The white-rot fungi *Phlebia radiata* and *Dichomitus squalens* in wood-based cultures: expression of laccases, lignin peroxidases, and oxalate decarboxylase

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ACADEMIC DISSERTATION

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Front cover White-rotted wood (photo: Miia Mäkelä)

Contents

List of original publications

This thesis is based on the following publications, which are referred in the text by Roman numerals I-IV. In addition, unpublished data is presented.

I Hildén, K.S., Mäkelä, M.R., Hakala, T.K., Hatakka, A. and Lundell, T. 2006. Expression on wood, molecular cloning and characterization of three lignin peroxidase (LiP) encoding genes of the white rot fungus *Phlebia radiata*. Current Genetics 49, 97-105.

II Mäkelä, M.R., Hildén, K.S., Hakala, T.K., Hatakka, A. and Lundell, T.K. 2006. Expression and molecular properties of a new laccase of the white rot fungus *Phlebia radiata* grown on wood. Current Genetics 50, 323-333.

III Mäkelä, M., Galkin, S., Hatakka, A. and Lundell, T. 2002. Production of organic acids and oxalate decarboxylase in lignin-degrading white rot fungi. Enzyme and Microbial Technology 30, 542-549.

IV Mäkelä, M.R., Hildén, K., Hatakka, A. and Lundell, T.K. 2009. Oxalate decarboxylase of the white-rot fungus *Dichomitus squalens* demonstrates a novel enzyme primary structure and non-induced expression on wood and in liquid cultures. Microbiology 155, 2726-2738.

Author's contribution

I The author participated in planning and conducting the laboratory work, in particular in RT-PCR, gene expression and cloning. She did the fungal submerged and semi-solid cultivations and measured enzyme activities. She collaborated in the analysis and the interpretation of the results, and the writing of the article.

II The author planned the experiments and did the laboratory work except for the solid wood cultivation. She did the phylogenetic analysis together with T. Lundell. The author analysed and interpreted the results and wrote the article.

III The author planned the experiments, did the laboratory work except for the HPLC analysis. CZE method used in this work was developed by S. Galkin. The author analysed and interpreted the results, and took principal action in writing the article.

IV The author planned the experiments and did the laboratory work. She did the phylogenetic analysis together with T. Lundell. The author analysed and interpreted the results and wrote the article.

Abbreviations

AAO	aryl alcohol oxidase
ATP	adenosine triphosphate
bp	base pair(s)
CAZyme	carbohydrate-active enzyme
CDH	cellobiose dehydrogenase
cDNA	complementary DNA
CZE	capillary zone electrophoresis
3D	three dimensional
DNA	deoxyribonucleic acid
eHN	extra high nitrogen
FDH	formate dehydrogenase
FOLy	Fungal Oxidative Lignin enzymes
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GLOX	glyoxal oxidase
3-HAA	3-hydroxyanthranilate
HN	high nitrogen
HPLC	high performance liquid chromatography
kDa	kiloDalton
LC	low carbon
LiP	lignin peroxidase
LME	lignin-modifying enzyme
LN	low nitrogen
MCO	multicopper oxidase
MnP	manganese peroxidase
mRNA	messenger RNA
NAD	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
ODC	oxalate decarboxylase
OXA	oxalyl-CoA decarboxylase
OXO	oxalate oxidase
PAH	polycyclic aromatic hydrocarbons
PCB	polychlorinated biphenyl
PCR	polymerase chain reaction
p*I*	isoelectric point
PY	peptone-yeast extract
qRT-PCR	quantitative reverse transcriptase-PCR
RACE-PCR	rapid amplification of cDNA ends-PCR
RNA	ribonucleic acid
ROS	reactive oxygen species
RT-PCR	reverse transcriptase-PCR
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
ThDP	thiamin pyrophosphate
VA	veratryl alcohol
VP	versatile peroxidase

Abstract

Basidiomycetous white-rot fungi are the only organisms that can efficiently decompose all the components of wood. Moreover, white-rot fungi possess the ability to mineralize recalcitrant lignin polymer with their extracellular, oxidative lignin-modifying enzymes (LMEs), i.e. laccase, lignin peroxidase (LiP), manganese peroxidase (MnP), and versatile peroxidase (VP). Within one white-rot fungal species LMEs are typically present as several isozymes encoded by multiple genes.

This study focused on two efficient lignin-degrading white-rot fungal species, *Phlebia radiata* and *Dichomitus squalens*. Molecular level knowledge of the LMEs of the Finnish isolate *P. radiata* FBCC43 (79, ATCC 64658) was complemented with cloning and characterization of a new laccase (*Pr-lac2*), two new LiP-encoding genes (*Pr-lip1, Pr-lip4*), and *Pr-lip3* gene that has been previously described only at cDNA-level. Also, two laccase-encoding genes (*Ds-lac3, Ds-lac4*) of *D. squalens* were cloned and characterized for the first time.

Phylogenetic analysis revealed close evolutionary relationships between the *P. radiata* LiP isozymes. Distinct protein phylogeny for both *P. radiata* and *D. squalens* laccases suggested different physiological functions for the corresponding enzymes. Supplementation of *P. radiata* liquid culture medium with excess Cu^{2+} notably increased laccase activity and good fungal growth was achieved in complex medium rich with organic nitrogen.

Wood is the natural substrate of lignin-degrading white-rot fungi, supporting production of enzymes and metabolites needed for fungal growth and the breakdown of lignocellulose. In this work, emphasis was on solid-state wood or wood-containig cultures that mimic the natural growth conditions of white-rot fungi. Transcript analyses showed that wood promoted expression of all the presently known LME-encoding genes of *P. radiata* and laccase-encoding genes of *D. squalens*. Expression of the studied individual LME-encoding genes of *P. radiata* and *D. squalens* was unequal in transcript quantities and apparently time-dependent, thus suggesting the importance of several distinct LMEs within one fungal species.

In addition to LMEs, white-rot fungi secrete other compounds that are important in decomposition of wood and lignin. One of these compounds is oxalic acid, which is a common metabolite of wood-rotting fungi. Fungi produce also oxalic-acid degrading enzymes of which the most widespread is oxalate decarboxylase (ODC). However, the role of ODC in fungi is still ambiguous with propositions from regulation of intra- and extracellular oxalic acid levels to a function in primary growth and concomitant production of ATP.

In this study, intracellular ODC activity was detected in four white-rot fungal species, and *D. squalens* showed the highest ODC activity upon exposure to oxalic acid. Oxalic acid was the most common organic acid secreted by the ODC-positive white-rot fungi and the only organic acid detected in wood cultures.

The ODC-encoding gene *Ds-odc* was cloned from two strains of *D. squalens* showing the first characterization of an *odc*-gene from a white-rot polypore species. Biochemical properties of the *D. squalens* ODC resembled those described for other basidiomycete ODCs. However, the translated amino acid sequence of *Ds-odc* has a novel N-terminal primary structure with a repetitive Ala-Ser-rich region of ca 60 amino acid residues in length. Expression of the *Ds-odc* transcripts suggested a constitutive metabolic role for the corresponding ODC enzyme. According to the results, it is proposed that ODC may have an essential implication for the growth and basic metabolism of wood-decaying fungi.

Tiivistelmä (Abstract in Finnish)

Kantasieniin kuuluvat valkolahosienet ovat ainoita organismeja, jotka hajottavat tehokkaasti puun kaikkia komponentteja. Tämän lisäksi ne pystyvät mineralisoimaan vaikeasti hajoavaa ligniinipolymeeriä solujensa ulkopuolelle erittyvien, hapettavien ligniiniä muokkaavia entsyymien avulla. Näitä entsyymejä, joista valkolahosienet tuottavat tyypillisesti useita eri geenien koodaamia isoentsyymejä, ovat lakkaasi, ligniiniperoksidaasi (LiP), mangaaniperoksidaasi (MnP) ja versatiiliperoksidaasi (VP).

Tässä tutkimuksessa keskityttiin kahteen ligniiniä tehokkaasti hajottavaan valkolahosieneen: rusorypykkään (*Phlebia radiata*) ja salokääpään (*Dichomitus squalens*). Molekyylitason tietoa Suomesta eristetyn *P. radiata* FBCC43 (79, ATCC 64658) –sienen ligniiniä muokkaavista entsyymeistä täydennettiin kloonaamalla ja karakterisoimalla yksi lakkaasi (*Pr-lac2*) ja kolme *lip*-geeniä (*Pr-lip1*, *Pr-lip3*, *Pr-lip4*), joista vain *Pr-lip3* on aiemmin kloonattu cDNA:sta. *D. squalens*ista kloonattiin ja karakterisoitiin ensimmäisen kerran kaksi lakkaasigeeniä (*Ds-lac3*, *Ds-lac4*).

Fylogeneettinen analyysi osoitti *P. radiata*n LiP-isoentsyymien olevan evolutiivisesti hyvin lähellä toisiaan. *P. radiata*n ja *D. squalens*in lakkaasit ovat puolestaan kaukaista sukua toisilleen, joten ne saattavat osallistua erilaisiin fysiologisiin toimintoihin. Lakkaasiaktiivisuus nousi huomattavasti, kun *P. radiata*n liemiviljelmiin lisättiin ylimäärin Cu^{2+}-ioneja. Sieni kasvoi hyvin ravinteikkaissa, runsaasti orgaanista typpeä sisältävissä liemiviljelmissä.

Puu on valkolahosienten luonnollinen kasvualusta, jolla sienet tuottavat kasvuunsa ja lignoselluloosan hajottamiseen tarvittavia entsyymejä ja aineenvaihduntatuotteita. Tässä työssä valkolahosieniä kasvatettiin puulla ja puuta sisältävillä, luonnollisia kasvuolosuhteita jäljittelevillä alustoilla. Transkriptianalyysit osoittivat kaikkien *P. radiata*n tunnettujen ligniiniä muokkaavia entsyymien ja *D. squalens*in lakkaasien ilmentyvän sienten kasvaessa puulla. Yksittäisten *P. radiata*n *lip*- ja lakkaasigeenien sekä *D. squalens*in lakkaasigeenien transkriptimäärissä oli eroja kasvun aikana. Näiden tulosten perusteella useilla ligniiniä muokkaavilla isoentsyymeillä on tärkeä merkitys valkolahosienten kasvaessa puulla.

Entsyymien lisäksi valkolahosienet erittävät puun ja ligniinin hajotukseen osallistuvia yhdisteitä, kuten oksaalihappoa, joka on puuta lahottavien sienten yleinen aineenvaihduntatuote. Sienet tuottavat myös oksaalihappoa hajottavia entsyymejä, joista yleisin on oksalaattidekarboksylaasi (ODC). ODC:n merkitystä sienissä ei toistaiseksi tunneta tarkasti. Entsyymin on ehdotettu säätelevän solunsisäistä ja -ulkoista oksaalihappopitoisuutta sekä osallistuvan ATP:n tuottoon.

Tässä tutkimuksessa solunsisäistä ODC-aktiivisuutta havaittiin neljällä uudella valkolahosienikannalla. Korkein ODC-aktiivisuus mitattiin *D. squalens*illa kasvualustoihin tehdyn oksaalihappolisäyksen jälkeen. Oksaalihappo oli ODC:a tuottavien sienten yleisimmin erittämä ja ainoa puualustalla havaittu orgaaninen happo.

Työssä kahdesta *D. squalens* –kannasta kloonattu *odc*-geeni on ensimmäinen valkolahottajakäävästä kuvattu *odc*-geeni. *D. squalens*in ODC oli biokemiallisilta ominaisuuksiltaan samankaltainen muista kantasienistä kuvattujen ODC-proteiinien kanssa. *Ds-odc* -geenistä käännetyn aminohapposekvenssin mukaan sen koodaaman proteiinin N-terminaalissa on kuitenkin uudenlainen, noin 60 aminohapon pituinen paljon alaniinia ja seriiniä sisältävä toistojakso. *Ds-odc* –geenin ilmentymisen perusteella sen koodaamaa entsyymiä tuotetaan konstitutiivisesti. ODC saattaakin olla tärkeä entsyymi puuta lahottavien sienten kasvulle ja perusaineenvaihdunnalle.

1 Introduction

1.1 Wood composition

Wood is a porous plant material made up of various types of xylem cells. Softwoods (in gymnosperm trees) consist mainly of long tracheids and smaller ray parenchyma cells. Water transport and stem strength are mainly sustained by the dead tracheid cells. In addition, longitudinal resin ducts exist (Kuhad et al. 1997). Hardwoods (in angiosperm plants and deciduous trees) have more diverse types of xylem cells including fibers, vessels, and ray parenchyma cells. These cells are responsible for support and nutrient storage as well as water and nutrient transport between plant roots and the photosynthesizing leaves or needles (Eriksson et al. 1990). Wood cell walls, in particular the long tracheids and fibers, consist of several layers which differ in their structure and chemical composition (Fig. 1a). The major components of the wood cell walls are three biopolymers, namely cellulose, hemicellulose, and lignin (Harris and Stone 2008).

Lignin is an aromatic and amorphous polymer present in all layers of woody cell walls. In fibers and tracheids, the thin middle lamella has the highest lignin content, whilst most of the lignin exists in the thick secondary wall layers embedded with cellulose microfibrils and hemicellulose (Eriksson et al. 1990). Lignin mechanically strengthens vascular plants and aids in water transportation since it physically attaches the xylem cells together. At the same time, lignin protects the more easily degradable cellulose and hemicellulose polymers from microbial attack.

The heterogenic lignin polymer is synthesized in the plant xylem cells from phenylpropanoid precursors i.e. *p*-coumaryl, coniferyl, and sinapyl alcohol. During lignin biosynthesis these monolignols are polymerized to *p*-hydroxyphenyl, guaiacyl, and syringyl type of lignin subunits by the action of laccases and peroxidases (Higuchi 2006). Lignin subunits are joined together by diverse carbon-carbon and ether bonds of which the *β*-aryl-ether (*β*-O-4) bond is the most common (Sjöström 1993). The composition and amount of lignin varies between softwood and hardwood, and between plant species. The lignin content of softwoods (25-33% of xylem dry weight) consists mainly of guaiacyl subunits while hardwood lignins (20-25% of xylem dry weight) contain both guaiacyl and syringyl subunits (Adler 1977, Higuchi 2006). In grass plants, xylem cell wall lignin also contains *p*-hydroxyphenyl subunits and e.g. esterified ferulic acid (Eriksson et al. 1990, Hatfield et al. 1999). Knowledge of the chemical structure of diverse plant lignins is still incomplete, although several lignin models have been presented (Fig. 1b).

Most wood species contain approximately 40-45% (as dry weight) cellulose which is the main component of wood (Eriksson et al. 1990). In the linear cellulose polymer, repeating glucose units are linked together by β-1,4-glucosidic bonds and the degree of polymerization is up to about 15000 glucose units within one polymeric chain (Kuhad et al. 1997). In wood cell wall the long, contiguous cellulose chains are stabilized by hydrogen bonds into microfibrils and further into cellulose fibers. The majority of cellulose is situated in the thick S_2 layer of xylem secondary wall (Fig. 1a) where its fibrillous structure gives mechanical strength to wood (Argyropoulos and Menachem 1997). Usually, the highly organized crystalline cellulose dominates whereas only a

small portion exists as amorphous non-organized cellulose which is more susceptible to enzymatic degradation (Kuhad et al. 1997).

Hemicelluloses are a diverse group of branched heteropolysaccharides consisting of different hexose, pentose, and sugar acid units. Most of the hemicelluloses act as a supporting material and usually comprise 20-30% of wood dry weight (Sjöström 1993). Hemicelluloses are amorphous and have a moderate degree of polymerization (100-200 units) and thus are more easily biodegradable than cellulose. The composition and structure of hemicelluloses differ in softwood and hardwood: galactoglucomannans are the main hemicelluloses in softwood while glucuronoxylan dominates in hardwood (Eriksson et al. 1990). In the woody cell walls, hemicelluloses are bound to cellulose microfibrils via hydrogen bonds. Hemicelluloses and lignins are covalently linked forming the so called lignin-hemicellulose matrix (Fig. 1a, Kuhad et al. 1997, Harris and Stone 2008).

Depending on the wood species, 2-5% of the wood dry weight is made up of extractives (Sjöström and Westermark 1998). Extractives are non-structural constituents of wood. They may be broadly divided into terpenes, resins, and phenols (Kuhad et al. 1997). These various organic compounds have several roles, acting as a nutrition reserve for the living wood cells as well as giving protection against microbial degradation (Sjöström 1993). In addition, low amounts of proteins and inorganic compounds are present in the wood (Fengel and Wegener 1989).

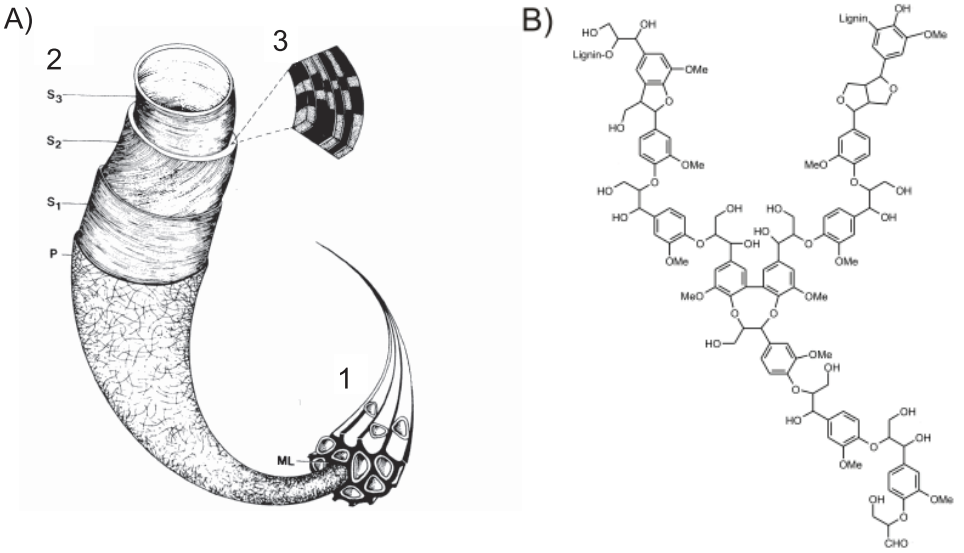


Figure 1. A) Composition of wood showing 1) tracheids, 2) wood cell wall layers, 3) distribution of lignin-hemicellulose matrix (black), hemicellulose (white) and cellulose (grey) in the secondary cell wall. ML: middle lamella, P: primary wall, S1-S3: secondary cell wall layers. Reprinted from Eriksson et al. 1990 with kind permission from Springer Science+Business Media. B) Structural model of lignin after Brunow et al. 2001.

1.2 Wood degradation by fungi

Wood, being poor in nutrients other than organic carbon, is a demanding growth environment for microorganisms. In addition, the lignocellulose complex efficiently hinders the access of microbes and their enzymes into wood cell walls. Other wood components, such as extractives, also restrict the growth of many microbes. Of all organisms, fungi are the most powerful degraders of the wood polymers (Eriksson et al. 1990, Carlile et al. 2001). As vascular plants form the vast reservoir of photosynthetically fixed carbon on earth, wood-decaying fungi have enormous ecological impact on the global carbon cycling.

Three different types of wood decay caused by fungi can be distinguished: white-rot, brown-rot, and soft-rot (Eriksson et al. 1990). Basidiomycetous white-rot fungi are saprotrophs mostly living on dead wood. White-rot fungi have a unique ability to efficiently mineralize lignin to CO_2 with their oxidative lignin-modifying enzymes (LMEs) (Kirk and Farrell 1987, Hatakka 2001, Hammel and Cullen 2008). Also wood-colonizing ascomycetous fungi are capable of mineralizing lignin to some extent (Liers et al. 2006). Basidiomycetous litter-decomposing fungi have been reported to mineralize lignin as well, but their growth and degradation capacity is usually restricted to the soil environment (Steffen 2003).

Following the action of white-rot fungi, the decayed wood is characteristically white and fibre-like. Most white-rot fungi are able to degrade all the wood polymers. However, there are so called selective white-rot fungi which preferentially degrade lignin and hemicellulose leaving cellulose polymer almost intact (Eriksson et al. 1990, Kuhad et al. 1997). These species include e.g. *Dichomitus squalens, Physisporinus rivulosus,* and *Ceriporiopsis subvermispora* (Hakala et al. 2004, Fackler et al. 2006). The model white-rot fungus, *Phanerochaete chrysosporium* (Martinez et al. 2004), is efficient in wood and lignocellulose decay but less selective for depolymerization of lignin over cellulose utilization (Hatakka and Uusi-Rauva 1983, Akhtar et al. 1997, Hatakka 2001, Hakala et al. 2004).

Another group of wood-decaying basidiomycetes is the brown-rot fungi which rapidly depolymerize cellulose in the early stage of wood decay. As wood polysaccharides are degraded and some modification of lignin occurs, mostly by demethoxylation, the decayed wood remains brown and has lost its strength (Akhtar et al. 1997, Hammel 1997). The mechanisms which brown-rot fungi use in cellulose degradation are still poorly understood, and the first brown-rot fungal genome sequenced (*Postia placenta*) (Martinez et al. 2009) has offered new insights into future studies on the decomposition of wood polysaccharides.

Some ascomycetes (e.g. *Trichoderma* and *Xylaria* species) cause a third type of wood-rot known as soft-rot. These fungi typically attack wood in wet environments. As a result of soft-rot, cavities or complete erosion of tracheid secondary cell walls is detected, and the decayed wood loses its mechanical strength due to cellulose breakdown (Akhtar et al. 1997, Carlile et al. 2001). Some ascomycetes, so called sap-staining fungi, degrade mainly wood extractives. These species are primary wood colonizers that characteristically discolour the sapwood with their dark pigmented hyphae, which leads to mainly cosmetic rather than structural damage (Breuil et al. 1998).

1.3 Lignin-modifying enzymes

Lignin-modifying enzymes (LMEs) considered to be involved in lignin biodegradation include oxidative enzymes that catalyze unspecific reactions, i.e. laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.2), lignin peroxidase (LiP, diarylpropane peroxidase, EC 1.11.1.14), manganese peroxidase (MnP, EC 1.11.1.13), and versatile peroxidase (VP, EC 1.11.1.16) (Martínez 2002, Hammel and Cullen 2008). Also several H_2O_2-generating enzymes such as aryl alcohol oxidase (AAO, EC 1.1.3.7), glyoxal oxidase (GLOX), and pyranose-2 oxidase (EC 1.1.3.10) are regarded as members of white-rot fungal lignin-degrading machinery (Lundell 1993, de Jong et al. 1994, Kersten and Cullen 2007). Recently, oxidases potentially involved in the degradation of lignin and related aromatic compounds have been classified into enzyme families according to their protein sequence and biochemical properties, and integrated into FOLy (Fungal Oxidative Lignin enzymes) database (Levasseur et al. 2008).

LMEs generate by oxidative reactions highly reactive free radicals due to which degradation of lignin by white-rot fungi is known as "enzymatic combustion" (Kirk and Farrell 1987). LMEs are expressed by white-rot and litter-decomposing fungi in different combinations (Hatakka 1994, 2001) and typically, several LME isozymes are encoded by multiple genes and their alleles within one fungal species (Martinez et al. 2004, Kersten and Cullen 2007, Pezzella et al. 2009). It has been considered that the multiple, structurally related LME-encoding genes and heterogeneity of their regulation can provide flexibility which white-rot fungi need for adaptation to for example, changing environmental conditions and during growth on different wood species (Conesa et al. 2002, Kersten and Cullen 2007). On the other hand, this genetic diversity may barely represent functional redundancy (Hammel and Cullen 2008).

Current knowledge of lignin-degradation supports the view that lignin is depolymerized outside the fungal hyphae by the combined oxidative action of LMEs, oxygen radicals, and small metabolites after which at least some of the resulting fragments are mineralized intracellularly (Lundell 1993, Hofrichter et al. 1999b, Kapich et al. 1999, Hatakka 2001, Hammel and Cullen 2008). The importance of lignin-modifying peroxidases and H_2O_2 production in lignin breakdown has been highlighted in recent transcriptome and proteome studies of the white-rot model fungus *Phanerochaete chrysosporium* (Sato et al. 2009, Vanden Wymelenberg et al. 2009). During the growth of *P. chrysosporium* in nutrient limited liquid cultures that mimic lignin-degrading conditions the increased expression of LiPs, MnPs, and various extracellular oxidases was observed (Vanden Wymelenberg et al. 2009). On the stationary wood cultures, *P. chrysosporium* genes encoding LiPs and alcohol oxidase were reported to be highly expressed (Sato et al. 2009).

In addition to this, the whole genome sequence of *P. chrysosporium* revealed a large number of putative genes encoding extracellular oxidative enzymes which can also be connected to lignocellulose degradation (Martinez et al. 2004, Kersten and Cullen 2007). The transcriptome and secretome analyses under lignin-degrading conditions have showed a set of expressed genes and secreted proteins of *P. chrysosporium* with unknown function (Sato et al. 2009, Vanden Wymelenberg et al. 2009). These data suggest that the whole complexity of the white-rot fungal process of lignin degradation is yet to be unraveled.

1.3.1 Lignin-modifying peroxidases

Lignin-modifying heme peroxidases (lignin peroxidase, LiP; manganese peroxidase, MnP; versatile peroxidase, VP) are extracellular glycoproteins that belong to the class II heme-containing, fungal secretory peroxidases within the plant peroxidase superfamily (Welinder 1992, Martínez 2002). The class II peroxidases are structurally related globular proteins predominantly consisting of 11-12 α-helixes divided to two domains. These peroxidases carry Fe-containing heme (protoporphyrin IX) as their prosthetic group coordinated by two highly conserved histidine residues (distal and proximal histidines) in a central cavity between the two domains (Martínez 2002).

Recently, the significance of lignin-modifying peroxidases in lignin degradation has been supported by comparison of the genomes of *Phanerochaete chrysosporium*, a model white-rot fungus for lignin degradation (Martinez et al. 2004), and *Postia placenta*, which has become a model brown-rot fungus for wood polysaccharide degradation (Martinez et al. 2009). The haploid genome of *P. chrysosporium* strain RP78, derived from dicaryotic strain BKM-F-1767 (ATCC 24725), contains multiple lignin-modifying-peroxidase-encoding genes (10 *lip* and 5 *mnp* genes). In contrast, the dicaryotic genome of *P. placenta* totally lacks lignin-modifying-peroxidase-encoding genes, and contains only one putative class II-fungal secretory peroxidase-encoding gene. This *P. placenta* peroxidase gene possibly encodes a low-redox potential type of peroxidase related to the CIP-enzyme of the basidiomycete *Coprinopsis cinerea* (*Coprinus cinereus*) not capable of lignin degradation (Martinez et al. 2009).

The first class II heme peroxidase gene of a brown-rot fungus was recently cloned from *Antrodia cinnamomea* and the corresponding enzyme was reported to decolorize and oxidize some phenolic dyes (Huang et al. 2009). Although *A. cinnamomea* peroxidase may represent a new group of extracellular class II heme peroxidases from previously unstudied brown-rot basidiomycetes (Huang et al. 2009), its role in lignin modification is still unclear. Preliminary genomic PCR data indicate that also some species of the ectomycorrhizal basidiomycetes may possess genes coding for class II heme peroxidases (Bödeker et al. 2009). However, so far no lignin-modifying-peroxidase-encoding genes have been annotated in the whole genome sequence of the ectomycorrhizal model fungus *L. bicolor* (Martin et al. 2008, Table 4).

1.3.1.1 Occurrence and properties of lignin-modifying peroxidases

Active LiP isozymes, first found in the cultures of *P. chrysosporium* (Glenn et al. 1983, Tien and Kirk 1983), have been described from only a few genera of white-rot fungi including *Phlebia* (e.g. *P. radiata* and *Phlebia (Merulius) tremellosa*) (Niku-Paavola et al. 1988, Lundell et al. 1990, Vares et al. 1994), *Trametes* (e.g. *T. versicolor* and *T. trogii*) (Jönsson et al. 1987, Vares and Hatakka 1997), and *Bjerkandera* (e.g. *B. adusta* and *Bjerkandera* sp.) (Heinfling et al. 1998, ten Have et al. 1998).

In contrast, MnPs are widespread among lignin-degrading fungi including both white-rot and litter-decomposing basidiomycetous species (Hatakka 2001, Hofrichter 2002, Steffen et al. 2002, Lankinen et al. 2005). The lignin-modifying peroxidase described latest is VP, an enzyme that combines the catalytic properties of LiP and MnP, while its 3D structure resembles more LiP than MnP (Martínez 2002). Currently, VPs are characterized from two genera, *Pleurotus* and *Bjerkandera*, and evidence for their production has been reported for *Panus*, *Trametes*, and *Spongipellis* species (reviewed by Ruiz-Dueñas et al. 2009).

The molecular masses of the LiP, MnP, and VP proteins vary between 35-48 kDa, 38-62 kDa, and 42-45 kDa, respectively. White-rot fungal lignin-modifying peroxidases have typically acidic p*I* values of 3.0-4.0 (Mester and Field 1998, Camarero et al. 1999, Hatakka 2001), while also neutral MnPs have been detected from litter-decomposing fungi (Steffen et al. 2002).

The overall amino acid sequences of fungal class II secretory peroxidases are well conserved. For example, two Ca^{2+}-binding sites and eight cysteine residues that form four disulfide bridges are present to stabilize the protein structure and active site (Fig. 2) (Piontek et al. 1993, Poulos et al. 1993, Petersen et al. 1994, Sundaramoorthy et al. 1994, Martínez 2002).

The crystal structure of *P. chrysosporium* LiP H8 has been described in detail (Piontek et al. 1993, Poulos et al. 1993). The outmost residue needed for LiP activity is an invariant tryptophan, Trp171 in the isozyme LiPA (LiP H8) of *P. chrysosporium*. Necessity of the residue has been confirmed by several site-directed mutagenesis studies (Doyle et al. 1998, Choinowski et al. 1999, Gold et al. 2000, Mester and Tien 2001).

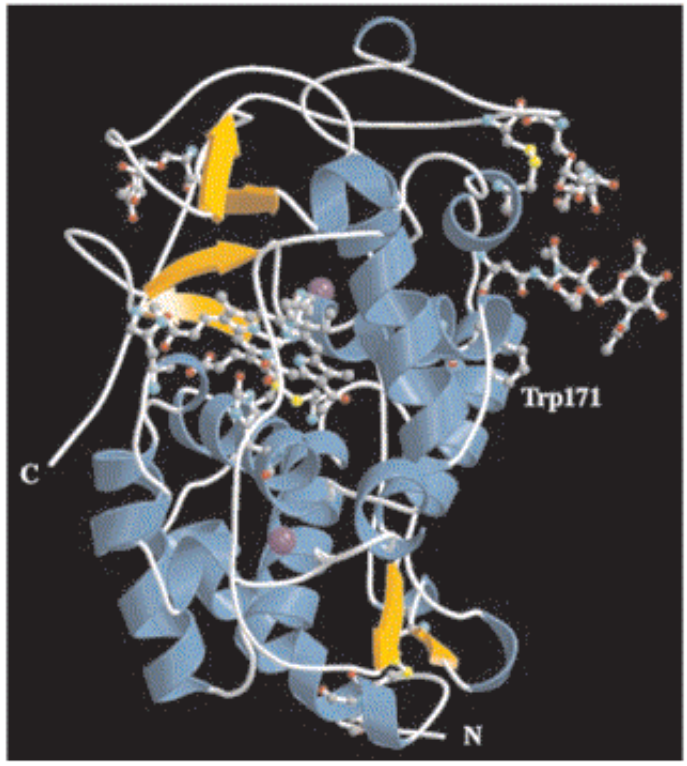


Figure 2. 3D model of *Phanerochaete chrysosporium* lignin peroxidase (LiP415) (Choinowski et al. 1999). Secondary structure elements (α- and 3_{10}-helices, blue; β-strands, orange arrows), two Ca^{2+} ions (purple spheres), and N- and C-termina are indicated. Heme group with proximal and distal histidine residues, four carbohydrate groups, Trp171, and disulfide bridges (S atoms, yellow spheres) are represented as ball and stick models. Reprinted with permission from Elsevier.

This tryptophan residue is situated in an exposed region of the enzyme surface (Fig. 2) and therefore it is thought to participate in the so-called long-range electron transfer from bulky aromatic substrates that cannot directly contact the oxidized heme in the active centre of LiP (Doyle et al. 1998). A similar solvent-exposed tryptophan residue is conserved in all the cloned VP-encoding genes of *Pleurotus* and *Bjerkandera* spp., and it is needed for the LiP-like activity of VPs (Martínez 2002, Pérez-Boada et al. 2005).

1.3.1.2 Catalytic reactions of lignin-modifying peroxidases

In a H_2O_2-dependent reaction, LiPs catalyze the initial one-electron oxidation of both phenolic and non-phenolic aromatic compounds, including the substructures of lignin, and several other substrates like veratryl alcohol (Fig. 3). This leads to C_α-C_β cleavage, aromatic ring oxidation, and cleavage reactions within the dimeric lignin-like model compounds (Kirk and Farrell 1987, Lundell et al. 1993a, Schoemaker et al. 1994, Hammel and Cullen 2008).

MnP catalyzes the specific oxidation of Mn^{2+} to Mn^{3+} in the presence of H_2O_2. Mn^{3+} ions are stabilized in chelated form to perform oxidative reactions that yield organic radicals from several phenolic substrates, carboxylic acids, and unsaturated lipids (Wariishi et al. 1992, Gold et al. 2000, Hammel and Cullen 2008). The natural chelators of Mn^{3+} are thought to be dicarboxylic acids, e.g. oxalic acid which is a common extracellular metabolite of white-rot fungi (Wariishi et al. 1992, Kuan and Tien 1993, Dutton and Evans 1996, Galkin et al. 1998). The chelated Mn^{3+} can diffuse even into the intact wood cell wall, the low porosity of which hinders the access from enzyme molecules (Blanchette et al. 1997).

The Mn^{3+} ions produced in MnP catalysis are not able to directly oxidize non-phenolic structures that comprise approximately 90% of lignin subunits in wood (Hammel and Cullen 2008). This may be avoided by the subsequent reactions of Mn^{3+}, which can result with e.g. lipid peroxidation, the radical chain reaction that has been shown to generate peroxyl radicals from lipids and also lead to the cleavage of non-phenolic synthetic lignin (Bao et al. 1994, Jensen et al. 1996, Enoki et al. 1999, Kapich et al. 1999).

VPs share the Mn^{2+}-oxidizing activity with MnPs. Both MnP and VP have three conserved acidic amino acid residues, two glutamates and one aspartate, which together with one of the heme propionates are involved in Mn^{2+}-binding (Gold et al. 2000, Martínez 2002). However, VP has been shown to efficiently oxidize Mn^{2+} in the presence of only two acidic amino acid residues reflecting certain differences between these two enzymes (Ruiz-Dueñas et al. 2009).

$$\text{LiP-P-Fe(III)} + H_2O_2 \rightarrow \text{LiP-}P^{\bullet+}\text{-Fe(IV)=O (compound I)} + H_2O$$
$$\text{LiP-}P^{\bullet+}\text{-Fe(IV)=O} + AH \rightarrow \text{LiP-P-Fe(IV)=O (compound II)} + H^+ + A^\bullet$$
$$\text{LiP-P-Fe(IV)=O} + H^+ + AH \rightarrow \text{LiP-P-Fe(III)} + H_2O + A^\bullet$$

Figure 3. Catalytic reactions of lignin peroxidase (LiP). P = porphyrin, $P^{\bullet+}$ = porphyrin cation radical, AH = aromatic compound, $A^\bullet$ = aromatic radical. During the catalytic cycle, two enzyme intermediate states (compound I and II) are detected. Adopted from Choinowski et al. 1999.

1.3.1.3 Evolutional relations of lignin-modifying peroxidases

Lignin-modifying peroxidases are evolutionarily closely related and phylogenetic analyses divide them into several clearly defined main groups or subfamilies discriminated by certain key amino acid residues (Martínez 2002, Hildén et al. 2005, Ruiz-Dueñas et al. 2009). The first phylogenetic main cluster includes the typical *mnp* genes that code for proteins with long C-terminal tails and are found in e.g. *Ceriporiopsis subvermispora*, *Dichomitus squalens*, *Phlebia radiata*, and *Phanerochaete chrysosporium*. The second main group is formed by short MnPs, from e.g. *Trametes versicolor* and *Pleurotus* species, and VPs. LiPs are closely related to the short MnP-VP group reflecting similar structural features between the short MnPs and LiPs (Martínez 2002, Hildén et al. 2005). The third main cluster of fungal class II peroxidases are the non-lignin-modifying, CIP-like peroxidases (Hildén et al. 2005). Interestingly, the same white-rot fungal species can express functionally similar but evolutionarily divergent MnPs as shown with *P. radiata* (Hildén et al. 2005) and *Physisporinus rivulosus* (Hakala et al. 2006).

1.3.1.4 Regulation of lignin-modifying peroxidase expression

Expression of the lignin-modifying peroxidases of white-rot fungi is often divergently regulated. The effect of different culture conditions and various supplements has been thoroughly investigated both at protein and transcript level. Expression of lignin-modifying peroxidases is commonly triggered e.g. by depletion of nutrients, oxidative stress, and heat shock (Stewart et al. 1992, Gold and Alic 1993, Li et al. 1994, Belinky et al. 2003).

Substrate-dependent expression of *P. chrysosporium* LiP-encoding genes has been detected on aspen wood chips (Janse et al. 1998), in defined liquid medium (Broda et al. 1995, Stewart and Cullen 1999), and in soil cultures (Lamar et al. 1995, Bogan et al. 1996). Spruce sawdust was shown to have a distinct effect on the transcript levels of *P. rivulosus mnp* genes (Hakala et al. 2006). Disparate regulation of *P. radiata lip* genes on different wood species and Mn^{2+} was observed in this work (publ. II). In accordance, production of *P. radiata* LiP isozymes has shown to be dependent on the lignocellulose materials used as carbon source (Niku-Paavola et al. 1990, Vares et al. 1995).

Nitrogen concentration (Gold and Alic 1993, Li et al. 1994, Hakala 2007) and source, i.e. organic or inorganic nitrogen (Kaal et al. 1993) are factors that affect fungal lignin-modifying peroxidase expression. For example, *Pleurotus eryngii* expresses one VP-encoding gene in peptone-containing liquid cultures while two allelic variants encoding another VP isozyme are expressed on lignocellulose cultures (Ruiz-Dueñas et al. 1999, Camarero et al. 2000).

Regulation of MnP expression by Mn^{2+} has been observed repeatedly in white-rot fungi. The levels of different *mnp* transcripts vary in response to Mn^{2+} e.g. in *P. chrysosporium* (Gettemy et al. 1998), *Pleurotus ostreatus* (Cohen et al. 2001), *C. subvermispora* (Manubens et al. 2003), *P. radiata* (Hildén et al. 2005), *P. rivulosus* (Hakala et al. 2006), and *Phlebia* sp. MG-60 (Kamei et al. 2008). Putative metal response elements (MREs) are present in the promoter regions of several white-rot fungal *mnp* genes (Gold and Alic 1993, Johansson and Nyman 1996, Gold et al. 2000, Tello et al. 2000, Hildén et al. 2005) although the functionality of these elements needs still to be proven. Also aromatic compounds, such as veratryl alcohol and syringic acid may

promote MnP production in white-rot fungal cultures (Niku-Paavola et al. 1990, Scheel et al. 2000, Hofrichter 2002, Manubens et al. 2003, Hakala et al. 2006).

One fungal species typically harbours several genes for the lignin-modifying peroxidases. Close genomic organization of eight LiP and two MnP -encoding genes in *P. chrysosporium* (Martinez et al. 2004) and tandemly arranged LiP and MnP -encoding genes in *T. versicolor* (Johansson and Nyman 1996) have been reported. However, the relationship between LME gene clustering and transcriptional regulation is not apparent (Stewart and Cullen 1999, Vanden Wymelenberg et al. 2009).

1.3.2 Laccase

Laccases catalyze the oxidation of a variety of phenolic compounds with the concomitant reduction of molecular oxygen to water. Laccase is one of the oldest known enzyme. The activity was originally found in the exudate from the Japanese lacquer tree, *Rhus vernicifera*, over one hundred years ago (Yoshida 1883).

Laccases belong to the large and diverse superfamily of multicopper oxidases (MCOs) (Hoegger et al. 2006). In addition to fungi, similar types of MCOs exist in plants, insects, and bacteria (Alexandre and Zhulin 2000, Martins et al. 2002, Mayer and Staples 2002, Claus 2004) showing their wide distribution in nature. Phylogenetically, MCOs are shown to be classified into true fungal laccases separated e.g. from insect laccases, fungal pigment MCOs, fungal ferroxidases, ascorbate oxidases, and plant laccase-like MCOs (Hoegger et al. 2006).

The interest towards fungal laccases has been enormous mostly owing to their potential applicability in industrial processes (discussed in section 1.10). The crystal structures of laccases from several fungal species are available, e.g. from the ascomycete *Melanocarpus albomyces* (Hakulinen et al. 2002) and the basidiomycetes *Coprinopsis cinerea (Coprinus cinereus)* (Ducros et al. 1998), *Trametes versicolor* (Bertrand et al. 2002, Piontek et al. 2002), *Rigidoporus lignosus* (Garavaglia et al. 2004), *Cerrena maxima* (Lyashenko et al. 2006), and *Lentinus (Panus) tigrinus* (Ferraroni et al. 2007).

1.3.2.1 Biochemical and molecular properties of fungal laccases

Fungal laccases are mainly extracellular glycoproteins with a carbohydrate content of 10-30%, a typical molecular mass between 60 and 80 kDa, and an acidic p*I* value of 3-6 (Thurston 1994, Baldrian 2006). Typical fungal laccases contain four copper atoms located in two centres (T1, T2/T3). The T1 site contains the mononuclear “blue” copper, while the T2/T3 site contains one T2 copper and two T3 copper ions (Solomon et al. 1996, Morozova et al. 2007a). The copper atoms are coordinated by ten conserved histidine residues and one conserved cysteine residue. Some “non-blue” fungal laccases (*Pleurotus ostreatus* POXA1w, *Phellinus ribis* laccase) are reported to harness other metals (Zn, Fe, Mn) instead of some of the mentioned copper atoms (Palmieri et al. 1997, Min et al. 2001). It is assumed that four electrons are transferred from the T1 reducing-substrate-binding site to the T2/T3-copper site during redox reactions (Solomon et al. 1996).

Laccase proteins are highly conserved at the amino acid sequence level (Fig. 4). The conserved amino acid residues are located in four amino acid regions considered as fungal laccase signature sequences (L1, L2, L3, L4) (Thurston 1994, Kumar et al.

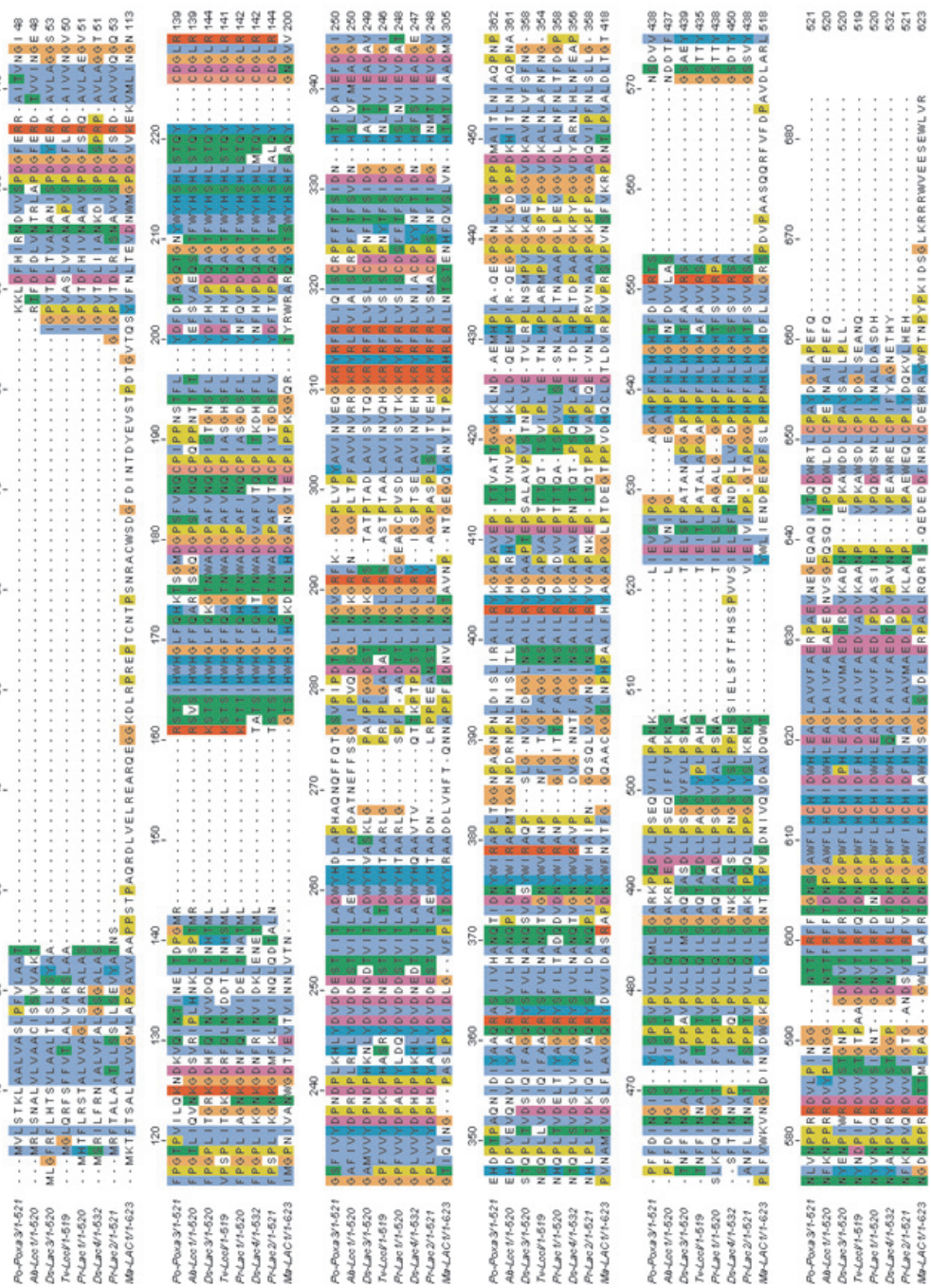


Figure 4. Protein sequences of selected fungal laccases. ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/) alignment illustrated by Jalview (Waterhouse et al. 2009). Colours refer to conserved or similar amino acids. Fungi and Uniprot sequence accessions: *Phlebia radiata* Pr-Lac1 (X52134), *P. radiata* Pr-Lac2 (Q0KHD1), *Trametes versicolor* Tv-LccI (U44851), *Pleurotus ostreatus* Po-Poxa3 (Q96TR4), *Agaricus bisporus* Ab-Lcc1 (Q12541), *Melanocarpus albomyces* Ma-LAC1 (Q70KY3). *Dichomitus squalens* Ds-Lac3 and Ds-Lac4, this study.

2003, Morozova et al. 2007a). These signature sequences also carry amino acid residues needed for the maintenance of protein fold. Furthermore, laccases have four substrate-binding loops revealed by the three-dimensional structure analysis of crystallized proteins (Hakulinen et al. 2002, Piontek et al. 2002, Larrondo et al. 2003b, Lyashenko et al. 2006).

1.3.2.2 Roles of fungal laccases

Fungal laccases are involved in the synthesis of melanin and other pigments, formation of fruiting bodies, conidiation, sporulation, and plant pathogenesis (Alexandre and Zhulin 2000, Mayer and Staples 2002). Due to their ability to catalyze the unspecific oxidation of phenolic and, in the presence of charge-transfer mediators, also non-phenolic compounds, fungal laccases have been repeatedly connected to lignin degradation.

The basis of the laccase mediator system is the use of low molecular weight compounds that are oxidized by laccase to organic radicals or positively charged intermediates which in turn act as redox mediators (Morozova et al. 2007b). The charged mediators are capable of oxidizing compounds that are not substrates for laccase and, furthermore, it is assumed that they can diffuse away from the fungal hyphae and oxidize larger molecules like lignin moieties in wood (Hatakka et al. 2003).

Examples of the laccase mediator molecules are 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonate (ABTS), 1-hydroxybenzotriazole (HBT), and 3-hydroxyanthranilate (3-HAA), of which the last one is found to occur naturally in the cultures of *Pycnoporus cinnabarinus* (Eggert et al. 1996, Morozova et al. 2007b). Also some phenolic lignin precursors and degradation products might act as laccase mediators in nature (Camarero et al. 2005).

The function of laccase in lignin degradation is still controversial. *Sensu stricto* laccase-encoding genes were not found in the genome of *Phanerochaete chrysosporium* (Martinez et al. 2004), proving that laccase is not essential for white-rot decay of wood. The recent whole genome sequence analysis of *Postia placenta* revealed for the first time the likely presence of true laccases in a brown-rot fungus although their function or expression pattern is presently not known (Martinez et al. 2009).

1.3.2.3 Fungal laccase-encoding genes

Fungi typically produce several laccase isoenzymes encoded by distinct laccase genes that have been suggested to be evolved through independent duplication-divergence events during evolution (Valderrama et al. 2003). This genetic multiplicity is proposed to be related to the diverse biochemical roles of laccase (Morozova et al. 2007a). Also, the phylogenetic clustering of fungal laccase sequences is at least partially in accordance with the function of the respective enzymes (Hoegger et al. 2006).

The number of laccase-encoding genes in different fungal species varies enormously. The largest taxon-level family of laccase-encoding genes so far identified (17 genes) is found in the non-lignin-degrading basidiomycete *Coprinopsis cinerea* (Kilaru et al. 2006). In another non-lignin-degrading basidiomycete, the ectomycorrhizal symbiotic species *Laccaria bicolor*, 9 laccase-encoding genes are present (Courty et al. 2009). White-rot fungi that have been shown to possess multiple laccase genes belong to the genera *Pleurotus* (Soden and Dobson 2001, Rodríguez et al. 2008, Pezzella et al.

2009) and *Trametes* (Yaver and Golightly 1996, Jönsson et al. 1997, Mansur et al. 1997, Mikuni and Morohoshi 1997, Cassland and Jönsson 1999, Necochea et al. 2005). Spatially close genomic organization of laccase genes has been recognized in *Pleurotus ostreatus* (Pezzella et al. 2009), *Agaricus bisporus* (Smith et al. 1998), *Rhizoctonia solani* (Wahleithner et al. 1996), and *C. cinerea* (Kilaru et al. 2006).

1.3.2.4 Regulation of laccase expression

Laccase expression in fungal cultures may generally be induced by addition of lignin-related and other aromatic compounds, e.g. 2,5-xylidine, guaiacol, ferulic acid, veratryl alcohol, veratric acid, and syringic acid (Lundell et al. 1990, Collins and Dobson 1997, Mansur et al. 1998, D'Souza et al. 1999, Soden and Dobson 2001, Manubens et al. 2007). For instance, it was early on suggested that some of the laccase isozymes of *Trametes versicolor* are inducible with aromatic compounds while some are so-called constitutive laccases (Mosbach 1963, Evans 1985, Rogalski et al. 1990).

Copper is a well-known inducer of laccase activity in fungi. Since the active site of laccase contains four copper atoms, the promoting effect of copper on enzyme activity may be basically explained at the protein level. For example, soluble copper has been found to play a post-transcriptional role in heterologously expressed *Ceriporiopsis subvermispora* laccase by activating the secreted apoprotein (Larrondo et al. 2003a). In addition, transcriptional induction of laccase genes by copper has been shown with several white-rot fungi, e.g. *T. versicolor* (Collins and Dobson 1997), *C. subvermispora* (Karahanian et al. 1998), *Pleurotus ostreatus* (Palmieri et al. 2000), *Pleurotus sajor-caju* (Soden and Dobson 2001), and *Trametes pubescens* (Galhaup et al. 2002).

Recently, it was shown that in *C. subvermispora*, the transcriptional activation of a laccase-encoding gene by copper is mediated by an ACE1-like copper-fist transcription factor (Álvarez et al. 2009). Similar putative ACE elements have been recognized in the promoter regions of the laccase-encoding gene *lac1* of basidiomycete PM1 (Coll et al. 1993) and *lac4* from *P. sajor-caju* (Soden and Dobson 2003).

In addition, putative metal response elements (MREs) have been identified within many laccase promoter regions (Mansur et al. 1998, Galhaup et al. 2002, Faraco et al. 2003), although their functionality has not been proven yet. Apart from copper, other heavy metal ions (Cd^{2+}, Ag^{2+}, and Hg^{2+}) have been reported as strong inducers of laccase activity or expression of laccase transcripts (Karahanian et al. 1998, Baldrian and Gabriel 2002, Galhaup et al. 2002). Also Mn^{2+} has been shown to regulate the laccase transcript levels in e.g. *P. sajor-caju* (Soden and Dobson 2001), *C. subvermispora* (Manubens et al. 2007), and *Phlebia* sp. *Nf* b19 (former *Nematoloma frowardii* b19) (Scheel et al. 2000, Hildén et al. 2008).

1.4 Fungal degradation of wood polysaccharides

1.4.1 Enzymatic decomposition of cellulose

In general, white-rot fungi express a set of hydrolytic enzymes for the degradation of cellulose. Endoglucanases (endo-1,4-*β*-glucanases, EC 3.2.1.4) hydrolyze internal glycosidic bonds of the cellulose polymer while cellobiohydrolases (exo-1,4-*β*-glucanases, EC 3.2.1.91) cleave the ends of cellulose chains resulting in the release of

cellobiose. Moreover, cellobiohydrolase I (Cel7A) -type enzymes act on non-reducing ends of the cellulose chains, while other cellobiohydrolase II (Cel6A) -type enzymes act on reducing ends. *β*-glucosidases (EC 3.2.1.21) finalize the concerted action of cellulases by cleaving the released disaccharides to glucose molecules (Baldrian and Valášková 2008).

Expression and production of fungal cellulases is controlled by induction and repression mechanisms, including carbon catabolite repression (Kuhad et al. 1997). For example, the white-rot fungi *Phlebia radiata* and *Dichomitus squalens* secrete endoglucanases, cellobiohydrolases, and *β*-glucosidases under cellulose-containing liquid cultures. (Rouau and Odier 1986, Rogalski et al. 1993c). Also, a full array of cellulases has been identified in the transcriptome and proteome of *Phanerochaete chrysosporium*, both on solid-state wood and in cellulose-grown cultures (Vanden Wymelenberg et al. 2005, Sato et al. 2007, 2009).

The ascomycete *Trichoderma reesei* (teleomorph *Hypocrea jecorina*) is the major model fungus for cellulose decomposition and soft-rot type of wood decay. Surprisingly, among the ascomycete whole genome sequences, the *T. reesei* genome reveals the smallest set of genes encoding enzymes involved in the decomposition of plant cell wall polysaccharides (Martinez et al. 2008). Furthermore, *T. reesei* harbours even fewer cellulolytic and hemicellulolytic enzyme-encoding genes than is recognized in the genome of the white-rot basidiomycete *P. chrysosporium*. Although still hyphothetical, efficient production of cellulases and control of gene expression have been suggested to explain the ability of *T. reesei* to cause powerful breakdown of cellulose and hemicellulose in natural lignocelluloses, regardless of the relatively low number of carbohydrate-active-enzyme (CAZyme) -encoding genes in the genome (Martinez et al. 2008).

Basidiomycetes and ascomycetes produce an additional extracellular enzyme, cellobiose dehydrogenase (CDH, EC 1.1.99.18), which oxidizes cellobiose to the corresponding lactone. The enzyme is often expressed by white-rot fungi but is so far identified only in a single brown-rot fungal species, *Coniophora puteana* (Hyde and Wood 1997, Henriksson et al. 2000). Furthermore, CDH is believed to play a role in degradation and modification of cellulose, hemicelluloses, and lignin by generating hydroxyl radicals in a Fenton-type reaction (Kremer and Wood 1992, Henriksson et al. 1995, Mansfield et al. 1997). CDH has been shown to be expressed during the growth of *P. chrysosporium* on solid-state wood and cellulose medium (Vanden Wymelenberg et al. 2005, Sato et al. 2009) further supporting the role of this particular enzyme in wood degradation.

1.4.2 Non-enzymatic decomposition of cellulose

Wood-decaying fungi, especially brown-rot fungi, are believed to degrade cellulose oxidatively by the means of hydroxyl radicals generated in the Fenton reaction ($H_2O_2 + Fe^{2+} + H^+ \rightarrow H_2O + Fe^{3+} + {}^{\bullet}OH$). The importance of Fenton chemistry in brown-rot fungal wood decay is recently emphasized by the whole genome sequence of *Postia placenta*, which harbours only two putative endoglucanases and several *β*-glucosidases, and totally lacks cellobiohydrolases (Martinez et al. 2009). In contrast, the *P. placenta* genome revealed a large variety of genes potentially involved in the generation of extracellular

reactive oxygen species (ROS). Furthermore, transcripts of several genes putatively involved in the extracellular generation of Fe^{2+} and H_2O_2 were also highly expressed during the growth of *P. placenta* on cellulose media (Martinez et al. 2009).

Some white-rot fungi produce ROS-quenching metabolites that can prevent the oxidative damage caused by active oxygen species. This may furthermore explain why these fungi leave wood cellulose more or less intact. In *Ganoderma* species, amino acids, polysaccharides, and methanolic extract from mycelia were observed to act as ROS-converting compounds (Yen and Wu 1999, Lee et al. 2001, Tseng et al. 2008). *Ceriporiopsis subvermispora* produces itaconic (ceriporic) acids, which may suppress the Fenton reaction leading to diminished cellulose depolymerization (Rahmawati et al. 2005).

Various fungal extracellular iron-chelating metabolites, e.g. siderophores and glycopeptides are thought to play a role in Fenton chemistry by reducing Fe^{3+} back to Fe^{2+} (Kuhad et al. 1997, Xu and Goodell 2001). Quinones produced by fungi are also able to reduce Fe^{3+} and contribute to a complete Fenton system in the so-called quinone redox cycling (reviewed by Baldrian and Valášková 2008) that has been shown to be a significant mechanism for cellulose cleavage in the brown-rot fungus *Gloeophyllum trabeum* (Suzuki et al. 2006).

Oxalic acid, secreted in relatively high concentrations by brown-rot fungi, is also proposed to participate in decomposition of cellulose (Espejo and Agosin 1991, Shimada et al. 1997). Oxalic acid strongly chelates Fe^{3+} into soluble complexes which predominate in brown-rot wood decay (Suzuki et al. 2006). Iron can be sequestered from Fe^{3+}-oxalate complexes and reduced back to Fe^{2+} thus promoting the continuation of Fenton reaction (Jensen et al. 2001, Xu and Goodell 2001, Varela and Tien 2003). In addition, the autooxidation of Fe^{2+}-oxalate complexes can lead to the slow production of hydroxyl ions even when quinones are not available (Hyde and Wood 1997, Suzuki et al. 2006). On the other hand, abundance of oxalic acid is believed to suppress Fenton reaction and protect the fungal hyphae from oxidative damage by scavenging hydroxyl ions (Shimada et al. 1994, 1997).

1.4.3 Decomposition of hemicellulose by basidiomycetous fungi

Due to the heterogeneous structure and organization of hemicellulose, a number of different CAZymes are required for its degradation. White-rot fungi secrete various glycoside hydrolases that cleave glycosidic bonds in the hemicellulose polymers, as well as carbohydrate esterases that hydrolyze ester linkages of acetate and the ferulic acid side groups. Carbohydrate esterases include e.g. feruloyl esterases (EC 3.1.1.73) which catalyze the hydrolysis of ester bond between arabinose subunits and ferulic acid involved in cross-linking of xylan to lignin (Kuhad et al. 1997, Shallom and Sholam 2003).

Endo-1,4-β-xylanases (EC 3.2.1.8) and endo-1,4-β-mannanases (EC 3.2.1.78) are the two main enzymes degrading the backbone of wood hemicelluloses. Several enzymes are responsible for further hydrolysis of the formed oligosaccharides (e.g. β-1,4- xylosidase, EC 3.2.1.37) and side groups (e.g. α-L-arabinosidase, EC 3.2.1.55) (Kuhad et al. 1997).

The recent brown-rot fungal transcriptome and secretome analysis of *Postia placenta* grown on cellulose revealed the expression of several hemicellulases (Martinez et al. 2009). Hemicellulase activities in white-rot fungi have been detected for example in the cultures of *Dichomitus squalens* (Rouau and Odier 1986), and have been studied in wheat bran cultures of *Phlebia radiata* (Rogalski et al. 1993a, b).

In the cultures mimicking lignin-degrading conditions, the *Phanerochaete chrysosporium* secretome has been shown to contain several hemicellulases together with LMEs (Vanden Wymelenberg et al. 2006). This may be related to the degradation of covalently linked lignin-hemicellulose matrix in the wood cell walls. However, a somewhat narrower selection of hemicellulases was shown in the proteome and transcriptome studies of *P. chrysosporium* when the fungus was cultivated on solid-state wood (Sato et al. 2007, 2009) as compared to cellulose-containing cultures (Vanden Wymelenberg et al. 2005).

1.5 Fungal low molecular weight compounds and wood degradation

Wood-decaying fungi produce several chemically diverse low molecular weight compounds, which have an impact on lignocellulose degradation. Low molecular weight compounds, such as phenols synthesized by fungi may be oxidized as substrates by the fungal LMEs. In consequence, this may lead to formation of free radicals which furthermore transfer oxidative reactivity to the lignocellulose matrix. Low-molecular weight compounds may also promote LME activity by stabilizing the reactive oxidants formed during enzyme catalytic action. Small organic molecules which readily diffuse away from the fungal hyphae are suggested to be important especially in the beginning of wood decay since the extracellular LMEs (laccases, LiPs, MnPs, VPs) are too large in size in order to penetrate into the intact wood cell walls (Evans et al. 1994, Blanchette et al. 1997, Goodell et al. 1997).

Veratryl (3,4-dimethoxybenzyl) alcohol (VA), which is a substrate for LiP, is a natural metabolite of a few white-rot fungi, e.g. *Phanerochaete chrysosporium* (Lundquist and Kirk 1978), *Pycnoporus cinnabarinus* (Hatakka 1985), and *Phlebia radiata* (Kantelinen et al. 1989). VA cation radical is most likely too short-lived to act as a far-diffusing redox-mediator upon LiP-catalysis under natural wood-decaying conditions (Candeias and Harvey 1995). However, updated with the current knowledge of the LiP 3D structure, VA is oxidized by LiP at a very specific site (i.e. exposed tryptophan residue) on the enzyme surface (Choinowski et al. 1999, Mester and Tien 2001, Johjima et al. 2002, Sollewijn Gelpke et al. 2002), which does not rule out the role of VA as a potential protector of LiP from an inactivation caused by H_2O_2. In addition to LiP, the fungal H_2O_2-producing enzyme aryl-alcohol oxidase (AAO) may use VA as a reducing substrate (Ferreira et al. 2005). In the case of laccase, the natural redox-mediator is also an aromatic compound, 3-hydroxyanthranilate (3-HAA), which is found to occur in the cultures of *P. cinnabarinus* (Eggert et al. 1996). 3-HAA expands the oxidation capacity of laccase to non-phenolic and polymeric compounds.

Cultures of many white-rot fungi including *Bjerkandera adusta, Pleurotus pulmonarius,* and *Phlebia radiata* become accumulated with fatty acids generated by the fungus (Gutiérrez et al. 2002). Unsaturated fatty acids participate in MnP-catalyzed lipid peroxidation reactions resulting with oxidation and even carbon-carbon bond cleavage of non-phenolic lignin substructures (Bao et al. 1994, Kapich et al. 1999).

Involvement of phospholipids and membrane-released fatty acids may have a versatile regulatory impact on fungal decay of wood and lignocellulose. Several alkyl- and alkenylitaconic acids (ceriporic acids), produced by the selective lignin-degrading white-rot fungus *Ceriporiopsis subvermispora* were shown to repress Fenton reaction and concomitantly diminish depolymerization of cellulose (Enoki et al. 2002, Amirta et al. 2003, Rahmawati et al. 2005). Quenching of cellulose degradation may in turn explain why this fungus leaves most of the wood cellulose intact while decaying lignin and hemicelluloses (Akhtar et al. 1997, Fackler et al. 2006).

White- and brown-rot fungi produce various iron-chelating compounds, e.g. glycopeptides, siderophores, oxalic acid, phenolates, and other monomeric aromatic compounds which are important for example in Fenton-type reactions (discussed in section 1.4.2). Oxalic and other carboxylic acids are generally secreted metabolites of fungi, and diverse functions of oxalic acid in wood degradation are discussed in the section 1.6.1.2.

1.6 Organic acids secreted by wood-decaying fungi

Wood-decaying fungi typically acidify their growth environment quickly by secreting organic acids. Several organic acids have been detected on defined liquid media and in lignocellulose-containing cultures of white-rot fungi. Oxalic acid is the most commonly secreted fungal acid (Table 1). In brown-rot fungi, production of other organic acids than oxalate has not so far been reported. The amount and diversity of organic acid production vary between fungal species, and secretion of carboxylic acids depends on the cultivation conditions (Galkin et al. 1998, Aguiar et al. 2006, publ. III). Organic acids originating from e.g. tricarboxylic (TCA) cycle are secreted as waste compounds of fungal cellular metabolism. The smallest organic acids, such as formic and oxalic acid, may also accumulate in fungal cultures as by-products of the cleavage of lignin substructures, such as side-chains and aromatic rings (Umezawa and Higuchi 1987, Hofrichter 2002).

1.6.1 Oxalic acid

Oxalic acid is a compound that is toxic to almost all organisms. It is the strongest dicarboxylic acid and has two pK_a values at 1.23 and 4.26. Oxalic acid is a major chelator of metal cations, e.g. Fe^{2+}, Mn^{2+}, Ca^{2+}, and Al^{3+}, and participates in various environmental and biological processes. Interestingly, oxalic acid plays several important roles in fungal growth and metabolism and is also connected to biological mechanisms underlying fungal pathogenesis (reviewed by Dutton and Evans 1996).

1.6.1.1 Fungal synthesis of oxalic acid

Fungi synthesize oxalic acid as a metabolic waste compound through the TCA cycle in mitochondria and by the so called glyoxylate cycle that operates in glyoxysomes (Dutton and Evans 1996, Munir et al. 2001b). More recently, the glyoxylate cycle has been proposed to take place in other organelles, the peroxisomes, after the studies of the brown-rot fungus, *Fomitopsis (Tyromyces) palustris* (Sakai et al. 2006). The biosynthesis of oxalic acid is catalyzed by the intracellular enzymes oxaloacetase (EC

3.7.1.1) (Akamatsu et al. 1993), glyoxylate oxidase (Akamatsu and Shimada 1994), and cytochrome *c*-dependent glyoxylate dehydrogenase (Tokimatsu et al. 1998).

Carbon catabolite repression of the glyoxylate cycle by glucose that is typically observed in bacteria does not seem to operate in wood-rotting basidiomycetes, thereby allowing fungi to secrete substantial amounts of oxalic acid (Munir et al. 2001b). A unique metabolic linkage between the TCA and glyoxylate cycles has been shown to be central in the oxalic acid biosynthesis of *F. palustris* (Munir et al. 2001a) and the brown-rot model fungus *Postia placenta* (Martinez et al. 2009). Furthermore, this metabolic shunt has been proposed to be a general feature of wood-rotting fungi, a means of acquiring energy for growth during wood decay by oxidizing released glucose to oxalic acid (Munir et al. 2001a). In contrast to this hypothesis, exposure of the white-rot fungus *Phanerochaete chrysosporium* to vanillin that is structurally related to lignin subunits, caused a drastic change from glyoxylate cycle to TCA cycle, and a flow of TCA cycle metabolites into the heme biosynthesis pathway was observed (Shimizu et al. 2005).

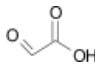
1.6.1.2 Roles of fungal-produced oxalic acid

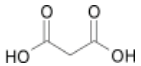
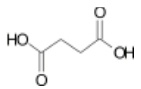
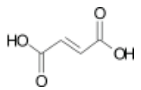
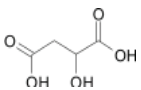
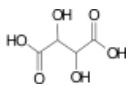
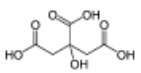
Fungal species belonging to Ascomycota, Basidiomycota, and Zygomycota are known to secrete considerable quantities of oxalic acid. Factors that affect the fungal production of oxalic acid include carbon and nitrogen sources, and pH of the growth environment (Dutton and Evans 1996). Several plant pathogenic fungi secrete oxalic acid to aid in defeating their host plant. In fact, oxalic acid secretion by *Sclerotinia sclerotiorum* was reported to induce a programmed cell death response in plant tissue (Kim et al. 2008a). Calcium oxalate crystals formed by fungi are frequently found in decayed wood and in soil (Hintikka et al. 1979, Dutton et al. 1993). Oxalic acid may sequester Ca^{2+} from the middle lamella of plant cell wall resulting with calcium oxalate crystal formation (Traquiar 1987). During wood decay, this can lead to increased pore size within the wood tracheids and fibers, which facilitates penetration of fungal extracellular enzymes into the inner layers of wood cell walls (Kuan and Tien 1993, Dutton et al. 1993).

Oxalic acid may inhibit the growth of more sensitive fungi, thus having an impact on competition between fungal species. For plant pathogenic fungi, secretion of oxalic acid is one of the factors promoting hyphal penetration and weakening of host defence. Secretion of oxalic acid by litter-decomposing and mycorrhizal fungi also increases the availability of soil nutrients (Dutton and Evans 1996). Leaching by oxalate has been shown to have an important role in solubilization of radioactive uranium oxides (Fomina et al. 2007). On the other hand, fungi can tolerate high environmental concentrations of toxic metals by secreting oxalic acid for chelation of cationic metals into insoluble form (Sayer and Gadd 1997, Jarosz-Wilkolazka and Graz 2006).

Oxalic acid is a common metabolic product of wood-rotting fungi, including both white- and brown-rot fungal species (Kuan and Tien 1993, Galkin et al. 1998, Urzúa et al. 1998, Green and Clausen 2003, Hakala et al. 2005, Aguiar et al. 2006, publ. III). White-rot fungi typically accumulate oxalic acid to their growth medium in millimolar quantities whereas in the cultures of brown-rot fungi, even several ten folds higher oxalic acid quantities are often detected (Espejo and Agosin 1991, Dutton et al. 1993). Previously, such high concentrations of oxalic acid were explained by the lack of oxalate decarboxylase (ODC) enzyme in the brown-rot fungi. However, at least in

Table 1. Organic acids produced by white- and brown-rot fungi on lignocellulose-containing and submerged liquid media.

Organic acid	Fungus	Decay type	Growth medium	References
Formic acid 	*Dichomitus squalens*	WR[a]	LM[c]	publ. III
Acetic acid 	*Phlebia tremellosa (Merulius tremellosus)*	WR	LM	Takao 1965
Glyoxylic acid 	*Ceriporiopsis subvermispora*	WR	LM	Urzúa et al. 1998
	Dichomitus squalens	WR	LM	publ. III
	Phanerochaete chrysosporium	WR	LM	Wariishi et al. 1992
Oxalic acid 	*Abortiporus biennis*	WR	LM	Galkin et al. 1998, Grąz et al. 2009
	Agaricus bisporus	WR	LM	Dutton et al. 1993
	Amyloporia xantha	BR[b]	LM	Dutton et al. 1993
	Ceriporiopsis subvermispora	WR	LM, pine wood chips	Urzúa et al. 1998, Aguiar et al. 2006
	Cerrena unicolor	WR	LM	Galkin et al. 1998
	Coniophora marmorata	BR	LM	Dutton et al. 1993
	Coniophora puteana	BR	LM, pine wood blocks	Dutton et al. 1993, Green and Clausen 2003
	Dichomitus squalens	WR	LM, spruce wood chips	publ. III
	Fomitopsis (Tyromyces) palustris	BR	LM, pine wood blocks	Munir et al. 2001a, Green and Clausen 2003
	Ganoderma applanatum	WR	wheat straw	Galkin et al. 1998
	Gloeophyllum trabeum	BR	LM, semisolid cellulose media, pine wood blocks	Espejo and Agosin 1991, Green and Clausen 2003
	Heterobasidion annosum	WR	LM	Dutton et al. 1993
	Phanerochaete chrysosporium	WR	LM	Wariishi et al. 1992, Kuan and Tien 1993
	Phanerochaete sanguinea	WR	LM, wheat straw, spruce wood chips	Galkin et al. 1998, publ III
	Phlebia sp.b19[d]	WR	wheat straw	Hofrichter et al. 1999a
	Phlebia radiata	WR	wheat straw	Galkin et al. 1998
	Phlebia tremellosa	WR	LM, wheat straw	Dutton et al. 1993, Galkin et al. 1998

		Physisporinus rivulosus	WR	spruce wood chips	Hakala et al. 2005
		Pleurotus florida	WR	LM	Dutton et al. 1993
		Poria vaporaria	BR	LM	Dutton et al. 1993
		Postia (Poria) placenta	BR	LM, pine wood blocks	Dutton et al. 1993, Jordan et al. 1996
		Serpula lacrymans	BR	pine wood blocks	Green and Clausen 2003
		Trametes gibbosa	WR	wheat straw	Galkin et al. 1998
		Trametes hirsuta	WR	wheat straw	Galkin et al. 1998
		Trametes trogii	WR	LM, wheat straw	Galkin et al. 1998
		Trametes (Coriolus) versicolor	WR	LM, semisolid cellulose media, wheat straw, spruce wood chips	Espejo and Agosin 1991, Galkin et al. 1998, publ. III
Malonic acid		*Ceriporiopsis subvermispora*	WR	pine wood chips	Aguiar et al. 2006 Wariishi et al. 1992
		Phanerochaete chrysosporium	WR	LM	
Succinic acid		*Phlebia tremellosa (Merulius tremellosus)*	WR	LM	Takao 1965
Fumaric acid		*Phlebia* sp. b19[d]	WR	wheat straw	Hofrichter et al. 1999a
		Phlebia tremellosa (Merulius tremellosus)	WR	LM	Takao 1965
Malic acid		*Bjerkandera adusta*	WR	LM	Galkin et al. 1998
		Ceriporiopsis subvermispora	WR	pine wood chips	Aguiar et al. 2006
		Cerrena unicolor	WR	LM	Galkin et al. 1998
		Phlebia sp. b19[d]	WR	wheat straw	Hofrichter et al. 1999a
		Phlebia tremellosa (Merulius tremellosus)	WR	LM	Takao 1965, Galkin et al. 1998
		Trametes gibbosa	WR	LM	Galkin et al. 1998
		Trametes trogii	WR	LM	Galkin et al. 1998
		Trametes versicolor	WR	LM	Galkin et al. 1998
Tartaric acid		*Ceriporiopsis subvermispora*	WR	pine wood chips	Aguiar et al. 2006
Citric acid		*Phanerochaete chrysosporium*	WR	LM	Wariishi et al. 1992

[a]WR = white-rot; [b]BR = brown-rot; [c]LM = liquid medium; [d]former *Nematoloma frowardii* b19 (Hildén et al. 2008)

Postia placenta, production and expression of ODC has been shown (Micales 1997, Martinez 2009). Fungal ODCs and their distribution in fungi are discussed in detail in section 1.6.3.

One explanation for the production of substantial amounts of oxalic acid by cellulose-degrading brown-rot fungi is the fact that wood as growth substrate is rich with carbon and scarce with nitrogen (Eaton and Hale 1993). By secreting oxalic acid the wood-decaying fungi can get rid of the excess wood carbon and keep their nutritional C/N ratio in balance (Shimada et al. 1997). Also, the difference in accumulation of extracellular oxalic acid between brown- and white-rot fungi may be a result of the lignin-degrading activity of white-rot fungi in which oxalic acid is consumed (Urzúa et al. 1998).

A number of roles for oxalic acid in fungal degradation and conversion of lignin have been proposed. Oxalic acid lowers the pH of fungal extracellular environment to the optimal levels (pH 2-5) that are usually needed for the activity of LMEs. Popp et al. (1990) demonstrated that oxalic acid is capable of mediating the oxidation of Mn^{2+} to Mn^{3+} via LiP and veratryl alcohol, thus enabling the oxidation of compounds that are not preferred substrates or directly oxidized by LiP. On the other hand, oxalic acid may also inhibit the LiP-catalyzed oxidation of veratryl alcohol (Akamatsu et al. 1990, Popp et al. 1990, Ma et al. 1992). Physiological concentrations of oxalic acid have been shown to stimulate MnP activity by chelating unstable Mn^{3+} (Kuan and Tien 1993). MnP can generate H_2O_2 by oxidation of oxalic and also glyoxylic acid thus providing an endogenous source for extracellular H_2O_2 (Urzúa et al. 1998).

During biopulping process with *Ceriporiopsis subvermispora* the oxalic acid secreted by the fungus forms oxalate esters that contribute to the softening of wood fibers (Hunt et al. 2004). The same mechanism has been suggested to be involved in naturally occurring white-rot decay process (Hunt et al. 2004). Oxalic acid is also shown to contribute to the decrease of wood carbohydrate content (Espejo and Agosin 1991, Shimada et al. 1994, Suzuki et al. 2006). As an indication, the brown-rot model fungus *Postia placenta* accumulates oxalic acid when colonizing wood, whereas non-decay isolate of *P. placenta* is unable to secrete oxalic acid (Micales 1997). The role of oxalic acid in cellulose degradation is discussed more in detail in section 1.4.2.

1.6.2 Oxalic-acid degrading enzymes

Three types of enzymes that catalyze oxalic acid degradation have been described from microbes and plants: oxalate decarboxylases (ODC, EC 4.1.1.2), oxalate oxidases (OXO, EC 1.2.3.4), and oxalyl-CoA decarboxylases (OXC, EC 4.1.1.8) (Fig. 5) (reviewed by Svedružić et al. 2005). ODC, isolated from fungi and bacteria, decomposes oxalic acid to formic acid and CO_2 via electron withdrawal in a very specific single-step reaction (Fig. 5a). The enzyme contains catalytical Mn^{2+} ions and requires O_2 for catalysis, although the overall reaction does not stoichiometrically utilize oxygen (Fig. 6a) (Just et al. 2004).

The evolutionarily closely related enzyme, OXO, is oxidized by O_2 but cleaves oxalic acid to two CO_2 molecules with generation of H_2O_2 (Fig. 5b). OXO is known mainly from plants, and the only two fungal OXOs are from the white-rot fungi *Ceriporiopsis subvermispora* (Aguilar et al. 1999) and *Abortiporus biennis* (Grąz et

al. 2009). In fact, *C. subvermispora* is the first fungus in which both ODC and OXO activities have been detected (Aguilar et al. 1999, Watanabe et al. 2005).

The third oxalate-cleaving enzyme, OXC, which is a bacterial enzyme, converts activated oxalyl-CoA to formyl-CoA and CO_2 (Fig. 5c). It has thiamin pyrophosphate as a cofactor. A number of bacterial species like *Bifidobacterium lactis, Lactobacillus acidophilus, Oxalobacter formigenes*, and *Thiobacillus novellus* use OXC for the breakdown of oxalate (Chandra and Shethna 1977, Anatharam et al 1989, Federici et al. 2004, Turroni et al. 2007), and the enzyme is connected to oxalate-dependent ATP synthesis at least in *O. formigenes* (Anatharam et al 1989).

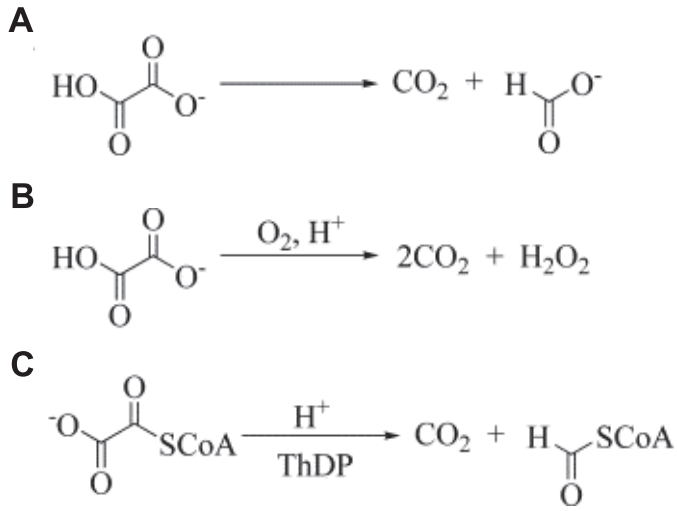


Figure 5. Schematic presentation of oxalate-degrading reactions catalyzed by A) oxalate decarboxylase, B) oxalate oxidase, and C) oxalyl-CoA decarboxylase. ThDP, thiamin pyrophosphate. The figure is modified from Svedružić et al. (2005) and reprinted with permission from Elsevier.

1.6.3 Oxalate decarboxylase

Oxalate decarboxylase (ODC) activity was first described over 50 years ago in the mycelial extracts of the white-rot fungi *Trametes (Coriolus) hirsutus* and *Flammulina (Collybia) velutipes* (Shimazono 1955, Shimazono and Hayaishi 1957). Since then ODC activity has been described for several fungal and bacterial species (Table 2). ODCs belong to the functionally diverse superfamily of cupin proteins that are present in all the three domains of life. Cupin proteins are characterized by similar primary and tertiary structure with two conserved histidine-containing, Mn^{2+}-binding motifs separated by an intermotif region, which varies in length. The cupin domain forms a conserved protein structure with a six-stranded β-barrel fold (Dunwell et al. 2000, 2004, Khuri et al. 2001). ODCs have a duplication of the cupin domain and are further classified as members of the bicupin subclass thus possessing two distinct Mn^{2+}-binding sites. The functional ODC enzyme is shown to be a hexamer comprised of two trimers of bicupin subunits (Fig. 6b) (Anand et al. 2002).

Table 2. Oxalate decarboxylases (ODC) reported for fungi and bacteria either in the level of enzyme activity, gene sequence, or protein.

Organism	Enzyme activity	Gene(s)	Protein	Reference
Ascomycetes				
Aspergillus niger	+	+[a]	n.r.[b]	Emiliani and Bekes 1964
Aspergillus phoenices	+	+[a]	n.r.	Emiliani and Bekes 1964
Myrothecium verrucaria	+	n.r.	n.r.	Lillehoj and Smith 1965
Sclerotinia sclerotiorum	+	n.r.	n.r.	Magro et al. 1988
Basidiomycetes				
Agaricus bisporus	+	n.r.	+	Kathiara et al. 2000
Coprinopsis cinerea (Coprinus cinereus)	n.r.	+[c]	n.r.	*C. cinereus* genome homepage (http://www.broad.mit.edu/annotation/genome/coprinus_cinereus/MultiHome.html)
Ceriporiopsis subvermispora	+	n.r.	n.r.	Watanabe et al. 2005
Dichomitus squalens	+	+[a]	+	publ. III and IV
Flammulina sp.	n.r.	+[a]	n.r.	Dias et al. 2006
Flammulina velutipes	+	+[a]	+	Mehta and Datta 1991
Gloeophyllum trabeum	+	n.r.	n.r.	Micales 1997
Laccaria bicolor	n.r.	+[d]	n.r.	*L. bicolor* genome homepage (http://genome.jgi-psf.org/Lacbi1/Lacbi1.home.html)
Phanerochaete chrysosporium	n.r.	+[d]	+	Sato et al. 2007
Phanerochaete sanquinea	+	n.r.	n.r.	publ. III
Pleurotus ostreatus	n.r.	+[d]	n.r.	*P. ostreatus* genome homepage (http://genome.jgipsf.org/PleosPC15_1/PleosPC15_1. home.html)
Postia placenta	+	+[d]	n.r.	Micales 1997, Martinez et al. 2009
Schizophyllum commune	n.r.	+[d]	n.r.	*S. commune* genome homepage (http://genome.jgi-psf.org/Schco1/Schco1.home.html)
Trametes ochracea	+	n.r.	n.r.	publ. III
Trametes versicolor	+	+[a]	+	Dutton et al. 1994, Zhu and Hong 2009, publ. III
Trametes hirsuta	+	n.r.	+	Shimazono 1955
Bacteria				
Agrobacterium tumefaciens	+[e]	+	+[e]	Shen et al. 2008
Bacillus subtilis	n.r.	+	+	Tanner and Bornemann 2000
Pandorea sp.	+	n.r.	n.r.	Jin et al. 2007
Synechocystis sp. PCC 6803	n.r.	n.r.	+	Kurian et al. 2006
Thermotoga maritima	n.r.	+	+[e]	Schwarzenbacher et al. 2004

[a]gene sequence(s) available in nucleotide sequence databases
[b]n.r. = not reported
[c]gene sequence(s) available at Broad Institute (http://www.broad.mit.edu/)
[d]gene sequence(s) available at DOE Joint Genome Institute (http://genome.jgi-psf.org/)
[e]heterologously produced protein in *Escherichia coli*

The most thoroughly studied ODC so far is the bacterial OxdC from *Bacillus subtilis* with high-resolution X-ray crystal structure available (Anand et al. 2002). Another crystal structure exists for the putative bacterial ODC of *Thermotoga maritima* (Schwarzenbacher et al. 2004). *B. subtilis* OxdC is the model enzyme in the catalytic mechanism studies of ODC enzymes. In the *B. subtilis* OxdC, a pentapeptide loop (amino acid residues 161-165) contains a specific amino acid residue, Glu-162, which forms a so called lid structure that controls the enzyme activity, and is shown to determine the reaction specificity (Burrell et al. 2007, Svedružić et al. 2007).

Conversion of oxalic acid to formic acid is believed to take place in the N-terminal domain of *B. subtilis* OxdC (Just et al. 2004, 2007, Burrell et al. 2007, Svedružić et al. 2007) but recent data suggests that both N- and C-terminal domains can catalyze the decarboxylation reaction (Moomaw et al. 2009). *B. subtilis* OxdC is one of the most abundant cell wall proteins expressed under acid stress conditions. However, the mechanism of translocation of the protein to the bacterial cell wall is unclear since the primary sequence lacks N-terminal signal peptide and conserved cell-wall-binding domains (Antelmann et al. 2007). In addition, the role of *B. subtilis* OxdC is still poorly understood, although it has been proposed that the enzyme protects bacterial cells against low-pH-stress by consuming protons via decarboxylation of oxalic acid, in particular in soil where oxalate-rich plant biomass is present (Tanner and Bornemann 2000, MacLellan et al. 2009).

At both gene and protein level, the currently best-described fungal ODC is from a hardwood and litter-decomposing white-rot basidiomycete *Flammulina velutipes* (Mehta and Datta 1991, Kesarwani et al. 2000, Azam et al. 2001, 2002, Chakraborty et al. 2002). Within the few other basidiomycetous species from which ODC has been purified and characterized, the molecular mass of the ODC bicupin subunit ranges from ca 50 to over 60 kDa, and the protein shows typically two isoforms with acidic p*I* values (Table 3).

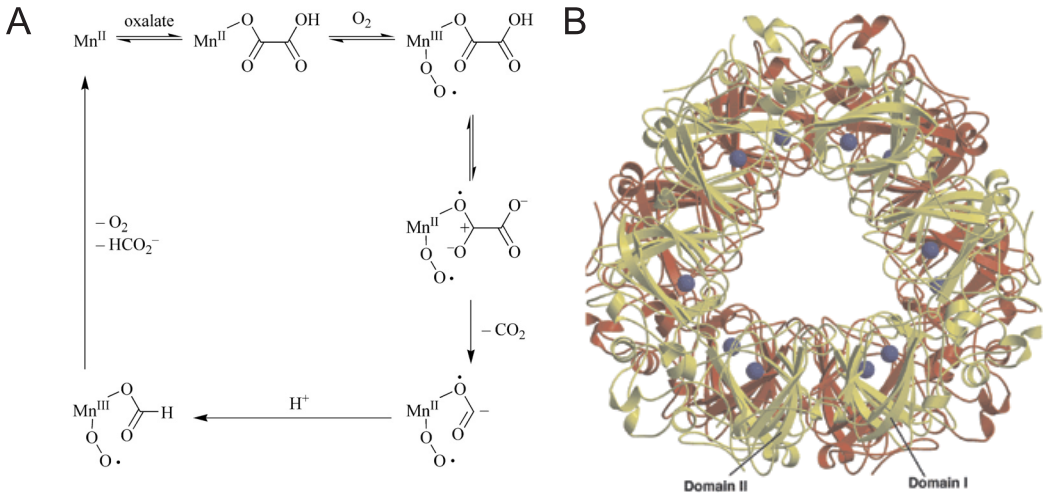


Figure 6. A) Proposed catalytic mechanism of oxalate decarboxylase. Modified and reprinted from Just et al. (2004) with permission from American Society for Biochemistry and Molecular Biology. B) 3D protein model showing the hexameric structure of functional *Bacillus subtilis* ODC (Anand et al. 2002). Two trimeric layers are shown with different colouring and Mn^{2+} ions are depicted with blue spheres. Reprinted with permission from American Chemical Society.

Table 3. Biochemical properties of oxalate decarboxylase (ODC) proteins from basidiomycetous fungi. The molecular mass accounts for one bicupin subunit of the active enzyme.

Fungal species	Molecular mass (kDa)	p*I*	Reference
Agaricus bisporus	64, deglycosylated protein 55	3.0, 3.4	Kathiara et al. 2000
Dichomitus squalens	52-55	2.6, 4.2	publ. IV
Flammulina (Collybia) velutipes	64	2.5, 3.3	Mehta and Datta 1991
Trametes (Coriolus) versicolor	59	2.3, 3.0	Dutton et al. 1994
Phanerochaete chrysosporium	52	nd[a]	Sato et al. 2007

[a]not determined

Recently, the whole genome sequencing and annotation projects have revealed several new putative ODC sequences from both bacteria and fungi, but the genes and their protein products have remained mostly uncharacterized. For example, annotation of the whole genome sequence of *Phanerochaete chrysosporium* revealed up to seven putative ODC-encoding sequences (http://genomeportal.jgi-psf.org/Phchr1/Phchr1.home.html), and the brown-rot fungus *Postia placenta* whole genome sequence shows three putative ODC-encoding genes and their allelic forms (Martinez et al. 2009).

Fungal ODCs are considered mainly as intracellular enzymes predominantly located close to the plasma membrane and in vesicles (Dutton et al. 1994, Kathiara et al. 2000). However, small amount of ODC protein and active enzyme was observed to be secreted either to the fungal cell wall and extracellular polysaccharide layers, or to culture medium (Dutton et al. 1994, Micales 1997, Kathiara et al. 2000, Sato et al. 2007).

F. velutipes ODC contains a functional N-teminal secretion signal sequence (Azam et al. 2002) and a putative secretion signal is similarly present in the primary amino acid sequence of *P. chrysosporium* ODC (Sato et al. 2007). In addition to the liquid cultivations, extracellular ODC from *P. chrysosporium* (Sato et al. 2007) and intra- and extracellular ODC from *Trametes versicolor* (Dutton et al. 1994) have been detected from solid-state cultures of red oak and beech wood, respectively.

Earlier, the main role of fungal ODCs has been assumed to be controlling of the intracellular levels and the secretion of oxalic acid. Secondly, ODC has been proposed to maintain steady pH levels and oxalate anions outside the fungal hyphae by decomposing extracellular oxalic acid (Micales 1997). More recently, ODC has been proposed to act sequentially with its reaction product, i.e. formic acid, degrading enzyme formate dehydrogenase (FDH, EC 1.2.1.2.) inside the fungal cells (Watanabe et al. 2005). FDH, which has been shown to operate in higher plants, yeasts, and bacteria, catalyzes NAD^+-dependent, oxidative degradation of formic acid resulting in the formation of CO_2 and NADH (reviewed by Popov and Lamzin 1994). The produced NADH could thereby be used for ATP synthesis during the fungal vegetative growth (Watanabe et al. 2005). A corresponding energy-producing mechanism has been described in methanol-utilizing yeasts (Popov and Lamzin 1994). In basidiomycetes, this hypothesis is supported by recent results from the brown-rot fungus *P. placenta* that showed simultaneous up-regulation of one putative *odc* and three *fdh* genes when grown on cellulose medium (Martinez et al. 2009). ODC protein and *fdh* transcripts have also been detected in *P. chrysosporium* when the fungus is cultivated on solid wood (Sato et al. 2007, 2009). Similar enzymatic cooperation is probably common in all the ODC-producing fungi, and intracellular FDH activity has in fact been observed in *F. velutipes*, *Trametes versicolor*, and *Schizophyllum commune* (Watanabe et al. 2005). The biotechnological applications of ODC enzymes are discussed in section 1.10.

1.7 Basidiomycete genomes and lignocellulose decay

At the moment (August 2009), nine complete genome sequences of different basidiomycetous fungal species are available. The representatives of divergent basidiomycetous subphyla and order have been among the first fungi selected for whole genome sequencing. These include the crop plant pathogens *Ustilago maydis* (Kämper et al. 2006) and *Puccinia graminis* (http://www.broad.mit. edu/annotation/genome/puccinia_graminis/MultiHome.html) and the coniferous tree pathogen *Heterobasidion annosum* spp. (http://www.jgi.doe.gov/genome-projects/), which cause remarkable economic losses, as well as a human pathogen, *Cryptococcus neoformans* (Loftus et al. 2005). Also species which have been studied as model organisms for fungal genetics and development, i.e. *Coprinopsis cinerea* (*Coprinus cinereus*) and *Schizophyllum commune* have been targets of whole genome sequencing projects (Table 4).

Current sequencing efforts are turning from yeasts and pathogens to other filamentous fungi due to their applicability for diverse biotechnological processes, in particular in conversion of lignocellulose and plant material for production of biofuels and sustainable energy. The whole genome sequence of the ecologically interesting ectomycorrhizal symbiotic species *Laccaria bicolor* has been annotated (Martin et al. 2008). The first published white-rot fungal and also basidiomycetous whole genome sequence was from *Phanerochaete chrysosporium* (Martinez et al. 2004), and it promoted vital progress in the molecular genetics of lignin-degrading white-rot fungi. Very recently, genome sequence data of two other wood-colonising, saprobic white-rot fungi, i.e. *S. commune* (http://genome.jgi-psf.org/Schco1/Schco1.home.html) and *Pleurotus ostreatus* (http://genome.jgi-psf.org/PleosPC15_1/PleosPC15_1.home.html), came available. In addition, the first published whole genome sequence of a brown-rot fungus, *Postia placenta* (Martinez et al. 2009), adds up to the pool of fungi which have biotechnological interest e.g. for biomass conversion. Table 4 summarizes genomic characteristics of the saprobic, wood and soil-inhabiting, and symbiotic basidiomycetes, and some of their annotated genes which are relevant to degradation and conversion of lignocellulose.

Present genomic data clearly suggests a specific role for the lignin-modifying peroxidases (LiPs, MnPs, VPs) in decomposition of lignin since the corresponding genes are found solely in the white-rot fungi (Table 4). Laccases are absent from *P. chrysosporium* genome and therefore they might be unessential for lignin degradation. Instead, the *P. chrysosporium* genome contains other types of multicopper oxidases, such as Fe-oxidoreductases (Larrondo et al. 2007), which are essential for other cellular functions. For laccases, roles in processes other than lignin decay are probable while even up to 17 laccase genes are found in the genome of the non-lignin-decaying, soil-inhabiting basidiomycete *C. cinerea* (Kilaru et al. 2006). In another non-lignin-degrading basidiomycete *L. bicolor*, 9 laccase-encoding genes have been annotated (Courty et al. 2009). However, the importance of laccases is emphasized also for lignin-degrading white-rot fungi that harbour multiple laccase genes (discussed in section 1.3.2), which is further confirmed by whole genome sequence data from *P. ostreatus* with 12 putative laccase-encoding genes (Table 4).

On the contrary, the oxalic-acid decaying ODC and formic-acid decaying FDH are ubiquitous among the basidiomycetous genomes, which points to a more universal role

Table 4. Characteristics of the sequenced genomes of wood- and soil-inhabiting and symbiotic basidiomycetes (situation in August 2009).

Fungus	Genome size[a] (Mbp)	Number of (putative) genes encoding								Number of CAZymes[b]			Reference
		Proteins	Laccase	LiP	MnP	VP	ODC	FDH	OXO	Glycoside hydrolase	Containing carbo-hydrate-binding module	Containing-cellulose-binding module	
Coprinopsis cinerea (Coprinus cinereus)	37.5	13 544	17	-[c]	-[c]	-[c]	1	1	0	211	26	1	*C. cinereus* genome homepage (http://www.broad.mit.edu/annotation/genome/coprinus_cinereus/MultiHome.html)
Laccaria bicolor	64.9[d]	20 614	9	-[c]	-[c]	-[c]	1	1	1	163	45	30	Martin et al. 2008 (http://genome.jgi-psf.org/Lacbi1/Lacbi1.home.html)
Phanerochaete chrysosporium	35.1	10 048	-[c]	10	5	-[c]	7	2	0	180	89	46	Vanden Wymelenberg et al. 2006 (http://genome.jgi-psf.org/Phchr1/Phchr1.home.html)
Pleurotus ostreatus	34.3	11 603	12	several	several	several	2	5	0	?[e]	?[e]	?[e]	*P. ostreatus* genome homepage (http://genome.jgi-sf.org/PleosPC15_1/PleosPC15_1.home.html)
Postia placenta	90.9[d]	17 173	2	-[c]	-[c]	-[c]	3	3	0	144	6	-[c]	Martinez et al. 2009 (http://genome.jgi-psf.org/Pospl1/Pospl1.home.html)
Schizophyllum commune	38.5	13 181	8	?[e]	?[e]	?[e]	5	7	5	?[e]	?[e]	?[e]	*S. commune* genome homepage (http://genome.jgi-psf.org/Schco1/Schco1.home.html)

[a]haploid, if not otherwise depicted
[b]adapted from Martinez et al. 2009
[c]not present
[d]dikaryon
[e]not yet determined

of these enzymes in fungal physiology. Putative genes coding for ODC and FDH are found in all the lignocellulose-converting basidiomycetes so far annotated (Table 4). The oxalic-acid degrading enzyme OXO is until now reported from only two fungi, *Ceriporiopsis subvermispora* (Aguilar et al. 1999) and *Abortiporus biennis* (Grąz et al. 2009). The rareness of OXO in fungi is supported by the available whole genome sequence of lignocellulose-converting basidiomycetes showing the presence of putative OXO-encoding genes only in *L. bicolor* and *S. commune* (Table 4).

According to the current genomic data, strategies for cellulose degradation obviously differ between white- and brown-rot fungi. The genome of the brown-rot fungus *P. placenta* harbours several *β*-glucosidase-encoding genes, but totally lacks the type of carbohydrate-active enzymes (CAZymes) that contain cellulose-binding modules, and has only two putative endoglucanase-encoding genes (Martinez et al. 2009). On the contrary, the whole repertoire of multiple cellulose-decomposing enzymes (endoglucanases, cellobiohydrolases, *β*-glucosidases), with and without cellulose-binding modules, is present in the *P. chrysosporium* genome (Martinez et al. 2004) (Table 4). In this respect, the *P. chrysosporium* genome resembles that of the efficient cellulose-decaying, soft-rotting ascomycetous fungus *Trichoderma reesei* (Martinez et al. 2008).

Despite the accumulating genomic data, physiologically related but taxonomically divergent white-rot fungal species are still needed to be sequenced in order to reveal more of the genetic factors required for efficient lignin-degradation. With multiple whole genome sequences from near- and far-related fungal taxons, comparative genome analyses may be carried out to investigate fungal wood-decay strategies. In addition, more whole genome sequences of white-rot fungi representing selective and non-selective degraders of lignin are needed in order to understand the fundamental differences in decomposition of lignocelluloses. The forthcoming whole genome sequence from the selective lignin-degrading white-rot fungus *Ceriporiopsis subvermispora*, the brown-rot fungus *Serpula lacrymans,* and the litter-decomposing, edible fungus *Agaricus bisporus*, are currently under refining and annotation (http://www.jgi.doe.gov/genome-projects/). Together with the accumulating whole genome sequence data, novel genetic transformation systems for basidiomycetous species together with efficient and precise gene knock-out and silencing systems are needed to untangle the mechanisms of lignin and lignocellulose degradation. In fact, encouraging progress in fungal gene silencing by RNA interference technique was recently shown in *P. chrysosporium* (Matityahu et al. 2008).

1.8 *Phlebia radiata*

Phlebia radiata Fr. ("rusorypykkä" in Finnish) is a common white-rot fungus that typically grows on dead deciduous trees as a saprotroph (Fig. 7a). *P. radiata* was previously systematically classified to the basidiomycetous family Corticiaceae under the order Aphyllophorales (Hibbett and Thorn 2001). It has recent proposed to be positioned within a separate Phlebioid clade of the subphylum Agaricomycotina (Binder et al. 2005, Hibbett et al. 2007). Molecular analysis of 18S and ITS rDNA have positioned the *P. radiata* strains into a coherent species-level clade near to *P. acerina* but well separated from e.g. *P. brevispora*, *P. tremellosa (Merulius tremellosus), Phlebiopsis gigantea*,

and *Phanerochaete chrysosporium* (Nakasone and Sytsma 1993, Dresler-Nurmi et al. 1999, de Koker et al. 2003, Hildén et al. 2008). Physiologically, *P. radiata* belongs to the rare group of white-rot fungi which express a multitude of extracellular LMEs including isozymes of laccases, LiPs, and MnPs (Lundell 1993, Hatakka 1994). Also H_2O_2-producing glyoxal oxidase (GLOX) has been detected in the cultures of *P. radiata* (Lundell 1993, Vares et al. 1995).

The wood and lignin-decomposing properties of this fungal species and its ability to degrade complex aromatic compounds have been the targets of various examinations. Special interest has been focused on the efficiently lignin-degrading, Finnish isolate of *P. radiata* FBCC43 (79, ATCC 64658) (Hatakka and Uusi-Rauva 1983). Table 5 summarizes the current knowledge of LMEs and LME-encoding genes of the *P. radiata* FBCC43, including the results of the current study and thesis work.

Table 5. Lignin-modifying enzymes, their biochemical properties, and corresponding genes described from *Phlebia radiata* FBCC43 (isolate 79).

Enzyme	Molecular mass (kDa)	p*I*	Corresponding gene	Reference
Lignin peroxidases				
LiP1	38-40	4.1-4.2	*Pr-lip1*	Niku-Paavola et al. 1988, Lundell and Hatakka 1994, publ. I
LiP2	44-45	3.9-4.0	-[a]	Niku-Paavola et al. 1988, Lundell and Hatakka 1994, Vares et al. 1995
LiP3/LIII	44-47	3.2-3.4	*Pr-lip3/lgp3*	Niku-Paavola et al. 1988, Saloheimo et al. 1989, Lundell and Hatakka 1994, publ. I
LiP4	-[b]	-[b]	*Pr-lip4*	publ. I
Manganese peroxidases				
MnP1 (MnPx)	46-47	4.7-4.9	-[a]	Lundell and Hatakka 1994, Moilanen et al. 1996
MnP2 (MnP)	47-50	3.7-3.8	*Pr-mnp2*	Karhunen et al. 1990a, Lundell and Hatakka 1994, Moilanen et al. 1996, Hildén et al. 2005
MnP3	43-45	3.6	*Pr-mnp3*	Vares et al. 1995, Moilanen et al. 1996, Hildén et al. 2005
Laccases				
Laccase1/Lacc1	63-64	3.4-3.5	*Pr-lac1*	Niku-Paavola et al. 1988, Saloheimo et al. 1991, Lundell and Hatakka 1994, Vares et al. 1995
Lac2	-[b]	-[b]	*Pr-lac2*	publ. II
Glyoxal oxidases				
GLOX1-2	67-68	4.0-4.8	-[a]	Lundell 1993, Vares et al. 1995

[a]gene(s) not characterized

[b]protein not characterized

The ability of *P. radiata* FBCC43 to efficiently decompose lignocellulose and produce enzymes required for that was studied already in the 1980's (Hatakka 1986). The fungus decomposes spruce wood (Hakala et al. 2004) and converts and mineralizes natural-type wood and milled-wood lignins (Hatakka and Uusi-Rauva 1983, Hofrichter et al. 2001), synthetic lignins, and various lignin model compounds (Lundell et al. 1990, Hatakka et al. 1991, Lundell 1993, Moilanen et al. 1996, Niemenmaa et al. 2006). It grows well in artificial liquid medium (Lundell et al. 1990, Niku-Paavola et al. 1990, Rogalski et al. 1991) and in bioreactors as immobilised mycelium (Kantelinen et al. 1989, Lankinen et al. 1991), as well as on solid lignocelluloses, such as chipped or milled softwood and hardwood (Hakala et al. 2004, Hildén et al. 2005, this study: publications I and II) and on wheat straw (Vares et al. 1995).

Amendment of even small concentration of glucose to the cultures of *P. radiata* FBCC43 reduces cellulose degradation and simultaneously enhances lignin degradation (Cho et al. 2009). Also, the degradation of hemicellulose and lignin by the fungus has been noticed to be linked together (Cho et al. 2009). Another strain of *P. radiata* (from the culture collection of the Institute of Chemical Engineering, University of Technology, Vienna, Austria) significantly reduces the amount of spruce wood lignin during the first four days of cultivation indicating that the degradation of lignin occurs already at the primary metabolic phase of this species (Fackler et al. 2006).

Of the various LMEs of *P. radiata*, three LiPs, three MnPs, and one laccase isozyme have been isolated and studied at protein level. The purified LiP fractions were shown to be efficient biocatalysts with similar biochemical properties as reported for the *Phanerochaete chrysosporium* LiPs (Lundell et al. 1992, 1993b). In particular, the *P. radiata* LiP3 (LIII), which is the dominant LME isozyme expressed by the fungus in lignocellulose-containing media (Niku-Paavola et al. 1990), was demonstrated to be an efficient oxidant causing carbon-carbon bond cleavage of a dimeric, non-phenolic lignin model compound (Lundell et al. 1993a).

The partially purified MnP fraction of *P. radiata* is similarly capable of decomposing lignin model compounds via lipid peroxidation (Kapich et al. 1999) and furthermore, the species has been shown to produce unsaturated lipids during wood decay (Gutiérrez et al. 2002). In agreement with these results, oxidation of the non-phenolic lignin model compound by Pr-MnP3 was observed in the presence of pine wood (Hofrichter et al. 2001). *P. radiata* produces a number of CAZymes including diverse cellulases and hemicellulases (Rogalski et al. 1993a, b, c, 2001, Prendecka et al. 2003, 2007, Mierzwa et al. 2005, Table 6) confirming that this fungus is able to decompose all the major components of wood.

The unspecific oxidation ability of the white-rot fungal LMEs, which apparently evolved to decompose the complex lignin polymer, may also be exploited e.g. in bioremediation applications, to degrade recalcitrant xenobiotics and environmental pollutants. The ability of several *Phlebia* species (e.g. *P. radiata*, *P. brevispora*, *P. tremellosa*, and *P. lindtneri*) to convert and even detoxify a variety of chemical pollutants including trichloroanisole (Campoy et al. 2009), trinitrotoluene (Van Aken et al. 1999), dioxins (Kamei et al. 2009), polycyclic aromatic hydrocarbons (PAHs) (Mori et al. 2003), polychlorinated biphenyls (PCBs) (Kamei et al. 2006), and phthalates (Kim et al.

2008b) has been reported. These data implicate the usefulness of *P. radiata* FBCC43 and other strains belonging to the genus *Phlebia* in environmental biotechnology.

Table 6. Wood polysaccharide degrading enzymes produced by *P. radiata* FBCC43 (isolate 79).

Enzyme	Molecular mass (kDa)	p*I*	Reference
Cellulases			
endo-*β*-1,4-glucanase, exo-*β*-1,4-glucanase, aryl-*β*-1,4-glucosidase, *β*-1,4-glucosidase	-[a]	-[a]	Rogalski et al. 1993c
Hemicellulases			
β-mannosidases	89.5, 100.3, 104.6	3.8, 4.7, 4.8	Prendecka et al. 2007
endo-1,4-*β*-xylanases	15.8, 18.6	4.1, 6.7	Rogalski et al. 2001
β-xylosidase	27	5.9	Rogalski et al. 2001
α-galactosidases	55-64	3.5-7.2	Prendecka et al. 2003
α-D-glucuronidase	110	4.4	Mierzwa et al. 2005
endo-1,4-*β*-mannanase, exo-1,4-*β*-mannanase, *α*-L-arabinofuranosidase, acetyl esterase, ferulic acid esterase	-[a]	-[a]	Rogalski et al. 1993a

[a]not determined

1.9 *Dichomitus squalens*

Dichomitus squalens (P. Karst.) D.A. Reid ("salokääpä" in Finnish, synonyme *Polyporus anceps*) is an effective lignin-degrading white-rot basidiomycete. The basidiocarps of *D. squalens* are usually found on old coniferous trunks fallen by storm and on charred tree after forest fire (Fig. 7b) (Niemelä 2005). In Finland, *D. squalens* has become rarer apparently due to more efficient forest fire prevention, and it is defined as a near-threatened polypore species (Niemelä 2005).

D. squalens belongs to the large group of white-rot fungi that express a set of laccases and MnPs as their LMEs (Périé and Gold 1991, Hatakka 1994). In various studies, it has been shown that different strains of the fungus efficiently degrade both natural and synthetic lignins (Blanchette et al. 1987, Périé and Gold 1991, Lang et al. 2000, Tuomela et al. 2002) and decompose softwood (spruce) lignin (Hakala et al. 2004, Fackler et al. 2006). *D. squalens* quickly diminishes the amount of spruce wood lignin

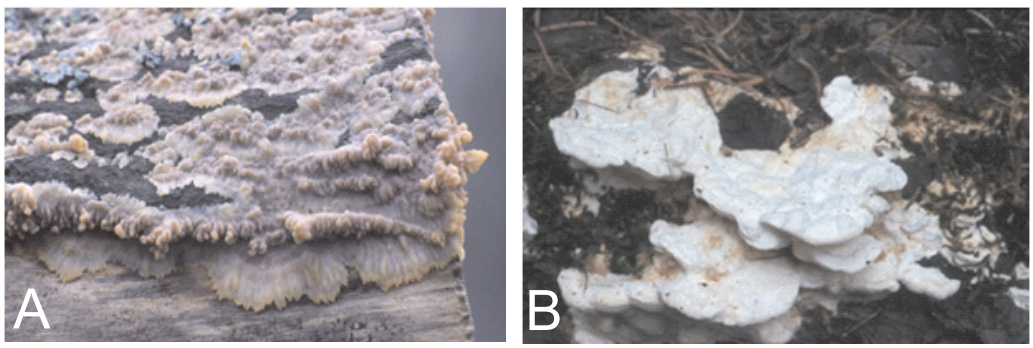


Figure 7. Fruiting body of A) *Phlebia radiata* (photo:Yu-Cheng Dai) and B) *Dichomitus squalens* (photo: Kari Steffen).

within the first days of cultivation. This points out to the rapid induction of the lignin-degrading machinery of the fungus already at the primary growth phase (Fackler et al. 2006).

Up to four chromatographic forms of extracellular *D. squalens* laccases have been demonstrated in the strains CBS 432.34 (Périé et al. 1998) and CCBAS 750 (Šušla et al. 2007). Two MnP isozymes of *D. squalens* CBS 432.34 have been isolated, biochemically characterized, and the genes have been cloned (Périé et al. 1996, Li et al. 1999). The gene encoding the thermostable MnP of *D. squalens*, *mnp2*, was heterologously expressed in *Phanerochaete chrysosporium* (Li et al. 2001). Recently, three additional chromatographic isoforms of *D. squalens* MnP were reported from the strain CCBAS 750 (Šušla et al. 2008). Table 7 summarizes the existing data of *D. squalens* LMEs and LME-encoding genes. Also cellulase and hemicellulase-degrading enzymes are secreted by *D. squalens* (Rouau and Odier 1986).

D. squalens has been studied due to its possible use in several biotechnological applications. *D. squalens* decomposes lignin selectively, having potential in biopulping, i.e. the fungal pretreatment of wood chips prior to paper manufacture with intention to reduce the consumption of electricity or chemicals (Blanchette 1984, Fackler et al. 2006). In extensive studies, this fungus has been demonstrated to be effective decolourizer of various synthetic dyes, which makes *D. squalens* promising for bioremediation of textile waste waters (Gill et al. 2002, Chander et al. 2004, Eichlerová et al. 2005, 2006, 2007, Chander and Arora 2007, Šušla et al. 2008). Furthermore, purified laccases of *D. squalens* decolorize the anthraquinone dye Remazol Brilliant Blue R without addition of redox mediator compounds (Šušla et al. 2007).

D. squalens degrades toxic compounds like polycyclic aromatic hydrocarbons (PAHs) (in der Wiesche et al. 1996, Martens and Zadrazil, 1998) and dibenzo-*p*-dioxin (Rosenbrock et al. 1997). The ability of *D. squalens* (strain CBS 432.34) to produce natural flavour compounds such as benzaldehyde (bitter almond aroma) and benzyl alcohol from L-phenylalanine has been noticed (Lapadatescu et al. 1997), and

Table 7. Lignin-modifying enzymes, their biochemical properties, and their corresponding genes described from *Dichomitus squalens*.

Enzyme	Molecular mass (kDa)	p*I*	Corresponding gene	Reference
Manganese peroxidases				
MnP1	48	4.1	*mnp1*	Périé et al. 1996, Li et al. 1999
MnP2	48.9	3.8-3.9	*mnp2*	Périé et al. 1996, Li et al. 1999
MnP3-5	50	3.5	-[a]	Šušla et al. 2008
Laccases				
Laccases 1 & 2	66	3.5-3.6	-[a]	Périé et al. 1998
Lc1	68	3.2	-[a]	Šušla et al. 2007
Lc2	68	3.1	-[a]	Šušla et al. 2007
Lac3	-[b]	-[b]	*Ds-lac3*	this work
Lac4	-[b]	-[b]	*Ds-lac4*	this work

[a]gene(s) not characterized
[b]protein not characterized

the biocidal activity of *D. squalens* (strain SC0197) sesquiterpenes against pine wood nematodes has been reported (Huang et al. 2004).

1.10 White-rot fungi and their enzymes in biotechnological applications

Several biotechnological applications take advantage of white-rot fungi and their LMEs. For example the utilization of fungi in the pulp and paper industry has been intensively studied. Biopulping has gained a lot of interest in the past decades (Kirk and Chang 1990, Akhtar et al. 1997). Selectively lignin-degrading white-rot fungi, e.g. *Ceriporiopsis subvermispora*, *Physisporinus rivulosus*, and *Dichomitus squalens*, have been regarded as the most suitable organisms for biopulping, to minimize cellulose loss during the fungal pretreatment (Blanchette 1984, Hakala 2007).

The direct use of white-rot fungal LMEs in pulp and paper industry could result in easier optimization and applicability as compared to fungal treatment. For example the use of MnP has been demonstrated to improve pulp bleaching (Moreira et al. 2003, Feijoo et al. 2008) and decrease consumption of refining energy (Maijala et al. 2008) during paper manufacturing.

The unspecific nature and high redox potential of the lignin-modifying peroxidases (LiPs, MnPs, VPs) enable them to convert various recalcitrant compounds. Therefore lignin-modifying peroxidases have been extensively studied in bioremediation of e.g. soil and industrial effluents contaminated with various harmful compounds of natural and anthropogenic origin (reviewed by Hofrichter 2002, Husain 2006, Raghukumar et al. 2008, and Haritash and Kaushik 2009). However, the industrial use of fungal peroxidases is still hindered by their high cost and low yields so far gained using heterologous expression systems. Also the limited availability and low stability of the enzymes as well as their inactivation by H_2O_2 and elevated temperatures are problems in large-scale production and in industrial applications (reviewed by Martínez et al. 2009).

Laccases, with prospects in diverse industrial areas, are the most studied fungal oxidoreductases for biotechnological applications. Laccases have potential e.g. in food industry to improve dough properties, and in cosmetic industry laccases may be added in products intended for skin lightening and hair dyeing. In biotechnological applications laccases have potential as biosensors and as enzyme-electrodes in biofuel cells (reviewed by Couto and Toca Herrera 2007). Attention is paid also to the exploitation of laccases in synthesis of new biomaterials and polymers (reviewed by Mikolasch and Schauer 2009).

In forest products industry, laccases could be used in biografting of low molecular weight compounds onto lignocellulosic materials, and in cross-linking of fibers and lignin moieties for the production of wood composite products (Widsten and Kandelbauer 2008a). Furthermore, laccases could be used in biopulping, pitch control, deinking, and process water treatment, among many other applications (reviewed by Widsten and Kandelbauer 2008b). In fact, commercial laccase and laccase-mediator applications e.g. for pulp and paper (delignification) and textile industry (bleaching) are already available (Morozova et al. 2007b).

Since specific enzyme properties are needed for different applications, molecular characterization of new fungal laccases has been in focus. One property often desired in industrial processes is enzyme thermotolerance, and some thermotolerant and thermostable laccases from basidiomycetes have been described (reviewed by Hildén et

al. 2009). The commercial use of laccase mediators is still often hindered by their high cost and lack of information on their toxicological safety. Recently, efforts have been made to find efficient naturally occurring and cost-effective laccase mediators, which could remove these obstacles (Camarero et al. 2005, 2007).

The ascomycetous fungal genera *Aspergillus* and *Trichoderma* comprise several isolates demonstrating excellent protein production and secretion capacity, and are the most promising host organisms for the heterologous expression of basidiomycetous enzymes, such as the LMEs. Furthermore, the use of fungal hosts has an advantage over bacterial systems because fungi perform the correct post-translational modifications needed for enzyme activity. Although a few studies show successful heterologous production of e.g. MnP in ascomycetous hosts, the main problem is the incorporation of heme to achieve a reasonable yield of active recombinant peroxidase (Stewart et al. 1996, Conesa et al. 2000, 2002).

Heterologous expression of laccases by filamentous fungi with increased enzyme production levels has been demonstrated in some cases (Record et al. 2002, Kiiskinen et al. 2004). Still, the lack of an efficient heterologous production system for laccases at bioreactor scale is constraining more bulky and industrial applications (Couto and Toca-Herrera 2007). Fungal laccases with improved catalytic properties and increased stability have been achieved by the use of molecular evolution techniques (Hu et al. 2007, Festa et al. 2008). Future studies will concentrate on improvement of the catalytic properties of fungal LMEs by mutagenesis, and to increase the production yield of active recombinant enzyme in order to fit the requirements of large-scale production.

The global demand for the use of renewable materials for production of energy and consumables has been expanded. So called biorefinery concept aims at the co-production of various value-added end products like biofuels and chemicals in advanced biotechnological processes (e.g. enzymatic hydrolysis followed by microbial fermentation) from renewable biomass. Forest and agricultural waste lignocelluloses form a massive source of renewable biomass that can be used as a feedstock for biorefining (reviewed by Kamm and Kamm 1994 and Kumar et al. 2008, Foust et al. 2008). Since lignin in plant cell walls prevents the efficient use of cellulose and hemicellulose, its removal is a key step for the use of cellulosic biomass. This highlights the role of white-rot fungi and their enzymes as environmentally friendly biocatalysts for the pretreatment of lignocelluloses (Ruiz-Dueñas and Martínez 2009).

In addition to the LMEs, other fungal enzymes are biotechnologically promising as well. For example, oxalic-acid degrading enzymes like ODC that catalyze highly specific reactions have several potential and established uses in diverse biotechnological applications. One common problem in many industrial processes is the formation of oxalate salt deposits which may harmfully clog pipeworks and filters. In order to prevent formation of calcium oxalate, the so called scaling, fungal ODC has been tested for removal of oxalic acid from the bleaching filtrates of the pulp and paper factories (Nilvebrant et al. 2002, Sjöde et al. 2008).

Commercial assays are available to use ODC for determination of oxalic acid concentrations in clinical and food samples. Excessive excretion of urinary oxalate (hyperoxaluria) can lead to the formation of calcium oxalate precipitates which end up with the formation of kidney stones. To find the treatment for hyperoxaluria, oral therapy

with crystalline cross-linked formulation of ODC has been shown to reduce symptoms in experiments with mice (Gruijic et al. 2009). Furthermore, lactic acid bacteria expressing heterologous ODC could be used as possible probiotics for depletion of intestinal, dietary oxalic acid (Kolandaswamy et al. 2009).

In diverse application studies on fungal ODC, one goal has been the construction of *odc*-expressing transgenic crop plants. Contributing to their reduced content of oxalic acid, transgenic plants have proved to be resistant to certain oxalic-acid secreting pathogenic fungi. These plants are also less toxic to herbivores, which lack oxalate-degrading enzymes and thereby are dependent on intestinal bacteria to catabolize the dietary oxalic acid (Kesarwani et al. 2000, Dias et al, 2006).

2 Objectives of the study

The main focus of this work was the ecologically and biotechnologically interesting white-rot fungi *Phlebia radiata* and *Dichomitus squalens*. One purpose was to increase the molecular-level data on the lignin-modifying enzymes of the well-studied Finnish isolate *P. radiata* FBCC43 (79, ATCC 64658). With the selectively lignin-degrading species *D. squalens*, two distinct strains (Finnish isolate FBCC184 and FBCC312) were investigated.

It was postulated that the use of the natural substrate wood would promote the production of enzymes and compounds needed for lignocellulose breakdown. While the lignin-modifying enzymes of white-rot fungi have been intensively studied for almost 30 years, knowledge of the expression of the corresponding genes on wood is at the moment limited to the white-rot model fungus *Phanerochaete chrysosporium*. Therefore there was a special interest to deepen the understanding of the expression of lignin-modifying enzymes at mRNA level during the growth of *P. radiata* and *D. squalens* on wood.

The second main objective was to follow the production of organic acids when fungi grow both in liquid medium and on wood. The significance of fungal secreted organic acids, especially oxalic acid, in biodegradation of lignocellulose is becoming more and more noticed. However, regulation of oxalic-acid metabolism is not widely studied among wood-decaying fungi, and so far, only a few reports of fungal production of organic acids on lignocellulose substrates are available. To clarify the occurrence of the oxalate-degrading enzyme oxalate decarboxylase (ODC) and its role in white-rot fungi, the ODC-producing *D. squalens* strains were chosen for a more detailed study on ODC expression and cloning of the corresponding genes, to facilitate primary protein-level characterization and further work on this enzyme.

The specific aims of this study were:

1. To clone and characterize at molecular level the various lignin-peroxidase-encoding genes of *P. radiata* (Publication I).
2. To find out whether *P. radiata* harbours more than one laccase-encoding gene, and to characterize a new *P. radiata* laccase at molecular level (Publication II).
3. To clone and characterize laccase-encoding genes of *D. squalens*, and to follow their expression when the fungus grows on wood.
4. To follow expression of lignin-modifying-enzyme-encoding genes of *P. radiata* during growth in wood-containing cultures (Publications I and II).
5. To determine culture conditions for the production of high laccase activity in *P. radiata*.
6. To evaluate production of organic acids and oxalate decarboxylase (ODC) activity among well wood-decaying white-rot fungi (Publication III).
7. To characterize ODC of *D. squalens* at the protein and gene level.
8. To study the expression of *Ds-odc* on wood and in liquid cultures in order to clarify *odc* gene regulation at transcriptional level (Publication IV).

3 Materials and methods

The fungal strains used in this study were obtained from the culture collection situated at the Department of Applied Chemistry and Microbiology, University of Helsinki, Finland, and they are listed in Table 8. The culture collection is from 2008 reorganized as Fungal Biotechnology Culture Collection (FBCC, University of Helsinki, Helsinki, Finland, fbcc@helsinki.fi)

The methods used in this study are summarized in Table 9 and are described in more detail in the original publications I-IV and in the chapters 3.1-3.4.

Table 8. White-rot basidiomycete strains used in this study. Systematic fungal names together with their current FBCC identifier (number) and original numbers and codes in other culture collections are shown.

Fungal strain	FBCC number	Original strain number	Used in publication no.
Ceriporiopsis subvermispora	FBCC314	CZ-3-FPL	III
Dichomitus squalens	FBCC184	PO114	III, IV
Dichomitus squalens	FBCC312	A-670	IV
Haploporus odorus	FBCC804	T154	III
Phanerochaete chrysosporium	FBCC283	F1767, ATCC 24725	III
Phanerochaete sanguinea	FBCC712	T51	III
Phlebia radiata	FBCC43	79, ATCC 64658	I, II
Phlebia sp. (Nf b19)	FBCC464	DSM 11239; previously *Nematoloma frowardii* b19	III
Phlebiopsis gigantea	FBCC719	T55	III
Radulodon erikssonnii	FBCC752	T84	III
Trametes ochracea	FBCC682	T7	III
Trametes versicolor	FBCC298	PRL 572	III
Trametes versicolor	FBCC324	R/7, ATCC 44308	III
Trichaptum fuscoviolaceum	FBCC691	T21	III

3.1 Cultivation of *Phlebia radiata*

In order to promote expression of laccase isozymes, *Phlebia radiata* FBCC43 was cultivated in stationary submerged cultures. Complex peptone-yeast extract (PY) liquid medium (pH 4.5), with total nitrogen concentration of 70 mM, was buffered with succinate and contained 0.5% (wt/vol) peptone, 0.2% (wt/vol) yeast extract, and 0.1% (wt/vol) glucose (Hildén et al. 2007). The PY medium was supplemented with $CuSO_4$ ranging from 25 µM to 3 mM (Fig. 9). Defined succinate-buffered (pH 4.5) low glucose (0.1% wt/vol) liquid medium with ammonium nitrate and asparagine as nitrogen sources in varying concentrations was used (modified from Hatakka and Uusi-Rauva 1983). Low nitrogen-asparagine-succinate (LN-LC, total N concentration 2 mM), high nitrogen-asparagine-succinate (HN-LC, total N concentration 20 mM), and extra high nitrogen-asparagine-succinate (eHN-LC, total N concentration 70 mM) (Hildén et al. 2007) liquid media were prepared and half of the flasks were supplemented with 1.5 mM $CuSO_4$ (Fig. 9). In all cases, $CuSO_4$ was added to the cultures on the third day of incubation. Mycelial dry weight was determined for submerged cultures after 32 days of cultivation (Fig. 10).

Table 9. Experimental methods used in this study.

Method	Described and used in
Cultivation of fungi	
liquid cultures	I, II, III, IV, chapters 3.1 and 3.3
semi-solid wood cultures	I, II, chapter 3.3
solid-state wood cultures	I, II, III, IV
High performance liquid chromatography (HPLC)	III
Capillary zone electrophoresis (CZE)	III
Enzyme activity measurements	I, II, III, IV
Extraction of mycelial proteins	II, III, IV
Fast protein liquid chromatography (FPLC)	
anion exchange chromatography	Chapter 3.2
chromatofocusing	IV
hydrophobic interaction chromatography (HIC)	II, chapter 3.2
SDS-PAGE	II, IV
Western blotting with immunodetection	II, IV
Isoelectric focusing	Chapter 3.2
Extraction of DNA and RNA	I, II, IV, chapter 3.4
Northern hybridization	I, II
Genome walking PCR	I, II, IV
Inverse PCR	IV, chapter 3.4
RACE-PCR	I, II
RT-PCR	I, II, IV
Competitive RT-PCR	I, II
Real time quantitative RT-PCR	IV, chapter 3.4
Cloning	
pCR2.1 TOPO vector	I, II
pJET1.2/blunt vector	IV, chapter 3.4
DNA sequencing	I, II, IV, chapter 3.4
Phylogenetic sequence analysis	I, II, IV, fig. 8
Protein 3D homology modelling	Fig. 15

Semi-solid liquid cultures with the low nitrogen-asparagine-succinate (total N concentration 2 mM, pH 4.5) medium were prepared by supplementation of 3 g (dry weight) milled alder wood as carbon source (Fig. 11). Fungal culture conditions are described in publication II. Total liquid volume was kept at 100 ml in all submerged and semi-solid cultures and the fungus was cultivated in three parallel 250-ml conical flasks at 28°C. The activity of LMEs in the extracellular culture liquid was followed spectrophotometrically (assays as described in publications I, II, and III).

3.2 Purification of *P. radiata* laccases

Laccases were purified and fractionated from the *P. radiata* mycelial extracts and concentrated culture liquids originating from the semi-solid cultures and liquid PY cultures amended with 1.5 mM $CuSO_4$. Preparation of mycelial extracts is described in publication II. Concentrated mycelial extracts and culture liquids from the semi-solid milled alder wood cultures were fractionated with hydrophobic interaction chromatography (HIC) (method described in publication II).

Mycelial extracts from the PY liquid cultures were concentrated and dialyzed against 10 mM Na-acetate buffer (pH 7.0). The extracts were first prepurified by anion-exchange chromatography with a Resource Q column (1 ml, Pharmacia Amersham). The pooled and concentrated fractions demonstrating laccase activity were further separated by using a HiTrap Q FF column (1 ml, Pharmacia Amersham). Proteins were eluted from both columns with a stepwise gradient from 10 mM to 1 M Na-acetate in pH 7.0.

Concentrated PY culture liquid was purified by hydrophobic interaction chromatography (HIC) with a Butyl FF column (1 ml, Pharmacia Amersham). The concentrate was dialyzed against 10 mM Na-acetate buffer (pH 6.5) and solid $(NH_4)_2SO_4$ was added to a concentration of 1 M. Proteins were eluted with a linear gradient of $(NH_4)_2SO_4$ from 1 M to 0 M in 10 mM Na-acetate buffer (pH 6.5). Prior to further purification with a HiTrap Q FF column (1 ml), pooled fractions containing laccase activity were concentrated and dialyzed against 10 mM Na-acetate buffer (pH 6.5). Proteins were eluted from the column with a stepwise gradient from 0 M to 1 M NaCl in Na-acetate buffer. Protein purification and fractionation were performed using the Äkta Explorer apparatus (GE Healthcare). Protein elution was followed as absorbance at 280 nm, 405 nm and, 610 nm. Fractions containing 200 µl - 1 ml were collected.

To determine the quantity and isoelectric points of the purified laccases, the protein samples were concentrated and introduced to 0.5 mm thin 3% acrylamide isoelectric focusing (IEF) gels of pH range 2.5-7 using a mixture of ampholytes (Pharmalyte 2.5-5 and Ampholine 3-10, GE Healthcare) and the Pharmacia Multiphore apparatus. IEF gels were run at constant power (25 W) and temperature (+10 ºC) according to the instructions of the manufacturer (Pharmacia Amersham), and they were activity stained with guaiacol to visualize laccases (Hildén et al. 2007).

3.3 Cultivation of *Dichomitus squalens*

Dichomitus squalens FBCC184 was cultivated as submerged liquid cultures in a Na-succinate-buffered medium, pH 4.5, containing 0.5% (wt/vol) peptone and 0.2% (wt/vol) yeast extract supplemented with 2 g (dry weight) of milled spruce (*Picea abies*) wood and 1 g of crushed charcoal. The culture medium was originally adopted for *Physisporinus rivulosus* (Hildén et al. 2007). LME activities in the extracellular culture liquid were followed spectrophotometrically (assays described in publications I, II, and III).

3.4 Cloning and expression of *D. squalens* laccases

Two laccase-encoding genes, *Ds-lac3* and *Ds-lac4*, were amplified from the extracted total DNA of *D. squalens* FBCC312 with degenerate laccase primers (publication II) and inverse PCR (method described in publication IV). The full-length gene fragments were amplified from the total DNA and the PCR products were sequenced (Macrogen Ltd., Korea), and their translated amino acid sequences were used in phylogenetical analyses. Relative amounts of the transcripts of *Ds-lac3* and *Ds-lac4* genes were determined on solid-state spruce wood cultures by adopting real time qRT-PCR (method described in publication IV).

4 Results and discussion

4.1 Characterization and expression of *Phlebia radiata* lignin peroxidase encoding genes (I)

Phlebia radiata belongs to the group a few white-rot fungi which are reported to produce lignin peroxidase (LiP) (Niku-Paavola et al. 1988, 1990, Hatakka 1994, Lundell and Hatakka 1994). Together with *P. radiata*, *Trametes versicolor* is the other well-characterized white-rot basidiomycete that secretes a set of multiple lignin-modifying peroxidases (LiPs and MnPs) with concomitant laccase production (Johansson and Nyman 1993, Jönsson et al. 1995).

In this study, two new LiP-encoding genes of *P. radiata* FBCC43, *Pr-lip1* and *Pr-lip4*, and the genomic fragment (*Pr-lip3*) for the previously described cDNA (*lgp3*) coding for the LiP3 isozyme (Saloheimo et al. 1989) were cloned and characterized. According to the translated amino acid sequences, *P. radiata* LiPs possess N-terminal secretion signal peptides and are synthesized as pre-proenzymes. They show typical LiP primary structures with all the essential amino acid residues needed for enzyme structure and catalysis. Of the *Pr-lip* gene protein products, the isozyme Pr-LiP3 has been biochemically characterized (Lundell et al. 1993b), and it presents kinetic properties similar to the first isolated *Phanerochaete chrysosporium* LiPH8 enzyme (Tien and Kirk 1983). The biochemical properties of Pr-LiP2 have also been described and its production has been shown under different culture conditions (Niku-Paavola et al. 1988, 1990, Lundell and Hatakka 1994, Vares et al. 1995) but the gene encoding this isozyme is not yet cloned or characterized.

The intron-exon organization of the three *P. radiata lip* genes is highly conserved (Fig. 1 in publication I). *Pr-lip* genes with 11 conserved introns differ from most of the cloned *lip* genes in that they have a higher intron number (Gold and Alic 1993, Cullen 1997, Martínez 2002). Phylogenetic analyses of translated amino acid sequences indicate that the three *P. radiata* LiPs cluster together with all the other fungal LiP-encoding genes so far sequenced (Fig. 3 in publication I) forming a branch very near to the short MnP-VP subgroup within the family of fungal class II secretory heme peroxidases (Martínez 2002, Hildén et al. 2005, Morgenstern et al. 2008). In addition, Pr-LiPs form a fungal species-based sub-branch together with a *Bjerkandera adusta* LiP.

All three *P. radiata lip* genes were expressed both in softwood (spruce) and hardwood (alder) containing cultures. However, the *Pr-lip* genes showed individual response to the supplement of extracellular Mn^{2+} and time-dependent regulation (Figs. 4 and 5 in publication I). Recent transcriptome and proteome studies on *P. chrysosporium* have further pointed out that LiP, which is capable of oxidizing both phenolic and non-phenolic lignin substructures, is an essential part of lignin-modifying enzyme (LME) machinery of this fungus (Ravalason et al. 2008, Vanden Wymelenberg et al. 2009). Furthermore, high expression of three *lip* genes has been reported for *P. chrysosporium* grown on red oak wood (Sato et al. 2009).

Addition of a high concentration (480 μM) of soluble Mn^{2+} to the semi-solid wood cultures inhibited expression of the *Pr-lip3* gene (Fig. 5 in publication I). LiP3 was previously found to be the predominant isozyme in lignocellulose-containing cultures of *P. radiata* (Niku-Paavola et al 1990). However, this isozyme was not identified by

IEF within the set of detectable LiPs when *P. radiata* was grown on wheat straw (Vares et al. 1995). The latter may be explained by p*I* variations caused by the complex solid lignocellulose medium.

Supplementation with a 50% lower Mn^{2+} concentration (240 μM) increased the amount of the *Pr-lip1* and *Pr-lip4* transcripts. It has been shown that LiP production and activity was enhanced slightly in liquid cultures of *P. radiata* amended with Mn^{2+} of similar concentration range (180 μM) (Moilanen et al. 1996). However, suppressed LiP production in the presence of 180 μM Mn^{2+} and malonate has been detected (Moilanen et al. 1996). To further elucidate the effect of soluble Mn^{2+} ions on the transcriptional level, the promoter regions of the *P. radiata lip* genes need to be analyzed for the presence of putative regulatory sequences like metal response elements (MREs).

Composition of the wood species used in fungal cultivations probably affected expression of the *Pr-lip* genes. Previously, disparate production of LiP isozymes by *P. radiata* has been observed in lignocellulose-containing cultures (Niku-Paavola et al. 1990, Vares et al. 1995). In addition, substrate-dependent expression was noticed for the LiP-encoding genes of *P. chrysosporium* (Broda et al. 1995, Lamar et al. 1995, Bogan et al. 1996, Janse et al 1998, Stewart and Cullen 1999). Distinct regulation of *lip* genes of *P. radiata* supports the view that several LME-encoding genes are needed under changing growth conditions as their transcription may be triggered differently (Kersten and Cullen 2007). These results confirm that, similarly with *P. chrysosporium*, *P. radiata* produces multiple LiP isozymes, which obviously play an important role during the decay of wood and other lignocelluloses.

4.2 Characterization and expression of *P. radiata* laccase-encoding genes (II)

A new laccase-encoding gene of *P. radiata* FBCC43, *Pr-lac2*, was cloned and characterized (publication II). Previously, one *P. radiata* laccase-encoding gene, *Pr-lac1*, and the corresponding protein have been described in detail (Niku-Paavola et al. 1988, Saloheimo et al. 1991, Lundell and Hatakka 1994), and a recombinant *Phlebia* laccase protein was expressed using *Trichoderma reesei* as host organism (Saloheimo and Niku-Paavola 1991). Expression of the *Pr-lac1* and *Pr-lac2* transcripts was demonstrated during growth of *P. radiata* FBCC43 on softwood (spruce) and hardwood (alder) (Figs. 5 and 7 in publication II).

4.2.1 *P. radiata* Lac1 and Lac2 encoding genes and proteins

The two *P. radiata* laccase genes have distinct intron-exon structures as has also been reported for the multiple laccases of *Coprinopsis cinerea* (Kilaru et al. 2006) and *Pleurotus ostreatus* (Pezzella et al. 2009). *Pr-lac1* and *Pr-lac2* carry 9 and 12 introns, respectively, sharing 7 conserved introns (Fig. 1 in publication II). Moreover, *Pr-lac1* carries a notably long (582 nt) last exon which in turn is interrupted by 3 introns in the *Pr-lac2* gene.

Both of the *P. radiata* laccases have putative N-terminal signal peptides and all conserved amino acid residues for copper-binding (Fig. 2 in publication II) within the four laccase signature sequences L1-L4 (Kumar et al. 2003). The translated Pr-Lac1 and Pr-Lac2 amino acid sequences demonstrate a high degree of homology (amino acid level identity 66-73%) with other basidiomycete laccases indicative of typical four-

copper laccase fold and 3D structure (see chapter 1.3.2 in Introduction). An earlier study suggested a combination of only two copper atoms and the prosthetic group pyrroloquinoline quinone to be the cofactor structure in *Phlebia* laccase (Karhunen et al. 1990b), now depicted the isozyme Pr-Lac1. However, the enzyme shows true laccase catalytic activity without e.g. catechol oxidase or tyrosinase side-activities (Niku-Paavola et al. 1988).

Non-blue laccases have been previously reported for several wood-decaying basidiomycetes, including *P. radiata* Lac1 during solid-state cultivation on wheat straw (Leontievsky et al. 1997). The authors proposed that the yellow colour resulted from interfering lignin-derived compounds bound to typical blue laccases. However, the presence of several different metal atoms (Cu, Fe, and Zn) in the non-blue derivative of *P. ostreatus* POXA1 (POXA1w, Palmieri et al. 1997) leaves the question open if the predicted Pr-Lac2 may accordingly contain other metal atoms in addition to Cu.

Recently, Kaneko et al. (2009) purified an extracellular laccase-like protein with an N-terminal amino acid sequence that is highly similar to the putative beginning of the mature Pr-Lac2 (Fig. 2 in publication II). The fungal source is reported to be another isolate of *P. radiata* (Kaneko et al. 2009). According to the absorption spectrum, this new laccase-like protein seemingly lacks at least the type I copper atom, and thereby further studies are needed to determine if the novel protein is a true laccase or another type of MCO enzyme.

Updated protein phylogenetic analysis re-demonstrates the distant grouping of Pr-Lac1 and Pr-Lac2 (Fig. 8), as was already depicted in publication II (Fig. 3 in publication II). Multiple laccases of white-rot fungi commonly show divergent phylogeny, which might reflect distinct enzyme properties (Hoegger et al. 2006, Rodríguez et al. 2008). Within the evolutionary tree of laccases (Fig. 8) Pr-Lac2 clusters with *Pleurotus* and *Laccaria bicolor* laccases, and close with *P. ostreatus* POXA1b, which is probably an intracellular or cell wall associated enzyme (Palmieri et al. 2000). *P. ostreatus* POXA1b shows a high p*I* value (p*I* 6.9) is also estimated for Pr-Lac2 (calculated p*I* 5.7, publication II). These values are closer to neutral pH than is typical for fungal laccases with p*I* values usually ranging from pH 3 to 4 (reviewed by Baldrian 2006).

4.2.2 Expression of *P. radiata* laccase transcripts

There are only a few reports where expression of laccase genes on lignocellulosic substrates has been studied, including the litter-decomposing basidiomycete *Agaricus bisporus* (Smith et al. 1998, Ohga et al. 1999), and the white-rot basidiomycetes *Ceriporiopsis subvermispora* (Lobos et al. 1994) and *Lentinula edodes* (Ohga and Royse 2001). The two *Pr-lac* genes were expressed simultaneously on wood cultures, but *Pr-lac1* transcripts were more abundant as judged by RT-PCR (Figs. 4, 5, and 7 in publication II). Accordingly, Pr-Lac1 is repeatedly observed as the main laccase isozyme secreted by *P. radiata* when cultivated in liquid medium or on solid lignocellulose substrate (Fig. 12, Niku-Paavola et al. 1988, Lundell and Hatakka 1994, Vares et al. 1995). In this respect, *P. radiata* deviates from *T. versicolor* (and other *Trametes* species) that generally secretes several laccase isozymes (Evans et al. 1985, Baldrian 2006, Hildén et al. 2009).

In the present work, evidence of at least two additional laccase-encoding genes of *P. radiata* was detected, both at cDNA and genomic level. This refers to the existence of

multiple laccase genes also in *P. radiata*, as has been earlier reported for the white-rot basidiomycete genera *Pleurotus* (Soden and Dobson 2001, Rodríguez et al. 2008, Pezzella et al. 2009) and *Trametes* (Yaver and Golightly 1996, Jönsson et al. 1997, Mansur et al. 1997, Mikuni and Morohoshi 1997, Cassland and Jönsson 1999, Necochea et al. 2005). In addition, expression of a partially processed *P. radiata* laccase transcript, which contains one intron including a stop codon was amplified from submerged and solid-state wood cultures of *P. radiata* (data not shown). Likewise, one intron-containing laccase transcript has been detected in *Pleurotus ostreatus* (Pezzella et al. 2009) and partially processed laccase transcripts resulting from altered or incomplete intron splicing have been reported for *Coprinopsis cinerea* (Kilaru et al. 2006) and *Cryptococcus neoformans* (Missall et al. 2005). Three truncated genes with homology to laccases are present in the

Figure 8. Minimum evolution neighbor-joining tree of 79 deduced laccase amino acid sequences from selected basidiomycetous fungi generated with Mega 4.0 software (Tamura et al. 2007). Bootstrap values for the branches were obtained with 1,000 replications. The scale bar represents a distance equivalent to 0.1 amino acid substitutions per site. The sequences were retrieved with SRS (www.ebi.ac.uk) and their UniProt accessions are shown. The *Phlebia radiata* and *Dichomitus squalens* laccases are depicted in frames.

genome of *Laccaria bicolor* (Courty et al. 2009) and in *Phanerochaete chrysosporium* partially processed transcripts have been observed for its non-laccase MCO (Larrondo et al. 2004).

4.3 Effect of copper and nitrogen supplements on submerged cultures of *P. radiata*

With the specific interest to optimize cultivation conditions for production of *P. radiata* laccase activity, defined low glucose (0.1%) asparagine-succinate liquid medium with varying inorganic nitrogen (as NH_4NO_3) concentration was compared with complex PY liquid medium which contains a rich source of organic nitrogen (peptone and yeast extract). Nitrogen-rich cultures often enhance laccase activities in white-rot fungi (Gianfreda et al. 1999), and a complex nitrogen source like peptone is generally required for efficient laccase production (Galhaup et al. 2002, Hess et al. 2002, Hou et al. 2004). The effect of addition of copper (Cu^{2+}), as the common inducer of fungal laccases, was also studied in the *P. radiata* FBCC43 cultures. At the same time, the activities of ligninolytic peroxidases were followed from the cultivations. As *P. radiata* obviously possesses several laccase genes (chapter 4.2), the second aim was to detect new laccases also at protein level. The laccases from *P. radiata* mycelial extracts and culture liquids originating from different culture conditions were partially purified and their isoelectric points were determined.

4.3.1 *P. radiata* laccase production

Addition of $CuSO_4$ strongly stimulated extracellular laccase activity in the liquid cultures of *P. radiata* (Fig. 9a, c). In the PY cultures, supplementation of 1.5 mM $CuSO_4$ caused over 20-fold increase in laccase activity within 14 days of cultivation when compared to the cultures without extra addition of copper (Fig. 9a). In comparison to the semi-solid cultures with milled alder wood (MAW), up to 30-fold higher laccase activity was observed (Fig. 11).

Similarly, 2 mM copper induced laccase transcription and activity in *Trametes pubescens* (Galhaup et al. 2002), and 1 mM copper significantly increased laccase activity in the cultures of *Trametes trogii* (Levin et al. 2002) and *Pleurotus ostreatus* (Baldrian and Gabriel 2002). The optimal concentration of Cu^{2+} for stimulation of laccase production obviously varies greatly between the fungal species, since notably lower $CuSO_4$ concentrations, 150-200 μM, already promoted extracellular laccase activity in another strain of *P. ostreatus* (Giardina et al. 1999), and in *Coriolopsis rigida* (Saparrat et al. 2002) and *Volvariella volvacea* (Chen et al. 2003).

The highest laccase activity peaked after addition of 1.5 mM $CuSO_4$ when *P. radiata* was grown with 2 mM (LN) and 20 mM (HN) additional inorganic nitrogen (Fig. 9c) in spite of the moderate amount of fungal mycelium produced under these conditions (Fig. 10). This indicates that vigorous growth and increase in cell mass does not necessarily correlate with elevated production of laccase. However, laccase activity was retained longer and was more stable in the PY cultures containing ca 70 mM organic nitrogen (Fig. 9a, b) which in turn probably was due to the more extended growth of the fungus in this medium.

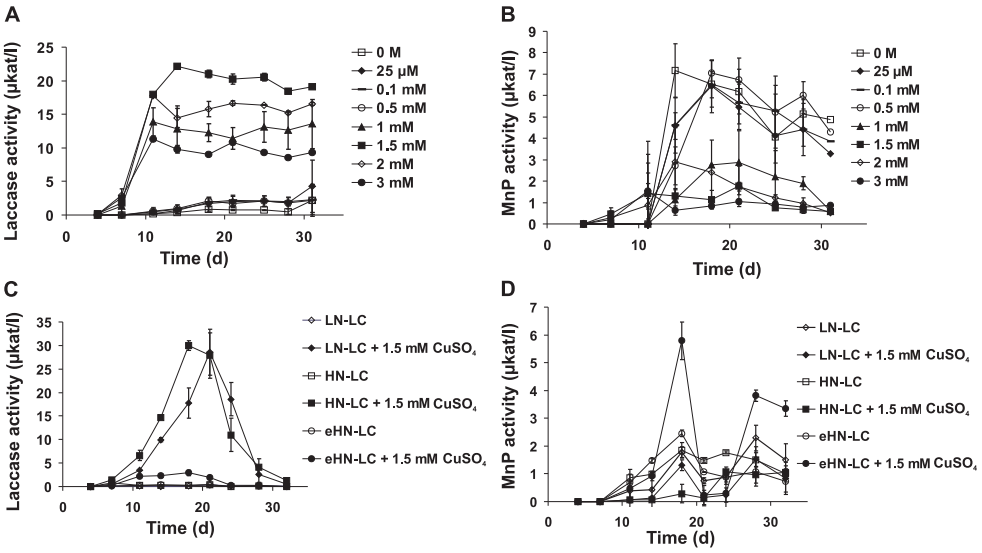


Figure 9. Extracellular LME activities secreted by *Phlebia radiata* FBCC43 in submerged liquid cultures. Complex peptone-yeast extract (PY) medium was supplemented with 0 to 3 mM $CuSO_4$ (A, B). Defined liquid medium with varying amounts of inorganic nitrogen was supplementated with 0 or 1.5 mM $CuSO_4$ (C, D). LN-LC: low nitrogen (2 mM N), low carbon (0.1% glucose); HN-LC: high nitrogen (20 mM N), low carbon; eHN-LC: extra high nitrogen (70 mM N), low carbon. Standard deviations represent the variation of the mean activity value obtained from three replicate cultivations.

Addition of copper to the PY liquid cultures caused no large decline in the mycelial dry weight of *P. radiata* (Fig. 10). In fact, a slight increase in the mycelial mass was observed after supplementation of 0.5 mM and 1 mM $CuSO_4$. The amount of *P. radiata* mycelium in the defined liquid cultures amended with 70 mM inorganic nitrogen (eHN) was at the same level as in the PY liquid cultures, but addition of 1.5 mM $CuSO_4$ apparently restricted fungal growth in the former case (Fig. 10) and lower activity of laccase was then observed (Fig. 9c). The tolerance to copper varies significantly in wood-decaying basidiomycetes, and 2 mM concentration as Cu^{2+} is reported to be toxic to some strains (Sierra-Alvarez et al. 2007).

The two copper centres situated in the active site of laccases play the key role in enzyme catalysis. This most probably explains the copper-promoted enhancement and stabilization of laccase activity at the protein level, which has been reported e.g. for purified laccases from *P. ostreatus* (Baldrian and Gabriel 2002). In addition, copper often induces mRNA levels of laccase-encoding genes in white-rot fungi (Collins and Dobson 1997, Karahanian et al. 1998, Palmieri et al. 2000, Soden and Dobson 2001, Galhaup et al. 2002).

Recently, the increase of *Ceriporiopsis subvermispora* laccase transcripts by copper was shown to be mediated by an ACE1-like copper-fist transcription factor (Álvarez et al. 2009). Putative ACE elements are present also in the promoter regions of the laccase-encoding genes *lac1* of basidiomycete PM1 (Coll et al. 1993), and *lac4* of *Pleurotus sajor-caju* (Soden and Dobson 2003). Revised analysis of promoter regions of the

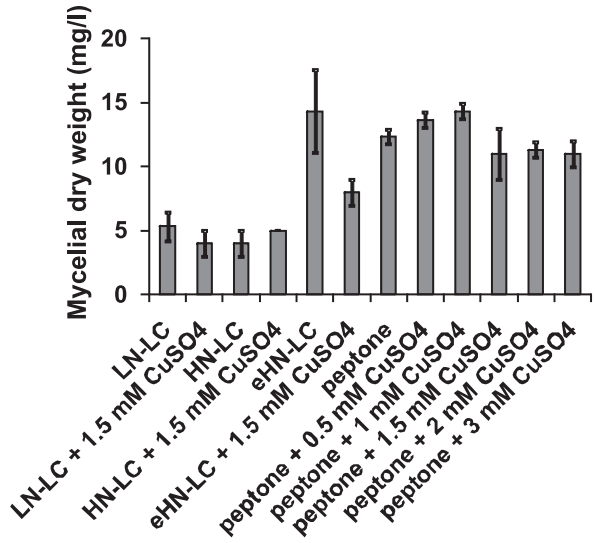


Figure 10. Dry weight of the *Phlebia radiata* FBCC43 mycelia from submerged cultures after 32 days of cultivation. For culture media and supplements, see legend for Fig. 9. Standard deviations represent variation of the mean value of three replicate cultivations. PY, peptone-yeast extract liquid medium.

P. radiata FBCC43 *Pr-lac1* and *Pr-lac2* genes also revealed putative ACE elements situated at -462 to -476 bp and -96 to -108 bp upstream from the start codon, respectively. Therefore, it is likely that stimulation of laccase activity observed in the various *P. radiata* cultures supplemented with $CuSO_4$ may be achieved at the transcriptional level leading to higher amount of enzyme produced.

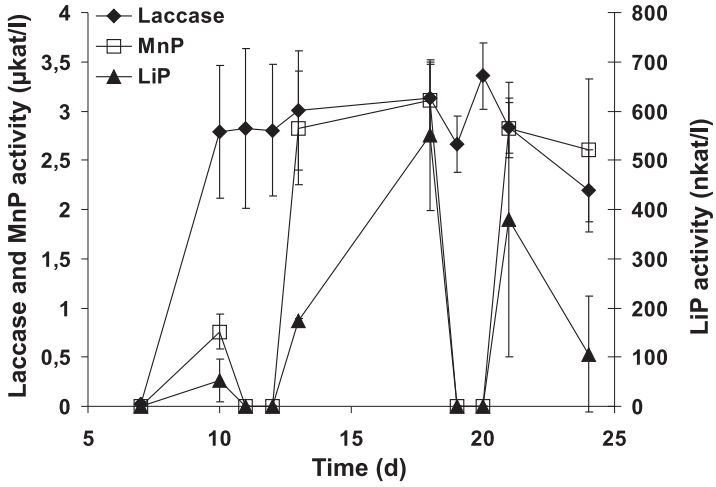


Figure 11. Extracellular lignin-modifying enzyme activities from semi-solid *Phlebia radiata* FBCC43 cultures. Defined low-nitrogen liquid medium was amended with 3 g milled alder wood as carbon source. Standard deviations represent the variation of the mean value of three replicate cultivations.

Based on protein purification experiments, *P. radiata* most often expresses one or two laccase isoforms with p*I* values around 3.5, irrespective of the cultivation substrate, that is nutrient rich PY liquid medium or semi-solid, milled wood containing cultures (Fig. 12). In addition, similar acidic laccase isoforms were present both in the extracellular culture fluid as well as in the mycelial extracts obtained from these cultures. The acidic laccase most probably corresponds to the Pr-Lac1 protein of *P. radiata* with p*I* 3.4-3.5 (Niku-Paavola et al. 1988, 1990, Lundell and Hatakka 1994). Production of several acidic laccase isoforms with slightly different p*I* values possibly results from post-translational protein processing like differential glycosylation, as has been earlier reported for *P. radiata* FBCC43 when cultivated on wheat straw (Vares et al. 1995). Accordingly, the transcripts of the *Pr-lac1* gene were dominating on solid and semi-solid wood cultures (Figs. 4, 5, and 7 in publication II).

Another possible laccase isozyme with a slightly lower molecular mass (58 kDa) than Pr-Lac1 was distinguished in the mycelial extracts of *P. radiata* obtained from semi-solid wood cultures (Fig. 6b in publication II). In addition to Pr-Lac1, a less acidic extracellular laccase isoform with p*I* of 4.9 was detected in the PY liquid culture amended with 1.5 mM $CuSO_4$ (Fig. 12). This is the first report of another extracellular laccase than Pr-Lac1 demonstrated from the *P. radiata* FBCC43. Also a faint ladder of several other proteins with p*I* values under 4.9 was present. However, further studies are needed to elucidate if one of these additional guaiacol-stained protein bands may correspond to the new Pr-Lac2 with calculated molecular mass of 56.5 kDa and calculated p*I* value of 5.8.

Comparable results were reported for *Physisporinus rivulosus* which secretes several laccase isoforms with p*I* values between 3.5 and 4.8 when cultivated in peptone-containing liquid medium (Hakala 2007). As with *P. radiata*, only the most acidic isoforms (with p*I* values under 3.5) showing identical N-terminal amino acid sequences are present on wood (Hakala et al. 2005). As a conclusion from the protein purification and gene expression studies, the predominant laccase of *P. radiata* is Pr-Lac1, regardless of whether the culture medium contains wood or glucose as carbon source, or a high or low concentration of nutrient nitrogen.

4.3.2 Production of lignin-modifying peroxidases by *P. radiata*

Addition of Cu^{2+} to the defined and complex liquid cultures of *P. radiata* caused no increase in the extracellular MnP activity, and $CuSO_4$ concentrations over 0.5 mM notably decreased the MnP activity (Fig. 9b, d). The stimulative effect of metal cations,

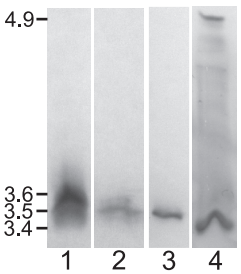


Figure 12. Zymograms of guaiacol stained isoelectric focusing gels showing laccases expressed by *Phlebia radiata* FBCC43 grown under different culture conditions. Culture liquid (lane 1) and mycelial extract (lane 2) from semi-solid liquid cultures supplemented with 2 g of milled alder wood. Culture liquid (lane 3) and mycelial extract (lane 4) from peptone-yeast (PY) liquid cultures supplemented with 1.5 mM $CuSO_4$.

especially Mn^{2+}, on MnP expression is well established. Copper has been reported to enhance MnP activity in *Trametes trogii* (Levin et al. 2002) and increase transcription of the *C. subvermispora* MnP-encoding genes through promoter region ACE elements (Álvarez et al. 2009). Also other metals (Zn^{2+}, Cd^{2+}, and Ag^{+}) elevate the transcript levels of *Cs-mnp* genes (Manubens et al. 2003).

In a previous study, addition of Mn^{2+} caused no obvious induction of the two distinct *P. radiata mnp* genes (*Pr-mnp2* and *Pr-mnp3*) at transcriptional level, although putative metal response elements (MREs) were found in their promoter regions (Hildén et al. 2005). Moreover, one putative ACE element, situated at -407 to -421 bp upstream from the start codon was found after re-analysis of the *Pr-mnp2* promoter region (data not shown). Explanation for the lack of *mnp* gene response by Mn^{2+} or Cu^{2+} in *P. radiata* may be that the putative transcription factors are dysfunctional, or that other metal concentrations than those used in the experiments are required for stimulation of transcription.

Probably due to the more vigorous growth, *P. radiata* secreted over two times higher MnP activity levels in the nutrient rich PY medium than in semi-solid cultures with alder sawdust as a carbon source (Figs. 6b and 7). In the defined liquid cultures with total nitrogen concentration of 70 mM, MnP activity peaked twice, possibly correlating with the good growth of the fungus, but remained at lower level than in the PY medium (Fig. 9d). As with laccase activity, the MnP activity was retained longer in the complex PY cultures.

P. radiata produced no detectable LiP activity in defined liquid cultures supplemented with various concentrations of nutrient nitrogen. While *P. radiata* typically secretes LiP under nitrogen-limited conditions with glucose as carbon source (Lundell 1993), LiP activity has been reported also from similar high nitrogen submerged cultures of *P. radiata* (Hatakka et al. 1987). LiP was secreted in this study on the semi-solid cultures of *P. radiata* with alder sawdust as carbon source (Fig. 11 and Fig. 6a in publication II). This notion is supported by earlier reports of *P. radiata* LiP production on lignocellulosic materials (Niku-Paavola et al. 1990, Vares et al. 1995, Lankinen 2004).

Wood-containing cultures indicated that the highest LiP and MnP activities of *P. radiata* are detected approximately after two weeks of cultivation (Fig. 11). Accordingly, intense expression of *Pr-lip* and *Pr-mnp* transcripts was shown after two and three weeks cultivation on wood cultures (Figs. 4 and 5 in publication I, Fig. 8 in publication II). Furthermore, synchronous expression of all the known *P. radiata* LiP-, MnP-, and laccase-encoding genes was shown for the first time on solid-state wood cultures indicating the importance of the LME-encoding genes and their enzyme products for the wood and lignin-degrading strategy of *P. radiata*.

4.4 Laccases of *Dichomitus squalens*

To examine the laccase activity produced by *D. squalens* in wood-containing cultures, the fungus was grown in semi-solid liquid medium supplemented with spuce sawdust and charcoal. In order to study the *D. squalens* laccases on molecular level, total DNA was extracted and used for the amplification of two laccase genes, *Ds-lac3* and *Ds-lac4*. *D. squalens* was grown on solid state spruce wood cultures and the expression of the two *D. squalens* laccase genes was quantified at mRNA level with real time quantitative RT-PCR.

4.4.1 Production of laccases by *D. squalens*

The nitrogen rich PY liquid medium supplemented with both spruce sawdust and charcoal promoted extracellular laccase activity of *D. squalens* FBCC184 (Fig. 13). Concomitantly, minor production of MnP was detected. Previously, with different strains of *D. squalens*, contradictory results for the production of laccase and MnP have been reported from cultures made in nitrogen-rich media (Arora and Gill 2000, Gill and Arora 2003, Šušla et al. 2007).

In sawdust and charcoal-containing PY medium cultures of the selective white-rot fungus *P. rivulosus*, laccase activity was even more vigorously stimulated (Hildén et al. 2007). Basidiocarps of *D. squalens* and *P. rivulosus* are typically found on burned tree after forest fire (Niemelä 2005). Thus, the culture conditions which mimic the natural growth environment may in turn explain promotion of laccase activity in the cultures of *D. squalens*.

With several other white-rot fungal species, LME-production increases on lignocellulose substrates (Vares et al. 1995, Hofrichter et al. 1999a, Pickard et al. 1999, Giardina et al. 2000, Lankinen et al. 2005, Hildén et al. 2007). Also, immobilization of white-rot fungi on supporting material, such as polyurethane foam, commonly enhances production of LMEs (Rodríquez Couto et al. 2004, Rogalski et al. 2006, Šušla et al. 2007).

Recently, with the *D. squalens* CCBAS750, extracellular laccase activity increased in the presence of an inert carrier material, but the inductive effect was significantly higher when the fungus was immobilized on pine wood blocks (Šušla et al. 2007). Although the specific factors are not yet known, these results further emphasize the promoting effect of solid woody substrates for white-rot fungal production of laccase.

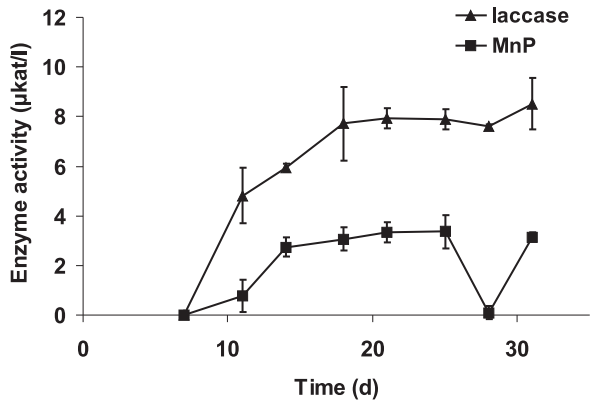


Figure 13. Secretion of laccase and MnP in the submerged peptone-yeast extract liquid cultures of *Dichomitus squalens* FBCC184 supplemented with 2 g of spruce sawdust and 1 g of crushed charcoal. Standard deviations represent the variation of the mean value of three replicate cultivations.

4.4.2 Laccase-encoding genes of *D. squalens*

Two laccase genes, *Ds-lac3* and *Ds-lac4*, of *D. squalens* FBCC312 were amplified from total DNA, and cloned and characterized at sequence level. The putative amino acid sequences show typical fungal laccase primary structure with conserved copper-binding residues in the four laccase signature sequence regions L1-L4 (Fig. 4) (Kumar et al. 2003). According to the N-terminal amino acid sequences after cleavage of the putative 5' secretion leader peptide, these genes seem to encode different laccase isozymes than the two previously isolated and characterized from *D. squalens* (Table 10). The present study suggests that there is a laccase multi-gene family in *D. squalens*. This has been reported for several other laccase-expressing basidiomycete species like *Coprinopsis cinerea (*Kilaru et al. 2006) and *Pleurotus ostreatus* (Pezzella et al. 2009).

Table 10. Comparison of N-terminal amino acid sequences of *Dichomitus squalens* laccases. Identical amino acids between the proteins are in boldface.

Fungal strain	Protein name	Source	N-terminal amino acid sequence	Reference
D. squalens CBS 432.34	Laccases 1 and 2	Protein	G**IGPVTDL**TIT**N**AD**I**A**PD**a[1]F	Périé et al. 1998
D. squalens FBCC312	Ds-Lac3	gDNA	A**IGPVTDL**TVA**N**AN**I**S**PD**GY[2]	this work
D. squalens FBCC312	Ds-Lac4	gDNA	S**IGPVTDL**IIA**N**KD**I**S**PD**GS[2]	this work

[1]Lower-case letter indicates preliminary data.

[2]Sequence determined after removal of putative 5' secretion signal leader peptide.

The real time qRT-PCR study showed that the *Ds-lac3* and *Ds-lac4* genes are expressed during the growth of *D. squalens* on solid-state spruce wood cultures (Fig. 14), further emphasizing the inductive effect of lignocellulose on the production of laccase by this fungus, as was also noted by Šušla et al. (2007). However, the distinct expression pattern of *D. squalens* laccase-encoding genes may implicate different functions for the corresponding laccase isozymes, as has been shown with plant laccases (Sato et al. 2001).

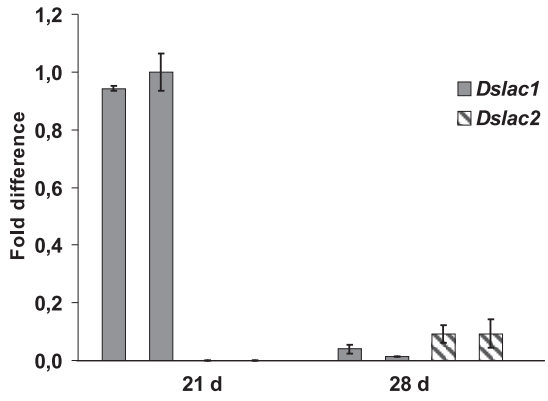


Figure 14. Amount of *Dichomitus squalens* FBCC312 laccase transcripts after three and four weeks of cultivation on solid-state spruce wood as determined by real time qRT-PCR. Two biological replicates were analyzed with three parallel PCR reactions. Standard deviation of the mean value of the three parallel fold differences is depicted. Glyceraldehyde-3-phosphate dehydrogenase encoding gene (*Ds-gapdh*) transcript was used as a reference in normalization. For the 21-day sample amplification, *Ds-lac4* transcripts were not detected.

Phylogenetic analysis of the deduced laccase amino acid sequences of basidiomycetous fungi shows separated positioning of the two new *D. squalens* laccases which is an indication of their divergent evolution (Fig. 8). Phylogenetic clustering of laccases is also proposed to partially correspond with enzyme function (Hoegger et al. 2006) reflecting the diverse biochemical roles of laccases among the superfamily of MCO enzymes (Hoegger et al. 2006, Morozova et al. 2007a).

4.5 Production of oxalate decarboxylase and organic acids in wood-decaying white-rot fungi (III)

Selected white-rot fungal strains were screened for intracellular oxalate decarboxylase (ODC) activity. The inductive effect of oxalic acid on the ODC activity was studied by adding oxalic acid to fungal liquid cultures to make 5 mM concentration. ODC activity was measured with a spectrophotometric assay from mycelial extracts. The production of organic acids by ODC-producing fungi was followed during the fungal growth in malt extract liquid medium and on solid-state spruce wood chips. Lignin-modifying enzyme activities were also measured from the cultures.

4.5.1 Oxalate decarboxylase activity

Intracellular oxalate decarboxylase (ODC) activity was detected for the first time in the mycelial extracts of four white-rot fungal strains representing different species, i.e. *Dichomitus squalens* FBCC184, *Phanerochaete sanguinea* FBCC712, *Trametes ochracea* FBCC682, and *Trametes versicolor* FBCC324 (Table 1 in publication III). *P. sanguinea* FBCC712 and *D. squalens* FBCC184 showed constitutive ODC activity irrespective of supplementation of oxalic acid to the culture liquid. This corresponds to the observations made with *Ceriporiopsis subvermispora* (Watanabe et al. 2005). The highest response in ODC activity after exposure to 5 mM oxalic acid was measured from the mycelial extracts of *D. squalens* FBCC184 and it was six-fold as compared to the non-induced cultures.

With the spectrophotometric assay method used in the experiments, ODC activity was not observed in *Phlebia radiata* FBCC43, *Phanerochaete chrysosporium* FBCC283 (F1767) or *T. versicolor* FBCC298 (PRL 572). However, in the whole genome sequence of *P. chrysosporium* strain RP78, derived from the dicaryotic strain BKM-F-1767, up to seven putative ODC-encoding genes have been annotated (http://genome.jgi-psf.org/Phchr1/ Phchr1.home.html) and the fungus was shown to express ODC protein during growth on beech wood (Sato et al. 2007). On the other hand, ODC activity was demonstrated from another species of the genus *Phanerochate*, *P. sanguinea* (publication III). With an HPLC method, ODC activity has been detected from the mycelial extracts and culture liquid of *T. versicolor* strain 28A (Dutton et al. 1994). Recently, with comparable spectrophotometric assay as used in this work, ODC activity was observed from the washed mycelium of *T. versicolor* strain PRL 572 (Zhu and Hong 2009). In this work another strain of *T. versicolor*, FBCC682 (R/7), showed ODC activity (publication III). The currently available whole genome sequence data of wood- and soil-inhabiting and symbiotic basidiomycetes support the prevalence of ODC (Table 4). This indicates that either the culture conditions or oxalic acid stimulation used in the present study were not suitable to promote ODC activity in many of the fungi.

The ODCs of different fungal species studied so far are also differentially upregulated. Low pH induces ODC activity in the basidiomycetes *Flammulina velutipes* (Mehta and Datta 1991) and *Agaricus bisporus* (Kathiara et al. 2000), and in the ascomycetes *Sclerotinia sclerotiorum* (Magro et al. 1988) and *Aspergillus niger* (Emiliani and Bekes 1964). In the white-rot basidiomycete *T. versicolor* (strains 28A and PRL 572) the increase in ODC activity may as well be due to decrease in environmental pH since amendment of the growth media by addition of oxalic or inorganic acids promoted ODC activity (Dutton et al. 1994, Zhu and Hong 2009).

In the brown-rot fungus *Postia placenta* the low pH levels caused no increase in ODC activity (Micales 1997). While *P. placenta* produces very low levels of the enzyme constitutively, the addition of high (over 100 mM) concentrations of oxalic acid to the culture medium enhanced ODC activity suggesting specific induction (Micales 1997). In the study with *D. squalens* (this work), the effect of pH on ODC activity cannot be ruled out as addition of oxalic acid also caused acidity and decreased pH values in liquid cultures (Fig. 1b in publication IV). These results confirm that conditions which promote ODC activity vary significantly between fungal species and even between strains of the same species.

4.5.2 Secretion of organic acids

The four fungi with noticeable ODC activity, *Dichomitus squalens* FBCC184*, Trametes ochracea* FBCC682, *Trametes versicolor* FBCC324, and *Phanerochaete sanguinea* FBCC712, were cultivated in malt extract liquid medium and on solid-state spruce wood chips in order to follow their production of organic acids. In the submerged cultures, millimolar concentrations of oxalic acid were secreted by all four fungi after one-week of incubation (Fig. 1 in publication III). This is comparable with the amounts of oxalic acid detected in liquid cultures of other white-rot fungi (Espejo and Agosin 1991, Dutton et al. 1993, Galkin et al. 1998).

The lowest concentration of oxalic acid, 0.55 mM, was detected with *D. squalens* FBCC184 and the oxalic acid disappeared from the submerged cultures after 17 days of growth (publication III). Simultaneously, this fungus produced the highest ODC activity levels. Another strain of *D. squalens*, CSIC 361.11, has been shown to accumulate up to 15.7 mM of oxalic acid in defined liquid cultures with 1% glucose as carbon source (Sierra-Alvarez 2007). Furthermore, this isolate tolerated even 8 mM Cu^{2+} in the culture liquid. As oxalate efficiently chelates toxic metal cations into insoluble salt compounds (Sayer and Gadd 1997, Jarosz-Wilkolazka and Graz 2006), the high capacity of oxalic acid production of the *D. squalens* CSIC 361.11 is probably connected with its tolerance of elevated metal concentrations.

In addition to oxalic acid, formic and glyoxylic acids were detected in the submerged cultures of *D. squalens* FBCC184 (publication III). Glyoxylic acid was present in the most abundant quantities (Fig. 2 in publication III). Accordingly, *Ceriporiopsis subvermispora* has been reported to secrete oxalic and glyoxylic acid in submerged cultures with chemically defined medium (Urzúa et al. 1998). In the present study small concentrations of formic acid were detected also in the cultures of *T. ochracea* FBCC682, *T. versicolor* FBCC324, and *P. sanguinea* FBCC712 after addition of 5 mM oxalic acid (Table 2 in publication III) as an evidence of ODC activity.

Oxalic acid was the only organic acid secreted on spruce wood with all the four ODC-producing fungi studied (Fig. 3 in publication III). Similarly, oxalic acid was the only organic acid detected in the spruce wood cultures of *Physisporinus rivulosus* (Hakala et al. 2005), while its taxonomically close relative, *C. subvermispora* secretes mainly oxalic, but also malic, malonic, and tartaric acids during growth on pine wood chips (Aguiar et al. 2006).

In the spruce wood chip cultures of *D. squalens*, MnP activity increased with the decrease in the pH values while oxalic acid accumulated. This is as well consistent with the results of *P. rivulosus* cultivated on wood (Hakala et al. 2005). On the contrary, in wood-containing cultures of *C. subvermispora*, decrease in accumulation of oxalic acid has been correlated with high MnP activity suggesting MnP-promoted oxidation of oxalic acid leading to the formation of H_2O_2 (Urzúa et al. 1998, Aguiar et al. 2006). In addition, in the wheat straw cultures of *Phlebia* sp. Nf b19, former *Nematoloma frowardii* b19 (Hildén et al. 2008), the MnP-catalyzed decomposition of fungal-produced oxalic, malic, and fumaric acids has been suggested to enhance MnP activity (Hofrichter et al. 1999a). These results support the important role of oxalic acid in particular for MnP-catalyzed degradation of lignocellulose and lignin.

4.6 Oxalate decarboxylase of *D. squalens* (IV)

The white-rot fungus *D. squalens* FBCC184 readily showed high response in mycelial ODC activity upon exposure to oxalic acid (publication III), and thus proved to be the most suitable fungus to study ODC at the protein and gene level. Ds-ODC protein was partially purified from mycelial extracts obtained from submerged cultures of the two strains of *D. squalens* (FBCC184 and FBCC312) (publication IV). One novel *Ds-odc* gene was amplified and cloned from cDNA (strain FBCC312) and total DNA (both strains), and characterized in detail. Effect of oxalic acid concentrations on ODC activity and gene expression was followed in submerged cultures, and the amount of *Ds-odc* transcripts was quantified from spruce wood after the solid-state growth of *D. squalens* FBCC312.

4.6.1 Characteristics of *D. squalens* oxalate decarboxylase

The partially purified ODC protein from *D. squalens* FBCC184 and FBCC312 was detected as two isozymes with p*I* values of 4.2-4.25 and 2.6, and a molecular mass of 52-55 kDa, which most probably corresponds to one monomeric ODC bicupin subunit (Fig. 2 in publication IV). Comparably, the molecular mass of the few other so far characterized basidiomycetous ODC monomers varies from about 50 to over 60 kDa, and the protein typically shows two acidic isoforms (Mehta and Datta 1991, Dutton et al. 1994, Kathiara et al. 2000, Sato et al. 2007).

For the first time, an *odc* gene of a white-rotting polypore species, *D. squalens*, was cloned and characterized in detail from genomic DNA and cDNA (Publication IV). Interestingly, the Ds-ODC shows a unique primary structure with the longest polypeptide (493 aa) for any ODC enzyme characterized so far. Furthermore, it possesses a novel N-terminal alanine-serine-rich region with a length of approximately 60 amino acid residues (Fig. 5 in publication IV). A similar but shorter N-terminal stretch is present within the putative amino acid sequence of *Trametes versicolor* ODC and one of the

Phanerochaete chrysosporium ODCs. The function of this region is presently not known.

The amino acid sequence of Ds-ODC has a typical bicupin domain structure, which is also depicted in the 3D homology model (Fig. 15). The Ds-ODC carries all the conserved histidine and glutamate residues needed for Mn^{2+}-binding (Fig. 4 in publication IV). Furthermore, the Ds-ODC sequence contains the pentapeptide loop, which apparently comprises the so-called lid structure controlling enzyme activity, and a glutamate residue (corresponding to Glu-162 in *B. subtilis* OxdC), which defines enzyme specificity (Burrell et al. 2007).

Previously, bacterial and fungal ODCs clustered into one functional group and formed a monophyletic clade when shorter stretches of the conserved regions of selected cupin superfamily proteins instead of full-length amino acid sequences were analyzed (Khuri et al. 2001). In the phylogenetic analysis of translated full-length ORF sequences of ODC and oxalate oxidase (OXO), an indication of the diverse ODC enzyme family was obtained in this study (Fig. 6 in publication IV). As expected, Ds-ODC clustered with the basidiomycetous ODCs, and was clearly separated from the ascomycetous and bacterial proteins.

Two out of the seven putative ODC-encoding gene sequences that are present in the haploid *P. chrysosporium* whole genome sequence are separated from the other fungal

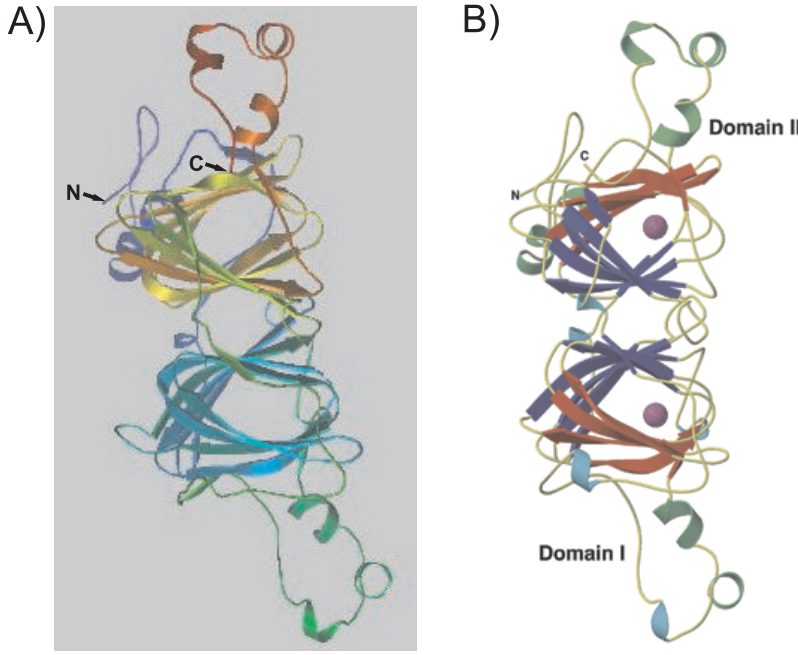


Figure 15. A) 3D homology model of the monomeric *Dichomitus squalens* oxalate decarboxylase (Ds-ODC) bicupin subunit. Protein model was created in SWISS-MODEL Workspace (http://swissmodel.expasy.org/workspace/index.php) (Arnold et al. 2006) against B) 3D crystal structure model of *Bacillus subtilis* OxdC monomer. Reprinted from Anand et al. (2002) with permission from American Chemical Society. Homology between the amino acid sequences is 51%. N- and C-termina of the protein subunits are shown. The upper and lower cupin subunits are depicted in different colouring (A). Purple spheres depict Mn^{2+} ions (B).

sequences leaving the true identity of these predicted proteins still open. As has been proposed earlier (Escutia et al. 2005), the *C. subvermispora* OXO with a conserved bicupin core structure showed close evolutionary relationship with the basidiomycete ODCs, whereas the monocupin OXO from wheat (*Triticum aestivum*) with a shorter translated protein sequence formed a clearly distinct outgroup in the neighbour-joining tree (Fig. 6 in publication IV).

4.6.2 Activity and expression of *D. squalens* oxalate decarboxylase

Notable induction of ODC activity in the submerged cultures of *D. squalens* FBCC184 was demonstrated after addition of 5 mM oxalic acid, which simultaneously lowered the extracellular pH (Table 1 in publication III, Fig. 1 in publication IV). Most of the ODC activity was detected in mycelial extracts obtained from both non-induced and oxalic-acid amended cultures, thus supporting the mainly intracellular localization of *D. squalens* ODC. The major part of the detected ODC activity was present in the mycelial extracts of *Trametes versicolor*, while a minor amount was secreted to the fungal cell wall or extracellular polysaccharide layers (Dutton et al. 1994). Furthermore, the primary location of the *Agaricus bisporus* ODC protein has been reported to be intracellular (Kathiara et al. 2000).

Extracellular ODC protein was recently detected in solid wood cultures of *Phanerochaete chrysosporium* (Sato et al. 2007). The brown-rot fungus *Postia placenta* produces some extracellular as well as intracellular ODC activity, while the highest activity is associated to the surface of the fungal hyphae (Micales 1997). Accordingly, the translated amino acid sequences of ODCs from *D. squalens*, *T. versicolor*, and two of the seven putative ODCs of *P. chrysosporium* (Fig. 5 in publication IV), and all the predicted ODCs of *P. placenta* (Martinez et al. 2009) possess putative N-terminal secretion signal peptides. It has been suggested that fungal ODC is exported from the fungal cells at specific times of growth instead of continuously, due to the observation that ODC proteins were localized in intracellular vesicles close to the plasma membrane (Dutton et al. 1994, Kathiara et al. 2000).

Quantification of the *Ds-odc* gene expression by qRT-PCR succeeded after three and four weeks of cultivation of *D. squalens* on spruce wood (Fig. 8a in publication IV). The transcript of one putative *odc* of *P. placenta* was present in cultures on cellulose medium (Martinez et al. 2009). Previously, ODC protein has been reported from the beech wood cultures of *T. versicolor* after four weeks of growth (Dutton et al. 1994), and from the lignocellulosic straw compost cultures of *A. bisporus* (Kathiara et al. 2000). Furthermore, extracellular ODC protein was detected by a proteomic approach after cultivation of *P. chrysosporium* on red oak wood (Sato et al. 2007).

In accordance with the *Ds-odc* expression, *D. squalens* secreted detectable amounts of oxalic acid after three and four week cultivation on spruce wood (Fig. 3a in publication III). The amount of *Ds-odc* transcripts detected from wood declined after four weeks concurrently with the decrease in oxalic acid concentration. Surprisingly, supplementation of excess oxalic acid caused no upregulation of *Ds-odc* at the transcriptional level in the submerged cultures since the highest relative transcript quantity was detected without supplementation of oxalic acid, when the extracellular pH value was 4.4 (Fig. 8 in publication IV). Addition of 5 mM oxalic acid dropped the

extracellular pH to 2.7 but no increase in the amount of *Ds-odc* transcripts was observed. On the contrary, in *F. velutipes* low pH (3.0) induces *odc* expression at the transcriptional level (Azam et al. 2002). In fact, a protein complex that binds specifically to a low pH responsive element in the promoter region of the *Flammulina velutipes odc* gene has been identified (Azam et al. 2002). A similar element is absent in the predicted promoter region sequence upstream of the *D. squalens odc* gene (Fig. 4 in publication IV).

The increase in *D. squalens* ODC activity observed after supplementation of oxalic acid may also be a result of protein level activation caused either un-specifically by increased acidity, or specifically by oxalic acid, as has been proposed for the ODC of *P. placenta* (Micales 1997). It is possible that *D. squalens* has several ODC-encoding genes considering the recent whole genome sequence data of other basidiomycetous species (Table 4). Therefore it is also likely that the observed promotion of ODC activity may be a consequence of upregulation of other, yet uncharacterized *odc* gene(s) of *D. squalens*.

In *Ceriporiopsis subvermispora*, the sequential action of ODC and the catabolic enzyme formate dehydrogenase (FDH) has been suggested leading to the complete conversion of oxalic acid to CO_2 (Watanabe et al. 2005). The simultaneously formed NADH may in turn serve as an electron source for ATP synthesis during fungal vegetative growth. In addition to *C. subvermispora*, FDH activity or the corresponding *fdh* gene has been detected in several other ODC-possessing white-rot fungi such as *F. velutipes*, *P. chrysosporium*, *T. versicolor*, *Schizophyllum commune*, and *P. placenta* (Watanabe et al. 2005, Martinez et al. 2009), thus suggesting a common physiological relationship between ODC and FDH.

Interestingly, *C. subvermispora* is the first fungus in which both ODC and oxalate oxidase (OXO) activities have been detected. While ODC and FDH activities were found during the fungal vegetative growth, H_2O_2-producing OXO activity appeared at later stage of cultivation, thereby suggesting a specific function for OXO in secondary metabolism and e.g. lignin degradation (Aguilar et al. 1999, Watanabe et al. 2005). The occurrence of OXO in white-rot fungi needs to be further investigated since the enzyme has so far been described only in *C. subvermispora* and *Abortiporus biennis* (Aguilar et al. 1999, Grąz et al. 2009).

Detection of the *Ds-odc* transcripts in marked amounts in submerged liquid and solid-state wood cultures suggests constitutive expression of the gene, thus pointing to general metabolic role of the corresponding Ds-ODC enzyme, most probably to be involved in energy production and growth. Studies with different white- and brown-rot fungal species are necessary to address these hypotheses, and to clarify the roles of the different kinds of oxalic-acid decomposing enzymes in wood-degrading fungi.

5 Summary and conclusions

This thesis describes the genes coding for the lignin-modifying enzymes of the well-studied white-rot fungus *Phlebia radiata* FBCC43 (79, ATCC 64658) and their expression in wood-based media. The secretion of organic acids and the prevalence of the oxalic-acid degrading enzyme oxalate decarboxylase (ODC) in white-rot fungi were determined. The knowledge of fungal ODCs was further advanced by a molecular study of the *Dichomitus squalens* ODC. The main findings and conclusions obtained in this work were:

1. Two new *P. radiata* LiP-encoding genes (*Pr-lip1* and *Pr-lip4*) and one gene previously studied at cDNA level (*Pr-lip3*) were cloned and characterized. According to protein phylogenetic analysis, *P. radiata* has multiple *lip* genes encoding evolutionarily very closely related LiP enzymes.
2. A new laccase-encoding gene of *P. radiata* (*Pr-lac2*) was cloned and characterized, and evidence of additional laccase genes in the fungus was obtained. The two *P. radiata* laccases characterized at present demonstrate distinct gene structure and protein phylogeny, thus indicating different physiological functions for the enzymes.
3. Wood promoted expression of all the presently known *P. radiata* LME-encoding genes, including the three LiPs, two divergent MnPs, and the two laccases, as judged by transcript-level analyses. Expression of individual LiP- and laccase-encoding genes on wood was variable and apparently time-dependent.
4. *P. radiata* laccase activity was significantly increased with supplementation of excess Cu^{2+} ions. The complex peptone-yeast extract medium rich with organic nitrogen supported fungal growth and laccase production as well, with the evidence of an additional extracellular laccase isoform of *P. radiata*.
5. Cloning and molecular characterization of two laccase-encoding genes from *D. squalens* was shown for the first time. The two *Ds-lac* genes, which encode evolutionary distinct proteins, were differently expressed when the fungus grew on wood.
6. ODC-activity was demonstrated for the first time in the mycelial extracts of four strains of wood-decaying white-rot fungi.
7. Oxalic acid was the most common organic acid secreted by the ODC-positive white-rot fungi. Especially with regard to the *D. squalens* wood cultures, the occurrence of MnP activity suggests the importance of oxalic acid in MnP-catalyzed degradation of wood and lignin.
8. A new ODC-encoding gene (*Ds-odc*) was cloned from two strains of *D. squalens*, demonstrating the first characterization of an *odc* gene in a white-rot polypore. Expression of *Ds-odc* suggests a constitutive metabolic role for the corresponding Ds-ODC.

9. The biochemical properties and the amino acid sequence of the Ds-ODC resemble those described for other basidiomycetous ODCs, but the translated protein comprises a novel N-terminal primary structure with unknown function.

The results obtained in this study refer to complex regulation of multiple LME-encoding genes during the growth of white-rot fungi on wood, their natural lignocellulosic substrate. The data obtained with two different species of wood-decaying, white-rot basidiomycetes emphasizes the importance of individual genes and specific functions of the corresponding protein products, which are probably needed at diverse phases of fungal growth and wood decay. The role of oxalic acid as an important and commonly secreted metabolite on fungal wood cultures was supported by the results of this study. The oxalic-acid decomposing enzyme ODC seems to be important during the primary growth of white-rot fungi, and as has been proposed, the enzyme may even play a role in concomitant production of ATP. Overall, the organic acid metabolism is closely linked to the lignocellulose-degrading machinery of white-rot fungi with further emphasis on fungal growth and energy metabolism. In Fig. 16 the intra- and extracellular reactions as a combination of the action of the white-rot fungal LMEs and the enzymes involved in organic acid metabolism are summarized.

In future, to complement the whole array and functions of lignin-modifying enzymes of *P. radiata* and *D. squalens*, characterization of new genes and their protein products, and extracellular metabolites is required. Eventually, whole genome sequencing followed by transcriptome and proteome studies will evaluate the significance of fungal LMEs in biological decomposition of wood. In order to clarify the roles of fungal produced oxalic acid during the decay process, it is necessary to study the prevalence and regulation of oxalic-acid converting enzymes (ODC, OXO, FDH) in several wood-decomposing fungal species. The next phase towards biotechnological applications on fungal ODCs is to clarify protein 3D structure and catalytical properties.

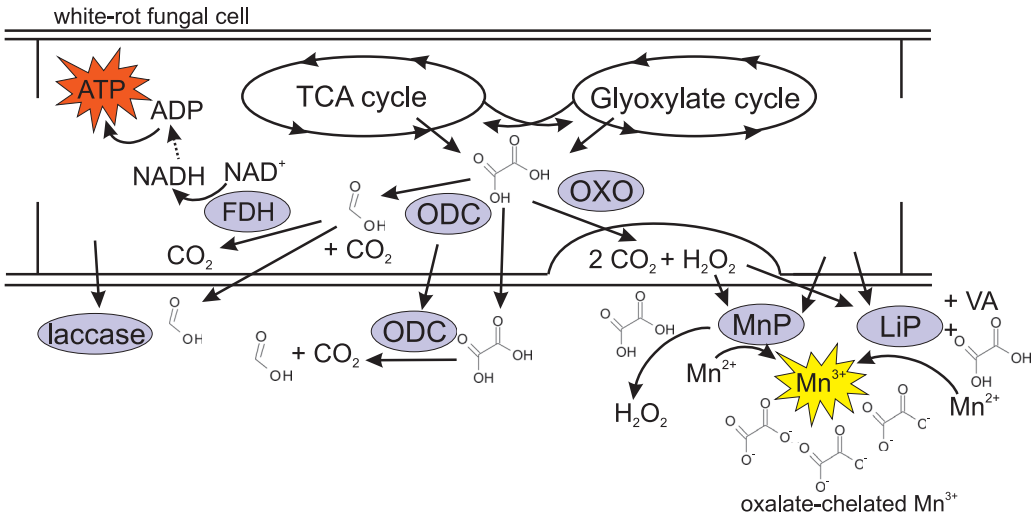


Figure 16. Proposed intra- and extracellular reactions of the lignin-modifying and oxalic-acid converting enzymes generally occurring in the lignin-degrading white-rot basidiomycetes. The scheme is illustrated according to Watanabe et al. (2005), Munir et al. (2001a), and original data obtained in this study.

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7 References

Adler E. (1977) Lignin chemistry – past, present and future. Wood Sci. Technol. 11:169-218.

Aguiar A., Brazil de Souza-Cruz P., Ferraz A. (2006) Oxalic acid, Fe^{3+}-reduction activity and oxidative enzymes detected in culture extracts recovered from *Pinus taeda* wood chips biotreated by *Ceriporiopsis subvermispora*. Enzyme Microb. Technol. 38:873-878.

Aguilar C., Urzúa U., Koenig C., Vicuña R. (1999) Oxalate oxidase from *Ceriporiopsis subvermispora*: Biochemical and cytochemical studies. Arch. Biochem. Biophys. 366:275-282.

Akamatsu Y., Ma D.B., Higuchi T., Shimada M. (1990) A novel enzymatic decarboxylation of oxalic acid by the lignin peroxidase system of white-rot fungus *Phanerochaete chrysosporium*. FEBS Lett. 269:261-263.

Akamatsu Y., Shimada M. (1994) Partial purification and characterization of glyoxylate oxidase from the brown-rot basidiomycete *Tyromyces palustris*. Phytochem. 37:649-653.

Akamatsu Y., Takahashi M., Shimada M. (1993) Cell-free extraction of oxaloacetase from white-rot fungi, including *Coriolus versicolor*. Wood Res. 79:1-6.

Akhtar M., Blanchette R.A., Kirk T.K. (1997) Fungal delignification and biomechanical pulping of wood. In: Eriksson K.-E.L., editor. Advances in Biochemical Engineering/Biotechnology. Vol. 57. Biotechnology in the Pulp and Paper Industry. Springer Verlag, Berlin, Germany, pp. 159-196.

Alexandre G., Zhulin I.B. (2000) Laccases are widespread in bacteria. Trends. Biotech. 41-42.

Álvarez J.M., Canessa P., Mancilla R.A., Polanco R., Santibáñez P.A., Vicuña R. (2009) Expression of genes encoding laccase and manganese-dependent peroxidase in the fungus *Ceriporiopsis subvermispora* is mediated by an ACE1-like copper-fist transcription factor. Fungal Genet. Biol. 46:104-111.

Amirta R., Fujimori K., Shirai N., Honda Y., Watanabe T. (2003) Ceriporic acid C, a hexadecenylitaconate produced by a lignin-degrading fungus, *Ceriporiopsis subvermispora*. Chem. Phys. Lipids 126:121-131.

Anand R., Dorrestein P.C., Kinsland C., Begley T.P., Ealick S.E. (2002) Structure of oxalate decarboxylase from *Bacillus subtilis* at 1.75 Å resolution. Biochem. 41:7659-7669.

Anantharam V., Allison M.J., Maloney P.C. (1989) Oxalate:formate exchange. The basis for energy coupling in *Oxalobacter*. J. Biol. Chem. 13:7244-7250.

Antelmann H., Töwe S., Albrecht D., Hecker M. (2007) The phosphorus source phytate changes the composition of the cell wall proteome in *Bacillus subtilis*. J. Prot. Res. 6:897-903.

Argyropoulos D.S., Menachem S.B. (1997) Lignin. In: Eriksson K.-E.L., editor. Advances in Biochemical Engineering/Biotechnology. Vol 57. Biotechnology in the Pulp and Paper Industry. Springer-Verlag, Germany, pp. 127-158.

Arnold K., Bordoli L., Kopp J., Schwede T. (2006) The SWISS-MODEL workspace: A web-based environment for protein structure homology modelling. Bioinformatics 22:195-201.

Arora D.S., Gill P.K. (2000) Laccase production by some white rot fungi under different nutritional conditions. Biores. Technol. 73:283-285.

Azam M., Kesarwani M., Chakraborty S., Natarajan K., Datta A. (2002) Cloning and characterization of the 5'-flanking region of the oxalate decarboxylase gene from *Flammulina velutipes*. Biochem. J. 367:67-75.

Azam M., Kesarwani M., Natarajan K., Datta A. (2001) A secretion signal is present in the *Collybia velutipes* oxalate decarboxylase gene. Biochem. Biophys. Res. Com. 289:807-812.

Baldrian P. (2006) Fungal laccases - occurence and properties. FEMS Microbiol. Rev. 30:215-242.

Baldrian P., Gabriel J. (2002) Copper and cadmium increase laccase activity in *Pleurotus ostreatus*. FEMS Microbiol. Lett. 206:69-74.

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

Bao W., Fukushima Y., Jensen K.A., Moen M.A., Hammel K.E. (1994) Oxidative degradation of non-phenolic lignin during lipid peroxidation by fungal manganese peroxidase. FEBS Lett. 354:297-300.

Belinky P.A., Flikshtein N., Lechenko S., Gepstein S., Dosoretz C.G. (2003) Reactive oxygen species and induction of lignin peroxidase in *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. 69:6500-6506.

Bertrand T., Jolivalt C., Briozzo P., Caminade E., Joly N., Madzak C., Mougin C. (2002) Crystal structure of a four-copper laccase complexed with an arylamine: Insights into substrate recognition and correlation with kinetics. Biochem. 41:7325-7333.

Binder M., Hibbett D.S., Larsson K.-H., Larsson E., Langer E., Langer G. (2005) The phylogenetic distribution of resupinate forms across the major clades of mushroom-forming fungi (homobasidiomycetes). Syst. Biodivers. 3:1-45.

Blanchette R.A. (1984) Screening wood decayed by white rot fungi for preferential lignin degradation. Appl. Environ. Microbiol. 48:647-653.

Blanchette R.A., Otjen L., Carlson M.C. (1987) Lignin distribution in cell walls of birch wood decayed by white rot basidiomycetes. Phytopath. 77:684-690.

Blanchette R.A., Krueger E.W., Haight J.E., Akhtar M., Akin D.E. (1997) Cell wall alterations in loblolly pine wood decayed by the white-rot fungus, *Ceriporiopsis subvermispora*. J. Biotechnol. 53:203-213.

Bogan B.W., Schoenike B., Lamar R.T., Cullen D. (1996) Expression of *lip* genes during growth in soil and oxidation of anthracene by *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. 62:3697-3703.

Breuil C., Iverson S., Gao Y. (1998) Fungal treatment of wood chips to remove extractives. In: Young R.A., Akhtar M., editors. Environmentally Friendly Technologies for the Pulp and Paper Industry. John Wiley & Sons, Inc., New York, pp. 541-565.

Broda P., Birch P.R.J., Brooks P.R., Sims P.F.G. (1995) PCR-mediated analysis of lignocellulolytic gene transcription by *Phanerochaete chrysosporium*: substrate-dependent differential expression within gene-families. Appl. Environ. Microbiol. 61:2358-2364.

Brunow G. (2001) Methods to reveal the structure of lignin. In: Hofrichter M., Steinbüchel A., editors. Biopolymers. Vol 1: Lignin, Humic Substances and Coal.Wiley-VCH, Weinheim, Germany, pp. 89-116.

Burrell M.R., Just V.J., Bowater L., Fairhurst S.A., Requena L., Lawson D.M., Bornemann S. (2007) Oxalate decarboxylase and oxalate oxidase activities can be interchanged with a specificity switch of up to 282 000 by mutating an active site lid. Biochem. 46:12327-12336.

Bödeker I.T.M., Nygren C.M.R., Taylor A.F.S., Olson Å., Lindahl B.D. (2009) ClassII peroxidase-encoding genes are present in a phylogenetically wide range of ectomycorrhizal fungi. ISME J. DOI:10.1038/ismej.2009.77

Camarero S., Ibarra D., Martínez Á.T., Martínez M.J., Romero J., Gutíerrez A., del Rio J. (2007) Paper pulp delignification using laccase and natural mediators. Enzyme Microb. Technol. 40:1264-1271.

Camarero S., Ibarra D., Martínez M.J., Martínez Á.T. (2005) Lignin-derived compounds as efficient laccase mediators for decolorization of different types of recalcitrant dyes. Appl. Environ. Microbiol. 71:1775-1784.

Camarero S., Ruiz-Dueñas J., Sarkar S., Martínez M.J., Martínez A.T. (2000) The cloning of a new peroxidase found in lignocellulose cultures of *Pleurotus eryngii* and sequence comparison with other fungal peroxidases. FEMS Microbiol. Lett. 191:37-43.

Camarero S., Sarkar S., Ruiz-Dueñas F.J., Martinez M.J., Martinez A.T. (1999) Description of a versatile peroxidase involved in the natural degradation of lignin that has both manganese peroxidase and lignin peroxidase substrate interaction sites. J. Biol. Chem. 274:10324-10330.

Campoy S., Álvarez-Rodrígues M.L., Recio E., Rumbero A., Coque J.-J.R. (2009) Biodegradation of 2,4,6-TCA by the white-rot fungus *Phlebia radiata* is initiated by a phase I (O-demethylation)-phase II (O-conjugation) reactions system: Implications for the chlorine cycle. Environ. Microbiol. 11:99-110.

Candeias L.P., Harvey P.J. (1995) Lifetime and reactivity of the veratryl alcohol cation. Implications for lignin peroxidase catalysis. J. Biol. Chem. 270:16745-16748.

Carlile M.J., Watkinson S.C., Gooday G.W. (2001) The Fungi. Academic Press, London, UK. 588 p.

Cassland P., Jönsson L.J. (1999) Characterization of a gene encoding *Trametes versicolor* laccase A and improved heterologous expression in *Saccharomyces cerevisiae* by decreased cultivation temperature. Appl. Microbiol. Biotechnol. 52:393-400.

Chakraborty S., Chakraborty N., Jain D., Salunke D.M., Datta A. (2002) Active site geometry of oxalate decarboxylase from *Flammulina velutipes*: Role of histidine-coordinated manganese in substrate recognition. Prot. Sci. 11:2138-2147.

Chander M., Arora D.S. (2007) Evaluation of some white-rot fungi for their potential to decolourise industrial dyes. Dyes Pigm. 72:192-198.

Chander M., Arora D.S., Bath H.K. (2004) Biodecolourisation of some industrial dyes by white-rot fungi. J. Ind. Microbiol. Biotechnol. 31:94-97.

Chandra T.S., Shethna Y. (1977) Oxalate, formate, formamide, and methanol metabolism in *Thiobacillus novellus*. J. Bacteriol. 131:389-398.

Chen S., Ma D., Ge W., Buswell J.A. (2003) Induction of laccase activity in the edible straw mushroom, *Volvariella volvacea*. FEMS Microbiol. Lett. 218:143-148

Cho N.S., Hatakka A.I., Rogalski J., Cho H.Y., Ohga S. (2009) Directional degradation of lignocellulose by *Phlebia radiata.* J. Fac. Agr. Kyushy U. 54:73-80.

Choinowski T., Blodig W., Winterhalter K.H., Piontek K. (1999) The crystal structure of lignin peroxidase at 1.70 Å resolution reveals a hydroxy group on the C^{β} of tryptophan 171: A novel radical site formed during the redox cycle. J. Mol. Biol. 286:809-827.

Claus H. (2004) Laccases: Structure, reactions, distribution. Micron 35:93-96.

Cohen R., Hadar Y., Yarden O. (2001) Transcript and activity levels of different *Pleurotus ostreatus* peroxidases are differentially affected by Mn^{2+}. Environ. Microbiol. 3:312-22.

Coll P.M., Tabernero C., Santamaria R., Perez P. (1993) Characterization and structural analysis of the laccase I gene from the newly isolated ligninolytic basidiomycete PM1 (CECT 2971). Appl. Environ. Microbiol. 59:4129-4135.

Collins P.J., Dobson A.D.W. (1997) Regulation of laccase gene transcription in *Trametes versicolor*. Appl. Environ. Microbiol. 63:3444-3450.

Conesa A., Punt P.J., van den Hondel C.A.M.J.J. (2002) Fungal peroxidases: Molecular aspects and applications. J. Biotechnol. 93:143-158.

Conesa A. van den Hondel C.A.M.J.J., Punt P.J. (2000) Studies on the production of fungal peroxidases in *Aspergillus niger*. Appl. Environ. Microbiol. 66:3016-3023.

Couto S.R., Toca-Herrera J.L. (2007) Laccase production at reactor scale by filamentous fungi. Biotechnol. Adv. 25:558-569.

Courty P.E., Hoegger P.J., Kilaru S., Kohler A., Buée M., Garbaye J., Martin F., Kües U. (2009) Phylogenetic analysis, genomic organization, and expression analysis of multi-copper oxidases in the ectomycorrhizal basidiomycete *Laccaria bicolor*. New Phytol. 182:736-750

Cullen D. (1997) Recent advances on the molecular genetics of ligninolytic fungi. J. Biotechnol. 53:273-289.

de Jong E., Field J.A., de Bont J.A.M. (1994) Aryl alcohols in the physiology of ligninolytic fungi. FEMS Microbiol. Rev. 13:153-187.

de Koker T.H., Nakasone K.K., Haarhof J., Burdsall H.H., Janse B.J.H. (2003) Phylogenetic relationships of the genus *Phanerochaete* inferred from the internal transcribed spacer region. Mycol. Res. 107:1032-1040.

Dias B.B.A., Cunha W.G., Morais L.S., Vianna G.R., Rech E.L., de Capdeville G., Aragão F.J.L. (2006) Expression of an oxalate decarboxylase gene from *Flammulina* sp. in transgenic lettuce (*Lactuca sativa*) plants and resistance to *Sclerotinia sclerotiorum*. Plant Pathol. 55:187-193.

Doyle W.A., Blodig W., Veitch N.C., Piontek K., Smith A.T. (1998) Two substrate interaction sites in lignin peroxidase revealed by site-directed mutagenesis. Biochem. 37:15097-15105.

Dresler-Nurmi A., Kaijalainen S., Lindström K., Hatakka A. (1999) Grouping of lignin degrading corticioid fungi based on RFLP analysis of 18S rDNA and ITS regions. Mycol. Res. 103:990-996.

D'Souza T.M., Merritt C.S., Reddy C.A. (1999) Lignin-modifying enzymes of the white rot basidiomycete *Ganoderma lucidum*. Appl. Environ. Microbiol. 65:5307-5313.

Ducros V., Brzozowski A.M., Wilson K.S., Brown S.H., Oestergaard P., Schneider P., Yaver D.S., Pedersen A.H., Davies G.J. (1998) Crystal structure of the type-2 Cu depleted laccase from *Coprinus cinereus* at 2.2 Å resolution. Nat. Struct. Biol. 5:310-316.

Dunwell J.M., Khuri S., Gane P.J. (2000) Microbial relatives of the seed storage proteins of higher plants: Conservation of structure, and diversification of function during evolution of the cupin superfamily. Microbiol. Mol. Biol. Rev. 64:153-179.

Dunwell J.M., Purvis A., Khuri S. (2004) Cupins: The most functionally diverse protein superfamily? Phytochem. 65:7-17.

Dutton M.V., Evans C.S. (1996) Oxalate production by fungi: Its role in pathogenicity and ecology in the soil environment. Can. J. Microbiol. 42:881-895.

Dutton M.V., Evans C.S., Atkey P.T., Wood D.A. (1993) Oxalate production by basidiomycetes, including the white-rot species *Coriolus versicolor* and *Phanerochaete chrysosporium*. Appl. Microbiol. Biotechnol. 39:5-10

Dutton M.V., Kathiara M., Gallagher I.M., Evans C.S. (1994) Purification and characterization of oxalate decarboxylase from *Coriolus versicolor*. FEMS Microbiol. Lett. 116:321-326.

Eaton R.A., Hale M.D.C. (1993) Wood decay, pests, and protection. Chapman & Hall, Cambridge, UK. 546 p.

Eichlerová I., Homolka L., Benada O., Kofroňová O., Hubálek T., Nerud F. (2007) Decolorization of orange G and remazol brilliant blue R by the white rot fungus *Dichomitus squalens*: Toxicological evaluation and morphological study. Chemosphere 69:795-802.

Eichlerová I., Homolka L., Lisá L., Nerud F. (2005) Orange G and remazol brilliant blue R decolorization by white rot fungi *Dichomitus squalens*, *Ischnoderma resinosum* and *Pleurotus calyptratus*. Chemosphere 60:398-404.

Eichlerová I., Homolka L., Nerud F. (2006) Synthetic dye decolorization capacity of white rot fungus *Dichomitus squalens*. Biores. Technol. 97:2153-2159.

Eggert C., Temp U., Dean J.F., Eriksson K.-E.L. (1996) A fungal metabolite mediates degradation of non-phenolic lignin structures and synthetic lignin by laccase. FEBS Lett. 391:144-148.

Emiliani E., Bekes P. (1964) Enzymatic oxalate decarboxylation in *Aspergillus niger*. Arch. Biochem. Biophys. 105:488-493.

Enoki M., Honda Y., Kuwahara M., Watanabe T. (2002) Chemical synthesis, iron redox interactions and charge transfer complex formation of alkylitaconic acids from *Ceriporiopsis subvermispora*. Chem. Phys. Lipids **120:** 9-20.

Enoki M., Watanabe T., Nakagame S., Koller K., Messner K., Honda Y., Kuwahara M. (1999) Extracellular lipid peroxidation of selective white-rot fungus, *Ceriporiopsis subvermispora.* FEMS Microbiol. Lett. 180:205-211.

Eriksson K.-E.L., Blanchette R.A., Ander P. (1990) Microbial and enzymatic degradation of wood and wood components. Springer-Verlag, Berlin, Germany, 407 p.

Escutia M.R., Bowater L., Edwards A., Bottrill A.R., Burrell M., Polanco R., Vicuña R., Bornemann S. (2005) Cloning and sequencing of two *Ceriporiopsis subvermispora* bicupin oxalate oxidase allelic isoforms: Implications for the reaction specificity of oxalate oxidases and decarboxylases. Appl. Environ. Microbiol. 71:3608-3616.

Espejo E., Agosin E. (1991) Production and degradation of oxalic acid by brown rot fungi. Appl. Environ. Microbiol. 57:1980-1986.

Evans C.S. (1985) Laccase activity in lignin degradation by *Coriolus versicolor in vivo* and *in vitro* studies. FEMS Microbiol. Lett. 27:339-343.

Evans C.S., Dutton M.V., Guillén F., Veness R.G. (1994) Enzymes and small molecular mass agents involved with lignocellulose degradation. FEMS Microbiol. Rev. 13:235-240.

Fackler K., Gradinger C., Hinterstoisser B., Messner K., Schwanninger M. (2006) Lignin degradation by white rot fungi on spruce wood shavings during short-time solid-state fermentations monitored by near infrared spectroscopy. Enzyme Microb. Technol. 39:1476-1483.

Faraco V., Giardina P., Sannia G. (2003) Metal-responsive elements in *Pleurotus ostreatus* laccase gene promoters. Microbiology 149:2155-2162.

Federici F., Vitali B., Gotti R., Pasca M.R., Gobbi S., Peck A.B., Brigidi P. (2004) Characterization and heterologous expression of the oxalyl coenzyme A decarboxylase gene from *Bifidobacterium lactis*. Appl. Environ. Microbiol. 70:5066-5073.

Feijoo G., Moreira M.T., Álvarez P.A., Lú-Chau T., Lema J.M. (2008) Evaluation of the enzyme manganese peroxidase in an industrial sequence for the lignin oxidation and bleaching of eucalyptus kraft pulp. J. Appl. Polym. Sci. 109:1319-1327.

Fengel D., Wegener E. (1989) Wood: chemistry, ultrastructure, reactions. Walter de Gruyter, Berlin, Germany. 613 p.

Ferraroni M., Myasoedova N., Schmatchenko V., Leontievsky A.A., Golovleva L.A., Scozzafava A., Briganti F. (2007) Crystal structure of a blue laccase from *Lentinus tigrinus*: Evidences for intermediates in the molecular oxygen reductive splitting by multicopper oxidases. BMC Struct. Biol. 7:60-72.

Ferreira P., Medina M., Guillén F., Martínez M.J., van Berkel W.J.H., Martínez Á.T. (2005) Spectral and catalytic properties of aryl-alcohol oxidase, a fungal flavoenzyme acting on polyunsaturated alcohols. Biochem. J. 389:731-738.

Festa G., Autore F., Fraternali F., Giardina P., Sannia G. (2008) Development of new laccases by directed evolution: Functional and computational analyses. Proteins 72:25-34.

Fomina M., Charnock J.M., Hillier S., Alvarez R., Gadd G.M. (2007) Fungal transformations of uranium oxides. Environ. Microbiol. 9:1696-1710.

Foust T.D, Ibsen K.N, Dayton D.C., Hess J.R., Kenney K.E. (2008) The biorefinery. In: Himmel M., editor. Biomass recalcitrance: deconstructing the plant cell wall for bioenergy. Blackwell Pub., Oxford, UK, pp. 7-37.

Galhaup C., Wagner H., Hinterstoisser B., Haltrich D. (2002) Increased production of laccase by the wood-degrading basidiomycete *Trametes* pubescens. Enzyme Microb. Technol. 30:529-536.

Galkin S., Vares T., Kalsi M., Hatakka A. (1998) Production of organic acids by different white rot fungi as detected using capillary zone electrophoresis. Biotechnol. Tech. 12:267-271.

Garavaglia S., Teresa Cambria M., Miglio M., Ragusa S., Iacobazzi V., Palmieri F., D'Ambrosio C., Scaloni A., Rizzi M. (2004) The structure of *Rigidoporus lignosus* laccase containing a full complement of copper ions, reveals an asymmetrical arrangement for the T3 copper pair. J. Mol. Biol. 342:1519-1531.

Gettemy J.M., Ma B., Alic M., Gold M.H. (1998) Reverse transcription-PCR analysis of the regulation of the manganese peroxidase gene family. Appl. Environ. Microbiol. 64:569-574.

Gianfreda L., Xu F., Bollag J. (1999) Laccases: a useful group of oxidoreductive enzymes. Biorem. J. 3:1-25.

Giardina P., Palmieri G., Fontanella B., Rivieccio V., Sannia G. (2000) Manganese peroxidase isoenzymes produced by *Pleurotus ostreatus* grown on wood sawdust. Arch. Biochem. Biophys. 376:171-179.

Giardina P., Palmieri G., Scaloni A., Fontanella B., Faraco V., Cennamo G., Sannia G. (1999) Protein and gene structure of a blue laccase from *Pleurotus ostreatus*. Biochem. J. 341:655-663.

Gill P.K., Arora D.S. (2003) Effect of culture conditions on manganese peroxidase production and activity by some white rot fungi. J. Ind. Microbiol. Biotechnol. 30:28-33.

Gill P.K., Arora D.S., Chander M. (2002) Biodecolourization of azo and triphenylmethane dyes by *Dichomitus squalens* and *Phlebia* spp. J. Ind. Microbiol. Biotechnol. 28:201-203.

Glenn J.K., Morgan M.A., Mayfield M.B., Kuwahara M., Gold M.H. (1983) An extracellular H_2O_2-requiring enzyme preparation involved in lignin biodegradation by the white rot basidiomycete *Phanerochaete chrysosporium*. Biochem. Biophys. Res. Comm. 114:1077-1083.

Gold M.H., Alic M. (1993) Molecular biology of the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. Microbiol. Mol. Biol. Rev. 57:605-622.

Gold M.H., Youngs H.L., Sollewijn Gelpke M.D. (2000) Manganese peroxidase. In: Sigel A., Sigel H., editors. Metal Ions in Biological Systems. Marcel Dekker, New York-Basel, pp. 559-586.

Goodell B., Jellison J., Liu J., Daniel G., Paszczynski A., Fekete F., Krishnamurthy S., Jun L., Xu G. (1997) Low molecular weight chelators and phenolic compounds isolated from wood decay fungi and their role in the fungal biodegradation of wood. J. Biotechnol. 53:133-162.

Grąz M., Jarosz-Wilkołazka A., Pawlikowska-Pawlęga B. (2009) *Abortiporus biennis* tolerance to insoluble metal oxides: oxalate secretion, oxalate oxidase activity, and mycelia morphology. Biometals 22:401-410.

Green F., Clausen C.A. (2003) Copper tolerance of brown-rot fungi: time course of oxalic acid production. Int. Biodet. Biodeg. 51:145-149.

Grujic D., Salido E.C., Shenoy B.C., Langman C.B., McGrath M.E., Patel R.J., Rashid A., Mandapati S., Jung C.W., Margolin A.L. (2009) Hyperoxaluria is reduced and nephrocalcinosis prevented with an oxalate-degrading enzyme in mice with hyperoxaluria. Am. J. Nephrol. 29:86-93.

Gutiérrez A., del Río J.D., Martínez-Íñigo M.J., Martínez M.J., Martínez Á.T. (2002) Production of new unsaturated lipids during wood decay by ligninolytic basidiomycetes. Appl. Environ. Microbiol. 68:1344-1350.

Hakala T.K. (2007) Characterization of the lignin-modifying enzymes of the selective white-rot fungus *Physisporinus rivulosus*. Dissertationes bioscientiarum molecularium Universitatis Helsingiensis in Viikki, 21/2007. Ph.D. Thesis, Department of Applied Chemistry and Microbiology, University of Helsinki, Helsinki, 60 p.

Hakala T.K., Hildén K., Maijala P., Olsson C., Hatakka A. (2006) Differential regulation and characterization of two variable MnP encoding genes in the white-rot fungus *Physisporinus rivulosus*. Appl. Microbiol. Biotechnol. 73:839-849.

Hakala T.K., Lundell T., Galkin S., Maijala P., Kalkkinen N., Hatakka A. (2005) Manganese peroxidases, laccases and oxalic acid from the selective white-rot fungus *Physisporinus rivulosus* grown on spruce wood chips. Enzyme Microb. Technol. 36:461-468.

Hakala T.K., Maijala Konn J., Hatakka A. (2004) Evaluation of novel wood-rotting polypores and corticioid fungi for the decay and biopulping of Norway spruce (*Picea abies*) wood. Enzyme Microb. Technol. 34:255-263.

Hakulinen N., Kiiskinen L.-L., Kruus K., Saloheimo M., Paananen A., Koivula A., Rouvinen J. (2002) Crystal structure of a laccase from *Melanocarpus albomyces* with an intact trinuclear copper site. Nat. Struct. Biol. 9:601-605.

Hammel K. (1997) Fungal degradation of lignin. In: Cadisch G., Giller K.E., editors. Driven by Nature: Plant Litter Quality and Decomposition. CAB International, Wallingford, UK, pp. 33-45.

Hammel K.E., Cullen D. (2008) Role of fungal peroxidases in biological ligninolysis. Curr. Opin. Plant Biol. 11:349-355.

Haritash A.K., Kaushik C.P. (2009) Biodegradation aspects of polycyclic aromatic hydrocarbons (PAHs): A review. J. Hazard. Mater. 169:1-15.

Harris P.J., Stone B.A. (2008) Chemistry and molecular organization of plant cell walls. In: Himmel M., editor. Biomass recalcitrance: deconstructing the plant cell wall for bioenergy. Blackwell Pub., Oxford, UK, pp. 61-93.

Hatakka A. (1986) Degradation and conversion of lignin, lignin-related aromatic compounds and lignocellulose by selected white-rot fungi. Ph.D. Thesis, Department of Microbiology, University of Helsinki, Helsinki, 99 p.

Hatakka A. (1994) Lignin-modifying enzymes from selected white rot fungi: production and role in lignin degradation. FEMS Microbiol. Rev. 13:125-135.

Hatakka A. (2001) Biodegradation of lignin. In: Hofrichter M., Steinbüchel A., editors. Biopolymers. Vol 1: Lignin, Humic Substances and Coal. Wiley-VCH, Weinheim, Germany, pp. 129-180.

Hatakka A.I. (1985) Degradation of veratric acid and other lignin-related aromatic compounds by the white-rot fungus *Pycnoporus cinnabarinus*. Arch. Microbiol. 141:22-28.

Hatakka A.I., Lundell T.K., Tervilä-Wilo A.L.M., Brunow G. (1991) Metabolism of non-phenolic β-O-4 lignin model compounds by the white-rot fungus *Phlebia radiata*. Appl. Microbiol. Biotechnol. 36:270-277.

Hatakka A., Kantelinen A., Tervilä-Wilo A., Viikari L. (1987) Production of ligninases by *Phlebia radiata* in agitated cultures. In: Odier E., editor. Lignin enzymic and microbial degradation. INRA Publications, Paris, pp.185-189.

Hatakka A., Lundell T., Hofrichter M., Maijala P. (2003) Manganese peroxidase and its role in the degradation of wood lignin. In: Mansfield S.D., Saddler J.N., editors. ACS Symposium series 855, Applications of Enzymes to Lignocellulosics. American Chemical Society, Washington DC, USA, pp. 230-243.

Hatakka A., Uusi-Rauva A. (1983) Degradation of ^{14}C-labelled poplar wood lignin by selected white-rot fungi. Eur. J. Appl. Microbiol. Biotechnol. 17:235-242.

Hatfield R.D., Ralph J., Grabber J.H. (1999) Cell wall cross-linking by ferulates and diferulates in grasses. J. Sci. Food Agric. 79:403-407.

Heinfling A., Martínez M.J., Martínez A.T., Bergbauer M., Szewzyk U. (1998) Purification and characterization of peroxidases from the dye-decolorizing fungus *Bjerkandera adusta*. FEMS Microbiol. Lett. 165:43-50.

Henriksson G., Ander P., Pettersson B., Petterson G. (1995) Cellobiose dehydrogenase (cellobiose oxidase) from *Phanerochaete chrysosporium* as a wood-degrading enzyme. Studies on cellulose, xylan and synthetic lignin. Appl. Microbiol. Biotechnol. 42:790-796.

Henriksson G., Johansson G., Pettersson G. (2000) A critical review of cellobiose dehydrogenases. J. Biotechnol. 78:93-113.

Hess J., Leitner C., Galhaup C., Kulbe K.D., Hinterstoisser B., Steinwender M., Haltrich D. (2002) Enhanced formation of extracellular laccase activity by the white-rot fungus *Trametes multicolor*. Appl. Biochem. Biotechnol. 98-100:229-241.

Hibbett D.S., Binder M., Bischoff J.F., Blackwell M., Cannon P.F., Eriksson O.E., Huhndorf S., James T., Kirk P.M., Lücking R., Thorsten Lumbsch H., Lutzoni F., Matheny P.B., McLaughlin D.J., Powell M.J., Redhead S., Schoch C.L., Spatafora J.W., Stalpers J.A., Vilgalys R., Aime M.C., Aptroot A., Bauer R., Begerow D., Benny G.L., Castlebury L.A., Crous P.W., Dai Y., Gams W., Geiser D.M., Griffith G.W., Gueidan C., Hawksworth D.L., Hestmark G., Hosaka K., Humber R.A., Hyde K.D., Ironside J.E., Kõljalg U., Kurtzman C.P., Larsson K., Lichtwardt R., Longcore J., Miądlikowska J., Miller A., Moncalvo J., Mozley-Standridge S., Oberwinkler F., Parmasto E., Reeb V., Rogers J.D., Roux C., Ryvarden L., Sampaio J.P., Schüßler A., Sugiyama J., Thorn R.G., Tibell L., Untereiner W.A., Walker C., Wang Z., Weir A., Weiss M., White M.M., Winka K., Yao Y., Zhang N. (2007) A higher-level phylogenetic classification of the fungi. Mycol. Res. 111:509-547.

Hibbett D.S., Thorn R.G. (2001) Basidiomycota: Homobasidiomycetes. In: McLaughlin D.J., McLaughlin E.G., Lemke P.A., editors. The Mycota. Vol. VII. Springer-Verlag, Berlin, Germany, pp. 121-168.

Higuchi T. (2006) Look back over the studies of lignin biochemistry. J. Wood Sci. 52:2-8.

Hildén K., Martinez A.T., Hatakka A., Lundell T. (2005) The two manganese peroxidases Pr-MnP2 and Pr-MnP3 of *Phlebia radiata*, a lignin-degrading basidiomycete, are phylogenetically and structurally divergent. Fungal Genet. Biol. 42:403-419.

Hildén K., Hakala T.K., Lundell T. (2009) Thermotolerant and thermostable laccases. Biotechnol. Lett. 31:1117-1128.

Hildén K., Hakala T.K., Maijala P., Lundell T.K., Hatakka A. (2007) Production and characterization of novel laccases of the selective white-rot fungus *Physisporinus rivulosus*. Appl. Microbiol. Biotechnol. 77:301-309.

Hildén K.S., Bortfeldt R., Hofrichter M., Hatakka A., Lundell T.K. (2008) Molecular characterization of the basidiomycete isolate *Nemataloma frowardii* b19 and its manganese peroxidase places the fungus in the corticioid genus *Phlebia*. Microbiology 154:2371-2379.

Hintikka V., Korhonen K., Näykki O. (1979) Occurence of calcium oxalate in relation to the activity of fungi in forest litter and humus. Karstenia 19:58-64.

Hoegger P.J., Kilaru S., James T.Y., Thacker J.R., Kües U. (2006) Phylogenetic comparison and classification of laccase and related multicopper oxidase protein sequences. FEBS J. 273:2308-2326.

Hofrichter M. (2002) Review: Lignin conversion by manganese peroxidase (MnP). Enzyme Microb. Technol. 30:454-466.

Hofrichter M., Lundell T., Hatakka A. (2001) Conversion of milled pine wood by manganese peroxidase from *Phlebia radiata*. Appl. Environ. Microbiol. 67:4588-4593.

Hofrichter M., Vares T., Kalsi M., Galkin S., Scheibner K., Fritsche W., Hatakka A. (1999a) Production of manganese peroxidase and organic acids and mineralization of ^{14}C-labelled lignin (^{14}C-DHP) during solid-state fermentation of wheat straw with the white rot fungus *Nematoloma frowardii*. Appl. Environ. Microbiol. 65:1864-1870.

Hofrichter M., Vares T., Scheibner K., Galkin S., Sipilä J., Hatakka A. (1999b) Mineralization and solubilization of synthetic lignin by manganese peroxidases from *Nematoloma frowardii* and *Phlebia radiata*. J. Biotechnol. 67:217-228.

Hou H., Zhou J., Wang J., Dua C., Yan B. (2004) Enhancement of laccase production by *Pleurotus ostreatus* and its use for the decolorization of anthraquinone dye. Process Biochem. 39:1415-1419.

Hu M.-R., Chao Y.-P., Zhang G.-Q., Yang X.-Q., Xue Z.-Q., Qian S.-J. (2007) Molecular evolution of *Fome lignosus* laccase by ethyl methane sulfonate-based random mutagenesis in vitro. Biomol. Eng. 24:619-624.

Huang S.T., Tzean S.S., Tsai B.Y., Hsieh H.J. (2009) Cloning and heterologous expression of a novel ligninolytic peroxidase gene from poroid brown-rot fungus *Antrodia cinnamomea*. Microbiology. 155:424-433.

Huang Z., Dan Y., Huang Y., Lin L., Li T., Ye W., Wei X. (2004) Sesquiterpenes from the mycelial cultures of *Dichomitus squalens*. J. Nat. Prod. 67:2121-2123.

Hunt C., Kenealy W., Horn E., Houtman C. (2004) A biopulping mechanism: Creation of acid groups on fiber. Holzforschung 58:434-439.

Husain Q. (2006) Potential applications of the oxidoreductive enzymes in the decolorization and detoxification of textile and other synthetic dyes from polluted water: A review. Crit. Rev. Biotechnol. 26:201-221.

Hyde S.M., Wood P.M. (1997) A mechanism for production of hydroxyl radicals by the brown-rot fungus *Coniophora puteana:* Fe(III) reduction by cellobiose dehydrogenase and Fe(II) oxidation at a distance from the hyphae. Microbiology 143:259-266.

in der Wiesche C., Martens R., Zadrazil F. (1996) Two-step degradation of pyrene by white-rot fungi and soil microorganisms. Appl. Microbiol. Biotechnol. 46:653-659.

Janse B.J.H., Gaskell J., Akhtar M., Cullen D. (1998) Expression of *Phanerochaete chrysosporium* genes encoding lignin peroxidases, manganese peroxidases, and glyoxal oxidase in wood. Appl. Environ. Microbiol. 64:3536-3538.

Jarosz-Wilkolazka A., Graz M. (2006) Organic acids production by white rot basidiomycetes in the presence of metallic oxides. Can. J. Microbiol. 52:779-785.

Jensen K.A., Bao W., Kawai S., Srebotnik E., Hammel K.E. (1996) Manganese-dependent cleavage of nonphenolic lignin structures by *Ceriporiopsis subvermispora* in the absence of lignin peroxidase. Appl. Environ. Microbiol. 62:3679-3686.

Jensen K.A., Houtman C.J., Ryan Z.C., Hammel K.E. (2001) Pathways for extracellular Fenton chemistry in the brown rot basidiomycete *Gloeophyllum trabeum*. Appl. Environ. Microbiol. 67:2705-2711.

Jin Z.-X., Wang C., Chen W., Chen X., Li X. (2007) Induction of oxalate decarboxylase by oxalate in a newly isolated *Pandoraea* sp. OXJ-11 and its ability to protect against *Sclerotinia sclerotiorum* infection. Can. J. Microbiol. 53:1316-1322.

Johansson T., Nyman P.O. (1996) A cluster of genes encoding major isozymes of lignin peroxidase and manganese peroxidase for the white-rot fungus *Trametes versicolor*. Gene 170:31-38.

Johansson T., Nyman P.O. (1993) Isozymes of lignin peroxidase and manganese(II) peroxidase from the white-rot basidiomycete *Trametes versicolor*: I. Isolation of enzyme forms and characterization of physical and catalytic properties. Arch. Biochem. Biophys. 300:49-56.

Johjima T., Wariishi H., Tanaka H. (2002) Veratryl alcohol binding sites of lignin peroxidase from *Phanerochaete chrysosporium*. J. Molec. Catal. B 17:49-57.

Jordan C.R., Dashek W.V., Highley T.L. (1996) Detection and quantification of oxalic acid from the brown-rot decay fungus, *Postia placenta*. Holzforschung 50:312-318.

Jönsson L., Johansson T., Sjöström K., Nyman P.O. (1987) Purification of ligninase isozymes from the white-rot fungus *Trametes versicolor*. Acta Chem. Scand. B41:766-769.

Jönsson L.J., Saloheimo M., Penttilä M. (1997) Laccase from the white-rot fungus *Trametes versicolor*: cDNA cloning of *lcc1* and expression in *Pichia pastoris*. Curr. Genet. 32:425-430.

Jönsson L., Sjöström K., Häggström I., Nyman P.O. (1995) Characterization of a laccase gene from the white-rot fungus *Trametes versicolor* and structural features of basidiomycete laccases. Biochim. Biophys. Acta 1251:210-215.

Just V.J., Burrell M.R., Bowater L., McRobbie I., Stevenson C.E.M., Lawson D.M., Bornemann S. (2007) The identity of the active site of oxalate decarboxylase and the importance of the stability of active site lid conformations. Biochem. J. 407:397-406.

Just V.J., Stevenson C.E.M., Bowater L., Tanner A., Lawson D.M., Bornemann S. (2004) A closed conformation of *Bacillus subtilis* oxalate decarboxylase OxdC provides evidence for the true identity of the active site. J. Biol. Chem. 279:19867-19874.

Kaal E.E.J., DE Jong E., Field J.A. (1993) Stimulation of ligninolytic peroxidase activity by nitrogen nutrients in the white rot fungus *Bjerkandera* sp. strain BOS55. Appl. Environ. Microbiol. 59:4031-4036.

Kamei I., Daikoku C., Tsutsumi Y., Kondo R. (2008) Saline-dependent regulation of manganese peroxidase genes in the hypersaline-tolerant white rot fungus *Phlebia* sp. strain MG-60. Appl. Environ. Microbiol. 74:2709-2716.

Kamei I., Sonoki S., Haraguchi K., Kondo R. (2006) Fungal bioconversion of toxic polychlorinated biphenyls by white-rot fungus, *Phlebia brevispora*. Appl. Microbiol. Biotechnol. 73:932-940.

Kamei I., Watanabe M., Harada K., Miyahara T., Suzuki S., Matsufuji Y., Kondo R. (2009) Influence of soil properties on the biodegradation of 1,3,6,8-tetrachlorodibenzo-p-dioxin and fungal treatment of contaminated paddy soil by white rot fungus *Phlebia brevispora*. Chemosphere 75:1294-1300.

Kamm B., Kamm M. (1994) Principles of biorefineries. Appl. Microbiol. Biotechnol. 64:137-145.

Kaneko S., Cheng M., Murai H., Takenaka S., Murakami S., Aoki K. (2009) Purification and characterization of an extracellular laccase from *Phlebia radiata* strain BP-11-2 that decolorizes fungal melanin. Biosci. Biotechnol. Biochem. 73:939-942.

Kantelinen A., Hatakka A., Viikari L. (1989) Production of lignin peroxidase and laccase by *Phlebia radiata*. Appl. Microbiol. Biotechnol. 31:234-239.

Kapich A., Hofrichter M., Vares T., Hatakka A. (1999) Coupling of manganese peroxidase-mediated lipid peroxidation with destruction of nonphenolic lignin model compounds and ^{14}C-labeled lignins. Biochem. Biophys. Res. Commun. 259:212-219.

Karahanian E., Corsini G., Lobos S., Vicuña R. (1998) Structure and expression of a laccase gene from the ligninolytic basidiomycete *Ceriporiopsis subvermispora*. Biochim. Biophys. Acta 1443:65-74.

Karhunen E., Kantelinen A., Niku-Paavola M.-L. (1990a) Mn-dependent peroxidase from the lignin-degrading white-rot fungus *Phlebia radiata.* Arch. Biochem. Biophys. 279:25-31.

Karhunen E., Niku-Paavola M.-L., Viikari L., Haltia T., van der Meer R.A., Duine J.A. (1990b) A novel combination of prosthetic groups in a fungal laccase. PQQ and two copper atoms. FEBS Lett. 267:6-8.

Kathiara M., Wood D.A., Evans C.S. (2000) Detection and partial characterization of oxalate decarboxylase from *Agaricus biosporus*. Mycol. Res. 104:345-250.

Kersten P., Cullen D. (2007) Extracellular oxidative systems of the lignin-degrading basidiomycete *Phanerochaete chrysosporium.* Fungal Genet. Biol. 44:77-87.

Kesarwani M., Azam M., Natarajan K., Mehta A., Datta A. (2000) Oxalate decarboxylase from *Collybia velutipes*. Molecular cloning and its overexpression to confer resistance to fungal infection in transgenic tobacco and tomato. J. Biol. Chem. 275:7230-7238.

Khuri S., Bakker F.T., Dunwell J.M. (2001) Phylogeny, function, and evolution of the cupins, a structurally conserved, functionally diverse superfamily of proteins. Mol. Biol. Evol. 18:593-605.

Kiiskinen L.-L., Kruus K., Bailey M., Ylösmäki E., Siika-aho M., Saloheimo M. (2004) Expression of *Melanocarpus albomyces* laccase in *Trichoderma reesei* and characterization of the purified enzyme. Microbiology 150:3065-3074.

Kilaru S., Hoegger P.J., Kües U. (2006) The laccase multi-gene family in *Coprinopsis cinerea* has seventeen different members that divide into two distinct subfamilies. Curr. Genet. 50:45-60.

Kim K.S., Min J., Dickman M.B. (2008a) Oxalic acid is an elicitor of plant programmed cell death during *Sclerotinia sclerotiorum* disease development. Mol. Plant Microbe Interact. 21:605-612.

Kim Y., Yeo S., Kim M.K., Choi H.T. (2008b) Removal of estrogenic activity from endocrine-disrupting chemicals by purified laccase of *Phlebia tremellosa*. FEMS Microbiol. Lett. 284:172-175.

Kirk T.K., Chang H.-m. (1990) Overview of biotechnology in pulp and paper manufacture. In: Kirk T.K., Chang H.-m., editors. Biotechnology in Pulp and Paper Manufacture. Applications and Fundamental Investigations. Butterworth-Heinemann, Boston, USA pp. 1-13.

Kirk T.K., Farrell R.L. (1987) Enzymatic "combustion": The microbial degradation of lignin. Ann. Rev. Microbiol. 41:465-505.

Kolandaswamy A., George L., Sadasivam S. (2009) Heterologous expression of oxalate decarboxylase in *Lactobacillus plantarum* NC8. Curr. Microbiol. 58:117-121.

Kremer S.M., Wood P.M. (1992) Production of Fenton's reagent by cellobiose oxidase from cellulolytic cultures of *Phanerochaete chrysosporium.* Eur. J. Biochem. 208:807-814.

Kuan I., Tien M. (1993) Stimulation of Mn peroxidase activity: A possible role for oxalate in lignin biodegradation. Proc. Natl. Acad. Sci. USA 90:1242-1246.

Kuhad R.C., Singh A., Eriksson K.-E.L. (1997) Microorganisms and enzymes involved in the degradation of plant fiber cell walls. In: Eriksson K.-E.L., editor. Advances in Biochemical Engineering Biotechnology. Vol 57. Biotechnology in the pulp and paper industry. Springer-Verlag, Germany, pp. 45-125.

Kumar R., Singh S., Singh O.V. (2008) Bioconversion of lignocellulosic biomass: biochemical and molecular perspectives. J. Ind. Microbiol. Biotechnol. 35:377-391.

Kumar S.V.S., Prashant S.P., Durani S., Wangikar P.P. (2003) Combined sequence and structure analysis of the fungal laccase family. Biotechnol. Bioeng. 83:386-394.

Kurian D., Phadwa K., Mäenpää P. (2006) Proteomic characterization of acid stress response in *Synechocystis* sp. PCC 6803. Proteomics 6:3614-3624.

Kämper J., Kahmann R., Bölker M., Ma L.-J., Brefort T., Saville B.J., Banuett F., Kronstad J.W., Gold S.E., Müller O., Perlin M.H., Wösten H.A.B., de Vries R., Ruiz-Herrera J., Reynaga-Peña C.G., Snetselaar K., McCann M., Pérez-Martín J., Feldbrügge M., Basse C.W., Steinberg G., Ibeas J.I., Holloman W., Guzman P., Farman M., Stajich J.E., Sentandreu R., González-Prieto J.M., Kennell J.C., Molina L., Schirawski J., Mendoza-Mendoza A., Greilinger D., Münch K., Rössel N., Scherer M., Vrane M., Ladendorf O., Vincon V., Fuchs U., Sandrock B., Meng S., Ho E.C.H., Cahill M.J., Boyce K.J., Klose J., Klosterman S.J., Deelstra H.J., Ortiz-Castellanos L., Li W., Sanchez-Alonso P., Schreier P.H., Häuser-Hahn I., Vaupel M., Koopmann E., Friedrich G, Voss H., Schlüter T., Margolis J., Platt D., Swimmer C., Gnirke A., Chen F., Vysotskaia V., Mannhaupt G., Güldener U., Münsterkötter M., Haase D., Oesterheld M., Mewes H.-W., Mauceli E.W., DeCaprio D., Wade C.M., Butler J., Young S., Jaffe D.B., Calvo S., Nusbaum C., Galagan J., Birren B.W. (2006) Insights from the genome of the biotrophic fungal plant pathogen *Ustilago maydis.* Nature 444:97-101.

Lamar R.T., Schoenike B., Vanden Wymelenberg A., Stewart P., Dietrich D.M., Cullen D. (1995) Quantitation of fungal mRNAs in complex substrates by reverse transcription PCR and its application to *Phanerochaete chrysosporium*-colonized soil. Appl. Environ. Microbiol. 61:2122-2126.

Lang E., Gonser A., Zadrazil F. (2000) Influence of incubation temperature on activity of ligninolytic enzymes in sterile soil by *Pleurotus* sp. and *Dichomitus squalens*. J. Basic Microbiol. 1:33-39.

Lankinen P. (2004) Ligninolytic enzymes of the basidiomycetous fungi *Agaricus bisporus* and *Phlebia radiata* on lignocellulose-containing media. Dissertationes Biocentri Viikki Universitatis Helsingiensis, 36/2004. Ph.D. Thesis, Department of Applied Chemistry and Microbiology, University of Helsinki, Helsinki, 54 p.

Lankinen P., Hildén K., Aro N., Salkinoja-Salonen M., Hatakka A. (2005) Manganese peroxidase of *Agaricus bisporus*: grain bran-promoted production and gene characterization. Appl. Microbiol. Biotehcnol. 66:401-407.

Lankinen P., Inkeröinen M., Pellinen J., Hatakka A. (1991) The onset of lignin-modifying enzymes, decrease of AOX and color removal by white-rot fungi grown on bleach plant effluents. Water Sci. Technol. 24:189-198.

Lapadatescu C., Feron G., Vergoignan C., Djian A., Durand A., Bonnarme P. (1997) Influence of cell immobilization on the production of benzaldehyde and benzyl alcohol by the white-rot fungi *Bjerkandera adusta*, *Ischnoderma benzoinum* and *Dichomitus squalens*. Appl. Microbiol. Biotechnol. 47:708-714.

Larrondo L.F., Avila M., Salas L., Cullen D., Vicuña R. (2003a) Heterologous expression of laccase cDNA from *Ceriporiopsis subvermispora* yields copper-activated apoprotein and complex isoform patterns. Microbiology 149:1177-1182.

Larrondo L.F., Canessa P., Melo F., Polanco R., Vicuña R. (2007) Cloning and characterization of the genes encoding the high-affinity iron-uptake protein complex Fet3/Ftr1 in the basidiomycete *Phanerochaete chrysosporium.* Microbiology 153:1772-1780.

Larrondo L.F., Gonzalez B., Cullen D., Vicuña R. (2004) Characterization of a multicopper oxidase gene cluster in *Phanerochaete chrysosporium* and evidence of altered splicing of the *mco* transcripts. Microbiology 150:2775-2783.

Larrondo L.F., Salas L., Melo F., Vicuña R., Cullen D. (2003b) A novel extracellular multicopper oxidase from *Phanerochaete chrysosporium* with ferroxidase activity. Appl. Environ. Microbiol. 69:6257-6263.

Lee J.-M., Kwon H., Jeong H., Lee J.W., Lee S.Y., Baek S.J., Surh Y.-J. (2001) Inhibition of lipid peroxidation and oxidative DNA damage by *Ganoderma lucidum*. Phytother. Res. 15:245-249.

Leontievsky A.A., Vares T., Lankinen P., Shergill J.K., Pozdnyakova N.N., Myasoedova N.M., Kalkkinen N., Golovleva L.A., Cammack R., Thurston C.F., Hatakka A. (1997) Blue and yellow laccases of ligninolytic fungi. FEMS Microbiol. Lett. 156:9-14.

Levasseur A., Piumi F., Coutinho P.M., Rancurel C., Asther M., Delattre M., Henrissat B., Pontarotti P., Asther M., Record E. (2008). FOLy: an integrated database for the classification and functional annotation of fungal oxidoreductases potentially involved in the degradation of lignin and related aromatic compounds. Fungal Genet. Biol. 45:638-45.

Levin L., Forchiassin F., Ramos A.M. (2002) Copper induction of lignin-modifying enzymes in the white-rot fungus *Trametes trogii.* Mycologia 94:377-383.

Li D., Alic M., Gold M.H. (1994) Nitrogen regulation of lignin peroxidase gene transcription. Appl. Environ. Microbiol. 60:3447-3449.

Li D., Heather L., Youngs L., Gold M.H. (2001) Heterologous expression of a thermostable manganese peroxidase from *Dichomitus squalens* in *Phanerochaete chrysosporium*. Arch. Biochem. Biophys. 385:348-356.

Li D., Li N., Ma B., Mayfield M.B., Gold M.H. (1999) Characterization of genes encoding two manganese peroxidases from the lignin-degrading fungus *Dichomitus squalens*. Biochim. Biophys. Acta 1434:356-364.

Liers C., **Ullrich R., Steffen K.T., Hatakka A., Hofrichter M.** (2006) Mineralization of ^{14}C-labelled synthetic lignin and extracellular enzyme activities of the wood-colonizing ascomycetes *Xylaria hypoxylon* and *Xylaria polymorpha*. Appl. Microbiol. Biotechnol. 69:573-579.

Lillehoj E.B., Smith F.G. (1965) An oxalic acid decarboxylase of *Myrothecium verrucaria*. Arch. Biochem. Biophys. 109:216-220.

Lobos S., Larrain J., Salas L., Cullen D., Vicuña R. (1994) Isoenzymes of manganese-dependent peroxidase and laccase produced by the lignin-degrading basidiomycete *Ceriporiopsis subvermispora.* Microbiology 140:2691-2698.

Loftus B.J., Fung E., Roncaglia P., Rowley D., Amedeo P., Bruno D., Vamathevan J., Miranda M., Anderson I.J., Fraser J.A., Allen J.E., Bosdet I.E., Brent M.R., Chiu R., Doering T.L., Donlin M.J., D'Souza C.A., Fox D.S., Grinberg V., Fu J., Fukushima M., Haas B.J., Huang J.C., Janbon G., Jones S.J.M., Koo H.L., Krzywinski M.I., Kwon-Chung J.K., Lengeler K.B., Maiti R., Marra M.A., Marra R.E., Mathewson C.A., Mitchell T.G., Pertea M., Riggs F.R., Salzberg S.L., Schein J.E., Shvartsbeyn A., Shin H., Shumway M., Specht C.A., Suh B.B., Tenney A., Utterback T.R., Wickes B.L., Wortman J.R., Wye N.H., Kronstad J.W., Lodge J.K., Heitman J., Davis R.W., Fraser C.M., Hyman R.W. (2005) The genome of the basidiomycetous yeast and human pathogen *Cryptococcus neoformans.* Science 307:1321-1324.

Lundell T. (1993) Ligninolytic system of the white-rot fungus *Phlebia radiata*: lignin model compound studies. Ph.D. Thesis, Department of Applied Chemistry and Microbiology, University of Helsinki, Helsinki, 90 p.

Lundell T., Hatakka A. (1994) Participation of Mn(II) in the catalysis of laccase, manganese peroxidase and lignin peroxidase from *Phlebia radiata*. FEBS Lett. 348:291-296.

Lundell T., Kilpeläinen I.A., Brunow G., Schoemaker H.E., Hatakka A.I. (1992) Degradation of macromolecular lignin models by lignin peroxidase from *Phlebia radiata* and catalytic properties of the enzyme. In: Kuwahara M., Shimada M., editors. Biotechnology in Pulp and Paper Industry, pp. 315-320.

Lundell T., Leonowicz A., Rogalski J., Hatakka A. (1990) Formation and action of lignin-modifying enzymes in cultures of *Phlebia radiata* supplemented with veratric acid. Appl. Environ. Microbiol. 56:2623-2629.

Lundell T., Schoemaker H., Hatakka A., Brunow G. (1993a) New mechanism of the C_α-C_β cleavage in non-phenolic arylglycerol β-aryl ether lignin substructures catalyzed by lignin peroxidase. Holzforschung 47:219-224.

Lundell T., Wever R., Floris R., Harvey P., Hatakka A., Brunow G., Schoemaker H. (1993b) Lignin peroxidase L3 from *Phlebia radiata*. Pre-steady-state and steady-state studies with veratryl alcohol and a non-phenolic lignin model compound 1-(3,4-dimethoxyphenyl)-2-(2-methoxyphenoxy)propane-1,3-diol. Eur. J. Biochem. 211:391-402.

Lundquist K., Kirk T.K. (1978) De novo synthesis and decomposition of veratryl alcohol by a lignin-degrading basidiomycete. Phytochem. 17:1676.

Lyashenko A.V., Bento I., Zaitsev V.N., Zhukhlistova N.E., Zhukova Y.N., Gabdoulkhakov A.G., Morgunova E.Y., Voelter W., Kachalova G.S., Stepanova E.V., Koroleva O.V., Lamzin V.S., Tishkov V.I., Betzel C., Lindley P.F., Mikhailov A.M. (2006) X-ray structural studies of the fungal laccase from *Cerrena maxima*. J. Biol. Inorg. Chem. 11:963-973.

Ma D., Hattori T., Akamatsu Y., Adachi M., Shimada M. (1992) Kinetic analysis of the noncompetitive inhibition of the lignin-peroxidase-catalyzed reaction by oxalic acid. Biosci. Biotech. Biochem. 56:1378-1381.

MacLellan S.R., Helmann J.D., Antelmann H. (2009) The YvrI alternative δ factor is essential for acid stress induction of oxalate decarboxylase in *Bacillus subtilis.* J. Bacteriol. 191:931-939.

Magro P., Marciano P., Di Lenna P. (1988) Enzymatic oxalate decarboxylation in isolates of *Sclerotinia sclerotiorum*. FEMS Microbiol. Lett. 49:49-52.

Maijala P., Kleen M., Westin C., Poppius-Levlin K., Herranen K., Lehto J.H., Reponen P., Mäentausta O., Mettälä A., Hatakka A. (2008) Biomechanical pulping of softwood with enzymes and white-rot fungus *Physisporinus rivulosus.* Enzyme Microb. Technol. 43:169-177.

Mansfield S.D., De Jong E., Saddler J.N. (1997) Cellobiose dehydrogenase, an active agent in cellulose depolymerization. Appl. Environ. Microbiol. 63:3804-3809.

Mansur M., Suárez T., Fernández-Larrea J.B., Brizuela M.A., González A.E. (1997) Identification of a laccase gene family in the new lignin-degrading basidiomycete CECT 20197. Appl. Environ. Microbiol. 63:2637-2646.

Mansur M., Suarez T., Gonzalez A.E. (1998) Differential gene expression in the laccase gene family from basidiomycete I-62 (CECT 20197). Appl. Environ. Microbiol. 64:771-774.

Manubens A., Avila M., Canessa P., Vicuña R. (2003) Differential regulation of genes encoding manganese peroxidase (MnP) in the basidiomycete *Ceriporiopsis subvermispora.* Curr. Genet. 43:433-438.

Manubens A., Canessa P., Folch C., Avila M., Salas L., Vicuña R. (2007) Manganese affects the production of laccase in the basidiomycete *Ceriporiopsis subvermispora.* FEMS Microbiol. Lett. 275:139-145.

Martens R., Zadrazil F. (1998) Screening of white-rot fungi for their ability to mineralize polycyclic aromatic hydrocarbons in soil. Folia Microbiol. 43:97-103.

Martin F., Aerts A., Ahrén D., Brun A., Danchin E.G.J., Duchaussoy F., Gibon J., Kohler A., Lindquist E., Pereda V., Salamov A., Shapiro H.J., Wuyts J., Blaudez D., Buée M., Brokstein P., Canbäck B., Cohen D., Courty P.E., Coutinho P.M., Delaruelle C., Detter J.C., Deveau A., DiFazio S., Duplessis S., Fraissiner-Tachet L., Lucic E., Frey-Klett P., Fourrey C., Feussner I., Gay G., Grimwood J., Hoegger P., Jain P., Kilaru S., Labbé J., Lin Y.C., Legué V., Le Tacon F., Marmeisse R., Melayah D., Montanini B., Muratet M., Nehls U., Niculita-Hirzel H., Oudot-Le Secq M.P., Peter M., Quesneville H., Rajashekar B., Reich M., Rouhier N., Schmutz J., Yin T., Chalot M., Henrissat B., Kües U., Lucar S., Van de Peer Y., Podila G.K., Polle A., Pukkila P.J., Richardson P.M., Rouzé P., Sanders I.R., Stajich J.E., Tunlid A., Tuskan G., Grigoriev I.V. (2008) The genome of *Laccaria bicolor* provides insights into mycorrhizal symbiosis. Nature 452:88-92

Martínez A.T. (2002) Molecular biology and structure-function of lignin-degrading heme peroxidases. Enzyme Microb. Technol. 30:425-444.

Martínez Á.T., Ruiz-Dueñas F.J., Martínez M.J., del Rio J.C., Gutiérrez A. (2009) Enzymatic delignification of plant cell wall: From nature to mill. Curr. Opin. Biotechnol. 20:348-357.

Martinez D., Berka R.M., Henrissat B., Saloheimo M., Arvas M., Baker S.E., Chapman J., Chertkov O., Coutinho P.M., Cullen D., Danchin E.G.J., Grigoriev I.V., Harris P., Jackson M., Kubicek C.P., Han C.S., Ho I., Larrondo L.F., Lopez de Leon A., Magnuson J.K., Merino S., Misra M., Nelson B., Putnam N., Robbertse B., Salamov A.A., Schmoll M., Terry A., Thayer N., Westerholm-Parvinen A., Schoch C.L., Yao J., Barabote R., Nelson M.A., Detter C., Bruce D., Kuske C.R., Xie G., Richardson P., Rokhsar D.S., Lucas S.M., Rubin E.R., Dunn-Coleman N., Ward M., Brettin T.S. (2008) Genome sequencing and analysis of the biomass-degrading fungus *Trichoderma reesei* (syn. *Hypocrea jecorina*). Nat. Biotechnol. 26:553-560.

Martinez D., Challacombe J., Morgenstern I., Hibbett D., Schmoll M., Kubicek C.P., Ferreira P., Ruiz-Dueñas F.J., Martinez A.T., Kersten P., Hammel K.E., Vanden Wymelenberg A., Gaskell J., Lindquist E., Sabat G., BonDurant S.S., Larrondo L.F., Canessa P., Vicuña R., Yadav J., Doddapaneni H., Subramanian V., Pisabarro A.G.,

Lavín J.L., Oguiza J.A., Master E., Henrissat B., Coutinho P.M., Harris P., Magnuson J.K., Baker S.E., Bruno K., Kenealy W., Hoegger P.J., Kües U., Ramaiya P., Lucas S., Salamov A., Shapiro H., Tu H., Chee C.L., Misra M., Xie G., Teter S., Yaver D., James T., Mokrejs M., Pospisek M., Grigoriev I.V., Brettin T., Rokhsar D., Berka R., Cullen D. (2009) Genome, transcriptome, and secretome analysis of wood decay fungus *Postia placenta* supports unique mechanisms of lignocellulose conversion. Proc. Natl. Acad. Sci. USA 106:1954-1959.

Martinez D., Larrondo L.F., Putnam N., Sollewijn Gelpke M.D., Huang K., Chapman J., Helfenbein K.G., Ramaiya R., Detter J.C., Larimer F., Coutinho P.M., Henrissat B., Berka R., Cullen D., Rokhsar D. (2004) Genome sequence of the lignocelluloses degrading fungus *Phanerochaete chrysosporium* strain RP78. Nat. Biotech. 22:695-700.

Martins L.O., Soares C.M., Pereira M.M., Teixeira M., Costa T., Jones G.H., Henriques A.O. (2002) Molecular and biochemical characterization of a highly stable bacterial laccase that occurs as a structural component of the *Bacillus subtilis* endospore coat. J. Biol. Chem. 277:18849-18859.

Matityahu A., Hadar Y., Dosoretz C.G., Belinky P.A. (2008) Gene silencing by RNA interference in the white rot fungus *Phanerochaete chrysosporium.* Appl. Environ. Microbiol. 74:5359-5365.

Mayer A.M., Staples R.C. (2002) Laccase: new functions for an old enzyme. Phytochemistry 60:551-565.

Mehta A., Datta A. (1991) Oxalate decarboxylase from *Collybia velutipes*. Purification, characterization, and cDNA cloning. J. Biol. Chem. 266:23548-23553.

Mester T., Field J.A. (1998) Characterization of a novel manganese peroxidase-lignin peroxidase hybrid isozyme produced by *Bjerkandera* species strain BOS55 in the absence of manganese. J. Biol. Chem. 273:15412-15417.

Mester T., Tien M. (2001) Engineering of a manganese-binding site in lignin peroxidase isozyme H8 from *Phanerochaete chrysosporium.* Biochem. Biophys. Res. Commun. 284:723-728.

Micales J.A. (1997) Localization and induction of oxalate decarboxylase in the brown-rot wood decay fungus *Postia placenta*. Int. Biodet. Biodeg. 39:125-132.

Mierzwa M., Tokarzewska-Zadora J., Deptuła T., Rogalski J. (2005) Purification and characterization of an extracellular α-D-glucuronidase from *Phlebia radiata.* Prep. Biochem. Biotechnol. 35:243-256.

Mikolasch A., Schauer F. (2009) Fungal laccases as tools for the synthesis of new hybrid molecules and biomaterials. Appl. Microbiol. Biotechnol. 84:605-624.

Mikuni J., Morohoshi N. (1997) Cloning and sequencing of a second laccase gene from the white-rot fungus *Coriolus versicolor.* FEMS Microbiol. Lett. 155:79-84.

Min K.-L., Kim Y.-H., Kim Y.W., Jung H.S., Hah Y.C. (2001) Characterization of a novel laccase produced by the wood-rotting fungus *Phellinus ribis.* Arch. Biochem. Biophys. 392:279-286.

Missall T.A., Moran J.M., Corbett J.A., Lodge J.K. (2005) Distinct stress responses of two functional laccases in *Cryptococcus neoformans* are revealed in the absence of the thiol-specific antioxidant Tsa1. Eukar. Cell 4:202-208.

Moilanen A.M., Lundell T., Vares T., Hatakka A. (1996) Manganese and malonate are individual regulators for the production of lignin and manganese peroxidase isozymes and in the degradation of lignin by *Phlebia radiata*. Appl. Microbiol. Biotehcnol. 45:792-799.

Moomaw E.W., Angerhofer A., Moussatche P., Ozarowski A., García-Rubio I., Richards N.G.J. (2009) Metal dependence of oxalate decarboxylase activity. Biochem. 48:6116-6125.

Morgenstern I., Klopman S., Hibbett D.S. (2008) Molecular evolution and diversity of lignin degrading heme peroxidases in the agaricomycetes. J. Mol. Evol. 66:243-257.

Moreira M.T., Feijoo G., Canaval J., Lema J.M. (2003) Semipilot-scale bleaching of kraft pulp with manganese peroxide. Wood Sci. Technol. 37:117-121.

Mori T., Kitano S., Kondo R. (2003) Biodegradation of chloronaphthalenes and polycyclic aromatic hydrocarbons by the white-rot fungus *Phlebia lindtneri.* Appl. Microbiol. Biotechnol. 61:380-383.

Morozova O.V., Shumakovich G.P., Gorbacheva M.A., Shleev S.V., Yaropolov A.I. (2007a) "Blue" laccases. Biochem. (Moscow) 72:1136-1412.

Morozova O.V., Shumakovich G.P., Shleev S.V., Yaropolov Y.I. (2007b) Laccase-mediator systems and their applications: A review. Appl. Biochem. Microbiol. 5:523-535.

Mosbach R. (1963) Purification and some properties of laccase from *Polyporus versicolor*. Biochim. Biophys. Acta 73:204-212.

Munir E., Yoon J.J., Tokimatsu T., Hattori T., Shimada M. (2001a) A physiological role for oxalic acid biosynthesis in the wood-rotting basidiomycete *Fomitopsis palustris*. Proc. Natl. Acad. Sci. USA 98:11126–11130.

Munir E., Yoon J.J., Tokimatsu T., Hattori T., Shimada M. (2001b) New role for glyoxylate cycle enzymes in wood-rotting basidiomycetes in relation to biosynthesis of oxalic acid. J. Wood Sci. 47:368-373.

Nakasone K.K., Sytsma K.J. (1993) Biosystematic studies on *Phlebia acerina*, *P. rufa*, and *P. radiata* in North America. Mycologia 85:996-1016.

Necochea R., Valderrama B., Díaz-Sandoval S., Folch-Mallol J.L., Vázquez-Duhalt R., Iturriaga G. (2005) Phylogenetic and biochemical characterisation of a recombinant laccase from *Trametes versicolor.* FEMS Microbiol. Lett. 244:235-241.

Niemelä T. (2005) Käävät - puiden sienet. Norrlinia 13., 320 p. (In Finnish).

Niemenmaa O., Uusi-Rauva A., Hatakka A. (2006) Wood stimulates the demethoxylation of [$O^{14}CH_3$]-labeled lignin model compounds by the white-rot fungi *Phanerochaete chrysosporium* and *Phlebia radiata.* Arch. Microbiol. 185:307-315.

Niku-Paavola M.-L., Karhunen E., Kantelinen A., Viikari L., Lundell T., Hatakka A. (1990) The effect of culture conditions on the production of lignin modifying enzymes by the white-rot fungus *Phlebia radiata*. J. Biotechnol. 13:211-221.

Niku-Paavola M.-L., Karhunen E., Salola P., Raunio V. (1988) Ligninolytic enzymes of the white-rot fungus *Phlebia radiata*. Biochem. J. 254:877-884.

Nilvebrant N.-O., Reimann A., de Sousa F., Cassland P., Larsson S., Hong F., Jönsson L.J. (2002) Enzymatic degradation of oxalic acid for prevention of scaling. Prog. Biotechnol. 21: 231-238.

Ohga S., Royse D.J. (2001) Transcriptional regulation of laccase and cellulase genes during growth and fruiting of *Lentinula edodes* on supplemented sawdust. FEMS Microbiol. Lett. 201:111-115.

Ohga S., Smith M., Thurston C.F., Wood D.A. (1999) Transcriptional regulation of laccase and cellulase genes in the mycelium of *Agaricus bisporus* during fruit body development on a solid substrate. Mycol. Res. 103:1557-1560.

Palmieri G., Giardina P., Bianco C., Fontanella B., Sannia G. (2000) Copper induction of laccase isoenzymes in the ligninolytic fungus *Pleurotus ostreatus.* Appl. Environ. Microbiol. 66:920-924.

Palmieri G., Giardina P., Bianco C., Scaloni A., Capasso A., Sannia G. (1997) A novel white laccase from *Pleurotus ostreatus.* J. Biol. Chem. 272:31301-31307.

Pérez-Boada M., Ruiz-Dueñas F.J., Pogni R., Basosi R., Choinowski T., Martínez M.J., Piontek K., Martínez A.T. (2005) Versatile peroxidase oxidation of high redox potential aromatic compounds: Site-directed mutagenesis, spectroscopic and crystallographic investigation of three long-range electron transfer pathways. J. Mol. Biol. 354:385-402.

Périé F.H., Gold M.H. (1991) Manganese regulation of manganese peroxidase expression and lignin degradation by the white rot fungus *Dichomitus squalens*. Appl. Environ. Microbiol. 57:2240-2245.

Périé F.H., Reddy V.B., Blackburn N.J., Gold M.H. (1998) Purification and characterization of laccases from the white-rot basidiomycete *Dichomitus squalens*. Arch. Biochem. Biophys. 353:349-355.

Périé F.H., Sheng D., Gold M.H. (1996) Purification and characterization of two manganese peroxidase isozymes from the white-rot basidiomycete *Dichomitus squalens*. Arch. Biochem. Biophys. 1297: 39-148.

Petersen J.F.W., Kadziola A., Larsen S. (1994) Three-dimensional structure of a recombinant peroxidase from *Coprinus cinereus* at 2.6 Å resolution. FEBS Lett. 339:291-296.

Pezzella C., Autore F., Giardina P., Piscitelli A., Sannia G., Faraco V. (2009) The *Pleurotus ostreatus* laccase multi-gene family: Isolation and heterologous expression of new family members. Curr. Genet. 55:45-57.

Pickard M.A., Vandertol H., Roman R., Vazquez-Duhalt R. (1999) High production of ligninolytic enzymes from white rot fungi in cereal bran liquid medium. Can. J. Microbiol. 45:627-631.

Piontek K., Antorini M., Choinowski T. (2002) Crystal structure of a laccase from the fungus *Trametes versicolor* at 1.90-Å resolution containing a full complement of coppers. J. Biol. Chem. 277:37663-37669.

Piontek K., Glumoff T., Winterhalter K. (1993) Low pH crystal structure of glycosylated lignin peroxidase from *Phanerochaete chrysosporium* at 2.5 Å resolution. FEBS Lett. 315:119-124.

Popov V.O., Lamzin V.S. (1994) NAD(+)-dependent formate dehydrogenase. Biochem J. 301:625–643.

Popp J.L., Kalyanaraman B., Kirk T.K. (1990) Lignin peroxidase oxidation of Mn^{2+} in the presence of veratryl alcohol, malonic or oxalic acid, and oxygen. Biochem. 29:10475-10480.

Poulos T.L., Edwards S.L., Wariishi H., Gold M.H. (1993) Crystallographic refinement of lignin peroxidase at 2 Å. J. Biol. Chem. 268:4429-4440.

Prendecka M., Buczyńska A., Rogalski J. (2007) Purification and characterization of β-mannosidases from white rot fungus *Phlebia radiata*. Pol. J. Microbiol. 56:139-147.

Prendecka M., Szyjka K., Rogalski J. (2003) Purification and properties of α-galactosidase isozymes from *Phlebia radiata.* Acta Microbiol. Pol. 52:25-33.

Raghukumar C., D'Souza-Ticlo D., Verma A. (2008) Treatment of colored effluents with lignin-degrading enzymes: An emerging role of marine-derived fungi. Crit. Rev. Microbiol. 34:189-206.

Rahmawati N., Ohashi Y., Watanabe T., Honda Y., Watanabe T. (2005) Ceriporic acid B, an extracellular metabolite of *Ceriporiopsis subvermispora*, suppresses the depolymerization of cellulose by the Fenton reaction. Biomacromol. 6:2851-2856.

Ravalason H., Gwénaël J., Mollé D., Pasco M., Coutinho P.M., Lapierre C., Pollet B., Bertaud F., Petit-Conil M., Grisel S., Sigoillot J.-C., Asther M., Herpoël-Gimbert I. (2008) Secretome analysis of *Phanerochaete chrysosporium* strain CIRM-BRFM41 grown on softwood. Appl. Microbiol. Biotechnol. 80:719-733.

Record E., Punt P.J., Chamkha M., Labat M., van den Hondel C.A.M.J.J. (2002) Expression of the *Pycnoporus cinnabarinus* laccase gene in *Aspergillus niger* and characterization of the recombinant enzyme. Eur. J. Biochem. 269:602-609.

Rodríguez Couto S., Sanromán M.A., Hofer D., Gübitz G.M. (2004) Stainless steel sponge: A novel carrier for the immobilisation of the white-rot fungus *Trametes hirsuta* for decolourization of textile dyes. Biores. Technol. 95:67-72.

Rodríguez E., Ruiz-Dueñas F.J., Kooistra R., Ram A., Martínez Á.T., Martínez M.J. (2008) Isolation of two laccase genes from the white-rot fungus *Pleurotus eryngii* and heterologous expression of the *pel3* encoded protein. J. Biotechnol. 134:9-19.

Rogalski J., Dawidowicz A.L., Leonowicz A. (1990) Purification and immobilization of the inducible form of extracellular laccase of the fungus *Trametes versicolor*. Acta Biotechnol. 10:261-269.

Rogalski J., Hatakka A., Longa B., Leonowicz A. (1993a) Hemicellulolytic enzymes of the ligninolytic white-rot fungus *Phlebia radiata*. Determination of enzyme activities. Acta Biotechnol. 13:47-51.

Rogalski J., Hatakka A., Longa B., Wojtaś-Wasilewska M. (1993b) Hemicellulolytic enzymes of the ligninolytic white-rot fungus *Phlebia radiata*. Influence of phenolic compounds on the synthesis of hemicellulolytic enzymes. Acta Biotechnol. 13:53-57.

Rogalski J., Hatakka A., Wojtaś-Wasilewska M., Leonowicz A. (1993c) Cellulolytic enzymes of the ligninolytic white-rot fungus *Phlebia radiata*. Acta Biotechnol. 13:41-45.

Rogalski J., Lundell T.K., Leonowicz A., Hatakka A.I. (1991) Influence of aromatic compounds and lignin on production of ligninolytic enzymes by *Phlebia radiata*. Phytochem. 30:2869-2872.

Rogalski J., Oleszek M., Tokarzewska-Zadora J. (2001) Purification and characterization of two endo-1,4-β-xylanases and a β-xylosidase from *Phlebia radiata*. Acta Microbiol. Pol. 50:117-128.

Rogalski J., Szczodrak J., Janusz G. (2006) Manganese peroxidase production in submerged cultures by free and immobilized mycelia of *Nematoloma frowardii*. Biores. Technol. 97:469-476.

Rosenbrock P., Martens R., Buscot F., Zadrazil F., Munch J.C. (1997) Enhancing the mineralization of [U-^{14}C]dibenzo-*p*-dioxin in three different soils by addition of organic substrate or inoculation with white-rot fungi. Appl. Microbiol. Biotechnol. 48:665-670.

Rouau X., Odier E. (1986) Production of extracellular enzyme by the white-rot fungus *Dichomitus squalens* in cellulose-containing liquid culture. Enzyme Microb. Technol. 8:22-26.

Ruiz-Dueñas F.J., Martínez Á.T (2009) Microbial degradation of lignin: how a bulky recalcitrant polymer is efficiently recycled in nature and how we can take advantage of this. Microb. Biotechnol. 2:164-177.

Ruiz-Dueñas F.J., Martinez M.J., Martinez A.T. (1999) Molecular characterization of a novel peroxidase isolated from the ligninolytic fungus *Pleurotus eryngii*. Mol. Microbiol. 31:223-235.

Ruiz-Dueñas F.J., Morales M., Garcia E., Miki Y., Martinez M.J., Martinez A.T. (2009) Substrate oxidation sites in versatile peroxidase and other basidiomycete peroxidases. J. Exp. Bot. 60: 441-452.

Saparrat M.C.N., Guillen F., Arambarri A.M., Martinez A.T., Martinez M.J. (2002) Induction, isolation, and characterization of two laccases from the white rot basidiomycete *Coriolopsis rigida*. Appl. Environ. Microbiol. 68:1534-1540.

Sakai S., Nishide T., Munir E., Baba K., Inui H., Nakano Y., Hattori T., Shimada M. (2006) Subcellular localization of glyoxylate cycle key enzymes involved in oxalate biosynthesis of wood-destroying basidiomycete *Fomitopsis palustris*. Microbiology 152:1857-1866.

Saloheimo M., Barajas V., Niku-Paavola M.L., Knowles J.K.C. (1989) A lignin peroxidase-encoding cDNA from the white-rot fungus *Phlebia radiata*: characterization and expression in *Trichoderma reesei*. Gene 85:343-351.

Saloheimo M., Niku-Paavola M.-L. (1991) Heterologous production of a ligninolytic enzyme: expression of the *Phlebia radiata* laccase gene in *Trichoderma reesei*. Bio/Technology 9:987-990.

Saloheimo M., Niku-Paavola M.-L., Knowles J.K.C. (1991) Isolation and structural analysis of the laccase gene from the lignin-degrading fungus *Phlebia radiata*. J. Gen. Microbiol. 137:1537-1544.

Sato S., Feltus F.A., Iyer P., Tien M. (2009) The first genome-level transcriptome of the wood-degrading fungus *Phanerochaete chrysosporium* grown on red oak. Curr. Genet. 55:273-286.

Sato S., Liu F., Koc H., Tien M. (2007) Expression analysis of extracellular proteins from *Phanerochaete chrysosporium* grown on different liquid and solid substrates. Microbiology 153:3023-3033.

Sato Y., Wuli B., Sederoff R., Whetten R. (2001) Molecular cloning and expression of eight laccase cDNAs in loblolly pine (*Pinus taeda*). J. Plant Res. 114:147-155.

Sayer J.A., Gadd G.M. (1997) Solubilization and transformation of insoluble inorganic metal compounds to insoluble metal oxalates by *Aspergillus niger*. Mycol. Res. 101:653-661.

Scheel T., Höfer M., Ludwig S., Hölker U. (2000) Differential expression of manganese peroxidase and laccase in white-rot fungi in the presence of manganese or aromatic compounds. Appl. Microbiol. Biotechnol. 54:686-691.

Schoemaker H.E., Lundell T.K., Hatakka A.I., Piontek K. (1994) The oxidation of veratryl alcohol, dimeric lignin models and lignin by lignin peroxidase - the redox cycle revisited. FEMS Microbiol. Rev. 13:321-332.

Schwarzenbacher R., von Delft F., Jaroszewski L., Abdubek P., Ambing E., Biorac T., Brinen L.S., Canaves J.M., Cambell J., Chiu H.-J., Dai X., Deacon A.M., DiDonato M., Elsliger M.-A., Eshagi S., Floyd R., Godzik A., Grittini C., Grzechnik S.K., Hampton E., Karlak C., Klock H.E., Koesema E., Kovarik J.S., Kreusch A., Kuhn P., Lesley S.A., Levin I., McMullan D., McPhillips T.M., Miller M.D., Morse A., Moy K., Ouyang J., Page R., Quijano K., Robb A., Spraggon G., Stevens R.C., van den Bedem H., Velasquez J., Vincent J., Wang X., West B., Wolf G., Xu Q., Hodgson K.O., Wooley J., Wilson I.A. (2004) Crystal structure of a putative oxalate decarboxylase (TM1287) from *Thermotoga maritima* at 1.95 Å resolution. Proteins 56:392-395.

Shallom D., Shoham Y.I. (2003) Microbial hemicellulases. Curr. Opin. Microbiol. 6:219-228.

Shen Y.-H., Liu R.-J., Wang H.-Q. (2008) Oxalate decarboxylase from *Agrobacterium tumefaciens* C58 is translocated by a twin arginine translocation system. J. Microbiol. Biotechnol. 18:1245-1251.

Shimada M., Akamatsu Y., Tokimatsu T., Mii K., Hattori T. (1997) Possible biochemical roles of oxalic acid as a low molecular weight compound involved in brown-rot and white-rot wood decays. J. Biotechnol. 53:103-113.

Shimada M., Ma D.-B., Akamatsu Y., Hattori T. (1994) A proposed role of oxalic acid in wood decay systems of wood-rotting basidiomycetes. FEMS Microbiol. Rev 13:285-295.

Shimazono H. (1955) Oxalic acid decarboxylase, a new enzyme from the mycelium of wood destroying fungi. J. Biochem. 42:321-340.

Shimazono H., Hayaishi O. (1957) Enzymatic decarboxylation of oxalic acid. J. Biol. Chem. 227:151-159.

Shimizu M., Yuda N., Nakamura T., Tanaka H., Wariishi H. (2005) Metabolic regulation at the tricarboxylic acid and glyoxylate cycles of the lignin-degrading basidiomycete *Phanerochaete chrysosporium* against exogenous addition of vanillin. Proteomics 5:3919-3931.

Sierra-Alvarez R. (2007) Fungal bioleaching of metals in preservative-treated wood. Process Biochem. 42:798-804.

Sjöde A., Winestrand S., Nilvebrant N.-O., Jönsson L.J. (2008) Enzyme-based control of oxalic acid in the pulp and paper industry. Enzyme Microb. Technol. 43:78-83.

Sjöström E. (1993) Wood chemistry: Fundamentals and Applications. Academic Press, San Diego, USA, 293 p.

Sjöström E., Westermark U. (1998) Chemical composition of wood and pulps: Basic components and their distribution**.** In: Sjöström E., Alén R., editors. Analytical methods in wood chemistry, pulping, and papermaking. Springer-Verlag, Berlin, Germany, pp 1-35.

Smith M., Shnyreva A., Wood D.A., Thurston C.F. (1998) Tandem organization and highly disparate expression of the two laccase genes *lcc1* and *lcc2* in the cultivated mushroom *Agaricus bisporus*. Microbiology 144:1063-1069.

Soden D.M., Dobson A.D.W. (2001) Differential regulation of laccase gene expression in *Pleurotus sajor-caju*. Microbiology 147:1755-1763.

Soden D.M., Dobson A.D.W. (2003) The use of amplified flanking region-PCR in the isolation of laccase promoter sequences from the edible fungus *Pleurotus sajor-caju* J. Appl. Microbiol. 95:553-562.

Sollewijn Gelpke M.D., Lee J., Gold M.H. (2002) Lignin peroxidase oxidation of veratryl alcohol: effects of the mutants H82A, Q222A, W171A, and F267L. Biochem. 41:3498-3506.

Solomon E.I., Sundaram U.M., Machonkin T.E. (1996) Multicopper oxidases and oxygenases. Chem. Rev. 96:2563-2606.

Steffen K.T. (2003) Degradation of recalcitrant biopolymers and polycyclic aromatic hydrocarbons by litter-decomposing basidiomycetous fungi. Dissertationes Biocentri Viikki Universitatis Helsingiensis, 23/2003. Ph.D. Thesis, Department of Applied Chemistry and Microbiology, University of Helsinki, Helsinki, 68 p.

Steffen K.T., Hofrichter M., Hatakka A. (2002) Purification and characterization of manganese peroxidases from the litter-decomposing basidiomycetes *Agrocybe praecox* and *Stropharia coronilla*. Enzyme Microb. Technol. 30:550-555.

Stewart P., Cullen D. (1999) Organization and differential regulation of a cluster of lignin peroxidase genes of *Phanerochaete chrysosporium.* J. Bacteriol. 181:3427-3432.

Stewart P., Kersten P., Vanden Wymelenberg A., Gaskell J., Cullen D. (1992) Lignin peroxidase gene family of *Phanerochaete chrysosporium*: complex regulation by carbon and nitrogen limitation and identification of a second dimorphic chromosome. J. Bacteriol. 174:5036-5042.

Stewart P., Whitwam R.E., Kersten P.J., Cullen D., Tien M. (1996) Efficient expression of a *Phanerochaete chrysosporium* manganese peroxidase gene in *Aspergillus oryzae*. Appl. Environ. Microbiol. 62:860-864.

Sundaramoorthy M., Kishi K., Gold M.H., Poulos T.L. (1994) The crystal structure of manganese peroxidase from *Phanerochaete chrysosporium* at 2.06-Å resolution. J. Biol. Chem. 269:32759-32767.

Šušla M., Novotný Č., Erbanová P., Svobodová K. (2008) Implication of *Dichomitus squalens* manganese-dependent peroxidase in dye decolorization and cooperation of the enzyme with laccase. Folia Microbiol. 53:479-485.

Šušla M., Novotný Č., Svobodová K. (2007) The implication of *Dichomitus squalens* laccase isoenzymes in dye decolorization by immobilized fungal cultures. Biores. Technol. 98:2109-2115.

Suzuki M.R., Hunt C.G., Houtman C.J., Dalebroux Z.D., Hammel K.E. (2006) Fungal hydroquinones contribute to brown rot of wood. Environ. Microbiol. 8:2214-2223.

Svedružić D., Jónsson S., Toyota C., Reinhardt L., Ricagno S., Lindqvist Y., Richards N. (2005) The enzymes of oxalate metabolism: Unexpected structures and mechanisms. Arch. Biochem. Biophys. 433:176-192.

Svedružić D., Liu Y., Reinhardt L.A., Wroclawska E., Wallace Cleland W., Richards N.G.J. (2007) Investigating the roles of putative active site residues in the oxalate decarboxylase from *Bacillus subtilis*. Arch. Biochem. Biophys. 464:36-47.

Takao S. (1965) Organic acid production by basidiomycetes. I. Screening of acid-producing strains. Appl. Microbiol. 13:732-727.

Tamura K., Dudley J., Nei M., Kumar S. (2007) MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24:1596-1599.

Tanner A., Bornemann S. (2000) *Bacillus subtilis* YvrK is an acid-induced oxalate decarboxylase. J. Bacteriol. 182:5271-5273.

Tello M., Corsini G., Larrondo L.F., Salas L., Lobos S., Vicuña R. (2000) Characterization of three new manganese peroxidase genes from the ligninolytic basidiomycete *Ceriporiopsis subvermispora.* Biochim. Biophys. Acta 1490:137-144.

ten Have R., Hartmans S., Teunissen P.J.M., Field J.A. (1998) Purification and characterization of two lignin peroxidase isozymes produced by *Bjerkandera* sp. strain BOS55. FEBS Lett. 422:391-394.

Thurston C. (1994) The structure and function of fungal laccases. Microbiology 140:19-26.

Tien M., Kirk T.K. (1983) Lignin-degrading enzyme from the hymenomycete *Phanerochaete chrysosporium* burds. Science 221:661-663.

Tokimatsu T., Nagai Y., Hattori T., Shimada M. (1998) Purification and characteristics of a novel cytochrome *c* dependent glyoxylate dehydrogenase from a wood-destroying fungus *Tyromyces palustris.* FEBS Lett. 437:117-121.

Traquiar J.A. (1987) Oxalic acid and calcium oxalate production by *Leucostoma cincta* and *L. persoonii* in culture and in peach bark tissues. Can. J. Bot. 65:1952-1956.

Tseng Y.-H., Yang J.-H., Mau J.-L. (2008) Antioxidant properties of polysaccharides from *Ganoderma tsugae.* Food Chem. 107:732-738.

Tuomela M., Oivanen P., Hatakka A. (2002) Degradation of synthetic ^{14}C-lignin by various white-rot fungi in soil. Soil Biol. Biochem. 34:1613-1620.

Turroni S., Vitali B., Bendazzoli C., Candela M., Gotti R., Federici F., Pirovano F., Brigidi P. (2007) Oxalate consumption by lactobacilli: Evaluation of oxalyl-CoA decarboxylase and formyl-CoA transferase activity in *Lactobacillus acidophilus*. J. Appl. Microbiol. 103:1600-1609.

Umezawa T., Higuchi T. (1987) Mechanism of aromatic ring cleavage of β-O-4 lignin substructure models by lignin peroxidase. FEBS Lett. 218:255-260.

Urzúa U., Kersten P.J., Vicuña R. (1998) Manganese peroxidase-dependent oxidation of glyoxylic and oxalic acids synthesized by *Ceriporiopsis subvermispora* produces extracellular hydrogen peroxide. Appl. Environ. Microbiol. 64:68-73.

Valderrama B., Oliver P., Medrano-Soto A., Vazquez-Duhalt R. (2003) Evolutionary and structural diversity of fungal laccases. Antonie Van Leeuwenhoek 84:289-299.

Van Aken B., Hofrichter M., Scheibner K., Hatakka A.I., Naveau H., Agathos S.N. (1999) Transformation and mineralization of 2,4,6-trinitrotoluene (TNT) by manganese peroxidase from the white-rot basidiomycete *Phlebia radiata.* Biodegradation 10:83-91.

Vanden Wymelenberg A., Gaskell J., Mozuch M., Kersten P., Sabat G., Martinez D., Cullen D. (2009) Transcriptome and secretome analyses of *Phanerochaete chrysosporium* reveal complex patterns of gene expression. Appl. Environ. Microbiol. 75:4058-4068.

Vanden Wymelenberg A., Minges P., Sabat G., Martinez D., Aerts A., Salamov A., Grigoriev I., Shapiro H., Putnam N., Belinky P., Dosoretz C., Gaskell J., Kersten P., Cullen D. (2006) Computational analysis of the *Phanerochaete chrysosporium* v2.0 genome database and mass spectrometry identification of peptides in ligninolytic cultures reveal complex mixtures of secreted proteins. Fungal Genet. Biol. 43:343-356.

Vanden Wymelenberg A., Sabat G., Martinez D., Rajangam A.S., Teeri T.T., Gaskell J., Kersten P.J., Cullen D. (2005) The *Phanerochaete chrysosporium* secretome: Database predictions and initial mass spectrometry peptide identifications in cellulose-grown medium. J. Biotechnol. 118:17-34.

Varela E., Tien M. (2003) Effect of pH and oxalate on hydroquinone-derived hydroxyl radical formation during brown rot wood degradation. Appl. Environ. Microbiol. 69:6025-6031.

Vares T., Hatakka A. (1997) Lignin-degrading activity and ligninolytic enzymes of different white-rot fungi: Effects of manganese and malonate. Can. J. Bot. 75:61-71.

Vares T., Kalsi M., Hatakka A. (1995) Lignin peroxidases, manganese peroxidases, and other ligninolytic enzymes produced by *Phlebia radiata* during solid-state fermentation of wheat straw. Appl. Environ. Microbiol. 61:3515-3520.

Vares T., Niemenmaa O., Hatakka A. (1994) Secretion of ligninolytic enzymes and mineralization of ^{14}C-ring-labelled synthetic lignin by three *Phlebia tremellosa* strains. Appl. Environ. Microbiol. 60:569-575.

Wahleithner J.A., Xu F., Brown K.M., Brown S.H., Golightly E.J., Halkier T., Kauppinen S., Pederson A., Schneider P. (1996) The identification and characterization of four laccases from the plant pathogenic fungus *Rhizoctonia solani*. Curr. Genet. 29:395-403.

Wariishi H., Valli K., Gold M.H. (1992) Manganese(II) oxidation by manganese peroxidase from the basidiomycete *Phanerochaete chrysosporium*. Kinetic mechanism and role of chelators. J. Biol. Chem. 267:23688-23695.

Watanabe T., Hattori T., Tengku S., Shimada M. (2005) Purification and characterization of NAD-dependent formate dehydrogenase from the white-rot fungus *Ceriporiopsis subvermispora* and a possible role of the enzyme in oxalate metabolism. Enzyme Microb. Technol. 37:68-75.

Waterhouse A.M., Procter J.B., Martin D.M.A, Clamp M., Barton, G. J. (2009) Jalview Version 2 - a multiple sequence alignment editor and analysis workbench. Bioinformatics 25:1189-1191.

Welinder K.G. (1992) Plant peroxidases: structure-function relationships. In: Penel C., Gaspar T., Greppin H., editors. Plant Peroxidases, Topics and Detailed Literature on Molecular, Biochemical and Physiological Aspects, Université de Genève, Genève, pp. 1–24.

Widsten P., Kandelbauer A. (2008a) Adhesion improvement of lignocellulosic products by enzymatic pre-treatment. Biotechnol. Adv. 26:379-386.

Widsten P., Kandelbauer A. (2008b) Laccase applications in the forest products industry: A review. Enzyme Microb. Technol. 42:293-307.

Xu G., Goodell B. (2001) Mechanisms of wood degradation by brown-rot fungi: Chelator-mediated cellulose degradation and binding of iron by cellulose. J. Biotechnol. 87:43-57.

Yaver D.S., Golightly E.J. (1996) Cloning and characterization of three laccase genes from the white-rot basidiomycete *Trametes villosa*: genomic organization of the laccase gene family. Gene 181:95-102.

Yen G.-C., Wu J.-Y. (1999) Antioxidant and radical scavenging properties of extracts from *Ganoderma tsugae*. Food Chem. 65:375-379.

Yoshida H. (1883) Chemistry of lacquer (Urushi) Part 1. J. Chem. Soc. 43:472-486.

Zhu C.X., Hong F. (2009) Induction of an oxalate decarboxylase in the filamentous fungus *Trametes versicolor* by addition of inorganic acids. Appl. Biochem. Biotechnol. DOI 10.1007/s12010-009-8571-6