Characterization of the lignin-modifying enzymes of the selective white-rot fungus *Physisporinus rivulosus*

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Academic dissertation

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Contents

List of original publications

This thesis is based on the following publications, which are referred to in the text by Roman numerals I-IV.

I Hakala, T.K., Maijala, P., 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.

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

III 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.

IV 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. Published online.

Author's contribution:

I The author participated in planning and carrying out the laboratory work. She interpreted the results and participated in writing the article.

II The author planned and carried out the laboratory work except the analysis of organic acids and N-terminal sequencing of enzymes. She interpreted the results and wrote the article.

III The author planned and carried out the laboratory work, except the cloning and sequencing of *Physisporinus rivulosus mnp*A –gene. She interpreted the results and wrote the article.

IV The author participated in planning and carrying out the laboratory work, interpreting of results and writing of the article.

Abbreviations

ABTS	2,2´-azinobis(3-ethylbenzthiazoline-6-sulphonate)
cDNA	complementary DNA
CTAB	N-cetyl-N,N,N-trimethyl-ammonium-bromide
2,6-DMP	2,6-dimethoxyphenol
FPLC	fast protein liquid chromatography
HPLC	high performance liquid chromatography
IEF	isoelectric focusing
kDa	kilo Dalton
LiP	lignin peroxidase
MnP	manganese peroxidase
mRNA	messenger RNA
RT-PCR	reverse transcription polymerase chain reaction
p*I*	isoelectric point
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
VP	versatile peroxidase

Abstract

White-rot fungi are wood degrading organisms that are able to decompose all wood polymers; lignin, cellulose and hemicellulose. Especially the selective white-rot fungi that decompose preferentially wood lignin over wood polysaccharides e.g. cellulose are promising for biopulping applications. In biopulping the pretreatment of wood chips with white-rot fungi enhances the subsequent pulping step and substantially reduces the refining energy consumption. Because it is not possible to carry out biopulping in industrial scale as a closed process it has been necessary to search for new selective strains of white-rot fungi which naturally occur in Finland and cause selective white-rot of Finnish wood raw-material. A rare polypore *Physisporinus rivulosus* strain T241i, that was isolated from a forest burn research site, was found to be a selective lignin degrader in a screening of 300 fungal strains. In laboratory scale biopulping studies the pretreatment of spruce wood chips with *P. rivulosus* T241i resulted in a 20% reduction in the refining energy consumption in mechanical pulping, suggesting that it is applicable in biopulping.

Since selective lignin degradation is apparently essential for biopulping, knowledge on lignin-modifying enzymes and the regulation of their production by a biopulping fungus is needed. White-rot fungal enzymes that participate in lignin degradation are laccase, lignin peroxidase (LiP), manganese peroxidase (MnP), versatile peroxidase (VP) and hydrogen peroxide forming enzymes. In this study, *P. rivulosus* was observed to produce MnP, laccase and oxalic acid during growth on wood chips. In liquid cultures manganese and veratryl alcohol increased the production of acidic MnP isoforms detected also in wood chip cultures. Laccase production by *P. rivulosus* was low unless the cultures were supplemented with sawdust and charred wood, the components of natural growth environment of the fungus.

In white-rot fungi the lignin-modifying enzymes are typically present as multiple isoforms. In this study, two MnP encoding genes, *mnpA* and *mnpB,* were cloned and characterized from *P. rivulosus* T241i. Analysis of the N-terminal amino acid sequences of two purified MnPs and putative amino acid sequence of the two cloned *mnp* genes suggested that *P. rivulosus* possesses at least four *mnp* genes. The genes *mnpA* and *mnpB* markedly differ from each other by the gene length, sequence and intron-exon –structure. In addition, their expression is differentially affected by the addition of manganese and veratryl alcohol. *P. rivulosus* produced laccase as at least two isoforms. Both laccase isoforms had moderate thermal stability and one of them showed thermal activation at 50ºC. The results of this study revealed that the production of MnP and laccase was differentially regulated in *P. rivulosus*, which ensures the efficient lignin degradation under a variety of environmental conditions.

Tiivistelmä (Abstract in Finnish)

Valkolahosienet ovat puuta lahottavia sieniä, jotka kykenevät hajottamaan kaikkia puun polymeerejä; ligniiniä, selluloosaa ja hemiselluloosaa. Valkolahoa aiheuttavien ligniiniä hajottavien sienten käyttöä puuhakkeiden käsittelyssä ennen massanvalmistusta nimitetään biopulppaukseksi (engl. biopulping). Erityisesti ns. selektiivisiä valkolahosieniä eli sieniä, jotka hajottavat puuaineesta suhteellisesti enemmän ligniiniä kuin muita komponentteja kuten selluloosaa, on pidetty lupaavina biopulppaussieninä. Puuhakkeen jauhatusenergian kulutuksessa on voitu saavuttaa merkittävä energiansäästö käsittelemättömään puuhakkeeseen verrattuna. Koska biopulppausta ei teollisessa mittakaavassa ole mahdollista toteuttaa suljettuna prosessina, on ollut tarpeellista etsiä ja tutkia tähän tarkoitukseen soveltuvia Suomessa luonnonvaraisena esiintyviä sienikantoja, jotka kykenevät suomalaisen puuraaka-aineen selektiiviseen ligniininhajotukseen. Laajassa, noin 300 sienen vertailututkimuksessa erittäin harvinainen metsäpaloalueilla esiintyvä talikääpä, *Physisporinus rivulosus* kanta T241i, osoittautui selektiiviseksi ligniininhajottajaksi ja biopulppausominaisuuksiltaan lupaavaksi. Esikäsittelemällä kuusihakkeita *P. rivulosus* T241i:llä laboratoriokokeissa voitiin saavuttaa n. 20 % säästö mekaanisen massan jauhatusenergian kulutuksessa.

Koska selektiivinen ligniinin hajotuskyky liittyy ilmeisesti edulliseen biopulppauskäsittelytulokseen (energian säästöön jauhatuksessa), on tärkeää tuntea biopulppaukseen käytettävän sienen ligniiniä hajottavat entsyymit ja tekijät, jotka säätelevät niiden eritystä. Ligniinin hajotukseen osallistuvia solunulkoisia entsyymejä ovat lakkaasi, ligniiniperoksidaasi (LiP), mangaaniperoksidaasi (MnP), hybridityyppinen peroksidaasi (versatile peroxidase; VP) sekä vetyperoksidia tuottavat entsyymit. Tässä työssä *P. rivulosus* T241i:n havaittiin erittävän puuhakkeella kasvaessaan oksaalihappoa sekä MnP:a ja lakkaasia. *P. rivulosus* –sienen liuosviljelmissä korkein MnP-aktiivisuus saavutettiin, kun ravintoliuoksessa oli käytettävissä vain alhainen pitoisuus (0,1 %) glukoosia. Mangaani ja veratryylialkoholi edistivät liuosviljelmässä happamien MnP -isomuotojen eritystä, jollaisia havaittiin myös hakekasvatuksessa. Lakkaasia *P. rivulosus* eritti liuosviljelmässä melko vähän, jos alustassa ei ollut sen luontaiseen kasvuympäristöön kuuluvia kiinteitä substraatteja kuten sahanpurua tai hiiltynyttä puuta.

Valkolahosienille on tyypillistä, että niiden ligniiniin vaikuttavat entsyymit esiintyvät lukuisina isomuotoina. *P. rivulosus* T241i:lta karakterisoitiin kaksi eri MnP:a koodaavaa geeniä, *mnpA* ja *mnpB,* mutta MnP:n N-terminaalisten aminohapposekvenssien analyysin perusteella on todennäköistä, että sienellä on *mnpA*:n ja *mnpB*:n lisäksi ainakin kaksi muuta *mnp*-geeniä. Geenit *mnpA* ja *mnpB* poikkesivat merkittävästi toisistaan sekä geenin pituuden että introni-eksoni -rakenteen suhteen. Lisäksi veratryylialkoholi ja mangaani vaikuttivat *P. rivulosus*in *mnpA* ja *mnpB* geenien ilmentymiseen eri tavalla. *P. rivulosus*ista löydettiin kaksi lakkaasi-isomuotoa. Molemmat lakkaasit olivat melko lämpökestoisia ja niistä lämpöherkempi aktivoitui 50 °C asteen lämpötilassa. Tämän tutkimuksen tulosten mukaan *P. rivulosus* –sienellä erot kasvuolosuhteissa vaikuttivat eri entsyymien ja niiden isomuotojen erityksen eri tavalla, mikä takaa sienen tehokkaan ligniinin hajotuksen erilaisissa kasvuolosuhteissa.

1 Introduction

1.1 COMPOSITION OF WOOD

Wood cell walls consist of several layers of lignin and polysaccharides, namely cellulose and hemicellulose (Figure 1). Lignin is an aromatic, amorphous, heterogeneous polymer present in all cell wall layers. The highest lignin content is in the thin middle lamella, where lignin glues the adjacent cells together (Kuhad et al. 1997) and ensures the plant cell walls strength and resistance towards e.g. microbial attack. However, majority of wood cell wall lignin is situated in the thick secondary wall embedded in the lignin-carbohydrate complex. The cell types present in softwood include the vertical tracheids and resin ducts in the earlywood and latewood regions and the radial parenchyma cells. The softwood tracheids are connected together by bordered pits (Figure 1). The structure of hardwood is more complex than that of softwoods and consists of longitudinal fibers and vessel elements and radial parenchyma cells (Eriksson et al. 1990).

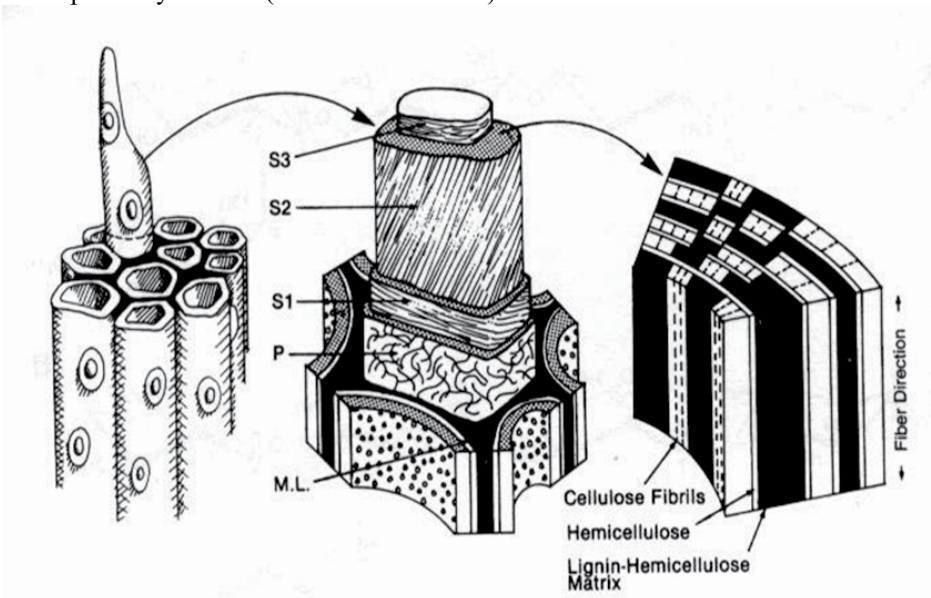

Figure 1. A schematic presentation of wood structure showing adjacent tracheids, diameter of each tracheid is approximately 30 μm (left), wood cell wall layers S1-S3: secondary cell wall layers, P: primary wall, M.L. middle lamella (middle) and lignin-carbohydrate complex of the secondary cell wall (right). Figure reprinted from Kirk and Cullen (1998) with permission of John Wiley & Sons, Inc.

Lignin comprises of phenylpropanoid units joined together in polymerization promoted by peroxidase and laccase action during lignin biosynthesis in the plant cell wall (reviewed by Boudet et al. 2003, Higuchi 2006). The three phenylpropanoid precursors of lignin i.e. p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol, form the three types of lignin subunits: hydroxyphenyl-, guaiacyl- and syringyl-types, respectively. The subunits are

joined together with a variety of bond types, mainly carbon-carbon and ether bonds the β-aryl-ether bonds being the most abundant (Adler 1977). Also heterogeneous ring structures such as dibenzodioxocin ring occur (Figure 2, Brunow et al. 1998b). Softwood (gymnosperm) lignin comprises mainly of coniferyl alcohol subunits, which makes its structure more dense and resistant towards microbial degradation than hardwood (angiosperm) lignin consisting of both guaiacyl and syringyl subunits (Faix et al. 1985, Blanchette 1995, Burlat et al. 1997). In addition, softwood has higher lignin content (25-33% of dry weight) than hardwood (20-25% of dry weight) (Sjöström and Westermark 1998).

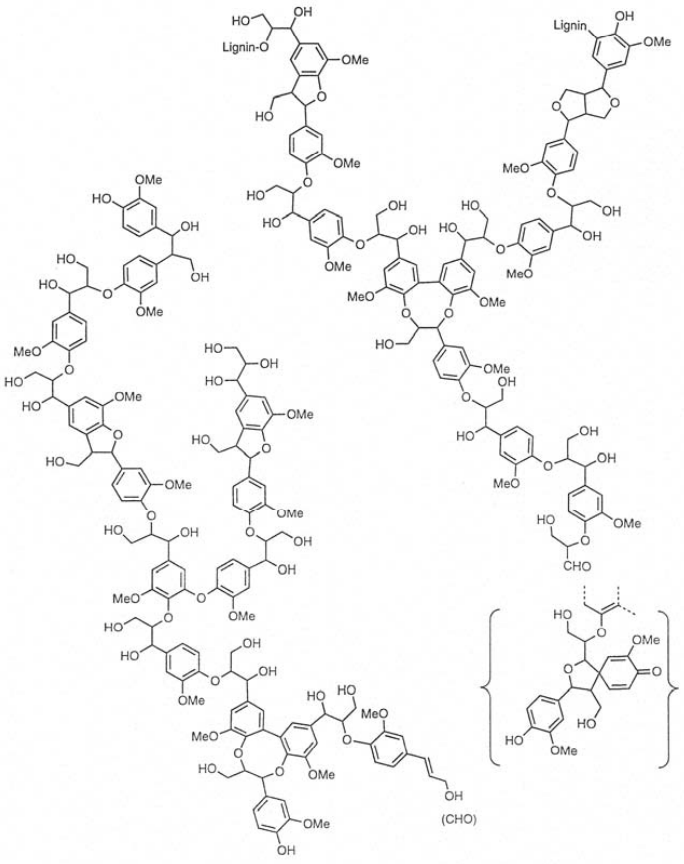

Figure 2. A structural model of lignin polymer according to Brunow et al. (1998a). The figure is reproduced by the permission of the American Chemical Society.

Cellulose is a linear polymer of glucose subunits linked together by β-1,4-glucosidic bonds with the degree of polymerization up to 15000. In wood cell wall cellulose forms microfibrils and fibers stabilized by hydrogen bonds between hydroxyl groups of the adjacent cellulose chains. Hemicelluloses are heteropolysaccharides, which comprise of β-1,4-linked polysaccharide backbone with different degree of substitution. The carboxyl groups of hemicellulose are covalently bonded with lignin via ether and benzyl ester linkages (Kuhad et

al. 1997). The main hemicelluloses in softwood and hardwood are galactoglucomannan and arabino-glucuronoxylan, respectively. Depending on the species, wood contains extractives from 2 to 5% of dry weight (Sjöström and Westermark 1998). Extractives comprise of triglycerides, fatty acids, resin acids, steryl esters, and phenolic substances, which provide the wood resistance towards microbial attack (Holmbom 1998).

1.2 WHITE-ROT FUNGI

Fungi involved in biodegradation of wood polymers can be divided into three main groups, namely white-rot, brown-rot and soft-rot fungi, according to the type of decay they cause. White-rot and brown-rot fungi both belong to the basidiomycetes, whereas soft-rot fungi are ascomycetes, and their activity is usually related to high or low moisture content of wood (Blanchette 1995). White-rot fungi are able to decompose all wood polymers, including lignin, which leaves the wood with a white, fibrous appearance. Brown-rot fungi efficiently degrade wood polysaccharides and are capable to only slightly alter, e.g. demethoxylate lignin, which leaves the wood brown, dry and with poor strength (Blanchette 1995). Formation of cavities and diffusion channels in wood cell wall has been found in a microscopic examination of soft-rotted wood (Anagnost 1998). Sap staining fungi are the primary colonizers of wood and decompose non-polymeric wood components such as extractives (Abraham et al. 1998).

Table 1. Typical features of selective and simultaneous white-rot

	Selective white-rot	**Simultaneous white-rot**	**Reference**
Degraded cell wall components	Initial stages of decay: hemicellulose and lignin Later stages: Hemicellulose, cellulose and lignin	Cellulose, hemicellulose and lignin	Adasgavek et al. 1995, Fackler et al. 2006
Anatomical features of decayed wood	Middle lamella dissolved Adjacent wood cells separated	Eroded cell walls, Degradation beginning from the secondary wall proceeding to middle lamella	Blanchette 1995
Lignin loss	Lignin loss diffusive throughout wood cell wall without major degradation of polysaccharides	Lignin loss together with wood cell wall polysaccharides starting progressively from lumen	Blanchette 1995
Representatives	*Ceriporiopsis subvermispora* *Pleurotus* spp. *Phlebia tremellosa* IZU 154	*Phanerochaete chrysosporium* *Trametes versicolor*	Blanchette 1995, Otjen et al. 1987, Nishida et al. 1988

White-rot fungi inhabit the wood cell lumen and the fungal hypha enters from cell to cell via bordered pits or directly through the cell wall. Depending on wood and fungal species different wood cell types may be decayed or resist fungal decay. For example in softwood the ray cells are more susceptible to fungal decay than tracheids and not all fungi are able to degrade the vessels in hardwood (Blanchette 1991, Kuhad et al. 1997). White-rot caused by fungi can be divided into simultaneous and selective lignin degradation types. In simultaneous white-rot the fungus degrades all wood cell wall polymers progressively, whereas in selective white-rot the fungus degrades preferably lignin and hemicellulose. The typical features of selective and simultaneous white-rot types are summarized in Table 1.

In selective white-rot the wood secondary cell wall is delignified diffusively starting from the lumen, followed with the delignification of the middle lamella. As white-rot fungi capable of selective lignin degradation prefer hemicelluloses as carbon source, the wood cell walls are enriched with cellulose (Blanchette 1991). Selective delignification can occur incompletely throughout wood substrate or merely in small, localized areas of complete lignin removal, which is called white pocket rot (Blanchette 1984, Otjen et al. 1987). In late stages of decay also cellulose is degraded and thus selective lignin degradation is usually limited to early stages of decay (Adasgavek et al. 1995). Selectivity of white-rot decay is dependent also on the physical and chemical environment in wood such as temperature, oxygen, nitrogen, and wood moisture content (Adasgavek et al. 1995, Blanchette 1995) and varies also between wood species (Blanchette et al. 1988). In addition to wood polymers, several white-rot fungi are able to degrade wood extractives (Gutiérrez et al. 1999, Dorado et al. 2000, Hatakka et al. 2003b, van Beek et al. 2007).

1.2.1 *PHYSISPORINUS RIVULOSUS*

Physisporinus rivulosus (Berk. & Curt.) Ryv. (talikääpä in Finnish), formerly known as *Polyporus rivulosus, Poria rivulosa, Poria albipellucida, Rigidopolus rivulosus, Ceriporiopsis rivulosa*, is a white-rot fungus widely distributed in North and Central America, and it is rarely found also in Europe (Kotiranta 1985) and Africa (Hjortstam and Ryvarden 1996). Other *Physisporinus* –species found in Finland are *Physisporinus vitreus* (maitovahakääpä in Finnish) and *Physisporinus sanguinolentus* (verivahakääpä in Finnish) (Niemelä 2005). According to a phylogenetic study based on the internal transcribed spacer (ITS) region sequence analysis, *P. rivulosus* and *Ceriporiopsis subvermispora* are closely related (Tomsovsky et al. 2006).

The fruiting bodies of *P. rivulosus* have been encountered from several species of softwood and hardwood, often on charred wood (Kotiranta 1985, Niemelä 2005). The occurrence of *P. rivulosus* became more prevalent after prescribed burning of a research forest site and thus it can be regarded as an anthracophile, i.e. favored by fire. Compared to old forests the environmental conditions after forest fire are more extreme: high insolation causes a low humidity and high maximal temperature, and thus fungal species tolerating extreme conditions can be enriched after fire (Kotiranta and Penttilä 1996). Also the wood substrate is altered during fire. Wood lignin is more resistant to pyrolysis than wood polysaccharides and thus wood is enriched with lignin. During pyrolysis the chemistry of wood polymers is altered and carbohydrates become anhydrated. In addition vanillins, guaiacols and other aromatic

hydrocarbons are formed from polymeric lignin (Alén et al. 1996). In Norway spruce (*Picea abies*) wood *P. rivulosus* grows preferentially in the less lignified earlywood and the hyphae pass through the pit membranes as the fungus colonizes the adjacent wood cells (Figure 3, Maijala et al. 2002). *P. vitreus* causes white pocket rot on water-saturated pine (*Pinus sylvestris*) wood, preferentially on earlywood (Schmidt et al. 1997). In Norway spruce (*P. abies*) and Douglas fir (*Pseudotsuga menziesii*) wood *P. vitreus* caused selective delignification and attacks on pit membranes of tracheids in early stages of wood decay (Schwarze and Landmesser 2000). *P. rivulosus* is also able to grow into unsterilized soil and efficiently mineralize chlorophenols, suggesting that the fungus is potentially useful also in bioremediation applications (Tuomela et al. 2007).

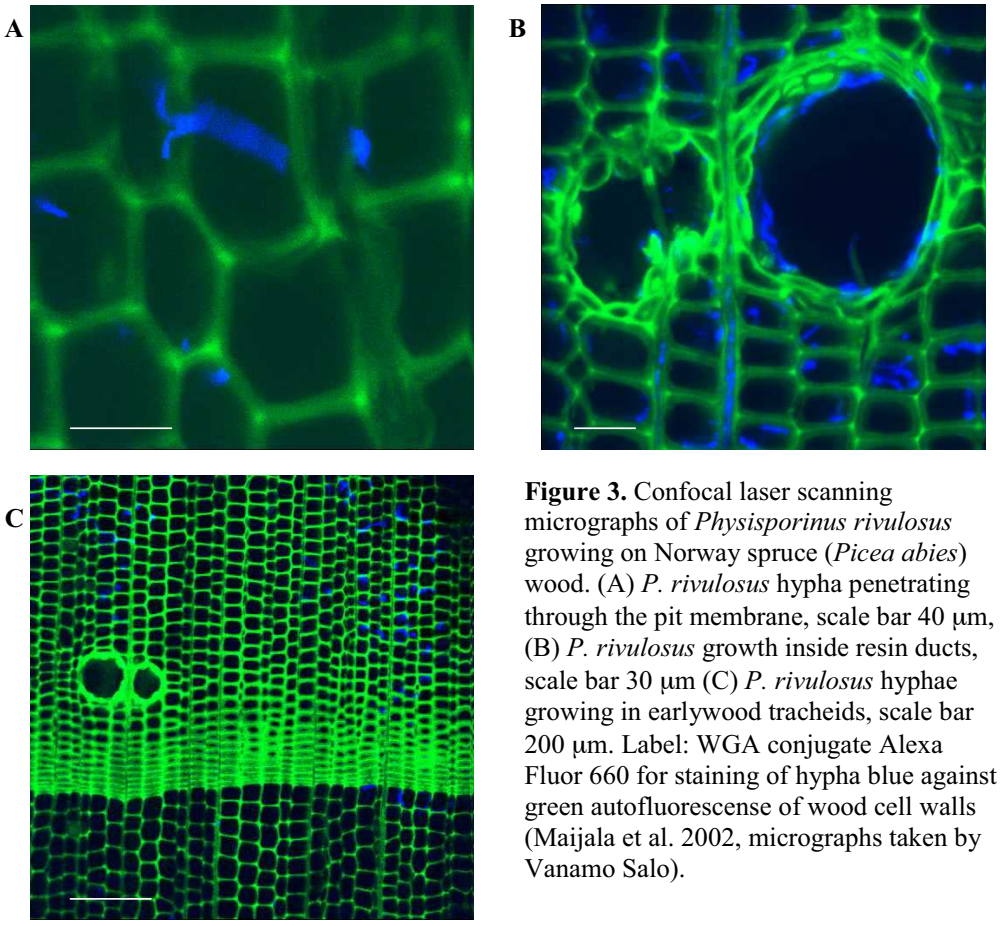

Figure 3. Confocal laser scanning micrographs of *Physisporinus rivulosus* growing on Norway spruce (*Picea abies*) wood. (A) *P. rivulosus* hypha penetrating through the pit membrane, scale bar 40 μm, (B) *P. rivulosus* growth inside resin ducts, scale bar 30 μm (C) *P. rivulosus* hyphae growing in earlywood tracheids, scale bar 200 μm. Label: WGA conjugate Alexa Fluor 660 for staining of hypha blue against green autofluorescense of wood cell walls (Maijala et al. 2002, micrographs taken by Vanamo Salo).

1.2.2 BIOTECHNOLOGICAL APPLICATIONS OF WHITE-ROT FUNGI

The ability of white-rot fungi to degrade recalcitrant molecules like lignin or even aromatic pollutants can be utilized in biotechnological applications. In biopulping, wood

chips or logs are pre-treated with fungi to enhance the subsequent pulping step (summarized in Table 2). According to an optimistic scenario of a recent technology evaluation, biopulping could be largely adopted by 2020. The biggest bottlenecks were considered to be the difficulties to control the process and the need for expensive investments (Kallioinen et al. 2003). Lignin-degrading white-rot and litter-decomposing fungi can be used in bioremediation to degrade toxic organic pollutants from contaminated soil or wood (reviewed by Pointing 2001, Steffen 2003). Wastewaters from dye, textile, and pulp manufacturing contain recalcitrant compounds such as synthetic dyes or halogenated organic compounds, which may be degraded or polymerized by white-rot fungi or their extracellular enzymes (Wesenberg et al. 2003). In bioethanol production white-rot fungi could be an alternative for chemical and physical pretreatment of lignocellulose material (Hatakka 1983, Itoh et al. 2003). Compared to other pretreatment alternatives the fungal treatment requires a long treatment time, but the energy requirement of the process is low and the treatment conditions are mild (Sun and Cheng 2002).

Table 2. The benefits of wood pretreatment with selected fungi

Fungus	Raw material	Benefits	Reference
Physisporinus rivulosus[b]	Sterilized wood chips	Selective lignin degradation Growth in a wide temperature range Reduced refining energy consumption Reduced wood pitch content	Hatakka et al. 2003b
Ceriporiopsis subvermispora[b]	Sterilized wood chips	Selective lignin degradation Reduced refining energy consumption Reduced wood pitch content Enhanced chemical pulping	Fischer et al. 1994, Akhtar et al. 2000, Bajpai et al. 2003
Phlebiopsis gigantea[b]	Wood logs	Reduced wood pitch content Enhanced chemical pulping Slightly reduced refining energy consumption Reduced staining of wood Enhanced debarking	Behrendt and Blanchette 1997
Ophiostoma piliferum[a]	Fresh, unsterilized wood chips, wood logs	Reduced wood pitch content Enhanced chemical pulping Reduced staining of wood	Messner et al. 1998, Breuil et al. 1998, Farrell 2007

b = Basidiomycete
a = Ascomycete

1.2.2.1 BIOPULPING

Biopulping, i.e. the fungal pretreatment of wood chips to enhance pulping, was invented in 1970´s (Eriksson et al. 1976, Eriksson and Vallander 1982), although the idea had been proposed already in the 1950´s (Lawson and Still 1957). To prevent yield loss caused by hydrolysis of cellulose during biopulping, fungi that degrade lignin selectively have been preferred as biopulping organisms (Akhtar et al. 1998a). In addition to selectivity, the thermotolerance of the fungus is of importance as the temperature in a wood chip pile may rise over 40°C as a result of fungal metabolism (Akhtar et al. 1998b).

In laboratory scale biopulping experiments two-week pretreatment with *C. subvermispora* has yielded up to 30 – 40% reduction in energy consumption in mechanical pulping. The energy savings obtained with fungal treatment of hardwood have been higher compared to softwood chips (Akhtar et al. 1998a). This is in agreement with softwood lignin being more resistant towards microbial degradation than hardwood lignin (Faix et al. 1985, Burlat et al. 1997). A fungal pretreatment of spruce wood chips in a 50-ton wood chip pile has verified energy savings obtained in laboratory scale. This pilot-scale process included (1) steam-sterilization of the wood chips, (2) cooling of the wood chips, (3) inoculation, and (4) two weeks incubation with continuous aeration to prevent self-heating (Figure 4, Akhtar et al. 2000). Recently similar experiments were conducted with *Eucalyptus grandis* as raw material with difficulties to prevent mold contamination when utilizing corn steep liquor as additive. These difficulties were overcome by inoculating the pile with pregrown wood chips (Ferraz et al. 2007). Controlling the process temperature is of importance for the establishment of the biopulping fungus and maintaining its selectivity (Adasgavek et al. 1995, Hatakka et al. 2003b). Compared to *C. subvermispora* the advantage of both *Phlebia subserialis* (Akhtar et al. 1998a) and *P. rivulosus* (Hatakka et al. 2003b) is that they grow at a wide temperature range and form less aerial hyphae than *C. subvermispora*, which can make the aeration and thus temperature control in the wood chip pile easier.

Biopulping can also be applied prior to chemical pulping to enhance cooking, to reduce cooking chemical consumption or to improve pulp quality (Messner et al. 1998, Bajpai et al. 2003). Combining the fungal treatment with Kraft pulping has recently gained more attention. Especially *Eucalyptus* spp. seem to be applicable to bio-Kraft process (Bajpai et al. 2003, Mardones et al. 2006). However, the acidification of wood chips by the fungal metabolites has been in some studies observed to increase the alkali consumption in Kraft pulping (Hatakka et al. 2004, Wolfaardt et al. 2004).

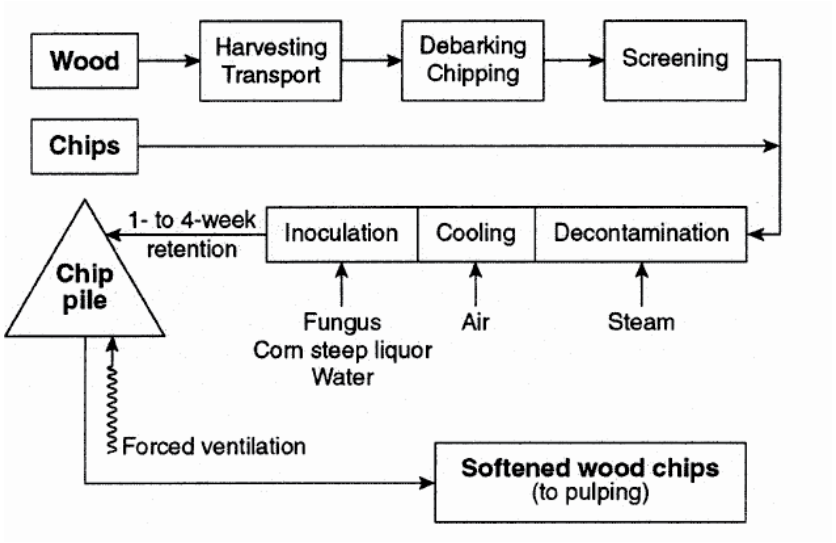

Figure 4. Overview of the biopulping process in pilot-scale trials. Figure reprinted from Akhtar et al. 2000 with the permission of Elsevier.

The wood extractives cause process problems during papermaking, and thus the ability to decrease the extractive content is desirable. During the biopulping of spruce (*P. abies*) and pine (*Pinus* sp.) *C. subvermispora* (Fischer et al. 1994, Hatakka et al. 2003b) and *P. rivulosus* (Hatakka et al. 2003b) degraded wood extractives. *Eucalyptus globulus* extractives were almost completely eliminated by *C. subvermispora* during 40 days of incubation (Gutiérrez et al. 1999). *Trametes versicolor* effectively degrades fatty acids, triglycerides, sterols and resin acids from *P. sylvestris* (Dorado et al. 2000) and *P. abies* (van Beek et al. 2007).

Another desired property of a biopulping fungus is the ability to efficiently colonize wood. If an efficient colonizer is chosen, the sterilization step can be omitted. The white-rot fungus *Phlebiopsis gigantea* can be inoculated to tree trunks at the time of harvest without sterilization. By this approach the debarking of the logs is enhanced and the wood extractive content, growth of sap-staining fungi and the energy consumption in refining are reduced (Behrendt and Blanchette 1997). However, the energy reduction is considerably lower than that achieved by *C. subvermispora* and the required treatment time is longer. Cartapip®/Sylvanex, a colorless mutant of the sap-staining ascomycete *Ophiostoma piliferum*, has been studied to reduce the pitch content and prevent the staining of wood chips since 1987 and today the technology is used in commercial scale (Breuil et al. 1998, Farrell 2007). Treatment of wood chips with Cartapip® does not reduce the energy consumption in mechanical pulping, but chemical pulping is enhanced probably due to the degradation of pitch components (reviewed by Messner 1998).

1.3 LIGNOCELLULOSE DEGRADATION BY WHITE-ROT FUNGI

1.3.1 LIGNIN-MODIFYING ENZYMES

Enzymes involved in lignin degradation are laccase (EC 1.10.3.2, benzenediol:oxygen oxidoreductase), lignin peroxidase (LiP, EC 1.11.1.14), manganese peroxidase (MnP, EC 1.11.1.13), versatile peroxidase (VP, EC 1.11.1.16), and H_2O_2-forming enzymes such as glyoxal oxidase (GLOX) and aryl alcohol oxidase (AAO, EC 1.1.3.7), which are produced by lignin-degrading white-rot and litter-decomposing fungi in different combinations (Hatakka 1994, 2001). Lignin-modifying enzymes oxidize substructures of lignin, are extracellular and typically they are present as multiple isoforms with similar function. Lignin degradation process by white-rot fungi has been termed "enzymatic combustion", because the causative agents are oxidative extracellular enzymes (Kirk and Farrell 1987). The production of lignin-modifying enzymes on defined media by several white-rot fungi has been widely studied. Several fungi, such as *Phanerochaete chrysosporium*, produce lignin-modifying enzymes efficiently during depletion of nutrients like nitrogen or sulfur (Kirk and Farrell 1987). On the other hand, enzyme production of some fungi, e.g. *Bjerkandera* spp., is dependent on the presence of organic forms of nitrogen (Kaal et al. 1993). Table 3 summarizes the wood lignin- and polysaccharide -degrading machinery of four well-studied white-rot fungi and shows that each fungus has a unique combination of enzymes performing the degradation of wood cell walls. In addition to the known lignin-degrading peroxidases, the sequenced *P. chrysosporium* genome (Martinez et al. 2004) contains hundreds of putative genes coding for extracellular oxidative enzymes, which might also play a role in the degradation of lignocellulose by the fungus (Kersten and Cullen 2007). This indicates that lignin degradation by white-rot fungi may be even more complex than previously considered.

1.3.1.1 LIGNIN-DEGRADING PEROXIDASES

Lignin-degrading peroxidases, i.e. MnP, LiP and VP, are structurally related heme-containing glycosylated peroxidases. MnP has been found from most of the lignin-degrading wood and litter inhabiting fungi studied so far (Hatakka 2001, Steffen 2003). The occurrence of LiP is less common; however, the fungi found to produce LiP are efficient lignin degraders, for example the corticioid fungi *P. chrysosporium* (Tien and Kirk 1983, Glenn et al. 1983), *Phlebia radiata* (Niku-Paavola et al. 1988) and *Phlebia tremellosa* (Vares et al. 1994) as well as the polypore *T. versicolor* (Jönsson et al. 1987). Versatile peroxidase has been found from species belonging to the genera *Pleurotus* (Camarero et al. 1999, Cohen et al. 2001) and *Bjerkandera* (Heinfling et al. 1998a, Mester and Field 1998). The whole genome sequencing of *P. chrysosporium* has confirmed the presence of ten LiP –encoding genes and five MnP –encoding genes (Martinez et al. 2004). The presence of multiple functionally related genes may have a role in maintaining lignin-degrading activity under changing environmental conditions and wood substrate during decay (Kersten and Cullen 2007). Recently it was reported that MnP is produced by the white-rot fungus *Pycnoporus cinnabarinus*, which was thought to lack lignin-modifying peroxidases and produce laccase as

Table 3. Enzymes involved in wood lignin and polysaccharide degradation by four selected white-rot fungi

Fungus	Lignin degradation	Polysaccharide degradation	References
Ceriporiopsis subvermispora	5 MnPs 2 laccases H_2O_2 formation by MnP	High hemicellulase/cellulase ratio Wide range of hemicellulases Low cellobiohydrolase activity	Lobos et al. 1994 Fukushima and Kirk 1995 Urzua et al. 1998 Sethuranaman et al. 1998b Tello et al. 2000 Ferraz et al. 2003 de Souza-cruz et al. 2004
Phanerochaete chrysosporium	10 LiPs 5 MnPs 2 GLOXs No laccase	Multiple cellulases (endoglucanase, cellobiohydrolases, β-glucosidase) Wide range of hemicellulases	Eriksson 1978 Kersten 1990 Vallim et al. 1998 Henriksson et al. 2000b
	1 cellobiose dehydrogenase (CDH) → $^{\cdot}OH$ formation in Fenton reaction → cellulose degradation, demethoxylation of lignin		Abbas et al. 2005 Kersten and Cullen 2007 Vanden Wymelenberg et al. 2005
Phlebia radiata	3 MnPs 2 Laccases 3 LiPs GLOX	Wide range of cellulases and hemicellulases	Vares et al. 1995 Rogalski et al. 1993a, b Hildén et al. 2005 Hildén et al. 2006 Mäkelä et al. 2006
Pleurotus ostreatus	1 MnP 3 VP 8 Laccases AAO	Endoglucanase β-glucosidase Xylanase β-mannosidase	Sannia et al. 1991 Valmaseda et al. 1991 Giardina et al. 1999 Giardina et al. 2000 Cohen et al. 2001 Palmieri et al. 2003 Baldrian and Gabriel 2003 Baldrian 2006

its only lignin modifying enzyme (Dölz et al. 2007). This information further points to the importance of peroxidases in lignin degradation.

MnP oxidizes Mn^{2+} to Mn^{3+} in a hydrogen peroxide dependent reaction (Gold et al. 2000, Hofrichter 2002). To stabilize the formed Mn^{3+} an organic chelator, e.g. a dicarboxylic acid that is produced by the fungus, is needed (Wariishi et al 1992, Gold et al. 2000). The oxidative reaction performed by chelated Mn^{3+} yields radical formation from various phenolic substrates, carboxylic acids, and unsaturated lipids. In phenolic substructures of lignin this leads to the formation of phenoxyl radical intermediates and thereafter to several reactions such as demethoxylation, quinone formation and C_α-C_β -cleavage (reviewed by Hofrichter 2002). When MnP of *C. subvermispora* oxidizes oxalate and glyoxylate H_2O_2 is formed and thus, in the presence of these organic acids, MnP is self-sufficient of H_2O_2 (Urzua et al. 1998). In lipid-mediated peroxidation reactions MnP oxidizes even the more recalcitrant non-phenolic substructures of lignin (Jensen et al. 1996, Kapich et al. 1999). In the presence of chelating organic acid and unsaturated lipids, fungal MnP is able to mineralize synthetic

lignin *in vitro* (Kapich et al. 1999, Hofrichter et al. 1999b), and to cause fragmentation of milled softwood (Hofrichter et al. 2001). The unsaturated lipids may be produced by the fungus (Elissetche et al. 2006) or cleaved by enzymatic action from triglycerides present in wood extractives (Dorado et al. 2000, Gutiérrez et al. 2002).

LiP oxidizes a variety of substrates, including veratryl alcohol, and both phenolic and non-phenolic substructures of lignin in a hydrogen peroxide dependent reaction to form a phenoxyl or aryl-cation radicals. The cation radical formation in non-phenolic lignin structures leads to unspecific reactions including cleavage of C_α-C_β -bond, demethoxylation, ring opening and depolymerization (Kirk and Farrell 1987, Hammel et al. 1993). It has been suggested that veratryl alcohol could act as a redox-mediator in reactions catalyzed by LiP, although the half-life of the radical is too short to be involved in oxidative reactions far from place of origin (Hatakka 2001). However, LiP has been demonstrated to oxidize Mn^{2+} to Mn^{3+} via radical reactions involving veratryl alcohol, oxalate and O_2 (Popp et al. 1990). Versatile peroxidase is able to oxidize both veratryl alcohol and Mn^{2+} in hydrogen peroxide dependent catalytic cycle, which means that it possesses the catalytic activity of both MnP and LiP (Mester and Field 1998, Camarero et al. 1999).

Multiple sequence analysis reveals, that lignin-degrading peroxidases, LiPs, MnPs and VPs, are genetically closely related, and most of them can be classified into three genetic groups (Figure 5, reviewed by Gold et al. 2000, Martínez 2002, Conesa et al. 2002). The *mnp* genes in the group I (corresponds to group B in Figure 5) found from e.g. *P. chrysosporium*, *C. subvermispora* and *Dichomitus squalens* code for proteins with long C-terminal tails. The short *mnp* genes, found in e.g. *T. versicolor*, are grouped together with versatile peroxidase genes as MnP group II (corresponds to the lower part of group A in Figure 5). The small MnPs shares structural features with LiP. Interestingly two MnP -encoding genes from *P. radiata* (Hildén et al. 2005), *Phlebia* sp. MG-60 (Kamei et al. 2007) and *P. rivulosus* (Publication III) belong to separate MnP-groups and share a higher homology with MnPs from other fungi than with each other. The third group consists of LiPs (corresponds to the upper part of group A in Figure 5) and is closely related with the MnP –group II (Martínez 2002).

The molecular weight of lignin-degrading peroxidases varies from 35 - 48 kDa for LiP to 38 - 62 kDa for MnP. The isoelectric points of LiPs and MnPs are generally between 3 and 4 for wood-inhabiting fungi (Hatakka 2001, Hofrichter 2002). However, even neutral MnPs have been found from litter-decomposing fungi (Steffen et al. 2002). Lignin-degrading peroxidases are globular proteins formed by 11-12 α-helices in two domains (Martínez 2002). Between the two domains situates the central cavity with heme bound by two histidine residues conserved in all lignin-degrading peroxidases (Conesa et al. 2002). Four to five disulfide bridges and two structural Ca^{2+} cations stabilize the structure of the protein and the active site (Martínez 2002, Conesa et al. 2002). Three acidic amino acids involved in binding of Mn^{2+} are conserved in MnPs and VPs but are absent from LiPs (Conesa et al. 2002). Trp171 (in *P. chrysosporium* LiPH8) is essential for binding of aromatic substrate. Trp171 is conserved in all LiP and VP encoding genes (Heinfling et al. 1998b, Martínez 2002) and the function of Trp171 in aromatic substrate oxidation has been verified by site-directed mutagenesis (Doyle et al. 1998) and using a tetrameric lignin model compound (Mester et al. 2001). LiP binds also polymeric lignin and His239 linked to Asp238 (in *P. chrysosporium* LiPH8) on the surface of LiP has been suggested to be involved in lignin binding (Johjima et

al. 1999). Thus, the catalytic activity of a lignin-degrading peroxidase can be predicted from the deduced amino acid sequence.

Addition of manganese has a stimulating effect on the expression of MnP in several fungi such as *P. chrysosporium* (Brown et al. 1993), *Pleurotus ostreatus* (Cohen et al. 2001), *T. versicolor* (Johansson et al. 2002), and *C. subvermispora* (Manubens et al. 2003). Manganese has been suggested to react via putative metal response elements (MRE), which have been found in the promotor region of several *mnp* genes (Li et al. 1999, Lobos et al. 1998, Tello et al. 2000, Hildén et al. 2005, Gold et al. 2000). The role of MREs is controversial, because a novel Mn^{2+} -responsive element has been found from the promotor region of *P. chrysosporium* gene *mnp1* (Ma et al. 2004) and Mn^{2+} markedly induces *T. versicolor mnp2* although it apparently lacks upstream MREs (Johansson et al. 2002). Manganese has been suggested to have a role also in the post-translational modification steps as the observed transcript levels and extracellular MnP activity do not correlate and Mn^{2+} is required for the production of active MnP by *C. subvermispora* (Manubens et al. 2003). Addition of manganese has been observed to repress the transcription of VP in *P. ostreatus* (Cohen et al. 2001) and the production of LiP in *P. chrysosporium* (Perez and Jeffries 1992). Instead, addition of veratryl alcohol or lignin induces LiP production in *P. chrysosporium* (Cancel et al. 1993, Kirk et al. 1986). Other factors influencing the expression of lignin-degrading peroxidases include oxidative stress (Li et al. 1995, Belinky et al. 2003), heat shock (Brown et al. 1993) and the availability of nutrients, such as nitrogen (Gold et al. 2000, Gettemy et al. 1998, Johansson et al. 2002, Kamitsuji et al. 2005). The heterological production of lignin-degrading peroxidases is discussed in Chapter 1.3.1.3.

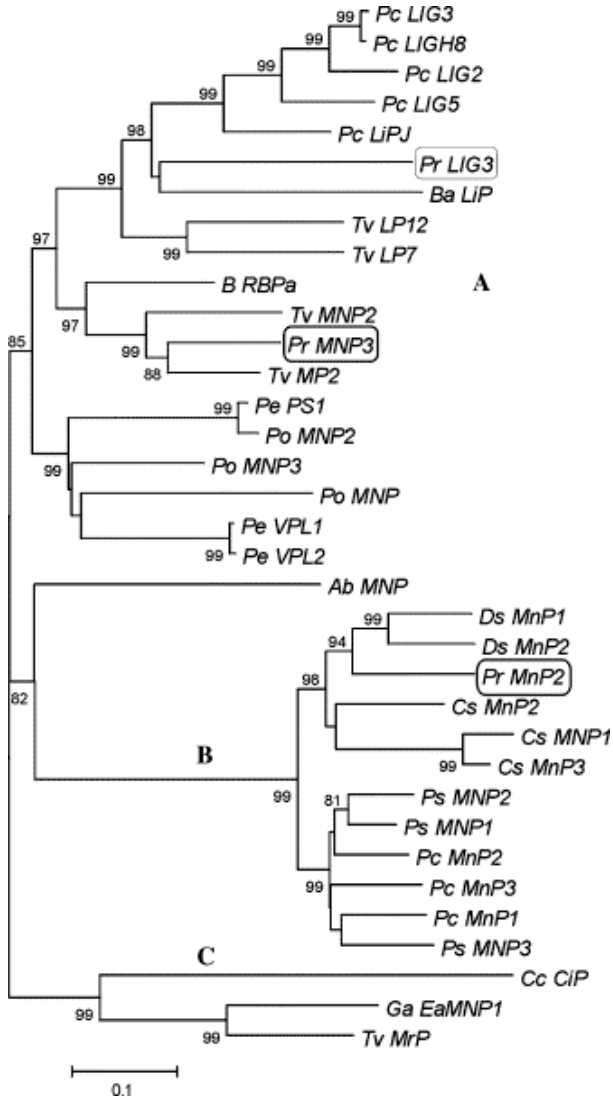

Figure 5. Phylogeny of fungal secreted heme peroxidases. Minimum evolution Neighbor-joining tree of the full-length MnP, VP, LiP protein sequences with 1000x bootstrapping was created with Mega 3.1 software. Scale bar presents a distance equivalent to 0.05 amino-acid substitutions per site. Values higher than 50% for the nodes are indicated. The tree has been rooted for *Armorica rusticana* peroxidase HRP, which belongs to plant heme peroxidases. Sequence accessions were retrieved from: GenBank (USA), EMBL (Europe), or DBJ (Japan). Initials refer to fungal species: *Ab* (*Agaricus bisporus*), *Ba* (*Bjerkandera adusta*), *B* (*Bjerkandera* sp.), *Cc* (*Coprinus cinereus*), *Cs* (*C. subvermispora*), *Ds* (*Dichomitus squalens*), *Ga (Ganoderma applanatum*), *Pc (P. chrysosporium*), *Pe* (*Pleurotus eryngii*), *Po* (*Pleurotus ostreatus*), *Pr* (*Phlebia radiata*), *Ps (Phanerochaete sordida*), and Tv (*Trametes versicolor*). Figure reprinted from Hildén et al. 2005 with permission of Elsevier.

1.3.1.2 LACCASE

Laccases are blue copper-containing oxidases that catalyze one-electron oxidations of aromatic amines and phenolic compounds such as phenolic substructures of lignin. The terminal electron acceptor in the catalytic reaction is molecular oxygen, which is reduced to water (Thurston 1994). The complete crystalline structure of laccase containing all four copper atoms in the active site has been published from the ascomycete *Melanocarpus albomyces* (Hakulinen et al. 2002), *T. versicolor* (Bertrand et al. 2002, Piontek et al. 2002) and *Cerrena maxima* (Lyashenko et al. 2006). The structure of laccase consists of three cupredoxin-like domains, and resembles that of ascorbate oxidase (Hakulinen et al. 2002, Bertrand et al. 2002). Laccases are glycoproteins and those of white-rot fungi generally have molecular weight between 60-80 kDa and p*I* 3-6 (Hatakka 2001, Badrian 2006). Although laccase has been extensively studied for decades the catalytic mechanism is not fully understood. Laccase catalyses the formation of phenoxyl radicals and their unspecific reactions leading finally to C_α-hydroxyl oxidation to ketone, alkyl-aryl –cleavage, demethoxylation and C_α-C_β -cleavage in phenolic lignin substructures, as well as polymerization reactions (Youn et al. 1995). Laccase is able to oxidize also non-phenolic substructures of lignin in the presence of a low molecular weight mediator like hydroxybenzotriazole (Call and Mücke 1987). Natural mediators could derive from lignin (Camarero et al. 2005, 2007) or be produced by the fungus (Eggert et al. 1996b).

In nature, the occurrence of laccase is widespread and laccase has been found in fungi, bacteria and plants. In the fungal kingdom laccase has been found in phytopathogenic, soil and fresh water inhabiting ascomycetes and in several basidiomycetes, including some mycorrhizal and brown-rot fungi. In lignin-degrading white-rot and litter-decomposing fungi laccase has been found in almost every species studied (Baldrian 2006). White-rot fungi usually have several laccase encoding genes and secrete laccases as multiple isoforms (Hatakka 2001) and for example *P. ostreatus* has at least eight laccase isoforms (reviewed by Baldrian 2006). An interesting exception in occurrence of laccase is the widely studied white-rot fungus *P. chrysosporium*. In the sequenced genome of *P. chrysosporium* no close match to known laccase encoding genes could be found (Kersten and Cullen 2007). There are few reports in laccase produced by *P. chrysosporium* (Srinivasan et al. 1995, Gnanamani et al. 2006) but these results can be partly explained by the unspecific nature of 2,2´-azinobis(3-ethylbenzthiazoline-6-sulphonate) (ABTS) oxidation reaction and the possibility of variation in strains. However, it seems evident that most *P. chrysosporium* strains do not produce laccase.

As laccase is able to oxidize lignin and is produced by most lignin-degrading fungi under ligninolytic conditions, it has been generally accepted to have a role in lignin degradation by white-rot fungi. Fungal laccase has been suggested to participate in morphogenesis, fungal plant pathogen interaction, stress defense and detoxification of by-products of lignin degradation (reviewed by Thurston 1994, Baldrian 2006). In plant cell wall laccase participates to the lignin biosynthesis (reviewed by Boudet et al. 2003, Higuchi 2006). Laccase production by white-rot fungi can be induced by the addition of Cu^{2+} (Collins et al. 1997, Michniewicz et al. 2006) or aromatic compounds such as veratryl alcohol (Kantelinen et al. 1989, D´Souza et al. 1999), other benzyl alcohols (González et al. 2003), and 2,5–xylidine (Eggert et al. 1996a, Collins et al. 1997, Perié et al. 1998). In some fungi such as *C.*

subvermispora (Fukushima and Kirk 1995, Salas et al. 1995) and *Ganoderma lucidum* (D´souza et al. 1999) laccase production is increased in the presence of lignocellulose material. The heterological production of laccase is discussed in next chapter.

1.3.1.3 BIOTECHNOLOGICAL APPLICATIONS OF LIGNIN-MODIFYING ENZYMES

The unspecific nature of the catalysis by fungal lignin-modifying enzymes makes the possibilities for their biotechnological applications numerous and include several fields of industry (Conesa et al. 2002, Rodríquez Couto and Toca Herrera 2006). Lignin-degrading peroxidases, especially MnP have been studied for pulp bleaching (Moreira et al. 2003), reduction of aromatic substances from textile and pulp industry effluents (Wesenberg et al. 2003) and enhanced refining of wood chips (Maijala et al. 2007) or interstage pulp (Kurek et al. 2001). In laboratory scale the consumption of refining energy in mechanical pulping was reduced with MnP pretreatment with a slight improvement in pulp properties (Kurek et al. 2001). MnP could be utilized for the degradation of recalcitrant molecules due to its ability to oxidize a wide range of substrates including polyaromatic hydrocarbons and chlorophenols (Hofrichter 2002).

The results obtained with MnP in Kraft pulp bleaching have been promising and the enzyme treatment has had only a minor effect on paper strength or yield (Moreira et al. 2003). Bleaching effect of MnP is enhanced by the addition of Mn^{2+}, H_2O_2, organic chelators and unsaturated lipids (Moreira et al. 2001b, Bermek et al. 2002). Organic chelators could be used to dissolve the Mn^{2+} present in pulp (Harazono et al. 1996, Moreira et al. 2001b) and the constant need of H_2O_2 could be fulfilled by glucose oxidase, which produces H_2O_2 from glucose (Moreira et al. 2001b). Immobilization of MnP into porous material increases the stability of the enzyme towards elevated temperature and excess hydrogen peroxide (Sasaki et al. 2001). The heterologous expression of lignin-degrading peroxidases has been challenging and in most cases heme needs to be added to the media to yield even low amounts of active enzyme (Conesa et al. 2002). Thus the obstacles in the commercialization of applications with lignin-degrading peroxidases include the lack of an efficient production system and the need for carefully optimized reaction conditions.

The heterologous expression of some laccases has been successful (Berka et al. 1997, Kiiskinen et al. 2004a) and today several enzyme suppliers produce laccase commercially for industrial applications. In industrial scale laccase is utilized at least in denim bleaching (Cavaco-Paulo and Gübitz 2003). For pulp and paper industry it is marketed for effluent control and increasing the strength properties of lignin containing paper products. However several other applications have been studied, for example in baking the ability of laccase to cross-link biopolymers is utilized to improve the properties of the dough and the baked product (Selinheimo et al. 2006). In addition to denim bleaching the ability of laccase to oxidize several dyes could be utilized in textile industry in the treatment of dye-containing wastewaters (Cavaco-Paulo and Gübitz 2003, Wesenberg et al. 2003). Utilization of laccase has been studied also in wood industry to increase fiberboard strength (Mai et al. 2004). One of the most studied application is the laccase-mediator bleaching of Kraft pulp (Call and Mücke 1997), the efficiency of which has been proven in mill-scale trials (Paice et al. 2002).

With the laccase-mediator system also the extractives from *Eucalyptus* and *P. abies* mechanical pulp can be efficiently removed (Gutiérrez et al. 2006). Together with a mediator, laccase is able to oxidize also the non-phenolic substructures of lignin and the accessibility of fiber lignin is higher for the low molecular weight mediator than laccase itself. The main obstacle for commercialization of laccase-mediator bleaching is the lack of safe and low-cost mediators. The search for natural mediators is in progress (Camarero et al. 2005, Camarero et al. 2007). Laccase could be also used to activate mechanical pulp fibers and subsequently graft different chemicals into the fibers to achieve functionality into the fibers (Chandra and Ragauskas 2002, Grönqvist et al. 2006).

1.3.2 WOOD POLYSACCHARIDE DEGRADATION BY FUNGI

White-rot fungi degrade wood cellulose by hydrolytic and oxidative reactions. Cellulases consist of endoglucanases (endo-1,4-β-glucanase, EC 3.2.1.4), cellobiohydrolases (1,4-β-cellobiosidase, EC 3.2.1.91) and β-glucosidases (EC 3.2.1.21), which degrade cellulose by a synergistic action. Endoglucanase catalyses the random cleavage of cellulose polymer, cellobiohydrolase I releases cellobiose units from the reducing end and cellobiohydrolase II from the non-reducing end of the cellulose polymer. The released oligosaccharides are hydrolyzed to glucose by β-glucosidase (reviewed by Lynd et al. 2002). Cellulases produced by white-rot fungi have been studied for decades and a wide range of cellulolytic activities have been found in white-rot fungi for example *P. chrysosporium* (Eriksson 1978, Abbas et al. 2005) and *P. radiata* (Rogalski et al. 1993a,b). The genome of *P. chrysosporium* contains up to 166 genes coding for glycosyl hydrolases, and in its secretome on cellulose containing media products of 32 of these genes could be identified (Vanden Wymelenberg et al. 2005). Production of cellulases is in many fungi, such as *Ceriporiopsis subvermispora* and *P. gigantea*, controlled by the carbon source by catabolite repression and is induced by the presence of cellulose (Eriksson 1978, Sethuranaman et al. 1998b, Niranjane et al. 2007).

Degradation of cellulose by brown-rot fungi is believed to occur via the oxidative action of hydroxyl radicals formed in the Fenton reaction: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\cdot}OH$. This reaction can be regulated by the fungal extracellular metabolites, such as siderophore-type Fe^{3+}–chelators that can reduce the formed Fe^{3+} back to Fe^{2+} (Xu and Goodell 2001) or by Fe^{3+}–reducing glycoproteins (Tanaka et al. 2007). Fe^{3+} -reducing activity has been detected also in wood chip cultures of *C. subvermispora* (Aguiar et al. 2006). However, it also produces several alkyl- and alkenylitaconates, such as ceriporic acid B, which represses the formation of hydroxyl radicals via Fenton reaction and thus the depolymerization of cellulose by hydroxyl radicals (Rahmawati et al. 2005). Cellobiose dehydrogenase (EC 1.1.99.18) is an enzyme, which oxidizes cellobiose to cellobionolactone. Cellobiose dehydrogenase has been found from brown- and soft-rot fungi (Henriksson et al. 2000a) and the white-rot fungi *P. chrysosporium* (Vallim et al. 1998, Henriksson et al. 2000b), *T. versicolor* (Roy et al. 1996) and *P. cinnabarinus* (Temp and Eggert 1999). The products of reductive reactions performed by cellobiose dehdyrogenase can react further in Fenton reaction to form hydroxyl radicals, which are reactive towards glycosidic bonds and some of the lignin substructures (Ander 1994, Henriksson et al. 2000a).

White-rot fungi degrade hemicellulose by both glycoside hydrolases and carbohydrate esterases (Shallom and Shoham 2003). Hemicellulases are involved in cleavage of hemicellulose backbone (e.g. endo-1,4-β-xylanase, EC 3.2.1.8), hydrolysis of oligosaccharides (e.g. 1,4-β-xylosidase, EC 3.2.1.37) and removal of side chains (e.g. α-glucuronidase, EC 3.2.1.139). Carbohydrate esterases (e.g. acetyl-xylan esterase, EC 3.1.1.72) hydrolyze the ester linkages of acetate and ferulic acid side groups, which are involved in formation of the linkage between hemicellulose and lignin (Kuhad et al. 1997, Shallom and Shoham 2003). Hemicellulases of white-rot fungi have been studied in detail in for example *P. radiata* (Rogalski et al. 1993 a,b). When *C. subvermispora* grows on wood the observed activity of hemicellulases exceeds that of cellulolytic enzymes (Heidorne et al. 2006), which indicates that the hemicellulolytic activity is of importance for the selectivity of white-rot decay.

1.3.3 SECRETION OF LOW MOLECULAR WEIGHT COMPOUNDS INVOLVED IN LIGNOCELLULOSE DEGRADATION

It is generally accepted that extracellular fungal enzymes, LiP, MnP and laccase, contribute to the lignin degradation process by fungi. However, the size of these enzymes is too large to penetrate wood cell walls during early stages of decay although the cell wall becomes more porous and accessible to enzymes as a result of the fungal decay (Blanchette et al. 1997). Accordingly it has been observed that LiP produced by *P. chrysosporium* is localized near the fungal hypha during growth on wood and penetrates only into strongly decayed wood cell wall (Srebotnik et al. 1988a, b). In highly degraded wood MnP of *P. chrysosporium* was shown to bind preferentially to the lignin-rich areas of the cell corners and middle lamella (Daniel et al. 1991). It is likely that especially in the beginning of the wood cell wall degradation low molecular weight compounds take part in lignin degradation. Lignin degradation by selective white-rot occurs in the middle lamella far from the fungal hyphae (Blanchette et al. 1995) and the diffusible low molecular weight compounds probably have an important role. The secreted low molecular weight compounds, which possibly are involved in the wood polymer degradation by the white-rot fungi, are summarized in Table 4.

Low molecular weight compounds may act as redox shuttles or stabilize the oxidation stage and thus oxidation brought about by the oxidative enzymes is transported deeper in the wood cell wall. Some LiP-producing fungi secrete veratryl alcohol, which participates in LiP–mediated lignin degradation (Lundquist and Kirk 1978, Hatakka et al. 1991, Mester et al. 1995). Veratryl alcohol is also a substrate for fungal aryl alcohol oxidase, which catalyses formation of H_2O_2 from a wide range of aromatic alcohols (Ferreira et al. 2005). Natural redox-mediators secreted by the fungus may also participate in lignin degradation by laccase (Eggert et al. 1996b). Lignin degradation triggered by MnP involves several low molecular weight compounds, e.g. unsaturated fatty acids and dicarboxylic acids, which may be secreted by the fungus (Dutton and Evans 1996, Kapich et al. 1999, Enoki et al. 1999, Elissetche et al. 2006). In a biomimetic approach lignin can be degraded non-enzymatically by the action of H_2O_2 and copper coordinated by pyridine containing compounds, mimicking the action of fungal laccase and peroxidases (Messner et al. 2003, Watanabe et al. 1998).

Table 4. Secretion of low molecular weight compounds involved in lignin and wood polysaccharide degradation by white-rot fungi

Secreted compound	Role in lignocellulose degradation	Occurrence	References
Carboxylic acids: Oxalate Glyoxylate Formate Malate	Chelator for Mn^{3+} pH control Substrate for H_2O_2 production Ca^{2+} solubilization to enhance pectin removal	Common among both white- and brown rot fungi	Dutton and Evans 1996 Hofrichter et al. 1999a Galkin et al. 1998 Urzua et al. 1998 Mäkelä et al. 2002
Veratryl alcohol	Redox –transfer in LiP catalyzed lignin degradation Substrate for H_2O_2 formation	LiP –producing fungi e.g. *P. radiata* *P. chrysosporium* *B. adusta*	Lundquist and Kirk 1978 Hatakka et al. 1991 Sannia et al. 1991 Mester et al. 1995
Unsaturated fatty acids	Lignin degradation via lipid peroxidation	*C. subvermispora* *Ganoderma australe*	Kapich et al. 1999 Enoki et al. 1999 Elissetche et al. 2006
Itaconic acids: Ceriporic acid	Suppression of Fenton reaction and cellulose degradation	*C. subvermispora*	Amirta et al. 2003 Rahmawati et al. 2005
3- hydroxyantranilate (3-HAA)	Redox mediator in laccase catalyzed lignin degradation	*Pycnoporus cinnabarinus*	Eggert et al. 1996b

Several wood-rotting fungi, causing both brown- and white-rot, have been observed to acidify their surroundings by secreting organic acids such as oxalic acid (Shimada et al. 1997, Galkin et al. 1998). For example the selective white-rot fungus *C. subvermispora* secretes oxalate as it grows on *Pinus taeda* wood chips (Aguiar et al. 2006). Oxalate originates from fungal intracellular biochemical reactions e.g. the tricarboxylic acid cycle in mitochondria and glyoxylate cycle in glyoxisomes (Dutton and Evans 1996, Munir et al. 2001). White-rot fungi have been observed to have specific enzymes such as oxalate decarboxylase and oxalate oxidase for the degradation of oxalate and thus the oxalate concentration may be strictly controlled (Dutton et al. 1994, Mäkelä et al. 2002, Escutia et al. 2005). Organic acids secreted by white-rot fungi, include also formate, glyoxylate and malate (Galkin et al. 1998, Hofrichter et al. 1999a, Mäkelä et al. 2002). Besides secretion by the fungus, formate can be formed as a by-product of lignin degradation after the opening of aromatic ring in MnP promoted reactions (Hofrichter 2002).

Oxalate and other organic acids have been proposed to have several roles in lignin and wood polysaccharide degradation. In lignin degradation one of the roles is to enhance the reactivity of ligninolytic enzyme by simply acidifying the pH of wood close to the optimum of the enzymes (Dutton and Evans 1996). In addition, organic acids are essential for MnP as they chelate and thus stabilize the formed Mn^{3+}-cations (Shimada et al. 1997) and can also act as a substrate for formation of H_2O_2 by the Mn^{3+}-mediated oxidation (Urzua et al. 1998). Oxalate facilitates pectin removal by solubilizing Ca^{2+} from pit membranes and middle

lamellae (Dutton and Evans 1996, Shimada et al. 1997). Wood is a nitrogen poor environment and the fungi may balance their intracellular C/N ratio by secreting oxalate (Shimada et al. 1997, Dutton and Evans 1996). The roles of fungal oxalate secretion are not limited to wood decay. Oxalate also participates in fungal pathogenesis, competition, controlling availability of environmental nutrients, and detoxification of metal cations (Dutton and Evans 1996).

2 Objectives of the present study

The aim of this study was to characterize the lignin-modifying enzymes of the novel biopulping fungus *Physisporinus rivulosus* T241i, which was found in a screening conducted as a part of this study. Although the enzymology of lignin degradation by white-rot fungi has been widely studied during the last decades, fairly little is known about the production of the different isoenzymes during fungal growth on wood. The aim of this study was to determine which factors affect the production of lignin-modifying enzymes by *P. rivulosus* under biopulping conditions and in defined media.

The aims addressed in four separate original publications were:

1. To evaluate Finnish white-rot fungi and to find a new selective fungus for the biopulping of Norway spruce (Publication I)

2. To find out which lignin-modifying enzymes and organic acids are produced on wood chip culture by the new interesting fungus, *P. rivulosus* (Publication II)

3. To characterize the lignin-modifying enzymes produced by *P. rivulosus* under different culture conditions (Publications II, III and IV)

4. To determine factors affecting the production of lignin-modifying enzymes, MnP and laccase, in *P. rivulosus* (Publications III and IV)

5. To clone, sequence and characterize genes coding for *P. rivulosus* MnP and to find out how they are regulated at transcriptional level (Publication III)

3 Materials and methods

The microbiological, analytical, biochemical and molecular biology methods used in this thesis are summarized in Table 5. The methods are described in detail in the original publications.

3.1 EVALUATION OF NOVEL WOOD-ROTTING FUNGI (I)

Over 250 strains of wood-rotting fungi were collected in Finland. The fungal strains were isolated from fruiting bodies identified by experts. The lignin-degrading ability, growth rate and thermotolerance of the novel fungal strains and additional strains from culture collections were evaluated to select the promising strains for further characterization. Among the 300 tested strains the thermotolerant strains, showing at least some Poly R-478 decolorization ability, and strains with efficient decolorization ability were selected for the next screening step. This resulted in 86 fungal strains chosen for the subsequent wood block decay test. The most promising strain, a dikaryotic isolate of *Physisporinus rivulosus* with strain number T241i (basidiocarp identified by Dr. Heikki Kotiranta), was selected for the following experiments. The strain was deposited to the culture collection Deutsche Samlung von Mikrooorganismen und Zellkulturen (DSM) by number DSM 14618.

3.2 CHARACTERIZATION OF LIGNIN-MODIFYING ENZYMES PRODUCED BY *P. RIVULOSUS* (II, III, IV)

The lignin-modifying enzymes produced by *P. rivulosus* T241i were extracted from the wood chip cultures and their activity was measured spectrophotometrically. The organic acids secreted by the fungus were analyzed by high performance liquid chromatography (HPLC). Manganese peroxidase and laccase produced by the fungus on wood chips were purified by fast protein liquid chromatography (FPLC). The purified enzymes were characterized electrophoretically by SDS-PAGE and isoelectric focusing (IEF). The enzymes were visualized in IEF gels by activity based staining methods, namely guaiacol (laccase), phenol red in the presence of H_2O_2 and Mn^{2+} (MnP) and phenol red in the presence of H_2O_2 without Mn^{2+} (LiP).

The effect of sawdust, nutrients, Mn^{2+} and veratryl alcohol on the production of MnP by *P. rivulosus* T241i was studied in liquid culture media in liquid media containing sawdust. Two MnP encoding genes were amplified, cloned, sequenced and characterized and their expression was studied using competitive reverse transcriptase polymerase chain reaction (RT-PCR).

The regulation of laccase production in *P. rivulosus* T241i was studied in media containing peptone together with sawdust and charcoal. Laccase isoforms were purified by FPLC and their molecular and kinetic characteristics as well as thermotolerance and pH optima were determined.

Table 5. Methods used in the original publications of this study.

Aim	Methodology	Publication
Cultivation of fungi:		
Growing the fungal inocula	Malt agar plates	I, II, III, IV
	Liquid culture	I, II, III, IV
Screening of fungi	Poly R -478 agar plates	I
	Wood block culture	I
Production of enzymes	Wood chip culture	II
	Liquid culture	III, IV
	Liquid culture with solid substrates	III, IV
Determination of wood components:		
Lignin	Klason – and acid soluble lignin	I
Cellulose	Acid hydrolysis	I
Hemicellulose	Acid-methanolysis	I
Determination of enzyme activity:		
Laccase	Oxidation of 2,6-dimethoxyphenol (2,6-DMP) or syringaldazine	II, III, IV
MnP	Oxidation of Mn^{2+} to Mn^{3+}	II, III, IV
LiP	Oxidation of veratryl alcohol	II, III, IV
Determination of organic acids:		
Oxalate	High performance liquid chromatography (HPLC)	II
Purification of enzymes:		
Fractionation of enzymes	Fast protein liquid chromatography (FPLC)	II, IV
Characterization of enzymes:		
Isoelectric point (p*I*)	Isoelectric focusing (IEF)	II, III, IV
Activity staining of IEF-gels	Laccase (guaiacol)	II, IV
	MnP (phenol red with Mn^{2+} and H_2O_2)	II, III
	LiP (phenol red with H_2O_2, no Mn^{2+})	II, III
Molecular mass determination	Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)	II, III, IV
Determination of pH optima	Oxidation of substrates at various pH	II, III, IV
Thermotolerance of laccase	Oxidation of 2,6-DMP after thermal treatment of enzyme	IV
Kinetic studies of laccase	Oxidation of 2,6-DMP, syringaldazine, guaiacol or ABTS	IV
Characterization of *mnp* genes:		
Extraction of DNA	N-cetyl-N,N,N-trimethyl-ammonium bromide (CTAB) buffer -based extraction	III
Extraction of RNA	CTAB buffer –based extraction and LiCl precipitation	III
Molecular weight determination	Agarose gel electrophoresis	III
Synthesis of cDNA	RT-PCR by Smart cDNA synthesis kit	III
Cloning	TOPO T/A cloning kit	III
Sequencing	ABI Prism 310 DNA analyzer	III
Quantitation of transcript levels	Competitive RT-PCR	III

4 Results and discussion

4.1 SELECTION OF *PHYSISPORINUS RIVULOSUS* (I)

The objective of a two-stage screening of 300 fungal strains was to discover a thermotolerant fungus that degrades softwood lignin selectively and could be applied for the biopulping of Norway spruce (*P. abies*). The white-rot fungus *Physisporinus rivulosus* T241i was found and chosen for further characterization.

4.1.1 POLY R-478 DECOLORIZATION AND GROWTH RATE OF FUNGI

The fungal strains (n = 300) were first screened for their ability to decolorize the polymeric dye Poly R-478 and for their growth rate at different temperatures (Figure 6). Poly R-478 decolorization has been reported to correlate with fungal lignin-modifying enzyme production, especially that of MnP (de Jong et al. 1992, Rodríquez Couto et al. 2000, Moreira et al. 2001a, Robinson et al. 2001). Therefore, it has been widely used for searching of fungi for efficient lignin or organic pollutant degradation (Gold et al. 1988, Freitag and Morrell 1992, Field et al. 1992) and as new sources of lignin-modifying enzymes (Kiiskinen et al. 2004b).

Totally 86 strains showing high or moderate decolorizing ability were selected to the subsequent decay test. The ability of the chosen strains to decolorize Poly R-478 did not correlate with either lignin degradation or wood weight loss (Chapter 4.1.2). It has been reported that also the availability of nutrient nitrogen has an impact on the Poly R-478 decolorization (Leung and Pointing 2000). In this study, the concentration and quality of carbon source affected Poly R-478 decolorization by some fungi (Figure 6). Cultivation in solid-state cultivation on wood or on defined media can result in the production of different enzyme isoforms and activities (Datta et al. 1991) and it is well known that the chemical composition of the media affects the production of lignin-modifying enzymes. Therefore the choice of the media in screening is critical. For example addition of Mn^{2+} into the media could have increased MnP production and thus dye decolorization. However, the first screening was successful and resulted the selection of 86 strains for further characterization.

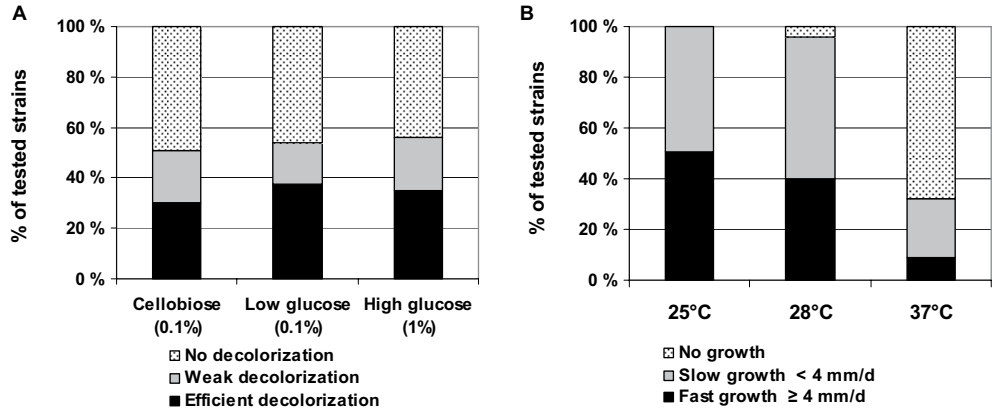

Figure 6. Results from the screening of 300 fungal strains in (A) Poly R -478 agar plates with different carbon sources and concentrations and (B) growth rate on malt agar plates at different temperatures.

4.1.2 DECAY TEST ON WOOD BLOCKS

Beneficial biomechanical pulping performance apparently correlates with selective removal of lignin (Ander and Eriksson 1977, Eriksson and Vallander 1982, Blanchette 1984, Scott et al. 2002). To assess the selectivity of lignin degradation the strains chosen in the first screening were grown on Norway spruce (*P. abies*) wood blocks for ten weeks. Weight loss and changes in chemical composition of the blocks caused by the fungi were determined. According to the results of the decay test, 15 of the novel strains showed selective lignin degradation ability (Table 6). Four of the selective strains were able to grow at 37ºC, whereas some, such as *Phellinus viticola* T255, had a relatively slow growth rate even at lower temperatures. Among the new selective fungi were also *P. rivulosus* T241i and two strains of *P. tremellosa*. All these three stains grew fast at the highest temperature tested. In earlier studies *P. tremellosa* has been found to be a selective white-rot fungus (Blanchette et al. 1988) that causes energy savings in biopulping (Akhtar et al. 1998a) and its lignin-modifying enzymes have been studied in detail (Vares et al. 1994, Robinson et al. 2001). Because there were no published results on lignin degradation or biopulping by *P. rivulosus*, and it showed selective degradation of lignin together with the ability to grow at 37ºC, it was chosen for further characterization.

Table 6. Wood weight loss, and relative loss of wood components after 10 weeks growth on Norway spruce (*Picea abies*) wood, and growth rate at 37°C of the new selective white-rot fungi.

Species and strain*	Selectivity[1]	Loss %					Growth mm/d
		Lignin	Glc[2]	Xyl[3]	Man[3]	Weight	at 37°C
C. subvermispora CZ-3	1.2	44	36	33	34	23	0.5
Climacocystis borealis T261i	2.9	11	3.9	14	11	8	-
Coltrichia perennis T272	1.4	16	12	20	20	11	-
Fibricium rude PO140i	2.6	9.1	3.5	18	15	7	-
Inocutis rheades T158	1.1	20	18	20	10	13	1
Onnia leporina T282	2.0	13	6.5	8.8	12	7	-
Phanerochaete velutina T244i	1.1	8.9	8.0	23	29	13	-
Phellinus viticola T24	1.1	28	27	26	26	13	-
Phellinus viticola T255	3.3	29	8.8	32	23	14	-
Phlebia centrifuga PO148i	1.3	25	20	24	22	19	-
Phlebia tremellosa PO171i	1.3	25	20	30	24	21	9
Phlebia tremellosa T186i	1.3	23	18	7.7	12	16	11
***Physisporinus rivulosus* T241i**	**2.0**	**39**	**20**	**18**	**20**	**19**	**8**
Resinicium furfuraceum PO175i	1.4	17	12	20	15	11	-
Tubulicrinis accedens T226i	1.3	33	25	3.9	5.4	11	-

* Letter "T" in the strain number indicates that the strain was isolated by the author. Letter "i" indicates that the strain was isolated from basidiospores, which were let to form dikaryotic hypha after germination

[1] The calculated ratio of lignin to cellulose loss

[2]Loss % of cellulose derived glucose (glc) determined by acid hydrolysis of wood

[3]Loss % of hemicellulose derived sugars, from xylan (xyl) or mannan (man), determined by acid methanolysis of wood

In this study the long incubation time (ten weeks) was chosen because the changes in wood chemical composition are difficult to detect in the early stages of decay, especially if selective lignin degradation is not uniformly distributed (Blanchette et al. 1985). By this approach it was possible to find fungi, which maintain the selectivity of lignin degradation even in long-term incubation. It has been shown that the selectivity of lignin degradation is temporally regulated and that a fungus can be selective in the beginning of the cultivation and become non-selective with time, as has been observed in *Ganoderma australe* (Ferraz et al. 2000) and *Ganoderma colossum* (Adasgaveg et al. 1995). Fourier transform near infrared spectroscopy (FTIR) has been found to be a powerful tool to analyze changes in the lignin content of wood during early stages of the white-rot decay. Using this method a 3% decrease in lignin content can be detected already after five days of incubation. It was also observed that some fungi, which have been found to be relatively non-selective in long decay tests, were selective in shorter incubations (Fackler et al. 2006). However, several white-rot fungi applicable for biopulping have been found with long decay tests. It has been observed in decay tests with *P. rivulosus*, *C. subvermispora* (Hatakka et al. 2003b) and *G. colossum* (Adasgavek et al. 1995) that selectivity of lignin degradation also depends on incubation temperature. All these three fungi caused most selective lignin degradation at the temperature close to the optimal growth temperature of the fungus, supporting the importance of temperature control of wood chip pile during biopulping. In addition, selecting the correct incubation time and temperature in a screening are of utmost importance and should correlate the conditions in the application.

4.2 CHARACTERISTICS OF GROWTH AND WOOD DECAY OF *PHYSISPORINUS RIVULOSUS* (I)

Tolerance of the fungus to high temperatures is of importance in biopulping, as the temperature in wood chip pile may rise as a result of the fungal metabolism (Akhtar et al. 1998b). The growth rate of *P. rivulosus* T241i and *C. subvermispora* was determined at different temperatures and it was observed that *P. rivulosus* grows in a wider temperature range (from 15 to 40°C) than *C. subvermispora* (from 15 to 37°C) (Publication I) supporting earlier results of Nakasone (1981). In addition *P. rivulosus* forms less aerial hyphae than *C. subvermispora*, which may make the aeration of wood chip pile easier. Thus, *P. rivulosus* shares the beneficial properties with *P. subserialis* (Akthar et al. 1998a), and caused an equal refining energy reduction with *C. subvermispora* after pretreatment of Norway spruce (Hatakka et al. 2003b).

In laboratory experiments *P. rivulosus* T241i caused decay of various hardwood and softwood species: Norway spruce (*P. abies*, Publication I), Scots pine (*P. sylvestris*), aspen (*Populus tremula*), silver birch (*Betula pendula*) and eucalyptus (*Eucalyptus dunnii* and *E. grandis*) (Hatakka et al. 2003b). Phloroglucinol staining revealed that on Norway spruce wood the lignin loss by both *P. rivulosus* (Figure 7), and *C. subvermispora* (Figure 2 in publication II) was most efficient in the earlywood area. The preference of earlywood as substrate by *P. rivulosus* has been confirmed by confocal laser microscopy, which showed that most of the fungal hyphae are localized in earlywood tracheids (Maijala et al. 2002, Figure 3, page 12). *P. rivulosus* T241i and three other *P. rivulosus* strains have been shown to colonize also ray parenchyma cells and resin ducts (Maijala et al. 2002). It has been observed earlier that white-rot fungi are able to degrade extractives (Dorado et al. 2000, van Beek et al. 2007). In addition to wood polymers *P. rivulosus* degraded extractives from both Norway spruce (32 % loss in one week) and Scots pine (*P. sylvestris*, 27% loss in one week) heartwood (Hatakka et al. 2003b). Action of enzymes like lipases and steryl esterases that could be responsible for the degradation of extractives by white-rot fungi remain to be studied in more detail.

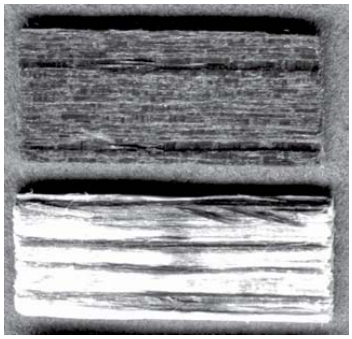

Figure 7. *Physisporinus rivulosus* grows and causes delignification preferentially in spruce earlywood. Lignin visualized by phloroglucinol staining, scale bar 5 mm.
above: control,
below: wood block after ten week incubation with *P. rivulosus*

4.3 LIGNIN-DEGRADING MACHINERY OF *PHYSISPORINUS RIVULOSUS* (II, III, IV)

Production of lignin-modifying enzymes by *P. rivulosus* T241i was studied in wood chip cultures (Publication II), in liquid cultures (Publication III), and in liquid cultures supplemented with solid lignocellulose substrates (Publication III and IV). The lignin-degrading machinery of *P. rivulosus* consisted of MnP and laccase, while LiP could not be detected. The objective was to find out which factors affect the production of lignin-modifying enzymes on wood chips. This was addressed by comparing isoenzyme profiles produced by the fungus during the cultivation on wood chips and in liquid cultures. Manganese and veratryl alcohol stimulated the production of MnP isoforms, whereas it remained unclear which individual factors trigger the laccase production in the presence of lignocellulose. The production of lignin-modifying enzymes in defined media by *P. rivulosus* was differentially regulated, which secures the lignin degradation by the fungus under changing environmental conditions during wood decay. Production of lignin-modifying enzymes as a combination of MnP and laccase is widely spread among white-rot fungi and has been supposed to be typical especially to the selective white-rot fungi (Hatakka 1994).

4.3.1 PRODUCTION AND EXPRESSION OF LIGNIN-DEGRADING PEROXIDASES ON WOOD CHIPS AND IN DEFINED MEDIA

MnP was the main ligninolytic enzyme activity detected in the extracts of *P. rivulosus* T241i wood chip cultures (Figure 1 in Publication II). MnP that has been found from almost every lignin-degrading fungus studied is suggested to be have a key role in the lignin degradation caused by white-rot and litter decaying fungi (Hatakka 2001, Hofrichter 2002). The selective white-rot fungi readily produce MnP on lignocellulose substrates (Hatakka et al. 2003a). Likewise, *C. subvermispora* produces MnP as its main lignin-modifying enzyme when it grows on *P. taeda* and *E. grandis* wood chips (Ferraz et al. 2003, Aguiar et al. 2006, Vicentim and Ferraz 2007).

In some white-rot fungi lignin degradation occurs as a part of secondary metabolism and the production of lignin-modifying enzymes is triggered by the depletion of nutrients (Kirk and Farrell 1987), whereas in some white-rot fungi the secretion depends on the presence of certain type of nutrients (Kaal et al. 1993). In *P. rivulosus* T241i the production of MnP was slightly increased by the presence of sufficient nutrient nitrogen (Figure 8), whereas the presence of excess glucose inhibited the MnP production. In *C. subvermispora* the production of MnP has been shown to be high in both nitrogen and carbon sufficient conditions (Rüttimann-Johnson et al. 1993). Accordingly, the supplementation of wood chips with nitrogen rich corn steep liquor increased the production of MnP in relation to fungal growth (Vicentim and Ferraz 2007) and enhanced the biopulping process by *C. subvermispora* (Akhtar et al. 1998a).

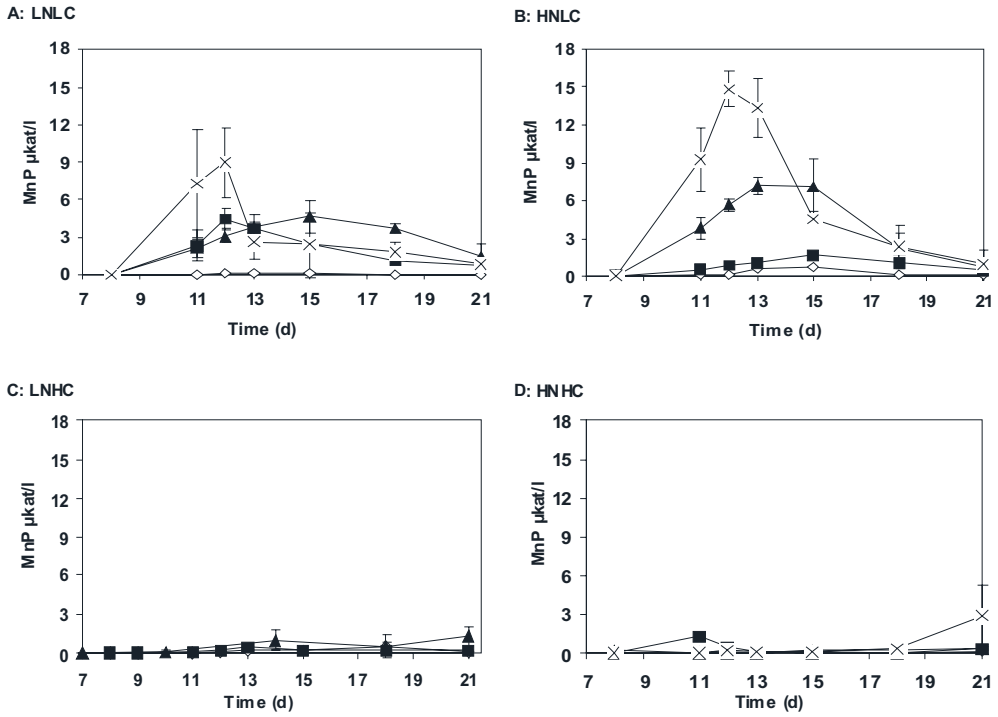

Figure 8. Extracellular MnP activity produced by *Physisporinus rivulosus* T241i under nutrient limited or sufficient culture conditions with various supplements. ◇ No supplements, ■ Mn^{2+}, ▲ veratryl alcohol, ✕ Mn^{2+} and veratryl alcohol. (A) low nitrogen low carbon, (B) high nitrogen, low carbon, (C) low nitrogen, high carbon and (D) high nitrogen, high carbon. LN: low nitrogen (2.0 mM N), HN: high nitrogen (20 mM N), LC: low carbon (0.1% w/v glucose), HC: high carbon (0.5% w/v glucose).

Enhanced MnP production in the presence of Mn^{2+} has been observed in a number of white-rot fungi (Brown et al. 1993, Cohen et al. 2001, Moilanen et al. 1996). In these experiments the transcription (Figure 6 in Publication III) and production (Figure 8) of MnP by *P. rivulosus* T241i in liquid cultures was induced by the addition of Mn^{2+} and/or veratryl alcohol. Low concentrations of Mn^{2+} (12-24 μM) induced production of MnP. At high concentration (120-240 μM) the observed MnP activity was lower and appeared later (Figure 9), moreover the pellet morphology and size were affected suggesting that high Mn^{2+} concentration can be toxic to the fungus. The inhibition of fungal growth by excess Mn^{2+} has been observed on wood chip cultivation of *C. subvermispora* (Vicentim and Ferraz 2007). In addition 40-160 μM concentration of Mn^{2+} has been shown to increase and 320 μM concentration to decrease the MnP production by *C. subvermispora* (Manubens et al. 2003). Manganese has an effect also on lignin degradation by *C. subvermispora* and too high manganese concentration has been observed to inhibit the lignin degradation rate although it did not inhibit fungal growth (Maijala 1996).

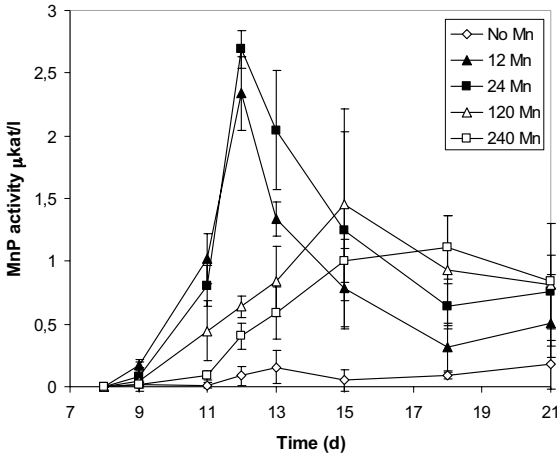

Figure 9. Effect of different concentrations (0-240 µM) of Mn^{2+} on extracellular MnP production by *Physisporinus rivulosus* T241i under nutrient limited culture conditions. Low nitrogen (2.0 mM N), low carbon (0.1% w/v glucose).

Interestingly, addition of veratryl alcohol increased the production of MnP by *P. rivulosus* T241i as much as or even more than the addition of Mn^{2+} (Publication III) The effect of veratryl alcohol on enzyme production has been studied for numerous white-rot fungi and it has seldom had any effect on MnP production, only in few reports a slight increase in MnP production has been observed (Scheel et al. 2000). In *C. subvermispora* aromatic compounds such as veratric acid and syringic acid induce MnP production (Sethuranaman et al. 1998a) and *Cs-mnp1* gene transcription (Manubens et al. 2003).

In wood chip cultures of *P. rivulosus* T241i a MnP isoform with p*I* 3.9 and a predominant form with p*I* 3.6 – 3.7 were observed (Figure 10, lane 5). A similar pattern, with the exception that the more neutral form had a higher p*I* (4.3), was observed in liquid cultures supplemented with either simultaneous addition of veratryl alcohol and Mn^{2+} or with sawdust as a carbon source (Figure 10, lanes 1 and 4). Interestingly, with Mn^{2+} or veratryl alcohol alone, only high or low p*I* isoforms, respectively, were produced by the fungus (Figure 10, lanes 2 and 3). Two new mnp encoding genes named *mnp*A and *mnp*B were cloned and sequenced from *P. rivulosus.* Differential regulation of MnP isoforms was observed also on transcriptional level (Figure 6 in Publication III). The transcription of *mnp*A was induced by addition of veratryl alcohol and sawdust but not by addition of Mn^{2+}, whereas the transcription of *mnp*B was induced by either veratryl alcohol or Mn^{2+} and only slightly by sawdust (Figure 6 in Publication III). Thus, Mn^{2+} and also aromatic compounds such as veratryl alcohol seem to have a role in regulation of MnP production in *P. rivulosus* T241i during growth on wood. In nature *P. rivulosus* is often found on burned wood, which contains a variety of monomeric aromatic compounds derived from lignin during pyrolysis (Alén et al. 1996). Slight induction of MnP production by veratryl alcohol has been observed only for few

fungi (Scheel et al. 2000), although the effect of veratryl alcohol has been widely studied to induce LiP production in white-rot fungi. Thus it seems that the regulatory system of *P. rivulosus* MnP has adapted to the conditions of its natural habitat and differs from that of most white-rot fungi.

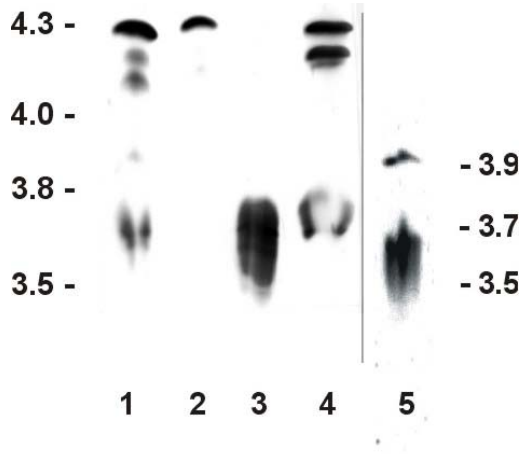

Figure 10. MnP isoforms produced by *P. rivulosus* T241i under different culture conditions characterized by analytical IEF stained by activity based staining. Lanes 1a-4 Low nitrogen – low carbon medium supplemented with: lane 1, Mn^{2+} and veratryl alcohol; lane 2, Mn^{2+}; lane 3, veratryl alcohol; lane 4, sawdust. Lane 5, extract from wood chip culture.

Veratryl alcohol has earlier been reported to induce LiP production in several fungi (Mester et al. 1995, Kirk et al. 1986, Kantelinen et al. 1989). No LiP activity could be found with either IEF analysis or enzyme activity assay in the cultures of *P. rivulosus* T241i, although in defined media the added veratryl alcohol was partly oxidized to veratraldehyde (Figure 7 in Publication III). In addition to oxidation by LiP veratryl alcohol oxidation to veratraldehyde could occur extracellularly by aryl alcohol oxidase (Ferreira et al. 2005), by MnP and lipid –mediated peroxidation (Kapich et al. 1999), by laccase together with a mediator (Arias et al. 2003) or by fungal intracellular metabolism. In general, measurement of enzyme activities from extracts derived from solid-state fermentation is challenging and detecting LiP activity is especially difficult (Vares et al. 1995, Datta et al. 1991). The IEF analysis and subsequent activity staining of the concentrated and dialyzed extracts should reveal LiP activity in the extracts. Thus, our results indicate that *P. rivulosus* does not possess LiP.

4.3.2 PRODUCTION OF LACCASE ON WOOD CHIPS AND IN DEFINED MEDIA

Production of laccase by *P. rivulosus* T241i was enhanced in liquid cultures by the presence of sawdust and/or charred wood, the components of natural habitat of the fungus. Rather low laccase activities could be detected in defined media with glucose as carbon source unless peptone was used as nitrogen source. According to the IEF analysis with activity staining *P. rivulosus* produced laccase in wood chip cultures mainly during the first week of the cultivation. The activity of laccase was barely detectable in the extracts derived from wood chip cultures of *P. rivulosus* (Publication II). However, at that time the characteristic pH optimum of the enzyme was not known and laccase activity was measured with syringaldazine at pH 5. Later (Publications II, IV), the pH optimum of laccase was determined and found to be more acidic, which revealed that the laccase activity in wood extracts had been underestimated. Likewise only a trace of laccase activity could be detected in the extracts of *C. subvermispora* on *P. taeda* and *Pinus radiata* wood chip cultures (Lobos et al. 1994, de Souza-Cruz et al. 2004, Aguiar et al. 2006), whereas on *E. grandis* wood chips a low laccase activity could be detected (Ferraz et al. 2003, Vicentim and Ferraz 2007). Like *P. rivulosus*, also *C. subvermispora* produces laccase mainly in the early phase of growth on wood (Lobos et al. 1994, Aguiar et al. 2006). Thus, laccase may have an important role in initiating lignin degradation or in colonization of wood by the fungus although the detected activities have been low. Laccase has also been suggested to have a role in detoxifying phenolic compounds formed during fungal decay (Thurston 1994, Baldrian 2006).

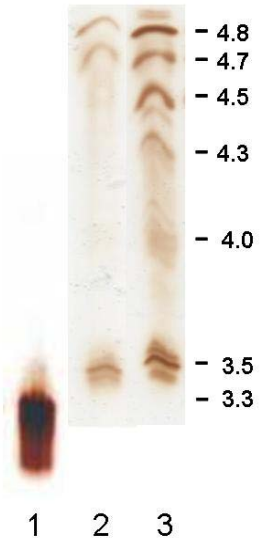

Figure 11. Activity stained isoelectric focusing gel of laccases produced by *Physisporinus rivulosus* under different cultural conditions, lane 1: extract from wood culture, lane 2: low nitrogen (2.0 mM) liquid culture with sawdust, lane 3: peptone liquid culture with sawdust

According to the immunoblot and IEF analyses, the laccase isoforms of *P. rivulosus* T241i were of similar p*I* value and size throughout the cultivation on wood. The four laccase isoforms, which were observed on wood chip cultures, had isoelectric points between 3.1 and 3.3 (Figure 11). In nutrient deficient culture conditions a less acidic laccase isoform, with p*I* 4.8, together with multiple laccase isoforms with p*I* values around 3.5 were detected. When peptone was used as nitrogen source more laccase isoforms could be detected in the culture media (Figure 11). Interestingly, the IEF profile of *P. rivulosus* laccases observed on nutrient deficient culture conditions strikingly resembles that of *C. subvermispora* (Fukushima and Kirk 1995).

Aromatic compounds, such as veratryl alcohol (Kantelinen et al. 1989, D´Souza et al. 1999) and Cu^{2+} (Collins and Dobson 1997, Michniewicz et al. 2006) are known inducers of laccase production. Lignocellulose substrates have been reported to enhance production of lignin-modifying enzymes in several white-rot fungi (D´Souza et al. 1999, Giardina et al. 2000, Lankinen et al. 2005). Laccase production by *P. rivulosus* T241i is clearly

stimulated by lignocellulose substrates, although it is not yet known which factors in lignocellulose cause the stimulation. In *C. subvermispora* laccase production has been observed to be enhanced by lignocellulose substrate (wheat bran), whereas both Cu^{2+} and 2,5 –xylidine had only minor effects on laccase production (Fukushima and Kirk 1995). In addition to stimulation by certain components, the effect might be due to the mechanical support that lignocellulose substrate offers to fungal hyphae during growth in submerged cultures. The immobilization of white-rot fungi on various materials such as polyurethane foam (Šušla et al. 2007), polypropylene carrier (Kantelinen et al. 1989) and stainless steel sponge (Rodríquez Couto et al. 2004) has been observed to enhance production of lignin-modifying enzymes by white-rot fungi. However, on *D. squalens* the effect of sawdust was considerably higher than that of polyurethane foam carrier material (Šušla et al. 2007).

4.3.3 SECRETION OF ORGANIC ACIDS ON WOOD CHIPS

In addition to lignin-modifying enzymes *P. rivulosus* T241i secreted extracellular oxalic acid during growth on spruce wood chips (Figure 1 in Publication II), which decreased wood pH from pH 5 to 4. Secretion of organic acids and a concomitant decrease in pH has been observed in lignocellulose cultures of several white-rot fungi including *C. subvermispora* (Galkin et al. 1998, Hofrichter et al. 1999a, Mäkelä et al. 2002, Aguiar et al. 2006, Elissetche et al. 2007). On wood chip cultures, MnP seems to be an important factor in the lignin-degrading machinery of *P. rivulosus* T241i and oxalate can act as a chelator for the MnP-generated Mn^{3+}-cations. In addition, secretion of oxalate lowers the pH of the wood chips closer to the pH optima of the lignin-modifying enzymes of *P. rivulosus*.

Secretion of oxalate has an impact also on the applicability of the fungus in biopulping. Acidic pH of wood chips from *P. rivulosus* cultures increased alkali consumption in laboratory scale Kraft cooking experiments (Hatakka et al. 2004), although positive effects on Kraft pulping have been obtained by pretreatment of wood chips with *C. subvermispora* (Bajpai et al. 2003). On the contrary, oxalate treatment of wood chips markedly reduces the energy consumption in mechanical pulping and improves the pulp physical properties (Akhtar et al. 2004). It has been suggested that the biopulping effect caused by white-rot fungi may be due to the incorporation of secreted oxalate into wood polysaccharides as oxalic acid esters (Hunt et al. 2004). Thus, it seems that due to the acidification of wood chips the pretreatment of wood chips with *P. rivulosus* is more advantageous prior to mechanical pulping than chemical pulping.

4.3.4 CHARACTERISTICS OF *P. RIVULOSUS* LACCASES

Laccases of *P. rivulosus* T241i were purified from wood chip cultivations and from peptone media containing sawdust and crushed charcoal. The purified laccases were characterized in terms of p*I*, molecular weight and pH optimum. In addition, N-terminal sequences and kinetic parameters on various substrates were determined. The molecular

characteristics of *P. rivulosus* laccases; i.e. the acidic p*I* (between 3.1 and 4.8) and molecular weight of 65-68 kDa (Table 7), correspond to those observed for laccases isolated from other lignin-degrading fungi (Baldrian 2006).

The two laccases purified from liquid cultures supplemented with sawdust and charcoal wood were named according to their p*I* as Lac-3.5 and Lac-4.8. The substrate specificity of the laccases was similar with each other (Table 7) and typical for fungal laccases (Baldrian 2006). The highest enzyme affinities and high turnover for the oxidation reaction were obtained with ABTS and syringaldazine. Also 2,6-DMP was observed to be a suitable substrate for both isoforms. On the other hand, the K_m values for guaiacol were high and the turnover number for guaiacol was rather low, indicating that the affinity of guaiacol to the enzyme is rather low and that its oxidation is slower than that of other tested substrates.

The pH optima of *P. rivulosus* T241i laccases for the oxidation of a variety of substrates including syringaldazine were low (Figure 4 in Publication IV). For many fungal laccases, the pH optimum for oxidation of ABTS is below 3 and shows a monotonic profile whereas a bell-shaped profile with a higher pH optimum (pH 5-6) is observed for the oxidization of syringaldazine (Baldrian 2006, Xu 1997). This can be explained by the inhibition of the internal electron transfer in laccase by OH^- , which leads to higher activity at low pH. However, the oxidation of syringaldazine depends on pH and thus a bell-shaped pH optimum curve is observed (Xu 1997). Low pH optimum for the oxidation of ABTS and guaiacol has been observed also for *C. subvermispora* L1, whereas the pH optimum for guaiacol oxidation by L2 is higher (Fukushima and Kirk 1995).

Both *P. rivulosus* laccases had a moderate thermal stability, while Lac-3.5 had a higher thermal stability than Lac-4.8 (Table 7). The isoform Lac-4.8 showed thermal activation at 50°C whereas Lac-3.5 was not activated at any of the tested temperatures. This suggests that the conformation of Lac-4.8 is more flexible than that of Lac-3.5 and allows a conformational change, which leads to enzyme activation at 50°C, and to total inactivation of the protein at 70°C. Thermal activation of laccase has earlier been described from Basidiomycete PM1 that was isolated from paper mill wastewater (Coll et al. 1993). The laccases of thermophilic ascomycetes *Myceliophthora thermophila* and *Scytalicium thermophilum* exhibit thermal stability and thermal activation especially in high pH (Xu et al. 1996). The thermal stability of enzymes is influenced by the presence of proline, hydrophobic or charged residues, and the number of disulphide bonds which increase enzyme rigidity and restrict conformational changes during substrate binding (Xu et al. 1996, Fields 2001, Somero 2004, Enguita et al. 2003).

N-terminal sequences of four *P. rivulosus* laccases with p*I* 3.1-3.4 purified from wood chip cultures were determined. These laccases probably correspond to Lac-3.5 from peptone media. The N-terminal sequences are typical for fungal laccases and differ only at three of the 20 first amino acids when compared to L1 from *C. subvermispora* CZ-3 (Fukushima and Kirk 1995). The first 15 amino acids in the N-terminal sequences obtained of four laccase isoforms purified from wood chip cultures were identical (Table 3 in Publication II). The observed differences in isoelectric point may result from differential post-translational modifications of the enzyme, namely glycosylation or phosphorylation (Kuan and Tien 1989). The multiple acidic laccases of *C. subvermispora* have been suggested to be encoded by two alleles, *lcs*-1A and *lcs*-1B. The *Aspergillus nidulans* transformant produces *lcs-1A* as multiple isoforms resembling the IEF profile of *C. subvermispora*, which further supports that these isoforms

are encoded by single gene (Larrondo et al. 2003). In addition, the analysis of the crystal structure of the acidic laccase isoform of *T. versicolor* indicated that the five isoforms differ from each other by the glycosylation (Bertrand et al. 2002). Thus, it seems likely that a single gene may encode the four acidic *P. rivulosus* laccase isoforms observed in wood chip cultures and in peptone charcoal cultivation. The less acidic laccase isoform, Lac-4.8, is probably encoded by another laccase gene.

Table 7. Summary on the properties of *P. rivulosus* laccases

Property		Lac-3.5	Lac-4.8
p*I*		3.5	4.8
mw (kDa)		67	65
Km (μM)	2,6-DMP	88	84
	ABTS	11	17
	Guaiacol	1095	1406
	Syringaldazine	27	13
T½ (min at 70˚C)		60	30

4.3.5 CHARACTERISTICS AND MOLECULAR BIOLOGY OF *P. RIVULOSUS* MANGANESE PEROXIDASE (MnP)

MnP is produced by *P. rivulosus* T241i as multiple isoforms, which is typical for white-rot fungi. The observed isoelectric points were acidic (between 3.4 and 4.3, Publications II and III) and the molecular weight between 47 and 52 kDa (Publication II), both of which are typical for MnPs produced by a wood inhabiting white-rot fungus (Hofrichter 2002). Altogether four different N-terminal amino acid sequences of MnPs were obtained from *P. rivulosus*, two of them from MnPs isolated from wood chips (Publication II) and two putative amino acid sequences from cDNA (Publication III). According to these data, it can be suggested that *P. rivulosus* has at least four different MnPs, which are encoded by separate genes. The four sequences shared similarities with those reported from other white-rot fungi (Table 8). The N-terminal sequences, MnP N1 and MnP N2, from *P. rivulosus* differ only slightly from the inferred MnP amino acid sequence of the selective white-rot fungus IZU 154 (Matsubara et al. 1996). In addition, the deduced amino acid sequence of *mnpA* from *P. rivulosus* shows similarity to IZU 154 *mnp*. The N-terminal sequences obtained from purified MnPs resemble more the inferred amino acid sequence of *P. rivulosus mnpA* than *mnpB*.

Table 8. Comparison of N-terminal amino acid sequences of manganese peroxidases of *Physisporinus rivulosus* T241i and other selective white-rot fungi.

Enzyme	Source	N-terminal amino acid sequence	Reference
P. rivulosus MnP N1	Protein	AVCSDGTRVS-NSA-C	Publ. II
P. rivulosus MnP N2	Protein	ATCSDGTRVS-NSA-C	Publ. II
*P. rivulosus mnp*A	cDNA	AVCPDGTRVN-NAV-C	Publ. III
*P. rivulosus mnp*B	cDNA	VTCPDGVNTATNAA-C	Publ. III
IZU154 *mnp*1	cDNA	AVCPDGTRVS-NSA-C	Matsubara et al. 1996
*C. subvermispora mnp*1	gDNA	VTCSDGTVVP-DSM-C	Lobos et al. 1998
*C. subvermispora mnp*2	gDNA	TICPDGTRVS-NHV-C	Tello et al. 2000
*C. subvermispora mnp*3	gDNA	VTCSDGTAVP-DAM-C	Tello et al. 2000
C. subvermispora MnP4	Protein	AIPPDGTRVS-NHVDX	Lobos et al. 1994
C. subvermispora MnP5-7	Protein	VTXSDGTAVP-DAM-X	Lobos et al. 1994

Two MnP-encoding genes of *P. rivulosus*, *mnp*A and *mnp*B, were cloned, sequenced and characterized. Interestingly, gene *mnpA* could be classified to the MnP group I, whereas the gene *mnp*B shared characteristics with the MnP group II. A similar divergence in *mnp* genes within the same fungus has been observed in the efficient lignin degrader *P. radiata* (Figure 5 page 20, Hildén et al. 2005). In both fungi, the two sequenced *mnp* genes are divergent in the primary structure, intron-exon splicing sites and gene length. The N-terminal sequences deduced from genomic DNA sequence and from isolated MnPs of *C. subvermispora* shows also variation (Table 8). However, the *C. subvermispora mnp* genes *Cs-mnp1-3* have only minor differences in the sequence and intron-exon splicing (Lobos et al. 1994, Lobos et al. 1998, Tello et al. 2000). The presence of lignin-degrading peroxidases as multiple genes has been observed from several fungi including *T. versicolor* (Jönsson et al. 1994, Johansson and Nyman 1996), *P. ostreatus* (Cohen et al. 2001), and *P. chrysosporium* (Kersten and Cullen 2007). In all these fungi, however, genes encoding for different isoforms with the same functionality are more closely related with each other and belong to one phylogenetic group. So far *mnp* –genes belonging to two different gene groups have been found in only three fungi: *P. rivulosus* T241i, *P. radiata* (Hildén et al. 2005) and *Phlebia* sp. (Kamei et al. 2007), however it is likely that similar divergence will be found in other white-rot fungi as well.

5 Summary and conclusions

In this thesis the lignin-modifying enzymes of a new interesting and biotechnologically promising polypore, *Physisporinus rivulosus* T241i, were characterized (results summarized in Figure 12). The main conclusions answering the specific aims of the study were:

1. A new promising fungus, *P. rivulosus* T241i, was found in an extensive screening of white-rot fungi. *P. rivulosus* degraded softwood lignin selectively and grew in a wide temperature range, suggesting that it could be applicable in biopulping.

2. During the growth on wood under biopulping conditions, *P. rivulosus* produced MnP and laccase as multiple acidic isoforms. Oxalate secreted by the fungus acidified the wood chips and could serve as an organic chelator for the manganese oxidized by MnP. No LiP production was detected. These results suggest that MnP, laccase and oxalate have a role in the lignin degradation by this fungus.

3. The biochemical characteristics of *P. rivulosus* MnP and laccase are typical for white-rot fungi and closely resemble those of selective white-rot fungi *C. subvermispora* and IZU 154. The laccases of *P. rivulosus* have acidic pH optima and moderate thermal stability.

4. Production of lignin-modifying enzymes is differentially regulated in *P. rivulosus*. MnP transcription and production is increased by addition of Mn and/or veratryl alcohol under carbon limited conditions. Components of the natural growth environment of *P. rivulosus* increased the production of laccase and MnP. Differential regulation of enzyme production enables efficient lignin degradation by *P. rivulosus* under changing environmental conditions.

5. The production of lignin-modifying enzymes by *P. rivulosus* is efficient in nitrogen sufficient culture conditions. Laccase production by *P. rivulosus* was enhanced by the presence of solid lignocellulose substrates, charcoal and sawdust with peptone as nitrogen source.

6. Two MnP encoding genes were cloned and sequenced from *P. rivulosus*. The two genes are differentially regulated at transcriptional level and belong to different gene groups. This phenomenon has earlier been observed in *P. radiata*, which suggests that divergent MnP genes might be found from other white-rot fungi as well.

The regulation of the production of lignin-modifying enzymes of *P. rivulosus* showed some unique features and also similarities to other white-rot fungi. A concise study on the polysaccharide hydrolyzing enzymes of *P. rivulosus* would be needed to conclude if the selectivity of this fungus is explained by the ineffectiveness of polysaccharide degradation or the effectiveness of lignin degradation. Based on the results obtained in this study a schematic model on the lignocellulose degradation by *P. rivulosus* is presented (Figure 12).

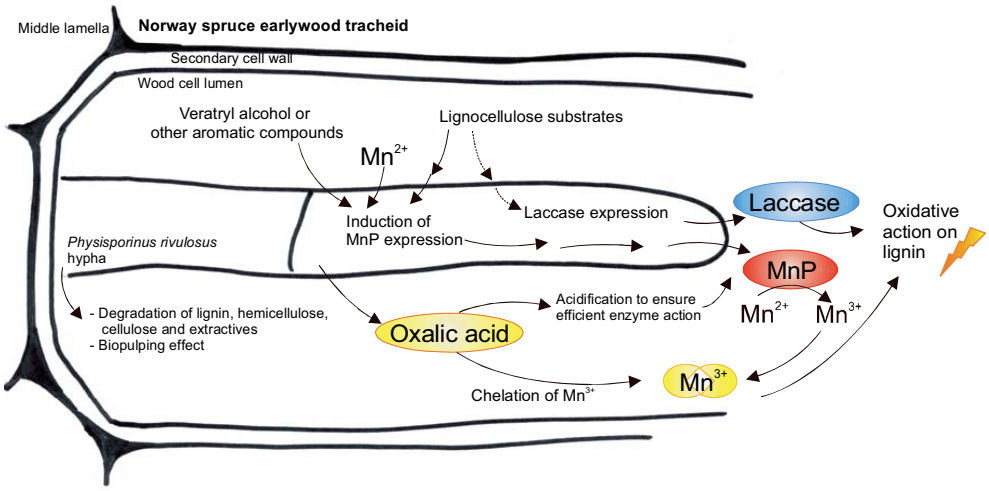

Figure 12. A schematic model of *Physisporinus rivulosus* T241i lignin-modifying enzymes and their regulation and action on wood based on the results in publications I, II, III, IV, Maijala et al. 2002, Hatakka et al. 2003b.

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7 References

Abbas, A., Koc, H., Liu, F., Tien, M. (2005) Fungal degradation of wood: initial proteomic analysis of extracellular proteins of *Phanerochaete chrysosporium* grown on oak substrate. Curr. Genet. 47:49-56.

Abraham, L., Hoffman, B., Gao, Y., Breuil, C. (1998) Action of *Ophiostoma piceae* proteinase and lipase on wood nutrients. Can. J. Microbiol. 44:698-701.

Adasgavek, J.E., Gilbertson, R.L., Dunlap, M.R. (1995) Effects of incubation time and temperature on *in vitro* selective delignification of Silver leaf oak by *Ganoderma colossum*. Appl. Environ. Microbiol. 61:138-144.

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

Aguiar, A., de Souza-Cruz, P.B., 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.

Akhtar, M., Blanchette, R.A., Myers, G., Kirk, T.K. (1998a) An overview of biomechanical pulping research, In: Young RA, Akhtar M, editors. Environmentally Friendly Technologies for Pulp and Paper Industries. John Wiley & Sons, Inc., New York, pp. 309-340.

Akhtar, M., Lentz, M.J., Lightfoot, E.N.Jr., Swaney, R.E., Scott, G.M., Kirk, T.K., Horn, E.G. (1998b) Method and apparatus for commercial scale biopulping, WO9842914A1.

Akhtar, M., Scott, G.M., Swaney, R.E., Shipley, D.F. (2000) Biomechanical pulping: a mill-scale evaluation. Resour. Conserv. Recycl. 28:241-252.

Akhtar, M., Swaney, R.E., Horn, E., Klungness, J., Sabourin, M. (2004) Oxalic acid pretreatment for mechanical pulping greatly improves paper strength and saves energy. In: Wolfaardt, F., Kock, M., editors. 9th International conference in biotechnology in the pulp and paper industry, 10.-14.10. 2004, Durban, South Africa, Book of abstracts, p. 169.

Alén, R., Kuoppala, E., Oesch, P. (1996) Formation of the main degradation compound groups from wood and its components during pyrolysis. J. Anal. Appl. Pyrolysis 36:137–148.

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

Anagnost, S.E. (1998) Light microscopy diagnosis of wood decay. IAWA J. 19:141-167.

Ander, P. (1994) The cellobiose-oxidizing enzymes CBQ and CbO as related to lignin and cellulose degradation – a review. FEMS Microbiol. Lett. 13:297-312.

Ander, P., Eriksson, K.-E. (1977) Selective degradation of wood components by white rot fungi. Physiol. Plant 41:239-248.

Arias, M.E., Arenas, M., Rodriquez, J., Soliveri, J., Ball, A.S., Hernandez, M. (2003) Kraft pulp bleaching and mediated oxidation of nonphenolic substrate by laccase from *Streptomyces cyaneus* CECT 3335. Appl. Environ. Microbiol. 69:1953-1958.

Bajpai, P., Bajpai, P.K., Akhtar, M. (2003) Process for producing pulp from *Eucalyptus* chips, US patent 6613192.

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

Baldrian, P., Gabriel, J. (2003) Lignocellulose degradation by *Pleurotus ostreatus* in the presence of cadmium. FEMS Microbiol. Lett. 220:235-240

Behrendt, C.J., Blanchette, R.A. (1997) Biological processing of pine logs for pulp and paper production with *Phlebiopsis gigantea*. Appl. Environ. Microbiol. 63:1995-2000.

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.

Berka, R.M., Schneider, P., Golightly, E.J., Brown, S.H., Madden, M., Brown, K.M., Halkier, T., Mondorf, K., Xu, F. (1997) Characterization of the gene encoding an extracellular laccase of *Myceliophthora thermophila* and analysis of the recombinant enzyme expressed in *Aspergillus oryzae*. Appl. Environ. Microbiol. 63:3151-3157.

Bermek, H., Li, K., Eriksson, K.-E. L. (2002) Studies on mediators on manganese peroxidase for bleaching of wood pulps. Biores. Technol. 85:249-252.

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

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

Blanchette, R.A. (1991) Delignification by wood-decay fungi. Annu. Rev. Phytopathol. 29:381-398.

Blanchette, R.A. (1995) Degradation of the lignocellulose complex in wood. Can. J. Bot. 73:S999-S1010.

Blanchette, R.A., Burnes, T.A., Leatham, G.F., Effland, M.J. (1988) Selection of white-rot fungi for biopulping. Biomass 15:93-101.

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

Blanchette, R.A., Otjen, L., Effland, M.J., Eslyn, W.E. (1985) Changes in structural and chemical components of wood delignified by fungi. Wood Sci. Technol. 19:35-46.

Boudet, **A.M., Kajita, S., Grima-Pettenati, J., Goffner, D.** (2003) Lignins and lignocellulosics: a better control of synthesis for new and improved uses. Trends Plant Sci. 8:576-581.

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 Pulp and Paper Industries. John Wiley & Sons, Inc., New York, pp. 541-565.

Brown, J.A., Li, D., Alic, M., Gold, M.H. (1993) Heat shock induction of manganese peroxidase gene transcription in *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. 59:4295-4299.

Brunow, G., **Kilpeläinen, I., Sipilä, J., Syrjänen, K., Karhunen, P., Setälä, H., Rummakko, P.** (1998a) Oxidative coupling of phenols and the biosynthesis of lignin. In: Lewis, N.G., Sarkanen, S., editors. Lignin and lignan biosynthesis. American chemical society, ACS symposium series 697, Washington DC, pp.131-147.

Brunow, G., Lundquist, K., Gellerstedt, G. (1998b) Lignin. In: Sjöström, E., Alén, R., editors. Analytical methods in wood chemistry, pulping, and papermaking. Springer-Verlag, Berlin, Germany, pp. 77-124

Burlat, V., Ambert, K., Ruel, K., Joseleau, J.-P. (1997) Relationship between the nature of lignin and the morphology of degradation performed by white-rot fungi. Plant Physiol. Biochem. 35:645-654.

Call, H.P., Mücke, I. (1987) History, overview and applications of mediated ligninolytic, systems, especially laccase-mediator-systems (Lignozym® -process). J. Biotechnol. 53:163-202.

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

Camarero S., Sarkar, S., Ruiz-Dueñas, F.J., Martínez, M.J., Martínez, 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.

Cancel, A.M., Orth, A.B., Tien, M. (1993) Lignin and veratryl alcohol are not inducers of the ligninolytic system of *Phanerochaete chrysosporium.* Appl. Environ. Microbiol. 59:2909-2913.

Cavaco-Paulo, A., Gübitz, G.M. (2003) Textile processing with enzymes. CRC Press, Boca Raton, USA, 228 p.

Chandra, R.P., Ragauskas, A.J. (2002) Evaluating laccase-facilitated coupling of phenolic acids to high-yield kraft pulps. Enzyme Microb. Technol. 30:855-861.

Cohen, R., Hadar, Y., Yarden, O. (2001) Transcripts and activity of different *Pleurotus ostreatus* peroxidases are differentially affected by Mn^{2+}. Environ. Microbiol. 3:312-322.

Coll, P.M., Fernández-Abalos, J.M., Villanueva, J.R., Santamaría, R., Perez, P. (1993) Purification and characterization of a phenoloxidase (laccase) from the lignin-degrading basidiomycete PM1 (CECT 2971). Appl. Environ. Microbiol. 59:2607-2613.

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.

Daniel, G., Jellison, J., Goodell, B., Paszczynski, A., Crawford, R. (1991) Use of monoclonal antibodies to detect Mn(II)-peroxidase in birch wood degraded by *Phanerochaete chrysosporium*. Appl. Microbiol. Biotechnol. 35:674-680.

Datta, A., Bettermann, A., Kirk, T.K. (1991) Identification of a specific manganese peroxidase among ligninolytic enzymes secreted by *Phanerochaete chrysosporium* during wood decay. Appl. Environ. Microbiol. 57**:**1453-1460.

de Jong, E., de Vries, F.P., Field, J.A., van der Zwan, R.P., de Bont, J.A.M. (1992) Isolation and screening of basidiomycetes with high peroxidative activity. Mycol. Res. 96:1098-1104.

de Souza-Cruz, P.B., Freer, J., Siika-Aho, M., Ferraz, A. (2004) Extraction and determination of enzymes produced by *Ceriporiopsis subvermispora* during biopulping of *Pinus taeda* wood chips. Enzyme Microb. Technol. 34:228-234

Dorado, J., Claassen, F.W., van Beek, T.A., Lenon, G., Wijnberg, B.P.A., Sierra-Alvarez, R. (2000) Elimination and detoxification of softwood extractives by white-rot fungi. J. Biotechnol. 80:231-240.

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

D´Souza, T., Merritt, C., Reddy, C.A. (1999) Lignin-modifying enzymes of the white rot basidiomycete *Ganoderma lucidum*. Appl. Environ. Microbiol. 65:5307-5313.

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

Dölz, F., Ullrich, R., Hofrichter, M., Nüske, J. (2007) Production of manganese peroxidase by *Pycnoporus cinnabarinus* (ATCC 200478) assumed to be an exclusive laccase producer In: 10th International congress on biotechnology in the pulp and paper industry. 10. -15.6.2007 Madison, WI, United States, Book of abstracts. pp.111-112.

Eggert, C., Temp, U., Eriksson, K.-E.L. (1996a) The ligninolytic system of the white rot fungus *Pycnoporus cinnabarinus*: Purification and characterization of the laccase. Appl. Environ. Microbiol. 62:1151-1158.

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

Elissetche, J.P., Ferraz. A., Freer, J., Mendonça, R., Rodríquez, J. (2006) Thiobarbituric acid reactive substrances, Fe^{3+} reduction and enzymatic activities in cultures of *Ganoderma australe* growing in *Drimys winteri* wood. FEMS Microbiol. Lett. 260:112-118.

Elissetche, J.P., Ferraz, A., Freer, J., Rodríguez, J. (2007) Enzymes produced by *Ganoderma australe* growing on wood and in submerged cultures. World J. Microbio. Biotechnol 23:429-434.

Enguita, F.J., Martins, L.O., Heniques, A.O., Carrondo, M.A. (2003) Crystal structure of a bacterial endospore coat component. J. Biol. Chem. 278:19416-19425.

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. (1978) Enzyme mechanisms involved in cellulose hydrolysis by the fungus *Sporotrichum pulverulentum*. Biotech. Bioeng. 20:317-332.

Eriksson, K.-E., Ander, P., Hennigsson, B., Nilsson, T., Goodell, B. (1976) Method for producing cellulose. US patent 3962033.

Eriksson, K.-E., Blanchette, R.A., Ander, P. (1990) Microbial and enzymatic degradation of wood and wood components, Chapter 1. Springer-Verlag, Berlin, Germany.

Eriksson, K.-E., Vallander, L. (1982) Properties of pulps from thermomechanical pulping of chips pretreated with fungi. Svensk Papperstidning 6:R33-38.

Escutia, M.R., Bowater, L., Edwards, A., Bottrill, A.R., Burrell, M.R., 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 oxidase and decarboxylases. Appl. Environ. Microbiol. 71:3608-3616.

Fackler, K., Gradingerm, 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.

Faix, O., Mozuch, M.D., Kirk, T.K. (1985) Degradation of Gymnosperm (Guaiacyl) vs. Angiosperm (Syringyl/guaiacyl) lignins by *Phanerochaete chrysosporium*. Holzforschung 39:203-208.

Farrell, R. (2007) Cartapip/Sylvanex^{TM}: Ophiostoma fungal product for commercial pulp and paper and solid wood applications. In: 10th International congress on biotechnology in the pulp and paper industry. 10. -15.6.2007 Madison, WI, United States, Book of abstracts. pp.63-64.

Ferraz, A., Córdova, A.M., Machuca, A. (2003) Wood biodegradation and enzyme productrion by *Ceriporiopsis subvermispora* during solid-state fermentation on *Eucalyptus grandis.* Enzyme Microbiol. Biotechnol. 32:59-65.

Ferraz, A., Guerra, A., Mendonca, R., Vicentim, M.P., Aguiar, A., Masarin, F., Seabra, G.G., Pavan, P.C. (2007) Mill evaluation of wood chips biotreated on a 50-ton biopulping pilot-plant and advances on understanding biopulping mechanisms. In: 10th International congress on biotechnology in the pulp and paper industry. 10. -15.6.2007 Madison, WI, United States, Book of abstracts. pp. 23-24.

Ferraz, A., Parra, C., Freer, J., Baeza, J., Rodriguez, J. (2000) Characterization of white zones produced on *Pinus radiata* wood chips by *Ganoderma australe* and *Ceriporiopsis subvermispora*. World J. Microbiol. Biotechnol. 16:641-645.

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

Field, J.A., de Jong, E., Feijoo Costa, G., de Bont, J.A.M. (1992) Biodegradation of polycyclic aromatic hydrocarbons by new isolates of white-rot fungi. Appl. Environ. Microbiol. 58:2219-2226.

Fields, A. (2001) Review: Protein function at thermal extremes: balancing stability and flexibility. Comp. Biochem. Physiol. Part A. 129:417-431.

Fischer, K., Akhtar, M., Blanchette R.A., Burnes, T.A., Messner, K., Kirk, T.K. (1994) Reduction in resin content in wood chips during experimental biological pulping processes. Holzforschung 48:285-290.

Freitag, M., Morrell, J.J. (1992) Decolorization of the polymeric dye Poly R-478 by wood-inhabiting fungi. Can. J. Microbiol. 38:811-822.

Fukushima, Y., Kirk, T.K. (1995) Laccase component of the *Ceriporiopsis subvermispora* lignin-degrading system. Appl. Environ. Microbiol. 61:872-876.

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

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.

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.

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. Commun. 114:1077-1083.

Gnanamani, A., Jayaprakashvel, M., Arulmani, M., Sadulla, S. (2006) Effect of inducers and culturing processes on laccase synthesis in *Phanerochaete chrysosporium* NCIM 1197 and the constitutive expression of laccase isozymes. Enzyme Microb. Technol. 38:1017-1021

Gold, M.H., Glenn, J.K., Alic M. (1988) Use of polymeric dyes in lignin biodegradation assays. Methods Enzymol. 161:74-78.

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 Inc, New York, pp. 559-586.

González, T., Terrón, M.C., Zapico, E.J., Téllez, A., Yagüe, S., Garbajo, J.M., González, A.E. (2003) Use of multiplex reverse transcription-PCR to study the expression of a laccase gene family in a bacidiomycetous fungus. Appl. Environ. Microbiol. 69:7083-7090.

Grönqvist, S., Rantanen, K., Alén, R., Mattinen, M.-L., Buchert, J., Viikari, L. (2006) Laccase-catalysed functionalisation of TMP with tyramine. Holzforschung 60:503-508.

Gutiérrez, A., Del Río J.C., Martínez M.J., Martínez, A.T. (1999) Fungal degradation of lipophilic extractives in *Eucalyptus globulus* wood. Appl. Environ. Microbiol. 65:1367-1371.

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

Gutiérrez, A., Del Río, J.C., Ibarra, D., Rencoret, J., Romero, J., Speranza, M., Camarero, S., Martínez, M.J., Martínez, A.T. (2006) Enzymatic removal of free and conjugated sterols forming pitch deposits in environmentally sound bleaching of eucalypt paper pulp. Environ. Sci. Technol. 40:3416-3422**.**

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.E., Jensen, K.A.Jr, Mozuch, M.D., Landucci, L.L., Tien, M., Pease, E.A. (1993) Ligninolysis by a purified lignin peroxidase. J. Biol. Chem. 268:12274-12281.

Harazono, K., Kondo, R., Sakai, K. (1996) Bleaching of hardwood kraft pulp with manganese peroxidase from *Phanerochaete sordida* YK-624 without the addition of $MnSO_4$. Appl. Environ. Microbiol. 62:913-917.

Hatakka, A. (1983) Pretreatment of wheat straw by white-rot fungi for enzymatic saccharification of cellulose. Appl. Microbiol. Biotechnol. 18:350–357.

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

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., Lundell, T., Hofrichter, M., Maijala, P. (2003a) 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., Lundell, T., 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., Maijala, P., Hakala, T.K., Hauhio, L., Ellmén, J. (2003b) Novel white-rot fungus and use thereof in wood pretreatment. International patent application WO 03/080812

Hatakka, A., Maijala, P., Hakala, T.K., Hildén, K., Hauhio, L., Ellmén, J. (2004) Potential and properties of *Physisporinus rivulosus*, a novel fungus for softwood biopulping. In: Wolfaardt, F., Kock, M., editors. 9th International conference in biotechnology in the pulp and paper industry, 10.-14.10 2004, Durban, South Africa., Book of abstracts, pp. 59-60.

Heidorne, F.O., Magalhães, P.O., Ferraz, A.L., Milagres, A.M.F. (2006) Characterization of hemicellulases and cellulases produced by *Ceriporiopsis subvermispora* grown on wood under biopulping conditions. Enzyme Microb. Technol. 38:436-442.

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

Heinfling, A., Ruiz-Dueñas, F.J., Martínez, M.J., Bergbauer, M., Szewzyk, U., Martínez, A.T. (1998b) A study on reducing substrates of manganese-oxidising peroxidases from *Pleurotus eryngii* and *Bjerkandera adusta*. FEBS Lett. 428:141-146.

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

Henriksson, G., Zhang, L., Li, J., Ljundquist, P., Reitberger, T., Pettersson, G., Johansson, G. (2000b) Is cellobiose dehydrogenase from *Phanerochate chrysosporium* a lignin degrading enzyme? Biochim. Biophys. Acta 1480:83-91.

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

Hildén, K., Martínez, 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 Gen. Biol. 42:403-419.

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

Hjortstam, K. and Ryvarden L. (1996) New and interesting wood-inhabiting fungi (Basidiomycotina – Aphyllophorales) from Ethiopia. Mycotaxon (LX) 181-190.

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.

Holmbom, B. (1998) Extractives. In: Sjöström, E., Alén, R., editors. Analytical methods in wood chemistry, pulping, and papermaking. Springer-Verlag, Berlin, Germany. pp. 125-148.

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

Itoh, H., Wada, M., Honda, Y., Kuwahara, M., Watanabe, T. (2003) Bioorganosolve pretreatments for simultaneous saccharification and fermentation of beech wood by ethanolysis and white rot fungi. J. Biotechnol. 103:273-280.

Jensen, K.A. Jr., 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.

Johansson, T., Nyman, P.O., Cullen, D. (2002) Differential regulation of *mnp2*, a new manganese peroxidase encoding gene from the ligninolytic fungus *Trametes versicolor* PRL 572. Appl. Environ. Microbiol. 68:2077-2080.

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

Johjima, T., Itoh, N., Kabuto, M., Tokimura, F., Nakagawa, T., Wariishi, H., Tanaka, H. (1999) Direct interaction of lignin and lignin peroxidase from *Phanerochaete chrysosporium*. Proc. Natl. Acad. Sci. 96:1989-1994.

Jönsson, L., Becker, H.G., Nyman, P.O. (1994) A novel type of peroxidase gene from the white-rot fungus *Trametes versicolor*. Biochim. Biophys. Acta 1207:255-259.

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

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

Kallioinen, A., Pere, J., Siika-Aho, M., Lehtilä, A., Mälkki, H., Syri, A., Thun, R. (2003) Biotechnical methods for improvement of energy economy in mechanical pulping, VTT research notes 2183.

Kamei, I., Daikoku, C., Tsutsumi, Y., Kondo, R. (2007) Expression analysis of manganese peroxidases from saline-tolerant white-rot fungus *Phlebia* sp. MG-60. In: 10th International congress on biotechnology in the pulp and paper industry. 10. - 15.6.2007 Madison, WI, United States, Book of abstracts. pp.42-43.

Kamitsuji, H., Honda, Y., Watanabe, T., Kuwahara, M. (2005) Mn^{2+} is dispensaple for the production of active MnP2 by *Pleurotus ostreatus*. Biochem. Biophys. Res Commun. 327:871-876.

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 with nonphenolic lignin model compounds and ^{14}C-labeled lignins. Biochem. Biophys. Res. Commun. 259:212-219.

Kersten, P.J. (1990) Glyoxal oxidase of *Phanerochaete chrysosporium*: Its characterization and activation by lignin peroxidase. Proc. Natl. Acad. Sci. 87:2936-2940.

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

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

Kiiskinen, L.-L., Rättö, M., Kruus, K. (2004b) Screening for novel laccase-producing microbes. J. Appl. Microbiol. 97:640-646.

Kirk, T.K., Croan, S., Tien, M., Murtagh, E., Farrell, R.L. (1986) Production of multiple ligninases by *Phanerochaete chrysosporium*: Effect of selected growth conditions and use of a mutant strain. Enzyme Microb. Technol. 8:27-32.

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

Kirk, T.K., Cullen, D. (1998) Enzymology and molecular genetics of wood degradation by white-rot fungi. In: Young RA, Akhtar M, editors. Environmentally Friendly Technologies for Pulp and Paper Industries. John Wiley & Sons, Inc., New York, pp. 273-307.

Kotiranta, H. (1985) *Physisporinus rivulosus*, an interesting polypore species, Karstenia 25:66-69.

Kotiranta, H., Penttilä R. (1996) Short-term effects of prescribed burning on wood-rotting fungi. Silva Fennica. 30:399-419.

Kuan, I., Tien, M. (1989) Phosphorylation of lignin peroxidases from *Phanerochaete chrysosporium*. Identification of mannose-6-phosphate. J. Biol. Chem. 264:20350-20355.

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 Biochem. Eng. Biotechnol. vol 57. Springer-Verlag, Germany. pp. 46-125.

Kurek, B., Petit-Conil, M., Sigoillot, J.-C., Herpoel, I., Ruel, K., Moukha, S., Joseleau, J.-P., Pennînckx, M., Asther, M., Gazza, G., de Choudens, C. (2001) Treatment of high-yield pulp with fungal peroxidases: from laboratory to pilot scale study. In: Argyropoulos, D., editor. ACS symposium series 785, Oxidative delignification chemistry. American Chemical Society, Washington DC, USA, 2001. pp. 474-486.

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. Biotechnol. 66:401-407.

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

Lawson, L.R., Still, C.N. (1957) The biological decomposition of lignin – a literature survey. Tappi J. 40:56A-80A.

Leung, P-C., Pointing, S.B. (2000) Effect of different carbon and nitrogen regimes on Poly R decolorization by white-rot fungi. Mycol. Res. 106:86-92.

Li, D., Alic, M., Brown, A., Gold, M.H. (1995) Regulation of manganese peroxidase gene transcription by hydrogen peroxide, chemical stress, and molecular oxygen. Appl. Environ. Microbiol. 61:341-345.

Li, D., Li, N., 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.

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

Lobos, S., Larrondo, L., Salas, L., Karahanian, E., Vicuña, R. (1998) Cloning and molecular analysis of a cDNA and the *CS-mnp1* gene coding a manganese peroxidase

isoenzyme from the lignin-degrading basidiomycete *Ceriporiopsis subvermispora*. Gene 206:185-193.

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

Lyashenko, A.V., Zhukhlistova, N.E., Gabdoulkhakov, A.G., Zhukova, Y.N., Voelter, W., Zaitsev, V.N., Bento, I., Stepanova, E.V., Kachalova, G.S., Koroleva, O.V., Cherkashyn, E.A., Tishkov, V.I., Lamzin, V.S., Schirwitz, K., Morgunova, E.Y., Betzel, C., Lindley P.F., Mikhailov A.M. (2006) Purification, crystallization and preliminary X-ray study of the fungal laccase from *Cerrena maxima*. Acta Cryst. (2006). F62, 954-957

Lynd, L.R., Weimer, P.J., van Zyl, W.H., Pretorius, I.S. (2002) Microbial cellulose utilization: fundamentals and biotechnology. Microbiol. Mol. Biol. Rev. 66:506-577.

Ma, B., Mayfield, M.B., Godfrey, B.J., Gold, M.H. (2004) Novel promoter sequence required for manganese regulation of manganese peroxidase isozyme 1 gene expression in *Phanerochaete chrysosporium*. Euk. Cell 3:579-588.

Mai, C., Kües, U., Militz, H. (2004) Biotechnology in the wood industry. Appl. Microbiol. Biotechnol. 63:477-494.

Maijala, P. (1996) Juurikäävän (*Heterobasidion annosum*) hemisellulolyyttiset entsyymit ja ligninolyyttinen mekanismi. Licenciate thesis. Unversity of Helsinki. 83 p. (In Finnish)

Maijala, P., Hakala, T.K., Salo, V., Lundell, T., Hatakka, A. (2002) Characteristics of a new white-rot fungus with potential in biopulping. In: Conference Proceedings of the Workshop of COST E23 Action Biotechnology in the Pulp and Paper Industry, "Biotechnology for improving pulp & paper processing", November 28-29, 2002, Centre Technique du Papier, Grenoble, France, p. 10.

Maijala, P., Mettälä, A., Kleen, M., Westin, C., Poppius-Levlin, K., Herranen, K., Lehto, J.H., Reponen, P., Mäentausta, O., Hatakka, A. (2007) Treatment of softwood chips with enzymes may reduce refining energy consumption and increase surface charge of fibres. In: 10th International congress on biotechnology in the pulp and paper industry. 10. -15.6.2007 Madison, WI, United States, Book of abstracts. p. 65.

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

Mardones, L., Gomide, J.L., Freer, J., Ferraz, A., Rodríguez, J. (2006) Kraft pulping of *Eucalyptus nitens* wood chips biotreated by *Ceriporiopsis subvermispora*. J. Chem. Technol. Biotechnol. 81:608-613.

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

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

Matsubara, M., Suzuki, J., Deguchi, T., Miura, M., Kitaoka, Y. (1996) Characterization of manganese peroxidases from the hyperligninolytic fungus IZU-154. Appl. Environ. Microbiol. 62:4066-4072.

Messner, K., Fackler, K., Lamaipis, P., Gindl, W., Srebotnik, E., Watanabe, T. (2003) Overview of white-rot research: where are we today. In: Goodell, B., Nicholas,

D.D., Schultz, T.P. editors. Wood deterioration and preservation. ACS symposium series 845. American Chemical Society, Washington DC, USA. pp. 73-96.

Messner, K., Koller, K., Wall. M.B., Akhtar, M., Scott, G.M. (1998) Fungal treatment of wood chips for chemical pulping. In: Young, R.A., Akhtar, M., editors. Environmentally Friendly Technologies for Pulp and Paper Industries. John Wiley & Sons, Inc., New York, pp:385-419.

Mester, T., de Jong, E., Field, J.A. (1995) Manganese regulation of veratryl alcohol in white-rot fungi and its indirect effect on lignin peroxidase. Appl. Environ. Microbiol. 61:1881-1887.

Mester, T., Ambert-Balay, K., Ciofi-Baffoni, S., Banci, L., Jones, A.D., Tien, M. (2001) Oxidation of a tetrameric nonphenolic lignin model compound by lignin peroxidase. J. Biol. Chem., 25:22985-22990.

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

Michniewicz, A., Ullrich, R., Ledakowicz, S., Hofrichter, M. (2006) The white-rot fungus *Cerrena unicolor* strain 137 produces two laccase isoforms with different physico-chemical and catalytic properties. Appl. Microbiol. Biotechnol. 69:682-688.

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. Biotechnol. 45:792-799.

Moreira, M.T., Feijoo, G., Canaval, J.M., Lema, J.M. (2003) Semipilot –scale bleaching of Kraft pulp with manganese peroxidase. Wood Sci. Technol. 37:117-123.

Moreira, M.T., Palma, C., Mielgo, I., Feijoo, G., Lema, J.M. (2001a) *In vitro* degradation of a polymeric dye (Poly R-478) by manganese peroxidase. Biotechnol. Bioeng. 75:362-368.

Moreira, M.T., Sierra-Alvarez, R., Lema, J.M., Feijoo, G., Field, J.A. (2001b) Oxidation of lignin in eucalyptus kraft pulp by manganese peroxidase form *Bjerkandera* sp. strain BOS55. Bioresour. Technol. 78:71-79.

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

Mäkelä, M.R, Galkin, S., Hatakka, A., Lundell, T. (2002) Production of oxalic acid and oxalate decarboxylase by lignin-degrading white rot fungi. Enzyme Microb. Technol. 30:542-549.

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

Nakasone, K.K. (1981) Cultural studies on *Poria cinerascens*, *Poria rivulosa* and *Poria subvermispora* (Aphyllophorales, Basidiomycotina). Mycotaxon (XIII) 105-111.

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

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.

Niranjane, A.P., Madhou, P., Stevenson, T.W. (2007) The effect of carbohydrate carbon sources on the production of cellulase by *Phlebia gigantea*. Enzyme Microb. Technol. 40:1464-1468.

Nishida, T., Kashino, Y., Mimura, A., Takahara, Y. (1988) Lignin biodegradation by wood-rotting fungi I. Screening of lignin-degrading fungi. Mokuzai gakkaishi 34:530-536.

Otjen, R., Blanchette, R.A., Effland, M., Leatham, G. (1987) Assessment of 30 white rot basidiomycetes for selective lignin degradation. Holzforschung 41:343-349.

Paice, M. Bourbonnais, R., Renaud, S., Labonté, S., Sacciadis,G., Berry, R., Amann, M., Candussio, A., Müller, R. (2002) Pilot plant bleaching trials with laccase and mediator. In: Viikari, L., Lantto, R., editors. Progress in biotechnology Vol 21: Biotechnology in the Pulp and Paper Industry, 8th ICBPPI meeting, Elsevier Science B.V., Amsterdam. pp: 203-211.

Palmieri, G., Cennamo, G., Faraco, V., Amoresano, A., Sannia, G., Giardina, P. (2003) Atypical laccase isoenzymes from copper supplemented *Pleurotus ostreatus* cultures. Enzyme Microb. Technol. 33:220-230.

Perez, J., Jeffries, T. (1992) Roles of manganese and organic acid chelators in regulating lignin degradation and biosynthesis of peroxidases by *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. 58:2402-2409.

Perié, 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.

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.

Pointing, S.B. (2001) Feasibility of bioremediation by white-rot fungi. Appl. Microbiol. Biotechnol. 57:20-33.

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

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.

Robinson, T., Chandran, B., Nigam, P. (2001) Studies on the production of enzymes by white-rot fungi for the decolorization of textile dyes. Enzyme Microb. Technol. 29:575-579.

Rodríquez Couto, S., Rivela, I., Sanromán, A. (2000) *In vivo* decolourization of the polymeric dye Poly R-478 by corncob cultures of *Phanerochaete chrysosporium*. Acta Biotechnol. 20:31-38.

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.

Rodriguez Couto S., Toca Herrera, J.L. (2006) Industrial and biotechnological applications of laccases: a review. Biotechnol. Adv. 24:500-513.

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

Rogalski, J., Hatakka, A., Longa, B., Wojtas-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.

Roy, B.P., Dumonceaux, T., Koukoulas, A.A., Archibald, F.S. (1996) Purification and characterization of cellobiose dehydrogenases from the white rot fungus *Trametes versicolor*. Appl. Environ. Microbiol. 62:4417-4427.

Rüttimann-Johnson, C., Salas, L., Vicuña, R., Kirk, T.K. (1993) Extracellular enzyme production and synthetic lignin mineralization by *Ceriporiopsis subvermispora*. Appl. Environ. Microbiol. 59:1792-1797.

Salas, C., Lobos, S., Larrain, J., Salas, L., Cullen, D., Vicuña, R. (1995) Properties of laccase isoenzymes produced by the basidiomycete *Ceriporiopsis subvermispora*. Biotechnol. Appl. Biochem. 21:323-333.

Sannia, G., Limongi, P., Cocca, E., Buonocore, F., Nitti, G., Giardina, P. (1991) Purification and characterization of a veratryl alcohol oxidase enzyme from the white-rot fungus *Pleurotus ostreatus*. Biochim. Biophys. Acta 1073:114-119.

Sasaki, T., Kajino, T., Li, B., Sugiyama, H., Takahashi, H. (2001) New pulp biobleaching system involving manganese peroxidase immobilized in a silica support with controlled pore sizes. Appl. Environ. Microbiol. 67:2208-2212.

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

Schmidt, O., Schmidt, U., Moreth, U. and Potsch, T. (1997) Wood decay by the white-rotting basidiomycete *Physisporinus vitreus* from a cooling tower. Holzforschung 51:193-200.

Schwarze, F.W.M.R., Landmesser, H. (2000) Preferential degradation of pit membranes within tracheids by the Basidiomycete *Physisporinus vitreus*. Holzforschung 54:461-462

Scott, G.M., Akhtar, M., Swaney, R.E., Houtman, C.J. (2002) Recent development in biopulping technology at Madison, WI. In: Viikari, L., Lantto, R., editors. Progress in Biotechnology Vol 21, Biotechnology in the pulp and paper industry: 8th ICBPPI Meeting, Elsevier, Amsterdam, pp. 61-72.

Selinheimo, E., Kruus, K., Buchert, J., Hopia, A., Autio, K. (2006) Effects of laccase, xylanase and their combination on the rheological properties of wheat doughs. J. Cereal Sci. 43: 152-159.

Sethuranaman, A., Akin, D.E., Eisele, J.G., Eriksson, K.-E.L. (1998a) Effect of aromatic compounds on growth and ligninolytic enzyme production by two white-rot fungi *Ceriporiopsis subvermispora* and *Cyathus stercoreus*. Can. J. Microbiol. 44:872-885.

Sethuranaman, A., Akin, D.E., Eriksson, K.-E.L. (1998b) Plant-cell-wall-degrading enzymes produced by the white-rot fungus *Ceriporiopsis subvermispora*. Biotechnol. Appl. Biochem. 27:37-47.

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

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.

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.

Somero, G.N. (2004) Adaptation of enzymes to temperatures: searching for basic "strategies". Comp. Biochem. Physiol. Part B. 139:312-333.

Srebotnik, E., **Messner, K., Foisner, R.** (1988a) Penetrability of white rot-degraded pine wood by the lignin peroxidase of *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. 54:2608-2614.

Srebotnik, E., Messner, K., Foisner, R., Pettersson, B. (1988b) Ultrastructural localization of ligninase of *Phanerochaete chrysosporium* by immunogold labeling. Curr. Microbiol. 16:221-227.

Srinivasan, C., D´souza, T.M., Boominathan, K., Reddy, C.A. (1995) Demonstration of laccase in the white-rot basidiomycete *Phanerochaete chrysosporium* BKM-F1767. Appl. Environ. Microbiol. 61:4274-4277.

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.* Enz. Microb. Technol. 30:550-555.

Sun, Y., **Cheng, J.** (2002) Hydrolysis of lignocellulosic material for ethanol production: a review. Biores. Technol. 83:1-11.

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.

Tanaka, H., **Yoshida, G., Baba, Y., Matsumura, K., Wasada, H., Murata, J., Agawa, M., Itakura, S., Enoki, A.** (2007) Characterization of a hydroxyl-radical-producing glycoprotein and its presumptive genes from the white-rot basidiomycete *Phanerochaete chrysosporium*. J. Biotechnol. 128:500-511.

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*. Biochem. Biophys. Acta. 1490:137-144.

Temp, U., Eggert, C. (1999) Novel interaction between laccase and cellobiose dehydrogenase durig pigment synthesis in the white-rot fungus *Pycnoporinus cinnabarinus.* Appl. Environ. Microbiol. 65:389-395.

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

Tomšovský, M., Kolarík, M., Pažoutová, S., Homolka, L. (2006) Molecular phylogeny of European *Trametes* (Basidiomycetes, Polyporales) species based on LSU and ITS (nrDNA) sequences. Nova Hedwigia 82:269-280.

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

Tuomela, M., Steffen, K., Valentin, L., Oivanen, P., Oesch-Kuisma, H., Kähkönen, M., Hatakka A. (2007) Wood-rotting fungi as degraders of contaminated material from old saw mill areas. In: 10th International congress on biotechnology in the pulp and paper industry. 10. -15.6.2007 Madison, WI, United States, Book of abstracts. p.130.

Urzua, U., Kersten, P.J., Vicuña, R. (1998) Manganese peroxidase-dependent oxidation of glyoxylic acid synthetized by *Ceriporiopsis subvermispora* produces extracellular hydrogen peroxide. Appl. Environ. Microbiol. 64:68–73.

Vallim, M.A., Jense, B.J.H., Gaskell, J., Pizzirani-Kleiner, A.A., Cullen, D. (1998) *Phanerochaete chrysosporium* cellobiohydrolase and cellobiose dehydrogenase transcripts in wood. Appl. Environ. Microbiol. 64:1924-1928.

Valmaseda, M., **Martínez, M.J., Martínez, A.T.** (1991) Kinetics of wheat straw solid-state fermentation with *Trametes versicolor* and *Pleurotus ostreatus* – lignin and polysaccharide alteration and production of related enzymes. Appl. Microb. Biotechnol. 35:817-823.

van Beek, T.A., Kuster, B., Claassen, F.W., Tienvieri, T., Bertaud, F., Lenon, G., Petit-Conil, M., Sierra-Alvarez, R. (2007) Fungal bio-treatment of spruce wood with *Trametes versicolor* for pitch control: Influence on extractive contents, pulping process parameters, paper quality and effluent toxicity. Biores. Technol. 98:302-311.

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.

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 enzyme and mineralization of ^{14}C-ring-labelled synthetic lignin by three *Phlebia tremellosa* strains. Appl. Microbiol. Biotechnol. 60:569-575.

Vicentim, M.P., Ferraz, A. (2007) Enzyme production and chemical alterations of Eucalyptus grandis wood during biodegradation by *Ceriporiopsis subvermispora* in cultures supplemented with Mn^{2+}, corn steep liquor and glucose. Enzyme Microb. Technol. 40:645-652.

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

Watanabe, T., Koller, K., Messner, K. (1998) Copper-dependent depolymerization of lignin in the presence of the fungla metabolite, pyridine. J. Biotechnol. 62:221-230.

Wesenberg, D., Kyriakides, I., Agathos, S.N. (2003) White-rot fungi and their enzymes for the treatment of industrial dye effluents. Biotechnol. Adv. 22:161-187.

Wolfaardt, F., Taljaard, J.L., Jacons, A., Male, J.R., Rabie, C.J. (2004) Assessment of wood-inhabiting basidiomycetes for biokraft pulping of softwood chips. Biores. Technol. 95:25-30.

Xu, F. (1997) Effects of redox potential and hydroxide inhibition on the pH activity profile of fungal laccases. J. Biol. Chem. 272:924-928.

Xu, F., Shin, W., Brown, S.H., Wahleithner, J.A., Sundaram, U.M., Solomon, E.I. (1996) A study of a series of recombinant fungal laccases and bilirubin oxidase that exhibit significant differences in redox potential, substrate specificity, and stability. Biochem. Biophys. Acta 1292:303-311.

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.

Youn, H.-D., Hah, Y.C., Kang, S.-O. (1995) Role of laccase in lignin degradation by white-rot fungi. FEMS Microbiol. Lett. 132:183-188.