# Ligninolytic enzymes of the basidiomycetous fungi *Agaricus bisporus* and *Phlebia radiata* on lignocellulose-containing media

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Front cover picture: *Agaricus bisporus* (white button mushroom, herkkusieni) on culture medium (photo: Mykora Oy)

## Abstract

The production of lignin degrading enzymes (laccase, manganese peroxidase (MnP) and lignin peroxidase (LiP)) of two basidiomycetous fungi, the white-rot fungus *Phlebia radiata* and the litter-degrading fungus *Agaricus bisporus* were examined on lignocellulose-containing (pulp mill wastewater, straw, bran, compost leachate) media.

In pulp bleaching wastewater and pulp mill wastewater-containing media *P. radiata* secreted LiP, MnP and laccase. Purification of the enzyme proteins was required to show the actual presence of ligninolytic enzymes in wastewater-containing media, since phenolic and coloured compounds from wastewater interfered with the spectrophotometric enzyme activity assays. The production of laccase and MnP were enhanced in wastewater-containing cultures. The production of LiP was also higher than expected from the results of activity measurement with the veratryl alcohol oxidation method. The consumption of glucose and the growth of the *P. radiata* increased with increasing amounts of pulp mill wastewater in the culture medium.

Laccase and MnP were the main ligninolytic enzymes secreted by *A. bisporus* in compost. The laccase from compost was a blue laccase. Addition of wheat and rye bran induced MnP production in liquid cultures. The MnP isolated from compost and bran-amended cultures, designated MnP1, was the same enzyme with p*I* 3.25 and MW 40 kDa. The *mnp1* gene expressed in bran-containing cultures was cloned and sequenced. It is the first MnP-encoding gene from a litter-degrading fungus. The predicted aa sequence of MnP is 328-aa-long polypeptide preceded by a 26-aa leader peptide. On the sequence level the MnP has the highest similarity to *Pleurotus ostreatus* MnP3 and *Pleurotus eryngii* versatile peroxidases VPL1 and VPL2. The intron-exon structure of the *mnp1* gene with 14 introns resembles that of *P. ostreatus* MnP1 and *P. eryngii* VPL2. The sequence of the main MnP1 of *A. bisporus* can be used to monitor the ligninolytic enzyme expression and thus substrate utilization in *A. bisporus* compost and thus correlate the substrate utilization to mushroom production.

## Tiivistelmä (abstract in Finnish)


### *Agaricus bisporuks*en (herkkusieni) ja *Phlebia radiata*n (rusorypykkä) ligninolyyttiset entsyymit lignoselluloosaa sisältävillä alustoilla

Ligniini on selluloosan jälkeen maapallon toiseksi yleisin uusiutuva polymeeri. Sitä muodostuu puuvartisissa kasveissa ja ruohokasveissa, joissa se sitoo selluloosakuituja yhteen liima-aineen tavoin. Puunjalostusteollisuudessa massanvalmistuksessa ligniiniä poistetaan puusta vahvoilla kemikaaleilla keittämällä korkeassa paineessa ja lämpötilassa. Lignoselluloosaa eli ligniiniä, selluloosaa ja hemiselluloosaa sisältävää jätemateriaalia ja sivutuotteita syntyy suuria määriä maataloudessa ja puunjalostusteollisuudessa. Tyypillisiä lignoselluloosaa sisältäviä jätteitä ovat mm. sahanpuru, olki, leseet ja muut kasvinjätteet. Tämän materiaalin hyötykäyttö on mahdollista esim. energiantuotannossa mutta myös syötävien sienten kasvatuksessa.

Valkolahosienet ovat ainoita tehokkaita ligniinin hajottajia luonnossa. Niiden kyky hajottaa ligniiniä perustuu solunulkoisiin hapettaviin entsyymeihin, ligninolyyttisiin entsyymeihin, jotka pilkkovat ligniinimolekyyliä pienemmäksi. Nämä entsyymit, mangaaniperoksidaasi (MnP), lakkaasi ja ligniiniperoksidaasi (LiP), pystyvät hajottamaan myös muita vaikeasti hajoavia yhdisteitä kuten värillisiä yhdisteitä puunjalostus- ja tekstiiliteollisuuden jätevesissä ja myrkkyjä kuten polyaromaattisia hiilivetyjä (PAH). Tyypilliset valkolahottajasienet ovat kantasieniä, useimmiten kääpiä, jotka kasvavat kuolleella puulla. Maassa esiintyvät ligniiniä hajottavat sienet ovat etupäässä karikkeenlahottajasieniä. Näiden sienten ligniininhajotuskykyä ja ligniiniä hajottavien entsyymien tuottoa on tutkittu vähän.

Työssä tutkittiin tehokkaan valkolahottajan *Phlebia radiata*n (rusorypykkä) ligniiniä hajottavien entsyymien tuottoa puunjalostusteollisuuden jätevettä ja siten hajonnutta ja muuntunutta lignoselluloosaa sisältävällä kasvualustalla. *P. radiata* tuotti runsaasti mangaaniperoksidaasia ja lakkaasia puunjalostusteollisuuden jätevettä sisältävällä alustalla. Ligniiniperoksidaasin tuotto oli myös runsaampaa kuin voitiin veratryylialkoholin hapetukseen perustuvalla spektrofotometrisellä aktiivisuusmäärityksellä havaita. Glukoosin kulutus ja sienen kasvu oli sitä runsaampaa mitä enemmän oli jätevettä alustassa. Voitiin siis päätellä, että lignoselluloosaa sisältävä jätevesi lisäsi ligninolyyttisten entsyymien tuottoa ja myös sienen kasvua. Lignoselluloosaa sisältävillä alustoilla spektrofotometriset entsyymiaktiivisuusmääritykset, erityisesti LiP:n ja MnP:n määritys, eivät toimi luotettavasti, koska lignoselluloosan sisältämät yhdisteet kuten fenolit ja värilliset yhdisteet häiritsevät entsyymien aktiivisuusmääritystä.

Taloudellisesti maailman tärkein karikkeenlahottaja, *Agaricus bisporus* (herkkusieni), kasvatetaan perinteisesti olkikompostissa, jossa sienirihmasto kasvaa ensin n. 5 viikkoa ennen kuin varsinaiset itiöemät muodostuvat. Ligniiniä hajottavien entsyymien merkitys on suuri erityisesti kasvualustan hyödyntämisessä ja sitä kautta sienen kasvussa. *Agaricus bisporuksen* ligniiniä hajottavia entsyymejä ja niiden tuottoa tutkittiin, kun sieni kasvoi lanta-olkikompostissa ja pullokasvatuksissa nestealustalla. Pullokasvatuksissa pyrittiin löytämään erityisesti herkkusienen mangaaniperoksidaasientsyymiä indusoiva kasvualusta, jotta pystyttäisiin karakterisoimaan herkkusienen MnP-entsyymi ja sen muodostumista aikaansaava geeni/t. Herkkusieni tuotti runsaasti lakkaasia olkikompostissa. Kompostista puhdistettiin MnP-entsyymi jonka molekyylipaino on 40 kDa ja isoelektrinen piste p*I* 3.25. MnP-entsyymin pH-optimi on pH 5. Leseiden, erityisesti vehnän ja ruisleseiden lisäyksen osoitettiin lisäävän MnP:n tuottoa, kun herkkusientä kasvatettiin nesteviljelmässä.

Lesealustalla kasvatetusta herkkusienestä eristettiin kokonais-RNA, josta syntetoitiin cDNA käänteistranskriptaasientsyymin avulla. Tätä cDNA:ta käytettiin templaattina, kun monistettiin *mnp1* -geeni. Alukkeina käytettiin degeneratiivisia, lahosienten mangaaniperoksidaasien konservoituneille alueille suunniteltuja alukkeita.

Herkkusienen *mnp1* -geenin koodaama aminohapposekvenssi osoittautui N-terminaalisekvenssiltään identtiseksi kompostista eristetyn MnP-entsyymin N-terminaalisekvenssin kanssa. Kun myös kompostista eristetyn MnP:n isoelektrinen piste oli sama kuin lesekasvatuksista eristetyn MnP:n, pääteltiin, että kyse oli samasta entsyymistä. Herkkusieni siis tuotti sekä kompostissa että leseitä sisältävissä nesteviljelmissä samaa MnP:a. Herkkusienen MnP1 osoittautui aminohapposekvenssiltään lähinnä osterivinokkaan (*Pleurotus ostreatus*) MnP3:n ja *Pleurotus eryngii*n VPL1 ja VPL2 -peroksidaasientsyymiproteiinien kaltaiseksi. *Mnp1* -geenin introni-eksoni-rakenne muistuttaa *P. ostreatukse*n MnP1:n ja *P. eryngii*n VPL2:n rakennetta. Herkkusienestä ei tässä työssä löydetty ligniiniperoksidaasia.

Herkkusienen *mnp1* geeni on ensimmäinen karikkeenlahottajasienesta karakterisoitu MnP:a koodaava geeni. Työssä eristettyä *mnp1* -geenisekvenssiä voidaan käyttää herkkusienen ligninolyyttisten entsyymien ilmentymisen seuraamiseen, kun sieni kasvaa kompostissa ja samalla saada tietoa sienen substraatin käytöstä. Näin voidaan selvittää kompostin koostumuksen vaikutusta ligninolyyttisten entsyymien tuottoon ja sitä kautta sienen kasvuun ja sadontuottoon.

## List of original publications

The thesis is based on the following articles referred to in the text by Roman numerals I-IV. In addition, unpublished data are also presented.

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

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

**III** **Lankinen, V.P**., Bonnen, A.M., Anton, L.H., Wood, D.A., Kalkkinen, N., Hatakka, A. and Thurston, C.F. 2001. Characteristics and N-terminal amino acid sequence of manganese peroxidase from solid substrate cultures of *Agaricus bisporus*. Applied Microbiology and Biotechnology 55:170-176.

**IV** **Lankinen, P.**, Hildén, K., Aro, N., Salkinoja-Salonen, M. and Hatakka, A. Manganese peroxidase of *Agaricus bisporus*: grain bran-promoted production and gene characterization 200x. Applied Microbiology and Biotechnology (in press).

## The author's contribution

**I** Pauliina Lankinen planned the experiments, did the laboratory work except for the cultivation of *Phanerochaete chrysosporium* and *Merulius tremellosa*. Pauliina Lankinen analysed and interpreted the results and wrote the article.

**II** Pauliina Lankinen purified the enzyme and planned and executed the analytical enzyme experiments of *Agaricus bisporus* laccase and interpreted the results. She contributed to the writing of the article.

**III** Pauliina Lankinen planned the experiments, did the laboratory work except the N-terminal amino-acid sequencing, analysed and interpreted the results and wrote the article. David Wood and Christopher Thurston contributed to the revision of the article.

**IV** Pauliina Lankinen planned the experiments, did the laboratory work except the RNA extractions, interpreted the results and wrote the article.

## Abbreviations

| | |
|---|---|
| **aa** | amino acid |
| **AAO** | aryl alcohol oxidase |
| **ABTS** | 2,2'-azinobis(3-ethylbenzthiazoline-6-sulphonate) |
| **AOX** | adsorbable organic halogen |
| **bp** | base pair |
| **cDNA** | complementary DNA |
| **DHP** | dehydrogenation polymer (synthetic lignin) |
| **DNA** | deoxyribonucleic acid |
| **ECF** | elemental chlorine free |
| **FPLC** | fast protein liquid chromatography |
| **GLOX** | glyoxal oxidase |
| **IEF** | isoelectric focusing |
| **kb** | kilobase(s) or 1000 bp |
| **kDa** | kiloDalton |
| **$K_m$** | Michaelis constant |
| **LDF** | litter-decomposing fungus, litter-degrading fungus |
| **LiP** | lignin peroxidase |
| **MnP** | manganese peroxidase |
| **mRNA** | messenger RNA |
| **MW** | molecular mass |
| **PAGE** | polyacrylamide gel electrophoresis |
| **PCR** | polymerase chain reaction |
| **p*I*** | isoelectric point |
| **RNA** | ribonucleic acid |
| **SDS** | sodium dodecyl sulphate |

## Table of Contents

# 1. INTRODUCTION

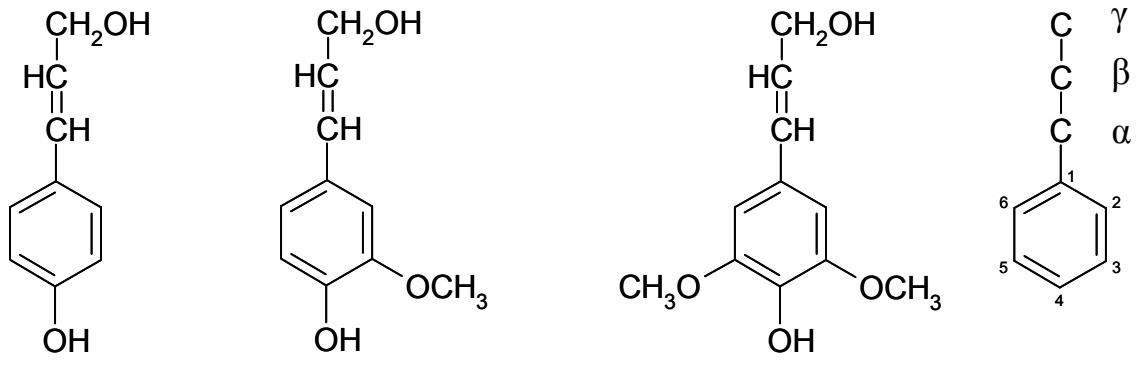
## 1.1 The ligninolytic enzymes of basidiomycetous fungi

### 1.1.1 Lignocellulose and lignin

The lignocellulosic biomass from plants is a renewable source of food, energy and chemicals. It accounts for more than 60% of the total biomass production (Kuhad *et al.* 1997). Lignocellulosic waste material is produced in huge amounts in agriculture, forestry and in the pulp and paper industry. The use of this waste is an important way to recycle carbon to energy and food.

The lignocellulosic material of plants consists of three main components, namely cellulose, hemicellulose and lignin. Cellulose is a linear homopolymer of glucose units linked with β-1,4-glucosidic bonds. Hemicelluloses are heteropolysaccharides consisting of short branched chains of hexoses, e.g. mannose units in mannans and pentoses such as xylose units in xylans (Kuhad *et al.* 1997). The hydrolytic breakdown of cellulose in nature is catalysed by extracellular enzymes: cellobiohydrolases, endoglucanases and β-glucosidases produced by fungi and bacteria (Kuhad *et al.* 1997, Teeri 1997). The major hemicellulose-degrading enzymes are endoxylanases and endomannanases.

After cellulose lignin is the second most abundant renewable biopolymer in nature. It is an essential part of the plant cell wall, imparting rigidity and protecting the easily degradable cellulose from attack by pathogens. Lignin is aromatic, 3-dimensional and amorphous. It is synthesized from phenyl propanoid precursors by polymerization in higher plants. The lignin precursors *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol consist of an aromatic ring and a 3-carbon side chain (Fig. 1.1) (Brown 1985). In the lignin molecule the precursors form 3 types of subunit: hydroxyphenol- (H-type), guaiacyl- (G-type) and syringyl subunits (S-type). A typical finding for the lignin polymer is that there is no single repeating bond between the subunits but a random distribution of at least 10 different types of bond, the most common being the β-aryl ether (*β*-O-4) bond (Argyropoulos and Menachem 1997) (Fig. 1.2).




*p*-coumaryl alcohol coniferyl alcohol sinapyl alcohol

**Fig. 1.1** Precursors of lignin (Buswell and Odier 1987) and model for numeration of the carbon skeleton in the lignin molecule.

Several structural models of lignin have been presented (Adler 1977, Brunow 2001) (Fig. 1.2). Due to its complicated structure and nonhydrolysable bonds, lignin is more difficult to break down than cellulose or hemicellulose. The molecular mass (MW) of lignin is high, about 100 kDa or more, which prevents its uptake inside the microbial cell (Eriksson *et al.* 1990). Thus, the biological degradation of macromolecular lignin must occur through the activity of extracellular enzymes. Detailed reviews of lignin are found in Adler (1977), Eriksson *et al.* (1990), Argyropoulos and Menachem (1997) and Kuhad *et al.* (1997).

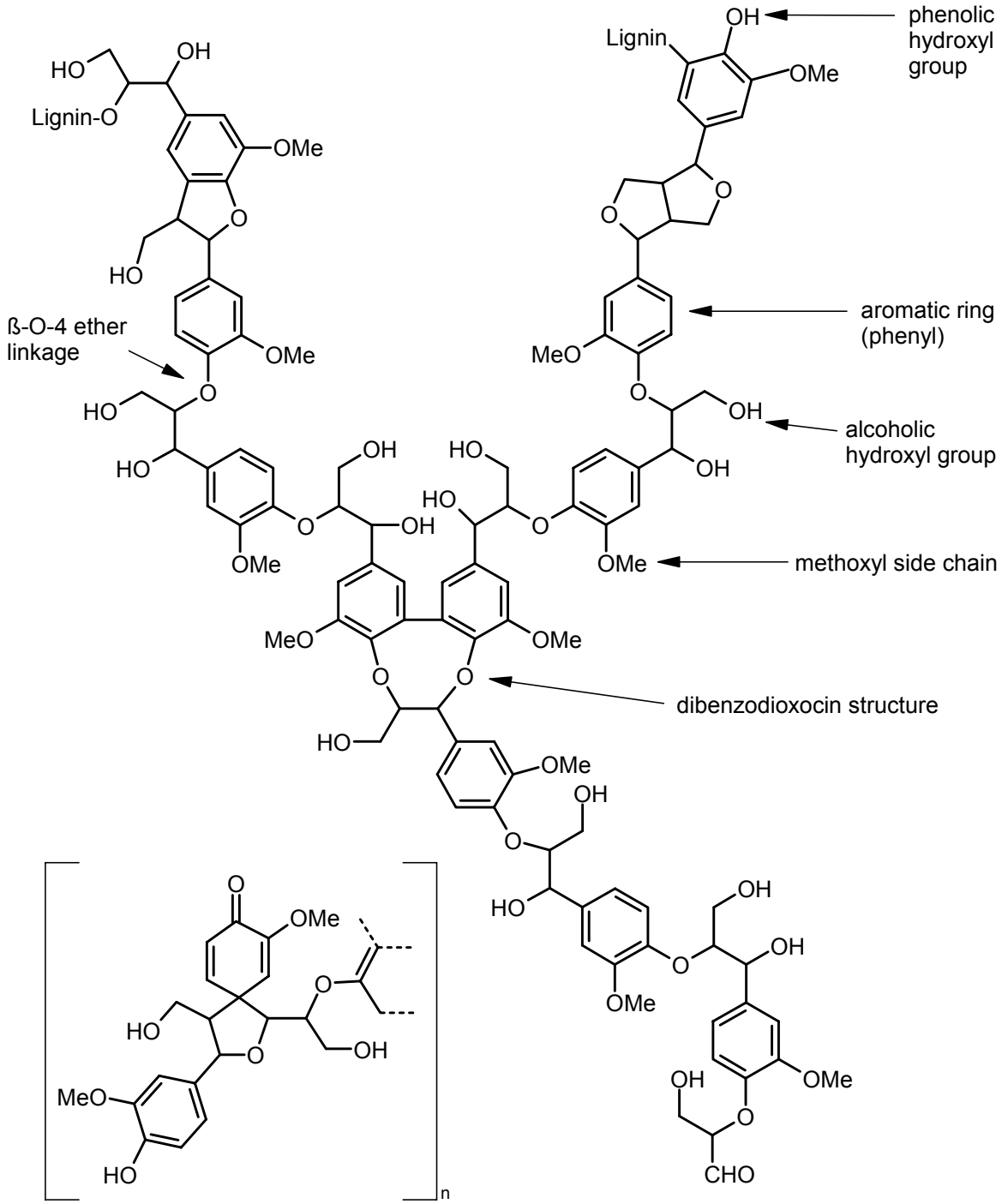



**Fig. 1.2** Structural model of lignin by Brunow (2001).

### 1.1.2 Ligninolytic enzymes

Lignin breakdown is thought to occur by concomitant action of ligninolytic enzymes. Table 1.1 summarizes the ligninolytic enzymes and their substrates and reactions. The main extracellular enzymes participating in lignin degradation are heme-containing lignin peroxidase (ligninase, LiP, EC 1.11.1.14), manganese peroxidase (MnP, EC 1.11.1.13) and Cu-containing laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) (review by Hatakka 2001). A new group of ligninolytic heme-containing peroxidases, combining structural and functional properties of the LiPs and MnPs, are the versatile peroxidases (VPs). The VPs are capable of oxidation of $Mn^{2+}$ and phenolic compounds, as well as nonphenolic aromatic compounds such as veratryl alcohol. These types of peroxidase were isolated from the white-rot fungi *Pleurotus eryngii* (Camarero *et al.* 1999), *Pleurotus ostreatus* (Cohen *et al.* 2001), *Bjerkandera adusta* (Heinfling *et al.* 1998, Wang *et al.* 2003) and *Bjerkandera* sp. BOS55 (Mester and Field 1997, Palma *et al.* 2000). *Lentinula edodes* Mn-dependent peroxidase also oxidizes veratryl alcohol (D'Annibale *et al.* 1996), while MnP from *Panus tigrinus* is able to degrade nonphenolic lignin model compounds (Maltseva *et al.* 1991). In addition, enzymes involved in hydrogen peroxide production such as glyoxal oxidase (GLOX) and aryl alcohol oxidase (AAO) (EC 1.1.3.7) are considered to belong to the ligninolytic system.

The lignin-degrading enzymes known so far are extracellular and nonspecific, participating in different oxidative reactions where the aromatic structure of lignin and bonds between the basic units are broken (Eriksson *et al.* 1990, Orth and Tien 1995, Kuhad

**Table 1.1** Ligninolytic enzymes and their main reactions (adapted from Hatakka 2001).

| Enzyme and abbreviation | Cofactor | Substrate, mediator | Reaction |
|---|---|---|---|
| Lignin peroxidase, **LiP** | $H_2O_2$ | Veratryl alcohol | Aromatic ring oxidized to cation radical |
| Manganese peroxidase, **MnP** | $H_2O_2$ | Mn, organic acids as chelators, thiols, unsaturated fatty acids | Mn(II) oxidized to Mn(III); chelated Mn(III) oxidizes phenolic compounds to phenoxyl radicals; other reactions in the presence of additional compounds |
| Versatile peroxidase, **VP** | $H_2O_2$ | Mn, veratryl alcohol, compounds similar to LiP and MnP | Mn(II) oxidized to Mn(III), oxidation of phenolic and non-phenolic compounds, and dyes |
| Laccase | $O_2$ | Phenols, mediators, e.g., hydroxybenzotriazole or ABTS | Phenols are oxidized to phenoxyl radicals; other reactions in the presence of mediators |
| Glyoxal oxidase, **GLOX** | | Glyoxal, methyl glyoxal | Glyoxal oxidized to glyoxal acid; $H_2O_2$ production |
| Aryl alcohol oxidase, **AAO** | | Aromatic alcohols (anisyl, veratryl alcohol) | Aromatic alcohols oxidized to aldehydes; $H_2O_2$ production |
| Other $H_2O_2$-producing enzymes | | Many organic compounds | $O_2$ reduced to $H_2O_2$ |

*et al.* 1997). The resulting small-molecular-weight compounds can then be transported inside the cell for further breakdown by fungi and also bacteria. Cell-free mineralization of synthetic $^{14}C$-labelled and natural lignin by one of the ligninolytic peroxidases MnP, was first reported by Hofrichter *et al.* (1999). This may point to the extracellular mineralization (outside the fungal cell) of lignin.

***Manganese peroxidases***

The MnPs (EC 1.11.1.13) are heme-containing peroxidases that are produced by lignin-degrading fungi. MnPs oxidize phenolic compounds to phenoxy radicals by oxidation of Mn(II) to Mn(III) with $H_2O_2$ as an oxidant. Mn(III) is chelated by organic acids (e.g. oxalate or malate in nature). Chelated Mn(III) oxidizes phenolic lignin compounds to phenoxy radicals that degrade spontaneously (Hofrichter 2002). MnP oxidizes a wide range of compounds from lignin to polycyclic aromatic hydrocarbons (PAHs; Steffen 2003). The MW of extracellular fungal MnPs varies from 40 to 50 kDa and the isoelectric point (p*I*) is usually acidic (p*I* 3-4), but neutral MnPs have also been found.

***Laccases***

The laccases (EC 1.10.3.2, benzenediol:oxygen oxidoreductase) are multicopper phenol oxidases, that oxidize phenolic compounds to phenoxyl radicals. In the presence of a mediator such as 2,2'-azinobis(3-ethylbenzthiazoline-6-sulphonate) (ABTS) or 1-hydroxybenzotriazole, laccases are capable of oxidation of nonphenolic compounds (Eggert *et al.* 1996a). Laccases were first isolated from plants but are also present in fungi and some bacteria (Thurston 1994). In plants laccases participate in formation of lignin by polymerization reactions. Fungal laccases contribute to several processes such as lignin degradation, sporulation, pigment production, fruiting body formation and plant pathogenesis (Thurston 1994, Mayer and Staples 2002). Laccases are usually the first ligninolytic enzymes secreted to the surrounding media by the fungus. The typical MW of fungal laccase is 60-80 kDa and the isoelectric point is between p*I* 3 and p*I* 4, but variations on these values occur.

***Lignin peroxidases***

The LiPs (EC 1.11.1.14) are heme-containing peroxidases. They were first found in the lignin-degrading fungus *Phanerochaete chrysosporium*. LiPs catalyse the oxidation of nonphenolic aromatic compounds. The resulting cation radicals are further decomposed chemically (review by Conesa *et al.* 2002, Martínez 2002).

### 1.1.3 White-rot fungi

The only organisms reported to degrade lignin efficiently are the white-rot fungi that under natural conditions mostly colonize dead or living wood (Eriksson *et al.* 1990). Wood-degrading fungi are divided in to 3 groups based on the type of rot they cause in wood: white-rot, brown-rot and soft-rot fungi. White-rot fungi attack the lignin component of wood and leave the cellulose and hemicellulose less affected. Those white-rot fungi that degrade lignin rather than cellulose, are called selective degraders. Selective lignin degraders are especially interesting from the standpont of biotechnological applications, since they remove lignin and leave the valuable cellulose intact. Lignin degradation by these fungi is thought to occur during secondary metabolism and typically under nitrogen starvation. However, a wide variety of lignin degradation efficiency and selectivity abilities, enzyme patterns and substrates enhancing lignin degradation are reported from these fungi (reviewed by Hatakka 2001, Hofrichter 2002).

Combinations of extracellular ligninolytic enzymes (LiP, MnP and laccase) are important for lignin degradation. Based on their ligninolytic enzyme patterns, wood-rotting fungi can be divided in to 3 groups (Hatakka 1994): 1. LiP-, MnP- and laccase-producing, 2. MnP- and laccase-producing and 3. LiP- and laccase-producing fungi. The most common group among the white-rot fungi is the MnP- and laccase-producing group (Hatakka 2001). A total of 6 LiP and 4 MnP isoenzymes have been characterized, some only on lignocellulose-containing media, from the best studied selective lignin degrader, the white-rot fungus *Phanerochaete chrysosporium* (Farrell *et al.* 1989, Stewart and Cullen 1999). *P. chrysosporium* has not been reported to produce laccase, although other selective degraders of wood produce a combination of MnP and laccase: *Pleurotus ostreatus* (Becker and Sinitsyn 1993, Martínez *et al.* 1994, Giardina *et al.* 1996), *Pleurotus eryngii* (Martínez *et al.* 1996, Muñoz *et al.* 1997) and *Dichomitus squalens* (Eriksson *et al.* 1990, Périé *et al.* 1996, 1998); a combination of MnP, LiP and laccase: *Phlebia radiata* (Lundell and Hatakka 1994) or only laccase: *Pycnoporus cinnabarinus* (Eggert *et al.* 1996b). *P. radiata* produces at least 3 LiPs, 3 MnPs and one laccase (Niku-Paavola *et al.* 1988, Moilanen *et al.* 1996).

### 1.1.4 Litter-decomposing fungi (LDFs)

The litter-decomposing fungi (LDFs) are those basidiomycetous and ascomycetous fungi that together with bacteria, other fungi and animal population participate in the decomposition of leaf litter to $CO_2$ and humus (Dix and Webster 1995). Their normal habitat is the ground litter layer of soil and the humus layer of forest and grasslands. Vartiovaara (1935) had already emphasized the remarkable role played by higher fungi in lignin degradation in soil. The basidiomycetous LDFs mostly belong to the families *Agaricaceae*, *Bolbitiaceae, Coprinaceae*, *Strophariaceae* and *Tricholomataceae* (Steffen 2003).

LDFs are able to degrade lignin and radiolabelled lignin model compounds (Durrant *et al.* 1991, Steffen *et al.* 2000). The overall lignin degradation rate by these fungi is, however, lower compared with that of white-rot fungi. The actual natural lignin degradation of LDFs may differ from results obtained with axenic cultures and with lignin model compounds since natural litter contains various compounds and organisms not present in defined media. Information regarding ligninolytic enzyme production and profiles of LDFs is still scarce compared with that on white-rot fungi.

Table 1.2 shows the properties of ligninolytic enzymes found in LDFs. The main ligninolytic enzymes produced by litter decomposers thus far studied are MnP and laccase (Hatakka 2001, Steffen 2003). The LDFs *Marasmius quercophilus* (Dedeyan *et al.* 2000) and *Volvariella volvacea* (Chen *et al.* 2004a,b) were reported to produce laccase, but no MnP has yet been found. *Coprinus cinereus, Coprinus friesii* and *Coprinus macrorhizus* are known to produce laccase and a peroxidase functionally and structurally resembling the well-known plant peroxidase, horse-radish peroxidase (Morita *et al.* 1988, Baunsgaard *et al.* 1993, Heinzkill *et al.* 1998).

### 1.1.5 Ligninolytic enzyme production in defined and natural media

Ligninolytic fungi have usually been cultured in defined liquid media low in N (Kirk *et al.* 1978) or in C (Haapala and Linko 1993) and containing supplements such as Mn, veratryl alcohol and Tween (Hatakka 2001). Lignin model compounds such as dimeric *β*-O-4 model compounds and synthetic lignin (dehydrogenation polymerizate, DHP) have been used in liquid cultures to study lignin mineralization or enzyme activities. The

**Table 1.2** Ligninolytic enzymes produced by litter-decomposing fungi. The Finnish name of the fungus is in parenthesis.

| Fungus | Enzymes | Properties p*I* | MW (kDa) | Reference |
|---|---|---|---|---|
| *Agaricus bisporus* (herkkusieni) | Two laccases<br>MnP | 3.4-4.0<br>3.5 | 65<br>n.r. | Perry *et al.* 1993a,b, Wood 1980b<br>Bonnen *et al.* 1994 |
| *Agrocybe praecox* (kesäpiennarsieni) | MnP1<br>MnP2<br>Laccase | 6.3-7.0<br>6.3<br>4.0 | 42<br>42<br>66 | Steffen *et al.* 2002c |
| *Collybia dryophila* (kalpeajuurekas) | MnP1<br>Laccase | 4.7<br>n.r | 44<br>n.r. | Steffen 2002a |
| *Lepista irina* (kalvasvalmuska) | VP | 3.75 | 50.5 | Zorn *et al.* 2003 |
| *Marasmius quercophilus* (etelännahikas) | Laccase | 3.6 | 63 | Dedeyan *et al.* 2000 |
| *Mycena galopus* (maitohiippo) | MnP<br>Laccase | n.r.<br>n.r. | n.r.<br>n.r. | Ghosh *et al.* 2003 |
| *Panaeolus sphinctrinus* (harmaakirjoheltta) | Laccase<br>MnP | <3.55<br>7.2 | 60<br>42 | Heinzkill *et al.* 1998 |
| *Stropharia coronilla* (nurmikaulussieni) | MnP1<br>MnP2<br>MnP3<br>Laccase | 6.3-7.1<br>3.5, 3.7<br>5.1<br>4.4 | 41<br>41<br>43<br>67 | Steffen *et al.* 2002c |
| *Stropharia rugosoannulata* (viljelykaulussieni) | MnP1<br>MnP2<br>Laccase | 3.2<br>3.2<br>3.3-3.4 | 41<br>43<br>67 | Steffen 2003 |
| *Volvariella volvacea* (olkisieni) | Laccase | 3.7 | 58 | Chen *et al.* 2004a,b |

n.r.: not reported

ligninolytic enzyme profile produced by the lignin degrading fungi in defined culture media is different from that produced in media, where lignocellulosic substrates such as wood, sawdust, straw or other natural substrates are used (Orth *et al.* 1993). The production of MnP and LiP isoenzymes by *P. chrysosporium* is strongly affected by medium composition (Cullen 1997). A lignocellulosic substrate (wheat straw and hemp woody core) promotes the production of MnP and LiP of *P. chrysosporium* under culture conditions in which N and C are nonlimiting (Kapich *et al.* 2004). The addition of bleaching plant wastewater in culture medium changes the enzyme production and enzyme profile of *P. chrysosporium* (Presnell *et al.* 1992). New MnP isoforms were reported from *Phlebia radiata* grown on straw (Vares *et al.* 1995). *Pleurotus ostreatus* produces MnP isoenzymes on sawdust different from those in defined culture media (Giardina *et al.* 2000, Kamitsuji *et al.* 2004) and *Pleurotus eryngii* VP was first found in straw-based cultures (Camarero *et al.* 1999). Enzyme profiles produced in lignocellulose-containing cultures are more likely to resemble the situation in the natural habitat of white-rot fungi than the enzyme profiles produced in defined liquid media.

Multiple ligninolytic peroxidases are products of multiple genes and/or results of differing posttranslational modifications (Conesa *et al.* 2002). The ecological role for the large variety of MnP, LiP or laccase isoenzymes in ligninolytic fungi is not yet clear. It is

assumed that the varying enzyme profile/production results from adaptation to different natural culture conditions and substrates (Conesa *et al.* 2002). In addition, the presence of multiple ligninolytic isoenzymes may reflect the efficiency of lignin degradation.

## 1.2 Cultivated edible fungi

Mushroom production has a history of over 1000 years. The edible mushroom *Auricularia auricula-judae* was cultivated in China as early as 600 A.D. (Chang 1999). The first shiitake (*Lentinula edodes*) mushrooms were also cultivated on logs in China around 1000 A.D. (Chang and Miles 1989). About 20 mushroom species are now produced commercially (review by Sánchez 2004), the annual production being 3.2 million tonnes (FAOSTAT data 2004). *Agaricus bisporus* (cultivated mushroom, white button mushroom), *Lentinula edodes* (shiitake) and *Pleurotus ostreatus* (oyster mushroom) are the mushrooms most cultivated worldwide. In Finland 1711.5 tonnes of *A. bisporus*, 308.5 tonnes of *L. edodes* and 0.4 tonnes of *P. ostreatus* were produced in 2003 (Information Centre of the Ministry of Agriculture and Forestry 2003). Other mushroom species produced for consumption include *Agrocybe aegerita* (black poplar mushroom), *Auricularia auricula, Flammulina velutipes, Ganoderma* spp., *Grifola frondosa* (maitake, hen-of-the-woods), *Hericium erinaceus* (lion's mane, bearded tooth), *Hypsizygus marmoreus* (beech mushroom), *Lepista nuda* (wood blewit), *Pholiota nameko* (nameko, golden needle mushroom), *Stropharia rugosoannulata (*Braunkappe, wine cap, king stropharia) and *Volvariella volvacea* (Royse 1996, Chang 1999) (see Table 1.3).

Saphrophytic basidiomycetes are the main group of cultivated edible fungi. However, the fungus most commonly used by the food and brewing industries, *Saccharomyces cerevisiae* (baker's yeast) and other *Saccharomyces* yeasts, belong to the *Ascomycota*. *Fusarium graminearum*, a filamentous ascomycete originally isolated from a crop field, has been grown in bioreactors and processed to a meatlike single-cell product Quorn® in the UK (Carlile *et al.* 2001). The valuable edible wild mushrooms such as the basidiomycetes *Boletus edulis*, *Cantharellus cibarius, Tricholoma matsutake* and the ascomycetous truffles and morels belong to the mycorrhizal fungi. Their commercial cultivation is uneconomic since they need a tree host for proper growth.

Cultivated mushrooms are decayers, mostly wood-degrading white-rot or litter-degrading fungi. The most common cultivated mushrooms and their ligninolytic properties are summarized in Table 1.3. Mushroom production is so far the most profitable way of utilizing lignocellulose-containing waste material (Carlile *et al.* 2001). Waste such as straw, sawdust, bran, corncobs, cotton stalks etc. from the agricultural, animal husbandry and manufacturing industries are used as substrates for mushroom production. Spent mushroom compost can be used as cattle feed, fertilizer or landfill (Cohen *et al.* 2002). *Pleurotus* spp. compost was used for bioaugmentation of tar-contaminated soils (Hestbjerg *et al.* 2003) and for experimental remediation of PAH-contaminated samples (Lau *et al.* 2003).

Cultivated edible mushrooms are a good source of nutrients and vitamins (Mattila *et al.* 2001). An average mushroom contains 10% dry matter of which 27-48% is protein (Sánchez 2004). In addition to the nutritional value, mushrooms are a good resource of bioactive compounds. Medical usage of mushrooms is widespread, especially in Asia. Species such as *Ganoderma* spp., *Lentinula edodes* and *Inonotus obliquus* have been used for medicinal purposes for hundreds of years (Wasser 2002). Fungal polysaccharides are the best-known mushroom substances to have been reported, possessing antitumour and immunomodulating properties (review by Wasser 2002).

### 1.2.1 *Agaricus bisporus* (white button mushroom, cultivated mushroom)

*A. bisporus* is economically the most important mushroom worldwide. It is the most common cultivated mushroom in Europe, North America, China and Australasia, with an annual production of 1.9 million tonnes (Chang 1999). *Agaricus bisporus* belongs to the family *Agaricaceae*, order *Agaricales* and subclass *Agaricomycetidae* (Kirk *et al.* 2001). It is a litter-decomposing basidiomycete that in nature usually grows on grassland and forest. Other *Agaricus* species such as *A. campestris* and *A. arvensis* are frequently seen on lawns and roadsides. The genome of *A. bisporus*, with an estimated size of 40 Mb in 13 chromosomes, has been nominated for sequencing by The Fungal Genome Initiative Steering Committee (Birren *et al.* 2003).

### 1.2.2 Cultivation of *A. bisporus*

Cultivation of *Agaricus bisporus* was first begun in France around the year 1650. Underground caves and cellars were found suitable for its cultivation, whereas today *A. bisporus* is grown in separate buildings where aeration, temperature and humidity are carefully controlled. The substrate used for *A. bisporus* cultivation is the most complex culture medium known among the edible mushrooms (Sánchez 2004). Production of *A. bisporus* compost is a 2-stage process in which the first stage includes composting of the raw material consisting of straw, horse or poultry manure and gypsum in a controlled windrow (Wood 1984). During composting the lignocellulose waste is modified by various bacteria and fungi to a better-digested form suitable for *A. bisporus*. The compost is pasteurized before inoculation with *A. bisporus* spawn. After growth of the mycelia for 2-3 weeks, a casing layer containing peat moss or soil with limestone is spread on the top of the compost. The temperature, humidity and $CO_2$ level are then adjusted to favour the development of mushrooms. The first pin initials begin to appear about 2 weeks after casing. One layer of compost produces 2-4 crops.

Production of *A. bisporus* is time-consuming due to the lengthy composting stage. The challenge in improving *A. bisporus* culture medium is to produce *A. bisporus* on a noncomposted substrate (review by Sánchez 2004). This innovation would greatly reduce the cultivation time and cost of mushroom production.

### 1.2.3 Breeding of *A. bisporus*

*A. bisporus* has been studied widely, since it is an economically important species. Research has focused on areas concerning productivity and quality of the mushroom. Currently the main targets in *A. bisporus* breeding research are:

- Genetic diversification to reduce the risks caused by monoculture
- Increased productivity and quality via strain development
- Improved utilization of compost nutrients, upgrading compost preparation
- Improved resistance to pathogens (bacterial, viral and fungal)

Strain improvement of *A. bisporus* thus far has been based on conventional breeding methods (Khush *et al.* 1995, Sonnenberg 2000). The latest increase in *A. bisporus* production was gained mainly by improving culture conditions rather than by obtaining better strains (review by Kothe 2001). Detection and development of genetic markers as well as the identification of mating-type genes have been an important step in developing the techniques for *Agaricus* breeding (Stoop and Mooibroek 1999, Sonnenberg 2000, Kothe 2001). The shortage of methods suitable for introducing genetic material into fungi

**Table 1.3** The most common cultivated edible mushrooms and their lignin-degrading properties. The Finnish names are in parentheses.

| Fungus | Decay type[1] | Main substrate for cultivation | Ligninolytic enzymes [2] | Reference |
|---|---|---|---|---|
| ***Agaricus bisporus*** *A. brunnescens, A. bitorquis* Cultivated mushroom, button mushroom (herkkusieni) | l | Straw and manure compost | laccase<br>MnP | Wood 1980a,b<br>Bonnen *et al.* 1994 |
| ***Lentinula edodes*** Shiitake (siitake) | w-r | Oak logs, sawdust- and bran-based logs | MnP<br>laccase | Forrester *et al.* 1990<br>Nagai *et al.* 2002 |
| ***Pleurotus ostreatus*** Oyster mushroom (osterivinokas) | w-r | lignocellulosic waste, cereal straw, sawdust, bagasse, waste cotton | laccase<br>VP<br>MnP | Giardina *et al.* 1996<br>Cohen *et al.* 2001<br>Becker and Sinitsyn 1993, Giardina *et al.* 2000 |
| ***Volvariella volvacea*** Paddy straw mushroom (viljelytuppisieni, olkisieni) | l | straw, water hyacinth, oil palm, banana, cotton or wood waste. | laccase | Chen *et al.* 2004a,b |
| ***Auricularia auricula*** Wood ear, jew's ear (juudaksenkorva) | w-r | logs composed of sawdust/bran or woodchips/bran | MnP and laccase (from *Auricularia* sp.) | Hofrichter and Fritsche 1997 |
| ***Flammulina velutipes*** Enoki, golden needle, velvet shank (talvijuurekas) | w-r | mixture of sawdust and rice bran | n.r. | |

[1]l : litter-degrading
w-r: white-rot
[2] MnP: manganese peroxidase
VP: versatile peroxidase
n.r.: not reported

restrained the molecular breeding until successful transformation of *A. bisporus* mycelia by *Agrobacterium tumefaciensis* was achieved (de Groot *et al.* 1998, Chen *et al.* 2000). Genetic engineering as a method for improving mushroom strains used for human food consumption faces public resistance. Production of bioactive or medically useful compounds is the most probable use for genetic engineering of basidiomycetous fungi.

### 1.2.4 Lignin degradation by *A. bisporus*

Lignin degradation by the litter decomposer *A. bisporus* was first reported by Waksman and Nissen (1932), who found that the lignin component of straw-based compost decreases towards the end of the mushroom production cycle. Wood and Leatham (1983) and also Durrant *et al.* (1991) showed that the lignin content in compost decreases from the time of spawning to the production of pin initials. Lignin degradation by *A. bisporus* is lowest during fruiting-body maturation.

Two ligninolytic enzymes, laccase and MnP, are secreted into compost by *A. bisporus* (Wood 1980a, Bonnen *et al.* 1994). Laccase forms about 2% of the total protein produced by the mycelia (Wood 1980b). Perry *et al.* (1993b) isolated 2 genes from *A. bisporus*

encoding differently expressed laccases (Smith *et al.* 1998). LiP has not been reported from *Agaricus*. Other lignocellulose-degrading enzymes described from *A. bisporus* include the endoglucanases and cellobiohydrolases (Raguz *et al.* 1992, Chow *et al.* 1994, Yagüe *et al.* 1994), xylanases (endoxylanase and ß-xylosidase) (Whiteford *et al.* 2000) and ß-mannanase (Tang *et al.* 2001).

## 1.3 Fungal applications in biotechnology (mycobiotechnology)

In addition to their important ecological role in carbon cycling, fungi have a history in biotechnology going back at least 3000 years. Currently, the roles played by fungi are indispensable to human civilization. Fig. 1.3 summarizes the beneficial and deleterious activities of fungi in relation to humans.

Brewing, winemaking, baking, cheesemaking and preparation of other fermented foodstuffs such as miso, soy sauce and tempeh together with mushroom cultivation for food are traditional applications of fungal biotechnology. When it was understood that micro- and macrofungi are a separate group of living organisms and cultivation techniques were developed, more sophisticated applications appeared. Production of antibiotics and other pharmaceuticals (penicillins, cephalosporins, cyclosporins, griseofulvin, mevalonin) (Endo and Hasumi 1997, Martin *et al.* 1997, Schreier 1997), enzymes (amylase, cellulase, glucose oxidase, invertase, laccase, lipase, pectinase, phytase, proteinases and rennin) and organic acids (citric acid and itaconic acid) by fungi were introduced (Carlile *et al.* 2001). Development of recombinant DNA technologies has modified traditional reaction routes and production-organisms. Heterologous expression systems have been introduced to aid in the manufacture of recombinant proteins and bioactive compounds (review by Bennet 1998). Production of antibiotics and enzymes for the textile and food industries is currently the aspect of fungal biotechnology demonstrating the highest economic value.

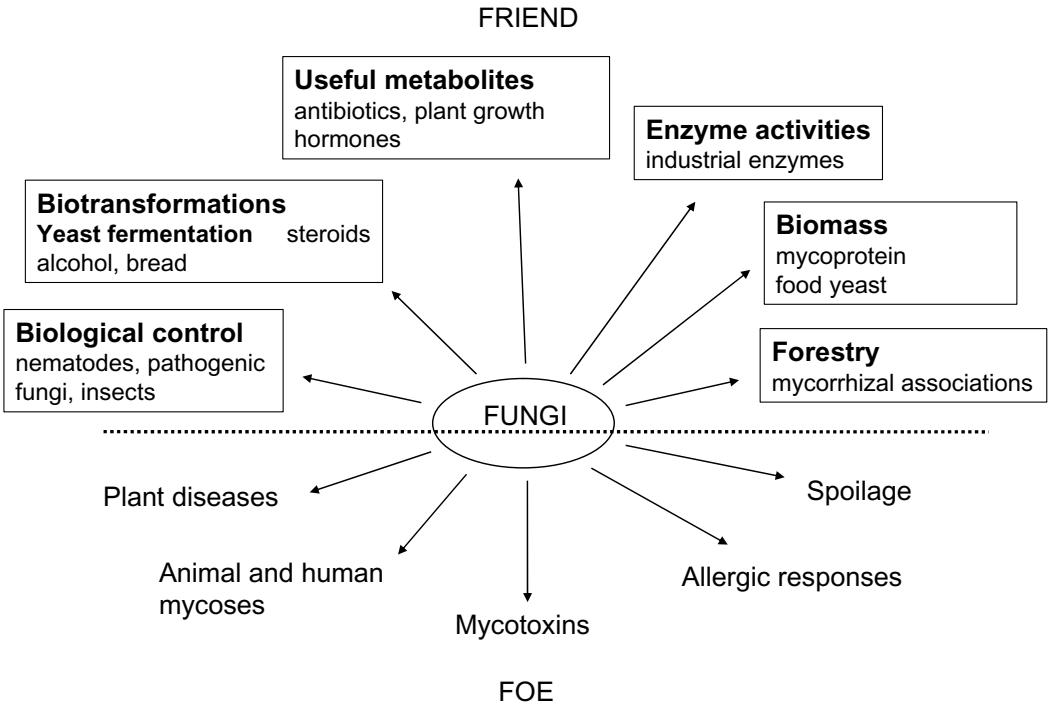



**Fig. 1.3** Activities of fungi in relation to humans (adapted from Moss in Alexopoulos *et al.* 1996).

Most biotechnological processes involving fungi exploit the yeasts or filamentous microfungi (ascomycetes, mitosporic fungi). Only a few products, such as edible mushrooms and ligninolytic enzymes, originate from basidiomycetous fungi. In addition, basidiomycetous fungi produce a variety of interesting secondary metabolites noted for their odours, tastes, colours or toxic properties (Alexopoulos *et al.* 1996). The traditional uses of some fungi, e.g. *Ganoderma lucidum*, *Lentinula edodes* and *Inonotus obliquus*, as pharmaceuticals have encouraged scientists to search for bioactive compounds from these and other basidiomycetous fungi (review by Wasser 2002).

### 1.3.1 Fungal enzymes of biotechnological interest

Under natural conditions fungi are able to grow on many substrates difficult to exploit by more rapidly growing organisms such as bacteria. Growth on complex substrates is based on the secretion of extracellular enzymes (e.g. ligninolytic enzymes). Fungal enzymes of biotechnological interest are shown in Table 1.4.

**Table 1.4** Fungal enzymes for biotechnological applications (based on Bennet 1998).

| Enzyme | Fungus | Application |
|---|---|---|
| Amylase | *Aspergillus niger, Aspergillus oryzae* | Malting enzyme, starch industry, bioethanol production |
| Catalase | *A. niger, Penicillium* spp. | Many applications |
| Cellulase | *Trichoderma reesei, A. niger, Humicola insolens, Trichoderma viride, Penicillium funiculosum* | Textile industry (stone washing, biopolishing), pulp and paper industry, food and feed industry, bioethanol production |
| Dextranase | *Penicillium* spp. | Food industry |
| Glucanase | *A. niger, Penicillium emersonii, T. reesei, T. viridae* | Brewing industry |
| Glucoamylase | *A. niger, A. oryzae, Aspergillus phoenicis, Rhizopus delemar, Rhizopus niveus* | Alcohol industry, starch industry |
| Glucose oxidase | *A. niger, Penicillium* spp. | Baking industry |
| Hemicellulase | *A. niger, A. oryzae, T. reesei, T. viridae, P. emersonii* | Pulp bleaching, baking industry, bioethanol production |
| Invertase | *A. niger, A. oryzae* | Food industry |
| Laccase | *Coriolus versicolor* | Textile industry (stone washing), pulp and paper industry, treatment of olive oil mill wastewaters, (Mayer and Staples 2002) |
| Lipase | Several species | Laundry detergents |
| Pectinase | Several species, *A. niger, Rhizopus oryzae, H. insolens* | Food processing industry, wine industry |
| Proteases | Several species, *A. niger, A. oryzae* | Detergents, ¼ of world enzyme market (Tobin *et al.* 1999) |
| Rennet | *Mucor miehei, Mucor pusillus, Endothia parasitica* | Cheesemaking |
| Tannase | *A. niger, A. oryzae* | Food industry (tea processing) |
| Xylanase | *A. niger, T. reesei* | Biobleaching |

### 1.3.2 Biotechnology of ligninolytic fungi and their enzymes

The ligninolytic enzymes except laccase were not listed in Table 1.4, because the commercial applications of MnP and LiP enzymes have not yet been developed. Some preparations such as LiP from *Phanerochaete chrysosporium* and *Phlebia radiata* and MnP from *P. chrysosporium* and *Nematoloma frowardii* are on the market for research purposes. An engineered form of *Coprinus cinereus* peroxidase was successfully produced in a heterologous system (Cherry *et al.* 1999) and sold as a bleaching agent for laundry detergents. Pure enzymes, crude enzyme preparations and cultures of ligninolytic fungi have been widely studied for biopulping (review by Messner and Srebotnik 1994), degradation of toxic compounds (review by Kremer and Anke 1997) and dye removal (review by Shah and Nerud 2002). These potential applications are based on the concomitant and nonspecific action of the fungal ligninolytic system (review by Pointing 2001, Ralph and Catcheside 2002). Pure enzymes have proved to be less effective for applications based on the use of ligninolytic activity than ligninolytic culture preparations. Table 1.5 lists some studies of ligninolytic fungi and their enzymes undertaken to determine their use in biotechnological applications.

Bleaching of industrial effluent and dye colours by white-rot fungi and their ligninolytic enzymes is a promising biotechnological application. Laccase was reported to be the enzyme responsible for decolourization of industrial dyes (Rodriguez *et al.* 1999, Mayer and Staples 2002). LiP was reported to be effective in decolourizing kraft pulp mill effluents (Ferrer *et al.* 1991). Moreira *et al.* (1998) considered MnP as the most important enzyme in biobleaching experiments with *Bjerkandera* sp. strain BOS55. Biopulping by lignin-degrading fungi has been studied at the pilot scale and development of commercial applications is in progress (Scott *et al.* 2000). Organopollutants such as 2,4,6-trinitrotoluene (TNT), polychlorinated biphenyls (PCBs), organochlorines, PAHs and wood preservatives were shown to be degraded by white-rot fungi (review by Pointing 2001).

The use of elemental chlorine and hypochlorite in pulp bleaching has caused environmental problems in the pulp and paper industry. High-molecular-weight chlorolignins are formed when active chlorine reacts with lignin during pulp bleaching (Kringstad and Lindström 1984). These chlorolignins are degraded extremely slowly under natural conditions (Kähkönen *et al.* 1998). The ecological implications of their accumulation in the sediments of recipient waters are not fully understood. White-rot fungi and their ligninolytic enzymes decolourize and degrade organic chlorine-containing material (measured as AOX, adsorbable organic halogen) from pulp mill wastewaters (Eriksson and Kirk 1985). In the 1980s methods called MYCOR (Eaton *et al.* 1981) and MYCOPOR (Messner *et al.* 1989) were developed to remove colour but they also decreased the total organic chlorine from pulp mill wastewaters. These processes were based on the white-rot fungus *P. chrysosporium*. Dehalogenation of organohalogen compounds was also reported in anaerobic pulp mill wastewater treatment (Jokela *et al.* 1993). Research on that topic in Finland was active in the late 1980s and early 1990s until the Finnish pulp industry in 1993 changed to the use of oxygen, enzymes and chlorine dioxide instead of elemental chlorine and hypochlorite in the bleaching processes (Suominen *et al.* 1999). Elemental chlorine is still used for pulp bleaching in the developing countries and also by some pulp manufactures in the USA and Canada, although most of the bleaching processes in North America are currently based on elemental chlorine-free (ECF) bleaching.

The potential for white-rot fungi and their ligninolytic enzymes has not yet been exploited. Factors limiting the biotechnological use of ligninolytic enzymes, especially

**Table 1.5** Potential biotechnological applications of ligninolytic fungi and their enzymes.

| Application | Fungus | Reference |
|---|---|---|
| Biopulping | *Ceriporiopsis subvermispora* | Messner and Srebotnik 1994, Akhtar *et al.* 1998 |
| | *Physisporinus rivulosus* T241i | Maijala *et al.* 2002, Hakala *et al.* 2004 |
| Decolourisation of synthetic dyes and dye industry waste waters | *Phanerochaete chrysosporium, Trametes versicolor, T. cingulata, T. hispida, T. pocas, Datronia concentrica, Bjerkandera sp.* BOS55*, Phanerochaete betulinus, Bjerkandera adusta, Pycnoporus cinnabarinus, P. sanguineus, Coriolus hirsutus, Funalia trogii, Irpex lacteus, Pleurotus ostreatus, P. sajor-caju, P. pulmonarius* | Review by Shah and Nerud 2002 and review by Wesenberg *et al.* 2003, Trupkin *et al.* 2003 |
| Biobleaching | *Phanerochaete sordida* | Kondo *et al.* 1994 |
| | *Trametes versicolor* | Archibald *et al.* 1997 |
| | *Bjerkandera* sp. BOS55 | Moreira *et al.* 1998 |
| Treatment of pulp mill waste waters (decrease of colour and organic halogen) | *Trametes elegans, Phlebia radiata, Panus crinitus, Trametes villosa, Phanerochaete chrysosporium* | Durán and Esposito 2000, Soares and Durán 2001, Lara *et al.* 2003 |
| Biodegradation of toxic compounds | *Pleurotus pulmonarius, Pleurotus ostreatus* | Review by Pointing 2001, Lau *et al.* 2003, Hestbjerg 2003 |
| Improvement of digestibility of lignocellulosic animal feed | *Pleurotus ostreatus, Ganoderma applanatum* | Zadrazil 1977, Hatakka *et al.* 1989 |

the peroxidases, have included their low production level and rather low stability (review by Conesa *et al.* 2002, Ikehata *et al.* 2004). Peroxidases also need the addition of $H_2O_2$ for their catalytic cycle. Heterologous expression of ligninolytic enzymes has been attempted, using several recombinant systems with various degrees of success (review by Cullen 1997, Conesa *et al.* 2002). The most successful approaches have been those using laccase; moreover, laccase is produced constitutively by many fungi and the homologous production of laccase may also be economically feasible (Lomascolo *et al.* 2003). The large-scale production of ligninolytic peroxidases is still under development. The challenge is to move from laboratory production to industrial-scale and economically feasible applications.

## 1.4 Molecular genetics of ligninolytic fungi

The white-rot fungus *P. chrysosporium* is the most studied ligninolytic fungus. The first LiP (Tien and Tu 1987) and MnP (Pribnow *et al.* 1989) were isolated and cloned from this fungus. In all 10 LiP genes in a cluster and 5 MnP genes were identified from the complete genome of *P. chrysosporium* but not all are expressed (Martinez *et al.* 2004). The first VPs were cloned from *Pleurotus eryngii* (Camarero *et al.* 2000). A versatile-type peroxidase-encoding gene from the LDF *Lepista irina* was reported by Zorn *et al.* (2003). Numerous laccase genes have been cloned from lignin-degrading fungi (review by Mayer and Staples

2002, comparison by Valderrama *et al.* 2003). The laccases appear to be encoded by complex families of structurally related genes (Cullen 1997). Laccase genes characterized from basidiomycetous LDFs include the 2 laccase genes of *A. bisporus* (Perry *et al.* 1993b), 3 laccase genes from *Coprinus cinereus* (Yaver *et al.* 1999) and that of *Volvariella volvacea* laccase (Chen *et al.* 2004b). White-rot fungus *Phlebia radiata*, used in the present work, has a LiP (Saloheimo *et al.* 1989), a laccase (Saloheimo *et al.* 1991) and 2 MnP (Hildén *et al.* 2004) genes identified.

Despite the increasing amount of information available on the genetic properties of the ligninolytic enzymes of the lignin degrading fungi, the relationship between expression of the genes and activity of the corresponding enzymes is not well understood. Regulation of ligninolytic enzyme production is assumed to occur mostly at the transcriptional stage (Ruiz-Dueñas *et al.* 1999a, Cohen *et al.* 2002). The factors inducing ligninolytic enzyme production include growth medium composition (high or low N, C or S concentrations, addition of Mn and veratryl alcohol or lignocellulose), growth in the dark or light, heat shock and oxidative or chemical stress (Conesa *et al.* 2002, Martínez 2002, Valderrama *et al.* 2003).

### 1.4.1 Molecular properties of ligninolytic enzymes

The molecular and genetic properties of ligninolytic enzymes were reviewed by Cullen (1997), Conesa *et al.* (2002) and Martínez (2002). Sequence data together with functional analysis of ligninolytic enzymes have revealed structural characteristics and conserved elements in peroxidases and laccases (Martínez 2002). Several 3-D models based on the amino acid sequence and crystal structures showed that all fungal extracellular peroxidases have similar structural elements such as disulphide bridges, glycans and structural calcium (Sundaramoorthy *et al.* 1994, Conesa *et al.* 2002, Martínez 2002). They all contain a single high-spin protoporphyrin IX (heme *b*) as the prosthetic group (Conesa *et al.* 2002). Iron coordination with proximal and distal histidines and the active site residues are conserved among most peroxidases (Conesa *et al.* 2002, Martínez 2002). Fig. 1.4 shows the 3-D homology model of the MnP3 isoenzyme of *Phlebia radiata* with common peroxidase elements (Hildén *et al.* 2004). In LiP protein the aromatic substrate-binding residue includes tryptophan (W171), which in MnPs is replaced by alanine or serine (Martínez 2002). A tryptophan is found at the same site in the veratryl alcohol-oxidizing VP from *P. eryngii*.

The multitude and short lengths of the introns is a typical characteristic for fungal peroxidase genes (Cullen 1997, Martínez 2002). Conservation in the intron-exon structure of peroxidases within clusters of ligninolytic fungi has been reported (Martínez 2002, Hildén *et al.* 2004). Peroxidases, like the other extracellular enzymes, are synthesized with an N-terminal signal peptide targeting them to the secretory pathway. This signal peptide and an additional propeptide found in some peroxidases (*P. chrysosporium*, *P. radiata* and *P. ostreatus*) are removed when the protein is secreted (Conesa *et al.* 2002).

The conserved features typically found in laccase structures include copper-binding sites (Kumar *et al.* 2003). The number of copper atoms per enzyme molecule can vary among the different laccases (Giardina *et al.* 1999). The catalytic site is also conserved through all laccases (Mayer and Staples 2002, Valderrama *et al.* 2003). Crystalline structures are available from laccases of the basidiomycetous fungi *Coprinus cinereus* and *Trametes versicolor* (Ducros *et al.* 1998, Piontek *et al.* 2002) and an ascomycetous fungus *Melanocarpus albomyces* (Hakulinen *et al.* 2002).

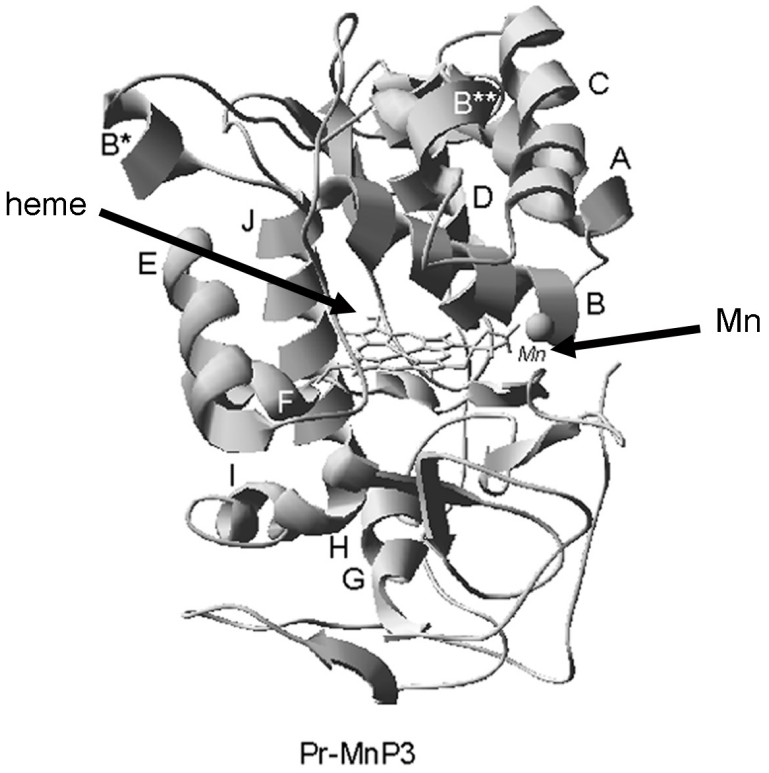



**Fig. 1.4** Molecular structure of *Phlebia radiata* MnP3. The position of the prosthetic heme group and $Mn^{2+}$ in the substrate-binding site are shown by the arrows (adapted from Hildén *et al.* 2004 with permission of the authors).

### 1.4.2 Fungal genomes

Knowledge of fungal genetics has been based on studies with the yeast *Saccharomyces cerevisiae* and filamentous microfungi such as *Trichoderma reesei, Aspergillus* spp. and *Neurospora crassa. Schizophyllum commune* has been used as a model organism in studies on the genetics of basidiomycetes. Genetic tools have provided new insight, especially on fungal taxonomy and the relationships of different species (Moncalvo *et al.* 2000). Total genomic sequences are available (June 2004) from 12 fungal species (Table 1.6), 4 of which are basidiomycetes: *Coprinus cinereus*, *Cryptococcus neoformas*, *Ustilago maydis* and *Phanerochaete chrysosporium*. *P. chrysosporium* is the only white-rot fungus from which the total genomic sequence is available (Martinez *et al.* 2004). Its genome size is 29.9 Mbp with a predicted number of 11 777 protein-encoding genes, which is a typical size and number of genes for fungi (Martinez *et al.* 2004). *C. cinereus* (inkcap) is a good model organism for development and regulation studies of basidiomycetes since it can complete its entire life cycle in the laboratory. *U. maydis* is a plant pathogen as are most of the other fungi for which the total genomic sequence is available. *C. neoformans* is a human pathogen, infecting especially immunosuppressed patients such as those with AIDS. The number of genomic sequences available from fungi is increasing rapidly; *Aspergillus fumigatus* and *Trichoderma reesei* will probably be the next fungi where genomic sequences will be made available.

**Table 1.6** Total genomic sequences available from fungi (June 2004).

| Fungus | Significance of the fungus | Reference / GenBank accession number |
|---|---|---|
| **Basidiomycetes** | | |
| *Coprinus cinereus* | Model for developmental and regulation studies, genetic source of commercial peroxidase | http://www.broad.mit.edu/annotation/fungi/coprinus_cinereus AACS01000000 |
| *Cryptococcus neoformans* | Human pathogen | AACO01000000 |
| *Phanerochaete chrysosporium* | White-rot fungus, potential applications in the pulp and paper industry and in bioremediation, source of potential secondary metabolites | Martinez *et al.* 2004 http://genome.jgi-psf.org/whiterot1/whiterot1.home.html |
| *Ustilago maydis* | Maize pathogen | http://www-genome.wi.mit.edu/annotation/fungi/ustilago_maydis/index.html AACP01000000 |
| **Ascomycetes, yeasts and mitosporic fungi** | | |
| *Aspergillus nidulans (Emericella nidulans)* | Model organism for fungal genetics, industrial significance | http://www-genome.wi.mit.edu/annotation/fungi/aspergillus/ AABX01000000 |
| *Candida albicans* | Yeast, pathogen | Jones *et al.* 2004 |
| *Fusarium graminearum (Gibberella zeae)* | Wheat and barley pathogen | AACM01000000 |
| *Magnaporthe grisea* | Rice pathogen | AACU01000000 |
| *Neurospora crassa* | Orange bread mould, model organism for fungal genetics | Galagan *et al.* 2003 AABX01000000 |
| *Podospora anserina* | Model organism of fungal genetics | http://www.genoscope.cns.fr/externe/English/Projets/Projet_GA/GA.html |
| *Saccharomyces cerevisiae* | Yeast, baking, beer, fermenting etc., model organism for fungal genetics | Goffeau *et al.* 1996 |
| *Schizosaccharomyces pombe* | Fission yeast | Wood *et al.* 2002 |

# 2. OBJECTIVES OF THE PRESENT STUDY

## 2.1 Background

Understanding the biochemistry of lignin-degrading fungi is needed for optimizing current and potential biotechnological applications such as improving the propagation of edible mushrooms or wastewater treatment by fungi. Factors such as the age of the culture, the growth substrate, culturing conditions and the composition of the media influence the production and profiles of extracellular ligninolytic enzymes. Ligninolytic enzymes are usually produced by lignin-degrading fungi in defined liquid media. However, inexpensive carbon sources are available for the production of fruiting bodies or enzymes. To examine the enzymes and isoenzymes produced during growth on natural substrates, ligninolytic fungi should be cultivated on lignocellulose-containing media (wood, straw, bran).

The biological role of LDFs in soil C cycling is well recognized. How, the lignin-degradation system of basidiomycetous LDFs degrades litter is not yet well understood. The economically most important litter degrader, *Agaricus bisporus*, is grown on lignocellulose-based compost for mushroom production. Understanding the ligninolytic system of *A. bisporus* would aid in optimizing compost composition for mushroom production.

## 2.2 Aims of the present study

The questions adressed were:

**Which ligninolytic enzymes are produced by the white-rot fungus *Phlebia radiata* and the LDF *Agaricus bisporus* during growth on lignocellulose substrates? Is there a difference between the profiles of extracellular ligninolytic enzymes in the (chemically) defined and in complex lignocellulose-containing media?**

**How to detect the activities of the ligninolytic enzymes secreted by the fungi in compost and on other complex substrates containing lignocellulose, e.g. pulp mill wastewaters?**

**Which ligninolytic enzymes are produced by the LDF *Agaricus bisporus*? Is the ligninolytic system of *A. bisporus* similar to that of the other lignin degraders? What are the enzymatic and molecular properties of MnP from *A. bisporus*?**

# 3. MATERIALS AND METHODS

## 3.1 Fungal strains used

The white-rot fungus *Phlebia radiata* 79 (ATCC 64658) was isolated originally at the Department of Microbiology (the present Department of Applied Chemistry and Microbiology), University of Helsinki (Hatakka and Uusi-Rauva 1983) and maintained on 2% malt agar slants. The compost inoculated with *A. bisporus* Horst U1 strain (III) was obtained from Dr. David Wood, Horticultural Research International, Wellesbourne, UK. *A. bisporus* ATCC 62459 (D649) was obtained from the culture collection of the Department of Applied Chemistry and Microbiology, University of Helsinki and maintained on 2% malt agar slants.

## 3.2 Experimental methods

The experimental setup as well as the methods used were described in detail (I-IV) and are summarized in Table 3.1.

***Previously unpublished data (Figs. 4.1-4.3 in the Results):***

*P. radiata* was cultivated in 2-liter aerated bioreactors in defined culture media with varying amounts of glucose and pulp mill effluent. The experimental setup was based on the central composite design to determine the optimal concentrations of glucose and pulp mill effluent needed for growth and for colour and AOX removal of *P. radiata*. Other methods were those described in the article (I).

**Table 3.1** Methods used in this study.

| Method | Described in article no. |
|---|---|
| **Liquid cultivation of fungi** | **I, III, IV** |
| bioreactor and fermenter cultivations | **I** |
| **Solid-state (compost) cultivation of *Agaricus bisporus*** | **II, III** |
| **Analytical methods** | |
| protein determination | **I - III** |
| glucose determination | **I** |
| AOX determination | **I** |
| colour determination | **I** |
| **Spectrophotometric measurements** | |
| enzyme activities (enzyme assays) | **I - IV** |
| enzyme properties (spectrum) | **II, III** |
| **Enzyme purification** | |
| ultrafiltration | **III, IV** |
| fast protein liquid chromatography, ion-exchange chromatography | **I, III, IV** |
| gel filtration | **III** |
| SDS-PAGE | **I, III** |
| isoelectric focusing | **III, IV** |
| **EPR spectroscopy** | **II** |
| **Molecular methods** | |
| DNA isolation | **IV** |
| RNA isolation | **IV** |
| plasmid isolation | **IV** |
| cloning by pCR2.1 TOPO | **IV** |
| RACE PCR | **IV** |
| genome walking PCR | **IV** |
| DNA sequencing | **IV** |
| amino acid sequencing | **III** |
| **Immunochemical methods** | |
| Western blotting | **III** |
| antiserum production | **III** |

# 4. RESULTS AND DISCUSSION

## 4.1 Production of ligninolytic enzymes by *Phlebia radiata* and *Agaricus bisporus* in lignocellulose-containing media (I-IV)

The white-rot fungi *Phanerochaete chrysosporium* (Pellinen *et al.* 1988), *Trametes versicolor* (Roy-Arcand and Archibald 1991), *Coriolus versicolor* and *Ganoderma lucidum* (Wang *et al.* 1992) were shown to remove organochlorine compounds from spent bleaching liquors of pulp mills. Ligninolytic enzymes are believed to be responsible for colour removal and chlorolignin degradation from wastewaters. In the present thesis the production of ligninolytic enzymes of the white-rot fungus *Phlebia radiata* 79 (ATCC 64658) was examined under conditions in which the fungus removed colour and AOX from wastewater. The culture media used for *P. radiata* contained (i) spent bleaching liquor ($E_1$ phase) from a Finnish kraft pulp mill (I) and (ii) wastewater from hardwood (50%) and softwood (50%) pulping (previously unpublished data). The LDF *Agaricus bisporus* ATCC 62459 was cultured for MnP and laccase production in (iii) mushroom compost (II, III) and in (iv) a basic liquid medium supplemented with wheat straw, wheat bran, rye bran, compost leachate and oat bran (IV).

### 4.1.1 Production of ligninolytic enzymes by *P. radiata* in pulp mill and bleaching plant wastewater-containing media (I)

***Production and activity of ligninolytic enzymes***

The production of ligninolytic (LiP, MnP and laccase) enzymes in bioreactors by immobilized *Phlebia radiata* 79 was investigated in wastewater (i and ii) -containing media. The results shown in Fig. 4.1a-b and in I (Figs. 3a and 4) revealed that the onset of LiP production, detected with the veratryl alcohol oxidation method, occurred later and that the enzyme activities were lower in the presence of wastewater from kraft bleaching or from kraft pulping than in its absence. Activity in the presence of wastewater was less than a quarter of that observed in a medium with no wastewater. When the culture liquids were fractionated by anion-exchange chromatography (Fig 4.2a-b and in I (Fig. 5)), high levels of LiP activity emerged. The activity yield of LiP after the first anion-exchange purification exceeded 100% compared with that obtained in raw culture fluid. Therefore, part of the LiP activity was probably masked by compounds present in the crude culture liquid. The veratryl alchohol oxidation assay resulted in likely underestimation of the LiP activity in the cultures containing wastewater, probably due to the presence of interfering compounds. These compounds may have competed with the veratryl alcohol as substrates or inhibited the enzyme activity. Interference in the LiP activity assay was observed by other authors in cultures containing sawdust (Orth *et al.* 1993), straw (Vares *et al.* 1995, Castillo *et al.* 1997), and phenolic compounds such as di-, tri- and tetrachloroguaiacols (Ono *et al.* 1992) present in wastewaters when kraft pulp is bleached with $Cl_2$ and/or with hypochlorite (Kringstad and Lindström 1984). Castillo *et al.* (1997) found that veratryl alcohol oxidation was delayed by straw-based compounds. By increasing the veratryl alcohol and $H_2O_2$ concentration in the reaction mixture, the veratryl alcohol oxidation increased and no delay was observed (Castillo *et al.* 1997). Coloured compounds from wood or straw can also interfere with spectrophotometric enzyme assays (Forrester *et al.* 1990, Vares *et al.* 1995).

The MnP activities assayed using phenol red as the substrate are shown in Fig. 3b in I. Mn-dependent activity was present in the cultures containing spent bleaching liquor.

However, part of the phenol red-oxidizing (Mn-independent) activity in the culture liquid in I was in fact due to laccase, since laccase is now known to oxidize phenol red (Li *et al.* 1999), a fact we were not aware of that time. The MnP activity of *P. radiata* was also found in solid-state cultures containing straw (Vares *et al.* 1995) and spruce wood chips (Hildén *et al.* 2004).

The influence of wastewater on the production of the third ligninolytic enzyme, laccase, of *P. radiata* is shown in Figs. 3a-b and 4 in I. These data for *P. radiata* laccase activity were obtained by assaying the oxidation of syringaldazine at pH 5 (laccase) and the oxidation

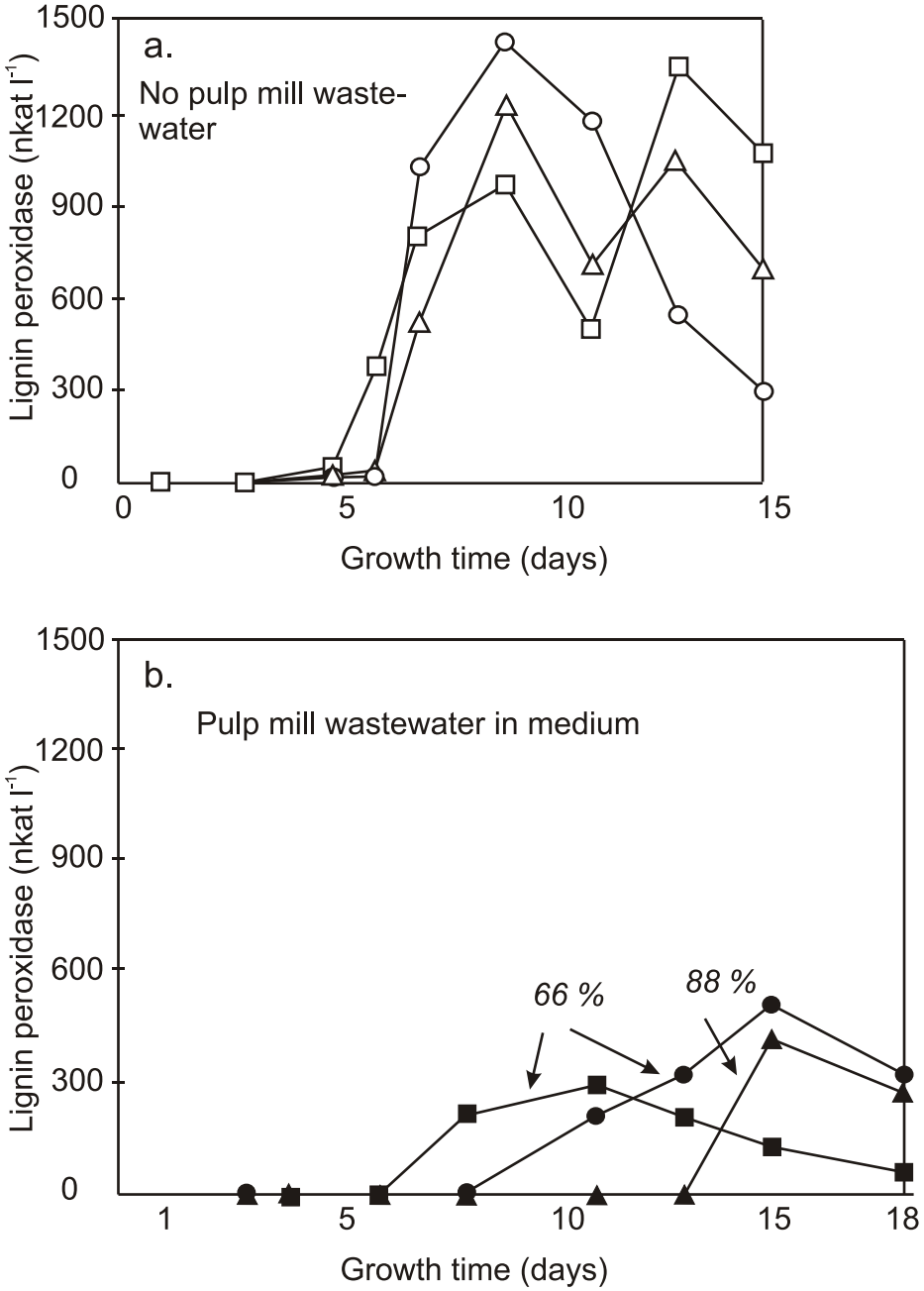



**Fig. 4.1** Lignin peroxidase activities of *Phlebia radiata* 79 cultivated in the presence of glucose without (a, open symbols) and with (b, filled symbols) pulp mill wastewater. Glucose concentrations: 1.37 g $l^{-1}$ (□), 2.75 g $l^{-1}$ (Δ), 4.13 g $l^{-1}$ (○), 1.37 g $l^{-1}$ (■), 2.75 g $l^{-1}$ (▲) and 4.13 g $l^{-1}$ (●). Wastewater content was 66% (v/v) except for the medium with 2.75 g $l^{-1}$ glucose where it was 80% (v/v).

of ABTS at pH 3 (oxidase). The enzyme activities obtained with these 2 substrates mainly reflected the different substrate affinities and pH optima of the *P. radiata* laccase enzymes, since with laccase the $K_m$ values for different substrates (phenolic compounds and dyes) share a high degree of variation (Li *et al.* 1999). The laccase activities increased after bleaching plant wastewater was added to the culture medium (Fig. 3a and 4 in I). Increased production of laccase by *P. radiata* may be a fungal response aimed to detoxifying and

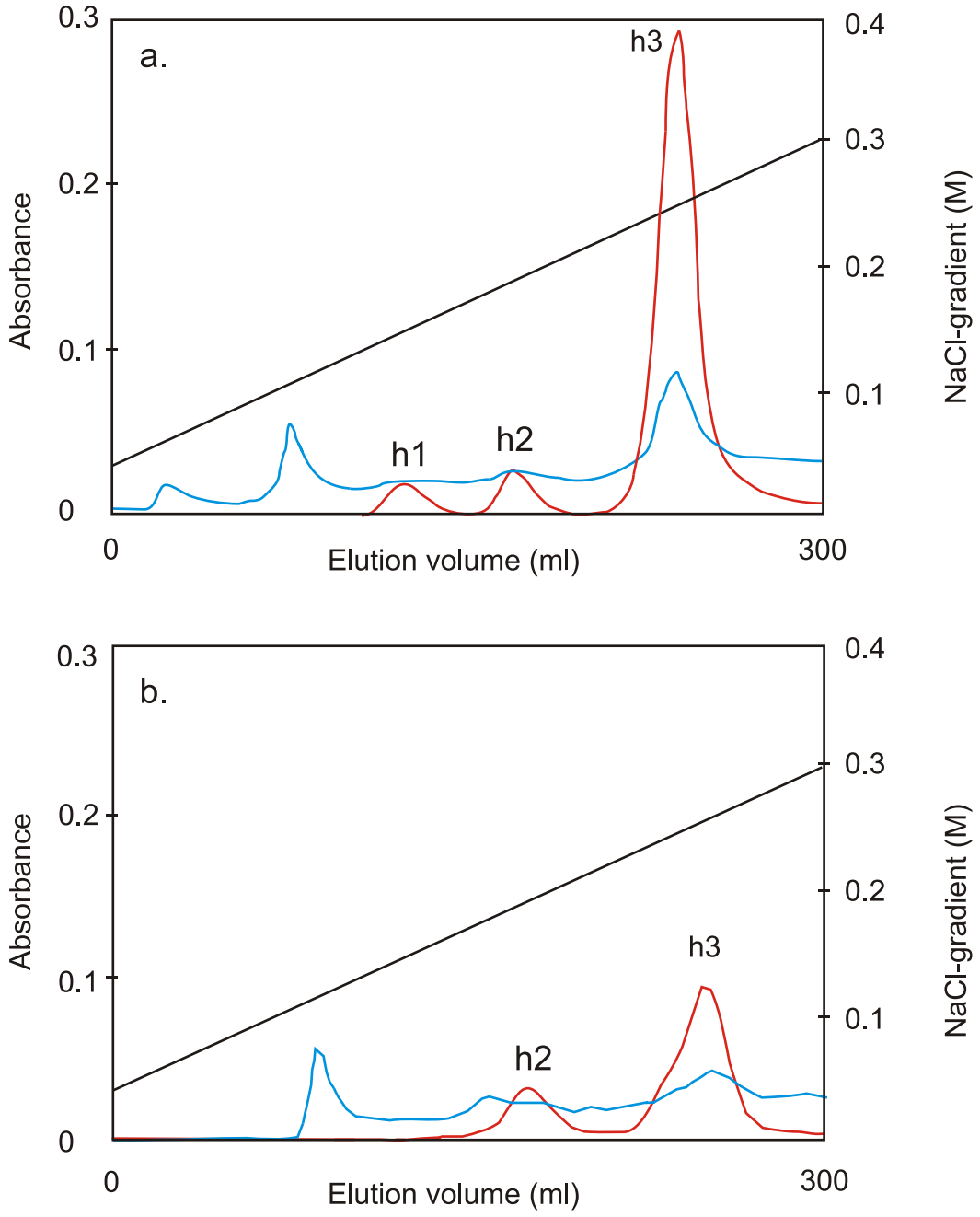



**Fig. 4.2** Separation of extracellular ligninolytic enzymes from *Phlebia radiata* 79 culture liquid by anion-exchange chromatography. Both culture liquids contained 4.13 g $l^{-1}$ of glucose. (a.) 15-d-old culture without pulp mill wastewater and (b.) 18-d-old culture with (66% v/v) pulp mill wastewater. The column was eluted with a gradient of NaCl (———), and the eluate monitored at 280 nm (blue line) and 409 nm (red line). The heme protein peaks are indicated as h1 (MnP1, LiP1), h2 (MnP2) and h3 (LiP2, 3 and MnP3).

polymerizing the toxic phenols that were present in the pulp mill wastewaters. These actions are typical for laccases in addition to the role played by laccase in delignification (Thurston 1994, Mayer and Staples 2002).

***Extracellular ligninolytic enzyme profile***

The culture liquid from fungal cultures with wastewater was fractionated using anion-exchange chromatography to examine the extracellular enzyme profile produced by *P. radiata*. The main heme protein peak (h3 in Fig. 4.2a-b and Fig. 5 in I) containing the *P. radiata* LiP isoenzymes LiP2,3 and possibly also MnP3 (Moilanen *et al.* 1996) was always detected in the *P. radiata* culture liquid after it was fractionated with anion-exchange chromatography. The relative amount of MnP2 (h2 in Fig 4.2a-b and Fig. 5 in I) increased but the peak h1 (containing MnP1) was not found in cultures containing wastewater compared with cultures containing no wastewater. Heme protein peak h1 (MnP1) reappeared when the culture liquid was fractionated on day 19, i.e. 6 d after the second addition of wastewater (I).

In conclusion, heme protein peaks h2 (MnP2) and h3 (LiP2,3 and MnP3) were the main heme protein peaks to occur when culture liquids of *P. radiata* containing pulp mill wastewater or bleaching liquors were fractionated. Production of MnP2 (h2) was higher in cultures supplemented with pulp mill wastewater than in cultures without wastewater, indicating an important role for MnP in wastewater-containing cultures. Further separation of the isoenzymes present in heme protein peak h3 may have revealed the relative amount of LiP2,3 to MnP3.

Heme protein peak h3 has also been reported in solid-state cultures of *P. radiata* with wheat straw as well as in liquid cultures grown in the basic medium, supplemented or not with insoluble lignocellulosic material (Niku-Paavola *et al.* 1988,1990, Vares *et al.* 1995, Moilanen *et al.* 1996). Vares *et al.* (1995) observed that the enzyme profile shifted towards h1 (MnP1) over time in straw-based solid-state cultures of *P. radiata*. It is noteworthy that in biobleaching studies MnP is considered to be the most important enzyme (Moreira *et al.* 1998).

Identification of the *P. radiata* heme protein peaks was based on the information available at the time (early 1990s) the results were presented (I). Since that time both the methodology and the level of expertise in ligninolytic enzyme versatility have greatly improved. Based on the information available the h1 protein peak most probably corresponded to MnP1, while h2 referred to MnP2 and h3 to both LiP3 and MnP3 (Taina Lundell, unpublished results).

***Glucose consumption***

The dry weight yield of *P. radiata* mycelia increased with increasing amounts of pulp mill wastewater in the medium (Fig. 4.3a). Accordingly, wastewater stimulates fungal growth which requires more glucose (Fig. 4.3b). It is interesting that the amounts of pulp mill wastewater used in this study caused no inhibition of growth of *P. radiata*.

Biodegradation of lignin occurs in most ligninolytic fungi during the secondary metabolic state when one or more of the indispensable nutrients such as C, N or P are depleted (review by Hatakka 2001). White-rot fungi are not able to use lignin as the sole source of carbon and energy (Kirk and Farrell 1987). Eaton *et al.* (1980) and Yin *et al.* (1989) found that addition of glucose as a cosubstrate was necessary for decolourization of bleaching plant wastewaters by *Phanerochaete chrysosporium*. Roy and Archibald (1993) reported that *Trametes versicolor* used glucose as the main source of carbon and energy in a medium containing hardwood kraft pulp.

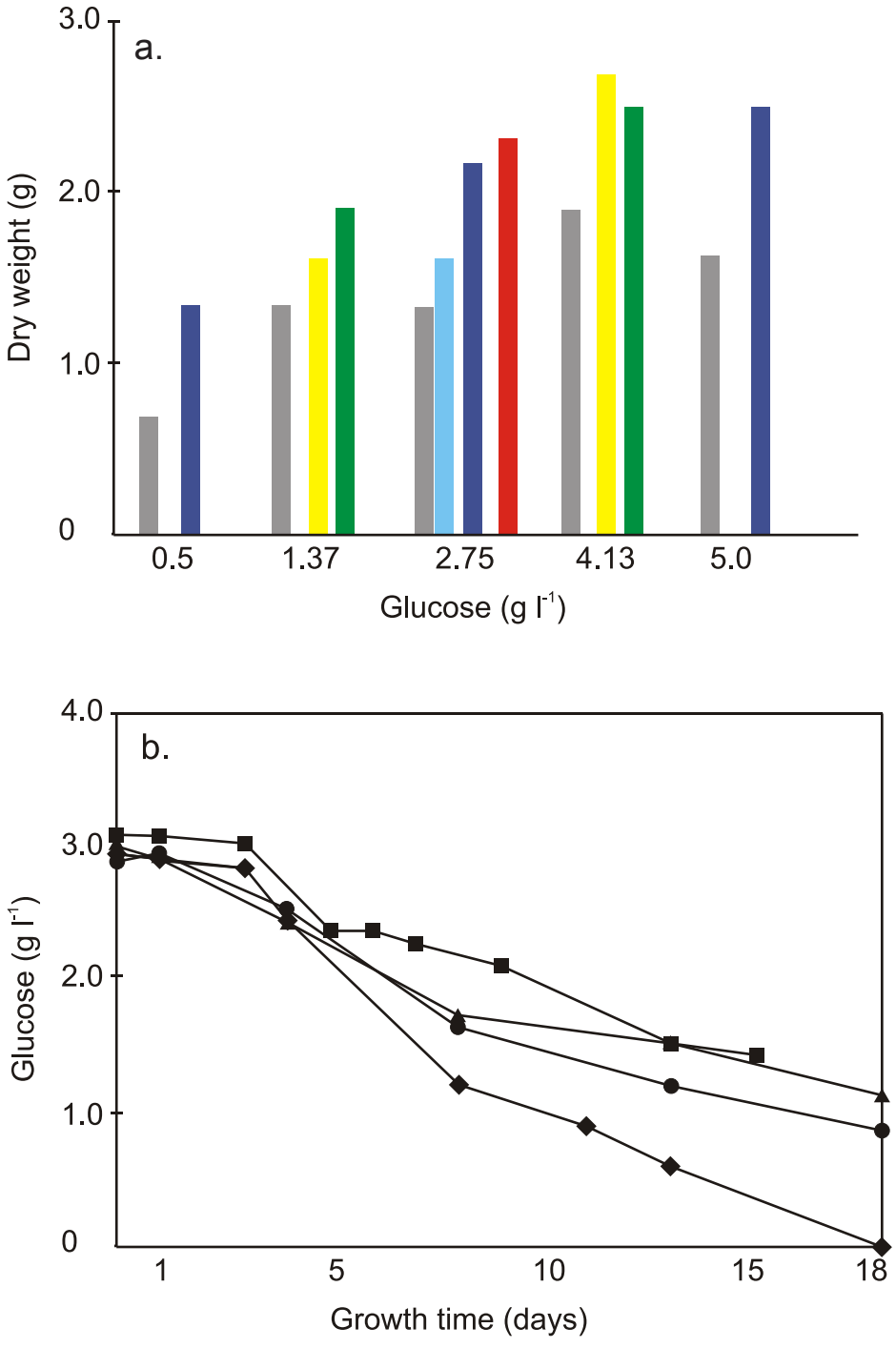



**Fig 4.3a-b** Mycelial production (a) and glucose consumption (b) by *Phlebia radiata* grown in 2 l bioreactors with different concentrations of pulp mill wastewater. (a.) 0 , 20 , 34 , 50 , 66 , 80 % (v $v^{-1}$) wastewater. (b.) Glucose consumption in medium containing 0 (■), 20 (▲), 50 (●) and 80 (♦) vol % of wastewater. The initial glucose concentration was 2.75 g $l^{-1}$.

### 4.1.2 Manganese peroxidase and laccase of *A. bisporus* from compost culture and liquid media with supplements (II-IV)

***Compost cultures of A. bisporus***

Wood (1980b) reported that laccase constituted about 2% of the total protein of the mycelia in *A. bisporus* compost. The high level of activity of laccase compared with MnP together with overlapping elutions in anion-exchange chromatography are likely the main reasons that the second ligninolytic enzyme of *A. bisporus,* MnP, was not discovered in *A. bisporus* compost until 14 years later, by Bonnen *et al.* (1994). The ligninolytic enzymes of *A. bisporus*, especially MnP from compost or basic media with lignocellulose supplements (compost leachate, wheat bran, rye bran and wheat straw) were characterized here.

The purification procedure of *A. bisporus* MnP from compost included concentration of compost leachate, 2 steps of anion-exchange chromatography followed by 2 gel filtration steps. The *A. bisporus* laccase fraction was separated after the 2 anion-exchange steps (II). The apparent yield of MnP activity from compost increased (Table 1 in III) after the first gel filtration step of the protein purification. Similar increases in activity yield were noted when the LiP of *Phlebia radiata* was purified from pulp mill wastewater-containing media (I). Compounds from the compost together with the dark colour may have influenced the MnP activity measurement and/or the activity itself in the crude culture filtrate. Wood and straw components such as phenols and extractives may inhibit the activity of ligninolytic enzymes (Forrester *et al.* 1990, Orth *et al.* 1993, Castillo *et al.* 1997, 2000).

***Liquid cultures of A. bisporus***

To better characterize the MnP and its production by *A. bisporus*, the next target was the production of MnP in defined media (IV). No MnP activity was found in liquid cultures containing defined carbon sources (Whatman CC-41 microcrystalline cellulose, D-glucose, glycerol or Solca floc cellulose), even though the enzyme protein was detected using Westernblotting (Fig. 6 in III), probably due to MnP activities that were under the detection limit. To determine the factors affecting the production of MnP by *A. bisporus*, the basic liquid medium was amended with different supplements (wheat straw, wheat bran, rye bran, oat bran, compost leachate, Mn and veratryl alcohol). The fungus grew in all 13 media tested but only 2, those containing rye and wheat bran, yielded MnP activity (Table 2 in IV). The MnP activity was found in media with 1% or 0.1% (w/v) glucose, irrespective of the presence of Mn or veratryl alcohol. Wheat bran appeared to stimulate the production of MnP slightly more than did rye bran, although rye bran was superior in promoting growth (Fig. 1 in IV). Interestingly, oat bran did not promote notable MnP activity although *A. bisporus* grew vigorously in oat bran-containing media. Wheat bran has been used to supplement the cultivation media of the edible fungi *Lentinula edodes* and *Pleurotus ostreatus* because bran as a lignocellulosic substrate resembles the natural substrates (wood and soil litter) favoured by these fungi. Cereal brans (bran flakes, oat bran, wheat bran and rice bran) were reported to increase ligninolytic enzyme production of two other white-rot fungi, *Coriolopsis gallica* and *Bjerkandera adusta* (Pickard *et al.* 1999). A promoting effect of lignocellulose on the production of MnP and LiP by the white-rot fungus *Phanerochaete chrysosporium* was reported by Kapich *et al.* (2004).

We tested whether the Mn content affected the production of MnP. No effect was apparent on MnP production by *A. bisporus* in the basic medium when 0.1-0.2 mM Mn was added (Table 2 and Fig. 1 in IV). Stimulation of MnP production by Mn has been observed in *Phanerochaete chrysosporium*, *Phlebia radiata, Dichomitus squalens*, *Pleurotus ostreatus* (reviews by Hatakka 2001, Martínez 2002), *Bjerkandera* sp. (Mester and Field 1997), and in

the LDFs *Agrocybe praecox, Stropharia coronilla*, *Stropharia rugosoannulata* and *Collybia dryophila* (Steffen *et al.* 2002a-c). Differential gene expression in response to Mn was shown for the MnPs of *P. chrysosporium* (Gettemy *et al.* 1998). Mn supplement improved MnP production in *P. ostreatus* when grown on its natural substrate, wood (Giardina *et al.* 2000), but inhibition of MnP isoenzyme production by Mn was also reported for *Pleurotus eryngii* (Martínez *et al.* 1996).

### 4.2 Laccase of *Agaricus bisporus* (II)

A blue laccase was found in *A. bisporus* compost (Table 1 in II). This is interesting, since Wood (1980b) previously reported the presence of a yellow laccase in *A. bisporus* compost. Comparison of the yellow and blue laccases from the white-rot fungi *Panus tigrinus*, *Phlebia radiata*, *Coriolus versicolor* and *Phlebia tremellosa* showed that these two forms of laccase may represent two different oxidation states of the same enzyme (II). We proposed that the yellow laccase may have resulted from the binding of lignin-derived (phenolic) molecules to the enzyme protein (II). This bound material can act as a laccase mediator, thus enhancing the oxidation of nonphenolic compounds (Leontievsky *et al.* 1997). However, the true nature of yellow laccases remains to be verified. The blue colour of compost-derived *A. bisporus* laccase (II) may have been resulted of the long (4 weeks) cultivation time, when the content of phenolic lignin-derived material in the compost has declined. The blue laccase was found in another litter decomposer, *Marasmius quercophilus*, in a synthetic culture medium (Dedeyan *et al.* 2000).

### 4.3 Manganese peroxidase of *Agaricus bisporus* (III, IV)

#### 4.3.1 Protein and enzyme properties of *A. bisporus* MnP1 (III,IV)

To further characterize the protein properties of *A. bisporus* MnP from compost, the active enzyme was successfully purified to homogeneity (III). The purified MnP1 from compost had an MW of 40 kDa (Fig. 2 in III). In isoelectric focusing the protein separated into 2 bands (Fig. 3 in III), indicating the presence of 2 isoforms of MnP in *A. bisporus* compost cultures. The absorption spectrum was typical of that of heme proteins, with a heme absorption peak at 408 nm and 2 minor peaks at 500 nm and 630 nm (Fig. 4 in III).

For further studies it was important to know if the MnP produced by *A. bisporus* in defined liquid cultures (IV) was the same enzyme as that representing the main MnP (MnP1) in compost (III). The properties of *A. bisporus* MnP enzyme protein are shown in Table 4.1.

**Table 4.1** Properties of *A. bisporus* MnP obtained from different culture media.

| Culture medium | Enzyme preparation | MnP isoforms | pH optimum | p*I* | MW (kDa) | Reference |
|---|---|---|---|---|---|---|
| Compost 14 d | Compost leachate | MnP | 5.4-5.5[a] | 3.5 (3.55) | Nd | Bonnen *et al.* 1994 |
| Compost 21 d | Purified enzyme | MnP1 (MnP2) | 5.5[b] | 3.25 3.3 | 40 | III |
| Wheat bran 34 d | Culture liquid | MnP1 | Nd. | 3.25 | Nd. | IV |

[a] guaiacol as substrate [b] $Mn^{2+}$ as substrate Nd.: Not determined

The value of *A. bisporus* main MnP (III) of compost origin (p*I* 3.25) was similar to that (p*I* 3.5) reported by Bonnen *et al.* (1994). The small difference, p*I* 3.25 vs. p*I* 3.5, may have resulted from culture medium components attached to the enzyme protein and affecting its ionic properties or differences in purification and analytical methods among laboratories. The attached compounds can be partially or totally removed during purification. The value (Fig. 2 in IV) of *A. bisporus* MnP from liquid cultures (p*I* 3.25) was identical to the p*I* value of the MnP1 from *A. bisporus* compost (Fig. 3 in III). The value of *A. bisporus* MnP (p*I* 3.25) is low compared with that of many fungal MnPs (review by Hatakka 2001). The MW of the *A. bisporus* MnP, 40 kDa (Fig. 2 in III), is in the lower MW range of fungal MnPs (Hatakka 2001). Similar acidic MnPs were found in white-rot fungi such as *Ceriporiopsis subvermispora* (p*I* 3.2 and 3.3; Lobos *et al.* 1994), *Lentinula edodes* (p*I* 3.2; Forrester *et al.* 1990), *Nematoloma frowardii* (p*I* 3.2; Schneegaβ *et al.* 1997), *Phanerochaete sordida* (p*I* 3.3; Rüttimann-Johnson *et al.* 1994) and *Trametes versicolor* (p*I* 2.9-3.2; Johansson and Nyman 1993) and from the LDF *Stropharia rugosoannulata* (p*I* 3.2; Steffen 2003). Two other LDFs *Stropharia coronilla* and *Agrocybe praecox* have MnPs with acidic p*I*s and an additional MnP with a nearly neutral p*I* (Steffen 2003). The pH optimum for $Mn^{2+}$ oxidation of *A. bisporus* MnP was pH 5 (Fig. 4.4) which is typical for fungal MnPs. Bonnen *et al.* (1994) found that for oxidation of guaiacol, the optimal pH was slightly higher: pH 5.4-5.5.

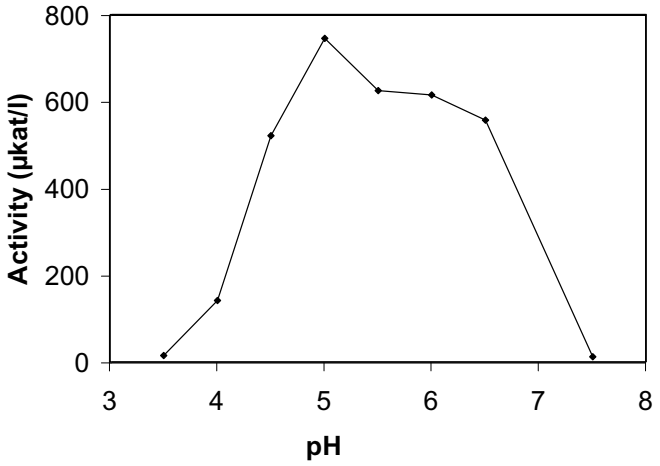



**Fig. 4.4** The pH optimum of $Mn^{2+}$ oxidation of *A. bisporus* MnP in 50 mM Na-malonate buffer, 25 °C.

We proposed the presence of a second MnP with p*I* 3.3 (III). Bonnen *et al.* (1994) also reported another MnP with a p*I* near that of the main MnP. The presence of a true isoenzyme encoded by a separate gene, not just an isoform of the same enzyme, was not confirmed. Modification of the p*I* and MW of a specific ligninolytic enzyme may result from 3 stages of protein production: distinct genes, variation in posttranslational modification e.g. glycosylation of the same gene product or from reactions of the enzyme protein with the medium.

No LiP activity was found in *A. bisporus* cultures with the veratryl alcohol oxidation method. This is similar to the situation in several other LDFs: *Agrocybe praecox*, *Collybia dryophila*, *Stopharia coronilla*, *Stropharia rugosoannulata* (Steffen 2003) and *Mycena galopus* (Ghosh *et al.* 2003). These fungi appear to produce a combination of laccase and MnP as ligninolytic enzymes.

### 4.3.2 Molecular properties of *A. bisporus* MnP1 (IV)

The main MnP (MnP1) of *A. bisporus* was found in compost cultures (III). The MnP found in cultures supplemented with rye and wheat bran had a p*I* value similar to that of MnP1 from the compost (Fig. 3a-c in III and Fig. 2 in IV). To evaluate the MnP gene/s expressed in bran-containing cultures, ribonucleic acid (RNA) was isolated from *A. bisporus* growing on bran agar and converted to complementary DNA (cDNA) by reverse transcriptase. The cDNA was amplified using degenerative primers designed for the conserved regions of known MnP genes and later with specific primers designed for the cloned and sequenced fragments of the cDNA of *A. bisporus* (Table 1 and methods in IV)(Fig. 4.5).

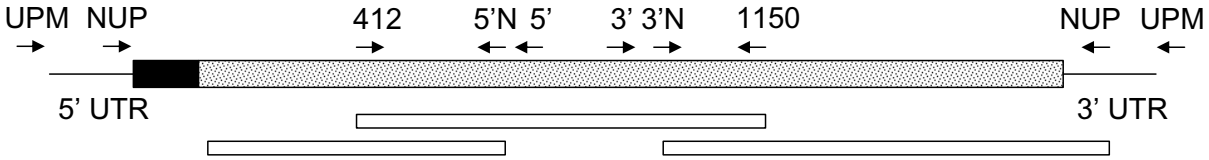



**Fig. 4.5** Amplification of the *A. bisporus* cDNA to obtain full-length cDNA clones of *mnp1*. The oligonucleotide primers are shown in Table 1 in IV. The signal sequence is in black.

The predicted aa sequence (Fig. 3 in IV) showed that *A. bisporus* MnP is a 328-aa-long polypeptide preceded by a 26-aa leader peptide. Conserved regions typically found among all peroxidases such as $Mn^{2+}$ binding sites and distal and proximal histidines were found in a predicted *A. bisporus* MnP sequence (Fig. 4.6). Prediction of the signal cleavage sites (Nielsen *et al.* 1997) (www.cbs.dtu.dk) suggests that *A. bisporus* MnP is synthesized as a preproenzyme as is *Pleurotus ostreatus* MnP1 (Asada *et al.* 1995) (Fig. 4.6). The MnP1 produced in the compost had the same p*I* (3.25) and was identical in a 25-aa N-terminal sequence (Fig. 5 in III) to the MnP produced in bran-containing cultures (Fig. 3 in IV), indicating they were the same enzyme.

The sequence similarity of MnP1 (Fig. 4.6) to VPs indicates similarities in enzymatic properties between VPs and *A. bisporus* MnP1. Veratryl alcohol oxidation involves tryptophan (W171) in the *Phanerochaete chrysosporium* LiP aa sequence (Doyle *et al.* 1998). Tryptophan is also present on that site in the VPs of *P. eryngii* (W164) and *L. irina* (W194). In the *A. bisporus* MnP1 sequence the tryptophan is replaced by alanine (A163). *A. bisporus* MnP1 also did not oxidize veratryl alcohol, thus supporting the postulated connection between tryptophan (W171) and veratryl alcohol oxidation.

Cloning of the *mnp1* gene was performed, using the genome-walking protocol (IV) in which the genomic DNA of *A. bisporus* was amplified with specific primers designed for the 5' and 3' ends of the sequenced cDNA. The sequenced region of *A. bisporus mnp1* was 1821 bp (Fig. 3 in IV) in length and was interrupted by 14 introns (Fig. 3 in IV). It is the first *mnp* gene characterized from an LDF. The intron-exon structure (Fig. 4 in IV) resembled that of the MnP1 of the wood-degrading white-rot fungus *Pleurotus ostreatus* (Asada *et al.* 1995) and of the versatile peroxidase VPL2 of *Pleurotus eryngii* (Ruiz-Dueñas *et al.* 1999b). The nucleotide sequence of *A. bisporus mnp1* showed highest similarity to *P. ostreatus* MnP3 (62.5%), *P. eryngii* versatile peroxidases VPL2 and VPL1 (61.9% and 61.2%, respectively) and *Lepista irina* VP (61.8 %). Both *Pleurotus* species are wood-degrading white-rot fungi, but *L. irina* is a litter decomposer.

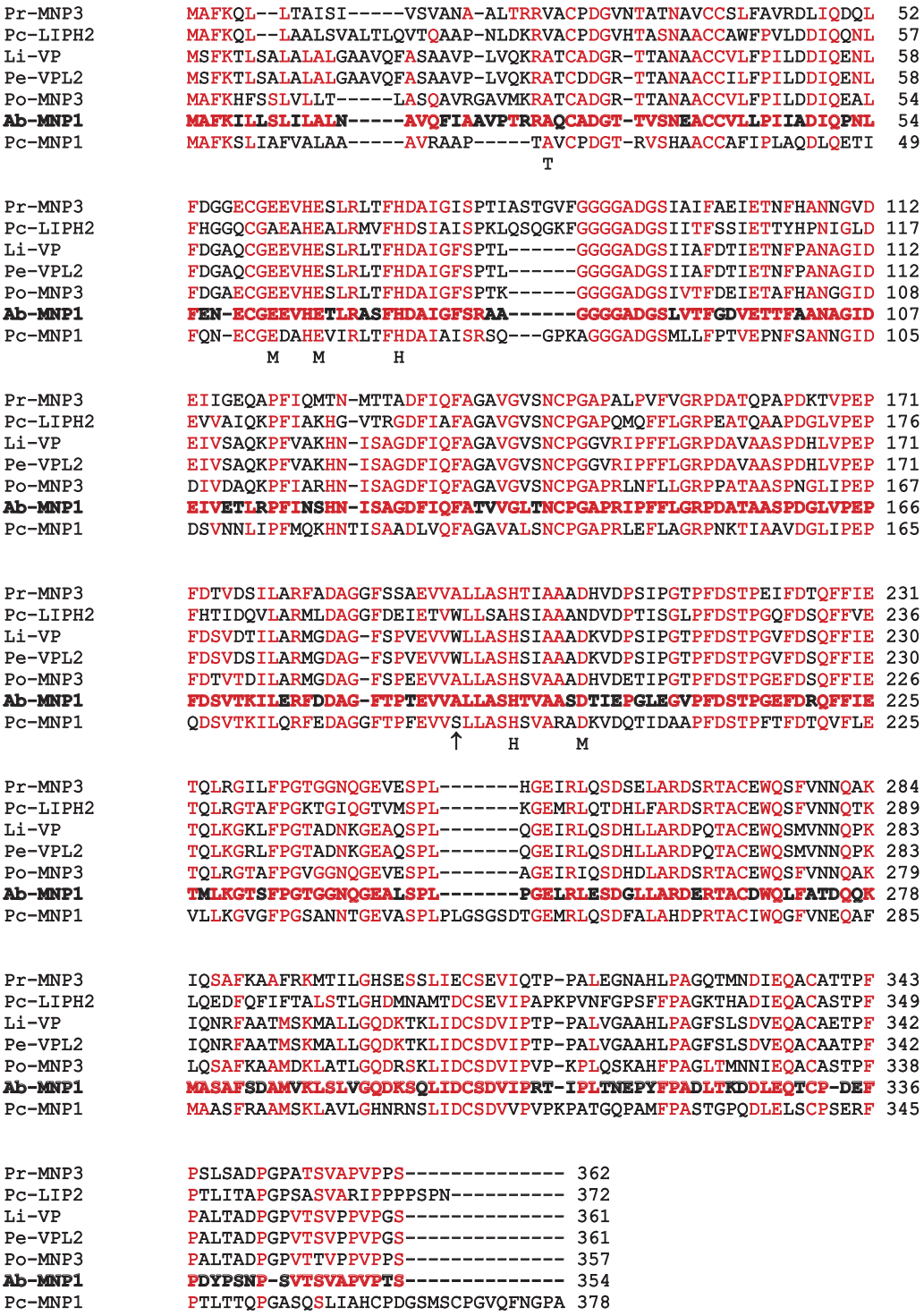



**Fig. 4.6** ClustalW (1.82) multiple sequence alignment of the translated aa sequences of *Agaricus bisporus* MnP1 (Ab-MNP1), *Phlebia radiata* MnP3 (Pr-MNP3), *Phanerochaete chrysosporium* MnP1 and LiP2 (Pc-MNP1 and Pc-LIP2), *Pleurotus ostreatus* MnP3 (Po-MNP3), *Pleurotus eryngii* VPL2 (Pe-VPL2) and *Lepista irina* VP (Li-VP). Consensus with Ab-MnP1 aa sequence marked in red. The site of the tryptophan involved in veratryl alcohol oxidation in LiPs and VPs is marked by arrow. T: N-terminus of the mature protein, H: distal and proximal histidines and M: $Mn^{2+}$ binding sites. The suggested propeptide cleavage site is 5 aa before the N-terminus of the mature protein (marked by T). EMBL databank accession numbers Ab-MNP1 (AJ699058), Li-VP (AJ515245), Pc-LIPH2 (M18743.1), Pc-MNP1 (J04624.1), Pe-VPL2 (AF007224), Po-MNP3 (AB011546) and Pr-MNP3 (AJ310930.1).

*A. bisporus* ATCC 62459 is a hybrid strain with a long history of cultivation (Chang 1999). It would be interesting to compare the naturally occurring *Agaricus* strains with this strain to determine if there are differences in the lignin-degrading machinery. Adaptation to a mushroom production medium could have changed the ligninolytic properties of *A. bisporus* possibly resulting in the presence of only one main MnP. Multiple MnP or LiP genes do not necessarily reflect the presence of isoenzymes produced by the lignin degrading fungus. In a fungal genome some genes may never produce a protein (Rajakumar *et al.* 1996). The appearance of multiple isoenzymes may also result from differences in glycosylation (Conesa *et al.* 2002). To be able to understand the ligninolytic system of a given fungus, information on the presence of genes, the corresponding ligninolytic enzyme proteins and their role in fungal growth is required.

## 5. SUMMARY

White-rot fungi are the only known organisms that effectively degrade lignin. In soil the LDFs participate in the overall lignin degradation. The ability to degrade lignin is connected with the extracellular ligninolytic enzymes LiP, MnP and laccase. The ligninolytic enzymes are nonspecific and take part in various oxidative reactions. The lignin-degrading fungi produce combinations of ligninolytic enzymes, the most typical being the production of MnP and laccase. The production of several isoenzymes is thought to relate to adaptation to different substrates and culture conditions.

Ligninolytic enzyme production by two ligninolytic fungi, namely the white-rot fungus *Phlebia radiata* and the edible LDF *Agaricus bisporus* was examined in cultures containing lignocellulosic material (pulp mill wastewater, compost, bran and straw). Purification of the proteins was required to show the actual presence of ligninolytic enzymes in these fungal cultures, since ligninolytic enzyme activities (especially LiP and MnP) in these culture fluids could not be reliably measured with spectrophotometric methods.

Ligninolytic enzyme production by *P. radiata* was shown to vary, depending on whether the cultures contained pulp mill wastewater; production of laccase and MnP isoenzyme were high in wastewater-containing cultures. More LiP was produced than expected, as shown from the enzyme activities obtained with the veratryl alcohol oxidation method. The consumption of glucose and growth of *P. radiata* increased with increasing amounts of pulp mill wastewater in the culture medium.

The most common edible fungus, the litter degrader *Agaricus bisporus* (button mushroom, cultivated mushroom), produced laccase and MnP in the compost from which the main MnP1 was purified and characterized. Its MW is 40 kDa and p*I* 3.25. The laccase purified from compost was a blue copper enzyme. The production of MnP by *A. bisporus* in liquid cultures was induced by wheat and rye bran. The *mnp1* gene expressed in bran-containing cultures was cloned and sequenced. MnP produced by *A. bisporus* in bran-amended cultures had the same p*I* and N-terminal sequence as MnP from compost cultures and was concluded to be the same enzyme.

*Mnp1* is the first MnP-encoding gene characterized from an LDF. The molecular properties of MnP1 showed its similarity to *P. ostreatus* MnP and *Pleurotus eryngii* VPs; however, *A. bisporus* MnP1 did not oxidize veratryl alcohol as VPs do. The intron-exon structure resembled that of the MnP1 of the wood-degrading white-rot fungus *P. ostreatus* and of VPL2 of *P. eryngii.*

The addition of lignocellulose (pulp mill wastewater) increased both the growth and production of ligninolytic enzymes by the lignin-degrading fungus *P. radiata* in liquid cultures. It may be possible to increase the growth of the cultivated fungus *A. bisporus* by changing the composition of the compost. The genetic sequence of the main ligninolytic MnP1 presented here can be used to follow the ligninolytic enzyme expression and thus substrate utilization in *A. bisporus* compost and thus correlate the substrate utilization to mushroom production. Comparison of ligninolytic enzyme production and profiles between wild and cultivated *Agaricus* strains would be interesting to determine if the long history of culturing has affected the ligninolytic enzyme profile of cultivated strains.

## 6. CONCLUSIONS

The main conclusions were:

1. Production of ligninolytic enzymes, especially laccase and MnP by the white-rot fungus *P. radiata* and the LDF *A. bisporus* was enhanced by the presence of lignocellulosic fraction in the culture medium.

2. Purification of the extracellular proteins was required to show the presence of ligninolytic enzymes in fungal cultures containing lignocellulose (pulp mill effluent, wheat straw, compost, bran). Ligninolytic enzyme (especially LiP and MnP) activities in these culture fluids cannot be reliably measured with spectrophotometric methods.

3. Addition of pulp mill wastewater increased the glucose consumption and production of mycelia in *P. radiata* cultures.

4. Laccase and MnP were the main ligninolytic enzymes secreted by *Agaricus bisporus* in cultures containing lignocellulose.

5. Wheat bran and rye bran promoted *A. bisporus* MnP production in liquid cultures.

6. MnP1 was isolated from *A. bisporus* and is the main MnP in the compost and in liquid cultures with added bran.

7. The *mnp1* gene of *A. bisporus* is the first MnP gene isolated from an LDF. It showed the closest similarity to *Pleurotus ostreatus* MnP3 and *Pleurotus eryngii* versatile peroxidases VPL1 and VPL2.

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Viikki, November 2004

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