 weeks.

CHAPTER 6

The effect of adding urea, manganese and linoleic acid to wheat straw and wood chips on lignin degradation by fungi and subsequent *in vitro* rumen degradation

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Abstract

The aim of this study was optimizing *Ceriporiopsis subvermispora* and *Lentinula edodes* pre-treatment of wheat straw and wood chips by adding urea, manganese and linoleic acid. Optimization was defined as more lignin degradation and an increase in *in vitro* gas production (IVGP), which is a model for rumen degradation, in comparison to fungal treatment without additives. First urea, manganese and linoleic acid were added separately to *C. subvermispora* or *L. edodes* treatment of wheat straw and wood chips. Only manganese and linoleic acid addition improved lignin degradation and IVGP compared to the same treatment without additives. Mn (150 µg /g wheat straw) influenced *C. subvermispora* treatment most. A combination of manganese and linoleic acid was also applied since both act on manganese peroxidase. This combination did indeed increase lignin degradation in wheat straw by *C. subvermispora*, but IVGP was not changed. None of the additions had a significant effect on the other fungus-substrate combinations tested here.

6.1 Introduction

Cellulose is a polymer consisting of glucose, and therefore a valuable ingredient in a ruminant's diet. Ruminants are specialists in digesting plant materials. However the cellulose in plant materials is unavailable for rumen degradation, since it is bound to lignin in the lignocellulosic matrix. Lignin is a polymer which can only be degraded by certain fungi and bacteria under aerobic circumstances (Ahmad et al., 2010; Isroi et al., 2011). As a consequence, lignin cannot be degraded by rumen microbes in the anaerobic rumen and the amount of lignin is negatively correlated to rumen degradability (Arora and Sharma, 2009). To make cellulose available for rumen microbes, lignin should be removed to increase the nutritional value of especially the plant cell walls.

Currently chemical and physical methods are used to pre-treat lignocellulosic materials (Sarnklong et al., 2010; Agbor et al., 2011). A cheap and environmental friendly alternative is a pre-treatment with white rot fungi (van Kuijk et al., 2015). Some white rot fungal species can selectively degrade lignin without degrading cellulose. In this way cellulose becomes available for further processing. Tuyen et al. (2012; 2013) demonstrated that *Ceriporiopsis subvermispora* and *Lentinula edodes* can selectively degrade lignin in lignocellulosic materials, such as wheat straw and sugarcane bagasse. The treatment described in those studies requires 6 to 7 weeks to obtain significant lignin degradation, while rumen degradability seemed not to have reached the optimum yet. This suggests a longer treatment time is needed to obtain optimal results. This long treatment time represents the major drawback of using fungal pre-treatment in practice. To make fungal pre-treatment competitive with current pre-treatments, optimization is needed to shorten the treatment time and to get more selective lignin degradation. Since white rot fungi also use hemicellulose during the vegetative growth, a minimization of the hemicellulose degradation will also improve the value of fungal treated lignocellulosic waste. There are several ways to improve the fungal treatment. One of the aspects is a faster colonization to speed up the process. A nitrogen source such as urea can be added to stimulate fast colonization and possibly fast delignification (Tripathi and Yadav, 1992). During and after colonization the selective lignin degrading fungi start to produce enzymes (Kirk and Farell, 1987). The main lignolytic enzyme produced by the very selective lignin degrading fungi *C. subvermispora* and *L. edodes* is manganese peroxidase (Saeki et al., 2011; Fernandez-Fueyo et al., 2012). This enzyme converts Mn^{2+} into Mn^{3+}, a radical that can attack the phenolic part of lignin (Hammel and Cullen 2008). Addition of manganese to the fungal pre-treatment may result in the formation of more Mn^{3+} radicals and consequently a faster attack of lignin (Kerem and Hadar, 1995). Previous research has shown that the Mn^{3+} generated by Mn peroxidase initiate the peroxidation of unsaturated lipids such as linoleic acid (Watanabe et al., 2000). These lipid hydroperoxides act as a MnP-oxidizing substrate in the place of H_2O_2 and can attack the non-phenolic parts of lignin (Enoki et al., 1999; Kapich et al., 1999). Addition of linoleic acid is shown to stimulate lipid peroxidation by manganese peroxidase (Cunha et al., 2010). However the effects of linoleic acid addition have not been tested under solid state fermentation conditions yet.

The aim of this study is to optimize *C. subvermispora* and *L. edodes* pre-treatment of wheat straw and wood chips using urea, manganese and linoleic acid as additives. The fungal pre-treatment will be regarded as optimized when more selective lignin degradation and an increase in *in vitro* rumen degradability are found within 4 weeks of treatment compared to the same treatment without additives.

6.2 Material and methods

6.2.1 Fungal strains and spawn preparation

The fungal strains *Ceriporiopsis subvermispora* (strain MES 13094) and *Lentinula edodes* (strain MES 11910) used in this experiment were preserved in liquid nitrogen. Initial culturing of the fungi was done on malt extract agar plates at 24 °C until mycelium was covering most of the plate surface. Spawn was prepared by adding pieces of colonized agar culture to sterilized sorghum grains and this was incubated at 24 °C until all grains were colonized by mycelium. The spawn was kept at 4 °C until further use.

6.2.2 Substrate preparation

Both wheat straw and wood chips (oak) were chopped into pieces of approximately 3 cm. Samples were taken as untreated non-autoclaved control. To the substrates an excess of water was added and left for 3 days to let the water fully penetrate into the material. After the excess of water was removed, the substrates were sprayed with each of the additives dissolved in ethanol (maximum 1 ml ethanol per kg wet substrate was added). Three experiments have been performed. In each experiment the treatments were performed in duplicate.

In the first experiment single additives were added to both substrates to investigate whether they have any effects on fungal treatment. Urea (108487, Merck, Darmstadt, Germany) was added in concentrations of 1 µg and 10 µg per g of dry substrate, manganese ($MnCl_2.4H_2O$, 105927, Merck, Darmstadt, Germany) was added in concentrations of 15 µg and 150 µg per g of dry substrate and linoleic acid (W338001, Sigma Aldrich, St. Louis, Missouri, USA) in concentrations of 0.5 mmol or 1 mmol per liter of water taken up by the substrate. Samples were taken after 4 weeks of incubation with the fungi.

Two follow up experiments were done in which the substrates were prepared in the same way as described above. Based on the lignin degradation results, Mn was added in a series of concentrations to *C. subvermispora* treatment of wheat straw. Mn was added in concentrations of 1.5, 5, 15, 30, 100 or 150 µg per g of dry wheat straw.

In another experiment combinations of Mn and linoleic acid, which both act via the enzyme manganese peroxidase, were added tested to investigate any addition effects. Combinations of Mn and linoleic acid were added to wheat straw and to wood chips in the following concentrations: 1.5 µg Mn with 0.5 mM linoleic acid, 1.5 µg Mn with 1 mM linoleic acid, 15 µg Mn with 0.5 mM linoleic acid and 15 µg Mn with 1 mM linoleic acid per g of dry substrate.

Substrates were weighed into 1.2 l polypropylene containers with a filter cover (model TP1200+TPD1200 XXL Combiness, Nazareth, Belgium). The material was sterilized by autoclaving for 1 hour at 121 °C. After cooling down, the containers with sterilized substrate were kept at room temperature until further use.

6.2.3 Inoculation substrate

To each g of dry substrate (wheat straw, wood chips) 0.1 g of spawn was added and mixed to distribute the spawn equally through the substrate. The inoculated samples were incubated in duplicate for 4 weeks at 24 °C. Autoclaved, uninoculated substrates were used as a control.
After incubation, the substrate was air-dried at 70 °C and ground through a 1 mm sieve.

6.2.4 Chemical analysis

Fiber analysis was done on air-dried, ground (1 mm) material according to Van Soest et al. (1991), using an Ankom fiber analyser 2000 (ANKOM Technology, Macedon, New York, USA). Neutral detergent fiber (NDF) was determined with heat stable amylase. All fiber fractions are expressed excluding residual ash. Hemicellulose content was calculated as the difference between NDF and acid detergent fiber (ADF). Cellulose content was calculated as the difference between ADF and acid detergent lignin (ADL). Ash was determined by combustion for 3 hours at 550 °C in a muffle furnace (ISO 5984). DM content was determined by drying at 103 °C for 4 hours (ISO 6496). All data are expressed as g per kg DM. Manganese was determined by dissolving the ash in acid and is then analyzed by inductively coupled plasma atomic emission spectroscopy.

6.2.5 *In vitro* gas production technique (IVGP)

IVGP was performed according to the procedure described by Cone et al. (1996). In summary rumen fluid from 2 fistulated non-lactating cows was collected 2 hours after the morning feeding. To each 60 ml buffered rumen fluid 500 mg air dried sample was added. Each replicate was analyzed in a separate run. During 72 hours of incubation at 39 °C the amount of gas produced by anaerobic fermentation was recorded continuously. Gas production is related to the amount of degraded OM of the samples.

6.2.6 Statistical analysis

The influence of fungus, incubation time and additives were analyzed for each substrate separately using the generalized linear model (GLM) analysis in SAS 9.3. The following model was used in the experiment in which single additives were tested and the experiment in which combinations of Mn and linoleic acid were tested:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \gamma_k + \alpha\beta_{ij} + \alpha\gamma_{ik} + \beta\gamma_{jk} + \omega_{ijk}$$

in which Y_{ijk} is the observation in treatment ijk; μ is the overall mean; α_i is the fixed effect of fungus i; β_j is the fixed effect of incubation time j; γ_k is the fixed effect of additive k; interactions are included; ω_{ijk} is the random error.
Customized comparisons between treatments in the presence of an additive and the same treatment without additive were done for each fungus and incubation time using the estimates statement in SAS 9.3. P-values lower than 0.05 were considered to be significantly different.
For the experiment in which different concentrations of Mn are tested, chemical composition and IVGP data of the fungal treatment at each concentration of Mn were compared to the same treatment without additives in a generalized linear model (GLM) analysis in SAS 9.3. Post-hoc multiple comparison with Tukey's significant test at a level of $\alpha = 0.05$ was performed to determine the significance between the treatments. The following model was used in the experiment in which different concentrations of Mn were tested:

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

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

6.3 Results

6.3.1 Fungal treatment without additives

Both *C. subvermispora* and *L. edodes* degraded significant amounts of ADL and hemicellulose in wheat straw and wood chips during 4 weeks of treatment (Table 6.1 and 6.2). For wood chips this also resulted in a higher IVGP after 4 weeks. For wheat straw, only the *L. edodes* treatment led to an increased IVGP, while treatment with *C. subvermispora* had no significant effect.
Although the IVGP was improved within the first 4 weeks of fungal treatment, the potential IVGP of wheat straw and wood chips after 12 weeks of fungal treatment is about 300 ml/g OM and 200 ml/g OM respectively (van Kuijk et al., 2015b). To reach the potential of fungal treatment within the first 4 weeks of incubation the effect of three additives were investigated.

6.3.2 Single additives

First single additives were added to investigate the most promising additive to improve lignin degradation and IVGP upon fungal treatment. The effect of the addition of urea, manganese or linoleic acid was measured on the extent of the lignin degradation and subsequent IVGP compared to fungal treatment without additives. Urea did not stimulate *C. subvermispora* or *L. edodes* to degrade more ADL and increase IVGP of wheat straw and wood chips (data not shown). For this reason, urea will not be discussed further in this paper.

Table 6.1 Chemical composition (g/kg DM) and *in vitro* gas production in rumen fluid (ml/g OM) of wheat straw treated with *C. subvermispora* or *L. edodes*. The composition was determined before treatment and after 4 weeks of treatment with the addition of different additives.

Nutrient[1]	Before treatment	*C. subvermispora*					*L. edodes*				
		No additive	Mn		Linoleic acid		No additive	Mn		Linoleic acid	
			15 µg/g	150 µg/g	0.5 mM	1 mM		15 µg/g	150 µg/g	0.5 mM	1 mM
ADL	91.2	66.9^{+}	45.9*	59.5*	69.0	60.7*	56.5^{+}	65.0	52.6	59.7	55.9
HC	301.1	237.4^{+}	170.4*	205.9*	227.6	209.2*	195.8^{+}	216.3	164.4*	200.2	193.2
Cell	471.1	468.4	479.3	468.1	469.7	472.1	488.0^{+}	479.5	504.9	488.0	494.5
IVGP	213.8	228.8	283.4	288.5*	219.7	268.3*	270.5^{+}	241.8	269.7	254.3	267.6

* Significant different from treatment with the same fungus without additive ($P<0.05$) $^{+}$ significant different from before treatment ($P<0.05$).
[1] Nutrients: ADL = acid detergent lignin, HC = hemicellulose, Cell = cellulose, IVGP = *in vitro* gas production after 72 hours.

Table 6.2 Chemical composition (g/kg DM) and *in vitro* gas production in rumen fluid (ml/g OM) of wood chips treated with *C. subvermispora* or *L. edodes*. The composition was determined before treatment and after 4 weeks of treatment with the addition of different additives.

Nutrient[1]	Before treatment	*C. subvermispora*					*L. edodes*				
		No additive	Mn		Linoleic acid		No additive	Mn		Linoleic acid	
			15 µg/g	150 µg/g	0.5 mM	1 mM		15 µg/g	150 µg/g	0.5 mM	1 mM
ADL	191.1	130.8^{+}	123.8	120.3	145.9	133.5	152.9^{+}	171.5	137.6	143.3	155.2
HC	216.0	124.0^{+}	156.0*	178.4*	159.3	140.3	143.1^{+}	161.8	156.4*	145.9	168.2*
Cell	409.2	406.6	387.7	420.3	408.4	401.1	368.3^{+}	382.7	395.0	389.3	354.0
IVGP	88.7	193.8^{+}	189.4	182.3	172.4	192.7	134.1^{+}	116.7	132.8	149.5	125.7

* Significant different from treatment with the same fungus without additive ($P<0.05$) $^{+}$ significant different from before treatment ($P<0.05$).
[1] Nutrients: ADL = acid detergent lignin, HC = hemicellulose, Cell = cellulose, IVGP = *in vitro* gas production after 72 hours.

Table 6.3 Chemical composition (g/kg DM) and *in vitro* gas production in rumen fluid (ml/g OM) of wheat straw treated with *C. subvermispora*. The composition was determined before treatment and after 4 weeks of treatment with no addition of with the addition of different combinations of Mn and linoleic acid (LA).

Nutrient[1]	Before treatment	*C. subvermispora*				
		No additive	Mn:LA 1.5:0.5	Mn:LA 1.5:1	Mn:LA 15:0.5	Mn:LA 15:1
ADL	93.6	56.0^{+}	45.0*	46.1*	44.3*	38.7*
HC	299.6	200.2^{+}	166.3*	171.9*	163.9*	147.9*
Cell	460.6	467.5	476.9	478.8	478.3	473.8
IVGP	193.6	250.1	271.7	272.7	275.5	280.0

* Significant different from treatment without additive ($P<0.05$) $^{+}$ significant different from before treatment ($P<0.05$).
[1] Nutrients: ADL = acid detergent lignin, HC = hemicellulose, Cell = cellulose, IVGP = *in vitro* gas production after 72 hours.

C. subvermispora degraded more ADL and hemicellulose in wheat straw in the presence of Mn or linoleic acid than without additives, while the cellulose content was not different. Addition of 150 µg Mn/g resulted in 10% more ADL degradation in wheat straw by *C. subvermispora*. Addition of 150 µg Mn /g or 1 mM linoleic acid resulted also in an increased ($P<0.05$) IVGP of *C. subvermispora* treated wheat straw (Table 6.1). On wheat straw, Mn has only effect on the *C. subvermispora* treatment, while *L. edodes* was not stimulated to degrade more ADL. Addition of Mn or linoleic acid separately to wood chips did not result in a lower ADL and a higher IVGP for both fungal species (Table 6.1 and 6.2).

6.3.3 Combination Mn and linoleic acid

Mn and linoleic acid both stimulate manganese peroxidase production and activity. It was hypothesized that a combination of Mn and linoleic acid would have additional effects on lignin degradation and thus IVGP. Since synergetic effects were expected, low concentrations of Mn and linoleic acid were chosen. To investigate any additional effects, a combination of Mn and linoleic acid was tested in different ratios. For Mn the concentrations were based on the results of single additives. Table 6.1 shows more ADL degradation in addition of 15 µg Mn per g wheat straw, compared to 150 µg Mn per g wheat straw, while there was no difference in wood chips. Any synergistic effect of linoleic acid would be expected to be visible at 15 µg/g or lower. All ratios of Mn and linoleic acid stimulated only *C. subvermispora* to degrade more ADL and hemicellulose in wheat straw, without changing the cellulose content. The IVGP, however, was not improved by any of the Mn/linoleic acid ratios compared to *C. subvermispora* treated wheat straw without additives (Table 6.3). There was also no effect on wood chips treated with both fungi neither on wheat straw treated with *L. edodes* (data not shown).

Table 6.4 Chemical composition (g/kg DM) and *in vitro* gas production in rumen fluid (ml/g OM) of wheat straw treated with *C. subvermispora*. The composition was determined before treatment and after 4 weeks of treatment with no addition and addition of different concentrations of Mn.

Nutrient[1]	Before treatment	No additive	Concentration Mn					
			1.5 µg/g	5 µg/g	15 µg/g	30 µg/g	100 µg/g	150 µg/g
ADL	93.6	56.0[a]	46.5[b]	42.8[b]	47.5[b]	48.3[b]	44.9[b]	32.8[c]
HC	299.6	200.2[a]	175.3[a]	173.3[a]	186.7[a]	177.8[a]	175.3[a]	125.0[b]
Cell	460.6	467.5	469.5	478.1	463.9	475.8	477.7	492.0
IVGP	193.6	250.1	251.5	250.6	256.4	243.4	261.2	288.7

[a-c] Significant different from treatment without additive (P<0.05).
[1] Nutrients: ADL = acid detergent lignin, HC = hemicellulose, Cell = cellulose, IVGP = *in vitro* gas production after 72 hours.

6.3.4 Optimization of Mn addition

Since the addition of Mn stimulates the IVGP after the *C. subvermispora* of wheat straw more than linoleic acid, a range of Mn concentrations (1.5 µg Mn/g to 150 µg Mn/g) was tested. This range was chosen based on ADL degradation patterns. Most ADL was degraded in addition of 15 µg Mn per g wheat straw, whereas 150 µg Mn per g wheat straw resulted in less ADL degradation (Table 6.1), indicating that this concentration was too high to be stimulatory. The range of concentrations was added to investigate whether a lower concentration of Mn would also result in an improved fungal treatment. All concentrations of Mn stimulated *C. subvermispora* to degrade more ADL in wheat straw to similar extend compared to the same treatment without additional Mn (Table 6.4). Only the highest concentration of 150 µg Mn per g wheat straw resulted in a significant better degradation of ADL and hemicellulose degradation, compared to the other concentrations of Mn. IVGP of wheat straw, however, was not increased by *C. subvermispora* in the presence of Mn, compared to no additives.

6.4 Discussion

The aim of this study was to improve the fungal treatment using additives in such a way that the time of the treatment can be reduced substantially. The fungal treatment is considered to be optimized if IVGP and lignin degradation are significantly higher than the same treatment without additives, without a significant degradation of cellulose.

The current study shows that *C. subvermispora* can only increase IVGP of wheat straw in presence of additives. Previous research has shown that *C. subvermispora* treatment of wheat straw without additives can result in a significant ADL degradation and subsequent increase in IVGP especially after long incubation (5-7 weeks) (Tuyen et al., 2012). This is also the case for *L. edodes*, a fungus that can increase the IVGP of wheat straw with approximately 35% in 7 weeks (Tuyen et al., 2012), while here only an increase of 26.5% was found. These results show that *C. subvermispora* and *L. edodes* pre-treatments were not optimal yet and improvement is possible.

Manganese addition (150 µg/g) stimulated *C. subvermispora* to degrade 10% more ADL in wheat straw, compared to without additives. Similarly, Kerem and Hadar (1995) found 10% more lignin degradation (measured as Klason lignin) in cotton stalks by *P. ostreatus* in the presence of 150 µg Mn/g. In the current study did Mn addition only have an effect on *C. subvermispora* treatment of wheat straw and on none of the other fungus-substrate combinations. One possible explanation for this is that enzyme production by fungi is substrate dependent (Elisashvili et al., 2008). This dependency can be explained by the Mn concentrations in the substrates used in the current study. The wood chips used in this study contained 112.8 mg Mn/kg dry material, while the wheat straw used contained 29.8 mg Mn/kg dry material. Mn might not be limiting in wood chips and therefore not have additional effects on the fungal treatment.

Secondly, Mn is involved in lignin degradation by manganese peroxidase excreted by these fungi. The genome of *C. subvermispora* contains 13 genes encoding for manganese peroxidase. The genome sequence of *L. edodes* is not publically available but research done so far indicate that this species has only 3 genes encoding for this enzyme (Nagai et al., 2007; Fernandez-Fueyo et al., 2012). This suggests that manganese peroxidase plays a larger role in lignin degradation by *C. subvermispora* than by *L. edodes*, which might explain why Mn has a larger stimulating effect on lignin degradation by *C. subvermispora*. It is thought that *C. subvermispora* does produce manganese peroxidase under low Mn conditions, but presence of Mn stimulates secretion of manganese peroxidase (Mancilla et al., 2010). Besides an effect on manganese peroxidase, Mn may also have an effect on growth as demonstrated by Kerem and Hadar (1995). The latter authors describe a higher respiration rate as a measure for growth of *P. ostreatus* on cotton stalks in the presence of Mn.

Both Mn and linoleic acid are involved in lignin degradation by manganese peroxidase. Manganese peroxidase attacks lignin via the conversion of Mn^{2+} to Mn^{3+} or via a lipid peroxidation pathway in which a lipid is converted to a lipid radical. Addition of Mn and linoleic acid might thus stimulate both pathways of manganese peroxidase and thus have an additional effect compared to the single additives. To test this, Mn and linoleic acid were added in various ratios to *C. subvermispora* or *L. edodes* treatment of wheat straw or wood chips. The latter experiment indicates that the addition of a combination of two components involved in lignin degradation by Mn peroxidase do not have a positive effect on the extent and selectivity of lignin degradation. This might indicate that there is no synergistic effect. In this study, Mn was the most promising additive to improve rumen degradability. The *in vitro* measurements did not show inhibiting effects of Mn on rumen microbes. The effect on the animal is expected to be low, since Mn is described to be poorly absorbable (less than 1% absorption) (Hidiroglou 1979; NRC 2001). NRC (2001) describes a maximum tolerable amount of manganese in the diet to be 1000 mg/kg. The maximum concentration in the current study was 150 µg Mn added per g wood chips containing 112.8 mg/kg, which was far below the maximum tolerable amount suggested.

There is a significant variation between the experiments in this study. The values of fiber analysis for wheat straw and IVGP before the fungal treatment shown in Tables 6.1 and 6.3 are well comparable, indicating that the methods used are reliable. After treatment with *C. subvermispora*, however, substantial differences are found in fiber composition and IVGP. Table 6.3 shows an increased degradation of hemicellulose, which possibly results in a reduced availability of carbohydrates for rumen microbes and counteract the effect of an increased availability of cellulose.

A likely explanation is the variation in fungal growth and subsequent enzyme production, which has an effect on lignin degradation, hemicellulose consumption and thus IVGP. Cellulose contents in both experiments were very similar, suggesting that *C. subvermispora* does not use large amounts of cellulose. The latter can be explained by the lower amount of cellulase genes in this fungus compared to other white-rot fungi (Fernandez-Fueyo et al., 2012). It is important to reduce the variation between experiments. Therefore the culture conditions should be controlled carefully in detail.

The choice of concentrations for the determination of the optimum levels of Mn was based on the lignin degradation described in Table 6.1. This table shows a higher lignin degradation when 15 µg Mn per g substrates was added compared to when 150 µg Mn per g substrate was added. Therefore we hypothesized that the optimal concentration for the highest lignin degradation would be below 150 µg Mn per g substrate. The higher lignin content in addition of 150 µg/g in experiment 1 suggests that this concentration is too high. For a proper dose-response analysis, ideally, the optimal concentration would be in the middle of the range. Due to the variation between the experiments the contrasts chosen were not large enough to perform a dose-response data analysis.

6.5 Conclusions

Additives had significant positive effects on the *C. subvermispora* treatment of wheat straw in 4 weeks under current experimental conditions. Mn or linoleic acid added separately resulted in an increased IVGP, and an increase in ADL and hemicellulose degradation, without changing the cellulose content. It is advised to add a concentration of 150 µg Mn/g substrate to obtain highest ADL degradation and IVGP. Addition of a combination of Mn and linoleic acid resulted in more ADL and hemicellulose degradation by *C. subvermispora* in wheat straw, compared to no additives, but did not increase the IVGP compared to the same treatment without additives. Under current experimental conditions no effects of the additives were found on the treatment of wheat straw and wood chips by *L. edodes*.

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CHAPTER 7

The effect of particle size and amount of inoculum added to wheat straw and wood chips on lignin degradation by fungi and subsequent *in vitro* rumen degradation

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Abstract

The aim of this study was to optimize the fungal treatment of lignocellulosic biomass by stimulating the colonization. Wheat straw and wood chips were treated with *Ceriporiopsis subvermispora* and *Lentinula edodes* with various amounts of colonized millet grains (0.5, 1.5 or 3.0% per g of wet weight of substrate) added to the substrates. Also, wheat straw and wood chips were chopped to either 0.5 or 2 cm. Effectiveness of the fungal treatment after 0, 2, 4, 6, or 8 weeks of incubation was determined by changes in chemical composition, *in vitro* gas production (IVGP) as a measure for rumen degradability, and ergosterol content as a measure of fungal biomass. Low growth was observed for *C. subvermispora* treated wheat straw and *L. edodes* treated wood chips. The different particle sizes and amounts of inoculum tested, had no significant effects on the chemical composition and the IVGP of *C. subvermispora* treated wood chips. Particle size did influence *L. edodes* treatment of wheat straw. The *L. edodes* treatment of 2 cm wheat straw resulted in a more selective delignification and a higher IVGP than the smaller particles. At least 1.5% *L. edodes* colonized millet grains should be added to result in an increased IVGP and acid detergent lignin (ADL) degradation.

7.1 Introduction

Cellulose is one of the most abundant carbohydrates in the world and is, next to starch, an important carbon source in a ruminant diet. In plant cell walls, cellulose is bound to hemicellulose and lignin in the lignocellulosic complex. Lignin is a polymer that is difficult to degrade, and only degradable under aerobic conditions (Isroi et al., 2011). In the anaerobic rumen, lignin is hardly affected and as a result, the lignocellulosic complex has a limited degradability (Jung and Allen, 1995). As a result, cellulose and hemicellulose in this complex are not available to the rumen microbes. To increase the availability of cellulose, and thus rumen degradability, lignin should be removed before feeding lignocellulosic biomass to ruminants. Lignin removal can be achieved using several pre-treatment methods (Sarnklong et al., 2010; Agbor et al., 2011). A biological pre-treatment using selective lignin degrading fungi is a relatively cheap and environment friendly alternative for chemical and physical pre-treatments (van Kuijk et al., 2015a). Pre-treatments using selective lignin degrading fungi, such as *Ceriporiopsis subvermispora* or *Lentinula edodes* were proven to increase the *in vitro* rumen degradability as a consequence of increased cellulose availability of wheat straw and sugarcane bagasse (Tuyen et al., 2012, 2013). The time needed to obtain maximum cellulose availability was 6 to 8 weeks (Tuyen et al., 2012, 2013; van Kuijk et al., 2015b). Although the substrates after the fungal pre-treatment were enriched in cellulose, hemicellulose was partially used by the fungi as lignin was degraded. This relatively long treatment time and consumption of part of the carbohydrate fraction are major drawbacks and optimization of both is needed to make this method competitive with current chemical and physical pre-treatments.

Fungal pre-treatment starts with the inoculation of the substrate. In scientific literature, inoculation for *C. subvermispora* or *L. edodes* treatments has been done using agar plugs (Okano et al., 2005; Sharma et al., 2010; Giles et al., 2011) or using liquid medium (Horta et al., 2011; Wan et al., 2011; Ciu et al., 2012). However, in the commercial mushroom production process spawn is produced from grains (Sanchez et al., 2004). Grain based spawn can be produced on a large scale and is easy to mix through the substrate. Previous studies have used grains spawn made from wheat grain, millet or sorghum (Okano et al., 2009; Gaitán-Hernández et al., 2011; Tuyen et al., 2012; 2013; van Kuijk et al., 2015b). Compared to other grains, sorghum grains are relatively large, meaning less inoculation points per g of inoculum added. The use of a smaller grain, like millet, would increase the amount of inoculation points per g of inoculum added. The latter can initiate a faster colonization of the lignocellulose biomass (Reid, 1989).

Rapid and complete colonization of the lignocellulosic material is key for a competitive fungal pre-treatment. During colonization, the fungus starts degrading the outer layer of the material before reaching the inside (Sachs et al., 1990). By decreasing the particle size of the substrate, the surface to volume ratio is increased. The latter will result in more contact points on the surface and reach the inner part of the material faster (Reid, 1989).

The aim of this study was to optimize the colonization conditions of the fungal pre-treatment to obtain the most selective delignification, with minimal carbohydrate degradation and a high *in vitro* rumen degradability. Based on the work of Tuyen et al. (2012), *C. subvermispora* and *L. edodes* were chosen as selective lignin degraders to treat wheat straw and wood chips. Two different particle sizes, 0.5 and 2 cm length, of both substrates were used with different amounts of inoculum (colonized millet) added.

7.2 Materials and methods

7.2.1 Fungal strains

Ceriporiopsis subvermispora (strain MES 13094) and *Lentinula edodes* (strain MES 11910) were initially cultured on malt extract agar until it was almost fully colonized. Agar pieces (approximately 1 cm^2) were added to sterilized millet grains. Inoculated millet grains were incubated at 24 ºC until full colonization. Fully colonized grains were used as spawn to inoculate the substrates.

7.2.2 Substrates

Wheat straw and wood chips (oak) were used as substrates. Both substrates were chopped to particles with average sizes of 0.5 cm or 2 cm. The substrates were submerged in water for 3 days where after the excess water was removed and the substrates divided over 1.2 l autoclavable polypropylene containers and covered with a lid containing a filter (model TP1200+TPD1200 XXL Combiness, Nazareth, Belgium) through which air can pass. Each container contained 200 g wet substrate, representing approximately 100 and 50 g dry matter of wood chips and wheat straw, respectively. The containers with substrate were sterilized for 1 hour at 121 ºC. The sterilized substrates were kept at room temperature until further use. Autoclaved, uninoculated samples were taken to serve as control.

7.2.3 Inoculation

Colonized millet grains were added to each container in a concentration of either 0.5, 1.5 or 3% of the weight of the wet substrate. The content of the containers were mixed under aseptic conditions to divide the inoculum equally over the substrate. All conditions were tested in triplicate. Samples were incubated for 2, 4, 6 and 8 weeks in a climate controlled chamber at 24 ºC and 70% relative humidity. After incubation, two containers of wheat straw were pooled to represent one sample. Part of each sample (approximately 10%) was freeze-dried for ergosterol measurements and the remaining part of the sample was air-dried at 70 ºC for 1 week to be used for chemical analyses and *in vitro* gas production measurements. The dried wheat straw was ground to 1 mm using a Peppink 100 AN cross beater mill (Peppink, Deventer, The Netherlands). The dried wood chips were finely ground over a 1 mm sieve using a Retch ZM100 centrifugal mill (Retsch, Haan, Germany) to obtain a homogenous sample.

The remaining colonized and uninoculated millet grains that were not used for inoculation were used for further analysis. Approximately 10% of each sample was

freeze-dried, and the remaining part was air-dried at 70 ºC for 1 week. The dried millet was ground to 1 mm using a Peppink 100 AN cross beater mill (Peppink, Deventer, The Netherlands).

7.2.4 *In vitro* gas production technique

In vitro gas production (IVGP) was measured in all samples by the IVGP technique, according to Cone et al. (1996). In summary, 60 ml of buffered rumen fluid, collected from non-lactating cows, was added to 0.5 g air-dried material. Incubations were done in shaking water baths at 39 ºC. Gas production was measured continuously for 72 hours. Results were expressed as ml gas produced after 72 hours per g organic matter (OM).

7.2.5 Chemical analysis

Fiber analysis was performed according to Van Soest et al. (1991), using an Ankom fiber analyser 2000 (ANKOM Technology, Macedon, New York, USA). The hemicellulose content was calculated as the difference between neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents. The cellulose content was calculated as the difference between ADF and acid detergent lignin (ADL) contents.
For dry matter (DM) determination air-dried material was dried for 4 hours at 103 ºC. Ash content was determined by combustion for 3 hours at 550 ºC in a muffle furnace. Starch content of the millet grains was determined enzymatically according to ISO15914.

7.2.6 Ergosterol

Ergosterol determination of freeze-dried material was based on Niemenmaa et al. (2008). In summary, 200 mg of the material was saponified in 10% KOH in methanol for 1 hour at 80 ºC. After cooling, hexane and distilled water were added for extraction. The samples were shaken for 10 minutes and centrifuged for 15 minutes at 4000 rpm. The hexane phase was collected and the hexane-water extraction was repeated once. The hexane phases of the 2 extractions were pooled and evaporated under vacuum. The extracted ergosterol was dissolved in 1 ml methanol before ergosterol content was determined by a high performance liquid chromatography (HPLC) fitted with a reversed phase C18 column (250x4.6 mm, Phenomex aqua 5 µm). The liquid phase was 90% methanol and 10% (1:1) 2-propanol/hexane. Areas under the peak were corrected for the extraction efficiency based on the internal standard cholecalciferol (vitamin D3) (9.6 µg added) (Sigma Aldrich, St. Louis, Missouri, USA), using Empower 2 software (Waters Corporation, Milford, Massachusetts, USA).

7.2.7 Statistical analysis

The effect of amount of inoculum, particle size and incubation on ergosterol content, detergent fiber content and IVGP was tested using the generalized linear model (GLM) analysis in SAS software version 9.3 (SAS Institute Inc., Cary, North Carolina, USA). The following model was used:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \gamma_k + \omega_{ijk}$$

in which Y_{ijk} is the observation at incubation time i; μ is the overall mean; α_i is the fixed effect of amount of inoculum i; β_j is the fixed effect of particle size j; γ_k is the fixed effect of incubation time k; ω_{ijk} is the random error.
The results of ergosterol measurements, detergent fiber analysis and IVGP at different incubation times of the fungal treatment of each substrate, for each amount of inoculum and particle size combination, were compared using the generalized linear model (GLM) analysis in SAS software version 9.3 (SAS Institute Inc., Cary, North Carolina, USA). Post-hoc multiple comparison with Tukey's significant test at a level of $\alpha = 0.05$ was performed to determine the significance of differences between the treatments. The following model was used:

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

in which Y_{ij} is the observation j at incubation time i; μ is the overall mean; α_i is the fixed effect of incubation time i; ω_{ij} is the random error.
Correlations between IVGP, enzymatic saccharification and ADL were subjected to correlation analysis using SAS software version 9.3. Correlations are provided as the Pearson correlation coefficient r.

7.3 Results

Only results of *C. subvermispora* treated wood chips and *L. edodes* treated wheat straw are presented. Visually no or limited growth was observed in *L. edodes* treated wood chips and *C. subvermispora* treated wheat straw. It is believed that this low growth was caused by external factors, other than the treatments.

Table 7.1 Chemical composition (g/kg DM) of spawn at the moment of inoculation.

Treatment	ADL	Hemicellulose	Cellulose	Starch	IVGP	Ergosterol
Control	14.4[a]	54.0[a]	76.3[a]	699.0[a]	319.7	0.0[c]
C. subvermispora	2.3[b]	30.3[b]	50.4[b]	680.2[a]	285.8	138.1[b]
L. edodes	3.5[b]	40.9[ab]	43.2[b]	408.2[b]	302.5	192.7[a]
SEM	1.58	3.31	2.65	5.56	16.00	10.61
P-value	0.003	0.0068	0.0003	<0.0001	0.3855	<0.0001

ADL = acid detergent lignin, IVGP = *in vitro* gas production in rumen fluid.

Table 7.2 Probability values for effects of the amount of inoculum added (0.5, 1.5 and 3.0 %), particle size (0.5 and 2.0 cm) and incubation time (2, 4, 6 and 8 weeks) on chemical composition, ergosterol content and *in vitro* gas production of two fungal treated substrates (wheat straw and wood chips).

Item	*C. subvermispora* treated wood chips			*L. edodes* treated wheat straw		
	Amount of inoculum	Particle size	Incubation time	Amount of inoculum	Particle size	Incubation time
ADL (g/kg DM)	0.1435	0.5688	<0.0001	0.0346	<0.0001	<0.0001
ADL (g)	0.4626	0.9148	<0.0001	0.1640	<0.0001	<0.0001
HC (g/kg DM)	0.3704	<0.0001	<0.0001	0.0003	<0.0001	<0.0001
HC (g)	0.2533	<0.0001	<0.0001	0.0054	<0.0001	<0.0001
Cell (g/kg DM)	0.0002	<0.0001	<0.0001	0.0026	0.0003	<0.0001
Cell (g)	0.0197	<0.0001	0.0012	0.4314	0.1179	0.0120
Ergosterol (mg/g)	0.5085	0.3220	<0.0001	<0.0001	<0.0001	<0.0001
IVGP (ml/g OM)	0.4508	0.6735	<0.0001	0.1910	<0.0001	<0.0001

ADL = acid detergent lignin, HC = hemicellulose, Cell = cellulose, IVGP = *in vitro* gas production after 72h in rumen fluid.

7.3.1 Inoculum

The chemical composition and the IVGP of the millet used as spawn at the moment of inoculation (5 weeks of colonization) are presented in Table 7.1. Ergosterol contents showed that the millet was colonized by both *C. subvermispora* and *L. edodes*. *C. subvermispora* contained 9.6 mg ergosterol/g mycelium and *L. edodes* contained 6 mg ergosterol/g mycelium. As such, each g of colonized millet contained approximately 14.3 mg *C. subvermispora* mycelium or 32.1 mg *L. edodes* mycelium at the moment of inoculation. However, it should be noted that the ergosterol contents of pure mycelium grown on malt extract agar may be different for the ergosterol contents of mycelium grown on millet grains. Colonized millet had a lower (P<0.05) ADL and cellulose content than the uninoculated grains (control). Although *L. edodes* did numerically decrease the hemicellulose content, this was not significant, whereas *C. subvermispora*

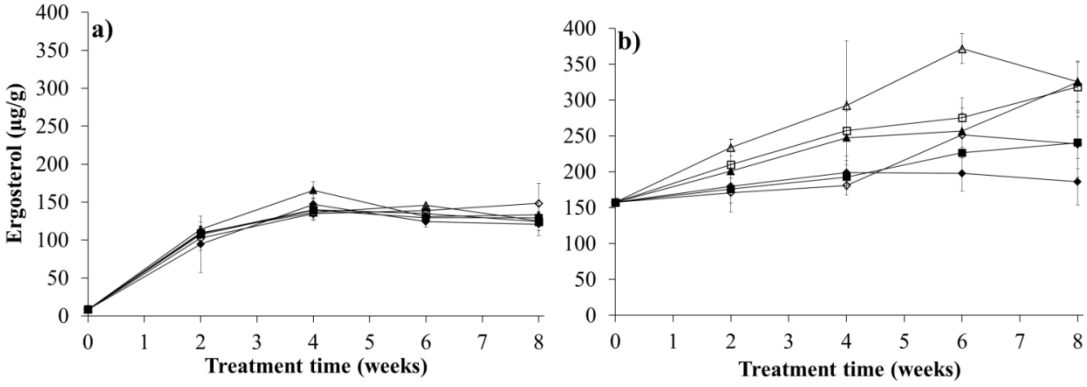

Figure 7.1 Results of ergosterol measurements. a) *C. subvermispora* on wood chips, b) *L. edodes* on wheat straw. ♦ 0.5% inoculum per g wet substrate (0.5 cm) ■ 1.5% inoculum per g wet substrate (0.5 cm) ▲ 3% inoculum per g wet substrate (0.5 cm) ◊ 0.5% inoculum per g wet substrate (2 cm) □ 1.5% inoculum per g wet substrate (2 cm) Δ 3% inoculum per g wet substrate (2 cm).

significantly decreased hemicellulose content. Interestingly, the starch content of the *C. subvermispora* spawn was not different from thecontrol, whereas *L. edodes* significantly degraded the starch in the millet grains. The spawn showed a high IVGP of approximately 300 ml/g OM, which suggests that the addition of spawn to the substrates contributed to the increase in IVGP at the start of fungal treatment.

7.3.2 *C. subvermispora* treatment of wood chips

The probability values of the effects of the amount of inoculum added, particle size and incubation time are shown in Table 7.2. The amount of inoculum had only a significant effect on the cellulose degradation during the *C. subvermispora* treatment of wood chips. Changing the particle size of wood chips had significant effects on the hemicellulose and cellulose degradation by *C. subvermispora*. No significant effects were observed for the amount of inoculum (content P=0.1435, absolute amounts P=0.4626) and particle size (content P=0.5688, absolute amount P=0.9148) on the ADL degradation. The time of incubation showed effects (P<0.05) on all variables measured. Tables 7.3 and 7.4 show that most changes occurred during the first 4 weeks of incubation. *C. subvermispora* colonized the wood chips within the first 4 weeks of incubation as the ergosterol content increased during the first 4 weeks, after which the growth rate reduced (Figure 7.1). Only after 4 weeks of incubation differences were seen between treatments. Addition of 3% inoculum to 0.5 cm wood chips resulted in a higher (P<0.05) ergosterol content compared to the other treatments. The low (P<0.05) cellulose content (g/kg DM) and the absolute amount of cellulose (g) using 3% inoculum and 0.5 cm wood chips suggests that *C. subvermispora* uses this carbohydrate for growth.

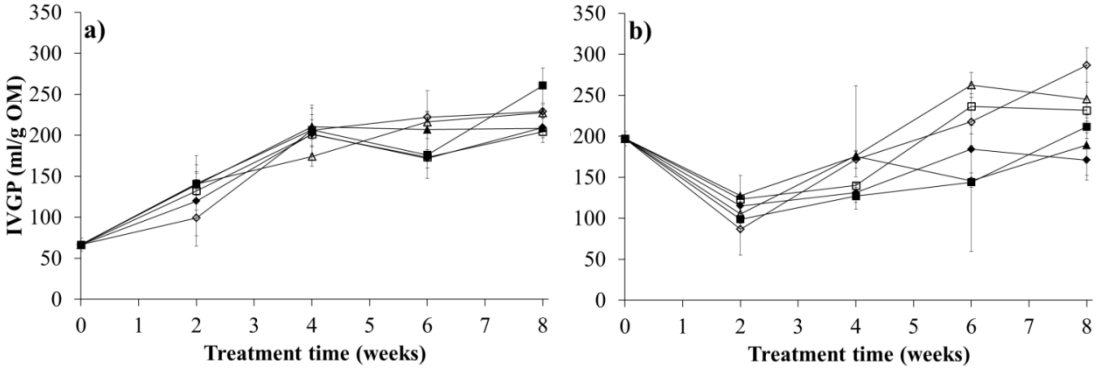

Figure 7.2 Results of *in vitro* gas production (IVGP) measurements. a) *C. subvermispora* on wood chips, b) *L. edodes* on wheat straw. ♦ 0.5% inoculum per g wet substrate (0.5 cm) ■ 1.5% inoculum per g wet substrate (0.5 cm) ▲ 3% inoculum per g wet substrate (0.5 cm) ◊ 0.5% inoculum per g wet substrate (2 cm) □ 1.5% inoculum per g wet substrate (2 cm) Δ 3% inoculum per g wet substrate (2 cm).

Table 7.3 Changes in detergent fiber content (g/kg DM) over an eight week period of 0.5 and 2 cm wood chips incubated with three different amounts of *C. subvermispora* inoculum.

Particle size		0.5 cm			2 cm		
Inoculum (%)		0.5	1.5	3.0	0.5	1.5	3.0
	Week						
ADL	0	214.8^{a}	214.8^{a}	214.8^{a}	214.8^{a}	214.8^{a}	214.8^{a}
	2	197.8^{a}	162.4^{b}	162.6^{b}	178.3^{b}	163.7^{b}	156.5^{b}
	4	127.0^{b}	130.0^{c}	126.3^{c}	133.2^{c}	126.8^{c}	134.3^{c}
	6	128.3^{b}	118.4^{d}	123.4^{c}	111.8^{d}	126.4^{c}	126.4^{c}
	8	112.0^{b}	123.6^{cd}	129.4^{c}	126.0^{c}	121.2^{c}	120.7^{c}
	SEM	9.95	2.07	2.18	2.31	5.41	2.95
	P-value	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
HC	0	151.5^{a}	151.5^{a}	151.5^{a}	151.5^{a}	151.5^{a}	151.5^{ab}
	2	154.1^{a}	160.7^{a}	170.7^{a}	151.7^{a}	156.2^{a}	158.3^{a}
	4	90.0^{b}	98.5^{b}	92.8^{b}	109.4^{bc}	114.1^{b}	129.9^{abc}
	6	107.4^{b}	85.6^{b}	87.0^{b}	126.8^{ab}	106.2^{b}	122.6^{bc}
	8	74.5^{b}	73.1^{b}	74.8^{b}	94.9^{c}	107.3^{b}	112.1^{c}
	SEM	8.21	10.80	9.82	5.50	4.32	6.55
	P-value	0.0001	0.0005	0.0001	<.0001	<.0001	0.0025
Cell	0	381.3^{a}	381.3^{b}	381.3^{ab}	381.3^{b}	381.3^{b}	381.3^{b}
	2	390.3^{ab}	393.1^{ab}	375.3^{b}	408.2^{b}	396.8^{b}	387.4^{b}
	4	417.0^{ab}	403.3^{ab}	384.5^{ab}	414.7^{ab}	430.3^{a}	422.2^{a}
	6	417.9^{ab}	415.4^{ab}	405.4^{a}	455.1^{a}	446.0^{a}	419.3^{a}
	8	430.3^{a}	419.1^{a}	397.1^{ab}	424.2^{ab}	438.4^{a}	419.2^{a}
	SEM	10.34	7.90	5.98	9.97	6.82	6.42
	P-value	0.0349	0.0362	0.0301	0.0054	0.0002	0.0019

Values with different superscripts within column are significantly ($P<0.05$) different. ADL = acid detergent lignin, HC = hemicellulose, Cell = cellulose,
SEM = standard error of the mean.

Table 7.4 Changes in absolute amounts of detergent fiber (g) over an eight week period of 0.5 and 2 cm wood chips incubated with three different amounts of *C. subvermispora* inoculum.

Particle size			0.5 cm			2 cm		
Inoculum (%)			0.5	1.5	3.0	0.5	1.5	3.0
	Weeks							
ADL	0		31.2^{a}	31.2^{a}	31.2^{a}	31.2^{a}	31.2^{a}	31.2^{a}
	2		27.5^{a}	22.3^{b}	22.4^{b}	24.3^{b}	23.0^{b}	21.3^{b}
	4		16.2^{b}	16.9^{c}	16.3^{c}	17.3^{c}	16.6^{c}	18.1^{c}
	6		16.4^{b}	14.9^{d}	15.8^{c}	14.0^{d}	16.1^{c}	16.5^{cd}
	8		13.8^{b}	15.1^{d}	16.4^{c}	15.5^{d}	15.5^{c}	15.2^{d}
		SEM	1.60	0.30	0.31	0.35	0.80	0.52
		P-value	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
HC	0		22.0^{a}	22.0^{a}	22.0^{a}	22.0^{a}	22.0^{a}	22.0^{a}
	2		21.4^{a}	22.1^{a}	23.5^{a}	20.7^{a}	21.9^{a}	21.6^{ab}
	4		11.5^{b}	12.8^{b}	12.0^{b}	14.2^{bc}	14.9^{b}	17.5^{bc}
	6		13.7^{b}	10.8^{b}	11.1^{b}	15.9^{b}	13.6^{b}	16.0^{c}
	8		9.2^{b}	9.0^{b}	9.5^{b}	11.7^{c}	13.7^{b}	14.1^{c}
		SEM	1.19	1.47	1.30	0.83	0.59	0.94
		P-value	<.0001	0.0001	<.0001	<.0001	<.0001	0.0005
Cell	0		55.5	55.5	55.5^{a}	55.5	55.5	55.5
	2		54.1	54.0	51.7^{ab}	55.7	55.7	52.7
	4		53.2	52.3	49.8^{b}	53.9	56.3	57.0
	6		53.2	52.3	51.9^{ab}	57.0	57.0	54.6
	8		52.9	51.3	50.5^{b}	52.1	55.9	52.9
		SEM	1.59	1.05	0.81	1.25	0.91	0.91
		P-value	0.7836	0.1072	0.0048	0.133	0.7735	0.0402

Values with different superscripts within column are significantly ($P<0.05$) different. ADL = acid detergent lignin, HC = hemicellulose, Cell = cellulose,
SEM = standard error of the mean.

The ADL and hemicellulose content and their absolute amounts also decreased during the first 4 weeks with *C. subvermispora* incubation. After 4 weeks, the lowest degradation ($P<0.05$) of hemicellulose and cellulose (contents and absolute amounts) occurred when 3% inoculum was added to 2 cm wood chips compared to the other treatments. The lower carbohydrate and unchanged ADL degradation suggest a more selective delignification process when 3% inoculum was added to 2 cm wood chips. The least selective delignification was observed when 0.5% inoculum was added to 0.5 cm wood chips, since most hemicellulose and a similar amount of ADL was degraded by *C. subvermispora.* The higher hemicellulose degradation did not result in a higher ergosterol content or a lower IVGP. The IVGP increased ($P<0.05$) during the first 4 weeks of incubation. Most treatments reached a plateau level, whereas addition of 1.5% inoculum to 0.5 cm wood chips and addition of 3% inoculum to 2 cm wood chips caused a continuous increase in IVGP. As a result the addition of 1.5% inoculum to 0.5 cm wood chips resulted in the highest IVGP after 8 weeks of *C. subvermispora* incubation.

7.3.3 *L. edodes* treatment of wheat straw

The effects of the amount of inoculum, particle size and incubation time are shown in Table 7.2. The main differences observed in *L. edodes* treatment of wheat straw were seen between particle sizes and incubation times. The particle size of wheat straw had a significant effect on all variables measured, except the absolute amounts of cellulose. The time of incubation had an effect ($P<0.05$) on all variables measured. The amount of inoculum had a significant effect on the ergosterol content, absolute amounts of hemicellulose and ADL and hemicellulose and cellulose contents.

Addition of 1.5 or 3% *L. edodes* inoculum to 0.5 cm wheat straw particles resulted in a continuous growth during 8 weeks, whereas addition to 2 cm wheat straw particles resulted in growth which stopped after 4 weeks of incubation. No changes in ergosterol content were observed throughout the entire incubation period when 0.5% inoculum was added to 0.5 cm wheat straw particles.

Regardless how much inoculum was added, the ADL content and absolute amounts of 0.5 cm wheat straw did not change after 4 to 6 weeks of *L. edodes* incubation (Tables 7.5 and 7.6). In contrast, the ADL in 2 cm wheat straw decreased continuously during the 8 weeks of incubation (Tables 7.5 and 7.6). After 8 weeks of incubation, the ADL content of 2 cm wheat straw was lower ($P<0.05$) than that of the 0.5 cm particles (Tables 7.5 and 7.6). The hemicellulose content decreased ($P<0.05$) during the first 4 to 6 weeks (Table 7.5). The final hemicellulose content and the absolute amounts after 6 and 8 weeks of incubation were lower for the 2 cm particles than for the 0.5 cm particles (Tables 7.5 and 7.6). The cellulose content increased more ($P<0.05$) for the 0.5 cm particles than for the 2 cm particles (Table 7.5). This effect was more visible by the absolute amounts of cellulose, where only a significant decrease in time was seen when 1.5% inoculum was added to 0.5 cm wheat straw particles (Table 7.6).

Six to eight weeks of *L. edodes* treatment increased the IVGP of the 2 cm wheat straw particles more ($P<0.05$) than that of the 0.5 cm wheat straw particles (Figure 7.2b).

Table 7.5 Changes in detergent fiber content (g/kg DM) over an eight week period of 0.5 and 2 cm wheat straw incubated with three different amounts of *L. edodes* inoculum.

Particle size			0.5 cm			2 cm		
Inoculum (%)			0.5	1.5	3.0	0.5	1.5	3.0
	Weeks							
ADL	0		90.8^{abc}	90.8^{ab}	90.8^{a}	90.8^{b}	90.8^{a}	90.8^{a}
	2		95.7^{a}	100.2^{a}	97.9^{a}	107.3^{a}	97.3^{a}	92.6^{a}
	4		92.7^{ab}	95.4^{ab}	82.6^{ab}	89.1^{b}	79.5^{b}	79.3^{b}
	6		81.9^{bc}	80.5^{ab}	95.6^{a}	68.2^{c}	64.1^{c}	64.5^{c}
	8		78.6^{c}	79.0^{b}	69.9^{b}	56.5^{d}	54.6^{d}	52.2^{d}
		SEM	2.74	4.40	3.82	1.80	1.63	2.36
		P-value	0.0056	0.0261	0.0025	<.0001	<.0001	<.0001
HC	0		287.1^{a}	287.1^{a}	287.1^{a}	287.1^{a}	287.1^{a}	287.1^{a}
	2		288.8^{a}	277.8^{a}	262.8^{ab}	286.9^{a}	273.6^{a}	262.1^{b}
	4		253.4^{b}	260.7^{ab}	235.4^{bc}	263.7^{a}	242.5^{b}	233.1^{c}
	6		224.7^{c}	228.9^{bc}	247.2^{bc}	211.4^{b}	204.8^{c}	190.7^{d}
	8		240.8^{bc}	223.6^{c}	221.0^{c}	199.9^{b}	188.2^{c}	196.2^{d}
		SEM	5.22	7.96	6.04	8.39	4.80	3.44
		P-value	<.0001	0.0006	0.0002	<.0001	<.0001	<.0001
Cell	0		474.1^{b}	474.1^{b}	474.1^{ab}	474.1	474.1^{b}	474.1^{bc}
	2		480.4^{ab}	470.5^{b}	461.1^{b}	493.5	475.6^{b}	467.1^{c}
	4		489.5^{ab}	503.1^{a}	485.5^{a}	481.3	500.3^{a}	486.9^{abc}
	6		493.8^{a}	491.2^{ab}	478.7^{ab}	500.7	498.3^{a}	503.0^{a}
	8		489.8^{ab}	479.6^{ab}	478.1^{ab}	508.3	507.4^{a}	494.1^{ab}
		SEM	4.04	6.20	4.63	8.32	4.17	4.62
		P-value	0.035	0.0221	0.0403	0.0835	0.0005	0.0016

Values with different superscripts within column are significantly (P<0.05) different. ADL = acid detergent lignin, HC = hemicellulose, Cell = cellulose,
SEM = standard error of the mean.

Table 7.6 Changes in absolute amounts of detergent fiber (g) over an eight week period of 0.5 and 2 cm wheat straw incubated with three different amounts of *L. edodes* inoculum.

Particle size			0.5 cm			2 cm		
Inoculum (%)			0.5	1.5	3	0.5	1.5	3
	Weeks							
ADL	0		17.7^{a}	17.7^{ab}	17.7^{a}	17.7^{b}	17.7^{a}	17.7^{a}
	2		18.4^{a}	19.5^{a}	19.2^{a}	20.6^{a}	18.8^{a}	18.0^{a}
	4		17.5^{a}	18.3^{ab}	16.0^{ab}	16.8^{b}	15.2^{b}	15.2^{b}
	6		15.7^{ab}	15.2^{b}	18.3^{a}	12.7^{c}	12.0^{c}	12.2^{c}
	8		14.6^{b}	14.7^{b}	13.2^{b}	10.4^{d}	10.1^{d}	9.7^{d}
		SEM	0.60	0.85	0.77	0.33	0.32	0.43
		P-value	0.0062	0.0113	0.0019	<.0001	<.0001	<.0001
HC	0		56.0^{a}	56.0^{a}	56.0^{a}	56.0^{a}	56.0^{a}	56.0^{a}
	2		55.6^{a}	54.0^{a}	51.6^{ab}	55.2^{a}	52.8^{a}	51.1^{b}
	4		47.9^{b}	50.1^{ab}	45.6^{c}	49.8^{a}	46.3^{b}	44.7^{c}
	6		43.1^{c}	43.2^{bc}	47.3^{bc}	39.3^{b}	38.2^{c}	36.1^{d}
	8		44.8^{bc}	41.7^{c}	41.8^{c}	36.8^{b}	34.8^{c}	36.4^{d}
		SEM	0.96	1.49	1.29	1.59	0.92	0.64
		P-value	<.0001	0.0001	0.0001	<.0001	<.0001	<.0001
Cell	0		92.5	92.5^{ab}	92.5	92.5	92.5	92.5
	2		92.5	91.4^{b}	90.6	94.9	91.7	91.1
	4		92.6	96.6^{a}	94.0	90.9	95.5	93.5
	6		94.7	92.8^{ab}	91.6	93.2	92.9	95.1
	8		91.2	89.5^{b}	90.3	93.6	93.9	91.7
		SEM	1.38	1.03	1.08	1.59	0.83	1.06
		P-value	0.5141	0.0076	0.1953	0.5183	0.0667	0.1257

Values with different superscripts within column are significantly ($P<0.05$) different. ADL = acid detergent lignin, HC = hemicellulose, Cell = cellulose,
SEM = standard error of the mean.

7.4 Discussion

The aim of this study was to optimize the colonization conditions of fungal treatments. To do so, the amount of inoculum added and the particle size of the substrate was varied. Here, millet was chosen as inoculation material, because of its small grain size. Addition of the same weight of spawn will result in more inoculation points when using a smaller grain. Analysis showed that the contribution of millet to the ADL content is negligible, whereas millet contributes to IVGP, and add up a few percentages of hemicellulose and cellulose to the substrates. The contribution to the IVGP is mainly because millet contains starch. The starch content in the uninoculated millet grains was not significantly different from that of millet grains colonized with *C. subvermispora*, while the starch content of the millet grains colonized by *L. edodes* was significantly lower. The finding that *C. subvermispora* did not utilize starch is interesting as the publically available genome data of *C. subvermispora* shows the presence of genes encoding for amylase. The ergosterol content data show that *C. subvermispora* grew on both millet and wood chips, suggesting millet can be used as spawn for *C. subvermispora*.

The spawn was added to the substrates in different amounts with all three resulting in full colonization and selective lignin degradation. The amount of *C. subvermispora* inoculum added to wood chips did not have a significant effect on the fungal treatment suggesting that the initial colonization rate is not the limiting factor for *C. subvermispora*.

C. subvermispora, in contrast to *L. edodes*, showed a low growth on wheat straw. In some treatments, such as addition of 0.5% inoculum to 0.5 cm wheat straw particles, also a low growth of *L. edodes* was found. A possible explanation for the low efficiency of the fungal treatment of wheat straw in the present study is the presence of fungicides. Analysis of the wheat straw showed that it contained 0.767 mg tebuconazole/kg dry substrate. Tebuconazole is a fungicide used in wheat production to control plant pathogenic fungi. The minimal inhibitory concentration of tebuconazole has been shown to be different for different basidiomycete species (Woo et al., 2010). Possibly *C. subvermispora* is more sensitive to this fungicide than *L. edodes*. Tebuconazole inhibits the formation of ergosterol in fungi (Woo et al., 2010). *C. subvermispora* contains more ergosterol per g of mycelium, which may be the reason why *C. subvermispora* is more sensitive to this fungicide. For future fungal treatments it is advised to use organically grown wheat straw. Although the growth of *L. edodes* was not fully inhibited, less degradation was observed in this study compared to other studies (Tuyen et al., 2012; van Kuijk et al., 2015b). A better performance of *L. edodes* can be expected when 'untreated' wheat straw is used for the fungal treatment.

However, *L. edodes* showed a reduced growth on wood chips. A possible explanation may be the different moisture content between both wood chips and wheat straw. The moisture content of wheat straw was around 60%, while that of wood chips was around 45%. This difference is inherent to the manner of moisturizing the substrates. After full penetration of the water, the 'free' water is removed by draining. The water holding capacity of wood chips is lower than that of wheat straw, causing a difference in

moisture content. However there may be a different water activity for both substrates, which was not measured. Possibly *L. edodes* is more sensitive to water activity than *C. subvermispora*.

The particle size of the substrate had only an effect on the *L. edodes* treatment of wheat straw. Sachs et al. (1990) showed that *P. chrysosporium* formed more mycelium on the surface of aspen wood chips compared to the inside of the wood chips. Therefore, it is expected that with a decreasing particle size, and thus a larger surface to volume ratio, the substrate is more accessible and more mycelium can be formed. Despite the different surface to volume ratio, particle size did not have a major influence on the *P. chrysosporium* growth patterns (Sachs et al., 1990). In the present study, particle size had a significant effect on the ergosterol content, although only addition of 0.5% inoculum to 0.5 cm wheat straw particles had a remarkable low ergosterol content. Probably the contrast between the surface to volume ratios between particle sizes was not large enough to see effects on colonization. Interestingly, 2 cm particles resulted in a lower ADL and a higher IVGP upon the fungal treatment. Gómez (2012) demonstrated an increased expression of cellulases and xylanases by *Trametes* sp., when grown on smaller particles of corn straw, compared to larger particles. Carbohydrates in smaller particles are possibly more exposed at the surface compared to large particles. To reach the carbohydrates in large particles, the fungi first have to degrade more lignin. On the other hand, not more carbohydrate degradation was observed in the smaller particles.

Particle size did not have an effect on the *C. subvermispora* treatment of wood chips. If a larger contrast between the particle sizes was used, maybe an effect would have been seen. In the scientific literature however, 15 mm size corn stover yielded less glucose upon enzymatic saccharification than 10 and 5 mm corn stover after a *C. subvermispora* treatment (Wan and Li, 2010). The same authors concluded that moisture content, time and temperature were also important factors influencing the effectiveness of the fungal treatment. Different substrates show different water holding capacities, which also depends on the particle size, explaining why the effect of particle size may be different for different substrates.

The results of this study show that the particle size (0.5 or 2 cm) tested does not have a major influence on the *C. subvermispora* treatment of wood chips. Using wheat straw particles smaller than 2 cm does not have beneficial effects on the *L. edodes* treatment.

7.5 Conclusions

The major changes in chemical composition, IVGP and ergosterol content occur during the first 4 weeks of *C. subvermispora* treatment of wood chips. The particle size of the wood chips and the amount of inoculum added do not have significant effects on the *C. subvermispora* treatment. Colonization does not seem to be the limiting factor for *C. subvermispora*. The *L. edodes* treatment of wheat straw was influenced by the particle size of the substrate. Larger (2 cm) particles resulted in a more selective delignification and a higher IVGP than smaller (0.5 cm) particles. The amount of inoculum to some

extent effects *L. edodes* treatment of wheat straw. At least 1.5% spawn should be added to obtain a more selective lignin degradation and an increased IVGP.

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CHAPTER 8

General discussion

In the coming decades, the demand for food, feed and fuel are expected to increase. The increasing demand for food will also require more animal products (Boland et al., 2013). An increasing demand for animal products requires an increased production of animal feed. On the other hand, fossil fuels are expected to become scarce, resulting in the development of fuels originating from plant materials. An increasing demand will require more arable land than available on our planet (FAO, 2009). One of the strategies to fulfil the increasing demand for feed and fuel without a competition with food is through using the whole crop. The part of the plant remaining after the harvest of products for human consumption is regarded as organic waste. Organic waste consists of plant parts that have a high concentration of plant cell walls. Plant cell walls contain carbohydrates such as hemicellulose and cellulose. These carbohydrates are highly degradable by the microbes in the rumen or by enzymes used in bioethanol production. However, in plant cell walls, these carbohydrates are bound to lignin, which is a recalcitrant polymer that is hard to degrade. As a consequence, lignin forms an obstacle for rumen microbes or enzymes used in bioethanol for saccharification. Lignin removal will increase accessibility of the carbohydrates in plant cell walls and several pre-treatments to remove lignin have been developed, among which chemical and physical. These pre-treatments are expensive, require special equipment and may cause toxic waste streams for the environment. Therefore, a biological pre-treatment using selective lignin degrading fungi has been investigated in this thesis.

8.1 Fungal treatment results in selective lignin degradation and increased cellulose accessibility

One of the aims of this thesis was to investigate the possibilities of a fungal pre-treatment to increase the utilization of lignocellulosic biomass. Based on the scientific literature (summarized in Chapter 2), four fungal species were selected to pre-treat four sources of biomass (Chapter 3). With this, the proof of principle was corroborated; fungal pre-treatment increases the *in vitro* rumen degradability of low quality biomass. Fungal pre-treated material was analyzed in more detail to reveal the mechanisms of delignification (Chapter 5).

In short, untreated wheat straw mainly consists of hemicellulose, cellulose and lignin. Furthermore, some crude protein, ash and other compounds such as water soluble compounds are present. Spawn inoculated with either *Ceriporiopsis subvermispora* or *Lentinula edodes* is added to moistened wheat straw to start the fungal treatment. Samples were taken weekly during incubation. The chemical composition obtained after 5 weeks of *C. subvermispora* and 8 weeks of *L. edodes* treatment of wheat straw are summarized in Figure 8.1. Both fungi degrade mainly hemicellulose, acid detergent lignin (ADL) and some cellulose. Degradation of these products results in the formation of mycelium and the fraction ‘other’ is increased. The composition of the fraction ‘other’ is not fully known, but it probably contains pectin and water soluble compounds. Possibly the carbohydrates in plant cell walls are converted to soluble sugars by the fungi. Also degradation products of lignin are formed (Chapter 4 and 5) and probably

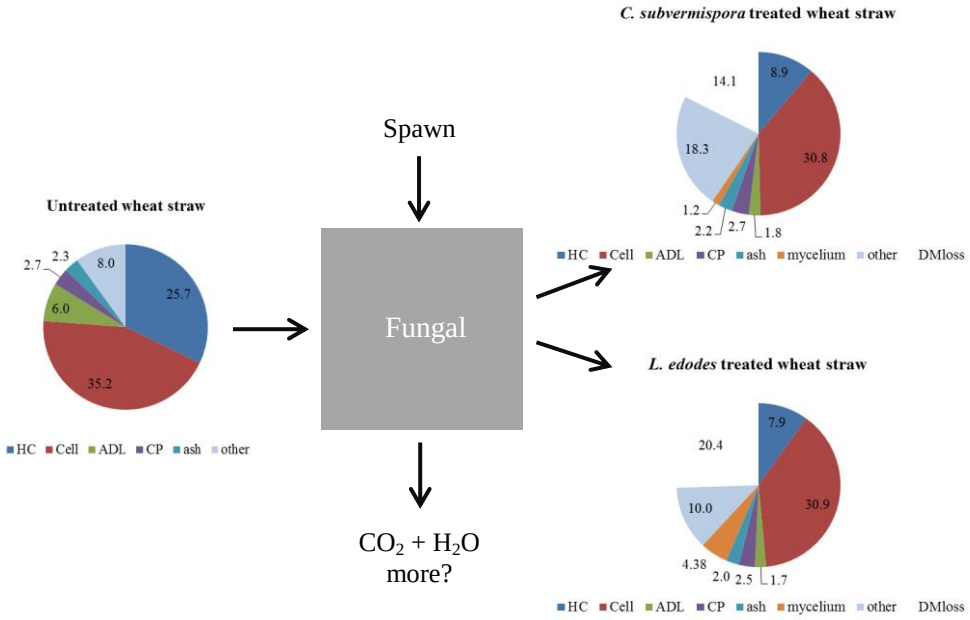

Figure 8.1 Mass balance of fungal treatment of wheat straw. HC = hemicellulose, cell = cellulose, ADL = acid detergent lignin, CP = crude protein, DMloss = dry matter loss.

these degradation products will not be measured as lignin due to the filtration step in ADL analysis. Also mycelium can significantly contribute to the detergent fiber contents, as in the scientific literature it is described that more than 50% of *Agaricus bisporus* mycelium is measured as Klason lignin (Jurak et al., 2015). Making a mass balance of a fungal treatment is even more complicated due to the production of gas upon lignocellulose degradation.

Although the changes in the chemical composition have been followed on a weekly basis in Chapter 5, the fungal treatment is still not fully understood. The composition of wheat straw that is the input of the fungal treatment is relatively well-known. The output of the fungal treatment can also be analyzed, although this is not routinely done for the gas fraction. The mode of action of the fungal treatment and the mechanisms of the enzymatic and non-enzymatic degradation are not fully understood. Also the intermediate products of other compounds than lignin (e.g. sugars) in wheat straw are not measured in this thesis.

Without fully understanding the mechanisms, it can be concluded that fungal treatment results in an increased accessibility of the plant cell wall carbohydrates. Both the *in vitro* rumen degradability and enzymatic saccharification are increased upon a fungal treatment (Chapter 5). The knowledge obtained in this thesis regarding fungal treatments can be used in applications as feed ingredients for ruminants and for biofuel production. In this Chapter, all data obtained in this thesis will be combined and discussed in the

perspective of feed and fuel applications. Also recommendations for future research will be provided in this Chapter.

8.2 Fungal treated biomass can be used in ruminant feed

The gastrointestinal tract of ruminants is adapted to a plant based diet. Digestion of plant material requires degradation of plant cell walls, which is a highly complex interaction involving multiple bacteria, anaerobic fungi and possibly protozoa species. These organisms produce several enzymes to degrade cellulose and hemicellulose, which requires direct adhesion of the microorganisms to the cell wall (Krause et al., 2003; Malherbe and Cloete, 2002). Upon adhesion, a complex of enzymes is produced to form cellulosomes, which degrades the cellulose and hemicellulose to which it is adhered (Krause et al., 2003). As such, direct contact between the cellulosome and the plant cell wall carbohydrates is required for effective degradation. As a consequence of the lack of lignin degrading enzymes in the rumen, cellulose and hemicellulose should be at the surface of the plant cell wall. Lignin is physically blocking the carbohydrates for degradation by the rumen microbiota. The removal of lignin during a fungal treatment, as shown in this thesis, resulted in an increased *in vitro* gas production (IVGP) by the rumen microbiota (Chapters 3, 4, 5, 6 and 7). IVGP is directly correlated to degradability of organic matter in the rumen (Menke et al., 1979), suggesting an increased accessibility of carbohydrates for the rumen microbiota after a fungal treatment. Plant cell wall degradation in the rumen is not yet fully understood, but many different organisms and enzymes are involved in this process. Until now, not all rumen microbes could be characterized and/or cultured (Dai et al., 2014; Krause et al., 2003). Understanding the mechanisms of plant cell wall degradation in the rumen can lead to a better targeted pre-treatment of plant cell wall material. The focus of the studies reported here was on selectively degrading lignin rather than modifying lignin to increase the accessibility of carbohydrates for rumen microbes. However, lignin degradation may not be necessary. Breaking the linkages between lignin and the carbohydrates may already be sufficient. Uncoupling of lignin from the plant cell wall will increase the accessibility of carbohydrates, but lignin will still be present in the material. Uncoupling of lignin will only result in an increased rumen degradability if lignin is not toxic for rumen microbes. Phenolics, such as cinnamic acid and vanillin, originating from lignin are described to depress the *in vitro* rumen degradability (Varel and Jung, 1986). These phenolics are also produced upon fungal delignification (Chapter 4 and 5). The same chapters describe an increased IVGP as more lignin is degraded into phenolics, suggesting that the degradation products of a fungal treatment are not toxic to the rumen microbiota. Chapters 4 and 5 also describe the degradation of ferulates in wheat straw by both *C. subvermispora* and *L. edodes*, which connects lignin to hemicellulose. Removal of the linkages between lignin and the carbohydrates may also contribute to the large improvement in IVGP upon a fungal treatment. The ferulate cross linkages have been described to contribute for 50% of the inhibitory effects of lignin (Grabber et al., 2009).

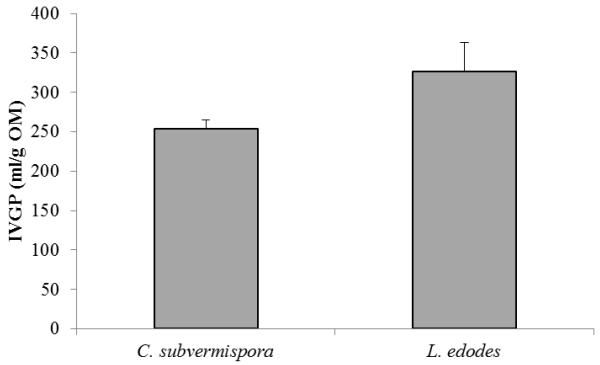

Figure 8.2 *In vitro* gas production in rumen fluid of pure mycelium.

Both *C. subvermispora* and *L. edodes* proved to be promising fungi for treatment of biomass containing a high lignin content. For the production of animal feed it is important to take the safety of the organisms used into consideration. *C. subvermispora* does not have a generally regarded as safe (GRAS) status, and is thus not yet approved for human or animal consumption. This fungus does not produce clear fruit bodies and, therefore, it is unclear whether this fungus is edible and thus safe for animals or humans to consume. To the author's knowledge, there are no data in the scientific literature that describe the toxicity of *C. subvermispora* or its metabolites. If the fungus would be inhibiting rumen microbes, then the *in vitro* gas production of fungal treated material is not expected to be able to increase to this extent. The IVGP of pure mycelium (grown on cellophane covered malt extract agar plates) of both *C. subvermispora* and *L. edodes* is very high (Figure 8.2), even comparable to grass (approximately 250 ml/g OM) and corn cobs (approximately 340 ml/g OM) (Cone et al., 1996). The high IVGP suggests that there is no inhibition by the mycelium itself. The growth of mycelium during the fungal treatment may even enhance the IVGP more than just increasing the accessibility of the carbohydrates. However, it must be noted that the excretion of secondary metabolites by the fungi can be different when grown on lignocellulosic substrates. These data are in line with the *in vivo* experiment of Okano et al. (2009) who fed sheep *C. subvermispora* treated bamboo and did not show any harmful effects to the animals. However, the latter study did not investigate any possible long term effects.

A good alternative for *C. subvermispora* is *L. edodes*. Although this fungus requires more time to reach optimal delignification, it is also highly effective (Chapter 5). The fruit bodies of *L. edodes* (shiitake) are consumed by humans all over the world, giving this organism the GRAS status and indicates they are safe for consumption. *L. edodes* has been used as a feed additive for poultry and is described to have a positive effect on the immune system and growth of broilers (Guo et al., 2004). A pre-treatment of lignocellulosic biomass with *L. edodes* may have additional positive effects to the animal's performance besides rumen degradability.

The data in Chapters 3, 4 and 5 show an increase in IVGP of wheat straw to similar levels as grass silage (approximately 250 ml/g OM) (Cone et al., 1996). As such, fungal treated wheat straw could be considered as replacement of (or part of) the roughage component in a ruminants ration. A point of concern is the low N content in fungal treated material and it is, therefore, unlikely that fungal treated material can be used as a complete ration.

In addition to wheat straw, wood chips are used in this thesis as a 'proof of principle' substrate, because of its high lignin content. The IVGP of wood chips significantly increased after a fungal treatment to values comparable to untreated wheat straw. Optimization of the fungal treatment of wood chips may even result in higher IVGP values. Wood chips are generally not seen as the most obvious choice to use in animal nutrition. However, Martínez et al. (2011) showed that wood degraded by *Ganoderma australe* is consumed by ruminants as a natural feed source. Degradability data show that wood chips can be used as a fibrous feed component, but care should be taken for splinters which can damage the gastrointestinal tract. Also, the palatability of wood chips should be studied in more detail. Generally, palatability of fungal treated materials, also other than wood chips, can be an issue as described in Chapter 2. This should be studied in more detail in *in vivo* experiments.

8.3 Fungal treated biomass can be used in bioethanol production

The effectiveness of fungal treatment has been measured using IVGP measurements. Chapter 5 describes enzymatic saccharification of fungal treated wheat straw, and shows a high correlation between enzymatic saccharification and IVGP. It can be assumed that an increase in IVGP will also result in an increased sugar yield upon enzymatic saccharification. Chapter 5 shows an increased accessibility of carbohydrates in the plant cell wall after a fungal treatment, since a higher release of glucose and xylose was found compared to untreated wheat straw. Although those results are very interesting, the biofuel production industry is interested in sugar yields. Additional data (not presented in this thesis previously) show the carbohydrate composition of untreated, *C. subvermispora* and *L. edodes* treated wheat straw after 8 weeks of treatment, and the conversions to monomers upon enzymatic saccharification (Table 8.1). From these data, sugar yields can be calculated according to the equation presented by Wan and Li (2011):

$$Sugar\ yield\ (\%) = \frac{Mmonosaccharide}{Mpolysaccharide \times f} \times 100$$

in which $Mmonosaccharide$ is the amount of glucose or xylose released upon enzymatic saccharification, $Mpolysaccharide$ is the amount of glucan or xylan present in the biomass and f is the conversion factor for polysaccharide to monosaccharide, which is 1.11 for glucan to glucose and 1.14 for xylan to xylose (Sluiter et al., 2008; Wan and Li, 2011).

The xylose yield increased from 24.3% in untreated wheat straw to 85.5 and 64.6% in *C. subvermispora* and *L. edodes* treated wheat straw, respectively. The glucose yield increased from 35.0% in untreated wheat straw to 71% in *C. subvermispora* treated and 72.6% in *L. edodes* treated wheat straw. These results show a numerical increase in sugar yield upon a fungal treatment, and confirm the increased accessibility of the carbohydrates.

The data presented here are obtained after 8 weeks of fungal treatment. The data in Chapter 5 show that the optimal fungal treatment (most selective lignin degradation and highest increase in IVGP) is reached after 5 weeks of *C. subvermispora* treatment, after which the fungus starts to degrade cellulose. The results presented in Table 8.1 could be improved by using material after a shorter *C. subvermispora* treatment. After 8 weeks of *C. subvermispora* treatment, more xylose and a similar glucose yield was obtained compared to 8 weeks of *L. edodes* treatment. Both xylose and glucose yields could be improved using material after 5 weeks of *C. subvermispora* treatment. For this reason, *C. subvermispora* would be a potential fungus for lignocellulose pre-treatment for biofuel production. In contrast to animal feed production, the criteria that the used organisms have to be safe for consumption is not the case for bioethanol production, making *C. subvermispora* the perfect candidate. If fungal treatment will be implemented in practice, a yeast strain that can convert both C5 (xylose) and C6 (glucose) sugars should be used for bioethanol production.

Yang and Wyman (2008) describe prerequisites for the pre-treatment of a low cost cellulosic bioethanol production. One important factor is the use of chemicals during the pre-treatment which can be expensive and results in waste streams that are difficult and expensive to dispose of. No chemicals are required for a fungal pre-treatment. This also means that the reactors do not need to be made of expensive materials. A second important factor determining the costs of the pre-treatment is the need for milling (Yang and Wyman, 2008). The effect of particle size on the fungal treatment was tested in Chapter 7. No significant effects of particle size of wood chips were observed. The use of wheat straw chopped to 2 cm length resulted in a significant higher increase in IVGP upon *L. edodes* treatment, compared to wheat straw of 0.5 cm. Himmel et al. (2007) recommended to use particle sizes between 1 and 15 cm in pre-treatments due to the economic and energetic costs of reducing particle size.

Table 8.1 Sugar yields before and after 8 weeks *C. subvermispora* or *L. edodes* treatment of wheat straw.

Sugar	Untreated	*C. subvermispora*	*L. edodes*
Xylan (g/kg sample)	185	125	132
Xylose (g/kg sample)	51	121	97
Xylose yield (%)	24.3	85.5	64.6
Glucan (g/kg sample)	386	435	425
Glucose (g/kg sample)	150	361	343
Glucose yield (%)	35.0	71.0	72.6

The disadvantage of fungal treatments is the use of carbohydrates by the fungi itself. Both *C. subvermispora* and *L. edodes* use hemicellulose, and to some extent cellulose. As a result, less sugars will be available for enzymatic saccharification and yeast fermentations to produce bioethanol. The hemicellulose and cellulose left in the materials are highly fermentable, which is beneficial for the biofuel production where the aim is 100% fermentability.

During the bioethanol production process the enzymatic saccharification and yeast fermentation steps occur at temperatures of approximately 50°C. To heat the production process, the lignin residues, which remain after bioethanol production, are burned. However, during the fungal pre-treatment, most of the lignin in the biomass is removed. As a result a low amount of lignin residue will be available after the bioethanol production for heating the process. This means that another source to heat the process is needed.

A suggestion to produce heat for the pre-treatment process is to use spent mushroom compost after *Agaricus bisporus* mushroom production, but not before the present xylanases are extracted (Textbox 8.1). The extraction process of xylanases is easy and cheap. The extracted xylanases can be used to convert hemicellulose into xylose (Textbox 8.1). After the enzyme extraction, the remaining material can be burned to obtain energy. In this way, the waste stream after mushroom production is reduced, cheap enzymes can be produced and a cheap source of energy to fuel the bioethanol production process is obtained. However, spent mushroom compost has a high moisture and ash content resulting in a low burning value.

8.4 Optimization of the fungal treatment is needed before it can be used in practice

The length of the fungal treatment and the use of carbohydrates by the fungi should both be minimized, while the lignin degradation should be maximized, to make the fungal treatment economically competitive with other pre-treatments. Optimization strategies can either target the improvement of the fungus or change the substrate in such a way that the fungal treatment is enhanced. This chapter will discuss optimization strategies to directly improve the fungal treatment or changes applied to the substrate to improve the effectiveness of the fungal treatment. In this thesis, three optimization strategies were tested, including additives to the fungal treatment (Chapter 6), decrease the particle size (Chapter 7) and the amount of inoculation points (Chapter 7). These optimization strategies will be discussed here and other optimization strategies will be suggested.

8.4.1 The choice of fungal species and strain is important for efficient fungal treatment

The selectivity of lignin degradation by *C. subvermispora* has been described by several authors (Okano et al., 2005; 2009; Sharma and Arora, 2010; Rahman et al., 2011; Hassim et al., 2012; Tuyen et al., 2012; 2013). The reason for this selectivity is thought to be an incomplete cellulose degrading system as demonstrated by the low ability of *C. subvermispora* to degrade filter paper consisting of cellulose (Tanaka et al., 2009). In

this thesis, cellulose degradation by *C. subvermispora* was observed (Chapters 5, 6 and 7). *C. subvermispora* needs 4 to 5 weeks to reach an optimum in lignin degradation before it starts degrading cellulose (Chapter 5). This optimum is similar for wheat straw and wood chips (Chapter 5).

The fungus *L. edodes* is a selective lignin degrader (Chapter 3, 4, 5, 6 and 7), but needs more time for the delignification process than *C. subvermispora* (Chapters 5 and 6), which was shown by Tuyen et al. (2012 and 2013). However, Zhao et al. (2015) reported *L. edodes* to be a better lignin degrader than *C. subvermispora*, when grown on rape straw. In the scientific literature, *L. edodes* treatment is often combined with mushroom production (Permana et al., 2000; Vane et al. 2003; Lin et al., 2005; Gaitan-Hernandez et al., 2006). Upon mushroom production, the fungus shifts to a cellulose degrading mechanism, whereas laccase is mainly produced just before the fruit body initiation, cellulase activities are highest during the fruit body development (Kües and Liu, 2000). It is thought that selective lignin degraders are degrading lignin to make cellulose accessible which is needed as carbon source during the fruit body production. To be prepared for reproduction, as much cellulose should be accessible as needed for the fruit body production. The required amount of cellulose for reproduction may be accessible after 4 weeks of treatment, explaining why the chemical composition of wood chips did not change further after this time (Chapter 5). If the amount of cellulose needed is the trigger to stop the delignification process, then the fungus can be stimulated by increasing the amount of cellulose needed. This can be achieved for example by breeding for larger fruit bodies. The production of a larger fruit body requires more carbon sources. However, laccase genes are described to be involved in the fruit body formation, especially during the formation of the cap of *L. edodes* (Kües and Liu, 2000). Breeding for larger fruit bodies might also change the laccase production, which is involved in the lignin degradation.

L. edodes continuously produces mycelium during the whole delignification period (Chapter 5). Mycelium growth is correlated to the actual acid detergent lignin (ADL) content ($r = -0.86$, $P<0.0001$, Chapter 5). This correlation suggests that stimulating the mycelium growth by *L. edodes* will stimulate lignin degradation. Addition of more inoculum at the start of the treatment as such, did not result in a faster fungal treatment (Chapter 7). This suggests that stimulation of mycelium growth should take place at a later stage of the fungal treatment. However, addition of urea to stimulate fungal growth did not result in more lignin degradation (Chapter 6).

Interestingly, *C. subvermispora* colonizes the material within the first week of treatment and does not grow until 5 weeks of treatment. Meanwhile the fungus is degrading lignin and hemicellulose and producing secondary metabolites such as alkylitaconic acids. The substrates used to produce the inoculum in this thesis were sorghum (Chapters 3 to 6) and millet (Chapter 7), which are both starch sources. Analysis of the colonized millet showed that *C. subvermispora*, in contrast to *L. edodes*, did not degrade the starch in the grains (Chapter 7). Similarly, sorghum grains contain 702.8 g/kg DM starch, whereas after colonization with *C. subvermispora* this is 658.8 g/kg DM and with *L. edodes*

483.2 g/kg DM (data not shown). The publically available genome of *C. subvermispora* shows that this fungus contains genes coding for amylase. Starch is apparently not the preferred nutrient for *C. subvermispora*. To obtain a faster start of the fungal treatment, a lignocellulosic inoculum should be considered. Using a lignocellulosic inoculum may result in a shorten lag time since a switch from a starch to a lignocellulose degrading enzyme system is not required. Stimulating the growth of *C. subvermispora* in later stages of the treatment is not necessary since the fungus degrades lignin without growing (Chapter 5 and 7).

For lignin degradation, mainly manganese peroxidase is produced (Lobos et al., 1994). *L. edodes* produces manganese peroxidase as main enzyme, but it is less important than for *C. subvermispora*. This is demonstrated by the fact that manganese (and linoleic acid) addition did not influence the delignification by *L. edodes* (Chapter 6). Janusz et al. (2013) report only 2 genes involved in manganese peroxidase production by *L. edodes*, while *C. subvermispora* contains 13 genes (Fernandez-Fueyo et al., 2012).

Manganese peroxidase can either convert Mn^{2+} into Mn^{3+} or fatty acids into lipid radicals via lipid peroxidation (Jensen et al., 1996). Delignification of wheat straw was stimulated in *C. subvermispora* cultures with manganese addition (Chapter 6), suggesting the production Mn^{3+} radicals. Manganese had only an effect after 4 weeks of treatment probably because in the first stages of delignification the cell wall structure is still too dense for enzymes to enter (Martínez et al., 2005), resulting in a first stage degradation by low-molecular-mass compounds (Tanaka et al., 2009). Addition of linoleic acid had limited effects on the delignification by *C. subvermispora*. This could be due to the presence of sufficient fatty acids in the substrates or production of fatty acid by the fungus. In contrast to *L. edodes*, *C. subvermispora* does produce fatty acids in the form of alkylitaconic acids (Chapter 5), which are thought to be involved in lipid peroxidation (Guiterrez et al., 2002; Nishimura et al., 2012). Lipid peroxidation involving alkylitaconic acids is described to be more effective than involving linoleic acid, due to the lifespan of the radicals (Nishimura et al., 2012). Addition of alkylitaconic acids to fungal treatments may result in more lignin degradation than the addition of linoleic acid. Another function proposed for these alkylitaconic acids is neutralization of products of the Fenton reaction (Rahmawati et al., 2005). The Fenton reaction is one of the few pathways in which *C. subvermispora* can degrade cellulose (Tanaka et al., 2009), and by blocking this with alkylitaconic acids, the fungus becomes very selective for lignin.

Both *C. subvermispora* and *L. edodes* mainly produce manganese peroxidase, although this enzyme is less important for the latter fungus. Breeding the fungi for more manganese peroxidase production might increase the lignin degradation.

Based on the results in this thesis and on data in the scientific literature, there are three reasons why only increasing manganese peroxidase production, or even a treatment with only manganese peroxidase (without fungus) may not have the desired effect. Firstly, in the initial phase of delignification, the cell wall structure is still too dense for enzymes to enter. Manganese peroxidase would not result in the optimal effect, since it cannot reach

Textbox 8.1: Xylanases from spent mushroom compost

Objective
Enzymes produced during mushroom production process of *Agaricus bisporus* may still be present in the spent mushroom compost. These enzymes can be used to convert carbohydrates in plant cell walls to sugars.

Materials and methods
The procedure for enzyme extraction was based on the method described by Ayala et al. (2011). In brief: spent mushroom compost (250 g) after 2 flushes of mushrooms was mixed with 375 ml sodium citrate buffer (50 mM, pH 5.3). This mixture was shaken during 30 minutes at 230 rpm on a rotary shaker at room temperature and filtrated through a double layer of cheesecloth. The filtrate was centrifuged for 25 minutes at 4000 rpm and the supernatant was centrifuged a second time for 20 minutes at 4200 rpm to remove particles from the extract. The protein concentration in the enzyme extract was determined using a BCA determination (Thermofisher Scientific Pierce BCA protein reagent kit, Waltham, Massachusetts, USA). Enzymatic saccharification was done at untreated, *C. subvermispora* or *L. edodes* treated wheat straw after 8 weeks (air-dried, ground to 1 mm) (Chapter 5). For statistical analysis, the sugar release data of the fungal treatment of each substrate were subject to the generalized linear model (GLM) analysis in SAS 9.3. The following model was used:
$Y_{ij} = \mu + \alpha_i + \omega_{ij}$
in which Y_{ij} is the observation j in treatment i; μ is the overall mean; α_i is the fixed effect of fungal treatment i; ω_{ij} is the random error.

Results
During 72 hours of enzymatic saccharification by spent mushroom compost extract, no glucose was released from both the fungal treated and untreated wheat straw (data not shown). The enzymes in the spent mushroom compost extract needed 8 hours or more to release significant ($P<0.05$) amounts of xylose per gram of hemicellulose in both *C. subvermispora* and *L. edodes* treated wheat straw (Figure T8.1.1). After 72 hours of enzymatic saccharification more ($P<0.05$) xylose was released from the fungal treated wheat straw, compared to untreated wheat straw, but there was no difference between both fungi (Figure T8.1.1).

Conclusions
In conclusion, no cellulases were extracted from spent mushroom compost. The xylanases extracted from spent mushroom compost released significantly more xylose from fungal treated wheat straw than from untreated wheat straw.

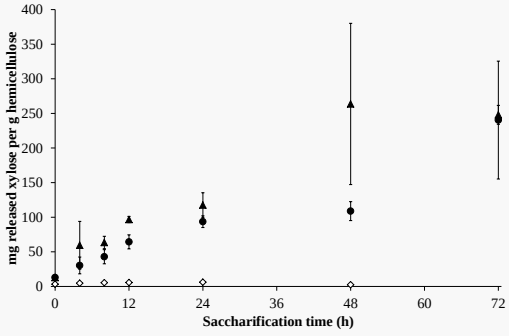

Figure T8.1.1 Xylose release upon enzymatic saccharification of untreated, *C. subvermispora* and *L. edodes* treated wheat straw. ● wheat straw treated for 8 weeks with *L. edodes*, ▲ wheat straw treated for 8 weeks with *C. subvermispora* ◊ untreated wheat straw. Error bars represent standard deviations (n=3).

Textbox 8.2: Fungal pre-treatment of sugarcane bagasse and spent brewery grains

Objective

Fungal treatment can potentially be used to increase the *in vitro* rumen degradability of all material with a high lignocellulose content. Here the effect of a fungal treatment by *C. subvermispora* and *L. edodes* of sugarcane bagasse and spent brewery grains on *in vitro* rumen degradability is investigated.

Materials and methods

Fungal strains and spawn preparation

Sugarcane bagasse and spent brewery grains were pre-treated with *Ceriporiopsis subvermispora* (strain MES 13094) and *Lentinula edodes* (strain MES 11910), according to the method described in Chapters 5 and 6. After incubation for 4 weeks, sugarcane bagasse and spent brewery grains were dried at 70ºC for further analysis. *In vitro* gas production (IVGP) was performed as described before in Chapters 3, 4, 5, 6 and 7. Results are expressed as ml gas produced per gram of organic matter (OM). For statistical analysis, the IVGP data of the fungal treatment of each substrate were subject to the generalized linear model (GLM) analysis in SAS 9.3. The following model was used:

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

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

Results

Sugarcane bagasse treated for 4 weeks with *C. subvermispora* or *L. edodes* resulted in a higher ($P<0.05$) IVGP, compared to the untreated control (Figure T8.2.1). The IVGP of spent brewery grains did not increase after the fungal treatment for 4 weeks. A longer pre-treatment of spent brewery grains might be considered, since other fungus/substrate combinations, described in this thesis, did not result in an increased IVGP after 4 weeks of treatment.

Conclusions

Fungal pre-treatment of sugarcane bagasse can be considered for future applications. The IVGP of spent brewery grains did not increase upon the fungal treatment, indicating that this substrate is unsuitable for a fungal pre-treatment.

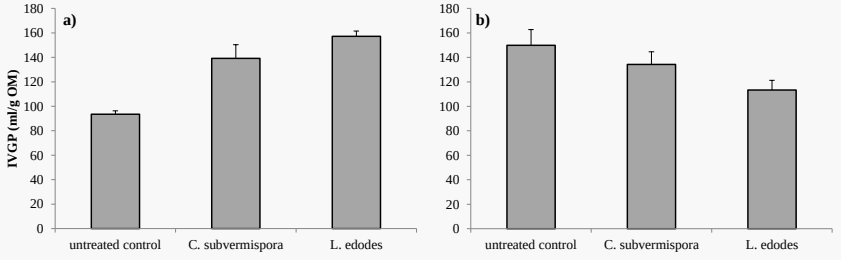

Figure T8.2.1 IVGP of sugarcane bagasse and spent brewery grains after 4 weeks of fungal treatment. a) sugarcane bagasse, b) spent brewery grains.

the lignin in the intact cell wall unless it produces radicals. Secondly, manganese peroxidase needs Mn and fatty acids to produce radicals that degrade lignin. The latter are present in the substrate, but are also produced by the fungus, such as alkylitaconic acids produced by *C. subvermispora* (Chapter 5). These fatty acids are not only involved in lignin degradation by manganese peroxidase but also in the prevention of Fenton reactions. A third reason why an enzyme treatment will not work is the fact that the lignin degrading enzymes produced by white rot fungi have their optimal activity at a low pH (Fernandez-Fueyo et al., 2014). The enzymes involved in lignin degradation are not stable at this pH, suggesting that the fungus must have a regulatory mechanism to ensure the enzymes work optimally.

Fernández-Fueyo et al. (2014) show that the lignolytic enzymes have their optimum around pH 2. The production of alkylitaconic acids (Chapter 5) may even contribute to the strategy of the fungus to decrease the pH of its environment. One of the described chemical pre-treatments for lignocellulosic biomass is a dilute acid pre-treatment. A dilute acid (often sulphuric acid) is added to the biomass and this mixture is heated (Mosier et al., 2005). The aim of a dilute acid pre-treatment is to remove hemicellulose and in this way the bindings with lignin, resulting in an increased accessibility of cellulose (Hendriks and Zeeman, 2009; Mosier et al., 2005). This may explain the loss of hemicellulose upon fungal treatments. Probably, the hemicellulose is used for growth of the fungus, although *C. subvermispora* degraded hemicellulose without growing (Chapter 5). An explanation can be the degradation of hemicellulose due to the acid environment, although the material is not heated like during a dilute acid pre-treatment.

An acid pre-treatment before a fungal treatment might decrease the treatment time needed. If the pH is already low before the fungal treatment, the fungus does not have to spent energy on the production of acids. On the other hand, the growth of the fungus might be hampered by the acid present in the substrate.

Having discussed the limitations of manganese peroxidase and the environment created by the fungus, breeding for alkylitaconic acid production may result in a better lignin degrading organism.

8.4.2 The choice of substrate is important for efficient fungal treatment

Chapter 3 shows that not only the fungal species but also the substrate is an important factor for the effectiveness of fungal treatment to decrease the lignin content. Chapter 4 confirms that the composition of the lignocellulosic biomass is influencing the delignification processes.

Several lignocellulosic materials have been used in this thesis for fungal treatment. These were substrates with a high cell wall content (NDF) of approximately 80%. Cell walls have a different composition in each plant species and can even differ within plants and tissues (Wei et al., 2009). A different composition of plant cell walls will require a different degradation strategy of the fungus. This thesis mainly describes the fungal treatment of wheat straw and wood chips, but also miscanthus (Chapter 3), rice straw (Chapter 3), sugarcane bagasse (Textbox 8.2) and spent brewery grains (Textbox 8.2) are

used. Not all substrates were found suitable for fungal treatment. For example, the IVGP of rice straw (Chapter 3) and spent brewery grains (Textbox 8.2) were not increased upon a fungal treatment. Other studies observed an increase in rumen degradability of rice straw after a fungal treatment using different fungal species (Bisaria et al., 1997; Karunanandaa et al., 1992; Tuyen et al., 2013). However, in those studies, different fungal species were used than described in Chapter 3. One of the reasons why the fungi used in Chapter 3 could not increase the IVGP of rice straw could be the relatively low ADL content (36 g/kg DM). A low ADL content is correlated to a higher IVGP (Chapter 3 and 4; Tuyen et al., 2012), suggesting a relatively good accessibility of the carbohydrates. In this case, lignin degradation is not required to obtain nutrients for fungal growth. Another possible explanation is the relatively high amount of silica in rice straw (Van Soest, 2006). Silica is present in cell walls of rice straw, but it is not bound to the cell wall carbohydrates. Silica reduces the organic matter digestibility, and is described as the main limiting factor for rice straw quality (Van Soest, 2006). This means that only lignin degradation during the fungal treatment is not sufficient to increase the digestibility of rice straw. Finally, rice straw composition and quality depend on the cultivar used and soil and weather conditions during cultivation (Van Soest, 2006). Possibly, the composition of the rice straw used in the current work was not optimal for a fungal treatment. The IVGP of spent brewery grains was not increased, probably due to the relatively high N content (about 38.9 g/kg DM, compared to 4.9 g/kg DM in wheat straw and 7.7 g/kg DM in wood chips). The C/N ratio is important in fungal treatments and fungal delignification is described to occur in an environment low in N (Reid, 1989).

The differences in the IVGP improvement upon a fungal treatment can be explained by a different composition of the substrates. Not only between substrates differences were observed, but also within the same substrate. The ADL content of the untreated wheat straw was significantly ($P<0.05$) different between the Chapters. However, ANOVA

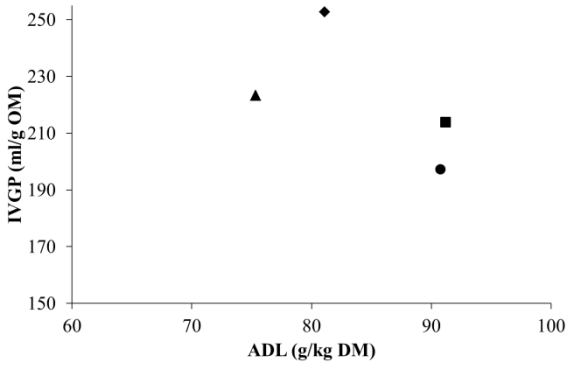

Figure 8.3 Acid detergent lignin (ADL) content and *in vitro* gas production (IVGP) in rumen fluid of untreated wheat straw used in ♦ Chapter 3 ▲ Chapter 5 ■ Chapter 6 and ● Chapter 7.

analysis of the IVGP data presented in Figure 8.3 showed that the IVGP was not significantly different between different batches of wheat straw used in this thesis.

A different chemical composition of the raw material might have an effect on the fungal treatment (Chapters 4 and 5; Skyba et al., 2013). To correct for the different composition at the start of the fungal treatment, changes in ADL and IVGP upon fungal treatment are expressed as percentage change compared to untreated material (Figure 8.4). Chapter 3 shows the importance of treatment time. Therefore, we can only compare results after 2 and 4 weeks of treatment, since these incubation times are described in Chapter 3, 4, 5 and 6. The wheat straw used in Chapter 7 had a different particle size and inoculum source than in the other Chapters. Secondly, as discussed in Chapter 7, wheat straw may contain chemicals inhibiting fungal growth (fungicides). Again this confirms the importance of raw material selection before the fungal treatment. As such, using organically grown wheat straw is advisable to avoid the presence of fungicides.

In all Chapters, a decrease in IVGP after 2 weeks of *C. subvermispora* or *L. edodes* treatment was observed (Figure 8.4b). This is probably due to the use of easily accessible nutrients present in the substrate for growth of the fungi (Zadrazil, 1977). However, in this thesis the substrate was moisturized by soaking in water for 3 days after which the excess of water was removed. With the removal of the excess of water, the water soluble compounds in the substrate may be, to a variable extent, removed. A different way of moisturizing the substrate may lead to more easy accessible nutrients. It is questionable if 2 weeks of treatment should be compared for ADL degradation, since degradation of easily accessible nutrients instead of delignification is taking place at this incubation time. Nevertheless, both fungi acted similar in all experiments, i.e. degradation of the easy accessible nutrients in the first weeks of treatment. Future research might determine what nutrients are used by the fungi at the start of the treatment. A suggestion to avoid this decrease in IVGP in the first phase of the fungal treatment would be to use a lignocellulose carrier as inoculum.

The changes in ADL content and IVGP after 4 weeks of fungal treatment as measured in the different chapters are shown in Figure 8.4. Changes in the ADL content were significantly ($P<0.05$) different between experiments of *C. subvermispora*, after 4 weeks of treatment, and of *L. edodes*, after both 2 and 4 weeks of treatment. Changes in the IVGP were only significant ($P<0.05$) between experiments for *L. edodes* after both 2 and 4 weeks.

One of the conclusions in Chapter 2 was to be careful with comparisons between studies due to differences in substrate batches, fungal strains and culture conditions. Throughout this thesis, strains and culture conditions were identical. Nevertheless, significant differences in changes in ADL and IVGP between studies were observed. This is also demonstrated in Chapter 6, which describes three different incubations of wheat straw with *C. subvermispora*.

There was a large variation between the experiments testing the same conditions. The above results show that the batch of a substrate is important. However, culture conditions of the fungal growth should be monitored. Controllable culture conditions,

such as temperature and moisture, were kept the same. Other culture conditions that might be of influence are the exposure to light, oxygen concentration or air quality. Changes in these culture conditions might influence the effectiveness of the fungal treatment. The effect of these culture conditions should be tested in future research, to find the optimal conditions.

Wood chips have a higher ADL content than wheat straw. Since ADL content and IVGP are negatively correlated (Chapter 3 and 5), the IVGP of untreated wood chips is relatively low. Chapter 4 shows that not only the lignin content of wood chips is higher than that of wheat straw, wood chips also have a different lignin composition. Whereas lignin in wheat straw contains S-, G- and H-units, lignin in wood chips contains mainly S- and G-units. Chapters 4 and 5 describe the preferential degradation of S-units by both *L. edodes* and *C. subvermispora*. S-units have a higher predominance of β-*O*-4 ether linkages than G-units (del Río et al., 2002). These β-*O*-4 ether linkages are a characteristic of linear lignins, which are easier to degrade than branched lignins (Grabber, 2005). Secondly, during cell wall formation, G- and H-units are formed in an earlier stage than S-units (Grabber, 2005). Possibly, S-units need to be removed before the fungus can reach the remaining lignin. Altogether, this means that lignin composition has an influence on lignin degradation efficiency by the fungi. Figure 8.5 combines changes in ADL to carbohydrate (hemicellulose + cellulose) ratio, as determined by the detergent fiber analysis, and IVGP upon fungal treatment obtained in this thesis. A similar figure was shown in Chapter 4, presenting the IVGP and detergent fiber contents of the treatments discussed in that study. Figure 8.5 combines data of all chapters and includes data from *C. subvermispora*. For both *C. subvermispora* and *L. edodes* treatment, the relationship between ADL to carbohydrate ratio and IVGP is shown for wheat straw and wood chips separately. With both fungi, the slope suggests that small changes in ADL to carbohydrate ratio have a slightly larger impact on the IVGP of wheat straw than of wood chips. This different effect on the substrate can be explained by the denser structure of wood chips (Chapter 6). Due to that density, both fungi and rumen microbes cannot enter the tissues. Within the dense tissue there is less oxygen

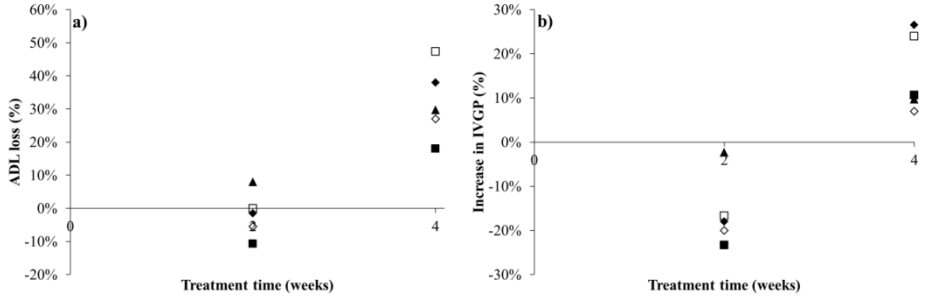

Figure 8.4 Fungal treatments compared between different chapters. a) acid detergent lignin (ADL) loss, b) increase in *in vitro* gas production (IVGP) in rumen fluid. ▲ *L. edodes* treated wheat straw Chapter 3, ■ *L. edodes* treated wheat straw Chapter 5, ♦ *L. edodes* treated wheat straw Chapter 6, □ *C. subvermispora* treated wheat straw Chapter 5, ◊ *C. subvermispora* treated wheat straw Chapter 6.

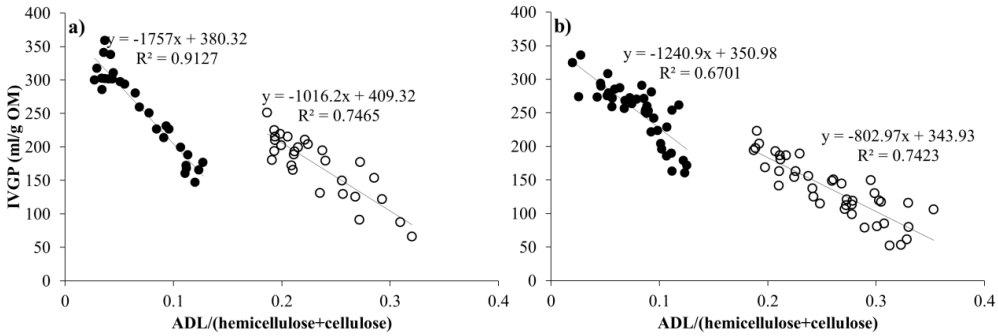

Figure 8.5 Relations between ADL to carbohydrate (hemicellulose + cellulose) ratio, as determined by the detergent fiber analysis, and IVGP. a) *C. subvermispora*, b) *L. edodes*. ● wheat straw ■ wood chips.

available for the fungi, compared to the relatively open structure of wheat straw. To overcome this problem, the particle size might be reduced. The effect of particle size of wood chips on the delignification by *C. subvermispora* has been tested in Chapter 7. However, no significant difference was observed between the fungal treatment of particles of 0.5 and 2 cm wood chips. Another explanation for the differences between the fungal treatment of wheat straw and wood chips might be the high lignin content of wood chips. A higher lignin content is negatively correlated to the IVGP (Chapter 3, Tuyen et al., 2012). Nevertheless, both wheat straw and wood chips have the same potential maximum IVGP when all ADL is removed. The maximum IVGP of both wheat straw and wood chips could theoretically be above 350 ml/g OM (intercept of regression equations Figure 8.5). The theoretical maximum IVGP is obtained after removal of all ADL. Since these substrates contain about 80% of cell walls, removal of ADL will result in almost pure cellulose and hemicellulose, which are both highly fermentable. It is not surprising that the theoretical maximum IVGP is similar to that of glucose as described by Cone et al. (1997).

Changes in lignin composition, i.e. S/G ratio, occurring during a fungal treatment are correlated to the IVGP (Chapter 4). In the same chapter, it is concluded that changes in the S/G ratio during a fungal treatment are a direct consequence of delignification. The effects of lignin content and lignin composition are difficult to separate. Literature data shows that lignin content is more important than lignin composition for both rumen degradability and enzymatic saccharification (Chen and Dixon, 2007; Grabber, 2005; Grabber et al., 2009). A major factor for the inhibition of the degradability of cell walls is ascribed to the linkages between lignin and the carbohydrates. The formation of ferulate cross-links accounts for half of the inhibitory effects of lignin, while p-coumarate esters have less inhibitory effects on the carbohydrate degradation (Grabber et al., 2009). Studies on the effects of lignin content, composition or cross-linkages use models consisting of modified cell walls which may have different properties from cell walls in plants. Due to their complexity, changing one feature may influence other characteristics of the cell walls.

8.4.3 Optimization of fungal treatment requires accurate analysis of plant cell wall components

Optimization is only useful if the effect of fungal treatments can be measured in a proper way. Since part of the thesis focuses on animal nutrition, analysis common in that field were used. In animal nutrition, cell walls are analyzed using the detergent fiber analysis according to Van Soest et al. (1991). Lignocellulosic biomass consists for a large part (>80%) of neutral detergent fiber (NDF). The NDF fraction, is insoluble in a neutral detergent solution and is described to consist of hemicellulose and acid detergent fiber (ADF) (Van Soest et al., 1991). The ADF fraction, is insoluble in acid detergent solution and is described to consist of cellulose and ADL. The ADL fraction is the fraction that is insoluble in 72% sulphuric acid and is described to consist of lignin. This means that the hemicellulose fraction can be calculated as NDF minus ADF and the cellulose fraction can be calculated as ADF minus ADL. The remaining part (lignocellulosic biomass minus NDF) consists of ash, proteins, fats and water soluble compounds. Using the detergent fiber analysis, fungal treated lignocellulosic biomass has a reduced hemicellulose and ADL content compared to the untreated material. The reduction in these fractions leads to a dry matter loss. Hemicellulose and ADL can be converted to fungal biomass, water soluble compounds or water and CO_2 (See also Figure 8.1). Both fungal biomass and water soluble compounds will still be measured as dry matter, but it is not known how this acts during the detergent fiber analysis. A mass balance should be made to study the fate of all compounds in the biomass. The conversion of each fraction of the biomass to water solubles and gas should be measured. As also discussed in Chapter 3, NDF contains some proteins and ADF contains some pectins and hemicellulose. As a result the hemicellulose fraction (NDF-ADF) represents only part of the hemicellulose and includes proteins. The cellulose fraction contains next to cellulose also pectin and acid soluble lignin. The terms hemicellulose and cellulose in the detergent fiber analysis do not represent the chemically defined polysaccharides, and both fractions are overestimated (Godin et al., 2015; Jung, 1997).

On the other hand, ADL is underestimating the total lignin content, since it does not take into account the acid soluble lignin (Godin et al., 2015; Jung, 1997). A change in acid soluble lignin may mistakenly be considered as a change in cellulose. Acid soluble lignin contents are described to be constant in beech and to increase in cedar during *C. subvermispora* treatment (Tanaka et al., 2009). Literature describing changes in the acid soluble lignin demonstrate the importance of analyzing the total lignin content. Changes in total lignin upon a fungal treatment have been studied in this thesis by pyrolysis gas chromatography/mass spectrometry (Py-GC/MS) (Chapter 4 and 6). Although the carbohydrate fractions are under estimated by Py-GC/MS due to charring upon pyrolysis, it is a powerful technique to measure lignin and lignin components. However, it is important to take into account that the results of Py-GC/MS are expressed as a ratio, and as such are not quantitative measurements. During a fungal treatment, lignin is degraded by the fungi to degradation products. Py-GC/MS shows the presence of these

degradation products (mainly Ph-C0-2) also before a fungal treatment. This could be inherent to the Py-GC/MS method. A second reason could be that upon handling maybe some part of the lignin is already degraded due to infections by other microorganisms. A last reason could be that these Ph-C0-2 compounds are still in development to become Ph-C3 compounds. It is not known in which fraction of the detergent fiber method these degradation products will end. Lignin degradation products such as phenolic monomers are described to inhibit cellulose and xylan degradability by fibrolytic rumen microbes (Varel and Jung, 1986). In this thesis, rumen microbes seemed to be not inhibited by the degradation products of a fungal delignification. The possibility of any inhibition by degradation products demonstrates the importance of analyzing total lignin in a correct manner.

Lignin content and cross-linking between lignin and carbohydrates are both influencing the *in vitro* rumen degradability. In Chapter 4 it is shown that pyrolysis of wheat straw released 4-vinylguaiacol and 4-vinylsyringol, which originates from ferulates and *p*-coumarates (del Río et al., 2012). During a fungal treatment, the relative amount of 4-vinylguaiacol and 4-vinylsyringol decreased using *C. subvermispora* (Chapter 6) and *L. edodes* (Chapter 4 and 6), suggesting that the fungi break down ferulates. Ferulate cross-linking is described to be responsible for half of the inhibition of carbohydrate degradation in the rumen by lignin (Grabber et al., 2009). Ferulates in the cell wall can be degraded by the enzyme feruloyl esterase, produced by the fungi. Feruloyl esterases are very difficult to measure due to an interference with laccase (Haase-Aschoff et al., 2013). Therefore, feruloyl esterase production is described for only a few white rot fungi, among which *Trametes versicolor*, *Bjerkandera. adusta*, *Ganoderma applanatum* and *Phlebia rufa* cultured on wheat straw (Dinis et al., 2009). Also *L. edodes* and *Pleurotus eryngii* are described to produce small amounts of feruloyl esterase (Haase-Aschoff et al., 2013). Although the production of feruloyl esterase by *C. subvermispora* has not been proven yet, this fungus is described to be able to remove ferulic acid and to a lesser extent *p*-coumaric acid (Akin et al., 1993). The increase in IVGP observed throughout this thesis may not only be a consequence of the total lignin degradation but also of breaking down cross-linkages between lignin and carbohydrates. Again it is questionable what the effect of breaking down cross-linkages is on the detergent fiber analysis. The high correlation between ADL and rumen degradability suggests that after breaking the cross-linkages, less of the material will end in the ADL fraction. Since literature describes that ferulates are affecting degradability more than the composition of lignin, future research should focus on changes in cross-linkages in the cell walls upon fungal treatments.

Rumen degradability of the cell wall has been studied intensively in the 1980s and 1990s. However, it is advised to study rumen degradability of plant cell walls in more detail using modern advanced molecular, physical and chemical techniques. Molecular techniques could be used to determine which genes in rumen microbes are expressed during plant cell wall degradation. Chemical techniques could be used to determine which part of the plant cell wall is degraded in the rumen, i.e. by measuring the chemical

composition before and after the IVGP measurements. The knowledge on how the plant cell wall is degraded in the rumen can lead to a more targeted pre-treatment of biomass, i.e. lignin degradation or breaking down cross-linking within the cell wall.
The detergent fiber analysis shows that at least some of the carbohydrates are degraded during a fungal treatment (Chapter 3, 4, 6 and 7). As discussed above, the hemicellulose and cellulose fraction in the detergent fiber analysis are overestimated. A more specific carbohydrate analysis would give more information about the changes in the substrate during the fungal treatment. It is important to know to what extent the different carbohydrates are degraded. Carbohydrate degradation will result in a lower sugar yield by enzymatic saccharification and a lower feeding value. Also, cellulose is partly present in the crystalline form, which is more difficult to degrade by microbes and enzymes due to strong hydrogen-bonds (Himmel et al., 2007). White rot fungi are described to degrade crystalline cellulose, which is another indication that lignin degradation is not the only mechanism to increase the degradability (Riley et al., 2014).

8.5 How to implement fungal treatment in practice?

When considering fungal treatments on an industrial scale, techniques and procedures could be based on the current mushroom industry. In the current mushroom industry, substrates are sterilized (pasteurized), inoculated and incubated on a large scale. Generally, the production cycle of the button mushroom includes the following steps: filling of compost, inoculation, growth, casing, induction of fruiting, harvesting, emptying and cleaning (Pardo et al., 2013). A fungal pre-treatment of lignocellulosic biomass could include similar steps, except casing, induction of fruiting and harvesting (Figure 8.6).
Applying this process for animal feed production systems, safety should be ensured. Pardo et al. (2013) suggest a HACCP system for the button mushroom industry. Similar interventions should be taken in fungal pre-treatment to prevent contaminations which hamper the fungal treatment or cause safety risks for the animal and in the end for consumers eating animal products.
Using fungal pre-treatments for the production of animal feed or fuel can be either done at a farm level or by specialized companies. In an on farm application, it would be ideal to both grow the biomass and feed the animals/produce the biofuel on the same farm, limiting transportation costs. An on farm application seems more appropriate in countries where small scale farming is common. To obtain a year round supply of the feed ingredient or fuel, the untreated biomass should be stored dry or the fungal treated biomass should be conserved (ensiled). In case the untreated biomass can be stored dry, it can be used directly to feed the animals or produce the biofuels, after the fungal treatment. In that case a year round supply of the feed ingredient or fuel can be obtained by parallel incubations. The major challenges in an on farm application are the sterilization and control of the culture conditions. For control of the culture conditions special equipment may be purchased. However, if the farm is located in the tropics, the climate may already supply ideal circumstances. Specialized companies could be

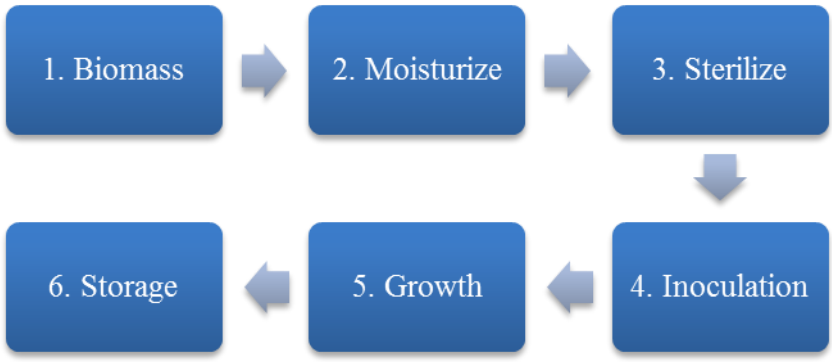

Figure 8.6 Steps in the process of fungal pre-treatment.

designed in the same way as mushroom production companies. All required equipment will be present. The major advantage of specialized companies is the control of the treatment, due to the presence of specialized equipment. One of the disadvantages will be the transportation costs and the need for storage after the treatment. Also costs may be higher because of the need for more special equipment. However the production of fungal treated material in specialized companies can be done in bulk, meaning that the price per kilo may be lower than in an on farm application.

8.6 Conclusions and recommendations

Fungal treatment increases the *in vitro* rumen degradability (and also enzymatic saccharification) of lignocellulosic biomass. *C. subvermispora* and *L. edodes* were found to be the most selective lignin degrading fungi leaving a substrate enriched in cellulose. Both fungi degrade more S-units than G-units in lignin. As a result, lignin degradation products, mainly originating from G-units are present in the fungal treated material.

Fungal treatment up to 8 weeks is required to reach an optimal *in vitro* rumen degradability. To make the fungal treatment economically interesting, the treatment should be shortened to less than 4 weeks. Decreasing treatment time has been attempted by the addition of manganese, linoleic acid or urea. Addition of manganese resulted in an increased lignin degradation and an increased *in vitro* rumen degradability of wheat straw after *C. subvermispora* treatment. Addition of linoleic acid, either separately or in combination with manganese, did not enhance the fungal treatment. It was observed that *C. subvermispora* produces alkylitaconic acids which are probably also involved in the lipid peroxidation by manganese peroxidase. Alkylitaconic acids should be considered as additive to the fungal treatment in future research.

Decreasing particle size to enhance the accessibility of fungal hyphae did not have an effect on the fungal treatment of wood chips, and had a negative effect on the *L. edodes* treatment of wheat straw. Decreasing the particle size even more may be too expensive to implement in practice, especially for small farm holders. For scientific reasons,

experiments with different particle sizes should be considered to study its effect on the of accessibility for fungal hyphae and the oxygen availability between the particles.
The addition of more inoculation points did not have an effect on the fungal treatment. The fungi colonize the material at the same rate, suggesting that colonization is not the limiting factor. Using a different inoculum, such as a lignocellulosic carrier, may decrease the lag time before the fungi start to degrade lignin.
It was shown that fungal treatments increase the utilization of lignocellulosic biomass. Future research should aim to optimize of the fungal treatment to make it economically attractive to be used in practice. The first steps should be the investigation of the lignin degrading mechanisms by these fungi, what happens with the lignin and which enzymes are involved. Also the limiting factors in lignocellulosic biomass for rumen degradation should be studied. In this way, a more targeted optimization strategy can be developed. During the investigation into the mechanisms, additional analysis than used in this thesis should be considered. A complete mass balance should be made, including information on the carbohydrate fractions and the gaseous products formed. Also molecular techniques should be used to study the fungal mechanisms and lignocellulose degradation in the rumen. The effectiveness of fungal treatment using different fungal strains should be investigated. The genetic background of fungal strains with different effectiveness may provide tools for breeding fungi for an optimal fungal treatment.

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SUMMARY

Carbohydrates in plant cell walls are highly fermentable and could be used as a source for ruminant nutrition or biofuel production. The presence of lignin in cell walls hampers the utilization of these carbohydrates and should thus be removed. In this thesis, the possibilities of a fungal treatment of lignocellulosic biomass are investigated.

A review of the scientific literature focusing on the potential of fungal treatments to increase the utilization of lignocellulosic biomass in ruminants feed ingredients is presented in Chapter 2. A prerequisite to the effective use of high lignocellulose feed ingredients is lignin removal, since lignin is negatively correlated to *in vitro* rumen degradability. Selective lignin degrading fungi have proven to increase *in vitro* rumen degradability with *Ceriporiopsis subvermispora* and *Pleurotus eryngii* showing the greatest potential. The effectiveness of fungal treatment is not only dependent on the choice of fungal strain, but also on the choice of substrate and culture conditions.

Based on the literature review, four different edible fungal species, i.e. *Ganoderma lucidum*, *Lentinula edodes*, *P. eryngii* and *P. ostreatus* were chosen to treat four different substrates, i.e. miscanthus, rice straw, wheat straw and wood chips. The results of these fungal-substrate combinations are described in Chapter 3 and confirm that fungal species, substrate and incubation time are important factors in fungal treatment. The most promising fungus-substrate combinations are *L. edodes* treatment of wheat straw and *L. edodes* treatment of wood chips. These two fungus-substrate combinations were used in a more detailed characterization of changes in lignin upon fungal treatment.

In Chapter 4, a study is described where *L. edodes* treated wheat straw and wood chips are analyzed by pyrolysis coupled to gas chromatography and mass spectrometry (Py-GC/MS) confirming the selective lignin degradation as determined with the detergent fiber analysis. Structural changes in lignin were observed with preferential degradation of syringyl (S) lignin units over guaiacyl (G) lignin units by *L. edodes*. Upon fungal delignification, a number of degradation products of lignin were observed. The building blocks in the original lignin consist of phenolic groups with 3 C-atoms in the side chain, while degradation products consist of phenolic groups with 0 to 2 C-atoms in the side chain. The ratio between side chain degradation products and original compounds was increasing in both wheat straw and wood chips upon *L. edodes* treatment. Besides side chain degradation, *L. edodes* modified the Cα-atom of the side chain by oxidation. Although correlations were found, a clear relationship between lignin composition and *in vitro* rumen degradability could not be demonstrated.

The safety of a fungal treatment of ruminant feed ingredients requires the used fungus to have a Generally Regarded As Safe (GRAS) status. The literature data compiled in Chapter 2 indicates *C. subvermispora*, which does not have a GRAS status, as one of the most promising fungi for fungal treatment. For this reason this fungus was included in the remaining chapters. This fungus was researched in Chapter 5 with the substrates wheat straw and wood chips and compared to *L. edodes*. Both fungi selectively degraded lignin and improved *in vitro* rumen degradability and the amount of sugar released upon enzymatic saccharification. *L. edodes* continuously grew on wheat straw and wood chips while degrading lignin and hemicellulose at the same time. *C. subvermispora* colonized

the wheat straw within the first week of treatment and starts degrading lignin and hemicellulose thereafter. Growth continued again after 5 weeks, which was accompanied by cellulose degradation. On wood chips, *C. subvermispora* stopped growing after the first week of treatment, while lignin degradation continued until 4 weeks of treatment. From 5 weeks onwards, no chemical changes were observed in wood chips. One of the explanations for this lack of change is the dense structure of the wood as observed with light microscopy. Both fungi degraded hemicellulose simultaneously with lignin. The loss of carbohydrates during fungal treatment and the long treatment times of up to 8 weeks represent a major disadvantage of fungal treatment of lignocellulosic biomass.

In Chapter 6, the incubation of *C. subvermispora* and *L. edodes* with wheat straw and wood chips were supplemented with urea to stimulate growth, and manganese and linoleic acid to stimulate lignin degradation via the enzyme manganese peroxidase produced by the fungi. Addition of manganese increased the selectivity of *C. subvermispora* treatment of wheat straw within the first 4 weeks of treatment. Addition of 150 µg manganese per g substrate improved lignin degradation and *in vitro* rumen degradability the most. A combination of manganese and linoleic acid did not show synergistic effects.

In Chapter 7 the particle size of wheat straw and wood chips, and the amount of *C. subvermispora* or *L. edodes* at the start of the treatment was varied. The amount of fungus added at the start of the treatment did not have an effect on colonization rate, lignin degradation or *in vitro* rumen degradability. *L. edodes* treatment of wheat straw chopped to 2 cm particles resulted in a higher lignin degradation and *in vitro* rumen degradability compared to *L. edodes* treatment of wheat straw chopped to a length of 0.5 cm. The particle size of wood chips did not have an effect on *C. subvermispora* treatment. In *C. subvermispora* treated wheat straw and *L. edodes* treated wood chips, a reduced growth was observed, which was unexpected based on results from previous experiments. A toxic compound to fungi (fungicide) was identified.

This thesis describes the potential of fungal treatment to increase utilization of lignocellulosic biomass. Fungal treatment resulted in an increased *in vitro* rumen degradability, and thus an increased cellulose accessibility. The same theory applies for biofuel production in which fungal treatment results in an increased accessibility of cellulose for enzymes. The major disadvantages of this low cost, relatively simple and environmentally-friendly biotechnological treatment are the loss of carbohydrates during the relatively long process of fungal incubation. Future studies should focus on optimization of the fungal treatment to enable large scale application.

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Anton en John, de oud studiegenoten die dan nu eindelijk samen aan een project mochten werken. Wat mij betreft is dit een goede combinatie en moeten jullie dit zeker zo blijven doen. Jullie hebben mij altijd heel veel vrijheid gegeven en daardoor heb ik een eigen draai aan dit project kunnen geven. Anton, jouw kritische vragen gaven mij vaak het idee dat ik mijn thesis zat te verdedigen. Toch moet ik je hier bedanken voor je kritische blik op de manuscripten en experimenten, want dit heeft mij tot nadenken aangezet en het uiteindelijke resultaat beter gemaakt. Hopelijk is dit ook een goede voorbereiding op de echte verdediging.

John, bij jou mag ik altijd binnenlopen en je hebt me altijd het gevoel gegeven dat je achter me stond. Je pakt dingen direct aan en dit heeft heel wat schot in de zaak gebracht. Bedankt hiervoor.

Johan, ondanks dat je niet op het podium komt te zitten, hoor je wat mij betreft echt bij de begeleiders. Bedankt dat je altijd tijd hebt gemaakt om mee te denken over experimenten, hoe ze statistisch geanalyseerd kunnen worden en hoe ze opgeschreven kunnen worden.

Wouter, hoewel het in het begin een heel nieuw onderwerp voor je was, is dit nu zeker niet meer het geval. Bedankt voor de leuke discussies over hoe de celwand nu in elkaar zit, en waarom schimmels deze op een bepaalde manier afbreken en natuurlijk bedankt voor je kritische blik op manuscripten.

Ik heb het geluk gehad om met twee groepen samen te werken. Mijn vaste werkplek was bij diervoeding, waar ik met veel plezier heb gewerkt. Dankzij jullie discussies tijdens de mamo/mami en de pauzes ben ik heel wat wijzer geworden over diervoeding. Ik was degene niet met dieren maar met schimmels werkte waardoor er soms wat sceptisch gekeken werd naar dit PhD project. De meest gestelde vraag na mijn presentaties was dan ook: “denk je dat de schimmelbehandeling in de praktijk voor diervoeding toepasbaar is?” Mijn antwoord daarop is en blijft: JA!

Om tot dit boekje te komen zijn er heel veel samples gegenereerd die allemaal gemalen en geanalyseerd moesten worden. Ik heb veel tijd op het lab door gebracht, maar het labpersoneel van ANU heeft mij ook veel geholpen met deze werkzaamheden. Erika, Adriaan, Joswita en Rik bedankt voor het malen! Joswita, Amine en Jane-Martine bedankt voor de vezelanalyses! Saskia en Inge voor de GPT metingen! De *in vitro* analyses konden niet zonder penssap gedaan worden, daarom wil ik Ries, Teus en Willem van Carus daarvoor bedanken! Xuan-Huang, bedankt voor de zetmeel analyses

(die nog even spoed op het laatste moment uitgevoerd moesten worden)! En Michel, bedankt voor je ongekende krachten als ik weer eens de flesjes na een GPT run niet meer open kreeg (oh ja, sorry voor de stank tijdens het klaarmaken van de buffers)! En natuurlijk ook Leon en Saskia bedankt voor alle administratie die bij al die samples kwamen kijken!
Diervoeding is een groep met veel PhDers, wat betekent dat je altijd wel je PhD ei kwijt kan bij iemand. Ik heb het altijd heel gezellig gevonden met iedereen! Een paar mensen zal ik hier nog specifiek noemen. Yvonne en Tetske bedankt voor alle gezelligheid tijdens de pauzes, het squashen, winkelen, wandelen, borrelen en wat we nog meer gedaan hebben! Myrthe bedankt dat ik al mijn vraagjes over de laatste loodjes van de PhD aan jou mocht stellen! Geronda, Henk, Kasper en Sanne, ik vond het erg gezellig om met jullie in Orlando te zijn.
Huyen, I really enjoyed you as my roommate! I feel privileged to get to know you better! Thank you for your smiles in the room and the nice talks!
Nazri, Lei Mao and Eli, when you started, I was finally not the only one working with fungi anymore. I really like your projects and I believe that you are doing an important job ;). I would like to wish you good luck with your PhD projects! And if you need any advice about this or how to manage your supervisors (I do have some experience with them…) please contact me!
I would like to thank all members of the ANU group of the last 4 years for their 'gezelligheid'!
De schimmelbehandeling zelf is uitgevoerd bij de Paddenstoelengroep, binnen Plant Breeding. Ondanks dat ik niet bij jullie op kantoor zat heb ik mij altijd welkom gevoeld. Uiteraard moet ik hier Marcel bedanken. Zonder jou zou ik nu nog stro in bakjes aan het wegen zijn en aan het vriesdrogen zijn. Bedankt dat je me bij veel van mijn praktische werk hebt bijgestaan!
Op jullie lab wordt alles voornamelijk op kleine schaal uitgevoerd, het was dan ook even wennen toen ik daar met balen stro het lab op kwam. Dit stro was dan vaak al geregeld en vakkundig gehakseld door Ed, bedankt daarvoor! José bedankt dat je steeds die stammen uit stikstof haalde en broed bereid hebt! Patrick bedankt dat je altijd meedacht als ik weer een probleem had dat een creatieve oplossing vereiste.
Bij het biochemische lab binnen Plant Breeding had ik veel lol met ergosterol. Hier wil ik dan ook Annemarie bedanken voor al haar hulp met de HPLC.
En natuurlijk de hele paddenstoelengroep: Anton, Brian, Ed, Johan, José, Karin, Marcel, Narges, Patrick bedankt voor alle gezelligheid en misschien heb ik zelfs nog wat geleerd over de champignonteelt en veredeling van schimmels.
During my PhD I got the chance to visit the IRNAS-CSIC in Seville, Spain. It was great to learn how things go in a different group and country. I would like to thank José Carlos and Ana for having me working in their group and Jorge for all his help with the pyrolysis measurements.
In dit boekje staan een aantal mooie microscopie plaatjes. Hierbij wil ik Norbert bedanken dat je me de weg hebt gewezen in deze, voor mij, nieuwe wereld.

Een PhD project wordt je leven voor 4 jaar. Dit betekent dat niet alleen mijn collega's aan dit boekje hebben bijgedragen maar ook mijn vrienden en (schoon)familie. Het is belangrijk om de nodige ontspanning te hebben, dus alle gezellige etentjes, feestjes, avondjes, dagjes uit zorgden dat ik het werk even van mij af kon zetten om daarna met een frisse blik weer terug te keren naar het werk.

Ik wil dan nog even specifiek de mannen (en hun vrouwen) in mijn leven noemen. Papa (en Maria), je staat altijd achter me en je gelooft in mij. Ondanks dat ik het over de mannen in mijn leven heb, kan ik er niet aan voorbij ook mijn moeder hier te noemen. Mama, je kan er niet meer bij zijn, maar toch zal je er altijd bij zijn. Marcel (en Helma), het begint toch tijd te worden om te zeggen dat kleine zusjes groot worden, maar toch blijf jij mijn grote broer.

Dennis, jij hebt de zware taak om het vriendje en dus degene te zijn die al mijn frustraties, twijfels en stress heeft moeten aanhoren en kalmeren. Daarnaast hebben mijn congressen de laatste jaren onze vakantiebestemmingen bepaald. Maar ik gelukkig heb ik je over het laatste nog niet horen klagen. Bedankt voor alle leuke dingen die we samen gedaan hebben en dat je mij altijd weer kunt opvrolijken. Ik hoop dat we samen nog heel veel leuke dingen gaan meemaken! Dennis, dan rest mij nog een ding om tegen je te zeggen: Dabadie!

Sandra

ABOUT THE AUTHOR

Curriculum vitae
List of publications
Training and supervision plan

Curriculum vitae

Sandra van Kuijk is born in Waalwijk, the Netherlands, on the 26th of September 1985. She graduated from secondary education (gymnasium) at Dr. Mollercollege, Waalwijk, the Netherlands, in 2003. Thereafter she started her BSc in Animal Sciences, after which she continued her MSc in Food Safety, both at Wageningen University. During her MSc studies, Sandra performed her thesis with the title 'Development of a method to detect testosterone esters in animal nutrition' at the RIKILT institute, Wageningen, the Netherlands. Sandra performed her MSc internship with the title 'Mode of action of the natural antimicrobial compounds curcumin and subtilosin against *Listeria monocytogenes*' at Rutgers University, New Brunswick, NJ, USA. After her graduation in 2009, Sandra started working in short research projects at the Dutch Vaccination Institute (NVI) and University of Utrecht. In 2010 she started working as a lab technician in a project to develop a dermal application of the (human) Hepatitis B vaccination at Dutch Vaccination Institute (NVI), later part of Governmental Institute of Public Health and Environment (RIVM), Bilthoven, the Netherlands

In 2012, Sandra started her PhD at the Animal Nutrition Group and the Plant Breeding group of Wageningen University. In her PhD she developed a fungal treatment of lignocellulosic biomass to increase the feeding value of ingredients originating from organic waste. This research was part of the STW waste to resource projects. In 2015 Sandra received a WIAS fellowship which allowed her to work for 3 months at the IRNAS-CSIC institute in Seville, Spain. The results of this PhD project are presented in this thesis.

List of publications

Peer reviewed scientific publications

van Kuijk, S.J.A., Sonnenberg, A.S.M., Baars, J.J.P., Hendriks, W.H., Cone J.W., 2015. Fungal treated lignocellulosic biomass as ruminant feed ingredient: A review. Biotechnology Advances 33, 191-202.

van Kuijk, S.J.A., Sonnenberg, A.S.M., Baars, J.J.P., Hendriks, W.H., Cone, J.W., 2015. Fungal treatment of organic waste: importance of colonization, time and fungal species on chemical composition and *in vitro* rumen degradability. Animal Feed Science and Technology 209, 40-50.

van Kuijk, S.J.A., Sonnenberg, A.S.M., Baars, J.J.P., Hendriks, W.H., Cone, J.W., 2016. The effect of adding urea, manganese and linoleic acid to wheat straw and wood chips on lignin degradation by fungi and subsequent *in vitro* rumen degradation. Animal Feed Science and Technology, in press.

van Kuijk, S.J.A., del Río, J.C., Rencoret, J., Gutiérrez, A., Sonnenberg, A.S.M., Baars, J.J.P., Hendriks, W.H., Cone, J.W. Preferential lignin degradation in wheat straw and wood chips by the white-rot fungus *Lentinula edodes* and its influence on *in vitro* rumen degradability. Submitted.

van Kuijk, S.J.A., Sonnenberg, A.S.M., Baars, J.J.P., Hendriks, W.H., del Río, J.C., Rencoret, J., Gutiérrez, A., de Ruijter, N.C.A., Cone, J.W. Characterization of wheat straw and oak wood chips treated with the white rot fungi *Ceriporiopsis subvermispora* and *Lentinula edodes*. To be submitted.

van Kuijk, S.J.A., Sonnenberg, A.S.M., Baars, J.J.P., Hendriks, W.H., Cone, J.W. The effect of particle size and amount of inoculum added to wheat straw and wood chips on lignin degradation by fungi and subsequent *in vitro* rumen degradation. Submitted.

Conference and symposia proceedings

van Kuijk, S.J.A., Tuyen, D.V., Sonnenberg, A.S.M., Baars, J.J.P., Hendriks, W.H., Cone, J.W., 2012. Biodegradation of lignocellulosic material by fungi – the route from organic waste to feed. Proceedings 37th ANR Forum, 18 April, Wageningen, the Netherlands.

van Kuijk, S.J.A., Sonnenberg, A.S.M., Baars, J.J.P., Hendriks, W.H., Cone, J.W., 2013. Effect of fungal treatment of organic waste on *in vitro* rumen degradability – a comparison with literature. Proceedings 38th ANR Forum, 21 May, Roeselare, Belgium.

van Kuijk, S.J.A., Sonnenberg, A.S.M., Baars, J.J.P., Hendriks, W.H., Cone, J.W., 2014. Shorten fungal treatment of lignocellulosic waste with additives to improve

rumen degradability. Proceedings 13th European Workshop on Lignocellulosics and Pulp (EWLP), 24-27 June, Sevilla, Spain. Pp. 847-850.

van Kuijk, S.J.A., Sonnenberg, A.S.M., Baars, J.J.P., Hendriks, W.H., Cone, J.W., 2014. Manganese and urea can increase lignin degradation by white rot fungi. Proceedings 39th ANR Forum, 03 April, Utrecht, the Netherlands. Pp 13.

van Kuijk, S.J.A., Sonnenberg, A.S.M., Baars, J.J.P., Hendriks, W.H., Cone, J.W., 2015. Fungal pre-treatment increases *in vitro* rumen degradability of wheat straw. WIAS Science day 2015, 5 February, Wageningen, the Netherlands.

van Kuijk, S.J.A., Sonnenberg, A.S.M., Baars, J.J.P., Hendriks, W.H., Cone, J.W., 2015. Fungal treatment of lignocellulosic biomass to selectively degrade lignin. Proceedings 1st International Workshop on Biorefinery of Lignocellulosic Materials, Córdoba, Spain.

van Kuijk, S.J.A., Sonnenberg, A.S.M., Baars, J.J.P., Hendriks, W.H., Cone, J.W., 2015. Rumen degradability of wheat straw is related to changes in lignin properties after fungal treatment. Proceedings Joint Annual Meeting of the American Dairy Science Association, Orlando, Fl., USA.

Training and Supervision Plan[1]

The Basic Package (3 ECTS[2])

WIAS Introduction Course	2012
Course on philosophy of science and/or ethics	2013

Scientific Exposure (15 ECTS)

International conferences

13th European Workshop on Lignocellulosics and Pulp, Sevilla, Spain, 24 june-27 June 2014	2014
1st International Workshop on Biorefinery of Lignocellulosic Materials, Cordoba, Spain, 9 June-12 June 2015	2015
ADSA-ASAS Joint Annual Meeting, Orlando, Florida, USA, 12 July-16 July 2015	2015

Seminars and workshops

Wias Science day 2012, Wageningen, The Netherlands, 2 February 2012	2012
Animal Nutrition Research forum, Wageningen, The Netherlands, 18 April 2012	2012
Wias Science day 2013, Wageningen, The Netherlands, 28 February 2013	2013
Animal Nutrition Research forum, Roeselare, Belgium, 21 May 2013	2013
Dairy Nutrition Symposium, Wageningen, The Netherlands, 21 November 2013	2013
Animal Nutrition Research forum, Utrecht, The Netherlands, 3 April 2014	2014
Wias Science day 2014, Wageningen, The Netherlands, 30 April 2014	2014
Fibre seminar, Wageningen, The Netherlands, 20 June 2014	2014
Seminar on calve nutrition, Wageningen, The Netherlands, 1 September 2014	2014
STW Annual Congress, Nieuwegein, The Netherlands, 2 October 2014	2014
Wias Science day 2015, Wageningen, The Netherlands, 5 February 2015	2015

Presentations

'Biodegradation of lignocellulosic material by fungi - the route from organic waste to feed', Wageningen, The Netherlands, 18 April 2012, oral presentation.	2012
'Effect of fungal treatment of organic waste on *in vitro* rumen degradability - a comparison with literature, Roeselare, Belgium, 21 May 2013, oral.	2013
'Manganese and urea can increase lignin degradation by white rot fungi', Utrecht, The Netherlands, 3 April 2014, oral presentation.	2014

[1]Completed in the fulfilment of the requirements for the education certificate of the Graduate School Wageningen Institute of Animal Sciences (WIAS).
[2]One ECTS equals a study load of 28 hours.

‘Shorten fungal treatment of lignocellulosic waste with additives to improve rumen degradability', Seville, Spain, 26 June 2014, poster presentation.	2014
‘Fungi turn waste into feed and fuel', Nieuwegein, The Netherlands, 2 October 2014, poster presentation.	2014
‘Fungal pre-treatment increases *in vitro* rumen degradability of wheat straw', Wageningen, The Netherlands, 5 February 2015, oral presentation.	2015
‘Fungal pretreatment of lignocellulosic biomass to selectively degrade lignin', Cordoba, Spain, 11 June 2015, oral presentation .	2015
‘Rumen degradability of wheat straw is related to changes in lignin composition after fungal treatment', Orlando, Fl., USA, 14 July 2015, oral presentation.	2015

In-Depth Studies (6 ECTS)

Disciplinary and interdisciplinary courses

Summer Course glycosciences (VLAG)	2014
Microscopy and Spectroscopy in Food and Plant Sciences (EPS-VLAG)	2014

Advanced statistics courses

Advanced Statistics course: Design of Experiments	2012
Statistics for the Life Sciences	2013

Professional Skills Support Courses (5 ECTS)

Course Techniques for Writing and Presenting a Scientific Paper	2013
Course 'Scientific Writing'	2014-2015
Recess College 'Our future leaders'	2015

Research Skills Training (2 ECTS)

External training period - 3 months at IRNAS-CSIC, Seville, Spain	2015

Didactic Skills Training (5 ECTS)

Supervising practicals and excursions

Supervising practicals "Toegepaste dierbiologie"	2012
Supervising 'praktijkproject Inleiding Dierwetenschappen'	2012
Supervising 'praktijkproject Inleiding Dierwetenschappen'	2013
Supervising practicals 'Principle of Animal Nutrition'	2014
Supervising practical 'Feed Formulation Science'	2013

Supervising theses	
Supervising MSc minor thesis	2013
Supervising BSc thesis	2014
Tutorship	
RMC	2015
Management Skills Training (2 ECTS)	
Membership of boards and committees	
WIAS science day committee	2013
Education and Training total:	**38 ECTS**

Colophon

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