Charles University in Prague
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Ph.D. Thesis

Physiology and ecology of saprotrophic basidiomycetes degrading dead plant biomass

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I declare that neither this thesis nor any of its part has been used to achieve identical or other academic degree.

Contents

Abbreviations

Ah	upper mineral soil horizon
BRF	brown-rot fungus/fungi
CBD	cellulose-binding domain
CBH	cellobiohydrolase
CDH	cellobiose dehydrogenase
CFU	colony forming units
DGGE	denaturing gradient gel electrophoresis
ECM	ectomycorrhizal fungi
EG	endo-1,4-β-glucanase
L	litter horizon of soil
L/C	lignin/carbohydrate ratio
Lac	laccase
LDF	litter-decomposing fungus/fungi
LiP	lignin peroxidase
MnP	manganese dependent peroxidase
O	organic horizon of soil
PCR	polymerase chain reaction
PLFA	phospholipid fatty acid
SRF	soft-rot fungus/fungi
TGGE	temperature gradient gel electrophoresis
VOC	volatile organic compounds
VP	versatile peroxidase
WRF	white-rot fungus/fungi

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Aims

In terrestrial ecosystems, the above- and belowground plant litter input and microbial exudates constitute the main resources of energy and organic matter for an extraordinarily diverse community of soil organisms. As much as 3.5 t ha^{-1} per year is the average input of aboveground plant litter in a broadleaved temperate forest (Bray and Gorham, 1964). Recycling of carbon and nutrients during litter decomposition is a fundamentally important ecosystem process that has major control over the carbon cycle, nutrient availability and, consequently, plant growth and community structure (Wardle, 2002; Bardgett, 2005).

Plant cell wall has three major constituents: lignin, cellulose and hemicellulose. Cellulose and hemicellulose can serve as a sole carbon and energy source. Lignin is cannot supply sufficient energy to maintain its own decomposition, so nonlignified carbon and energy sources such as cellulose are necessary as growth co-substrates for fungi to decompose lignin (Kirk et al., 1976). Lignin decomposition serves mainly to unmask cellulose and expose it to degradation (Cooke and Whipps, 1993) and it is also a tool for faster growth and primary nutrient source acquisition. List of organisms capable of lignocellulose decomposition is rather short: bacteria, fungi, protozoa (flagellata) and yeasts. Bacteria and fungi are, in terms of biomass and species numbers, the largest groups involved in lignocellulose and soil organic matter turnover. White-rot and litter-decomposing fungi efficiently degrade lignin and cause fast and complete decomposition of wood. Saprotrophic basidiomycetes are therefore the most efficient decomposers in the terrestrial ecosystems (Dix and Webster, 1995).

Biochemistry of lignocellulose degradation by wood-rotting fungi has been extensively studied since the middle of the last century. Attention has been mainly paid to the wood degrading microorganisms, considering also their biotechnological application in pulp, paper, textile and food industries and bioremediations. Ecophysiology of basidiomycetes from habitats other than wood, for example the litter decomposers and mycorrhizal species, attracted much less attention. Last, but not least, microbial interactions within wood and soil communities, important for the "living processes" in decaying wood and soil, have became only recently the scope of environmental scientists.

My study is focused on saprotrophic basidiomycetes, their role in dead plant biomass decomposition and interactions with other members of microbial community. The aims of this study were to isolate and characterize litter-decomposing saprotrophic basidiomycetes from temperate oak forest, to evaluate their ability to participate in degradation of litter

and in the turnover of soil organic mass, to evaluate the effect of isolated fungi on the abundance and community composition of soil fungi and bacteria and last, but not least, to describe the effect of fungi on wood environment and bacterial community composition in decomposed wood, a habitat markedly changed due to presence of the fungus, and evaluate the role of wood-associated bacteria in lignocellulose degradation and microbial community functioning.

Chapter 1

Introduction

1.1 Lignocellulose and its degradation

1.1.1 Wood and plant litter

Wood and dead plant material constitute more than 60% of the total biomass produced on the earth. The degradation of lignocellulosic materials belongs to important biological processes, leading to production of carbon dioxide, water and humic substances, in the earth's carbon cycle (Kuhad et al., 1997).

The wood cell wall is chemically composed mainly of cellulose, hemicellulose and lignin. Pectin, proteins and resin belong to minor cell wall components. Morphologically, it is composed of primary and secondary cell walls (Figure 1.1). Primary cell wall consists of cellulose fibrils (30%), hemicellulose (25%), pectin (35%) and proteins (10%). Centripetally present secondary wall has three distinct layers, thin outer (S1) and inner (S3) and thick middle layer (S2). It consists of cellulose (35%), hemicellulose and pectin (25%) and lignin (35%), but the composition is species-specific. Adjacent cells are separated by middle lamella rich in pectin and lignin. The highest amount of lignin is located in the S2 layer of secondary wall (Sjöström, 1993; Kuhad et al., 1997). The plant cell wall is due to its structure and complexity particularly resistant to microbial degradation.

1.1.2 Cellulose and its degradation

Cellulose is the main polymeric component of the plant cell wall and is the most abundant polysaccharide on earth. The physical structure and morphology of native cellulose are complex, and fine structural details are difficult to determine experimentally (O'Sullivan, 1997). The chemical composition of cellulose is simple: the polysaccharide consists of D-glucose residues linked by β-(1-4)-glycosidic bonds to form linear polymeric chains of over 10 000 glucose residues. The individual chains adhere to each other along their lengths by hydrogen bonding and van der Waals forces, and crystallize shortly after biosynthe-

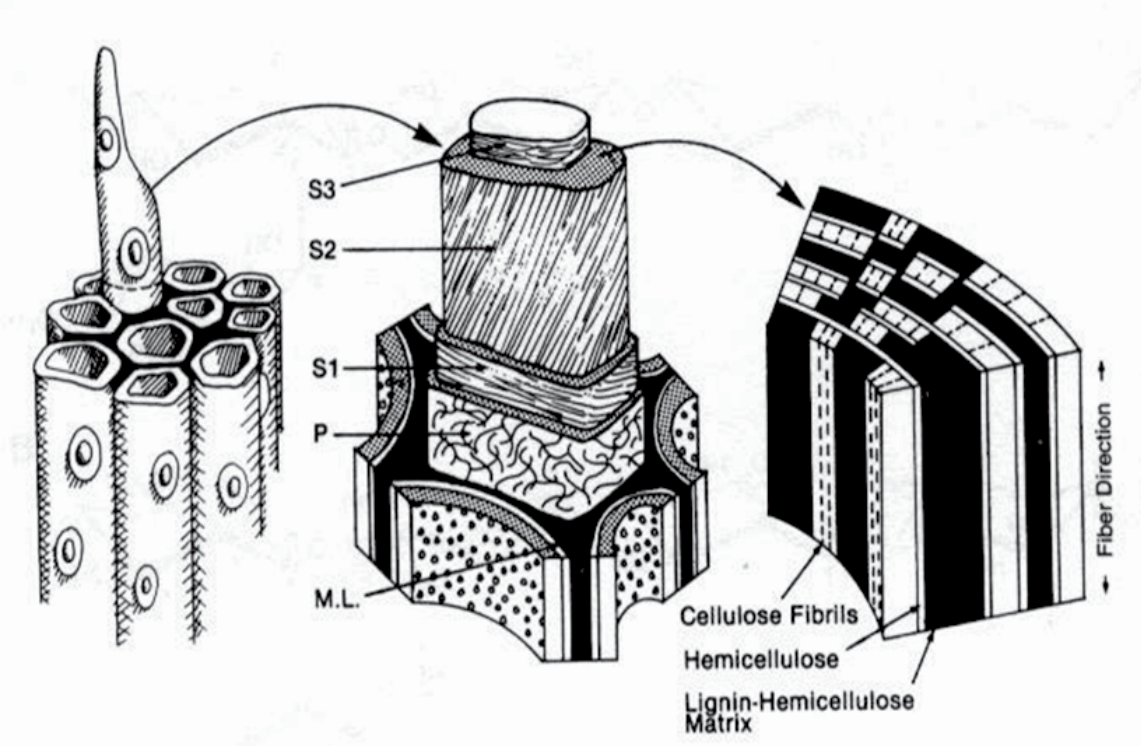

Figure 1.1: A schematic illustration of wood structure showing adjacent tracheids, each ca 30 μm in diameter (left), wood cell wall layers (middle): P - primary wall, S1 - S3 - secondary cell wall layers, M. L. - middle lamella, and lignocellulose complex of the secondary cell wall (right) (Kirk and Cullen, 1998).

sis. Although highly crystalline, the structure of cellulose is not uniform. Physical and chemical evidence indicates that cellulose contains both highly crystalline and less-ordered amorphous region (Hon, 1994) (Figure 1.2).

Although chemically simple, the extensive intermolecular bonding pattern of cellulose generates a crystalline structure that together with other cell wall components, such as hemicellulose and lignin, results in very complex morphologies. Multiple enzyme systems are thus required to efficiently degrade cellulose. The cellulose degradation pathways lead to release of CO_2 and water under aerobic conditions, or carbon dioxide, methane and water under anaerobic conditions (Béguin and Aubert, 1994).

The ability to digest cellulose is widely distributed among many genera in the domain *Bacteria* and in the fungal groups within the domain *Eucarya*, while no cellulolytic members of the domain *Archaea* have yet been identified. Within the eubacteria there is considerable concentration of cellulolytic capabilities among the predominantly aerobic *Actinomycetales* (phylum *Actinobacteria*) and the anaerobic *Clostridiales* (phylum *Firmicutes*) (Table 1.1).

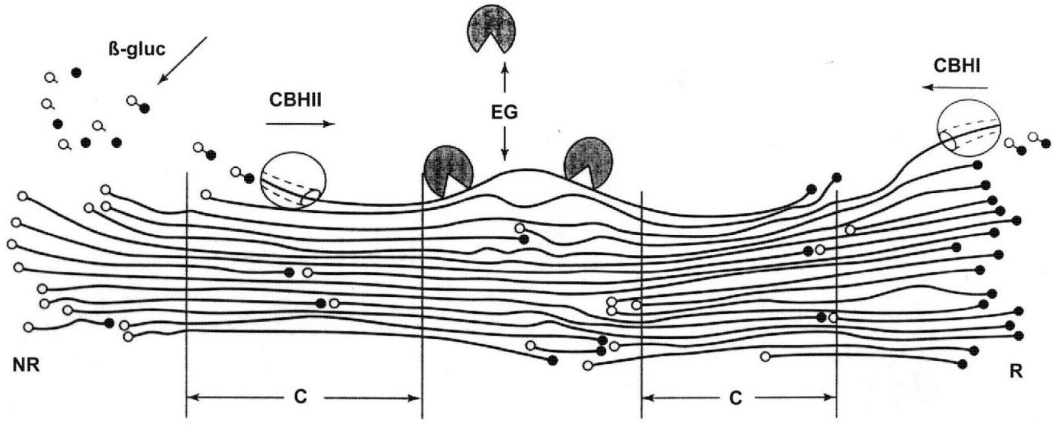

Figure 1.2: A schematic view of the cellulose structure and action of the cellulolytic enzymes: EG - endoglucanase, CBH - cellobiohydrolase, β-glucosidase in *Trichoderma reesei*. C defines the highly ordered crystalline region, R the reducing ends (filled circles), and NR the nonreducing ends (open circles) (Teeri, 1997).

Anaerobic bacteria degrade cellulose primarily via complex cellulase systems exemplified by the well-characterized polycellulosome organelles of the thermophilic bacterium *Clostridium thermocellum*. Cellulolytic enzymes are partially also released into the environment, although several anaerobic species utilizing cellulose do not release measurable amounts of extracellular cellulase, and instead have localized their complex cellulases directly on the surface of the cell or the cell-glycocalyx matrix. Most anaerobic cellulolytic species grow optimally on cellulose when attached to the substrate, and in at least a few species this adhesion appears to be obligate. In contrast, aerobic bacteria release enzymes into the environment. The enzymes do not form complexes but act synergistically. Adherence to substrate is probably not essential (Lynd et al., 2002). This strategy resembles fungal utilization of cellulose.

Fungal cellulose utilization is distributed across the entire kingdom, from the protist-like anaerobic chytridomycetes to the basidiomycetes (Table 1.1) (Carlile et al., 2001). Saprotrophic basiciomycetes belong to the most potent degraders. They often grow on wood or litter and their enzymatic and nonenzymatic systems are able to efficiently degrade all main component of wood: cellulose, hemicellulose and lignin.

Wood-rotting basidiomycetous fungi are usually divided into white-rot (WRF) and brown-rot (BRF). They are taxonomically closely related, and WRF and BRF can be found in the same genera. The most wood-rotters belong to the orders *Agaricales* and *Aphyllophorales*. Ascomycetes and deuteromycetes are also able to degrade wood, usually causing soft-rot decay of wood. WRF are able to degrade all major wood cell wall components, whereas BRF and SRF degrade mainly cellulose and hemicellulose and modify the lignin. Main features of wood-rotting fungi are described in more details in section 1.3.

Chytridiomycetes	*Neocallimastix*, *Piromyces*, *Caecomyces*, *Orpimomyces*, *Anaeromyces*
Zygomycetes	*Mucor*
Ascomycetes	*Bulgaria*, *Chaetomium*, *Helotium*
Basidiomycetes	*Trametes*, *Phanerochaete*, *Poria*, *Schizophyllum*, *Serpula*
Deuteromycetes	*Aspergillus*, *Cladosporium*, *Fusarium*, *Geotrichum*, *Myrothecium*, *Paecilomyces*, *Penicillium*, *Trichoderma*
Aerobic bacteria	*Acidothermus*, *Bacillus*, *Erwinia*, *Micromonospora*, *Pseudomonas*, *Rhodotermus*, *Streptomyces*
Anaerobic bacteria	*Acetivibrio*, *Clostridium*, *Eubacterium*, *Fibrobacter*, *Ruminococcus*, *Spirochaeta*, *Thermotoga*

Table 1.1: Examples of cellulose degrading fungi and bacteria (Carlile et al., 2001; Lynd et al., 2002).

An efficient fungal cellulolytic system (Figure 1.2) requires endocleaving (endoglucanase), exocleaving (cellobiohyhrolase, exocellulase) enzymes and β-glucosidase (detailed in Table 1.2). Some microbial cellulases display both endo- and exo-type attack features (Henrissat and Davies, 1997). The complementary activities of endo- and exo-type enzymes lead to synergy, an enhancement of activity, which is more than the added activities of individual enzymes. Exo-exo-synergy is also observed, and may indicate a low, inherent endoglucanase activity of exoglucanases (Shen et al., 1995). The resulting cellobiose and glucose can be taken up and assimilated by hyphae. The cellobiose taken up is probably hydrolyzed to glucose by cell wall bound or intracellular β-glucosidases. Enzymatic hydrolysis of cellulose is proposed to have mainly or exclusively nutritive role. Although fungal cellulolytic enzymes are extracellular, they are not able to freely diffuse into the compact substrate due to their size (Flournoy et al., 1991). Significant fraction of cellulolytic enzymes was also found to be associated with fungal (Cai et al., 1999; Valášková and Baldrian, 2006).

All cellulolytic enzymes share the same chemical specificity for ß-1,4-glycosidic bonds, which they cleave by a general acid-catalyzed hydrolysis. A common feature of most cellulases in different fungal genera is a domain structure with a catalytic domain linked with an extended linker region to a cellulose-binding domain (CBD) (Gilkes et al., 1991). Some enzymes of the cellulolytic system exhibit broader specificity and can thus also contribute to the degradation of hemicelluloses. Cellobiohydrolases and cellulose-binding endoglucanases participation in hemicellulose degradation is, however, limited because they specifically bind to cellulose. β-glucosidases generally exhibit low specificity and are able to cleave mannose, xylose or galactose units from oligosaccharides and thus participate in hemicellulose degradation.

Cellulose degradation in Figure 1.2 shows simplified cellulose degradation by the ascomycete

Enzyme	Properties
Endo–1,4–β–glucanase EC 3.2.1.4 endocellulase	▷ **internal cleavage in cellulose molecule** ▷ mainly directed towards amorphous regions ▷ progresive endoglucanases: cleave internally long chains and also shorter oligosaccharides ▷ monomeric, 22 - 45 kDa, glycosylated ▷ pI 2.6 - 4.9 ▷ catalytic optima: pH 4.0 - 5.0, 50 - 70 °C ▷ common among basidiomycetes, wood-rooting fungi, litter-decomposing fungi, ectomycorrhizal fungi, fungal plant pathogens, wood-associated yeasts
Cellobiohydrolase EC 3.2.1.91 exocellulase	▷ **release of cellobiose from reducing or nonreducing ends of cellulose** ▷ active on crystaline cellulose, enzymes are either specific for reducing or nonreducing ends, cleave cellotriose, cellotetraose, cellodextrins ▷ monomeric, 50 - 65 kDa, glycosylated ▷ pI 3.6 - 4.9 ▷ catalytic optima: pH 4.0 - 5.0, 37 - 60 °C ▷ competitive inhibitor: cellobiose ▷ white-rot fungi, litter-decomposing fungi, ectomycorrizal fungi, plant pathogens
β–**glucosidase** EC 3.2.1.21	▷ **cleaves cellobiose to glucose** ▷ extracellular , intracellular ▷ monomeric or homooligomeric 35 - 640 kDa, glycosylated ▷ catalytic optima: pH 3.5 - 5.5, intracellular pH neutral, 45 - 75 °C ▷ competitive inhibition: glucose, gluconolactone, cellobionolactone ▷ universally produced by basidiomycetes, wood rotting fungi, litter-decomposing fungi, plant pathogens, ectomycorrhizal fungi

Table 1.2: Cellulolytic enzymes of basidiomycetes and their main properties.

Trichoderma reesei, but this model is common for several other fungi. Not all fungi produce complete spectra of cellulolytic enzymes, e.g. brown-rot fungi generally do not produce cellobiohydrolase. On the other hand, processive endoglucanase of two brown-rot fungi *Gloeophyllum trabeum* and *Fomitopsis palustris* act as endoglucanase and also cellobiohydrolase (Cohen et al., 2005; Yoon and Kim, 2005). This group of endoglucanases was originally reported from cellulolytic bacteria. Similar cellulase and xylanase structures in fungi and bacteria suggest that cellulolytic genes could have spread to a significant extent by horizontal transfer across a wide range of organisms during the course of evolution (Béguin and Aubert, 1994).

Although cellulose degradation by basidiomycetes has been studied extensively since the middle of the last century, e.g. Reese and Levinson (1952), the view of cellulose degradation changed in the last two decades. In addition to hydrolytic enzymes, the role of oxidative reactions in the cleavage of cellulose polymer was described. The main role of radical-based mechanism can be in the structural degradation of wood to promote fungal colonization and resource capture. Owing to the diffusible nature of oxidants produced, cleavage by radical-based system can occur at larger distances from hyphae, radicals are small enough to penetrate into the substrate and to initiate polymer degradation.

The detection of hydrogen peroxide production by several fungi led to the proposal of a degradation pathway based on the Fenton reaction:

$$H_2O_2 + Fe^{2+} + H^+ \longrightarrow H_2O + Fe^{3+} + \bullet OH.$$

This reaction is a well-recognized route of •OH production in biological systems. Functioning of this system depends on production of H_2O_2. Hydrogen peroxide is generated via action of enzymes such as glyoxal oxidase, glucose oxidase, aryl alcohol oxidase, pyranose-2-oxidase, superoxide dismutase and other alcohol oxidases. In addition to free •OH radicals, certain states of hypervalent iron were also considered by some authors as potential oxidizing agents (Wood, 1994; Branchaud, 1999; Welch et al., 2002a; Welch et al., 2002b). Production of •OH can be affected by low concentration of oxalate, but its high concentration has opposite effect (Shimada et al., 1997).

Three oxidative systems operated by wood-rotting basidiomycetes have already received sufficient experimental evidence. These include (Hammel et al., 2002; Goodell, 2003; Tanaka et al., 2007):

1. cellobiose dehydrogenase (CDH) catalyzed reactions
2. redox cycling by small-molecular mass quinones or other redox compounds
3. •OH production catalyzed by small glycopeptides.

In all three oxidative systems, •OH is responsible for nonspecific scission of polysaccharides. Hydroxyl radicals can abstract hydrogen atoms from the sugar subunit of cellulose or other polysaccharides (Ek et al., 1989). These reactions produce transient carboncentred radicals that react rapidly with O_2 to give peroxyl radical species. If the ROO• carries a hydroxyl

group on the same carbon, it eliminates •OOH (Halliwell and Gutteridge, 1999). If there is no a-hydroxyl group present, the molecule undergoes a variety of oxidoreductions, some of which can result in the cleavage of the cellulose chain (Kirk et al., 1991). Oxidative degradation via reactive radical is nonspecific and can also degrade other polymers and compounds of cell wall.

The CDH-based decomposition differs from the other oxidative systems in two ways: (1) it depends on the presence of cellobiose and the action is more specific due to enzyme binding to cellulose and (2) in addition to oxidative cleavage of polysaccharides it also transforms cellobiose and cello-oligosaccharides, major products of cellulose hydrolysis. CDH acts as cellobiose oxidase reducing O_2 to give H_2O_2, but Fe^{3+} is better electron acceptor and thus CDH also acts as Fe^{3+} reductase. It oxidizes cellodextrines, mannodextrines and lactose. Cellobiose dehydrogenases are produced by basidiomycetes and ascomycetes. The enzyme is produced by white-rot basidiomycetes, brown-rot fungi from the class *Coniophoraceae* and some ascomycetes belonging to soft-rot fungi (Henriksson et al., 2000). Its activity has been also detected in some soil plant pathogens (e.g. *Sclerotium rolfsii*) and ectomycorrhizal fungi *Pisolithus tinctorius*, *Suillus variegatus* and *Cortinarius* sp. (Burke and Cairney, 1998)

The principle of the quinone redox cycling mechanism is in the fungal reduction of quinones to the corresponding hydroquinones, which then react with Fe^{3+} to give Fe^{2+} and semiquinone radicals. The semiquinones can reduce O_2 to give •OOH and the original quinones. Because •OOH is a source of O_2, this cycle will generate a complete Fenton system (Kerem et al., 1999). This reaction can be catalyzed by intracellular benzoquinone reductase (Brock et al., 1995) and extracellular sugar dehydrogenase such as CDH which have been shown to use quinones as alternate electron acceptors (Henriksson et al., 2000). Functioning of this model is dependent on sufficient production of quinones. Quinones are produced during lignin degradation, or can be synthesized by fungi. Low-molecular-mass glycopeptides catalyze •OH production through redox reactions between O_2 and electron donors. They reduce Fe^{3+} to give Fe^{2+} and strongly bind Fe^{2+} (Goodell et al., 1997). The size of glycopeptides does not allow them to penetrate the intact wood cell wall (Flournoy et al., 1991), and the reduction of their substrates thus probably occurs close to fungal hyphae although some diffusion into the cell wall was demonstrated (Hirano et al., 2000). Their production was reported from both brown-rot and white-rot basidiomycetes.

1.1.3 Hemicellulose and its degradation

Hemicellulose is a low molecular mass linear or branched polymer usually containing several different sugar units and substituted side chains. Xylans, consisting of D-xylose units, and glucomannans, consisting of D-glucose and D-mannose units, are the main hemicelluloses of angiosperm and conifer trees, respectively, while other lignocellulosic materials may additionally contain considerable amounts of arabinogalactans and galactans (Dekker, 1985). Branched polymers contain neutral and/or acidic side groups that render hemicelluloses noncrystalline or poorly crystalline. Hemicelluloses thus usually form a matrix together with pectins and proteins in primary plant cell walls and with lignin in secondary cell walls.

Enzymes degrading hemicellulose have been found widely distributed among bacteria and fungi. Similarly to cellulose, degradation of hemicellulose in terrestrial ecosystems is mainly ascribed to fungi. The enzymatic degradation of hemicelluloses requires a complex set of different enzymes reflecting the variability of the hemicellulose structure. Hemicellulose hydrolysis proceeds through the action of endo-type enzymes that liberate shorter fragments of substituted oligosaccharides, which are further degraded by side-group cleaving enzymes, and exotype enzymes. Alternatively, side-branches may be cleaved first. As a result, acetic acid and monomeric sugars are liberated and can be used as carbon sources for the fungal growth. Similarly to cellulose hydrolysis, hydrolases act synergistically to convert hemicellulose polymer into soluble units.

The effective native xylan degradation seems to involve probably multiple isoenzymes of five enzyme types. **Endo-1,4-β-xylanases** (EC 3.2.1.8) randomly hydrolyze β-1,4-glycosidic bonds in xylans, **1,4-β-xylosidases** (EC 3.2.1.37) release xylose monomers, and **1,4-β-glucuronidases** (EC 3.2.1.131), **α-arabinofuranosidases** (EC 3.2.1.55)/ **α-arabinosidases** (EC 3.2.1.99) and **acetyl xylan esterases** (EC 3.1.1.72) are involved in cleavage of side hemicellulose chains (Figure 1.3). They all differ in specificity with respect to the neighboring substituents and chain length (Tenkanen et al., 1995). For example, in conifers, where the xylan has arabinose as a substituent, xylan decomposition requires a β-arabinofuranosidase but not the esterase.

Xylanases have been widely studied due to their biotechnological importance (Subramaniyan and Prema, 2002). Their production has been described in microfungi (e.g. *Penicillium*, *Aspergillus*, *Thermoascus*, *Trichoderma*) and also wood-rotting basidiomycetes (e.g. *Phanerochaete chrysosporium*, *Schizophyllum commune*).

Complete hydrolysis of glucomannans also requires a wide set of enzymes (Figure 1.4). **Endo-1,4-β-mannanase** (EC 3.2.1.78) hydrolyses randomly the 1,4-β-mannopyranosyl linkages. **Acetyl(gluco)mannan** esterase removes the acetyl groups and **α-galactosidase** (EC 3.2.1.22) removes galactose. **1,4-β-mannosidase** (EC 3.2.1.25) and **β-glucosidase** cleaves the β-1,4 linkages between oligomeric fragments. Only few studies have been conducted on these enzymes from wood-rotting basidiomycetes, although they obviously effectively remove mannan from the cell walls of wood (Eriksson et al., 1992). Apparently, many species produce a wide set of hemicellulose-degrading enzymes. For example, endomannanase, endoxylanase, β-mannosidase and β-xylosidase were produced by the white-rotters *Pleurotus ostreatus*, *Trametes versicolor* and the brown-rotter *Piptoporus betulinus*, while the latter also produced β-galactosidase activity (Valášková and Baldrian, 2006). Hemicellulase production does not appear to be as strictly controlled by substrate induction as cellulase production (Eriksson et al., 1992; Valášková and Baldrian, 2006).

Hemicelluloses are bound to lignin by three types of covalent linkages (Španiková and Biely, 2006). The first involves *p*-coumaric or ferulic acid, linked by ether bonds to lignin, and esterically to hemicellulose sugars. This linkage could be cleaved by **feruloyl esterase** (EC 3.1.1.73) that is typical of filamentous fungi and has been demonstrated in the ligninolytic basidiomycetous yeast *Aureobasidium pullulans* (Rumbold et al., 2003), but not yet in other ligninolytic species. The second is represented by ether linkages be-

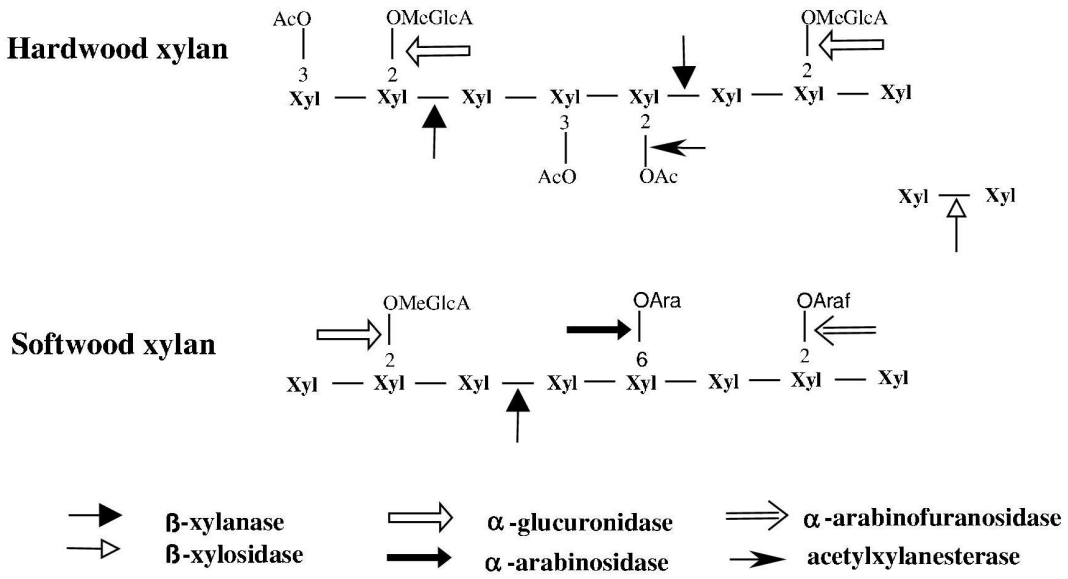

Figure 1.3: Schematic structure of hardwood and softwood xylan and the action of different hemicellulolytic enzymes

tween OH-groups of saccharides and lignin alcohols. The third are ester linkages between 4-O-methyl- D-glucuronic acid (MeGlcA) or D-glucuronic acid residues of glucuronoxylans and hydroxyl groups of lignin alcohols, which can potentially be cleaved by **glucuronoyl esterase** (Španiková and Biely, 2006).

Nonenzymatic radical-based systems for the cleavage of cellulose, already described in section 1.1.2, work due to its nonspecificity equally well for hemicellulose decomposition. Although the above definitions of enzymes with respect to the reactions they catalyze seem to be clear, in reality the substrate specificity often overlaps. Also the cellulolytic and hemicellulolytic enzymatic systems cannot be separated completely since the substrates are chemically analogous and individual enzyme molecules frequently exhibit activities with more than one substrate (Copa-Patino and Broda, 1994; Cohen et al., 2005). The classification according to the major activity can thus be misleading with respect to the actual physiological role, and even the purified enzymes described so far probably exhibit additional activities that have not yet been searched for.

1.1.4 Lignin and its degradation

Lignin is the second most abundant biopolymer next to cellulose in terrestrial vascular plants. Lignin comprises of phenylpropanoid units joined together in polymerization promoted by peroxidase and laccase action during lignin biosynthesis in the plant cell wall (reviewed by Boudet et al., 2003; Higuchi, 2006). The three phenylpropanoid precursors

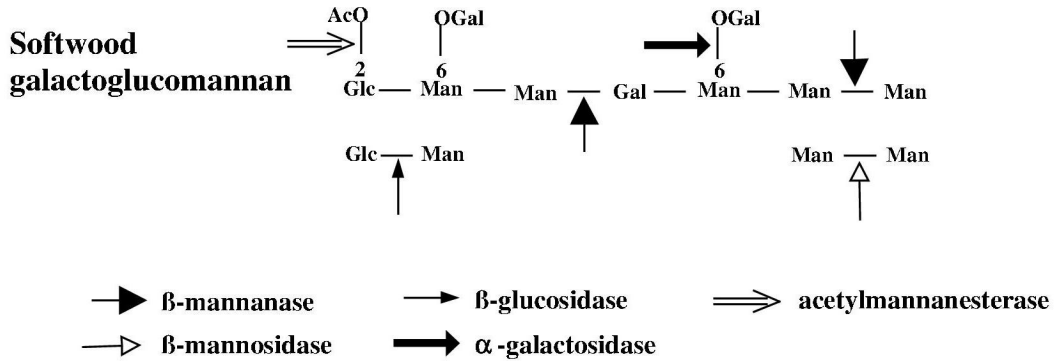

Figure 1.4: Schematic structure of softwood galactoglucomann and the action of different hemicellulolytic enzymes

of lignin: *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol, form the three types of lignin subunits: hydroxyphenyl-, guaiacyl-, and syringyl-types, respectively. The subunits are linked with variety of bond types; β-O-4 linkages are the most common and can account for 50% and 60% of the intermonomeric bonds in softwood and hardwood lignin, respectively (Sjöström, 1993).

Lignin is closely associated with cellulose and hemicelluloses in plant cell walls and attaches to polysaccharide polymers in the cell walls both physically and chemically. It binds fibrers of cell wall together and this gives wood the mechanical strength. Lignin is highly resistant against chemical and enzymatic degradation and makes wood resistant against microbial attack (Argyropoulos and Menachem, 1997).

Lignin alone cannot supply sufficient energy to maintain its own decomposition, so other carbon and energy sources such as cellulose are necessary as growth co-substrates for fungi to decompose lignin (Kirk et al., 1976). Lignin decomposition is thought to be a secondary metabolic function that serves mainly to unmask cellulose and expose it to cellulases from which it would otherwise have been protected (Cooke and Whipps, 1993). Its degradation also allows faster colonization and thus can be advantageous in primary resource capture in competition with other cellulolytic organisms (Boddy, 2000).

Although certain bacteria can degrade lignified cell walls, the evolution of the pathways for lignin degradation has been restricted largely to basidiomycetes and and xylariaceous ascomycetes, causing white-rot type of wood decay (Rayner and Boddy, 1988; Worrall et al., 1997).

Lignin polymer structure is irregular, which means that the degradative enzymes must show lower substrate specificity compared to the hydrolytic enzymes in cellulose or hemicellulose degradation. Because lignin consists of interunit carbon-carbon and ether bonds, the enzymes must be oxidative rather than hydrolytic. White-rot fungi produce extracellular peroxidases and phenoloxidases that act nonspecifically via generation of free radicals, which are unstable and undergo a variety of spontaneous cleavage reactions.

Table 1.3 summarizes the ligninolytic enzymes of white-rot fungi and their substrates and reactions. The main extracellular enzymes participating in lignin degradation are heme-containing **lignin peroxidase**, **manganese peroxidase** and Cu-containing **laccase** (reviewed by Hofrichter, 2002; Piontek et al., 2001; Baldrian, 2006). Lac and MnP shape the backbone of ligninolytic system. Whereas laccase is produced by many lignin degrading fungi, including ascomycetes (e.g. *Penicillium chrysogenum*, *Fusarium oxysporum* and *Fusarium solani* (Rodriguez et al., 1996)) and white-rot basidiomycetes (e.g. *Trametes versicolor*, *Pleurotus ostreatus*, *Phanerochaete chrysosporium*), MnP is produced only by basidiomycetes (Hofrichter, 2002; Hatakka et al., 2003). MnP producers often belong to saprotrophic litter decomposing fungi. Lignin peroxidase is minor enzyme produced only by few genera of white-rot basidiomycetes: *Phanerochaete*, *Trametes*, *Bjerkandera*, *Phlebia* and *Merilius* (Hatakka, 2001). A small group of ligninolytic heme-containing peroxidases, combining structural and functional properties of the LiPs and MnPs, are the **versatile peroxidases**, produced by some basidiomycetous genera, e.g. *Pleurotus* and *Bjerkandera* (Ruiz-Duenas et al., 2001; Moreira et al., 2005). Many white-rot fungi obviously do not produce all three, but only two or one of the enzymes (Hatakka, 2001).

Enzyme	Proposed role in lignin degradation	co–factors
Lignin peroxidase EC 1.11.1.14 LiP	degradation of non–phenolic units	H_2O_2, aryl alcohols
Manganese peroxidase EC 1.11.1.13 MnP	degradation of phenolic units and nonphenolic units	H_2O_2, lipids, Mn^{2+}
Versatile peroxidase EC 1.11.1.16 VP	combines functions of LiP and MnP	H_2O_2, aryl alcohols
Laccase EC 1.10.3.2 phenol oxidase	oxidation of phenolic units, and nonphenolic units	
Various H_2O_2 generating oxidases Glyoxaloxidase EC 1.2.3.5 Arylalcoholoxidase EC 1.1.3.7 pyranose-2-oxidase EC 1.1.3.10 and others	H_2O_2 production	

Table 1.3: Ligninolytic enzymes, their main reactions and cofactors (modified from Hatakka, 2001).

Peroxidase function requires extracellular hydrogen peroxide. Hydrogen peroxide-generating systems thus form an important part of the fungal delignification system. Hydrogen perox-

ide is extracellularly produced by several enzymes such as **glyoxal oxidase**, **aryl alcohol oxidase** and **glucose-2-oxidase** or by nonenzymatic systems via Fenton reaction. H_2O_2 may be also utilized by cellobiose dehydrogenase together with Fe^{3+} and Mn^{4+} reduction (Roy et al., 1994) for the generation of oxyradicals that are able to cause bond cleavages in lignin and cellulose as described in section 1.1.2. Fenton-type catalytic system producing hydroxyl radicals that attack wood components is particularly important for brown-rot fungi, as this group does not produce ligninolytic enzymes. The radicals formed by brown-rot fungi can remove methoxyl groups of lignin and produce metanol, and thus they leave a residue that consists mainly of modified lignin. Demethoxylation and methoxyl groups and aromatic hydroxylation make the modified lignin more reactive (Hatakka, 2001).

Ability of bacteria to mineralize lignin is very limited, bacterial attack usually leads only to lignin molecule modification. Only filamentous strains belonging to genera *Streptomyces* are well-known degraders of lignin producing lignin peroxidase-type of enzymes and phenol oxidase. Lignin degradation model studies demonstrated formation of monomeric compounds (i.e., vanilic acid, protocatechuic acid), indicating the cleavage of C_α-C_β bonds as well as demethylation and oxidation of C_α in the side chain to carbonyl group (Hatakka, 2001). Nonfilamentous bacteria are able to degrade only low-molecular-mass oligomers of lignin and degradation products of lignin. *Pseudomonas* spp. are the most efficient degraders among eubacteria. However, since these bacteria do not produce extracellular oxidoreductases, and large molecules apparently cannot be taken up into the cell, they are obviously unable to attack polymeric lignin (Zimmermann, 1990; Hatakka, 2001; Daniel, 2003).

1.1.5 Degradation of other wood cell wall components

It should be noted that plant cell wall contains other compounds like pectin, starch, proteins and suberin. **Pectin** (approximately 4% of wood mass) encompasses a group of amorphous polymers of 1,4-β-D-galacturonic acid and its methylesters. It is mostly present in primary wall and middle lamella and it is covalently bound to hemicellulose. It can serve as sole carbon source as demonstrated for e.g. *Gloeophyllum trabeum*, *Postia placenta* and *Serpula incrassata* (Green III et al., 1996). Pectinolytic activity has been found in plants, saprotrophic and mycorrhizal fungi, yeasts, and bacteria (Ramstedt and Soderhall, 1983; Garciaromera et al., 1992; Dahm et al., 1999; Uenojo and Pastore, 2007).

Cell wall **proteins** are typically glycoproteins rich in hydroxyproline. They contribute to the cell wall architecture and resistance against plant diseases (Evans and Hedger, 2001). **Suberin**, main component of bark tissue, consists of spatially separate polyaromatic (polyphenolic) and polyaliphatic (lipidic) domains, but its fine structure has not been clarified. It contributes to both cell wall strength and resistance to water loss during plant growth and development. Suberised tissue contains phenolics and acids which can be toxic to invading microorganisms, but there are fungi e.g. *Heterobasidion annosum* able to detoxify and penetrate these barriers during infection (Maijala, 2000).

1.2 Plant litter - lignocellulose source in soil

Plant litter input into broadleaved forest soils was estimated to be in the range of several tons per ha^{-1} per year (Bray and Gorham, 1964). In these ecosystems it is composed of approximately 78% leaves, 20% flowers, seeds and related organs, and 2% twigs and bark (Rihani et al., 1995). Litter forms clearly distinguishable uppermost soil horizon (litter horizon) that is typically 1 - 10 cm thick. Fresh litter, moderately decomposed organic matter, humus and well-decomposed organic residue shapes the structured vertical profile of the forest floor. Litter is inhabited by variety of soil organisms: bacteria, saprotrophic and opportunistic fungi and invertebrates that are involved in its degradation. Plant litter decomposition is one of the main processes responsible for the formation of temperate forest soils with developed organic (O) horizon characterized by a high content of humic compounds. Cambisol, typical soil type for temperate deciduous forests (Fitzpatrick, 1984), composes of litter horizon (L), organic horizon (O) and mineral soil horizon (Ah) (Figure 1.5).

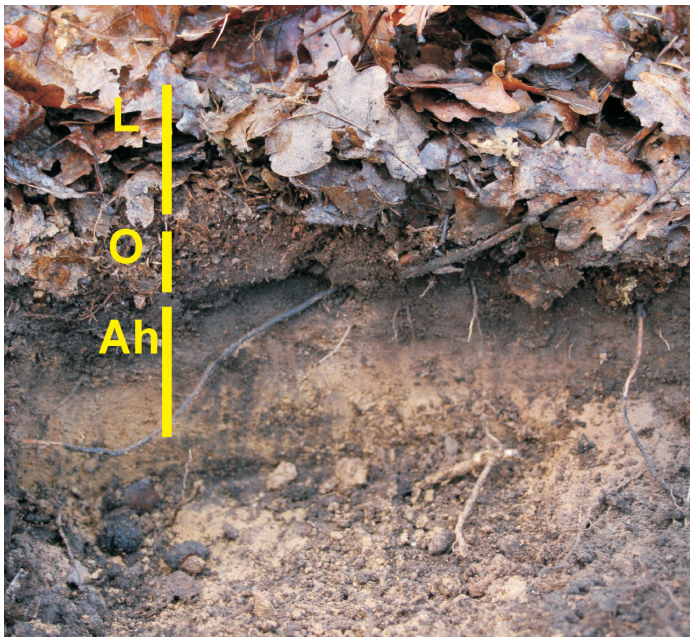

Figure 1.5: Cambisol soil profile of deciduous temperate forest, Xaverov, Czech Republic. L: litter horizon (11 mm), O: organic horizon (19 mm), Ah: upper mineral soil horizon (41 mm).

Litter is mainly composed of cellulose (10 - 50%), hemicellulose (30 - 40%) and lignin (generally 15 - 40%, in extremes as low as 4%, or as much as 50%) (Berg and McClaugherty, 2003). Other minor litter components are: polyphenols (tannins), microbial cell wall components (e.g. peptidoglycans and chitin), cutin, suberin, low-molecular-mass carbohydrates and amino-type compounds, typically exuded from roots and mycorrhiza, ether- and alcohol-soluble constituents including fats, oils, waxes, resins, and many pigments, and

proteins (Satchell, 1974; Horwath, 2007).

Plant litter together with the input from plant roots (root exudates and dead root biomass) represent the main sources of organic matter in forest soils (Litton et al., 2003). Two mechanisms are balanced in organic matter turnover in soil. Organic matter may be mineralized, resulting in production of CO_2, phosphate, sulphate, nitrate, etc. that are further available to soil microorganisms. On the other hand, it may be only partially decomposed into more or less complex organic radicals that polymerize and form humus, a very stable complex component of soils. Both the mineralization and humification processes are driven by soil bacteria and fungi, where a broad set of enzymes is involved (Buscot, 2005).

Among the hundreds of different enzymes released by microbes into the environment, soil enzymes directly involved in the decomposition of lignocellulose and the cycling of major nutrients have been the focus of recent studies. These soil enzymes include phenol oxidase (laccase), peroxidases, cellulases, hemicellulases, proteases, and phosphatases (Eriksson et al., 1992; Boddy, 2000; Sinsabaugh et al., 2005; Baldrian and Šnajdr, 2006; Baldrian et al., 2006).

1.3 Litter-decomposing fungi and bacteria

The dominant primary decomposers in temperate forest soils are the microorganisms, encompasing both fungi and bacteria. Both these main groups of microorganisms can degrade cellulose, hemicellulose and the different lignins. Polymer carbohydrates may be degraded both aerobically and anaerobically, but a complete degradation of lignin (white-rot type of decay) requires the action of aerobic organisms (fungi and/or aerobic bacteria). Partial lignin degradation (brown-rot type of decay) may also be caried out by anaerobic bacteria, but is mainly found among fungi and aerobic bacteria.

The functional concept white-rot, brown-rot and soft-rot have been already used during description of wood polymer degradation in section 1.1. The terms refer to the type of rot rather than to a taxonomic identification, thus these groups are ecological. As regards degradation by bacteria, it is described and discussed as such. The main characteristics of white-rot, brown-rot and soft-rot type of decay are listed in Table 1.4. The composition of the microbial population (e.g. cellulolytic *vs.* ligninolytic) may vary with general properties of the soil/litter ecosystem, such as nutrient status and pH (Berg and McClaugherty, 2003).

By tradition, soil animals such as collembola, mites and earthworm, have been considered important for litter decomposition. Different roles in decomposition have been ascribed to these groups, although these roles are not always clear. In recent decades, it has become increasingly clear that the microbial component is of absolute dominance in temperate forests (Berg and McClaugherty, 2003). Due to ecological and degradation abilities, litter decomposition in temperate forests is mainly driven by fungal activity (Hattenschwiler et al., 2005), and basidiomycetes dominate the litter degradation.

Fungal group	main features
White–rot fungi ligninolytic fungi WRF	▷ degrade lignin to CO_2 and H_2O, cellulose, hemicellulose ▷ produce ligninolytic, cellulolytic, hemicellulolytic and related enzymes ▷ selective delignifers – preferentially remove lignin from lignocellulose (SD) ▷ nonselective delignifers – remove all compounds equally (ND) ▷ *Basidiomycotina* degrading soft– and hardwood, *Ascomycotina* prefering hardwood ▷ *Ceriporiopsis subvermispora* (SD), *Physisporinus rivulosus* (SD),*Phlebia radiata* (SD), *Phanerochaete chrysosporium* (SD), *Trametes versicolor* (ND), *Ganoderma applanatum* (both SD, ND), *Heterobasidion annosum* (both SD, ND)
Brown–rot fungi cellulolytic fungi BRF	▷ degradation of cellulose and hemicellulose ▷ possible lignin modification (Kirk and Farrell, 1987; Eriksson et al., 1990) ▷ do not produce ligninolytic enzymes and cellobiohydrolase (except for *Coniophora puteana*) ▷ reactive oxygen species and hemicellulase as iniciators of degradation (Jellison et al., 1997) ▷ actively decrease pH, to lower values compare to WRF ▷ long lasting wood residues after degradation (Tuomela et al., 2000) ▷ *Basidiomycotina*, preferentially degrade softwood ▷ *Anthrodia carbonica*, *Gloeophyllum trabeum*, *Laetiporus sulfureus*, *Tyromyces palustris*, *Lentinus lepideus*, *Polyporus schweinitzii*, *Piptoporus betulinus*, *Poria placenta*, *Serpula lacrymans*
Soft-rot fungi cellulolytic fungi SRF	▷ degradation of cellulose and hemicellulose ▷ limited degradation (Tuomela et al., 2000) or only modification (Ward et al., 2004) of lignin ▷ low rate of degradation compare to WRF and BRF ▷ limited knowledge about lignocellulolytic system ▷ wet environment, lignocellulose in soil (Blanchette, 1995) ▷ *Ascomycotina* amd *Deuteromycotina*, although basidiomycete *Inonotus hispidus* ▷ *Phialophora*,*Ceratocystis*,*Hypoxolon*, *Ustulina*, *Chaetomium*

Table 1.4: Ecological groups of wood-rotting fungi and their important features.

1.3.1 Litter-decomposing fungi

Term litter-decomposing fungi (LDF) refers to saprotrophic fungi inhabiting forest floor. They efficiently decompose litter through action of wide variety of oxidoreductases and hydrolytic enzymes (Colpaert and van Laere, 1996; Hofrichter, 2002; Baldrian, 2006) and thus cause white-rot type decay of litter. Taxonomicaly, LDF mainly belong to basidiomycetes (particularly *Agaricales*, *Boletales* and *Poriales*), although some macroscopic fruiting body forming ascomycetes can be also considered as LDF (Table 1.5).

Basidiomycetes
Agaricus, Agrocybe, Clavaria, Clitocybe, Collybia, Coprinus, Cortinellus, Cudonia, Cystoderma, Galerina, Hypholoma, Lepiota, Lepista, Lyophyllum, Macrolepiota, Marasmius, Micromphale, Mycena, Phaelolepiota, Psalliota, Rhodocybe, Spathularia, Stropharia
Ascomycetes
Gyromitra, Morchella, Xylaria

Table 1.5: Litter-decomposing fungi.

Litter -decomposing fungi, as they have been defined above, comprise in temperate forest only a part of the entire decomposer fungal community. Additionaly to LDF, ascomycetes and deuteromycetes utilizing cellulose and simple sugars and ecological groups such as wood-rotting fungi and mycorrhizal fungi inhabit and transform forest litter. These fungi thus also play considerable role in litter degradation. The habitats of these three main eco-physiological groups of fungi may overlap, as is demonstrated in Figure 1.6.

Wood-decomposing fungi colonize dead or dying tree trunks and stumps, although some are capable of soil colonization. According to the type of decay caused, they are defined as white-rot, brown-rot and soft-rot (Table 1.4). **Mycorrhizal fungi** actively colonize soil and form a symbiotic relationship with the roots of trees and other plants and provide them with better access to water and nutrients in return for host carbon assimilates. Mycorrhizal community inhabiting roots of trees and soils in forest ecosystems is dominated by ectomycorrhizal fungi (ECM). These taxonomically belong to basidiomycetes, but minority is clasified as ascomycetes, zygomycetes and deuteromycetes (Gryndler et al., 2004). Litter-decomposing fungi and ectomycorrhizal fungi cannot be taxonomicaly separated. Members of one genera can belong to different ecological groups. Mycorrhizal fungi produce litter-degrading enzymes, however, the enzymatic activities of mycorrhizal fungi are generally lower than those of saprotrophic litter-decomposing basidiomycetes (Colpaert and van Tichelen, 1996; Burke and Cairney, 2002). Mycorrhizal fungi do not depend on litter-derived energy and can thus replace saprotrophic community in energy depleted substrate such as degraded litter and humus,. Lindahl et al. (2007) proposed the spatial separation of the degrading (saprotrophic) and nutrient-mobilizing (mycorrhizal) components of the fungal community in the forest floor of the boreal pone forest.

Litter decomposing fugi can degrade all constituents of litter. The degradation resem-

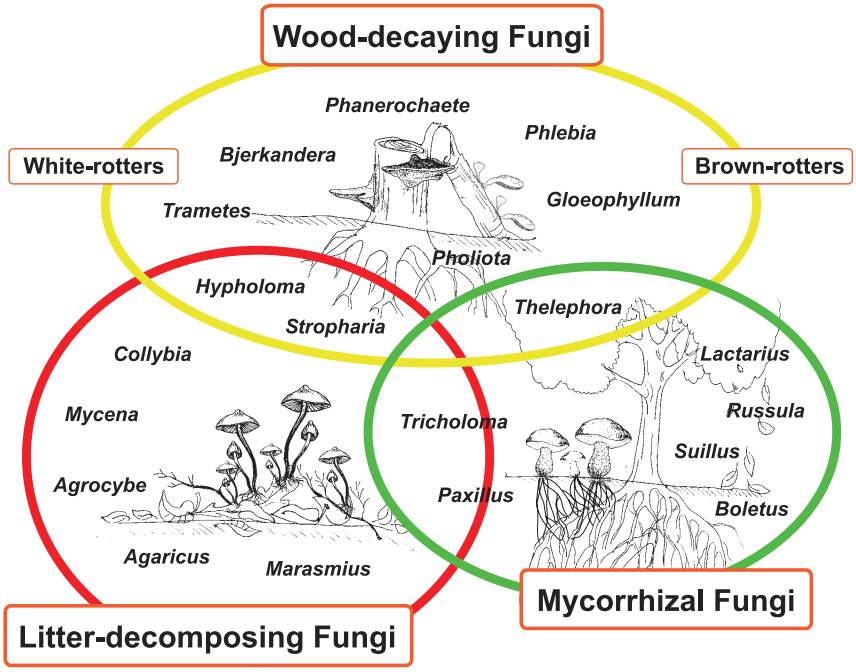

Figure 1.6: Ecophysiological division of basidiomycetous fungi into three partially overlapping groups according to their habitat and lifestyle (Steffen, 2003).

bles that of wood-rotting fungi. Ligninolytic enzymes of LDF have been studied extensively (Steffen et al., 2002a, b; Hatakka et al., 2003; Kapich et al., 2005; Ullrich et al., 2005; Baldrian and Šnajdr, 2006; Anh et al., 2007; Osono, 2007; Kahkonen et al., 2008). Manganese peroxidase (MnP), involved in humus degradation by recycling high molecular mass humic substances, and laccase are considered to be major enzymes involved in lignin degradation (Steffen et al., 2002b). Production of lignin peroxidase (LiP) has not been so far observed by LDF. Less attention has been paid to cellulolytic and hemicellulolytic

enzymes, though, LDF produce wide spectra of cellulolytic, hemicellulolytic and other enzymes (Rosenbrock et al., 1995; Steffen et al., 2007; Kahkonen et al., 2008). For instance, soil enriched by *Lepista nuda* exhibited higher activity of phosphatase, protease, cellulase, β-xylosidase, β-glucosidase and phenol oxidase (Colpaert and van Laere, 1996). Litter-decomposing fungi are next to main litter components able to degrade tannins, melanins, humic substances, and cutin (Osono, 2007).

The reactions controled by lignocellulolytic enzymes lead to release of carbon and energy. When carbon is released, relative amount of nitrogen increases and thus C/N ratio in litter decreases from 24-40 in fresh *Quercus* litter (Vane et al., 2003; Madritch and Hunter, 2005; Quideau et al., 2005), or 150 in fresh wood, to 13-17 in decomposed *Quercus petraea* litter (Steffen et al., 2007). High C/N ratio prevents substrate colonization by bacteria. Its decrease can be thus favorable for further degradation processes and also microbial community development (Dilly et al., 2001; Hattenschwiler et al., 2005). Next to C/N ratio, LDF can also decrease the lignin content (Steffen et al., 2007) that is typical for white-rot type of decay.

Chemical decomposition of leaf litter follows a sequential pattern with different classes of organic compounds dominating the process as it proceeds (Berg and McClaugherty, 2003). In general loss of soluble components occurs in the first phase, followed by holocellulose decomposition in the second phase. Finally lignin becomes a dominant component when litter mass loss slows down and litter decomposition approaches that of humus.

Changes in litter quality are reflected on the level of degrader community. Endophytic phyllosphere fungi frequently occur in early stages od decomposition. Their persistence from live leaves gives them advantage of gaining access to readily available organic compounds in freshly fallen leaves (Stone 1987). Hyphal growth of cellulolytic and sugar fungi is generally faster than that of ligninolytic fungi. First stages of litter decomposition are thus dominated by ascomycetes and deuteromycetes. As an example, early colonizer of *Swida* litter (*Phoma*, *Cladosporium* and *Pestalotiopsis*) were during early stages of degradation replaced by membrers of genera *Arthrinium*, *Trichoderma*, *Mucor*, *Umbelopsis* and *Mortierella* (Osono, 2005). In the initial phase of black alder (*Alnus glutinosa*) litter decomposition, a zymogenic microflora of the genera *Mucor, Alternaria, Epicoccum* colonized the litter. This flora was progressively replaced by autochtonous soil-inhabiting species, principally of the genus *Fusarium* (Rosenbrock et al., 1995).

The relative abundance of ligninolytic fungi increases in later stages when easily utilizable saccharides are not available and lignin content increases due to relative enrichment of litter with lignin during decomposition. Even within the ligninolytic fungi, ascomycetes are more abundant in freshly fallen leaves than basidiomycetes as they preferentialy remove cellulose (Osono and Takeda, 2002; Osono et al., 2003; Osono and Takeda, 2006). Ascomycetes abundance gradually decreases during decomposition. Ligninolytic basidiomycetes dominate in later stages of litter degradation. This does not exclude the possibility, however, that ligninolytic ascomycetes persist until later stages of decomposition. In fact, xylariaceous ascomycetes were frequently isolated from *Fagus crenata* and *Pinus densiflora* litter even after 4 years of decomposition (Osono, 2007).

The changes of chemical composition and fungal degraders community during the degradation could be transformed into vertical distribution in soil profile. Freshly fallen litter is deposited onto the top, whereas chemical changes acumulate towards deeper horizons. This hypothesis agree with an observation of Lindahl et al. (2007), who found a clear shift in fungal community composition between the surface litter (L) horizon and the underlying F horizon, in which the litter had lost its structural integrity in boreal pine forest. Endophytic fungi were frequently recorded in fresh litter horizon. They seem to be replaced by other fungi at a relatively early stage of decomposition. The "early" stage of degradation (surface of the soil) was dominated by ascomycetes within *Helotiales* and *Dothideomycetes*, who rarely extended below the surface layer of the forest floor. The only frequently recorded saprotrophic basidiomycetes were *Mycena* species. Ectomycorrhizal fungi within the genera *Cortinarius* and *Piloderma* dominated the nutrient depleted decomposed litter and humus, the "late" community.

1.3.2 Bacteria inhabiting litter

Bacteria commonly inhabit lignocellulose material such as wood and litter (Aneja et al., 2006), they are initial colonizers of wood (Clausen, 1996). Contrary to fungi, bacteria degrade cellulose and hemicellulose under both aerobic and anaerobic condition (section 1.1.2). Lignin mineralization is, however, very limited. Bacterial attack of lignin molecule more often leads only to its modification (Zimmermann, 1990; Hatakka, 2001; Daniel, 2003; Vargas-Garcia et al., 2007) (section 1.1.4). Bacterial lignocellulose decomposition seems to be more common in situation where fungi are under stress. Thus bacteria are believed to dominate wood decay in water, sediments and other anoxic environments (Holt, 1983; Landy et al., 2008).

Not all bacteria inhabiting litter are able to degrade cell wall polymer. Early colonizing bacteria are thought to grow on easily degradable substrates like sugars, organic acids, pectin and easily accessible cellulose (Schmidt, 2006). These compounds can be also utilized in later stages of decay as substrates produced by the action of fungi. Live (mycophagous bacteria), dead or damaged hyphae can also serve as carbon and energy sources.

In leaf litter of *Liriodentron tulipifera* bacteria accounted for 43% of total microbial biomass. Culturable bacteria were identified to 14 different bacterial species and *Bacillus* sp. predominated (Mikluscak and Dawson-Andoh, 2004). Aneja et al. (2006) studied bacterial community composition in beech and spruce litter. Sequences derived from beech litter were dominated by γ-proteobacteria of genera *Pseudomonas*, sequences of the members of actinobacteria and acidobacteria were also present. Spruce litter was inhabited by α-, β- and γ-proteobacteria. All of the above-mentioned genera are capable of degrading structural carbohydrates. Among proteobacteria, members of genus *Burkholderia* are probably the most diverse and environmentally adaptable soil- and plant-associated bacteria (Compant et al., 2008). *Cytophaga-Flavobacterium-Bacteroidetes* have been also implicated in the degradation of lignocellulose in plant litter (Lydell et al., 2004; Das et al., 2007), along with high proportion of actinobacteria and acidobacteria (Aneja et al., 2006; Dedysh et al., 2006). Other studies showed that the cellulolytic aerobes such as *Cytophaga* and

Cellvibrio, anaerobic species such as *Clostridium*, or ubiquitous species such as *Bacillus, Pseudomonas, Arthrobacter, Flavobacterium* and *Spirillum* can be isolated from decaying wood (Daniel et al., 1987; Clausen, 1996; Przybyl, 2001).

1.4 Factors affecting degradation of plant litter

The physicochemical environment, litter quality, and the composition of the decomposer community are the three main factors controlling litter decomposition (Hattenschwiler et al., 2005). Substrate moisture, pH and temperature are the most important physicochemical factors of the environment (Poole et al., 2001). Under given environmental conditions, the remaining two factors - litter quality and decomposers - are directly related to biological diversity. Litter-decay rates differ widely among species that decompose under identical environmental conditions. These differences in decomposition are attributed to variation in litter properties, such as leaf toughness, nitrogen, lignin, and polyphenol concentrations, and the carbon/nitrogen and lignin/nitrogen ratios and their consequences for microbial activity and substrate utilization (Hattenschwiler et al., 2005).

Soil microbial diversity has been hypothesized to correlate positively with process rates within soils. The experiments of Robinson et al. (1993), Setala and McLean (2004) and Tiunov and Scheu (2005) showed a clear positive effect of fungal diversity on litter decomposition, whereas Cox et al. (2001) documented faster pine-litter decomposition in the presence of single fungal species. Both observations have an explanation: competitive interactions among fungi in a diverse community can result in reduced decomposition rates, whereas e.g. niche differentiation seems to provide a likely explanation for positive effects of fungal-species richness on litter decomposition (Loreau, 2001).

Pretreatment of leaf litter with endophytic fungi and concomitant changes in litter quality were found to affect the substrate utilization patterns of *Mycena* spp., which shifted from simultaneous removal of lignin and carbohydrates to selective delignification. In turn, the removal of lignin by ligninolytic fungi enhanced the decomposition of leaf litter by succeeding fungi under both laboratory and field conditions (Osono, 2007).

1.5 Interactions between lignocellulose-degrading fungi and bacteria

Interactions generally occur, when organisms share the same environment and nutrient source. Interactions have important role in microbial community establishment, for instance when fungi colonize wood, soil or litter that are already inhabited by bacteria. Intensity of interactions depends on ecology of microorganisms and niche they inhabit. The fungi in general, and saprotrophic basidiomycetes in particular, are efficient degraders of recalcitrant organic compounds (lignin and plant cell wall polysaccharides), while bacteria in soil are more successful in the decomposition of simple substrates (de Boer et al., 2005; Baldrian, 2008b). Due to different preferences for substrate, fungi and bacteria oc-

cupy different decomposition niches. On the other hand, the presence of fungi in the soil creates an array of bacterial niches for exudate-consuming or mycophagous bacteria (de Boer et al., 2005; de Boer et al., 2008). In this case, microbial interactions can be indirect. In simple words, bacteria may profit from fungal presence due to increased amount of nutrients produced during fungal lignocellulose degradation, but fungus directly does not interact with bacteria. When fungi or bacteria deliberately affect the other interaction partner e.g. by production of antibiotic compounds, by stimulation of metabolism and growth or stimulation of defense mechanisms in the course of direct interaction. The final composition of microbial community in soil, wood or litter, of course, depends also on abiotic factor (e.g. moisture, pH, temperature, soil type), fauna and plants as well.

Fungi change the numbers of bacteria inhabiting soil or wood. The effect is species-specific. Increased or decreased numbers of culturable heterotrophic bacteria have been found in the mycosphere of the saprotrophic soil basidiomycetes *Hypholoma fasciculare, Recinicium bicolor, Anthrodia vaillantii* and others (Tornberg et al., 2003; Folman et al., 2008). Recently, differences in bacterial communities in bulk soil and the mycorrhizosphere of *Laccaria proxima* were reported (Warmink and van Elsas, 2008). Since wood-decomposing, litter-degrading and ectomycorrhizal basidiomycetes are closely related (Hibbett et al., 2000), similar effects can also be expected for basidiomycetes colonizing wood and litter. *Pleurotus ostreatus*, one of the most successful competitors, frequently decreased the counts of heterotrophic bacteria in colonized soils (Beltran-Garcia et al., 1997; Andersson et al., 2003; Otieno et al., 2003). It lyses bacterial colonies (Barron, 1988) and may even prevent soil microbes from utilizing lignocellulose substrate added to soil (in der Wiesche et al., 1996; Lang et al., 2000).

Fungi also change the composition of the soil bacterial community. The bacterial community of soil outside mycorrhizosphere of *Pinus sylvestris - Suillus bovinus* was dominated by *Paenibacillus* sp. isolates, whereas in the mycorrhizosphere was higher proportion of proteobacteria dominated by *Burkholderia* isolates (Timonen and Hurek, 2006). Bacterial PLFA pattern of soil colonized by basidiomycetes changed towards higher proportion of Gram-negative bacteria compare to noncoloniyed soil (Tornberg et al., 2003). Bacterial community composition of Douglas fir differs among root tips and seems not to be affected by taxonomical identity of fungus (Burke et al., 2008). However, species specific effect on bacterial community composition has been also described (Poole et al., 2001). Most bacteria in mycorrhizosphere are related to *Alphaproteobacteria, Bacteroidetes, Burkholderia, Paenibacillus, Pseudomonas* and *Rhodococcus* (Bianciotto et al., 2000; Poole et al., 2001; Burke et al., 2008; Warmink and van Elsas, 2008). Changes in community composition have been detected independently of the changes in total bacterial biomass abundance (Andersson et al., 2003).

Bacteria do also affect fungi in soil and wood. Bacteria isolated from spruce stumps had significant negative effect on the growth of *Heterobasidion annosum*, although effect of mixed bacterial polulation differed between different fungi (Murray and Woodward, 2003). Some strains of bacteria isolated from soil were inhibitory to the growth of *Phanerochaete chrysosporium* (Ali and Wainwright, 1994; Radtke et al., 1994; Tucker et al., 1995). Most were fluorescent pseudomonads although not all isolates from this group caused growth in-

hibition (Tucker et al., 1995). In contrast, bacteria associated with mycorrhizosphaere such as *Paenibacillus*, *Burkholderia* and *Rhodococcus* promoted mycorrhiza formation (Poole et al., 2001), they are known as "mycorrhiza helper bacteria" (Bianciotto et al., 2000). Some other bacteria belonging to genera *Pseudomonas* and *Bacillus* are also able to promote mycorrhiza formation, stimulate mycelial growth and production of secondary metabolisms (Gryndler et al., 2004).

The mechanisms leading to the suppression or promotion of bacteria by fungi and *vice versa* of fungi by bacteria are not very clear, but some possible explanations can be followed. Ligninolytic fungi typically respond to the presence of soil fungi or bacteria with an increase in laccase activity (Baldrian, 2006) and this enzyme catalyses the formation of antibacterial compounds in *Pycnoporus cinnabarinus* (Eggert, 1997), direct effects of laccase on soil bacteria were, however, not confirmed (Baldrian, 2004). Some wood-colonizing fungi produce toxic hydroxyl radicals when confronted with the antagonistic bacteria *Pseudomonas fluorescens* and *Bacillus subtilis* (Tornberg and Olsson, 2002) and the production of antibiotic compounds by fungi is another possible mechanism. With respect to the latter, *Hypholoma fasciculare* is known to produce high amounts of chlorinated anisylates, which have been suggested to be involved in the suppression of competing microorganisms (de Jong and Field, 1997). In the study of Folman et al. (2008) the negative effect of *Hypholoma fasciculare* on wood-inhabiting bacteria was ascribed to acidification of the environment next to already mentioned mechanisms.

From the other side, bacterial inhibition of fungal growth is ascribed to the production of chemicals that cause fungistatis or affect fungal growth and enzyme activities such as antibiotics, volatile compounds and siderophores (Radtke et al., 1994; Cornelis and Matthijs, 2002; Schouten et al., 2003; Zou et al., 2007; Compant et al., 2008). Some bacteria belonging to *Firmicutes* largely represented by *Paenibacillus* genus are known for their extracellular lytic enzyme production and have been shown to attack fungi *in vitro* (Budi et al., 2000).

The positive effect of fungi to bacteria abundance and changes in community composition can be a side effect of fungal metabolisms as bacteria may profit from nutrients released by fungal extracellular enzymes. Environments rich in fungal biomass and fungi-specific compounds such as chitin, manitol or trehalose can be suitable for mycophagous species (Leveau and Preston, 2008) and utilizers of hyphal exudates. The probably most important fungal exudate is oxalate that fungi actively produce to lower pH of the environment necessary for enzyme activity and that is also involved in lignocellulose degradation (Zapanta and Tien, 1997). Oxalotrophy is taxonomically widespread among bacteria (Sahin, 2003). Hendrickson (1991) documented increase of organotrophic nitrogen-fixing bacteria in moderately decayed wood. Their activity can increase the level of nitrogen available for fungi.

The stimulation of fungal growth may be simply due to bacterial production of vitamins and growth factors demanded by fungi (e.g. thiamin, biotin, water-soluble B vitamins, sterols, fatty acids, purines, pyrimidines and inositol). The importance of this phenomenon for the structure of decomposer fungal communities is, however, unclear (Carlile et al., 2001).

Fungal cellulolytic enzymes are inhibited by the reaction products, utilization of these "inhibitors" by bacteria may increase enzyme activities and further positively affect fungal growth.

1.6 Use of denaturing gradient gel electrophoresis in microbial community analysis

In soil microbial ecology, the effects of environmental factors and their gradients or temporal changes or the response to specific experimental treatments on microbial communities, including also microbial interactions, can only be studied using methods addressing the structural differences among whole communities. Fingerprinting methods are designed to allow rapid comparison of samples, finding the similarities or differences in composition or diversity. They are therefore the most appropriate, when multiple samples must be analyzed. None of them is, however, able to provide the identification of individual members of microbial community. Most of the fingerprinting methods are based on differences among DNA or RNA sequences and their properties and several of them use the PCR amplification step followed by electrophoretic separation (Kowalchuk et al., 2004; Oros-Sichler et al., 2007). Exception to this rule is the analysis of phospholipid fatty acids (PLFA) which is based on the molecular fingerprint of lipidic molecules of microbial membranes. Denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) are based on the electrophoretic separation of double stranded DNA molecules based on the differences in their melting behavior in a gradient of either a denaturing agent or temperature. Single-strand conformation polymorphism analysis (SSCP) uses the separation of single stranded DNA molecules differing in their secondary structures. Terminal restriction length polymorphism (T-RFLP) is based on the differences in the localization of restriction sites in DNA sequences. The principles of length heterogeneity PCR (LH-PCR) or automated ribosomal intergenic spacer analysis (ARISA) are in the separation of whole sequences differing by length. Microarray techniques use specific hybridization of environmental nucleic acids to a set (array) of probes which can be used for the detection and quantification of several sequences in the sample.

Denaturing gradient gel electrophoresis was firstly adapted for use in microbial community analysis by Muyzer et al. (1993). Since then, this cultivation-independent approach has been widely used in different environment for analysis both limited and very complex microbial communities. In this method, electrophoretic separation of PCR amplified fragments of DNA depends on sequence composition of individual fragment. The acrylamide gel contains linear gradient of the denaturants urea and formamide. DNA fragments differ in their denaturation properties due to variability in guanine (G) + cytosine (C) content among sequences. There are three hydrogen bonds between guanine and cytosine compared to two between adenine and thymine. The fragment with higher GC content thus needs higher concentration of denaturant to melt. When the DNA strands separate, the fragment migration slows down considerably. The complete strand separation is prevented by high a GC sequence (CG clamp) added to the 5' end of one primer (Sheffield et al.,

1989). Analysis of mixture of different PCR fragments from the environment results in a fingerprint characteristic for the community. The number of observed bands represents an approximation of the numbers of populations and the intensity of the band stands for the relative abundance in the PCR products community.

Molecular fingerprinting methods such as DGGE provide cost-effective analysis and comparisons of large numbers of samples within short time. This method can provide a fast opportunity in determination of subsequent analyses. Other advantage of this method is direct determination of nucleotide sequence of selected bands directly extracted from gel. However, the length of the studied fragment is usually between 200 - 500 bp, the taxonomical identification at species level is thus sometimes not possible. Next to the advantages mentioned, some drawbacks of this method should be also drawn. Care has to be taken while preparing the gel as the differences in gradient between gels would not allow between-gel comparisons and thus comparisons of the large sample sets. The studies on soil microbial community showed that soil can contain several tens of thousands of bacterial species per gram with different level of community evenness (Roesch et al., 2007). The very complex soil communities with equal abundance of populations are represented with rather smear in the DGGE profile (Nakatsu et al., 2000). Moreover, the number of bands does not represent the exact number of population. One population can be represented by more than one bands and from the other side, one band can comprise fragments of more than one population according to the sequence variability in fragment used for analysis.

This method is widely applied in community analysis as a "snapshot" of community structure providing and approximation of the diversity and proportional representation of the community members. This approach can also illustrate and compare the complexity of communities of various environments. A strength of the DGGE method is in its use in determining the effect of different treatments on community structure (Nakatsu, 2007). RNA can be used after reverse transcription instead of DNA as a template for PCR. This modification gain better understanding of the active population within a community (Bastias et al., 2007; Anderson et al., 2008).

The majority of bacterial community analysis has been performed on 16S rDNA. Choosing appropriate primer set can restrict the analysis to more specific bacterial groups such as actinobacteria (Heuer et al., 1997; Gelsomino et al., 2006; O'Callaghan et al., 2008), proteobacteria (Gelsomino and Cacco, 2006; O'Callaghan et al., 2008) or ammonia oxidizing bacteria (Kowalchuk et al., 1998; Bäckman et al., 2003; Innerebner et al., 2006; Sundberg et al., 2007). For analysis of fungal community 18S rDNA is commonly used, although analysis of ITS (internal transcribed spacers) is more discriminative (Anderson and Cairney, 2004). As in bacteria, group-specific primer were also used e.g. for analysis of arbuscular mycorrhizal fungi (Kowalchuk et al., 2002; Santos et al., 2006; Jaatinen et al., 2008). Some studies also successfully attempted to analyze gene diversity in soils (Gremion et al., 2004; Sakurai et al., 2007; Wartiainen et al., 2008).

Chapter 2

Publications

I Valášková, V., Šnajdr, J., Bittner, B., Cajthaml, T., Merhautová, V., Hoffichter, M., and Baldrian, P. (2007) Production of lignocellulose-degrading enzymes and degradation of leaf litter by saprotrophic basidiomycetes isolated from a *Quercus petraea* forest. Soil Biology and Biochemistry 39: 2651-2660.

II Valášková, V., Šnajdr, de Boer, W., Klein Gunnewiek, P., Pospíšek, M., Baldrian, P. Community composition and properties of wood-inhabiting bacteria associated with the white rot fungus *Hypholoma fasciculare*. Submitted for publication.

III Valášková, V., Baldrian, P. The use of DGGE and TGGE for the analysis of soil microbial communities. Submitted for publication.

IV Valášková, V., Baldrian P. (2006) Degradation of cellulose and hemicelluloses by the brown rot fungus *Piptoporus betulinus* - production of extracellular enzymes and characterization of the major cellulases. Microbiology 152:3613-3622.

V Baldrian, P., and **Valášková, V.** (2008) Degradation of cellulose by basidiomycetous fungi. FEMS Microbiology Reviews 32: 501-521.

VI Šnajdr, J., **Valášková, V.**, Merhautová, V., Herinková, J., Cajthaml, T., and Baldrian, P. (2008) Spatial variability of enzyme activities and microbial biomass in the upper layers of *Quercus petraea* forest soil. Soil Biology and Biochemistry 40: 2068-2075.

VII Šnajdr, J., **Valášková, V**., Merhautová, V., Cajthaml, T., and Baldrian, P. (2008) Activity and spatial distribution of lignocellulose–degrading enzymes during forest soil colonization by saprotrophic basidiomycetes. Enzyme and Microbial Technology 43: 186-192.

I

Valášková, V., Šnajdr, J., Bittner, B., Cajthaml, T., Merhautová, V., Hoffichter, M., and Baldrian, P. (2007) Production of lignocellulose-degrading enzymes and degradation of leaf litter by saprotrophic basidiomycetes isolated from a *Quercus petraea* forest. Soil Biology & Biochemistry 39: 2651-2660.

Litter is very important source of forest soil organic matter; the mean year deposit in temperate broadleaved forest is calculated to be 3.5 t ha^{-1}. In terrestrial environments, basidiomycetes are probably the ecologically most significant group of fungi involved in the breakdown and chemical conversion of litter components. Saprotrophic lignocellulose-degrading basidiomycetes are efficient litter-decomposing organisms producing a wide variety of oxidoreductases and hydrolytic enzymes. They are capable of degradation of all main lignocellulose components – cellulose, hemicellulose and lignin, and are, furthermore, involved in the formation, stabilization and degradation of humic substances (humus turnover) which can form a distinct layer in temperate forest soils.

The aim of this work was to obtain more detailed information about the production of extracellular lignocellulose-degrading enzymes by saprotrophic fungi. In this study we isolated and identified three saprotrophic basidiomycetes from *Quercus petraea* forest soil: *Hypholoma fasciculare*, *Gymnopus* sp. and *Rhodocollybia butyracea*. Attention was paid to overall degradation capability and to main hydrolytic and oxidative enzymes involved in lignocellulose degradation: endo-1,4-β-glucanase, endo-1,4-β-xylanase, cellobiose hydrolase, 1,4-β-glucosidase, 1,4-β-xylosidase, laccase, manganese peroxidase, Mn-independent peroxidase and lignin peroxidase. We also aimed at the description of composition changes of litter during after fungal degradation, with the main focus on lignin removal and modification and changes in litter quality.

II

Valášková, V., Šnajdr, de Boer, W., Klein Gunnewiek, P., Pospíšek, M., Baldrian, P. Community composition and properties of wood-inhabiting bacteria associated with the white rot fungus *Hypholoma fasciculare*. Submitted for publication.

Fungi, especially saprotrophic basidiomycetes, are the principal inhabitants of wood and litter and the main decomposers of their components in terrestrial ecosystems. Yet, bacteria are among the first inhabitants of freshly fallen wood and forest litter, although their ability to degrade wood is, contrary to wood-rotting and litter-decomposing fungi, generally limited to utilization of cellulose and hemicellulose. During lignocellulose degradation, fungi actively change this environment due to production of oxidative and hydrolytic enzymes and also by exuding different compounds, e.g. oxalic acid. Fungi establish strong selective pressure on wood- and litter-inhabiting bacteria, while on the other hand, new niche – microhabitats suitable for specialized bacteria are formed. Microbial interactions thus play an important role in microbial community establishment. Wood and litter have similar basic chemical composition but wood is more homogeneous and can be more easily defined as a model substrate. The composition and diversity of the bacterial community in wood is largely unexplored as well as the influence of fungi on wood-associated bacteria.

The aim of this study was to investigate the composition and properties of the bacterial community in wood undergoing degradation by a saprotrophic basidiomycete *Hypholoma fasciculare* – potent secondary colonizer of wood and litter, markedly changing the properties of colonized wood and thus capable of contributing to the formation of a specific niche characterized by low pH, partially removed lignin and the availability of utilizable sugars. We used cultivation-independent analyses based on DNA isolation to study whole bacterial community from naturally colonized wood, as only a minor part of bacterial strains are culturable. In addition to molecular analyses, selected nutritional and physiological adaptations of members of the bacterial community were addressed using cultivation-dependent studies of isolated strains. Namely we were interested in the ability to utilize degradation products of lignocellulose, fungal structural compounds and exudates, tolerance to low pH and fungal-bacterial antagonistic interactions. Our main queries were: Who are the most important members of the bacterial community in decaying wood? How dependent are the bacteria in wood on the presence of a fungus? Can they benefit from compounds specifically produced by the fungus? What kind of relationship do bacteria have with *Hypholoma fasciculare* during advanced stages of wood decay?

III

Valášková, V., Baldrian, P. The use of DGGE and TGGE for the analysis of soil microbial communities. Submitted for publication.

In soil microbial ecology, the effects of environmental factors and their gradients or temporal changes or the response to specific experimental treatments on microbial communities can only be studied by the methods addressing the structural differences among whole communities. Fingerprinting methods are the most appropriate for this task when multiple samples must be analyzed. Among the methods currently used to compare microbial communities based on nucleic acid sequences, the ones based on differences in melting properties of double-stranded molecules, the DGGE or TGGE are the most widely used. Their main advantage is the fact that they offer the possibility to further analyze whole sequences contained in fingerprints by molecular methods. In addition to the analysis of microbial communities based on DNA extracted from soils, DGGE/TGGE can also be used for the assessment of the active part of the community based on the analysis of RNA-derived sequences or for the analysis of sequences of functional genes encoding for proteins involved in important soil processes.

The aim of this review is to identify the posibilities of DGGE/TGGE for analysis of complex soil microbial communities, to compare it with other fingerprinting methods and to offfer suitable standardized protocol for use. We describe the main advantages and also disadvantages of this method and general recommendation for its application considering also sampling strategy. We also describe optimized methodology for analysis of community of soil basidiomycetes using nested PCR approach that has not been described before.

IV

Valášková, V., Baldrian P. (2006) Degradation of cellulose and hemicelluloses by the brown rot fungus *Piptoporus betulinus* - production of extracellular enzymes and characterization of the major cellulases. Microbiology 152: 3613-3622.

Cellulolytic enzymes of wood associated basidiomycetes attracted by far less attention than the ligninolytic enzymes. Only in few species the most important enzymes were characterized. There is also considerable lack of knowledge of properties of fungus colonized wood and the activity of their extracellular cellulolytic enzymes in this natural substrate. *Piptoporus betulinus* is a hardwood-specific parasite of birch (*Betula* species) trees in northern temperate forests and causes a very fast wood decay. It is also one of the very common brown rot species in central Europe (Baldrian and Gabriel, 2002) and has been reported as one of the most active cellulose decomposers (Bell and Burnett, 1966).

The aim of this study was to characterize cellulolytic degradation system of saprotrophic basidiomycete *Piptoporus betulinus*, detect these activities in the natural samples of wood colonized by the fungus and in the fruiting bodies themselves. We aimed at description of changes caused by physiology of this fungus in degraded substrates and the differences in extracellular enzyme distribution between colonized wood and fruiting bodies.

V

Baldrian, P., and Valášková, V. (2008) Degradation of cellulose by basidiomycetous fungi. FEMS Microbiology Reviews 32: 501-521.

Cellulose is the main polymeric component of the plant cell wall, the most abundant polysaccharide on Earth, and also an important renewable resource. Fungi and bacterial are the main organisms involved in its degradation, but basidiomycetous fungi with evolved hyphal growth are the driving force in cellulose degradation and carbon turnover in forest ecosystem. Although cellulose degradation by basidiomycetes has been studied extensively since the middle of the last century, the view of cellulose degradation changed in the last few years. The main reasons were the formulation of the contribution of oxidative systems to cellulose degradation, the detection of new types of cellulolytic enzymes and the first complete genome sequence of a wood-rotting basidiomycete, *Phanerochaete chrysosporium* enhancing greatly the power of proteomic and computational methods for detection of individual components of its model cellulolytic system. It should be made clear that the degradation of cellulose is a complex process where several components may be acting at the same time. Also basidiomycetes from habitats other than wood, for example, the litter-decomposers and mycorrhizal species, attracted more attention in the past years.

Previous reviews on cellulose degradation by fungi were usually limited to the description of the properties of enzymatic systems or focused only on one of the several redox-based systems active upon all plant cell wall components. The aim of this review is to present both the information about the composition and biochemical properties of enzymatic systems utilized by basidiomycetous fungi for cellulose degradation and the redox, radical-generating systems, and to point out the main differences. My contribution to the publication was the summarization and analysis of the data on the properties of individual components of enzymatic systems of cellulose degradation.

VI

Šnajdr, J., Valášková, V., Merhautová, V., Herinková, J., Cajthaml, T., and Baldrian, P. (2008) Spatial variability of enzyme activities and microbial biomass in the upper layers of *Quercus petraea* forest soil. Soil Biology and Biochemistry 40: 2068-2075.

Plant litter and the input from plant roots represents the main sources of organic matter in forest soils, which is further transformed by the action of extracellular lignocellulose-degrading enzymes produced mainly by saprotrophic fungi. The decomposition of litter is one of the main processes responsible for the formation of temperate forest soils with developed organic (O) horizon – cambisols. Several studies have focused on the measurement of enzyme activities and microbial biomass in forest litter and soils. However, these have largely neglected the small-scale spatial variability (i.e. <10 m) of enzyme distribution that might be related to localized differences in microbial biomass and community composition. Furthermore, although some studies described the differences in the activity of extracellular enzymes with soil depth, the usual sampling by soil horizons might underestimate any small-scale differences.

The aims of this study were to characterize the spatial variability and distribution of cellulolytic and ligninolytic enzyme activities in the L and O horizons of oak (*Quercus petraea*) forest soil at a 1-cm scale. Additionally to commonly estimated cellulolytic, hemicellulolytic and ligninolytic enzymes, we also tested activity of other enzymes involved in soil organic matter turnover: 1,4-β-*N*-acetylglucosaminidase (chitinase) and the phosphorus-mineralizing acid phosphatase. Last, but not least, we quantified soil fungal and bacterial biomass using PLFA and ergosterol measurement and investigated the relationship between microorganism abundance and enzyme activities as well as the spatial distribution of individual enzymes. My contribution to this publication was in the experiment planning, obtaining of the data of the microbial biomass and community composition and analysis of the results.

VII

Šnajdr, J., Valášková, V., Merhautová, V., Cajthaml, T., and Baldrian, P. (2008) Activity and spatial distribution of lignocellulose-degrading enzymes during forest soil colonization by saprotrophic basidiomycetes. Enzyme and Microbial Technology 43: 186-192.

Litterfall in temperate broadleaved forests is composed of approximately 78% leaves, 20% flowers, seeds and related organs, and 2% twigs and bark. A defined litter horizon is generally present on the soil surface and can be clearly distinguished from the underlying mineral layers. It provides a suitable habitat for litter decomposing fungi and it is often only 1-10 cm thick. Litter-decomposing fungi are contrary to wood-rotting fungi known to colonize not only litter layer, but also lower soil horizons: humic and even deeper soil horizons, to which they translocate litter derived carbon via their mycelial network. Its degradation is one of the crucial steps in global carbon turnover.

Different microorganisms contribute to organic matter degradation in the upper layers of forest soils under natural conditions. However, the variability in environmental system as temperature, moisture and pH, brings too much complexity to the system and makes it difficult to assign the relative roles in organic matter transformation to specific groups of soil microbes. The aim of this work was to estimate how the presence of litter-decomposing basidiomycetes affects enzyme activities in the forest soil environment. For this purpose, we introduced *Hypholoma fasciculare* and *Rhodocollybia butyracea*, previously demonstrated to efficiently degrade oak litter and to produce the lignocellulose-degrading enzymes, into nonsterile soil profile microcosms reconstructed using the material from their site of isolation – *Quercus petraea* forest. We estimated the effect of the two litter-decomposing fungi on the production of lignocellulolytic enzymes during *in vitro* incubation of microcosms in the upper layers of the forest floor. We also analyzed how saprotrophic basidiomycetes affect microbial community in soil during its colonization and litter degradation. I contributed to this study by the help with the project design, collection of the data on the qualitative and quantitative parameters of soil microbial populations and by contribution to data analysis.

Chapter 3

Discussion

Litter-decomposing fungi are the most efficient degraders of lignocellulose in the upper part of forest soils. Although their ecological importance is apparent, their physiology, ecology and degradation potential was studied to far less extend compared to the ecologically similar group of wood-rotting fungi. The ligninolytic, hemicellulolytic and cellulolytic systems of wood-rooting fungi are well described, even though the view on degradation of cellulose needs to be changed as we demonstrated in paper V. Previous reviews on cellulose degradation by fungi were usually limited to the description of the properties of the enzymatic systems or focused only on one of the several-redox based systems active upon all plant cell wall components. The degradation of cellulose is, however, a complex process, where several components may be acting at the same time. In this review we provide information about the composition and biochemical properties of enzymatic systems produced by basidiomycetes for cellulose degradation. We pointed also to the broader substrate specificity of cellulases that can be assumed from recently published studies as we also documented in paper IV. We tried to identify the different redox and radical generating systems and their role in lignocellulose degradation by white-rot and especially brown-rot fungi. We pointed out the main differences between enzymatic and redox-based cellulose degradation. Last, but not least, we attempted to describe cellulolytic systems of basidiomycetes from habitats different from wood such as litter-decomposers and mycorrhizal species.

In paper I we attempted to understand the ecophysiology of the litter-degrading basidiomycetes. We focused on basidiomycetes colonizing oak litter and isolated a set of litter-degrading basidiomycetes. We selected three isolates: *Hypholoma fasciculare*, *Rhodocollybia butyracea*, and *Gymnopus* sp. Selection was based on preliminary growth and positive oxidative enzyme activity tests. All three selected isolates were able to colonize *Quercus petraea* litter under sterile conditions. *H. fasciculare* and *R. butyracea* were also documented to colonize nonsterile litter and organic horizons of soil if provided with a suitable substrate for growth (paper VII). The mass loss of litter reported in paper I, up to 38% of litter dry mass in *Gymnopus* sp. cultures within 12 weeks, is comparable to previous results. For instance, *Collybia peronata* caused 24% mass loss of oak litter within 10 weeks (Dix and Simpson, 1984), *Mycena inclinata* decomposed 33%, *Marasmius quercophilus* 38%

and *Pholiota lenta* 48% of oak litter dry mass within 12 weeks (Steffen et al., 2007). *Mycena* and *Collybia* exhibited 19-44% mass loss of birch litter within three months and differed in their preference for lignin or polysaccharide removal (Osono and Takeda, 2006).

There are unfortunately only a few studies of enzyme production by litter-decomposing basidiomycetes in leaf litter so far, although the production of cellulases, Mn-peroxidases and laccases is often attributed to them (Colpaert and van Laere, 1996; Criquet et al., 2002; Ghosh et al., 2003). Recently, litter-decomposing fungi *Lepista nuda, Marasmius quercophilus* and *Mycena inclinata* were reported to produce laccase, Mn-peroxidase and a complete set of cellulose-degrading enzymes on *Quercus* sp. litter (Steffen et al., 2007). Our isolates produced wide spectra of cellulolytic and hemicellulolytic, as well as ligninolytic enzymes. Previous studies demonstrated the presence of the ligninolytic enzymes laccase and MnP in several species of litter-decomposing fungi (Steffen et al., 2000; Baldrian, 2006). Among 27 species of litter-decomposing fungi tested, almost all showed oxidase activity and approximately one-third were able to oxidize Mn^{2+} (Steffen et al., 2000). MnP is thought to play a key role in the breakdown of lignin by litter-decomposing fungi since only those fungi producing MnP were able to substantially mineralize synthetic lignin (Steffen et al., 2000), and even the purified MnP can cleave and mineralize lignin (Hofrichter et al., 1999). None of the litter-decomposing fungi was reported to produce lignin peroxidase so far as production of LiP was observed only by few genera of wood-rotting basidiomycetes such as *Phaneroachaete*, *Trametes*, *Phlebia* and *Bjerkandera* (Hatakka, 2001), that do not colonize litter and soil. Laccase has been reported to act in lignin degradation, resulting in both fragmentation and re-polymerization (Leonowicz et al., 2001) and is present in litter during fungal degradation (Ghosh et al., 2003). Laccase was also found in decaying oak litter while MnP was detected only occasionally in Mediterranean evergreen forest ecosystem (Criquet et al., 2000). All our isolates produced laccase and also Mn-peroxidase, and none of them produced lignin peroxidase or other Mn-independent peroxidases. Production of Mn-peroxidase is detected consistently in temperate forest litter (Baldrian, 2008b; Baldrian, 2008a).

However, it is not simple to link the enzyme activities and lignin removal. In our study (paper I), the highest lignin removal was observed for *Gymnopus* sp., which also showed the highest MnP activity, while the removal of lignin by *H. fasciculare* was low, as was also its MnP activity. The composition of lignocellulolytic enzyme systems did not differ among isolates, but there was a clear distinction in the enzyme production pattern. *R. butyracea* and *H. fasciculare* caused a marked mass loss during the initial phase of litter degradation (approximately 4 weeks) accompanied by a temporary production of ligninolytic enzymes and high production of hydrolytic enzymes. In contrast, *Gymnopus* sp. caused a continuous mass loss over the whole period similarly to *Lepista nuda, Marasmius quercophilus* and *Mycena inclinata* (Steffen et al., 2007), with a later onset of production of ligninolytic enzymes and a longer lasting production of both lignin- and cellulose-degrading enzymes. Although the production of endo-cleaving polysaccharide hydrolases in this fungus was relatively low, it produced the highest level of cellobiose hydrolase. The major endo-cleaving enzyme was endo-1,4-β-xylanase, as was also observed for *Lepista nuda, Marasmius quercophilus* and *Mycena inclinata* (Steffen et al., 2007), whereas *H. fasciculare*

produced mainly endo-1,4-β-glucanase and *R. butyrace* produced both enzymes in equal amount. Major exo-cleaving enzyme was 1,4-β-glucosidase for all above mentioned fungi.

The reactions controlled by lignocellulolytic enzymes lead to release of carbon and energy and are characterized also by chemical changes of the soil organic matter. This is important for further degradation processes and probably also for the structuring of the microbial community (Dilly et al., 2001; Hattenschwiler et al., 2005). The C/N ratio of fresh *Q. petraea* litter was 24, similar to the ratio of 30 - 40 of *Q. robur* litter and 27 of also *Q. petraea*, but far lower than the ratio in fresh oak wood (more than 150) and its decrease is in agreement with the observations about natural decay of other oak litter species (Vane et al., 2003; Madritch and Hunter, 2005; Quideau et al., 2005; Steffen et al., 2007). Chemical composition of decayed litter by *Gymnopus* sp. resembled decay caused by the white-rot basidiomycetes (Vane, 2003), but the lignin decay by *R. butyracea* and *H. fasciculare* seems to be more different from that by typical wood-inhabiting white-rot fungi. Litter colonized by any of the three fungi contained more lignin and less carbohydrates compared to the fresh litter. Production of humic and fulvic acids with low molecular masses by *Gymnopus* sp. probably indicates more frequent cleavage of the lignin polymer by this species.

Compared to sterile litter degraded by basidiomycete cultures, the degradation of litter *in situ* using litterbags resulted in a preferential decomposition of carbohydrates which was demonstrated by the increase of relative lignin content and the increase of the L/C ratio in litterbags incubated at the site of study (paper I). This is in agreement with previous studies. In *Q. ilex* litter, lignin content also increased during decomposition despite the presence of laccase and peroxidase activities (Fioretto et al., 2005; Fioretto et al., 2007). The same was found for beech litter by Osono and Takeda (2001), who also isolated fungi participating in different stages of litter degradation but did not isolate any basidiomycete species. It seems that the participation of non-basidiomycetous fungi in the natural decomposition of litter leads to a decreased lignin removal and probably also to differences in the chemical composition of degraded litter.

Forest soil is, however, very diverse and variable environment under its natural condition. To at least partly control variability of physicochemical background, we reconstructed soil environment in a microcosm experiment, where the soil profile and microbial community were better defined (paper VII). This approach facilitated the explanation of the effects of litter-degrading fungus on enzyme activities responsible for lignocellulose degradation.

Activity of several extracellular enzymes was significantly higher in the nonsterile soil colonized by *H. fasciculare* and *R. butyracea* that in control nonsterile soil and despite some small differences the changes were essentially the same. The most dramatic changes were found in the case of ligninolytic enzymes. This is not surprising in case of laccase: it was repeatedly documented that its activity increases during the interaction of different basidiomycete species including litter-decomposers from the genera *Agrocybe, Hypholoma* and *Mycena* with other fungi or bacteria (White and Boddy, 1992; Baldrian, 2004). The relative ratio of laccase to MnP activity was shifted towards MnP dominance which is atypical for the forest soil; on the site of collection laccase was always the dominant enzyme (Baldrian et al., 2006). Our system can be to some extend considered as artificial as the

sieving of the material during preparation of the microcosms could have caused damage of mycelia of cord-forming LDF and microcosms assembly prevented growth of mycorrhizal fungi. The increase in ligninolytic enzyme activity may thus be mainly ascribed to *H. fasciculare* and *R. butyracea*. Luis et al. (2004, 2005) observed high diversity of laccase genes that can be ascribed to different functional groups of fungi in brown (moder) and cambisol forest soil and thus the increase of activity under natural condition cannot be addresed to one species, as many microorganisms contribute to the process.

The increase of cellulose-degrading enzymes, *N*-acetylglucosaminidase (chitinase) and acidic phosphatase during microcosms colonization were also probably due to production by the LDF, as significantly increased activity of oxidases and peroxidases occurs in the soil under fairy rings of saprotrophic basidiomycetes compared to soil devoid of visible mycelia (Gramss, 1997; Gramss et al., 2005).

Although both *H. fasciculare* and *R. butyracea* colonized soil rapidly and their heavy mycelial growth was apparent in the soil microcosms, no significant effects on total fungal or bacterial biomass were found. This is in agreement with the results of Tornberg et al. (2003) who introduced several saprotrophic basidiomycetes including *H. fasciculare* into agricultural soil without quantitative response of microbial biomass, however, they detected changes in the composition of bacterial PLFA marker molecules. This was not found in our study where fungal and control treatments were similar. These results also correspond with additional DGGE analysis results of bacterial community composition, where significant differences were found between soil layers, but similar very complex community pattern was observed when comparing fungal and control treatments (data not shown).

On the other hand, both *H. fasciculare* and *R. butyracea* decreased fungal CFU in the litter layer but increased both fungal and heterotrophic bacterial CFU in the humus layer (paper VII). Since the CFU increase started already when the mycelia of colonizing fungi just only reached the humic layer, the reason may be in the transfer of nutrients liberated by the fungi from plant cell wall biopolymers in the litter layer.

In paper VI, we documented a vertical gradient of lignocellulose-degrading enzymes and microbial biomass in forest soil *in situ*. It was already reported from several forest soils including Mediterranean *Quercus* spp. soils, central European *Fagus sylvatica* or *Picea excelsa* soils as well as soils from boreal coniferous forests (Trasar-Cepeda et al., 2000; Prietzel, 2001; Andersson et al., 2004; Wittmann et al., 2004), but we demonstrated that the gradient is present even within individual soil horizons. Generally, the enzyme activities degreased with depth and the slope of activity decrease was enzyme-specific. The PLFA analysis showed that these differences are also reflected in the changes in microbial community composition. The deeper Ah horizon exhibits a less steep gradient of C_{org} content and smaller differences in enzyme activities and microbial biomass content and community composition.

It is very difficult to link enzyme activities in soil or litter to their producers, although there are some hints that saprotrophic basidiomycetes may be responsible for the production of ligninolytic enzymes and microbial biomass may be correlated with enzyme activities (Miller et al., 1998; Gramss et al., 1999; Moller et al., 1999; Sinsabaugh et al., 2002;

Gramss et al., 2005) (paper VI). For example Mn-peroxidase production is a clean sign of the presence of basidiomycete fungi that are the only known producers (Hofrichter, 2002). On the other hand, the weak correlation between laccase and Mn-peroxidase in our study (paper VI) can be ascribed to laccase production by a broader spectrum of laccase producers among basidiomycetes or by the production of soil ascomycetes (Baldrian, 2006). The correlations between enzyme activity and microbial biomass content were not present consistently. Ergosterol was significantly correlated with the activity of cellulolytic enzymes in August when total fungal biomass in soil was high. This is consistent with reports indicating that cellulolytic enzymes in beech litter microcosms are mainly of fungal origin (Moller et al., 1999). However, the correlation between ergosterol and cellulolytic enzymes was not significant in May when fungal biomass in soil was lower.

There can be several reasons why enzyme activities do not reflect the quantity of microbial biomass that apparently produce them. There can be a higher rate of biomass turnover in soils than that of enzymes or a significant fraction of enzyme activity can persist in soil for long time after the death of the producing cell. In the past, several extracellular enzymes were demonstrated to survive even a harsh drying (Bonmati et al., 1991). It is also possible that for much of the time a large part of soil microbial biomass is metabolically inactive (e.g. the hyphal cords used for nutrients translocation) or that some enzymes are produced only by a specific group of microorganisms. In this sense, enzymes produced by all groups of microorganisms, e.g. acid phosphatase (Criquet et al., 2004) can be expected to correlate with soil microbial biomass measures more closely. In our study, ergosterol and acid phosphatase correlation was observed only within litter horizon, the correlation was, however, not present consistently in both sampling times.

In paper III we resume the analysis of microbial communities using DGGE/TGGE fingerprinting method, its application, advantages and disadvantages. Fingerprinting methods represents widely used approach whenever multiple samples must be analyzed. The main advantage of DGGE or TGGE is the fact that it offers the possibility to further analyze fingerprints by molecular methods and DGGE is today probably the most commonly used method for typing and comparing microbial communities. In the future, the value of this method can be further increased when suitable primers are developed for further bacterial phyla occurring in soils or specific groups of fungi interesting from the viewpoint of soil ecology or agriculture. Next to protocol commonly used for analysis of fungal and bacterial community, we also describe optimized protocol for analysis of soil basidiomycetes that has not been described before. Nested PCR amplification approach allows direct comparisons of basidiomycetous community with results form already described method for analysis of total fungal community (Bastias et al., 2007).

Using DGGE approach combined with other molecular methods we also studied effect of saprotrophic basidiomycetes with *H. fasciculare* as s model fungus, on bacterial community of decayed wood (paper II). *H. fasciculare* is a strong competitor against fungi that also exhibits bactericidal activities (de Jong and Field, 1997; Koukol et al., 2006). Interestingly, various effects on bacterial and fungal communities have been reported: the presence of this fungus reduced the colonization of sterile wood by bacteria (Folman et al., 2008), but it increased bacterial CFU in the colonized soil (Gramss et al., 1999) and soil adjacent to

hyphae (Folman et al., 2008). In our study we found high numbers of culturable bacteria in wood colonized and degraded by this species. Comparable bacterial counts were reported in a leaf litter environment with similar chemical composition (Krivtsov et al., 2005), while the counts of culturable bacteria in rhizosphere and acidic soils were found to be at least 100 times lower (Matthies et al., 1997; de Boer et al., 2005; Timonen and Hurek, 2006).

Wood associated fungi change the physico-chemical properties of environment. *H. fasciculare* actively lowers pH by production of oxalate to 3.6-4.3 and produces reactive radicals and extracellular lignocellulose degrading enzymes (paper II). Similarly, low pH was also observed in wood colonized by other wood-associated fungus *Piptoporus betulinus*. The significant decrease of pH (2.8-3.6) corresponded with high activity of endocleaving enzymes observed in wood compared to *Piptoporus betulinus* fruiting bodies (pH 3.5-5.6) (paper IV). The considerably changed environment may affect the organisms inhabiting the same substrate. It seems that *H. fasciculare* decreases bacterial numbers during the colonization of wood (but not soil), where bacteria may be harmful to fungus as competitors for easily degradable compounds (Schmidt, 2006). It was thus expected that the diversity of bacteria able to cope with these changed conditions is low (paper II). However, this was not at all the case. The observed high bacterial diversity and relatively low similarity in community composition between samples, as studied by DGGE and confirmed by cloning and sequencing, point at a limited ability of the fungus to select for specific bacterial strains. Environmental conditions are thus probably the driving force in community development. As the bacterial community differed also among wood samples, the function of community may be more important than taxonomical diversity (Caldwell, 2005). Similar principle can be as well valid in wood environments.

Sequencing of 16S rDNA fragments of both isolates and directly extracted wood DNA revealed a dominance of *Proteobacteria* and *Acidobacteria* in decaying wood. *Alphaproteobacteria* dominated the clone library directly obtained from extracted wood DNA, whereas *Betaproteobacteria* and *Gammaproteobacteria* dominated among the isolates. Interestingly, members of the families *Burkholderiaceae* (*Betaproteobacteria*) and *Xanthomonadacaea* (*Gammaproteobacteria*) were also dominant among the forest soil bacteria that colonized sterile beech woodblocks (Folman et al., 2008). However, the numbers of bacteria belonging to these families were strongly reduced upon colonization of the woodblocks by *H. fasciculare*. The presence of *Acidobacteria* was already indicated in beech woodblocks after colonization by *H. fasciculare* (Folman et al., 2008). The dominance of *Acidobacteria* in the natural wood samples shows that they are well adapted to the conditions in decaying wood. *Acidobacteria* are known to thrive well in acid environments like peat (Dedysh et al., 2006). In addition to *Proteobacteria* and *Acidobacteria* several taxonomic groups were detected. These groups had a relatively low abundance and were in most cases not detected among the isolates. Direct sequencing yielded higher diversity of bacteria, but the general structure of the community remained unchanged.

Only few members of the phylum *Actinobacteria* and strains belonging to the *Cytophaga-Flavobacterium-Bacteroidetes*, previously reported to be involved in the degradation of lignocellulose in plant litter in terrestrial and aquatic environments (Lydell et al., 2004; Kirby, 2006; Das et al., 2007), were present in clone library derived from decayed wood.

This supports the suggestion that actinomycetes are not strongly involved in wood degradation (de Boer et al., 2005; de Boer and van der Wal, 2008). On the other hand, there were several sequences in our clone library belonging to bacteria potentially capable of cellulose and hemicellulose degradation, namely *Burkholderia, Planctomycetes, Sphingobacteria*, and *Xanthomonadaceae* (Lydell et al., 2004; Belova et al., 2006; Das et al., 2007). The bacteria inhabiting wood in the late phase of degradation can thus partly contribute to wood degradation. Interestingly, no sequences of bacteria from the genus *Pseudomonas*, frequently encountered in the plant litter environment (Aneja et al., 2006), were detected. We also detected members of genus *Paenibacillus* spp., that are known for production of extracellular lytic enzyme and have been shown to attack fungi *in vitro* (Budi et al., 2000).

Bacterial community composition in decayed wood closely resembled the community described for an acidic *Sphagnum* peat bog (Dedysh et al., 2006). The abundance of proteobacteria also resembles the community in spruce litter, although no acidobacteria were detected (Aneja et al., 2006) (Table 3.1). Since this is an environment with a very low pH, it seems that pH is one of the most important factors defining bacterial community composition. All but one of the bacterial isolates obtained from wood (pH 3.6-4.3) in the present study were tolerant to pH 4.0, indicating that this community is composed of low pH-tolerant and acidophilic bacteria.

Substrate	peat bog	wood	beech litter	spruce litter
Acidobacteria	29	23	–	4
α-proteobacteria	24	33	–	34
β-proteobacteria	–	14	–	23
γ-proteobacteria	–	10	82	20
δ-proteobacteria	5	1	–	2
unclasified proteobacteria	–	1	–	–
Verrumicriobia	15	3	–	–
Actinobacteria	10	2	–	–
Firmicutes	–	7	–	–
Planctomycetes	4	3	–	–
Chloroflexi	4	–	–	–
Bacteroidetes	2	2	–	–
Chlorobi	2	–	–	–
TM7	–	2	–	–
Gemnatimonadetes	–	–	–	2
Unclasified	6	–	18	15

Table 3.1: Comparison of bacterial communities in peat bog (Dedysh et al., 2006), wood (paper II) and beech and spruce litter (Aneja et al., 2006). Analysis based on 16S rDNA cloning and sequencing. Occurences of bacterial groups are listed in % of total abundance among clones.

Possible interactions between fungi and bacteria have been extensively discussed in several previous papers (Wheatley, 2002; Tornberg et al., 2003; de Boer et al., 2005; Wick et al., 2007; de Boer et al., 2008), although some of the results remain hypothetical and require further extensive research. Our results do not support a direct negative effect of bacteria on fungi; no growth antagonism towards fungi was detected among bacterial

isolates, although some isolated bacteria, namely *Rhizobiaceae, Xanthomonadaceae* and also *Burkholderiaceae*, are known to produce chemicals that cause fungistatis or to affect fungal growth and enzyme activities (Zou et al., 2007; Compant et al., 2008). Antibiotic production is a known strategy of bacteria to compete successfully with fungi for the same resources e.g. root exudates in the rhizosphere (de Boer et al., 2008). However, this appears not to be the case in the decaying wood samples. The apparent lack of antifungal activities of wood-inhabiting bacteria may be due to the fact that they rely on substrates released due to fungal activities. Caution with interpretation of the *in vitro* antagonism test should, however, been taken into consideration as conditions are different than those in wood.

Proteobacteria and acidobacteria did not contribute to the increase of nitrogen in the wood as all isolates were negatively tested for *nifH*-genes since the primers used to amplify these genes in at least betaproteobacteria (Rosch and Bothe, 2005). Hence, a mutualistic interaction between these bacteria and *H. fasciculare* based on carbon usage by the bacteria and nitrogen delivery to the fungus is not likely.

The alpha- and betaproteobacteria tested appeared to be metabolically versatile and could use several low molecular mass compounds that are released by extracellular fungal cellulases and hemicellulases. Small aromatic compounds, which could be released upon degradation of lignin, were less commonly used. Gammaproteobacteria apparently needed a growth factor that was present in yeast extract as they did not grow in mineral medium with a carbon substrate. Trehalose - the fungal energy storage compound, was not utilized by any of bacterial isolates derived from fungus-colonized wood. The same was true for oxalic acid - the major organic acid produced by saprotrophic basidiomycetes. On the other hand, *N*-acetylglucosamine, the component of the chitinous fungal cell wall, was used as an energy source by several bacterial strains.

Chapter 4

Conclusions

My study is focused on soil saprotrophic basidiomycetes, their role in leaf litter decomposition and interactions with other members of microbial community since these fungi play a particularly important role in biodegradation of soil organic matter and therefore also in the cycling of carbon and mineral nutrients. Although the current data are not sufficient to give a complete picture of the ecological role of litter degrading basidiomycetes and the relationship with other soil microorganisms some general conclusions can be drawn:

- The degradation of cellulose by basidiomycetes is more complex than have been expected. Except for hydrolytic enzymes, other systems such as radical based systems and lower specificity of some hydrolytic enzymes than was proposed, also play very important roles in degradation of cellulose and other cell wall polymers. This is particularly clear in brown-rot fungi that produce limited spectra of hydrolytic enzymes. More systems are unambiguously involved in degradation process at the same time.

- Litter-inhabiting species of saprotrophic basidiomycetes are able to efficiently degrade *Quercus petraea* leaf litter in pure culture and also as a part of microbial community in nonsterile soil.

- Litter-decomposing fungi can cause substantial litter transformation despite considerable differences in the production of lignocellulose-degrading enzymes.

- Lignocellulose degrading system of litter-degrading basidiomycetes *Hypholoma fasciculare*, *Rhodocolybia butyrace* and *Gymnopus* sp. consist of laccase, Mn-peroxidase and wide spectra of hydrolytic enzymes degrading cellulose and hemicellulose.

- Although litter differs from wood in its composition, chemical changes during its transformation resemble changes caused by white-rot fungi during wood decay.

- Saprotrophic basidiomycetes from oak forest soil can be responsible for spatial variability in extracellular enzyme activities in the upper soil horizons.

- Spatial differences in enzyme activities are accompanied by differences in the microbial community composition, where the relative amount of fungal biomass decreases with soil depth. The vertical gradients in soil occur at a small scale even within separate soil layer. We observed significant differences with respect to enzyme activities, microbial biomass content and community composition.

- Saprotrophic basidiomycetes considerably modify the environment of the wood during their growth. The most important changes are decrease of pH (2.8 - 4.3) and production of lignocellulose degrading enzymes.

- DGGE analysis is suitable for analysis of complex soil microbial communities on the level of DNA or RNA and can be used to describe the effects of interactions between microorganisms. Community of soil basidiomycetes can be successfully addressed by this method using nested PCR approach. Our protocol also allows direct comparison of basidiomycetous community and general fungal community.

- During soil colonization *H. fasciculare* and *R. butyracea* do not affect total soil bacterial or fungal biomass in a quantitative way.

- Bacterial community in *H. fasciculare*-colonized wood is rich and diverse, dominated by *Proteobacteria* and *Acidobacteria*.

- Selection of bacterial community is more likely due to selective pressure of environment rather than specific effects of *H. fasciculare*.

- Bacteria inhabiting decayed wood seem to be acid tolerant and rely on growth substrates released by fungal decay activities, although some may also utilize cellulose.

- No interference competition (antibiosis) or mutualism (nitrogen-fixation) became apparent in interaction between *H. fasciculare* and wood-inhabiting bacteria.

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