Role of Manganese and Veratryl Alcohol in the Ligninolytic System of *Bjerkandera* sp. Strain BOS55

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Tünde Mester

Proefschrift

ter verkrijging van de graad van doctor,
op gezag van de rector magnificus
van de Landbouwuniversiteit Wageningen,
Dr. C. M. Karssen,
in het openbaar te verdedigen
op woensdag 9 september 1998
des namiddags om half twee in de Aula.

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to my parents and my children

Contents

1. General Introduction

LIGNIN

Lignin is the second most abundant plant biopolymer after cellulose in the biosphere, accounting for up to 35 % of the dry weight of woody tissue. Lignin is an aromatic polymer providing plant cell walls with rigidity, water impermeability and resistance to microbial attack (43). It is synthesized by the random coupling of three different phenylpropanoid precursors *p*-coumaryl, coniferyl, and sinapyl alcohols (Fig. 1A) linked with irregular bonds (e.g., C_β-O-C_4, C_5-C_β linkages) resulting in a heterogeneous, three-dimensional, hydrophobic structure (2, 43) (Fig. 1B). Approximately 80-90 % of the phenolic hydroxyl groups found in lignin participate in intermolecular linkages which accounts for the predominantly nonphenolic character of lignin. The complex physical and chemical characteristics of lignin make this polymer inaccessible for hydrolytic mechanisms of degradation which are more typical for other biopolymers such as cellulose. Instead, the microbial attack of lignin proceeds via a nonspecific extracellular, oxidative process (13, 58).

LIGNIN DEGRADATION BY WHITE ROT FUNGI

Many microorganisms have been described which degrade woody materials; however, only one group of fungi can extensively degrade lignin (13, 58). These lignin degrading basidiomycetes are denominated as white rot fungi due to the white appearance of wood after fungal attack. Lignin is not utilized as a sole carbon source by white rot fungi, although they are able to completely mineralize it to carbon dioxide (58). The real purpose of ligninolysis is presumably to get better access to the polysaccharides protected by the lignin. Several hundred white rot species have been identified so far. The lignin degrading system of one species, *Phanerochaete chrysosporium* has been studied extensively (36, 91). Recently, more work has been done on characterizing the ligninolytic system of other species such as *Trametes versicolor, Ceriporiopsis subvermispora, Pleurotus spp., Phlebia radiata, Bjerkandera adusta* (13, 57, 55, 72, 75, 106). All of the ligninolytic fungal strains studied are characterized by their ability to produce extracellular oxidative enzymes.

LIGNINOLYTIC SYSTEM OF WHITE ROT FUNGI

Ligninolytic peroxidases. Three main types of extracellular oxidative enzymes; lignin peroxidase (LiP), manganese dependent peroxidase (MnP), and laccases have been shown to be produced by white rot fungi which potentially participate in lignin degradation (13, 58). This chapter will only focus on LiP and MnP since they are the best characterized

ligninolytic enzymes and both are produced by *Bjerkandera* sp. strain BOS55, the selected strain of this PhD study.

The two types of peroxidases were first discovered in *P. chrysosporium* (35, 67, 99). LiP and MnP are known to be families of isozymes occurring as extracellular glycosylated proteins (50, 68, 72, 75). The ratio between the isozymes changes with the culture age and culture conditions (9, 68, 72, 80, 106). They have molecular weights ranging from 35-47 kD and isoelectric points of 2.8-5.3 in various white rot fungi (26, 48, 68, 72, 75, 85). The absorption spectrum of the native enzyme has a very distinctive absorbance maximum at 406-409 nm due to the one heme group (protoporphyrin IX) (34, 75, 85, 100).

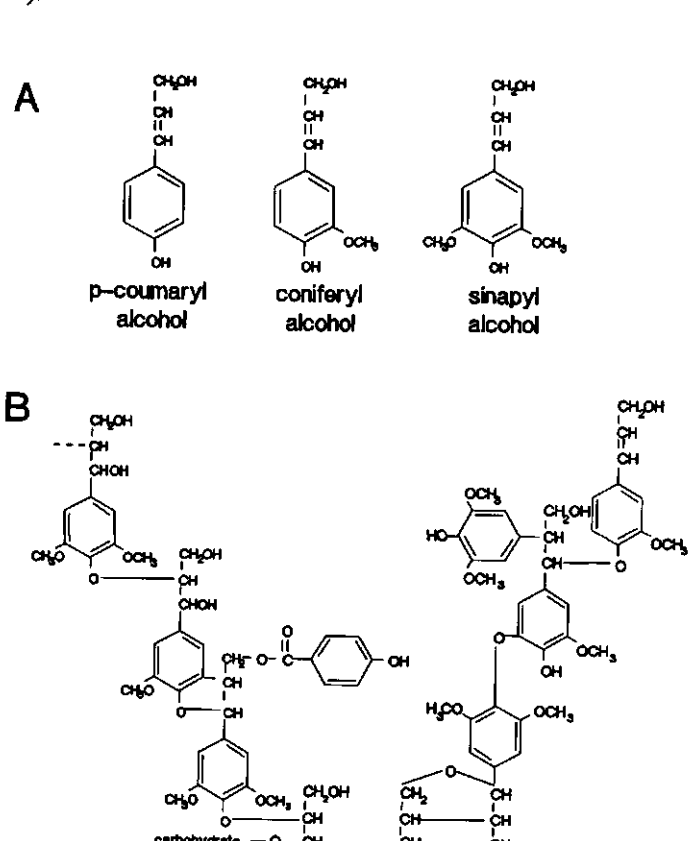

Figure 1. Lignin precursors (A) and structural model of lignin (B). Adapted from (2, 13)

LiP and MnP have a typical catalytic cycle, characteristic of other peroxidases (Fig. 2) (33, 107, 100, 108). One molecule of hydrogen peroxide oxidizes the native (ferric) enzyme withdrawing two electrons (compound I). Then compound I is reduced back in two one electron oxidation steps to the native form via compound II in the presence of appropriate substrate. Otherwise, compound II further reacts with hydrogen peroxide leading to an inactive enzyme form (dioxygen complex) compound III (14, 107).

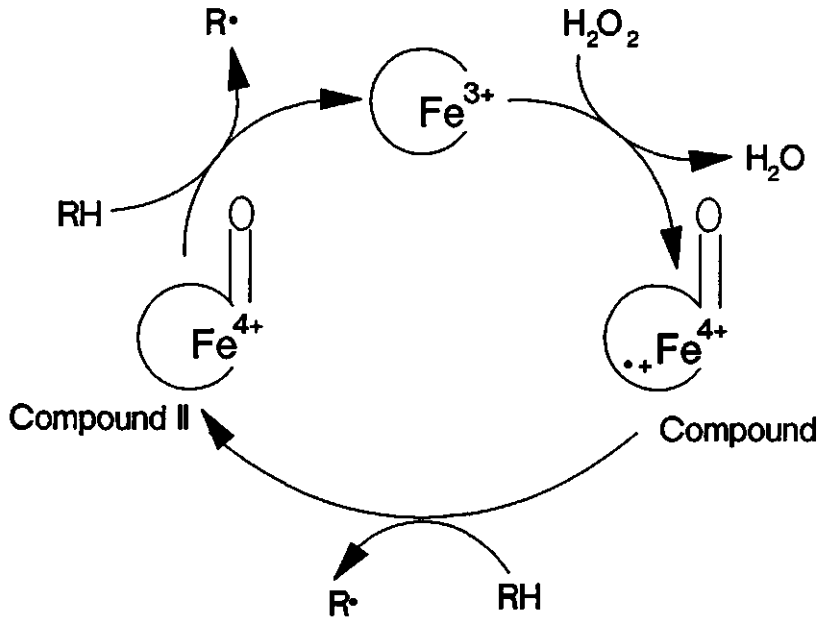

Figure 2. Catalytic cycle of peroxidases. Adapted from (5). (RH: reductive substrate; R •: oxidized substrate by one electron)

There are important differences in the substrate spectrum of LiP and MnP due to their different binding sites (Table 1). LiP unlike MnP is capable of oxidizing various nonphenolic substrates with redox potential up to approximately 9 eV. LiP has been shown to oxidize fully methylated lignin and lignin model compounds, as well as various polyaromatic hydrocarbons (5, 13, 40). The most typical oxidation reaction of LiP are the cleavage of C_α-C_β bond, aryl C_α bond, aromatic ring opening, demethylation, phenolic oxidation (13).

MnP is different from the other peroxidases due to the structure of its binding site. Recently, the crystal structure of MnP and site-directed mutants of MnP show a distinct manganese binding site of the enzyme (96, 97, 112). Therefore the principle function of MnP is to oxidize Mn(II) to Mn(III) which cannot be replaced by other metals at

physiological concentrations (33). Mn(III) generated by MnP can oxidize phenolic lignin model compounds and various phenols through phenoxy radical formation (40, 82).

Table 1. Typical examples for the direct substrates of LiP and MnP from *P. chrysosporium*. (62, 63, 82, 103, 109)

Substrates	LiP	MnP
Mn(II)	-	I, II[a]
anisyl alcohol	I	-
veratryl alcohol	I, II	-
1,4-dimethoxybenzene	I, II	-
guaiacol	I, II	I
syringic acid	I, II	I

[a] I and II indicate that the compound is substrate for compound I or II of LiP and MnP, respectively

Source of hydrogen peroxide. The required hydrogen peroxide for peroxidase activity is supplied by other enzymes in white rot fungi. As part of their ligninolytic system, white rot fungi produce hydrogen peroxide generating oxidases (22, 58). Glucose oxidases, glyoxal oxidase, aryl alcohol oxidase have all been identified as important examples of such enzymes utilizing glucose, glyoxal, and various aryl alcohols, respectively (24, 54, 56, 79). White rot fungi which lack H_2O_2-generating oxidases rely on the oxidation of physiological organic acid metabolites such as oxalate and glyoxylate which results in superoxide anion radicals that dismutate to hydrogen peroxide (104).

The role of low molecular weight cofactors. Various low molecular weight compounds play vital roles in the ligninolytic system as redox mediators or cofactors of the peroxidases. The importance of cofactors in the oxidation of lignin model compounds was observed early on during in vitro experiments. LiP and MnP were only able to oxidize various lignin model compounds and synthetic lignin if an appropriate cofactor was present (40, 103, 110). The de novo produced secondary metabolite 3,4-dimethoxybenzyl alcohol (veratryl alcohol) functions as an important cofactor for LiP. Three major roles of veratryl alcohol have been suggested so far. Veratryl alcohol acts as a cation radical redox mediator in the oxidation of many substrates having lower redox potential than the veratryl alcohol cation radical such as phenolic compounds, guaiacol, chlorpromazine and 4-methoxymandelic acid (37, 63, 101). Secondly, veratryl alcohol is a good substrate for compound II, therefore veratryl alcohol is essential for completing the catalytic cycle of LiP during the oxidation of compounds that are substrates of LiP compound I but not LiP compound II. The most studied example of such a compound is *p*-methoxybenzyl alcohol (*p*-anisyl alcohol) having a higher redox potential than veratryl alcohol itself (62). Thirdly, veratryl alcohol prevents the hydrogen peroxide inactivation of LiP (15, 102). This role of veratryl alcohol can partly be explained by the fact that veratryl alcohol is a good substrate for compound II. In addition, if the inactive LiP, compound III is formed, veratryl alcohol cation radical is capable of reducing LiP compound III back to its native form (6, 109). Only a few compound such as 3,4-dimethoxytoluene, 1,4-dimethoxybenzene, 3,4,5-trimethoxybenzyl alcohol, 2-chloro-1,4-dimethoxybenzene have been found to replace the function of veratryl alcohol as a cofactor of LiP (51, 62, 98). Since, veratryl alcohol is the

most frequently occurring secondary metabolite produced by white rot fungi, its physiological relevance has never been questioned.

Manganese is the essential cofactor of MnP. Mn(II) is the best reducing substrate for Compound I and II of all MnP isozymes tested (36, 75, 85, 105). Mn(III) generated by MnP acts as a mediator in the oxidation of various phenolic compounds (40, 82, 110). Although compound I can directly oxidize a few phenolic compounds, Mn(II) is absolutely required to reduce compound II back to the ferric form (107, 108). Thus, Mn(II) is implicated to play a role as cofactor of MnP in terms of completing the catalytic cycle. Consequently, Mn(II) is also important for preventing the H_2O_2 inactivation of the enzyme.

Only a few MnP reported are different from the classic MnP in terms of role of manganese as mediator and/or cofactor. A few MnP isozymes have been shown to have manganese independent activities for oxidizing some substrates. Various aromatic amines were directly oxidized by MnP from *C. subvermispora* without any manganese (105). In studies on *Pleurotus* spp., MnP isozymes were demonstrated to directly oxidize veratryl alcohol, however the very high K_m value makes this reaction unrealistic under physiological conditions (75, 90).

At the time of the discovery of MnP, the stimulatory effect of certain aliphatic organic acids such as lactate, oxalate, was demonstrated (33, 34, 65). These organic acids, e.g. oxalate, and to a lesser extent malonate and glyoxylate were shown to be produced as de novo metabolites by white rot fungi (23, 93, 104, 111). There are two theories which explain the stimulatory effect of these organic acids. According to Wariishi *et al.* (111), the organic acids accelerate the dissociation of Mn(III) from the Mn(III) - MnP complex by chelating Mn(III) resulting in an increased turnover of the enzyme. The other hypothesis described by Kuan *et al.* (66) is that Mn(II) can be bound by MnP only in the chelated form by strong chelators such as oxalate or malonate. Irregardless of which theory is right, the result of the reaction is the formation of a reactive Mn(III)-organic acid complex having sufficiently long life span to act as an diffusible mediator. The Mn(III)-organic acid complex is able to oxidize phenols dyes, polycyclic aromatic hydrocarbons (PAH), phenolic lignin (29, 34, 39, 40, 103, 110).

Recently several studies proposed that organic acids have additional roles in lignin degradation beside serving as chelators. The formate anion radical generated in the reaction of oxalate with Mn(III) or veratryl alcohol cation radical can react with dioxygen resulting in active oxygen radical formation such as superoxide (Fig. 3). This active oxygen species was suggested to participate in the oxidation of lignin. (32, 42, 92). Moreover, in the reaction of MnP or LiP together with the appropriate cofactors, and oxalate or glyoxylate, hydrogen peroxide accumulation via superoxide dismutation was also observed (Fig. 3). This reaction accounted for the catalysis of phenol red and kojic acid oxidation by MnP in the presence of Mn(II) and oxalate or glyoxylate without any hydrogen peroxide addition (64, 65, 105).

Evidences are accumulating showing the involvement of reductive processes during lignin degradation or in the elimination of highly oxidised xenobiotic compounds such as carbon tetrachloride, trichloroethylene, trichloroethane by ligninolytic peroxidases (5, 60). Obvious candidate intermediates which can participate in such a reductive degradation are formate anion radicals or superoxide anion radicals generated by either LiP-veratryl

alcohol or MnP - Mn(II) system from oxalate (5, 93). In in vitro experiments these system were demonstrated to reduce Fe(III) and cytochrome C (59, 86).

All experimental data indicate that the concentration of oxalate should be very much regulated during lignin degradation. The addition of oxalate in too high concentration inhibited the formation of veratraldehyde from veratryl alcohol, and degradation of other lignin model compounds due to the reduction of cation radicals by oxalate (94). In another study, the excess oxalate strongly inhibited the lignin decomposition in ligninolytic cultures of several white rot fungi (93). On the other hand, lignin content remarkably decreased in the biobleaching cultures of *Bjerkandera* sp. strain BOS55 under manganese deficiency if small amounts of oxalate were added (77). Manganese deficiency was shown to be unfavorable for the fungal oxalate production. The authors suggested that one of the reasons why little lignin content decrease occurred without oxalate addition was due to the low level of superoxide formation.

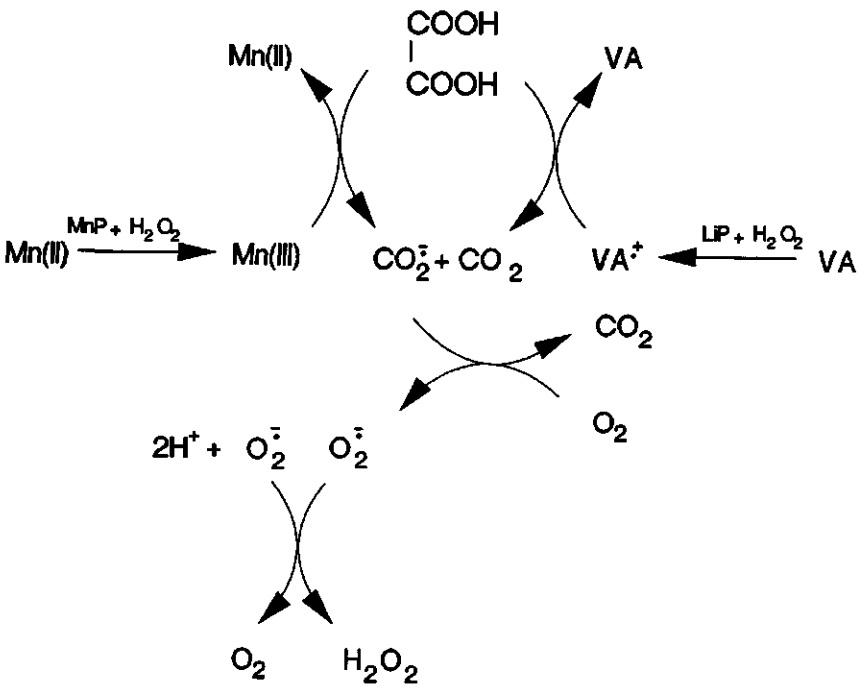

Figure 3. The formation of superoxide anion radical and hydrogen peroxide by the ligninolytic peroxidases of white rot fungi. LiP and MnP in the presence of their cofactors; veratryl alcohol (VA) and Mn(II), respectively can oxidize oxalate indirectly. In this reaction formate anion radical is formed. The formate anion radical undergoes several chemical reactions in the presence of dioxygen forming first superoxide anion radical. Then two superoxide anion radicals can dismutate resulting in the formation of hydrogen peroxide and dioxygen.

MECHANISM OF LIGNIN DEGRADATION

Mediators have been considered essential for the oxidation of lignin by ligninolytic peroxidases since native lignin in wood is inaccessible for large enzymes as LiP and MnP (30, 81). Although veratryl alcohol cation radical can mediate the oxidation of readily accessible lignin model compounds, the true mechanism of lignin degradation in natural substrates still remains a mystery. Unbound veratryl alcohol cation radical is too short lived to act as a diffusible mediator in wood (61). On the other hand, although, Mn(III) chelated by the naturally occurring organic acids, e.g., oxalate is more stable and can diffuse into wood cell walls, it is incapable of oxidizing nonphenolic lignin (40). In a few studies, it was shown that MnP was able to oxidize nonphenolic lignin or lignin model compounds as well as polyaromatic hydrocarbons with high ionization potential values such as phenanthrene if cooxidants such as unsaturated fatty acids were present (46, 76). Lipid peroxidation process are thought to be involved. However, the physiological relevance of these mechanisms has yet to be proven.

Recently, Abosharkh and Atalla (1) have demonstrated long distance electron abstraction in lignin suggesting that mediation for ligninolysis is not needed. Therefore, electrons abstracted by MnP or LiP at the surface of wood cell walls or at the phenolic moiety can be replaced by other electrons that move through lignin allowing for oxidation deep inside of the macropolymer.

REGULATION OF PEROXIDASE GENE EXPRESSION

Lignin degradation is a secondary metabolic event. From extensive studies with the model fungus *P. chrysosporium*, the expression of ligninolytic system was observed to be triggered by nutrient nitrogen, carbon, or sulphur depletion (58). After the discovery of the ligninolytic peroxidases, the isolation of the responsible genes from various white rot fungi has been attempted (4, 36 49, 57, 73). Various regulatory elements have been described in the promoter regions of LiP and MnP (Table 2). Promoter regions of *lip* or *mnp* genes studied so far contain cAMP response (CRE) elements as a general signal of starvation (36, 73). Additionally, there is evidence of differential regulation of certain isozymes of LiP and/or MnP expressed differently as a response of carbon or nitrogen limitation (44, 83).

Table 2. Regulatory elements found in the promoter region of *lip* and *mnp* genes (36, 73)

Elements of the promoter region	LiP isozymes	MnP isozymes
cAMP response elements (CRE)	+	+
Metal response elements (MRE)	-	+
Heat shock elements (HSE)	-	+
Xenobiotic response elements (XRE)	+/-[a]	-

[a] XRE was found in some of the tested *lip* gene promoter

Aside from starvation, the presence of manganese is essential for MnP gene expression in *P. chrysosporium* and *Dichomitus squalens* (11, 84). The observation is in

agreement with the general finding that elevated manganese levels are beneficial for the production of MnP in many white rot fungi (10). Putative metal response elements (MRE) were found in *P. chrysosporium* which are similar to those a gene encoding mouse metalloprotein (3, 36). However, the transcription of different MnP isozymes showed variable dependencies on the presence of manganese nutrition (31). The addition of manganese highly stimulated the gene expression of two MnP isozymes (*mnp1* and *mnp2*) but to a lesser extent that of the third one (*mnp3*). Moreover, in some white rot fungi from the genus *Pleurotus*, proteins with MnP activity were found in cultures lacking manganese nutrients and the addition of manganese inhibited the production of such manganese oxidizing peroxidases (75). These findings indicate that the role of manganese on *mnp* gene expression is diverse.

LiP production in various white rot fungi was also affected by manganese. LiP titres were considerably lowered as a response of manganese nutrient addition compared to those in manganese deficient conditions (10). However, manganese has no suppressive effect on LiP gene transcription in *P. chrysosporium* (70). A possible reason for the decreased LiP activity at extremely high manganese concentrations is the lowered oxygen stress due to the scavenging of reduced oxygen radicals by manganese (88). It is well established that LiP activity is highly stimulated under a pure oxygen atmosphere (25, 88). Manganese itself can be an oxygen scavenger in high concentrations (16, 17). Additionally it was shown that manganese induces manganese-superoxide dismutase (Mn-SOD) which further contributes to minimizing the oxidative stress when cultures contain manganese (88).

Unlike MnP, the expression of LiP genes is not stimulated by the presence of its cofactor. Although, the addition of veratryl alcohol is known to stimulate the LiP activities in the cultures of various white rot fungi (71, 80, 102), slight repression of *lip* gene transcription has been detected in *P. chrysosporium* (15). When veratryl alcohol was replaced by L-tryptophan in the cultures of *T. versicolor*, the same effect was observed (18). Both veratryl alcohol and L-tryptophan most probably resulted in higher LiP titres by preventing the inactivation of LiP compensating for the repressive effect on gene transcription.

Putative xenobiotic response elements and heat shock elements have been found in the promoter region of *lip* and *mnp* genes of various white rot fungi respectively (36, 73). The xenobiotic response elements are similar to those found in mammals, however, no xenobiotic chemicals have yet been tested to induce LiP gene expression so far. The addition of various chemicals such as hydrogen peroxide, ethanol, sodium arsenite, 2,4-dichlorophenol, as well as heat treatment stimulated the MnP production and *mnp* gene expression probably through the heat shock elements (12, 69). However, when manganese was omitted very little increase in *mnp* mRNA was detected due to those chemicals and the heat, and no MnP protein could be found in the culture fluid.

SOURCE AND BIOSYNTHESIS OF LOW MOLECULAR WEIGHT COFACTORS

Manganese and oxalate. Manganese is naturally present in wood. Manganese is the most abundant metal after Ca, K, Mg (27). The concentration of manganese is relatively high in wood (approximately 10 to 100 mg kg^{-1} dry wood), indicating that the availability

of manganese during the initial stages of wood decay is probably not a limiting factor in lignin degradation. It was observed that during white rot decay of wood the oxidation state and location of manganese changes resulting in black spots of insoluble MnO_2 precipitates in decayed wood (8). During lignin degradation manganese undergoes continuous changes in the oxidation stage. Mn(III) produced by MnP is reduced back to Mn(II) in the presence of reducing agents (e.g. oxalate, phenolic lignin). When Mn(III) complexing acids such as oxalate are present only in low concentrations, MnO_2 formation can occur via dismutation resulting in the insoluble black precipitates decreasing the bioavailable pool of manganese. If oxalate or cellobiose:quinone oxidoreductase (CBQase) is present Mn(IV) could be reduced back to the soluble Mn(II) making available again for MnP (89, 93).

White rot fungi produce oxalate, a strong chelator of manganese as a major aliphatic organic acid metabolite (23, 65, 104, 111). Two types of enzyme have been purified from basidiomycetes which are responsible for oxalate production using either oxaloacetate or glyoxylate as biosynthetic precursors that are derived from the tricarboxylic cycle. Oxaloacetase requires manganese for oxalate formation from oxaloacetate (23). Various types of glyoxylate oxidase using NAD^+, $NADP^+$ as cofactor and O_2 were identified which oxidize glyoxylic acid to oxalate (93).

In spite of oxalate production, oxalate does not accumulate in cultures of white rot fungi. This is an important distinguishing feature separating white rot fungi from brown rot fungi which accumulate oxalate in high concentrations. White rot fungi are unique since they contain several oxalate decomposing enzyme. Intracellular oxalate decarboxylase has been found in white rot fungi (23, 93). More importantly, extracellular enzymes of the ligninolytic system such as LiP and MnP can also decompose oxalate in the presence of veratryl alcohol and manganese, respectively (7, 94). This reaction is the important source of reduced oxygen radical species such as superoxide radicals with important roles in lignin degradation as was discussed earlier.

Biosynthesis of veratryl alcohol. Veratryl alcohol was shown to be synthesized de novo from glucose in many white rot fungi (22, 74). The production of veratryl alcohol begins during the early phases of secondary metabolism in parallel with the onset of LiP production. Veratryl alcohol originates from the shikimate pathway from L-phenylalanine (Fig. 4) (47). L-Phenylalanine is deaminated possibly by phenylalanine ammonia lyase (PAL) resulting in the production of cinnamate. Then cinnamate undergoes Claisen cleavage resulting in benzoate and/or benzaldehyde. Benzoate or benzaldehyde is hydroxylated and methylated forming veratrate which can be reduced back to veratryl alcohol presumably by intracellular dehydrogenases such as those found in *Bjerkandera* and *Pleurotus* species (21, 37).

The most thoroughly characterized step in the pathway of veratryl alcohol biosynthesis is the methylation of the OH groups at the meta and para positions. Two methylation systems have been discovered in *P. chrysosporium*. One works with chloromethane and the other uses S-adenosylmethionine (SAM) as methyl donor (19, 41). Two SAM dependent methyltransferases have already been purified each specific for the 3- or 4-hydroxy group, respectively (20, 45). The chloromethane dependent methylating system of *P. chrysosporium* can also methylate both hydroxy groups in meta and para

position (41). The enzyme(s) responsible for chloromethane dependent methylation have not yet been found.

Lignin degradation intermediates can be a possible source of veratryl alcohol precursors. A nonbiosynthetic precursor, 3,4-dimethoxycinnamyl alcohol was converted to veratryl alcohol by *P. chrysosporium* and *T. versicolor* (53, 95). Moreover, the de novo formation of secondary metabolites from glucose was repressed by vanillate a known lignin degradation product, addition to cultures of *P. radiata* (87).

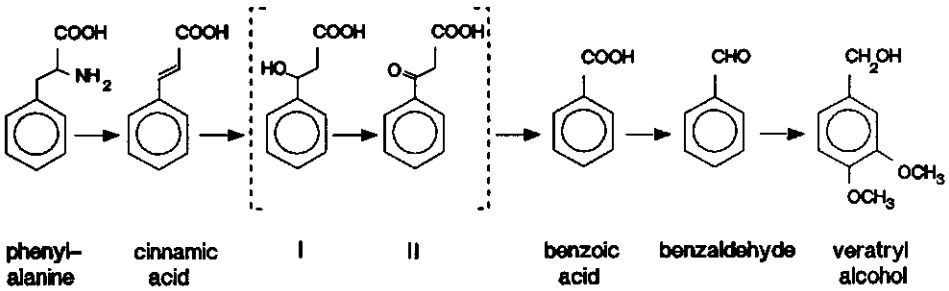

Figure 4. Biosynthetic pathway of veratryl alcohol biosynthesis in *P. chrysosporium*. (I and II are hypothetical precursors resulted by Claisen cleavage)

CONCLUSIONS

The investigations on ligninolysis by white rot fungi demonstrate the complexity of the mechanisms involving peroxidases, oxidases, and low molecular weight cofactors. After 20 years of extensive study, still no clear understanding of this mechanism has been achieved. However, it has been learned that the ratio between enzymes and cofactors should be strictly balanced for successful lignin degradation. Many studies emphasize the role of low molecular weight cofactors (e.g., Mn(II), veratryl alcohol, oxalate) which have multiple roles in the catalytic cycle of peroxidases, generation of hydrogen peroxide and even in the regulation of the gene expression of ligninolytic enzymes. The link between these low molecular weight cofactors/mediators for catalysis and their role on regulating the physiology of a white rot fungus *Bjerkandera* sp. strain BOS55 is the topic of this thesis.

SCOPE OF THE THESIS

For this study, *Bjerkandera* sp. strain BOS55 was selected which was shown to be an outstanding white rot fungus in previous studies in terms of production of ligninolytic peroxidases (52), degradation of polycyclic aromatic hydrocarbons (PAH) (28) and biobleaching of kraft pulp (78). During these studies, it was found that this fungus differs in many aspects from the well studied model fungus, *P. chrysosporium*. The objective of the PhD study was to gain insights on the physiology of the ligninolytic system of

Bjerkandera sp. strain BOS55. In particular, the roles of manganese and veratryl alcohol on the physiology of ligninolysis were examined.

In Chapter 2 the inhibitory effect of manganese on veratryl alcohol biosynthesis and its impact on LiP production were examined. In Chapter 3, the mechanism by which manganese inhibits veratryl alcohol biosynthesis was elucidated and methods of bypassing the inhibitory step with lignin degradation products are described. The production of veratryl alcohol and peroxidases on natural substrates was studied in Chapter 4 and compared with the bioavailability of manganese. The importance of the lignin degradation products on veratryl alcohol production is discussed. In Chapter 5, optimal conditions for MnP production were described. In Chapter 6 the production of a novel organic acid induced MnP in the complete absence of manganese is described. This novel enzyme behaves as a hybrid between LiP and MnP since it can oxidize manganese as well as directly oxidizes veratryl alcohol and other nonphenolic compounds without any manganese.

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2. Manganese Regulation of Veratryl Alcohol in White Rot Fungi and Its Indirect Effect on Lignin Peroxidase

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SUMMARY

Many white rot fungi are able to produce de novo veratryl alcohol which is known to be a cofactor involved in the degradation of lignin, lignin model compounds and xenobiotic pollutants by lignin peroxidase (LiP). In this study, Mn nutrition was shown to strongly influence the endogenous veratryl alcohol levels in the culture fluids of N-deregulated and N-regulated white rot fungi, *Bjerkandera* sp. strain BOS55 and *Phanerochaete chrysosporium* BKM-F-1767, respectively. Endogenous veratryl alcohol levels as high as 0.75 mM in *Bjerkandera* sp. strain BOS55 and 2.5 mM in *P. chrysosporium* were observed under Mn deficient conditions. In contrast, veratryl alcohol production was dramatically decreased in cultures supplemented with 33 or 264 μM Mn. The LiP titres which were highest in Mn deficient media were shown to parallel the endogenous veratryl alcohol levels, indicating that these two parameters were related. When exogenous veratryl alcohol was added to Mn sufficient media, high LiP titres could be obtained. Consequently, we must conclude that Mn does not regulate LiP expression directly. Instead, LiP titres are enhanced by the increased production of veratryl alcohol. The well known role of veratryl alcohol in protecting LiP from inactivation by physiological levels of H_2O_2 is postulated to be the major reason why LiP is apparently regulated by Mn. Provided that Mn was absent, LiP titres in *Bjerkandera* sp. strain BOS55 increased with enhanced fungal growth obtained by increasing the nutrient N concentration; while veratryl alcohol levels were similar in both N-limited and N-sufficient conditions.

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INTRODUCTION

White rot fungi and their ligninolytic enzymes have potential applications in biopulping and biobleaching (32, 40) as well as in the bioremediation of aromatic pollutants (1, 14). The extracellular lignin degrading machinery is typically composed of lignin peroxidase (LiP) and manganese dependent peroxidase (MnP) which function together with H_2O_2-generating oxidases and secondary metabolites (26). Veratryl alcohol is an important secondary metabolite involved in the ligninolytic system.

Many white rot fungi, especially those which secrete LiP, have been shown to produce veratryl alcohol de novo (11). This metabolite is formed by white rot fungal cultures where glucose is the only carbon source (8, 31). Labelled phenylalanine was shown to be the precursor to veratryl alcohol, indicating that this metabolite is derived from the shikimate pathway (23).

The occurrence of veratryl alcohol biosynthesis coincides with the physiological events associated with the appearance of LiP and lignin mineralization (8, 22). Exogenous addition of veratryl alcohol to white rot fungal cultures has generally been shown to stimulate the extracellular titres of LiP (12, 30, 34). Veratryl alcohol has been shown to prevent inactivation of LiP by excess H_2O_2 (7, 43). For many substrates of LiP, such as benzo[a]pyrene (18), azo dyes (36), Poly R-478 (35), p-anisyl alcohol (44), 4-methoxymandelate (20) and lignin (19), veratryl alcohol was found behave as a beneficial or essential cosubstrate for in vitro catalyzed reactions. The role of veratryl alcohol in stabilizing LiP against H_2O_2 inactivation has been suggested to account for the stimulation (44). More recently it has also been demonstrated that veratryl alcohol is responsible for the proper turnover of the enzyme cycle (e.g. by reducing compound II) enabling an improved oxidation of nonphenolic substrates (27). Considering that veratryl alcohol is produced endogenously together with LiP and has demonstrable physiological roles both *in vivo* and *in vitro* it therefore must be regarded as a physiological cofactor of LiP.

The ligninolytic machinery in white rot fungi, is highly regulated by nutrients. In particular, manganese (Mn) and nitrogen (N) have been shown to have strong regulating effects (16, 26). There are many literature reports which indicate that Mn has a potent inducing effect on the expression MnP in many white rot fungi (3, 6, 45). While Mn is an essential cofactor for the proper functioning of the MnP protein (15), the molecular regulation of MnP expression is also signaled by Mn (5). Many studies indicate that Mn addition can severely decrease LiP titres in white rot fungi (3, 37, 38). However, Mn up to 180 μM did not decrease the transcription *lip* mRNA in *P. chrysosporium* (30), indicating that the negative impact of Mn on LiP titres is not related to regulation at the molecular level.

N-sufficient conditions repress expression of both LiP and MnP in *P. chrysosporium* (16, 29). Likewise, the endogenous production of veratryl alcohol was repressed by N-sufficiency (13). However, it should be noted that strains from the genus *Bjerkandera* are N-deregulated as evidenced by the stimulated production of LiP and MnP in N-sufficient compared to N-limited medium (24, 25). In the case of *Bjerkandera* sp. strain BOS55, it was demonstrated that the endogenous production of veratryl alcohol was much higher in N-sufficient glucose-yeast extract-peptone medium than in N-limited glucose BIII medium (8). Since the glucose-yeast extract-medium does not contain Mn while BIII does, either N or Mn could have been responsible for the enhanced production of veratryl alcohol.

Here we report that Mn was responsible for regulating the endogenous production of veratryl alcohol. This type of regulation was discovered during our research evaluating the effect of Mn and N nutrients on the peroxidase titres in *Bjerkandera* sp. strain BOS55. Experiments were also carried out to demonstrate that the apparent regulation of LiP by Mn is an indirect result of the direct effect of this metal ion on veratryl alcohol biosynthesis.

MATERIALS AND METHODS

Microorganisms. *Bjerkandera* sp. strain BOS55 was isolated and determined as described before (9). This strain produces LiP, MnP, and manganese independent peroxidase (MIP) (8, 10). *Bjerkandera* sp. strain BOS55 and *P. chrysosporium* BKM-F-1767 (ATTC 24725) were maintained at 4°C on peptone/yeast slants (per liter: 20 g glucose, 5 g mycological peptone, 2 g yeast extract, 1 g KH_2PO_4, 0.5 g $MgSO_4 7H_2O$, 15 g agar) from which they were transferred to malt extract plates (per liter: 15.0 g of agar, 3.5 g of malt extract, 5.0 g glucose). They were incubated at 30°C for 4 to 6 days before use as inoculum in the experiments. The experiments were inoculated with agar plugs as described before (24).

Media. The standard basal medium used contained 2.2 mM N in the form of diammonium tartrate, Mn free BIII mineral medium modified from that of Tien and Kirk (42), 10 g liter^{-1} glucose, 2 mg liter^{-1} thiamine in 20 mM 2,2-dimethylsuccinate (pH 4.5) buffer. The KH_2PO_4 content of standard basal medium was 40 mM. Different media were prepared identically with either 33 or 264 μM Mn and with extra N from various sources. The extra N sources were NH_4^+ in the form of diammonium tartrate, peptone and a mixture of 20 L-amino acids (equal amounts of Ala, Arg, Asn, Asp, Cys, Gly, Glu, Gln, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val). The concentration of all extra N sources was 19.8 mM N, providing a total N content of 22 mM. In the experiment where the effect of the Mn and the N additions (2, 6, 20, 60 mM peptone-N) on peroxidase activity and on veratryl alcohol levels were studied, the basal medium did not contain diammonium tartrate. The pH of media used in the experiments with *P. chrysosporium* was 4.5, and the pH in media used in the experiments with *Bjerkandera* sp. strain BOS55 was adjusted to a value of 6 by NaOH addition.

All media were filter sterilized by Schleicher & Schuell FP 030/3 filters with a pore size of 0.2 μm (Dassel, Germany).

The background levels of Mn in Mn free basal and peptone media were measured with an atomic absorption spectrophotometer, Varian SprectrAA 300/400 System (Houten, The Netherlands). Background levels of 3.2 x 10^{-5} mM and 4.3 x 10^{-5} mM Mn were detected in basal medium and this medium supplemented with 19.8 mM peptone-N, respectively.

Culture conditions. Aliquots (5 ml) of media were placed in 250-ml serum bottles. Each bottle was loosely capped for passive aeration. The bottles were incubated statically under an air atmosphere. The incubation temperature was 30°C in experiments with *Bjerkandera* sp. strain BOS55 and 37°C in the case of *P. chrysosporium*. For measuring the CO_2 evolution, the 250-ml serum bottles were sealed with gas-impermeable rubber septa. After each sampling, the headspaces of the cultures were aseptically flushed with air.

Enzyme assays. All enzyme were determined spectrophotometrically (Perkin-Elmer Lambda 1 UV/VIS) (Norwalk, USA) at 30°C. For enzymatic assays, centrifuged extracellular fluids were utilized (12,000 g for 10 min). LiP activity was measured by oxidation of veratryl alcohol (42) with a correction for the background veratryl oxidase activity in the absence of

H_2O_2 and in the presence of 55 fold diluted catalase of *Aspergillus niger* (Sigma, St. Louis, USA). MnP and MIP activities were measured in a combined assay (8). The reaction mixture contained 50 mM sodium malonate (pH 4.5), 1 mM 2,6-dimethoxyphenol, 1 mM $MnSO_4$, and up to 0.6 ml of supernatant in a total volume of 1 ml. The reaction was initiated by adding 0.4 mM H_2O_2 and corrected for laccase activity. MIP activity was measured in the reaction mixture containing 50 mM sodium malonate (pH 4.5), 1 mM 2,6-dimethoxyphenol, 1 mM EDTA, and up to 0.55 ml supernatant in a total volume of 1 ml. The reaction was initiated by adding 0.4 mM H_2O_2 and corrected for laccase activity. MnP activity is expressed as a combined MnP/MIP activity minus MIP activity.

Carbon dioxide analysis. Carbon dioxide production was measured in the headspace by gas chromatography with a model 427 apparatus (Packard, Palo Alto, Calif.) apparatus fitted with a thermal conductivity detector (140°C). The column (Hayesep Q; Chrompack, Middelburg, The Netherlands) was maintained at 110°C, and the carrier gas was helium (30 ml min^{-1}). The injection port was maintained at 110°C. The injection volume was 100 µl.

Determination of the dry weight of mycelium. Mycelial mats were separated from the culture fluids by filtration. Mycelia were rinsed with distilled water and filtered through dried and tared glass fiber filters (Schleider & Schuell GF 50) (Dassel, Germany). Mycelial dry weight were determined after drying overnight at 105°C.

Determination of the secondary metabolites. 50 µl of centrifuged supernatant was analyzed by high pressure liquid chromatography (HPLC). A Pascal series HPLC ChemStation (Helwett Packard, Waldbronn, Germany) equipped with an HP1050 pumping station, an HP1040 M series II diode array detector and an HP9000-300 data processor was used. The column (200 by 3 mm) was filled with ChromoSpher C18-PAH (5 µm particles) (Chrompack, Middelburg, The Netherlands). Aromatic metabolites were analyzed with the following gradient (0.4 ml min^{-1}, 30°C): 90:10, 0:100, and 0:100 H_2O:CH_3CN at 0, 15 and 20 min, respectively. Compound identifications were based on matching retention times and UV spectra with standards of veratryl alcohol and veratraldehyde.

Determination of the extracellular protein profile by fast protein liquid chromatography (FPLC). Extracellular fluid was centrifuged at 20,000 × g for 10 min at 4°C to remove the mycelium. A 15 ml volume of supernatant was concentrated three fold by ultrafiltration through PM-10 membrane (Amicon, Rotterdam, The Netherlands) with a cutoff of 10 kDa. The concentrated supernatant was analyzed at 405 nm by anion exchange chromatography using a FPLC system (MonoQ HR 5/5, Pharmacia, Uppsala, Sweden). The column was equilibrated with 10 mM KP_i, pH 6.0. The enzymes were eluted with linear salt gradient from 0 to 300 mM KCl in starting buffer at a flow rate of 1 ml min^{-1} and 1 fraction min^{-1} for 40 min.

Chemicals. Mycological peptone was obtained from Oxoid Ltd. (Basingstoke, Hampshire, England). The L-amino acids were obtained from either Merck (Darmstadt, Germany) or Janssen Chimica (Geel, Belgium). Veratryl alcohol and veratraldehyde are purchased from Janssen Chimica (Geel and Beerse, Belgium). All other chemicals were commercially available and were used without further purification.

Statistical procedures. In all experiments, the measurements were carried out in triplicate parallel cultures. Values reported are means with standard deviation values.

RESULTS

Effect of manganese on peroxidase and veratryl alcohol production in *Bjerkandera* sp. strain BOS55. Extracellular titres of LiP, MnP, MIP as well as veratryl alcohol concentration were measured during 18 days in cultures receiving three levels of Mn (0, 33, and 264 μM). Figure 1 shows a typical example of the time course of MnP, LiP and veratryl alcohol production under Mn deficiency and Mn sufficiency. At a high Mn concentration, low LiP activity and traces of veratryl alcohol were detected, however MnP was remarkably stimulated. On the other hand, MnP activity was very low in the absence of Mn; whereas, LiP and veratryl alcohol production were enhanced.

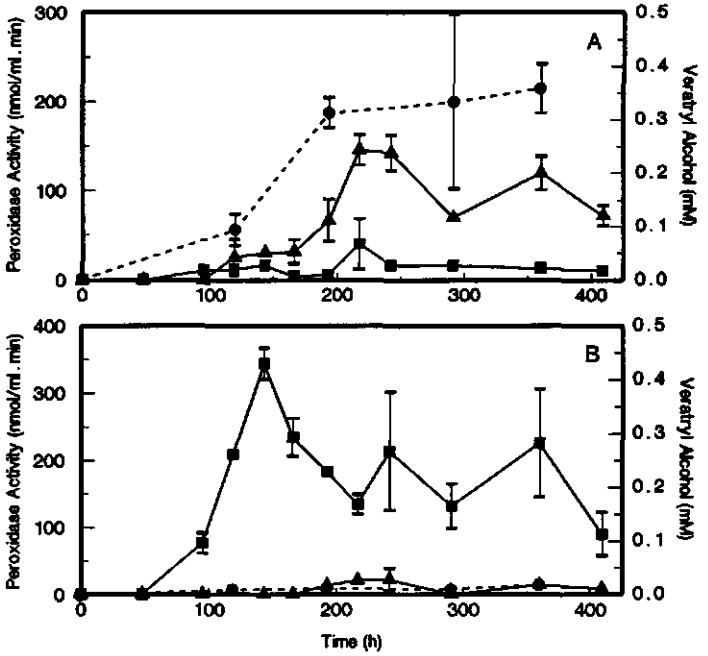

Figure 1. The effect of Mn on the time course of peroxidase titres and endogenous veratryl alcohol concentrations in the extracellular fluid of *Bjerkandera* sp. strain BOS55. Panels: A, Mn deficient medium; B, Mn sufficient medium (264μ M Mn). Symbols: ■, MnP; (▲), LiP; and (●), veratryl alcohol.

The Mn regulation of veratryl alcohol and peroxidase production at different nitrogen levels was also tested. Four levels of peptone-N were studied (2, 6, 20, and 60 mM). Figure 2 presents the peak concentrations of endogenous veratryl alcohol production and the peak titres of MnP and LiP measured during 18 days of culturing in media with different nitrogen and manganese levels. Veratryl alcohol production was greatly influenced by manganese at any level of nitrogen. In the Mn deficient media, irrespective of the peptone-N regimen, veratryl alcohol concentrations were high (0.40-0.75 mM) while the levels were much lower (< 0.075 mM) in media containing added Mn. An exception to this trend was observed in the 60 mM peptone-N media, where the negative impact of Mn on veratryl alcohol production was partially reversed. During the experiment, the concentrations of veratraldehyde were also

monitored. In Mn-deficient media, the veratraldehyde concentrations were very low (ranging from 0.007 to 0.044 mM) compared to the veratryl alcohol levels (Fig. 2C). The veratraldehyde levels were also significantly lowered by Mn supplementation.

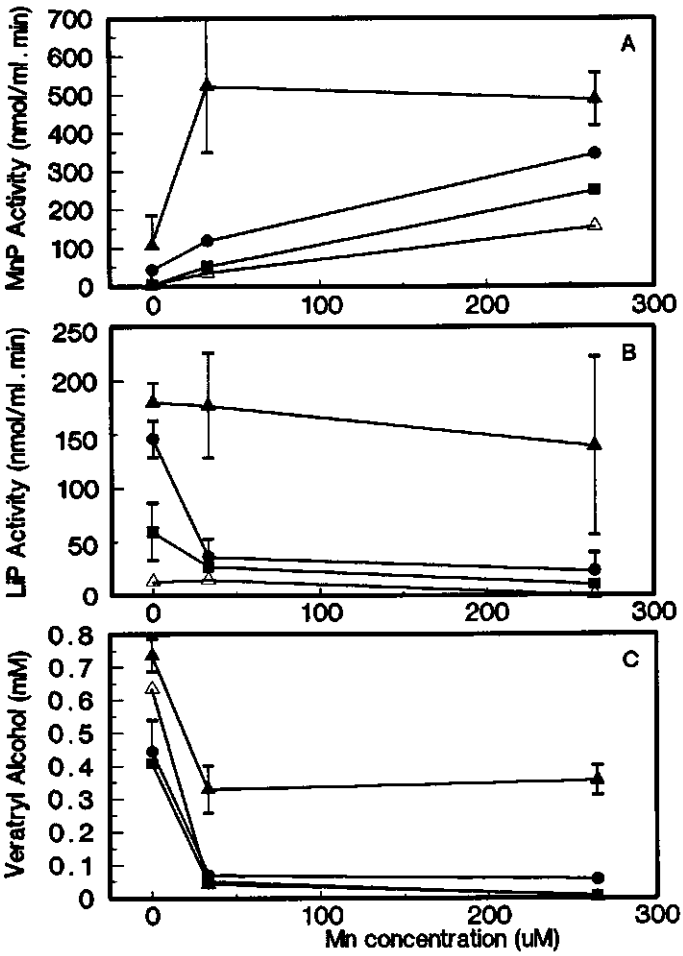

Figure 2. Effect of Mn and peptone-N supplements on the peak peroxidase titres and on the peak concentration of endogenous veratryl alcohol in *Bjerkandera* sp. strain BOS55. Panels: A, MnP; B, LiP; C, veratryl alcohol. Peptone-N was added at (Δ) 2; (■) 6; (●) 20; (▲) 60 mM.

Parallel to the trends observed for veratryl alcohol production, LiP titres were highest under Mn deficient conditions provided the N content was 6 mM or greater. The LiP titres were remarkably reduced by Mn sufficiency. Again it was observed that the negative effect of Mn could be diminished by 60 mM peptone N. MnP activity was consistently enhanced by Mn addition at any level of nitrogen. The highest MnP activities were detected in the 264 μM Mn media.

The influence of Mn and nitrogen on the third type of peroxidase in *Bjerkandera* sp. strain BOS55, MIP, was also examined. Mn had no effect on MIP production at any level of nitrogen (result not shown). However, increasing nitrogen contents in the media gave increasing MIP titres at all levels of Mn. For example at 33 μM Mn, 2, 6, 20, 60 mM peptone N gave peak MIP titres of 7.18±1.08, 21.98±12.62, 52.88±11.08, and 198.08±31.18 nmol ml^{-1} min^{-1}, respectively.

Other nitrogen sources, NH_4^+ and the amino acid mixture at 22 mM N were also studied to determine if the type of nitrogen source affected the Mn regulation of peroxidase and veratryl alcohol production. The trends described above for peptone N were the same for the other nitrogen sources (results not shown). However, the maximum veratryl alcohol concentrations achieved of 0.18±0.02 to 0.24±0.06 mM in Mn-deficient cultures were somewhat lower than those observed with peptone N. A part of the decrease could be accounted for by the fact that the veratraldehyde concentrations were higher (0.096 to 0.112 mM) when NH_4^+ and amino acids were used as a N-source. In the presence of Mn, both veratryl alcohol and veratraldehyde were only present at trace levels.

TABLE 1. Effect of manganese on the total CO_2 production after 18 days

N source (concn [mM])	Mean cumulative CO_2 Production (mmol) ± SD at Mn concn of:		
	0 μM	33 μM	264 μM
Peptone-N (2)	0.218±0.008	0.204±0.015	0.326±0.019
Ammonium-N (2.2)	0.258±0.021	0.348±0.015	0.429±0.013
Peptone-N (6)	0.424±0.015	0.508±0.018	0.546±0.012
Peptone-N (20)	1.104±0.049	1.101±0.014	1.030±0.028
Amino acids-N (22)	1.065±0.072	0.911±0.033	0.961±0.015
Peptone-N (60)	1.473±0.011	1.380±0.076	1.434±0.078

TABLE 2. Effect of Mn on the yield of mycelium dry weight on day 11

N source (concn [mM])	Mean mycelium dry wt (mg) ± SD at Mn concn of:		
	0 μM	33 μM	264 μM
Ammonium-N (2.2)	10.650±1.501	8.867±0.660	10.525±0.928
Peptone-N (20)	24.075±4.412	26.400±4.210	26.667±0.329
Peptone-N (60)	33.900±3.259	33.633±1.144	34.100±1.778

[a] The dry weight of the mycelium for cultures receiving 33 μM Mn was determined in a separate experiment from those using 0 and 264 μM Mn.

The differences observed in peroxidase and veratryl alcohol production due to Mn cannot be attributed to the role of manganese on fungal growth. Table 1 and 2 compare data on the total carbon dioxide production and the mycelial yield as a function of Mn and N nutrition. No significant differences in either of these parameters could be found when comparing Mn deficient and sufficient cultures under high N conditions. Likewise under low N conditions, Mn had no effect on the biomass yield. However, Mn increased the CO_2 production to a small extent.

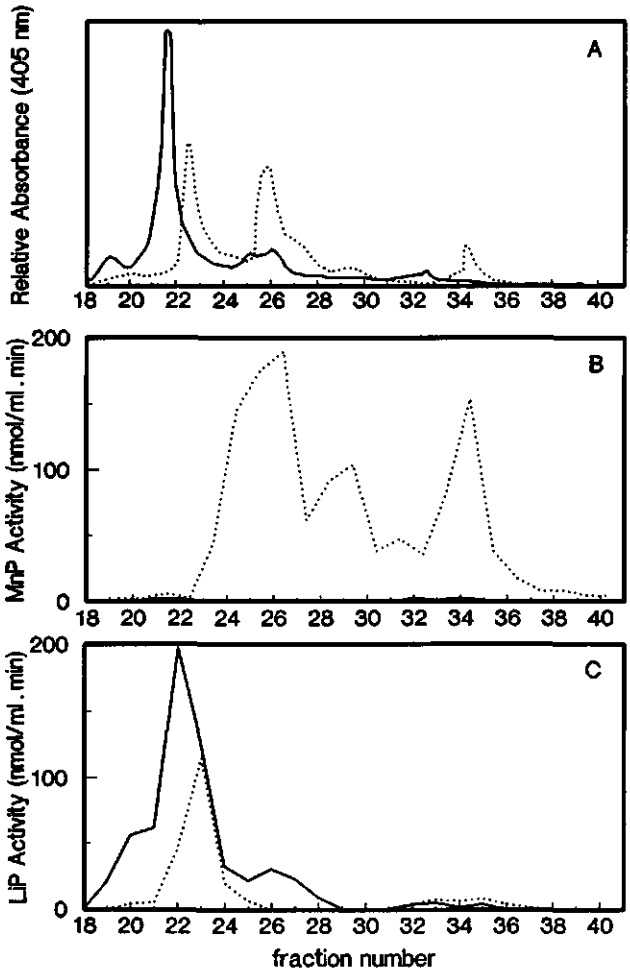

Figure 3. The FPLC protein profile of 7-day-old extracellular fluids of *Bjerkandera* sp. strain BOS55 grown in media containing 22 mM peptone-N. Panels: A, A_{405}; B, MnP activity; C, LiP activity. ____, Mn not added; ..... Mn added (264 μM).

Effect of manganese on extracellular heme-protein composition in *Bjerkandera* sp. strain BOS55. The changes in the extracellular heme-protein composition in the absence of Mn and in the presence of 264 μM Mn were followed by FPLC on day 4, 7, 10, and 14 in 22 mM peptone-N containing culture fluids. An example of these profiles is shown in Figure 3 demonstrating that the heme-protein profiles on day 7 differed depending on the Mn concentration. In the absence of Mn, LiP activity was dominant in those fractions giving higher A_{405} values. In the culture fluid containing 264 μM Mn, the absorbance of LiP containing fractions decreased and at the same time, new peaks were evident where mainly MnP activity occurred. During the entire time-course, FPLC peaks corresponding mainly to LiP activity were dominant in Mn free media; whereas peaks with MnP activities remained dominant in the high-Mn medium.

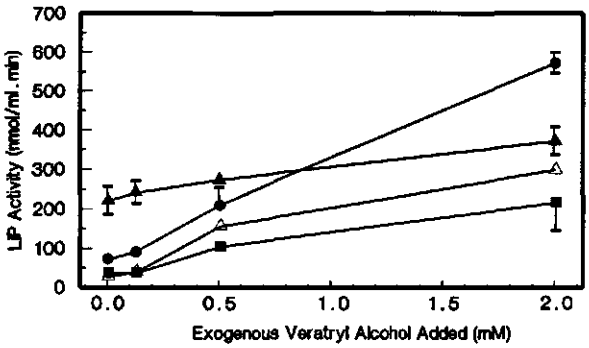

Figure 4. Effect of exogenous veratryl alcohol additions on peak LiP titres of *Bjerkandera* sp. strain BOS55 grown in media containing 33 μM Mn under varied conditions of N-nutrition. Symbols: Δ, 2.2 mM NH_4^+-N; ■, 22 mM NH_4^+-N; ●, 22 mM amino acids-N; ▲, 22 mM peptone N

Effect of exogenous veratryl alcohol addition on LiP production in *Bjerkandera* sp. strain BOS55. The effect of exogenous veratryl alcohol addition on LiP production was tested in the presence of 33 μM Mn. Veratryl alcohol was added at concentrations of 0.125, 0.5 and 2 mM at the time of inoculation, and LiP activity was measured during 18 days and compared to that of the control cultures without veratryl alcohol addition.

LiP production was greatly enhanced by increasing veratryl alcohol additions in Mn sufficient media irrespective of the nitrogen source and the concentration (Fig. 4). Surprisingly, high LiP titres were obtained in low-N media (2.2 mM NH_4^+-N) with addition of 0.5 and 2 mM veratryl alcohol. The most remarkable increase in LiP activity was measured in media with the amino acid mixture as a nitrogen source. Peptone medium gave the highest LiP activity in the controls (no veratryl alcohol added) in comparison to the other media, and with addition of veratryl alcohol, the increases in LiP production were less dramatic than with the other nitrogen sources.

The fate of the exogenous veratryl alcohol was followed at 2 mM veratryl alcohol addition (data not shown). The veratryl alcohol concentrations were reduced to about 1 mM on day 8. This level of veratryl alcohol remained more or less the same during the rest of the experiment in media with organic N-sources, while veratryl alcohol decreased further to 0.22

mM or less in the media with NH_4^+ as an N-source. In almost all cases, approximately half of the eliminated veratryl alcohol was recovered in the form of veratraldehyde.

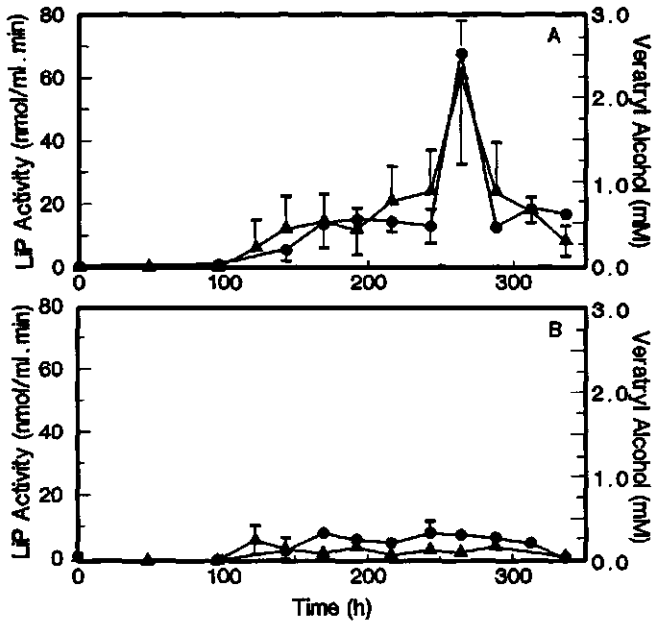

Figure 5. Effect of Mn on the time course of LiP titres and endogenous veratryl alcohol concentrations in extracellular fluids of *P. chrysosporium* BKM-F-1767. Panel: A, Mn-deficient medium; B, Mn sufficient medium (33 μM Mn). Symbols: ▲, LiP; ●, veratryl alcohol.

Effect of manganese on LiP and veratryl alcohol production in *P. chrysosporium*. The effect of Mn on LiP production by another white rot fungus was also investigated. *P. chrysosporium* was cultured under N-limited conditions either in Mn deficient or in Mn containing media (33 μM) to compare LiP titres and veratryl alcohol concentrations for 14 days. During the first 8 days, only slight increases in the LiP activity and veratryl alcohol level could be attributed to the Mn deficiency. However after day 8, both the LiP titres and veratryl alcohol concentrations started to become remarkably higher in the absence of Mn (Fig. 5). The endogenous veratryl alcohol reached a maximum concentration of 2.5 mM on day 11.

DISCUSSION

Mn regulation of peroxidases. The results of this study demonstrate that Mn has a strong regulatory effect on the appearance of MnP and LiP titres in the extracellular fluids of *Bjerkandera* sp. strain BOS55. In contrast to these peroxidases, the titre of the third type of peroxidase in *Bjerkandera*, MIP, was not influenced by Mn nutrition.

The MnP titres in *Bjerkandera* sp. strain BOS55 were highly stimulated in media containing 33 and 264 μM Mn(II) compared to those in medium lacking Mn(II). Similar results

have been reported previously with a wide variety of white rot fungi (3). Addition of Mn(II) to Mn-deficient cultures results in detectable *mnp* mRNA in *P. chrysosporium* (16), indicating that Mn has a regulatory role at the molecular level in white rot fungi.

In contrast to MnP, LiP titres in the extracellular fluids of *Bjerkandera* sp. strain BOS55 were dramatically lowered when the organism was cultivated in media containing 33 or 264 μM Mn(II) compared with those in Mn-deficient medium. However in 264 μM Mn medium, the LiP protein peak areas in FPLC profiles and the LiP activities in the extracellular protein mixture washed free of low MW material were equal to about a half of those obtained from cultures grown under Mn-deficiency. Similar observations were also reported in experiments with *Phlebia tremellosa* and *P. chrysosporium* (37, 47). These findings, together with the fact that Mn had no demonstrable effect in lowering *lip* mRNA levels in *P. chrysosporium* (29), suggest that Mn(II) additions somehow inhibit LiP activity but do not necessarily prevent LiP expression. Mn(II) itself is not toxic to LiP in vitro at concentrations below 1,000 μM (21). Thus the toxic effect of Mn(II) additions is most likely related to a product generated from Mn(II). One obvious candidate is Mn(III) which is known to rapidly deactivate LiP (39).

Mn regulation of veratryl alcohol biosynthesis. The absence of Mn(II) in the medium was found to strongly increase the physiological levels of endogenous veratryl alcohol in the two white rot fungi tested, *Bjerkandera* sp. strain BOS55 and *P. chrysosporum* BKM-F 1767. This is the first time that Mn nutrition has been shown to affect the veratryl alcohol levels in white rot fungi. Previously, it was claimed that Mn(II) nutrition had no influence on the endogenous veratryl alcohol levels in *P. chrysosporium* (6). However, this claim was based on a spot check in a 6-day-old culture. In our experiments, Mn-deficient medium provided an unprecedented high level of endogenously produced veratryl alcohol (2.5 mM) on day 11. Mn deficiency under a wide variety of experimental conditions consistently stimulated endogenous veratryl alcohol pools in *Bjerkandera* sp. strain BOS55. The appearance of veratryl alcohol was earlier; however, the peak levels of around 0.75 mM were not as high as those in *P. chrysoporium*.

The fact that Mn had such an important impact on the endogenous veratryl alcohol levels in white rot fungi suggests that it regulates enzymes involved in either the degradation, recycling or biosynthesis of veratryl alcohol. Even though veratryl alcohol is a de novo metabolite, it is also degraded by white rot fungi (11, 31). Intracellular reductases have been shown to occur in *Bjerkandera* sp. strain BOS55 which can recycle veratraldehyde back to veratryl alcohol (8). In this study, veratraldehyde was observed to be an important degradation product when high levels of exogenous veratryl alcohol were added to the culture medium. Endogenous veratraldehyde remained at trace levels and was not observed to increase as a result of the lowered endogenous veratryl alcohol levels in Mn sufficient culture conditions. On the contrary, veratraldehyde levels were even lower in Mn-sufficient medium. Consequently, we must conclude that Mn is not likely involved in the regulation of veratryl alcohol degradation nor recycling. Therefore a more plausible location for the regulation would be during the biosynthesis of veratryl alcohol. Veratryl alcohol is synthesized via phenylalanine, cinnamic acid, benzoic acid and benzylaldehyde (23). The regulation probably occurs in a step after phenylalanine. Mn had little or no effect on fungal growth in NH_4^+-N media lacking phenylalanine. Therefore, the fungus was apparently able to synthesize this essential amino acid in the presence of Mn.

Indirect effect of veratryl alcohol on LiP titres. The endogenous levels of veratryl alcohol were generally highly correlated to the titres of LiP. This finding suggested that the higher LiP titres found in Mn-deficient cultures were an indirect result of the enhanced veratryl alcohol production. LiP isozymes are known to be rapidly inactivated by physiological levels of H_2O_2 (7, 21, 43) and veratryl alcohol is known to protect LiP from this kind of inactivation (7, 18, 43, 44). Adding exogenous veratryl alcohol to Mn-sufficient cultures of *Bjerkandera* sp. strain BOS55 resulted in high LiP titres. The LiP titres were highly statistically correlated (R^2 = 0.767, $p < 0.001$) to the veratryl alcohol concentration irrespective of whether it was produced endogenously in Mn deficient cultures or added exogenously in Mn sufficient medium (Fig. 6), confirming that Mn(II) has no direct regulating effect on LiP expression. Instead LiP titres are only apparently higher due to the enhanced endogenous production of veratryl alcohol.

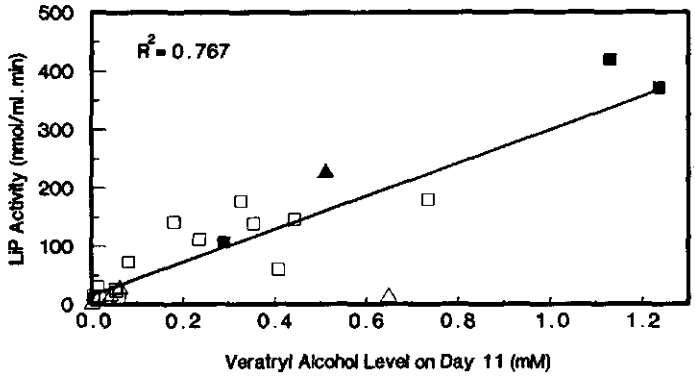

Figure 6. Correlation between LiP titres and veratryl alcohol concentrations in the extracellular fluids of 11-day-old *Bjerkandera* sp. strain BOS55 cultures. Shown are endogenous veratryl alcohol in response to various Mn concentrations in media supplemented with 2 (Δ) or 20 to 60 (□) mM N; exogenous veratryl alcohol remaining in Mn-sufficient cultures supplemented with 2.2 (▲) or 22 (■) mM N.

Exogenous veratryl alcohol supplements are commonly used in studies evaluating LiP production by white rot fungi. For this reason, Mn up to concentrations of approximately 200 μM had no major role in lowering LiP titres (3, 33, 38, 46). However when abnormally high concentrations of Mn were used (720 to 3618 μM), little or no LiP activity was detected (3, 33, 38). In these cases, Mn probably had an inhibitory effect on the LiP protein activity as suggested above.

While veratryl alcohol apparently induces LiP expression in *Bjerkandera* sp. strain BOS55 and many other white rot fungi (12, 34), no increases in *lip* mRNA levels were found to result from adding veratryl alcohol to cultures of *P. chrysosporium* (7, 41). Thus LiP secretion must be signalled by another mechanism. Under the N-sufficient conditions used here, a likely signal would be the cyclic AMP that results from limited energy resources during C-starvation and is known to induce *lip* gene expression in *P. chrysosporium* (4). The first appearance of LiP activity in *Bjerkandera* sp. strain BOS55 generally coincided with the cessation of growth, indicating that it occurred when the readily available substrates were being depleted.

Nitrogen regulation. *Bjerkandera* sp. strain BOS55 and other strains of *Bjerkandera* produce LiP and MnP in N-sufficient conditions (8, 24, 25). Therefore *Bjerkandera* spp. can be regarded as N-unregulated, in strong contrast to the model fungus, *P. chrysoporium* (16, 26). In the absence of N-regulation, increasing N would be expected to increase peroxidase production as a result of increased cell yields. *Bjerkandera* sp. strain BOS55 followed this behaviour. N-sufficiency remarkably increased growth with a parallel increase in LiP titres in Mn-deficient medium or with a parallel increase in MnP titres in Mn-sufficient medium. On the other hand, veratryl alcohol levels in any given Mn regimen were more or less similar, independently of the N supplied. Therefore N has no regulatory effect on veratryl alcohol biosynthesis in *Bjerkandera* sp. strain BOS55, in contrast to *P. chrysosporium* (13, 26).

Previously, it was reported that Mn and N regulation function independently of each other in both the wild type and N-deregulated mutants (45). Between 2 and 20 mM N, this pattern was also observed to be the case with *Bjerkandera* sp. strain BOS55. However, when 60 mM peptone N was used, the Mn sufficient media were no longer able to repress veratryl alcohol production or lower LiP titres. This effect may be due to the ability of some peptides in peptone to chelate Mn. Peptides containing glycine, cysteine and glutamate are powerful metal binding agents (17). In one study, Mn(II) chelated by polyglutamate was unable to enter the active site of MnP (28). Perhaps, peptides can also lower the bioavailability of Mn(II) for the molecular regulation of a key enzyme involved in veratryl alcohol biosynthesis or for a key enzyme that requires Mn as a cofactor.

Importance of veratryl alcohol regulation by Mn. Veratryl alcohol is an essential cofactor for the proper functioning of LiP. Nonphenolic lignin models and lignin itself can be degraded *in vitro* with LiP only if veratryl alcohol is present (19, 20, 44). Therefore, the biosynthesis of veratryl alcohol in response to Mn-deficiency must be regarded as a key physiological event enabling LiP to function as a ligninolytic enzyme. Mn-deficiency would be expected to result from decreases in available Mn lost to insoluble MnO_2 deposits occurring during white rot decay (2, 39).

ACKNOWLEDGEMENT

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3. Stimulation of Aryl Metabolite Production in the Basidiomycete *Bjerkandera* sp. Strain BOS55 with Biosynthetic Precursors and Lignin Degradation Products

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SUMMARY

Aryl metabolites are known to have an important role in the ligninolytic system of white rot fungi. The addition of known precursors and aromatic acids representing lignin degradation products stimulated the production of aryl metabolites (veratryl alcohol, veratraldehyde, p-anisaldehyde, 3-chloro-*p*-anisaldehyde) in the white rot fungus, *Bjerkandera* sp. strain BOS55. The presence of manganese (Mn) is known to inhibit the biosynthesis of veratryl alcohol (T. Mester, E. de Jong, and J. A. Field, Appl. Environ. Microbiol. 61:1881-1887, 1995) A new finding of this study was that the production of the other aryl metabolites, *p*-anisaldehyde and 3-chloro-*p*-anisaldehyde, was also inhibited by Mn. We attempted to bypass the Mn inhibited step in the biosynthesis of aryl metabolites by the addition of known and suspected precursors. Most of these compounds were not able to bypass the inhibiting effect of Mn. Only the fully methylated precursors (veratrate, *p*-anisate, 3-chloro-*p*-anisate) provided similar concentrations of aryl metabolites in the presence and absence of Mn, indicating that Mn does not influence the reduction of the benzylic acid group. The addition of deuterated benzoate and 4-hydroxybenzoate resulted in the formation of deuterated aryl metabolites indicating that these aromatic acids entered into the biosynthetic pathway and were common intermediates to all aryl metabolites. Only deuterated chlorinated anisyl metabolites were produced when the cultures were supplemented by deuterated 3-chloro-4-hydroxybenzoate. This observation combined with the fact that 3-chloro-4-hydroxybenzoate is a natural product of *Bjerkandera* spp. (H. J. Swarts, F. J. M. Verhagen, J. A. Field, and J. B. P. A. Wijnberg, Phytochemistry 42:1669-1701, 1996) suggests that it is a possible intermediate in chlorinated anisyl metabolite biosynthesis.

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INTRODUCTION

White rot fungi are basidiomycetes which effectively degrade lignin with their extracellular oxidative enzyme system (2, 12, 20). Evidence is accumulating indicating that aromatic secondary metabolites are involved in the ligninolysis process (8). *Bjerkandera* sp. strain BOS55 is an outstanding white rot fungus (11) well known for its production of aryl metabolites. Aside from the more commonly occurring veratryl (3,4-dimethoxybenzyl) and *p*-anisyl (4-methoxybenzyl) alcohol/aldehyde, it is also able to synthesize de novo 3-chloro-*p*-anisyl (3-chloro-4-methoxybenzyl) and 3,5-dichloro-*p*-anisyl (3,5-dichloro-4-methoxybenzyl) alcohol and aldehyde (7, 9, 10).

Veratryl alcohol has multiple functions in conjunction with lignin peroxidase (LiP). It is considered as a cofactor of LiP required for completing the enzymatic catalytic cycle (21). Recently it was demonstrated that veratryl alcohol cation radical generated by LiP can mediate the oxidation of a wide range of compounds possessing lower redox potential than the veratryl alcohol radical itself (13). Finally, the fact that veratryl alcohol apparently induces LiP production in white rot fungi; whereas, it has no influence on the *lip* mRNA synthesis can be explained by its role in protecting LiP against H_2O_2 mediated inactivation (4).

In many white rot fungi, *p*-anisyl alcohol, and to a lesser extent *p*-anisaldehyde, are thought to be involved in the H_2O_2 generation as substrates for the extracellular aryl alcohol oxidase (AAO) (8, 14). De Jong and coworkers (7) found that 3-chloro-*p*-anisyl alcohol and 3,5-dichloro-*p*-anisyl alcohol serve as even better substrates for H_2O_2 production by AAO than the other non-chlorinated aryl metabolites in *Bjerkandera* sp. strain BOS55. AAO together with its substrates may also have an important role in preventing the repolymerization of lignin degradation products by reducing the quinones formed during lignin degradation (24).

Little is known about the physiology and molecular biology of the secondary metabolite production in white rot fungi. The involvement of aryl metabolites in lignin degradation is suggested by the fact that their production coincides with the onset of ligninolytic activity. Factors which in general are crucial for lignin degradation, e.g. nutrient limitation, O_2 atmosphere, are also beneficial for veratryl alcohol production in the well studied white rot fungus, *Phanerochaete chrysosporium* (2). Recently we discovered that the presence of soluble manganese (Mn) inhibits the production of veratryl alcohol by white rot fungi (25).

The biosynthetic pathway of aryl metabolites is not entirely understood. Veratryl alcohol was shown to be completely synthesized de novo from glucose in several white rot fungi (8, 23). Veratryl alcohol biosynthesis originates from the shikimate pathway via phenylalanine with cinnamate, benzoate and benzaldehyde having been identified as biosynthetic intermediates in the case of *P. chrysosporium* (18). The precursors of anisyl and chloroanisyl metabolites also seem to be derived from aromatic amino acids since the addition of tyrosine increased the *p*-anisaldehyde and 3-chloro-*p*-anisaldehyde production in *Bjerkandera adusta*. (30).

The objective of the present study was to determine if aryl metabolite production could be stimulated by adding biosynthetic precursors or aromatic acids representing lignin degradation products. These compounds were added in the presence and absence of Mn in order to determine at which point along the biosynthetic pathway the Mn regulation of veratryl alcohol could be bypassed.

MATERIALS AND METHODS

Microorganism. *Bjerkandera* sp. strain BOS55 was isolated and maintained as described before (25). Inoculum was prepared on malt extract plates (per liter: 15 g agar, 5 g glucose, 3.5 g malt extract) and incubated at 30°C for 4 to 6 days. The experiments were inoculated with 6 mm diameter agar plugs obtained from the leading edge of mycelium.

Media and culture conditions. The standard basal medium used contained 22 mM nitrogen (N) (2.2 mM N as diammonium tartrate and 19.8 mM N as an L-amino acid mixture), 0, 33 μM or 100 μM $MnSO_4$ containing BIII mineral medium modified from Tien and Kirk (32), 10 g of glucose liter $^{-1}$ and 2 mg of thiamine liter $^{-1}$ in 20 mM 2,2-dimethylsuccinate and 40 mM KH_2PO_4 buffer (pH 6). The amino acid mixture was composed as described before (25) with the exception that L-phenylalanine and L-tyrosine were excluded. The media were sterilized with FP030/3 filters (Schleicher & Schuell, Dassel Germany) with a pore size of 0.2 μm. Different compounds were added individually to the cultures, these included: L-phenylalanine, L-tyrosine, *trans*-cinnamate, benzoate, benzaldehyde, 4-hydroxybenzoate, 3-hydroxybenzoate, protocatechuate (3,4-dihydroxybenzoate), *p*-anisate (4-methoxybenzoate), isovanillate (3-hydroxy-4-methoxybenzoate), vanillate (3-methoxy-4-hydroxybenzoate), veratrate (3,4-dimethoxybenzoate), veratraldehyde (3,4-dimethoxybenzaldehyde), 3-chloro-4-hydroxybenzoate, 3-chloro-*p*-anisate (3-chloro-4-methoxybenzoate). The timing of the addition and the final concentration of the additives are indicated in the results section.

Aliquots (5 ml) of media were placed in 250 ml, presterilized loosely capped serum bottles. Cultures were incubated statically under an air atmosphere at 30°C.

Determination of secondary metabolites. Culture supernatants were centrifuged for 10 min (1200 × g) and 50 μl samples were analyzed by high-pressure liquid chromatography (HPLC). Triplicate parallel cultures were analyzed on each sampling day. A Pascal series HPLC ChemStation (Hewlett-Packard, Waldbronn, Germany) equipped with HP1040 M series II diode array detector was used. The column (200 by 3 mm) was filled with ChromoSpher C18-PAH (5 μm particles) (Chrompack, Middelburg, The Netherlands). The following gradient (0.4 ml min^{-1}, 30°C) was used: 90:10, 0:100, and 0:100 H_2O:CH_3CN at 0, 15, 20 min, respectively. Compound identifications were based on matching retention times and UV spectra with standards.

Determination of deuterated metabolites. The deuterated metabolites were identified by GC-MS. After filtration of the extracellular fluid, pH of the filtrate was adjusted to 2 with 4 M H_2SO_4 followed by the extraction 3 times with freshly distilled ethylacetate. The combined organic layers were washed with distilled water and concentrated under reduced pressure at ambient temperature. The concentrate was filtered over silica gel 60 (230-400 mesh) (Merck) with ethylacetate as eluent. After removal of the solvent under reduced pressure, the remaining residue was redissolved in 0.5 ml ethylacetate and then subjected to GC-MS analysis. All the samples were analyzed on an HP5970B quadrupole mass spectrometer coupled to an HP5890 gas chromatograph equipped with a fused silica capillary column (DB17, 30 m × 0.25 mm i.d., film thickness: 0.25 μm). Carrier gas and flow were Helium (He) at 1.1 ml min^{-1}. Injector temperature 220°C; temperature program: 70 to 250°C at 7°C min^{-1} with holding for 20 min. Injection volume was 5 μl (split ratio 1:100). EI-MS were obtained at 70 eV. The identification of deuterated compounds was achieved by comparison of retention times and mass spectra to data from respective nondeuterated authentic compounds. The incorporation of deuterated

precursors was determined by monitoring the main ion peaks of the deuterated and non-deuterated metabolites.

Chemicals. Deuterated 4-hydroxy benzoate was prepared from deuterated phenol as described by Komiyama and Hirai (22). Deuterated 4-hydroxybenzoate was monochlorinated using sulfurylchloride in glacial acetic acid to give deuterated 3-chloro-4-hydroxybenzoate, as white powder (87%). The structures of the synthesized compounds were confirmed by ^{13}C NMR. The synthesis of 3-chloro-*p*-anisyl alcohol/aldehyde and 3,5-dichloro-*p*-anisyl alcohol/aldehyde are described elsewhere (9, 10). All other chemicals were commercially available and used without further purification.

Statistical procedures. In all experiments, the measurements were carried out with triplicated cultures incubated in parallel. The values reported are means with standard deviations.

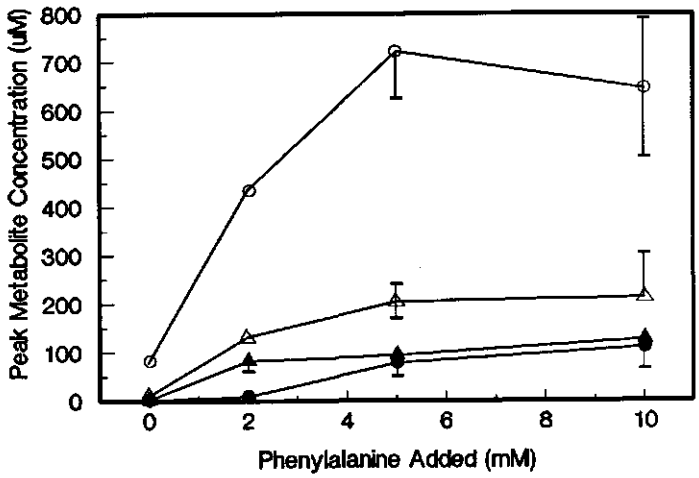

Figure 1. Effect of increasing concentrations of phenylalanine on the production of secondary metabolites. Symbols: O, veratryl alcohol; Δ, veratraldehyde; ●, *p*-anisaldehyde; ▲, 3-chloro-*p*-anisaldehyde.

RESULTS

Effect of phenylalanine and tyrosine on the aryl metabolite production. Phenylalanine was added to Mn deficient cultures of *Bjerkandera* sp. strain BOS55 at the time of inoculation and the aryl metabolites produced were measured daily over a period of 14 days. The peak concentrations of veratryl alcohol and veratraldehyde as a function of the initial phenylalanine concentration in the media are shown in Figure 1. The veratryl metabolite production was remarkably enhanced up to a phenylalanine concentration of 5 mM. Additionally, the phenylalanine supplementation stimulated the production of *p*-anisaldehyde and 3-chloro-*p*-anisaldehyde (Fig. 1).

The effect of tyrosine, as a potential alternative precursor on the secondary metabolite production was also examined under Mn deficiency. Tyrosine was added in a slow dosage

format of 1 mM every second day of culturing (providing a total addition of 6 mM) starting on day 2. This procedure was done in order to prevent an excessive polymerization of this phenolic compound. The addition of tyrosine increased the production of secondary metabolites but to a lesser extent than phenylalanine. The peak concentrations of veratryl alcohol, veratraldehyde, *p*-anisaldehyde, and 3-chloro-*p*-anisaldehyde were 347±50, 91±36, 1.6±0.1 and 23.4±6.2 μM, respectively.

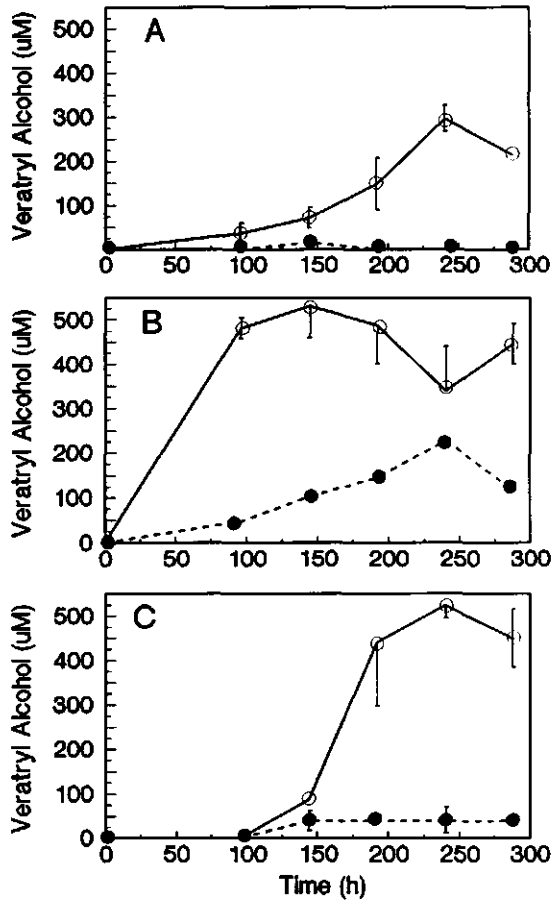

Figure 2. Effect of the addition of known veratryl alcohol precursors on the time course of veratryl alcohol production under manganese deficiency and sufficiency. Shown are veratryl alcohol concentrations in the control cultures (A), in those with 5 mM of phenylalanine (B) and in those with 5 mM of benzaldehyde (C). Symbols: O, veratryl alcohol under Mn deficiency; ●, veratryl alcohol in cultures containing 33 μM Mn.

Effect of biosynthetic precursors and lignin degradation products on veratryl alcohol production under Mn sufficiency and deficiency. Known biosynthetic precursors of veratryl alcohol in *Phanerochaete chrysosporium* (phenylalanine, cinnamate, benzoate, benzaldehyde)

were added at the time of inoculation to Mn sufficient (33 μM) and deficient cultures of *Bjerkandera* sp. strain BOS55 at a concentration of 5 mM. In cultures receiving cinnamate and benzoate, growth was completely inhibited. Phenylalanine and benzaldehyde stimulated the production of veratryl alcohol in both Mn nutrient regimes; however, the veratryl alcohol concentrations were much higher in the absence of Mn (Fig. 2). The peak concentrations occurred after 10 days cultivation in the control and benzaldehyde supplemented cultures, whereas cultures receiving phenylalanine produced a high concentration of veratryl alcohol much earlier, on day 4, and the high concentration was maintained during the whole experiment.

Veratraldehyde at 5 mM was also tested as a supplement in several experiments. This compound was readily reduced to high levels of veratryl alcohol (3000 to 4000 μM) independent of the Mn concentration (results not shown).

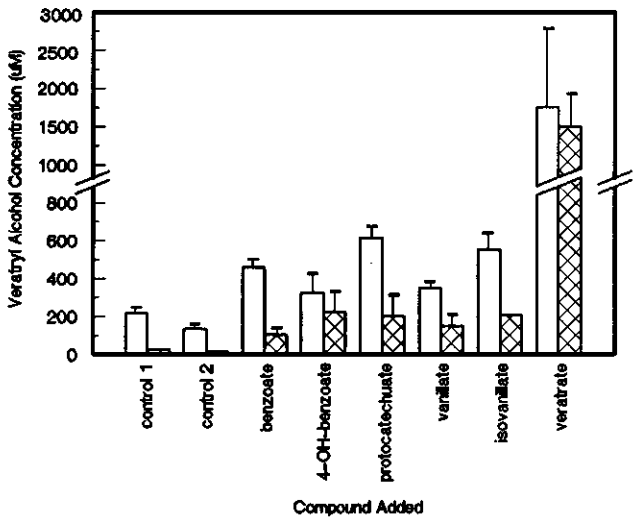

Figure 3. Effect of the addition of lignin degradation products on the peak concentration of veratryl alcohol produced in cultures under Mn deficiency (open bars) and sufficiency (crosshatched bars). The data originate from two separate experiments: Experiment 1 comprised control 1, benzoate, isovanillate, and veratrate, and experiment 2 comprised control 2, 3-hydroxybenzoate, 4-hydroxybenzoate, protocatechuate, and vanillate.

Aromatic acids representing monomeric lignin degradation products and suspect biosynthetic precursors of veratryl alcohol were tested for their ability to stimulate veratryl alcohol production in *Bjerkandera* sp. strain BOS55. The aromatic acids were added in a slow dosage format in order to minimize the growth inhibiting effect of certain aromatic acids (e.g. cinnamate, *p*-anisate) as well as prevent an excessive polymerization of phenolic compounds. As shown in Figure 3, a large variety of the aromatic acids significantly enhanced the peak concentration of veratryl alcohol under Mn-sufficient (33 μM) and deficient culture conditions, compared to control cultures from the same experiment. These included benzoate, 3- and 4-

hydroxybenzoate, protocatechuate, vanillate, isovanillate, and veratrate. For almost all of the compounds tested, the veratryl alcohol concentration was much higher in the Mn deficient medium compared to the parallel treatment in Mn sufficient medium. Only in the case of veratrate, was the veratryl alcohol production high in both Mn nutrient regimes (Fig. 3). For the most part, the trends outlined above for veratryl alcohol were also observed for the production of veratraldehyde. However, the concentration of veratraldehyde was generally 2- to 10-fold lower than the veratryl alcohol concentrations.

The addition of other compounds such as cinnamate and *p*-anisate were less successful for stimulating veratryl alcohol production. Cinnamate significantly enhanced the peak concentration of veratryl alcohol in the Mn sufficient medium but not in the Mn deficient culture (data not shown). However, even under Mn deficient conditions, cinnamate did stimulate veratraldehyde production the early production of veratryl alcohol resulting in 296±30 μM of veratryl alcohol on day 6 while only 92±9 μM was present in the controls. Additions of *p*-anisate did not significantly increase the production of veratryl alcohol (data not shown).

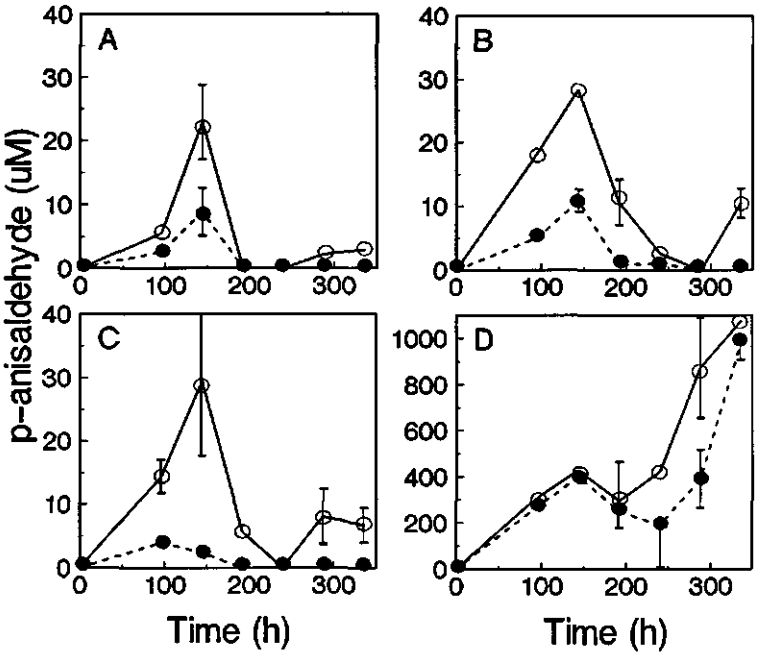

Figure 4. Effect of the addition of known veratryl alcohol precursors and suspected precursors on the production of *p*-anisaldehyde in the presence (●) and absence (○) of manganese. Cultures received cinnamate (A), benzoate (B), 4-hydroxybenzoate (C) as well as *p*-anisate (D). Cinnamate, benzoate and 4-hydroxybenzoate were added in the slow-dosage format, 1 mM of these compounds were added on days 2, 4, 6, 8 and 10. *p*-Anisate was added at 1 mM on day 2 only. In the control cultures (no precursors added) the production of *p*-anisaldehyde was below the detection level.

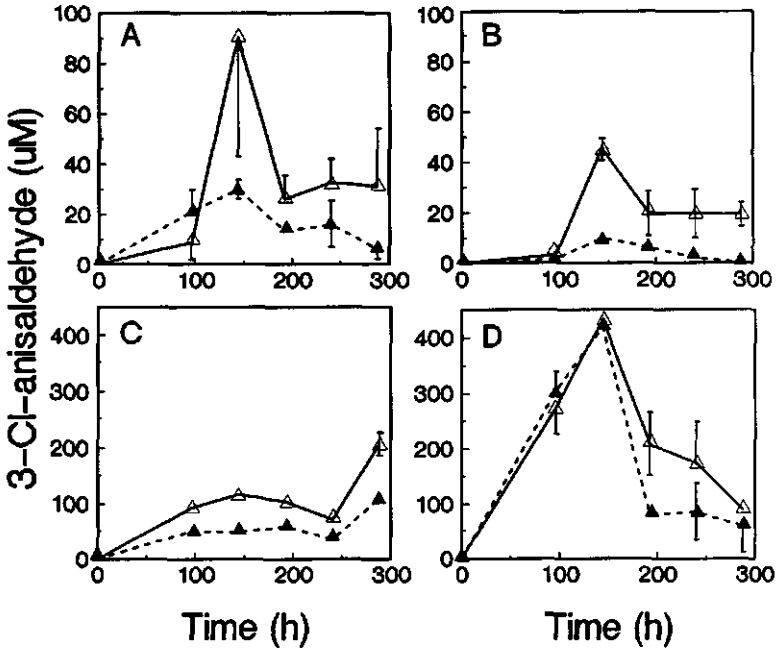

Figure 5. Effect of the addition of known veratryl alcohol precursors and suspected precursors on the production of 3-chloro-*p*-anisaldehyde in the presence (▲) and absence (Δ) of manganese. Cultures received cinnamate (A), benzoate (B), 3-chloro-4-hydroxybenzoate (C) as well as 3-chloro-*p*-anisate (D). Cinnamate, benzoate and 3-chloro-*p*-anisate were added at the concentration of 1 mM on day 2. 3-chloro-4-hydroxybenzoate was added in the slow-dosage format, 1 mM of this compound was added on day 2, 4, 6, 8 and 10. In the control cultures (no precursors added) corresponding to panel A, B, and D the production of 3-chloro-*p*-anisaldehyde was below the detection level in Mn sufficient and 0.8±0.7 μM in Mn deficient cultures. The highest concentration of 3-chloro-*p*-anisaldehyde produced in control cultures corresponding to panel C was 1.5 ±0.4 and 6.2±1.3 μM under Mn sufficiency and deficiency, respectively.

Effect of biosynthetic precursors of veratryl alcohol and the suspected precursors on anisyl metabolite production under Mn deficiency and sufficiency. As indicated in the first experiment, phenylalanine, an important precursor of veratryl alcohol biosynthesis stimulated the production of other aryl metabolites as well. In order to evaluate, whether other biosynthetic precursors of veratryl alcohol are also beneficial for the *p*-anisaldehyde and 3-chloro-*p*-anisaldehyde production, compounds were added either at a concentration of 1 mM on the second day of fungal growth or in the above described slow dosage format to Mn sufficient and Mn deficient cultures.

Figure 4 illustrates that the detection of *p*-anisaldehyde was enabled by supplementation of *Bjerkandera* sp. strain BOS55 cultures with cinnamate, benzoate, 4-hydroxybenzoate, and *p*-anisate; whereas, this metabolite remained below the detection limit in the control cultures. Similar to the observations with veratryl alcohol, most of the compounds provided higher *p*-anisaldehyde concentrations in the Mn-deficient compared to the Mn-sufficient medium.

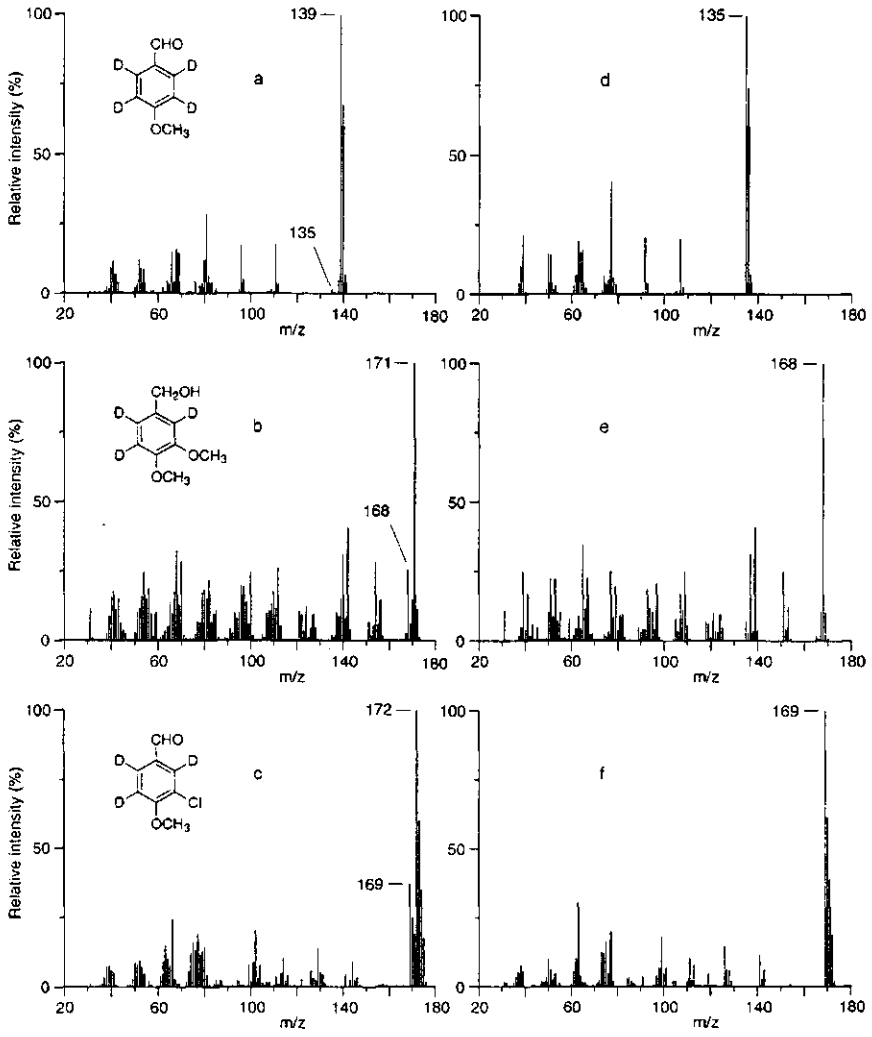

Figure 6. Shown are mass spectra of the deuterated *p*-anisaldehyde (A), veratryl alcohol (B), and 3-chloro-*p*-anisaldehyde (C) produced by the fungus when deuterated 4-hydroxybenzoate was added as a supplement. Also shown are the mass spectra of the nondeuterated *p*-anisaldehyde (D), veratryl alcohol (E) and 3-chloro-*p*-anisaldehyde (F) from standards.

Only *p*-anisate gave a high level of *p*-anisaldehyde irrespective of the Mn nutrient regime. Culture supplementation with *p*-anisate also enabled the production of *p*-anisyl alcohol at a concentration of 82±34 μM and 279±196 μM in Mn sufficient and deficient cultures, respectively.

Most of the aromatic acids such as cinnamate and benzoate which increased the production of *p*-anisaldehyde, were also found to significantly stimulate the production of 3-chloro-*p*-anisaldehyde (Fig. 5A and B). In control cultures, the concentration of this metabolite reached a peak concentration of only 1.5 to 4.3 μM and 6.2 to 15.1 μM in Mn sufficient and deficient cultures, respectively (results not shown). The highest production of 3-chloro-*p*-anisaldehyde was observed by the supplementation of the cultures with 3-chloro-4-hydroxybenzoate and 3-chloro-*p*-anisate (Fig. 5C and D). The addition of 3-chloro-*p*-anisate also resulted in the detection of 3-chloro-*p*-anisyl alcohol which reached 295±82 μM and 180±144 μM in the Mn deficient and sufficient cultures, respectively. Again it was observed that the production of 3-chloro-*p*-anisaldehyde was usually higher in Mn-deficient compared to Mn-sufficient cultures. However, Mn had no effect on the level of 3-chloro-*p*-anisaldehyde produced when 3-chloro-*p*-anisate was supplemented. While 4-hydroxybenzoate did not appear to enhance the peak concentration of 3-chloro-*p*-anisaldehyde, it did stimulate the early production of this metabolite on day 4 to 6 by two-fold (data not shown). However, the addition of the methylated form of this compound, *p*-anisate only enhanced the late production of 3-chloro-*p*-anisaldehyde up to 17±3 μM by day 14. Additionally, aromatic acids with hydroxy- or methoxy-substituents in the 3-position such as 3-hydroxybenzoate, protocatechuate, vanillate, isovanillate had no effect on the production on anisyl metabolites (results are not shown).

Incorporation of deuterated precursors into aryl metabolites. In order to exclude the possibility that the above tested aromatic compounds induce the aryl metabolite production instead of playing a role as precursors, deuterated compounds (benzoate, 4-hydroxybenzoate, 3-chloro-4-hydroxybenzoate) were added in the slow dosage format to the cultures. The production of aryl metabolites was monitored on day 6, 8, and 10 under Mn deficiency. The incorporation of deuterated precursors into aryl metabolite pools was measured by the GC-MS. The deuterated compounds were readily taken up and metabolized by the fungus. Figure 6A, B, and C show as examples the mass spectra of deuterated *p*-anisaldehyde, veratryl alcohol, 3-chloro-*p*-anisaldehyde synthesized by the fungus, respectively, and Figure 6D, E, and F show the corresponding nondeuterated compounds as references from standards. The increased mass number due to the deuterium atoms remaining in aryl metabolites resulted in a good distinction from the corresponding non-deuterated compounds. Based on this difference in mass number, the incorporation of deuterated precursors into each aryl metabolite pool was estimated.

Table 1 represents the deuterated aryl metabolites found when the cultures were supplemented by deuterated benzoate, 4-hydroxybenzoate, or 3-chloro-4-hydroxybenzoate. The addition of deuterated benzoate resulted in very high incorporation into *p*-anisaldehyde, veratryl alcohol, veratraldehyde, 3-chloro-*p*-anisaldehyde during the whole time period of the experiment. The reduction of deuterated benzoate to deuterated benzaldehyde was also observed. When deuterated 4-hydroxybenzoate was added, the incorporation into veratryl compounds was also high on day 6, however it decreased on day 8 and 10. Deuterated *p*-anisyl alcohol, *p*-anisaldehyde, 3-chloro-*p*-anisaldehyde, and 3,5-dichloro-*p*-anisaldehyde were also measured. Deuterated 3-chloro-4-hydroxybenzoate was readily methylated resulting in the

formation of deuterated 3-chloro-*p*-anisyl alcohol and aldehyde as well as chlorinated products such as 3,5-dichloro-*p*-anisaldehyde.

TABLE 1. The production of deuterated secondary metabolites by the addition of deuterated aromatic acids.

Deuterated Metabolite	Value for deuterated compound added					
	Benzoate		4-Hydroxybenzoate		3-Chloro-4-hydroxybenzoate	
	Incorp. (%)[a]	Day	Incorp. (%)[a]	Day	Incorp. (%)[a]	Day
Benzaldehyde	100	10	nd[b]		nd	
p-Anisyl alcohol	nd		81	6	nd	
p-Anisaldehyde	98	6	98	6	nd	
	98	10	93	8		
Veratryl alcohol	91	6	80	6	nd	
	86	8	54	8		
	90	10	57	10		
Veratraldehyde	96	6	82	6	nd	
	90	8	62	8		
	93	10	59	10		
3-Chloro-p-anisaldehyde	96	6	71[c]	6	98	6
	91	8	77	8	96	8
	93	10	74	10	98	10
3,5-Dichloro-p-anisaldehyde	100	8	31	8	92	6
			25	10	82	8
					84	10

[a] Incorporation (percent) = 100 × deuterated metabolite/(deuterated + nondeuterated metabolite).
[b] nd. not detected
[c] In the case of chlorinated metabolites, the incorporation (percent) was corrected for isotopic effects of chlorine atoms.

DISCUSSION

Veratryl compounds. Previously, phenylalanine, cinnamate, benzoate and benzaldehyde were shown to be biosynthetic precursors of veratryl alcohol in the white rot fungus, *P. chrysosporium* based on a ^{14}C-isotope trapping experiments (18). The results here demonstrate that when these precursors are added to the cultures of *Bjerkandera* sp. strain BOS55, the production of veratryl alcohol and veratraldehyde was significantly stimulated suggesting that they are readily taken up by the cells and enter freely into the biosynthetic route. Aside from the known biosynthetic precursors, a large number of aromatic acids such as 3- and 4-hydroxybenzoate, protocatechuate, vanillate, isovanillate, veratrate were also shown to stimulate the production of veratryl alcohol and veratraldehyde. Many of these aromatic acids are known to be intermediates in the degradation of lignocellulose, lignin, and lignin model

compounds by various white rot fungi (5, 34, 17, 26, 16). Consequently, we must conclude that there are several alternative pathways for the production of veratryl alcohol in white rot fungi. On the one hand, veratryl alcohol is a de novo product that can be completely synthesized from glucose as has been demonstrated using ^{14}C-glucose (23). The de novo synthesis probably proceeds via the shikimate pathway yielding aromatic amino acids such as phenylalanine as an important intermediate (33). Several research groups have demonstrated that ^{14}C-phenylalanine is converted to ^{14}C-veratryl alcohol by *P. chrysosporium* (29, 18). In this study, we have also shown that tyrosine could stimulate veratryl alcohol production suggesting that it also could possibly serve as an alternative biosynthetic precursor. On the other hand, aromatic acids representing lignin degradation products were shown to be readily converted to veratryl alcohol by *Bjerkandera* sp. strain BOS55. In a similar fashion, it was demonstrated that *P. chrysosporium* readily methylated 4-hydroxybenzoate, protocatechuate, vanillate, and isovanillate (15). Additionally it was previously observed that 4-hydroxybenzoate and vanillate were converted to veratryl alcohol albeit in minor yields by the white rot fungus *Pycnoporus cinnabarinus* (16). Also two white rot fungi, *P. chrysosporium* and *Coriolus (Trametes) versicolor*, were shown to convert 3,4-dimethoxycinnamyl alcohol to veratryl alcohol (29, 19), while this compound was determined not to be a biosynthetic precursor (18). *Bjerkandera* sp. strain BOS55 was likewise able to convert 3,4-dimethoxycinnamate to veratryl alcohol (results not shown). When lignin degradation products are used for aryl metabolite production, the de novo synthesis appears to be repressed as was observed in the case of *Phlebia radiata* (28). The addition of vanillate repressed de novo formation of secondary metabolites from ^{14}C-glucose.

Some of the aromatic acids derived from lignin degradation might coincide with the unknown biosynthetic precursors representing hydroxylated and partially methylated intermediates between benzoate and benzaldehyde on the one side and veratrate, veratraldehyde, and veratryl alcohol on the other. The formation of deuterated veratryl alcohol and veratraldehyde when deuterated 4-hydroxybenzoate was added supports this hypothesis. Compounds such as vanillate and isovanillate would be expected to be readily methylated to veratryl metabolites by the most effective methylating systems discovered in *P. chrysosporium* utilizing either L-methionine or chloromethane as a methyl donor (15). Once either veratrate or veratraldehyde is formed, these compounds would be reduced to veratryl alcohol by intracellular aryl dehydrogenases that are well known in white rot fungi (8).

Stimulation of *p*-anisyl and 3-chloro-*p*-anisyl compound production. The fact that the biosynthetic precursors of veratryl alcohol simultaneously stimulated the production of other aryl metabolites as well, indicates that there are common precursors. Phenylalanine, tyrosine, benzoate, and benzaldehyde represent the compounds that could enhance the production of *p*-anisaldehyde and 3-chloro-*p*-anisaldehyde in parallel with veratryl metabolites. The deuterated form of all these metabolites were obtained when deuterated benzoate and deuterated 4-hydroxybenzoate were added, indicating that they are common precursors. 4-Hydroxybenzoate is most likely the last common intermediate of veratryl alcohol and *p*-anisaldehyde biosynthesis in *Bjerkandera* sp. strain BOS55, which was also confirmed in the experiments where deuterated 4-hydroxybenzoate was used. The addition of deuterated 4-hydroxybenzoate also resulted in deuterated 3-chloro-*p*-anisaldehyde, and 3,5-dichloro-*p*-anisaldehyde; however, compounds with a propanoid side chain such as phenylalanine, tyrosine, or cinnamate stimulated the production of 3-chloro-*p*-anisaldehyde from four- to ten-fold better than 4-

hydroxybenzoate. This different effect of 4-hydroxybenzoate and the phenylpropanoid compounds was not observed for the production of veratryl and anisyl compounds. A probable explanation is that intermediates with longer side chains could be better substrates for the yet unidentified chlorinating enzyme. We have not yet been able to demonstrate the occurrence of 4-hydroxybenzoate as a de novo product in *Bjerkandera* sp. strain BOS55, probably because it is a fleeting intermediate. However, 4-hydroxybenzyl compounds have been identified as a natural product in the cultures of *Pleurotus* spp. grown on mineral-glucose medium indicating that this compound does occur as a de novo product in white rot fungi (14).

The isomer 3-hydroxybenzoate exclusively stimulated veratryl metabolites and had no effect on *p*-anisaldehyde nor 3-chloro-*p*-anisaldehyde probably because it is structurally incompatible with the latter. Similar trends were observed with protocatechuate, vanillate and isovanillate because these aromatic acids possibly represent precursors unique to the veratryl alcohol branch of biosynthetic pathway.

Beck and coworkers (1) suggested that during the biosynthesis of 3-chloro-*p*-anisaldehyde in *Bjerkandera adusta* methylation precedes the chlorination. Our results rather indicate that the chlorination probably takes place first because 4-hydroxybenzoate could significantly stimulate the early production of the chlorinated anisyl metabolite whereas *p*-anisate only enhanced the late production. Additionally, 3-chloro-4-hydroxybenzoate, previously identified as a naturally occurring metabolite in *Bjerkandera* spp. (31) stimulated the formation of 3-chloro-*p*-anisaldehyde by 30- to 70-fold. The direct incorporation of deuterated 3-chloro-4-hydroxybenzoate into chlorinated anisyl metabolites also supports the hypothesis that this compound is an intermediate in the biosynthesis.

Manganese regulation. Previously we have demonstrated that the presence of Mn in the medium inhibits the biosynthesis of veratryl alcohol in white rot fungi (25). A new finding of this study was that the production of the other aryl metabolites *p*-anisaldehyde and 3-chloro-*p*-anisaldehyde were also inhibited by the presence of Mn. We have attempted to bypass the Mn inhibited step in the biosynthesis of aryl metabolites by the addition of known and suspected precursors. Most of these compounds were not able to bypass the inhibiting effect of Mn on aryl metabolite formation in *Bjerkandera* sp. strain BOS55. Only the fully methylated precursors such as veratraldehyde/veratrate, *p*-anisate, and 3-chloro-*p*-anisate in the case of veratryl alcohol, *p*-anisaldehyde, 3-chloro-*p*-anisaldehyde biosynthesis, respectively, provided similar levels of aryl metabolites in both Mn-deficient and sufficient cultures. Consequently we can conclude with certainty that the reduction of the benzylic acid group by aryl dehydrogenases was not influenced by the presence of Mn. The results might suggest that the methylation of phenol groups is regulated by Mn. However, it should be noted that the addition of precursors consistently enhanced the production of aryl metabolites even in the presence of Mn; albeit, that the production was higher in the absence of Mn. Consequently, methylation was not *per se* the limiting factor in the biosynthesis of aryl metabolites in Mn sufficient medium. It seems more likely that the presence of Mn affected the stability of phenolic precursors. Manganese-dependent peroxidase (MnP) is known to be expressed at higher titres under Mn sufficient condition (25) and this enzyme is very efficient in oxidizing phenols (27). However aryl metabolite biosynthesis is an intracellular process. Although the localization of MnP in the peripheral regions of cytoplasm was observed (6) MnP is generally regarded as an extracellular enzyme. Therefore other intracellular enzymes that oxidize phenols should be considered such as vanillate hydroxylase which occurs in *Sporotrichum pulverulentum (P.*

chrysosporium) (3). Phenolic acids such as 4-hydroxybenzoate, protocatechuate, and vanillate are good substrate for this enzyme while substrates with protected phenol groups such as veratrate are by comparison poorly oxidized (3). Further studies therefore should investigate if vanillate hydroxylase production or enzyme activity is influenced by Mn.

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4. Peroxidase and Aryl Metabolite Production by the White Rot Fungus *Bjerkandera* sp. Strain BOS55 During Solid State Fermentation of Lignocellulosic Substrates

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SUMMARY

Ligninolytic enzymes and secondary metabolite production by *Bjerkandera* sp. strain BOS55 were monitored during solid state fermentation (SSF) on two lignocellulosic substrates, beech wood and hemp stem wood (HSW). After 6 weeks of SSF, the fungus was responsible for removing 27 and 39% of the Klason lignin as well as 43 and 70% of the apolar extractives on beech and HSW, respectively. The lignin degradation during beech wood decay was very selective. On both substrates, high activities of lignin peroxidase (LiP) and manganese peroxidase (MnP) were detected. The peak activity of LiP was 660 nmol $ml^{-1}min^{-1}$ on HSW and that of MnP was 1320 nmol ml^{-1} min^{-1} on beech wood. The presence of several LiP and MnP isoenzymes at different times during the SSF was demonstrated by FPLC profiles of these heme proteins. The production of the secondary aryl metabolites, veratryl alcohol and 3-chloro-*p*-anisaldehyde, reached peak concentrations of 820 and 90 μM, respectively. The enhanced production of these secondary metabolites compared to defined liquid cultures is suggested to be due to the release of lignin degradation products serving as alternative precursors for their biosynthesis. The high production of veratryl alcohol, which is a cofactor known to protect LiP from inactivation by physiological levels of H_2O_2, may account for the high production of active LiP on the lignocellulosic substrates.

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INTRODUCTION

White rot fungi are generally acknowledged as the most significant degraders of lignin. Some strains are able to selectively degrade lignin leaving cellulose largely unutilized (14, 27, 44). Ligninolytic fungi secrete various oxidative extracellular enzymes; lignin peroxidase (LiP), manganese-dependent peroxidase (MnP), laccase, hydrogen peroxide producing oxidases which together with secondary metabolites, and manganese form a complex extracellular system being responsible for the ligninolysis (21, 30). Previous research on wood ultrastructure suggested that the initial attack of lignin is not directly carried out by ligninolytic enzymes, but by unknown low molecular weight mediators of ligninolytic enzymes (35). The expression of the ligninolytic enzymatic system coincides with the onset of secondary metabolism triggered by C-, N-, or S-starvation (30); whereas, lignin itself is not an inducer (5).

White rot fungi and their oxidative enzymes have potential applications in the pulp and paper industry and in upgrading woody materials for animal feed. The application of lignin degrading fungi in pulping can save energy and/or chemicals (35), while in bleaching they can potentially replace, at least in part, chlorine or chlorine-dioxide (45). After fungal treatment, the improved digestibility of lignocellulosic by-products from agriculture has also been shown in many studies (1, 47, 51). However, most of the investigations concerned with the practical applications of white rot fungi have focused on a few strains, i.e. *Phanerochaete chrysosporium, Trametes versicolor, Phanerochaete sordida, Ceriporiopsis subvermispora, Pleurotus* spp., *Lentinus edodes* (32, 35, 45, 48, 54).

In different screening studies, *Bjerkandera* sp. strain BOS55 was found to be an outstanding ligninolytic strain (12, 17). It was shown to be the most efficient biobleacher of oxygen delignified eucalyptus kraft pulp resulting in brightness gains of up to 14 ISO units (40). *Bjerkandera* sp. strain BOS55 is a nitrogen unregulated white rot fungus producing high ligninolytic enzyme activities in submerged cultures composed of defined nitrogen-nutrient rich media (28, 38). The ligninolytic enzyme system of this fungus consists of three types of peroxidases: LiP, MnP and a lesser known peroxidase denominated manganese independent peroxidase (MIP) (8, 9). Also an H_2O_2-generating oxidase, aryl alcohol oxidase (AAO) and several de novo aryl secondary metabolites are produced (8). The production of LiP and MnP are dependent on the presence of Mn (36) as it was observed in many other white rot fungal strains (3); while, the appearance of MIP was not affected by the Mn level (36). *Bjerkandera* sp. strain BOS55 is also known as a good producer of various secondary metabolites. These include the more commonly occurring veratryl alcohol and veratraldehyde (3,4-dimethoxybenzyl alcohol/aldehyde) as well as *p*-anisyl alcohol and *p*-anisaldehyde (4-methoxybenzyl alcohol/aldehyde) (8, 36). Veratryl alcohol is an essential cofactor for LiP and *p*-anisyl alcohol is a substrate for AAO (10). Aside from these metabolites, chlorinated *p*-anisyl alcohol and aldehyde (3-chloro-4-methoxybenzyl and 3,5-dichloro-4-methoxybenzyl alcohol/aldehyde) have also been reported in *Bjerkandera* spp. (8, 50). The chlorinated anisyl alcohols are considered as the physiological substrates for AAO in this genus (8).

The applicability of the strain to biopulping, biobleaching, and upgrading woody feedstocks would greatly depend on its ability to produce ligninolytic enzymes and related secondary metabolites on natural lignocellulosic substrates. The objective of the present study was to investigate the production of peroxidases and secondary metabolites of *Bjerkandera* sp. strain BOS55 during solid state fermentation (SSF) on lignocellulosic substrates. Two different

substrates were chosen: beech wood, representing a commonly occurring substrate in nature, and hemp stem wood (HSW) as a model for annual fibre crops. The time course of peroxidases, AAO, secondary aryl metabolite production paralleled by the measurement of lignin degradation was followed for six weeks. The results were compared to those of previous studies in synthetic liquid media.

MATERIALS AND METHODS

Microorganism. *Bjerkandera* sp. strain BOS55 was isolated and maintained as described before (36). Inoculum was prepared on malt extract plates (per litre: 15 g agar, 5 g glucose, 3.5 g malt extract) and incubated at 30°C for 4 to 6 days. The experiments were inoculated with 6 mm-diameter agar plugs obtained from the leading edge of the mycelium.

Culture conditions. 5 g of air dry milled wood (< 2 mm; 8% moisture content) of hemp stem wood (*Cannabis sativa* L.) and beech wood (*Fagus sylvatica* L.) was placed to 250 ml serum bottles and autoclaved for 1 hour with either 5 or 3.5 ml demineralized water, respectively, resulting in unsubmerged conditions. Moisture content was corrected twice a week with sterilized demineralized water. All bottles were inoculated with one agar plug. They were loosely capped for passive aeration and were incubated at 30°C with parallel abiotic controls which did not receive inoculum.

Enzyme assays. For the preparation of enzyme assays, 50 ml of 10 mM KP_i buffer (pH 6) was added to each serum bottle and the substrates were extracted for 30 min with 100 rpm agitation at 20°C. Extracellular fluid extracts were centrifuged (20,000 × *g*), and the measured enzyme activities reported were corrected to the undiluted volume of the moisture in the colonized substrates.

All enzyme activities were determined spectrophotometrically at 30°C. LiP activity was measured by oxidation of veratryl alcohol to veratraldehyde at 310 nm (ε = 9,300 M^{-1} cm^{-1}) as described by Tien and Kirk (52) and corrected for background AAO activity. MnP and MIP were assayed by the oxidation of 2,6-dimethoxyphenol to its dimeric product at 469 nm (ε = 49,600 M^{-1} cm^{-1}) in the presence and absence of Mn, respectively (36).

The measurement of aryl alcohol oxidase (AAO) activity with the oxidation of *p*-anisyl alcohol monitored at 290 nm (8) was not feasible because of the interference of soluble lignin. Therefore, an indirect method was used to estimate the hydrogen peroxide production in the presence of *p*-anisyl alcohol. The reaction mixture contained: 40 mM KH_2PO_4 buffer (pH 5.7), 0.5 mM diammonium 2,2'-azinobis(3-ethyl-6-benzothiazoline sulfonate) (ABTS), 1 U ml^{-1} horseradish peroxidase (HRP type II; Sigma), and 1 mM *p*-anisyl alcohol. Absorbance of oxidized ABTS was measured at 420 nm (ε = 36,000 M^{-1} cm^{-1}). AAO measurements were corrected in the absence of *p*-anisyl alcohol for the background activity from other hydrogen peroxide producing oxidases. The relevance of the method was checked by adding 30 U ml^{-1} catalase of *Aspergillus niger* (Sigma, St. Louis, USA), which completely hindered the oxidation of ABTS.

Protease activity was measured with Azocoll substrate as described by Dosoretz *et al.* (13). One unit is defined as the amount of enzyme that catalyzes the release of the azo dye causing a change in absorbance of 0.001 min^{-1} at 520 nm.

Determination of extracellular peroxidase profile by fast protein liquid chromatography (FPLC). Extracellular fluid extracts were centrifuged at 20,000 × *g* for 10

min at 4°C to remove the wood particles and mycelium. Centrifuged extracts were concentrated four-fold to the original volume by ultrafiltration through a membrane with 10 kDa cut-off (Amicon Rotterdam, the Netherlands). A 0.5 ml sample of concentrated extracellular filtrates was applied to an FPLC equipped with an 1 ml analytical MonoQ column (Pharmacia, Roosendaal, The Netherlands) as described before (36). Samples were eluted with a linear salt gradient (0 to 0.3 M NaCl in 10 mM KP_i starting buffer, pH 6) at a flow rate of 1 ml min^{-1}, and absorbance was monitored at 405 nm. Fractions were collected each minute and were assayed for LiP, MnP, and MIP activity.

Determination of secondary metabolites. A 50 µl sample of extracellular fluid extract was analyzed by high pressure liquid chromatography as described before (36) using a column (200 mm by 3 mm) packed with ChromoSpher C18-PAH (5-µm particles) (Chrompack, Middelburg, The Netherlands). The following gradient was used: 90:10, 0:100, and 0:100 H_2O:CH_3CN at 0, 15, 20 min., respectively. The measurements were carried out at the eluent flow rate of 0.4 ml min^{-1}, at 30°C. Compound identification was based on matching retention times and UV spectra with standards. The concentration of the metabolites was corrected to the undiluted volume of moisture in the colonized substrates.

Analytical techniques. Total weight loss was estimated from the change in the dry weight of milled wood (105°C, overnight).

Klason (acid insoluble) and acid soluble lignin contents were measured after digesting milled wood with 72% sulphuric acid as described in method T222 of Technical Association of the Pulp and Paper Industry (TAPPI, Atlanta, GA., USA). The acid soluble lignin was estimated by measuring the absorbance at 280 nm, using 13 liter g^{-1} cm^{-1} as the extinction coefficient (16).

Wood extractives were determined by extracting 1 g of oven dried milled wood with 200 ml of ethanol-cyclohexane solvent in a Soxhlet extraction apparatus as described in TAPPI method T264.

Holocellulose was estimated from the difference in total weight less the sum of Klason lignin, acid soluble lignin and apolar extractives.

Soluble Mn in wood was determined by atomic absorption spectrometry (Varian SpectrAA 300-400) after extracting 5 g milled wood with 50 ml double distilled water for 20 minutes. Samples were filtered through filter paper and 10 ml of the separated liquid phase was acidified by 10 ml of 0.04 N H_2SO_4.

Statistical procedure. In all experiments, the measurements were carried out with triplicate parallel cultures. The values reported are means with standard deviation.

Chemicals. Standards of 3-chloro-*p*-anisyl alcohol and aldehyde as well as 3,5-dichloro-*p*-anisyl alcohol and aldehyde were synthesized as described earlier (11). All other chemicals were commercially available and used without further purification.

RESULTS

Colonization and decay of substrates. The two substrates of this study, HSW and beech wood had a very similar lignin content of approximately 25%. Most of the lignin was acid insoluble, only 2.5 to 3.5% of the total lignin content was acid soluble in the two lignocellulosic substrates used. The apolar wood extractives content was accounting for 2.3 and 0.7% of the dry weight in HSW and beech, respectively (Table 1).

Table 1. Change in wood composition during the wood decay by *Bjerkandera* sp. strain BOS55.[a]

Wood Components	HSW		Beech	
	day 0	day 42	day 0	day 42
Total dry matter (g/flask)	4.63±0.07[b]	3.42±0.03	4.61±0.02	4.09±0.03
Klason lignin (g/flask)	1.16±0.02	0.71±0.03	1.11±0.01	0.81±0.02
Acid soluble lignin (g/flask)	0.03±0.01	0.07±0.01	0.04±0.01	0.06±0.00
Extractives (mg/flask)	108±2	32±6	30±1	17±3

[a] In the abiotic controls of wood samples, the weight of total dry matter, Klason lignin, soluble lignin, and extractives did not change significantly during the 6 weeks of the SSF.
[b] Data presented here are means ±SD

The results of the fungal degradation of different wood components are summarized in Table 1. *Bjerkandera* sp. strain BOS55 readily colonized both lignocellulosic substrates. The total weight loss of HSW and beech wood was 26 and 11 %, respectively. After six weeks of SSF, 39 and 27% decreases in Klason lignin were observed in HSW and beech wood, respectively. There were little increases in acid soluble lignin; however, the acid soluble fraction only represented 7-9% of the total lignin at the end of the experiment. The fungal degradation of wood extractives was remarkable, accounting for 70% of losses on HSW and 43% of losses on beech wood.

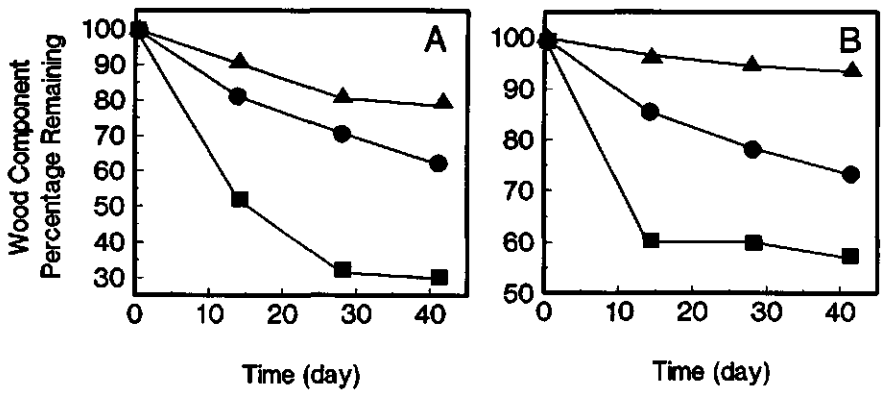

Figure 1. Time course of degradation of wood components by *Bjerkandera* sp. strain BOS55 on hemp stem wood (A) and beech wood (B). Symbols: ▲, estimated holocellulose; ●, lignin; ■, apolar extractives.

The time course of wood decay on the two lignocellulosic substrates is shown in Figure 1. In HSW, an extensive holocellulose degradation (up to 21.7 % after 6 weeks) was observed which slowed down only in the last two weeks of the SSF (Fig. 1A). The rates of lignin and holocellulose decomposition were similar resulting in very poor selectivity of lignin degradation. However, a selective degradation of apolar extractives was observed in this lignocellulosic substrate. In beech wood, the holocellulose consumption was slow (only 6.6% after 6 weeks). On the other hand, the percentage of lignin removed was by comparison high resulting in a very selective lignin degradation (Fig. 1B). The apolar extractives were also

selectively utilized in the first two weeks of SSF. Thereafter, the apolar extractives were degraded slowly.

The pH was also observed to change due to the fungal activity. The pH dropped by approximately 1.5 units by day 14 from the initial values of 6.6 and 5.2 in the case of HSW and beech, respectively. Thereafter, it remained constant in both substrates.

The initial concentration of soluble Mn was different in the two lignocellulosic materials. At the beginning of the experiment, 25 and 6 ppm of Mn were detected in beech wood and HSW, respectively. These values did not change significantly due to the fungal activity during the six weeks of SSF.

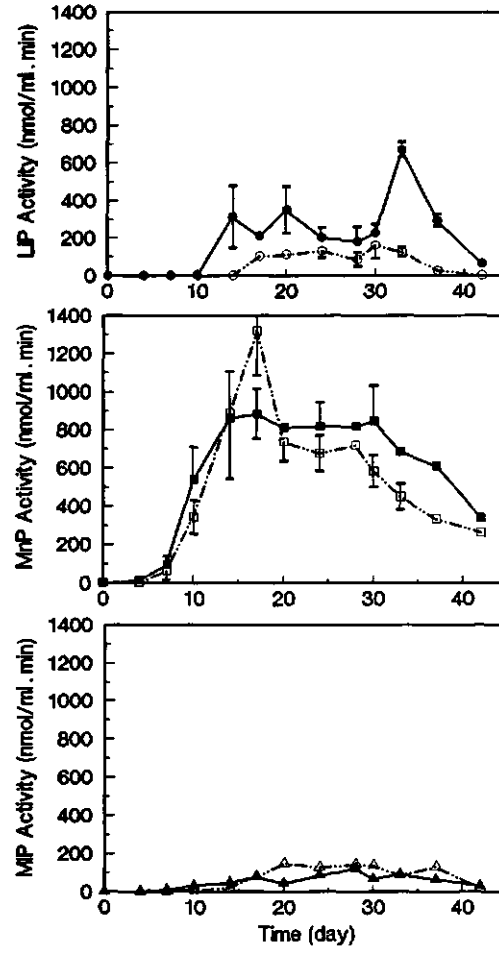

Figure 2. The time course of peroxidase activity during SSF on hemp stem wood (filled symbols) and on beech wood (open symbols). Panel A: Lignin peroxidase; Panel B: Manganese-dependent peroxidase; Panel C: Manganese-independent peroxidase.

The production of peroxidases and other extracellular enzymes. Figure 2 represents the time course of extracellular peroxidase activities secreted by *Bjerkandera* sp. strain BOS55. Ligninolytic enzyme activities were first detected on day 7 and were present at relatively high titres thereafter till the end of the experiment. The development of LiP activity occurred, starting on day 10 with HSW and somewhat later with beech. Higher LiP activity (Fig. 2A) was detected on HSW than on beech wood. The peak activity was 660 nmol ml^{-1} min^{-1} on HSW while 180 nmol ml^{-1} min^{-1} was obtained on beech. The peak activity of MnP was somewhat lower, 880 nmol ml^{-1} min^{-1} on HSW than that of 1320 nmol ml^{-1} min^{-1} on beech. However, very similar titres were achieved in the rest of the time course (Fig. 2B). The time course of the third peroxidase, MIP is presented in Figure 2C. The peak activities were 120 and 150 nmol ml^{-1} min^{-1} with HSW and beech wood, respectively.

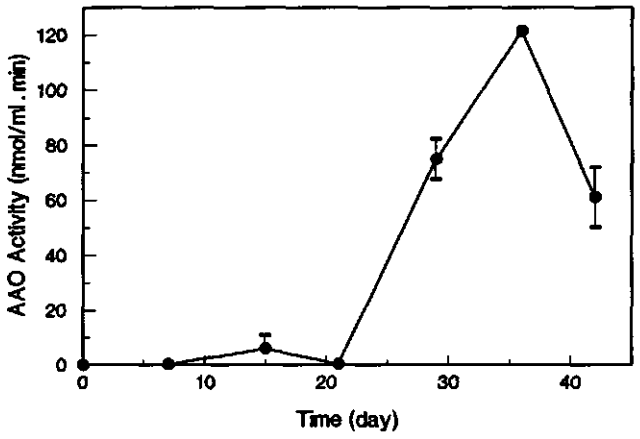

Figure 3. The time course of aryl alcohol oxidase activity on HSW during the SSF.

Aryl alcohol oxidase (AAO) activity was also detected during the experiment. On beech wood, AAO activity was very low. Incidentally 1-2 nmol ml^{-1} min^{-1} were detected but otherwise its activity was under the detection limit (data not shown). On HSW, AAO titres were slightly higher, about 4-5 nmol ml^{-1} min^{-1} in the first three weeks of incubation. Thereafter AAO activity was unprecedently high reaching a peak value of 120 nmol ml^{-1} min^{-1} on day 36 (Fig. 3).

On both substrates, protease activity was detected during the 6 week-time period reaching a peak activity of 39 U l^{-1} with HSW on day 10 and 25 U l^{-1} with beech wood on day 24 (data not shown).

FPLC enzyme profiles. The FPLC study was carried out in order to investigate the enzyme profiles of peroxidases produced on the two different natural substrates. Figure 4 shows the FPLC profiles of heme-proteins as monitored at 405 nm and the measured extracellular peroxidase activities in the fractions on day 14, 20, 33. On both substrates, all three enzyme activities were present in fractions corresponding to those containing heme proteins. At least 8 heme peaks could be distinguished. According to their elution pattern, they were numbered I through VIII from left to right. The first two peaks, I and II coincided with

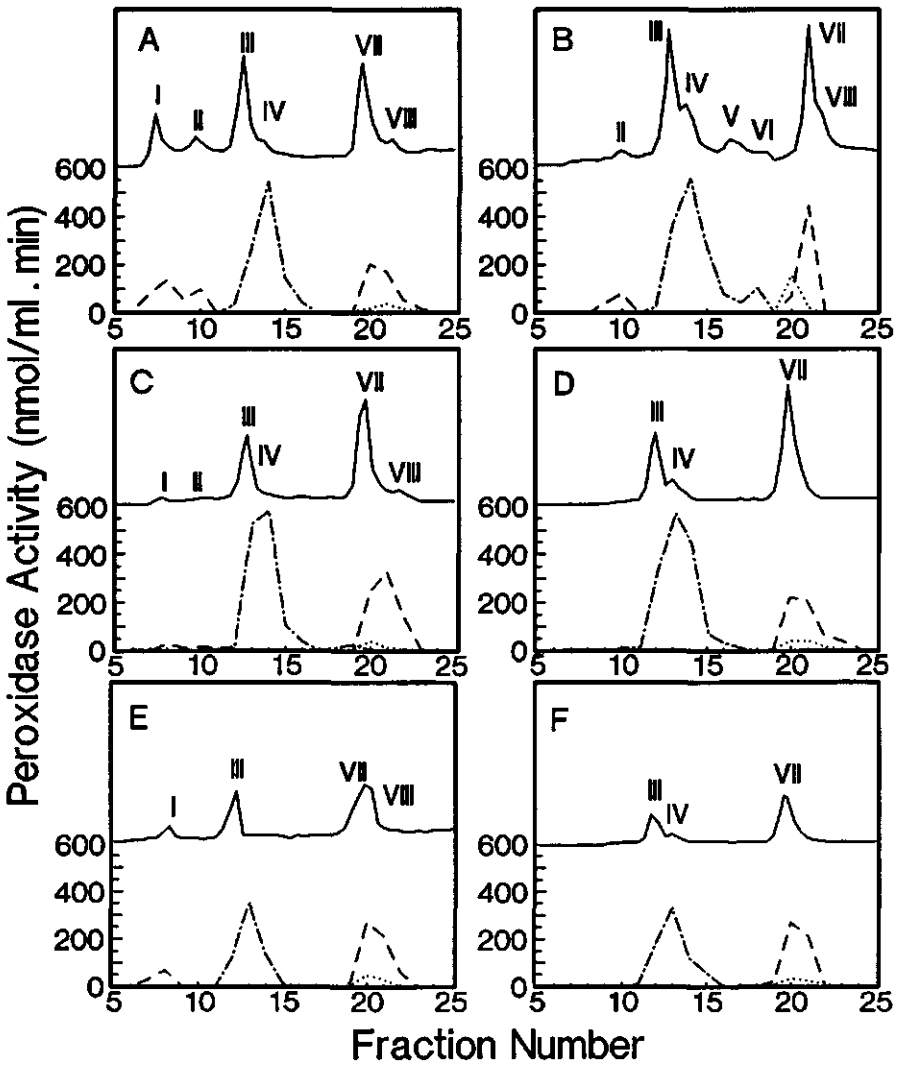

Figure 4. FPLC profiles of extracellular peroxidase on day 14 (A, B), day 20 (C, D) and day 33 (E, F). Panel A, C, E: peroxidases produced on hemp stem wood; Panel B, D, F: peroxidases produced on beech wood. The upper trace in each panel is the relative absorbance at 405 nm indicating heme proteins. The lower traces are the measured peroxidase activities in the fractions collected: LiP (_ _ _); MnP (_._._); MIP (.....).

LiP activity. The next two peaks, III and IV, which partially overlapped each other coincided with MnP activity. Most of the activity appeared to be associated with the smaller peak, IV. Additionally, fractions corresponding to peaks V and VI also showed MnP activity. The last two peaks, VII and VIII, coincided with LiP activity. Some MIP activity was also observed in the fraction under peak VII, indicating that MIP was not successfully separated from the LiP isoenzyme in peak VII. The results also demonstrate that MnP and LiP activities were successfully separated by the FPLC procedure used. LiP peaks I, II, VII and VIII contained no MnP activities and MnP peaks III, IV, V and VI had no LiP activity. The major peroxidase isoenzymes, which were detected in almost all of the samples, were MnP III and IV and LiP VII. Some of the peaks were unique to a given substrate, such as LiP I which was detected in HSW only and MnP V and VI which were incidentally detected on beech wood. In all cases, heme protein level, and the number of peroxidase isoenzymes decreased with culture age from day 14 to 33.

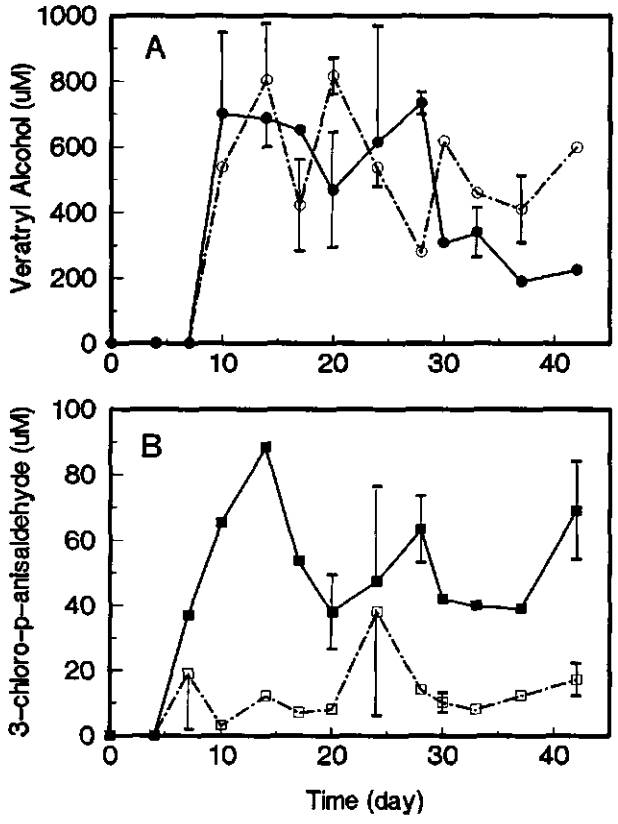

Figure 5. Production of veratryl alcohol (A) and 3-chloro-*p*-anisaldehyde (B) on hemp stem wood (filled symbols) and on beech wood (open symbols).

Secondary metabolites on lignocellulosic substrates. Figure 5 represents the time course of the production of the two major secondary aryl metabolites, veratryl alcohol and 3-chloro-*p*-anisaldehyde. These two metabolites together with veratraldehyde were present during the entire experiment. The concentration of veratryl alcohol was very similar on each substrate resulting in peak concentrations of 690 and 820 μM with HSW and beech wood, respectively (Fig. 5A). The concentration of veratraldehyde was ten-fold lower reaching peak concentrations of 20-80 μM (data not shown). The production of 3-chloro-*p*-anisaldehyde differed on the two substrates (Fig. 5B). On HSW, the fungus produced several-fold more of this secondary metabolite than on beech reaching peak concentrations of 88 and 38 μM, respectively.

Aside from the most abundant metabolites, both 3-chloro-*p*-anisyl alcohol and 3,5-dichloro-*p*-anisaldehyde were also detected. 3-chloro-*p*-anisyl alcohol was found in traces on one or two days (data not shown). The production of 3,5-dichloro-*p*-anisaldehyde started on day 24 and its concentration ranged from 5 to 10 μM during the rest of the experiment on both substrates. Anisaldehyde, which occurs in the early stages of secondary metabolism in defined liquid media was not found on these natural substrates.

DISCUSSION

In this study, two types of lignocellulosic material, milled HSW and beech wood were used to investigate the ligninolytic enzyme and secondary metabolite production on lignocellulosic substrates as well as the lignin degrading capability of *Bjerkandera* sp. strain BOS55. The substrates were readily colonized by the strain resulting in extensive losses of lignin and extractives in the woody materials. Extracellular protease activity was detectable on both substrates indicating the utilization of organic nitrogen in wood as a N source. The faster development of protease activity and the higher peak titres observed on HSW are probably due to the higher N content of HSW (approximately 0.5%) (F. P. de Vries, personal communication) compared to hardwoods with 0.03- 0.1% N (7).

Activities of LiP, MnP and MIP which were present in synthetic liquid media (28, 36, 38) were also found on the fungal decayed natural substrates. The LiP activities observed on both lignocellulosic substrate were very high, being either similar or higher than the activities observed before on defined liquid media (28, 38). HSW was apparently favorable for LiP production, resulting in more isoenzymes and approximately two-fold or higher enzyme activities.

Since the time of its discovering in *P. chrysosporium*, LiP has been considered as a crucial enzyme in the initialization of lignin degradation. Its high redox potential permits the oxidation of the non-phenolic lignin, the most representative of wood lignin (30). Purified LiP was shown to effectively oxidize in vitro various non-phenolic lignin model compounds (22, 33) as well as depolymerize synthetic lignin, including methylated lignin samples which were non-phenolic (22). The high production of LiP by *Bjerkandera* sp. strain BOS55 during wood decay is remarkable since in previous studies, it has been difficult to demonstrate the presence of LiP activity on lignocellulosic substrates with the commonly used enzyme assay based on the formation of veratraldehyde. This has been the case for many white rot fungi having *lip* genes and considered as good LiP producers in synthetic liquid media (12, 41, 43, 55). In more careful studies using either immunoblotting or protein purification techniques, LiP proteins

were detected in several fungal strains even if the enzyme assay was unsuccessful in the extracts (43, 55). Oak saw dust as well as several compounds such as phenols and organic acids were shown to inhibit the oxidation of veratryl alcohol to veratraldehyde by LiP in vitro (43, 49). These compounds may have accounted for the lack of LiP activity in 14-day old fungal treated beech wood extracts of this study when active LiP could be detected in the FPLC. The limitations of the enzymatic assays may have contributed to an underestimation of the importance of LiP for white rot fungi during lignin degradation on natural substrates.

Throughout SSF experiment, MnP was the dominant peroxidase as was found in liquid cultures with Mn-containing media (36). The appearance of MnP is dependent on the presence of Mn in many white rot fungi since Mn is a physiological inducer of the enzyme expression (3, 21). MnP titres reached very similar levels in this study as those obtained in nutrient rich defined liquid media supplied with optimal Mn concentrations of 6-28 ppm (37). The concentrations of soluble Mn in HSW and beech of 6 and 25 ppm, respectively, were within this optimal range and remained unchanged during the experiment. The fungus produced more types of MnP isoenzymes on beech wood than on HSW, although the enzyme activity levels were very similar.

The most ubiquitous peroxidase produced among white rot fungus is MnP (10) which is considered as a good oxidizer of phenolic but not the non-phenolic lignin model compounds (21, 46). Synthetic lignin but not fully methylated lignin could be depolymerized to some extent by purified MnP (22). Although 80-90% of wood lignin is non-phenolic, fungi which lack LiP producing ability like *Ceriporiopsis subvermispora*, degrade native wood lignin. These fungi probably use alternative mechanisms for the oxidation of non-phenolic lignin. Recently it has been demonstrated that unsaturated lipids can serve as powerful cooxidants enabling MnP to slowly oxidize non-phenolic lignin model compounds via a lipid peroxidation mechanism (25).

The production of MIP was also observed during the SSF on both substrates. MIP titres were considerably higher on natural substrates than in previous studies with synthetic liquid media (28, 38). MIP was apparently produced as a single isoenzyme; however, it was not purified from one of the LiP isoenzymes. The production of other types of peroxidase aside from MnP and LiP is not an unique characteristic of *Bjerkandera* sp. strain BOS55 since an increasing number of strains are reported to have peroxidases with MIP-like characteristics (41, 56, 57). Nonetheless, the true physiological role and the mechanisms of MIP has yet to be studied.

Important aryl secondary metabolites, veratryl alcohol, veratraldehyde and 3-chloro-*p*-anisaldehyde, were present during the SSF again in similar or higher concentrations than those found in defined liquid media (8, 36). The high LiP activity observed in this study is probably due to the high production of veratryl alcohol. Exogenous addition of veratryl alcohol is known to stimulate LiP activities in white rot fungi (15, 36, 42) although this metabolite has no inductive effect on *lip* gene expression (5). Rather it is the role of veratryl alcohol in protecting LiP from inactivation by physiological levels of H_2O_2 which is thought to account for the enhancement of LiP activity (5, 24, 53).

Aside from veratryl alcohol, 3-chloro-*p*-anisaldehyde was also present in significant concentrations on the natural substrates. Production of chlorinated anisyl compound was already observed under natural conditions by de Jong and coworkers (11). In our study, we followed the time course of their production. The appearance of 3-chloro-*p*-anisaldehyde coincided with the development of the ligninolytic enzymes. This compound was produced in

considerably higher concentrations on HSW than on beech wood. It was paralleled by higher AAO production as well, indicating that AAO and 3-chloro-*p*-anisyl compounds might participate in the lignin degradation by producing hydrogen peroxide, as it was suggested by de Jong and coworkers (8).

In spite of the early recognition that secondary metabolites are essential components of the ligninolytic system in white rot fungi, only a few studies have been conducted to understand the pathways and the regulation of their biosynthesis. Veratryl and chlorinated anisyl metabolites are produced de novo from glucose via the shikimate pathway by white rot fungi in synthetic liquid media (10, 20, 34, 50). In *P. chrysosporium*, phenylalanine, cinnamate and benzoate were identified as biosynthetic precursors of veratryl alcohol (26). On lignocellulosic materials, degradation products released from lignin; such as 4-hydroxybenzoate, protocatechuate, vanillate and isovanillate (6) can potentially serve as alternative precursors (23, 39).

The complete de novo production of the secondary aryl metabolites from glucose is repressed by the presence of Mn in defined liquid cultures. While Mn deficient cultures produce up to 400 μM of veratryl alcohol, the concentration was only 10-30 μM in cultures containing Mn (36). Likewise veratraldehyde and 3-chloro-*p*-anisaldehyde were only produced at low concentrations (0 to 5 μM) in Mn sufficient cultures (39). The exogenous addition of phenolic precursors to Mn sufficient cultures stimulated the production of these secondary metabolites (39). In this study, the production of secondary metabolites is 10- to 20-fold higher on lignocellulosic substrates than those in Mn-sufficient defined liquid media (36). The high aryl secondary metabolite production on woody materials in spite of the high Mn concentrations in wood, suggests that low molecular weight phenolic products released from lignin degradation were being utilized as alternative precursors.

Differences in the selectivities towards lignin degradation in the two substrates tested were observed. Lignin was selectively degraded only in beech wood but not in HSW. Kirk and Moore (31), and Blanchette (2) already reported that different degrees of lignin degradation were obtained on different types of lignocellulosic substrates. The lower selectivity on HSW might be due to the higher N content. It was shown for many other strains that N-supplements decreased the selectivity by accelerating the consumption of carbohydrates (47). Another reason for the observed higher selectivity towards lignin degradation in beech wood might be attributed to its five-fold higher Mn content. The addition of Mn increased the selectivity of cotton stalk degradation by *Pleurotus ostreatus* (29). Increased lignin degradation in wheat straw was also observed by *Pleurotus pulmonarius*, when exogenous Mn was added (4).

The application of white rot fungi in biopulping is also beneficial for the removal of wood extractives which cause pitch troubles and effluent toxicity in pulp and paper production (18, 19). Very rapid degradation of the extractives was achieved by *Bjerkandera* sp. strain BOS55 on both substrates. The extractives in HSW were more readily degraded than those in beech wood resulting in exceptionally high removal rates of 70%. Therefore, *Bjerkandera* sp. strain BOS55 should be considered as a potential agent for pitch control.

In conclusion, *Bjerkandera* sp. strain BOS55 extensively degrades lignin and extractives in natural lignocellulosic substrates. During the SSF, all ligninolytic peroxidases, AAO and major secondary metabolites were present for extended periods of time. The enzyme activities and secondary metabolite concentrations in lignocellulosic substrates were found in similar or even higher levels than those in defined liquid media. LiP and MnP isoenzymes and their

respective cofactors, veratryl alcohol and Mn were present during degradation of lignocellulosic materials, indicating their possible involvement in ligninolysis by *Bjerkandera* sp. strain BOS55.

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5. Optimization of Manganese Peroxidase Production by the White Rot Fungus *Bjerkandera* sp. Strain BOS55

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SUMMARY

Manganese dependent peroxidase (MnP) is the most ubiquitous peroxidase produced by white rot fungi. MnP is known to be involved in lignin degradation, biobleaching and in the oxidation of hazardous organopollutants. *Bjerkandera* sp. strain BOS55 is a nitrogen-unregulated white rot fungus which produces high amounts of MnP in the excess of N-nutrients due to increased biomass yield. Therefore, the strain is a good candidate for use in large scale production of this enzyme. The objective of this study was to optimize the MnP production in N-sufficient cultures by varying different physiological factors such as Mn concentration, culture pH, incubation temperature and the addition of organic acids. The fungus produced the highest level of MnP (up to 900 U l^{-1}) when the Mn concentration was 0.2 to 1 mM, the pH value was 5.2, and the incubation temperature was 30°C. A noteworthy finding was that MnP was also produced at lower levels in the complete absence of Mn. The addition of organic acids like glycolate, malonate, glucuronate, gluconate, 2-hydroxybutyrate to the culture medium increased the peak titres of MnP up to 1250 U l^{-1}. FPLC profiles indicated that the organic acids stimulated the production of all MnP isoenzymes present in the extracellular fluid of the fungus.

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INTRODUCTION

White rot fungi are the only organism which efficiently degrade the complex natural polymer, lignin. Extracellular peroxidases are the most important components of the extracellular lignin degrading system which is responsible for the initial attack of lignin by a nonspecific oxidation mechanism (13). The most ubiquitous extracellular peroxidase found among white rot fungi is manganese dependent peroxidase (MnP) which is a glycosylated heme-containing enzyme produced as a number of isoenzymes (13). MnP directly oxidizes Mn(II) to Mn(III) which is chelated by organic acids, such as oxalate (16, 35). The Mn(III)-organic acid complex is considered to act as a diffusible oxidant mediating the oxidation of lignin by MnP.

MnP is well know for oxidizing various phenols, phenolic lignin model compounds, and chlorophenols (7, 8, 9, 29. 34). Recently, MnP has also been shown to oxidize compounds with even higher ionization potential such as nonphenolic lignin model compounds, and certain polycyclic aromatic hydrocarbons (phenanthrene, and anthracene) in the presence of cooxidants (unsaturated lipids) or organosolvents (acetone) (6, 10, 25). Therefore, MnP is considered as a potential biocatalyst in environmental biotechnology. MnP has been implicated in the remediation of contaminated soils, decolorization of wastewaters, biopulping and biobleaching by different white rot fungi (6, 17, 20, 24, 28).

The production of MnP is a secondary metabolic event triggered by nutrient limitation (13). Based on studies with *Phanerochaete chrysosporium*, the presence of Mn was found to be crucial for the induction of gene expression (2). In agreement with this finding, the addition of increasing amounts of Mn to the cultures of many other white rot fungi stimulated the MnP production (1, 30).

Recently we have observed that the white rot fungus, *Bjerkandera* sp. strain BOS55 is a good producer of MnP (21, 22). In contrast to the well studied model organism *P. chrysosporium*, this fungus is N-unregulated. Sufficient or excess N-nutrients stimulate high MnP titres in parallel with the high biomass production (22). This characteristic of the fungus makes it an outstanding candidate for large scale fermentation in order to produce MnP in bulk.

In this study, we determined the optimal culture conditions for the maximal production of MnP by *Bjerkandera* sp. strain BOS55 under an N-sufficient nutrient regime. MnP titres were measured in cultures with varying manganese concentrations, pH values and incubation temperatures. The effect of organic acids on MnP production was also studied.

MATERIALS AND METHODS

Microorganism. *Bjerkandera* sp. strain BOS55 was maintained at 4°C on peptone-yeast-extract slants (21) from which it was transferred to glucose-malt extract plates (per liter: 15.0 g agar, 5.0 g of glucose, 3.5 g of malt extract). The plates were incubated at 30°C for 4 to 6 days and 6 mm diameter agar plugs from the leading edge were used as inocula in the experiments.

Media. The standard basal medium used contained 2.2 mM N as diammonium tartrate, 56 mM glucose, 2 mg liter^{-1} thiamine and BIII mineral medium (33), and 20 mM 2,2 dimethylsuccinate (pH 4.5) buffer. The standard basal medium was supplemented with 40 mM KH_2PO_4 for better buffer capacity at higher pH values. All experiments reported here were

conducted in N rich media. A mixture of 3 g liter^{-1} of mycological peptone, 3.5 g liter^{-1} malt extract and 2 g liter^{-1} yeast extract was used as the extra N source bringing the final N concentration of the medium to 32 mM. The variable culture parameters (pH, Mn concentration, incubation temperature) are indicated in the results section.

All media were filter sterilized by FP 030/3 filters with pore size of 0.2 μm (Schleicher & Schuell, Dassel, Germany).

Culture conditions. Aliquots (5 ml) of media were placed in 250 ml serum bottles. Each bottle was inoculated by one agar plug and loosely capped for passive aeration. The bottles were statically incubated under an air atmosphere at 30°C.

Enzymatic assay. The enzyme activities were determined spectrophotometrically (Lambda 1 UV/VIS, Perkin-Elmer, Norrwalk, Conn.) at 30°C. Extracellular fluids (0.3 ml) were sampled aseptically from the serum bottles and were centrifuged (12,000 × *g* for 10 min). MnP and manganese independent (MIP) peroxidase activity were measured in a combined assay (4). The reaction mixture contained 50 mM sodium malonate (pH 4.5), 1 mM $MnSO_4$, 1 mM 2,6-dimethoxyphenol, and from 20 to 100 μl extracellular culture fluid in 1 ml total volume. The reaction was initiated by adding 0.4 mM hydrogen peroxide and corrected for background laccase activity. MIP activity was measured in the same manner but Mn was excluded. Instead 1 mM EDTA was added to chelate the possible Mn contamination. MnP activity was expressed as combined MnP and MIP activity minus MIP activity. The formation of coerulignone (3,3',5,5'-tetramethoxydiphenoquinone) was monitored at the wavelength of 469 nm (ε = 49600 M^{-1} cm^{-1}). The unit definition used here refers to 1 μmol of coerulignone formed per minute, which is equivalent to 2 μmol Mn(II) oxidized.

Aryl alcohol oxidase activity was measured as described by de Jong *et al.* (4) using anisyl alcohol as substrate at the wavelength 290 nm (ε = 15000 M^{-1} cm^{-1}).

Determination of dry weight of mycelium. Mycelial mats were separated from the culture fluid by filtration through dried and tared glass fibre filters (GF50, Schleicher & Schuell, Dassel, Germany) and were rinsed with distilled water. The dry weights were determined after overnight drying (105°C).

Determination of extracellular heme-protein profile by fast protein liquid chromatography (FPLC). Extracellular fluid was centrifuged (20,000 × *g* for 20 min) to separate from the mycelial mat. Then it was washed and concentrated tenfold by ultrafiltration through a PM-10 membrane (Amicon, Rotterdam, The Netherlands) with a cut-off of 10 kDa. The concentrated supernatant was applied to a 1 ml anion-exchange column, MonoQ HR 5/5 (Pharmacia, Tilburg, The Netherlands). The column was equilibrated with 10 mM sodium acetate (pH 6). The enzymes were eluted with a linear salt gradient from 10 to 450 mM sodium acetate (pH 6). The flow rate was 1 ml min^{-1} and fractions for enzymatic assay were collected once every minute for 45 min.

Statistical Procedures. In all experiments the measurements were carried out in triplicate parallel cultures. The value reported in figures and tables are means with standard deviation.

RESULTS

Determination of optimal Mn concentration, pH, and incubation temperature. Cultures were incubated in the N rich medium supplemented with different concentrations of

Mn (0 to 2 mM) in order to investigate the effect of Mn level on the MnP production. Figure 1A shows the peak titres of MnP activity at different levels of Mn. The highest MnP titres were obtained at 0.5 mM Mn concentration (900 U l^{-1}). However MnP titres were not significantly different in the range of 0.2-1 mM Mn concentration. In these cases, the MnP activity was already as high as 300-400 U l^{-1} on day 4 and then the peak activity was reached on day 6 or 7. Thereafter, a slow decrease was observed resulting in 300-400 U l^{-1} activity by day 14. Concentrations of Mn below 0.2 mM resulted in lower peak MnP activities. A noteworthy finding was the production of MnP in the absence of Mn. Approximately one fourth of the optimum MnP activity was detected even in the complete absence of Mn. Extremely high Mn concentration of 2 mM gave slightly lower peak MnP activities than the optimal concentration.

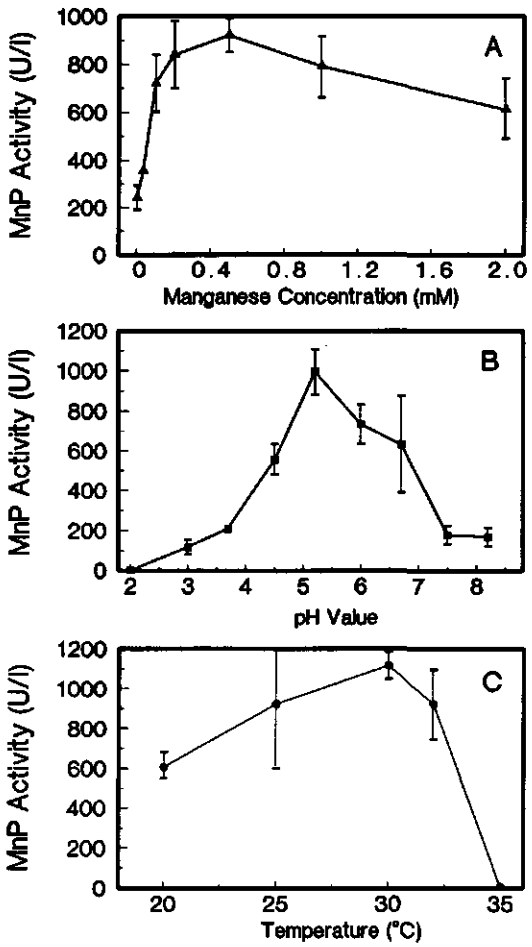

Figure 1. Effect of manganese concentration (A), initial pH (B), and incubation temperature (C) on the peak MnP titres in the cultures of *Bjerkandera* sp. strain BOS55.

Figure 1B represents the peak titres of MnP as a function of medium pH values. In this experiment the Mn concentration was 0.5 mM. The optimal pH value for MnP activity was pH 5.2 resulting in 990±110 U l^{-1} activity. All cultures from pH values of 3 to 8.2 produced MnP. The lowest pH value tested of 2 was completely growth inhibitory. The growth of fungus was optimal in the pH range of 5.2 to 6.7 as was indicated by mycelial dry weight measurements (approximately 40 mg per cultures) on day 10. At pH values from 3 to 4.5 and higher than 6.7, mycelial dry weights decreased by about 20-25 %. On day 10, the pH of the cultures was also measured and compared to the initial values. The cultures with an initial pH of 5.2 did not significantly change in pH after 10 days. The cultures set in with pH values lower than 5.2 increased the pH by 0.7-0.8 units, while those set in at pH values higher than 5.2 decreased the pH to a pH value of 5.2-5.9 units by day 10.

The fungal cultures were incubated at different temperatures in the range of 20-35 °C and the temperature optimum was determined for MnP production. The results are presented in Figure 1C. The temperature in the range of 25-32°C resulted in similar MnP titres. However, the peak activity was reached 3 days later at 32°C compared to those of cultures incubated at 25 and 30°C. At a temperature of 20°C, the growth of the fungus and the development of enzyme activity slowed down. At 35°C the fungus did not grow at all.

Table 1. Effect of organic acids on the peak titre of MnP activity in the cultures of *Bjerkandera* sp. strain BOS55.

Organic acid added[a]	Means of peak titre of MnP ± SD (U l^{-1})
none	816± 85
gluconic acid	1103± 24
glucuronic acid	1229± 63
glycolic acid	1248± 10
2-hydroxybutyric acid	1015±112
lactic acid	949± 59
mandelic acid	812± 61
oxalic acid	840± 57

[a] The acids were added in the final concentration of 5 mM after pH correction on day 3

Effect of organic acid addition on MnP production. The effect of various organic acids on MnP was also studied. The acids were added to the cultures containing 0.5 mM Mn on day 3 to a final concentration of 5 mM after correcting their pH to 5.2. The time course of MnP activity in the presence and absence of glycolic acid is shown in Figure 3. Two days after the addition of glycolic acid, a considerable increase in the MnP activity was observed compared to that of the control. The results obtained by other acids are summarized in Table 1. Most of the acids stimulated the peak MnP titres by approximately 50 %. Mandelic acid did not enhance the peak titre of MnP but increased the early production of the enzyme. On day 5, 812 ± 59 U l^{-1} activity was measured when 5 mM of mandelate was added, while the control culture produced only 480 ± 54 U l^{-1} enzyme activity at that time.

FPLC profile of heme-protein in the presence and absence of glycolic acid. An FPLC study was conducted in order to investigate the effect of organic acid on the production of different MnP isoenzymes. The extracellular fluids of 7 day old cultures which were

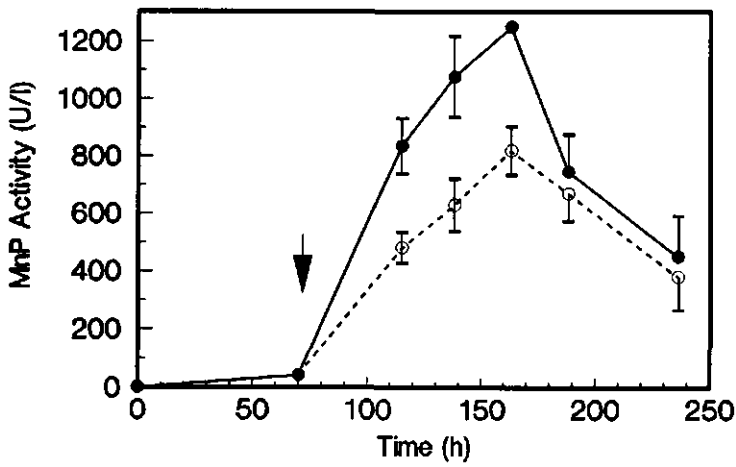

Figure 2. The time course of MnP production in the absence (○) or presence (●) of 5 mM glycolic acid added on day 3 to the cultures of *Bjerkandera* sp. strain BOS55. ↓ indicates the timing of glycolate addition.

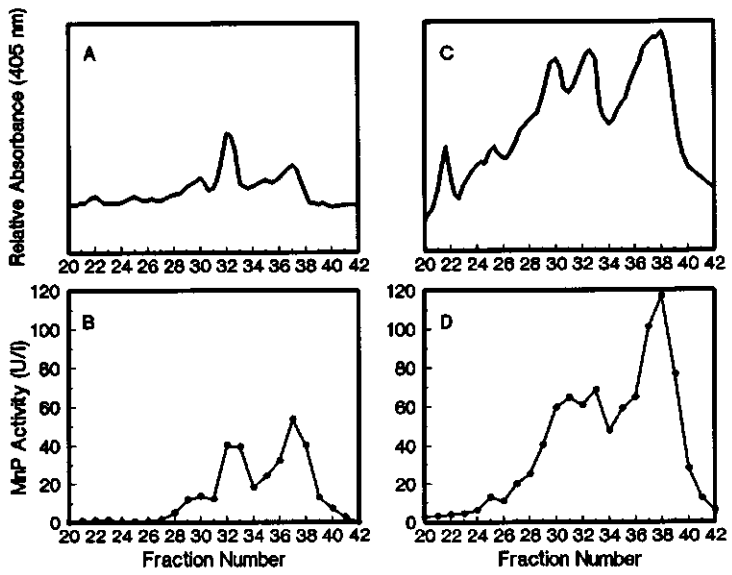

Figure 3. FPLC protein profile of 7-day-old extracellular fluid of *Bjerkandera* sp. strain BOS55 grown in medium supplemented with no glycolic acid (A, B) or with 5 mM of glycolic acid (C, D). Panel A, C: relative absorbance at 405 nm wavelength; Panel B, D: MnP activity measured in the collected fractions.

incubated under the optimal condition (0.5 mM Mn, pH 5.2, 30°C) in the presence or absence of 5 mM glycolic acid were collected and applied to an FPLC system in order to identify the heme-protein profile. The results are shown in Figure 3. Both the absorbance peaks of the heme proteins at 405 nm and the peroxidase activities in the collected fractions were measured. In this experiment, 2,2-dimethylsuccinate buffer was excluded from the basal media since the effect of adding organic acids was found to be even more remarkable in the absence of the buffer. The exclusion of 2,2-dimethylsuccinate slightly decreased the MnP activity measured in the control cultures, but the stimulatory effect of organic acids was much more pronounced. The pH of the media did not change significantly due to the lack of the buffer.

As was observed in the enzymatic assays of the culture supernatants, the predominate enzyme was MnP. Three smaller and one major peak were distinguished which corresponded to MnP activity in the control culture. The addition of glycolic acid considerably increased the absorbance of all peaks indicating that glycolate stimulated the production of all isoenzymes. In parallel with the increase in heme peaks, an enhanced MnP activity was measured in the corresponding fractions. Low MIP activity (< 2 U l^{-1}) was found in fractions from 22 to 30. No LiP activity was detected in any fraction. The peak from 21 to 23 ml elution volume corresponded to a FAD containing aryl alcohol oxidase (AAO) as was indicating by measuring its activity in those fractions. AAO production was also considerably stimulated (5-fold) by the addition of glycolic acid (data not shown).

DISCUSSION

Mn is a naturally occurring metal ion in wood and participates in the lignin degrading processes of white rot fungi. Mn is not only a substrate and important diffusible mediator produced by MnP but it also regulates the enzyme production by inducing gene transcription as was observed in *P. chrysosporium* (2). Increasing concentrations of Mn resulted in higher levels of *mnp* mRNA, MnP protein production, and enzyme activity in many white rot fungi (1, 18, 30). The enzyme titres in the cultures of *Bjerkandera* sp. strain BOS55 were also enhanced by the addition of extra Mn nutrient. The optimal Mn concentrations (0.2-1 mM) for MnP production are comparable to the soluble Mn concentration in natural lignocellulosic substrates (23).

It should be noted that there are few exceptional white rot fungi, in which the effect of Mn on MnP production does not follow the expected trends. *Pleurotus eryngii* produced MnP at the highest titres in the absence of Mn (19). In another study, the addition of extra Mn above to the basal level to the cultures of *Phlebia radiata* did not result in increased MnP production (26). In this study, we have observed that *Bjerkandera* sp. strain BOS55 produces MnP in the absence of Mn. The addition of Mn resulted in even higher MnP titres. Under Mn deficient conditions, the Mn(II) oxidizing activity cannot be attributed to one of LiP isoenzymes as was claimed to be the case for LiP isoenzyme H2 of *P. chrysosporium* which oxidizes Mn(II) without any veratryl alcohol mediation (14). Recent FPLC studies of *Bjerkandera* sp. strain BOS55 showed that MnP isoenzymes were produced in the absence of Mn and that these were distinguished from the LiP isoenzymes. The LiP isoenzymes were incapable of Mn(II) oxidation (23, 27).

Previous studies indicated that the initial pH values of culture media considerably affect the production of ligninolytic enzymes (11, 12). In this study, we found that the optimal pH

value for MnP production of *Bjerkandera* sp. strain BOS55 was 5.2. Interestingly, in almost all cultures with a different initial pH value, the pH changed in time resulting in very similar final values of approximately 5.2. The observed pH lowering activity of *Bjerkandera* sp. strain BOS55 in cultures with starting pH values of 6 to 8.2 was probably due to the production of organic acids. The de novo production of organic acids during the secondary metabolism has been described in many white rot fungi. Oxalate was found to be a major organic acid produced by white rot fungi during secondary metabolism (5, 16). Oxalate is considered to be involved in lignin degradation. Various roles are suggested by different researchers. Firstly, oxalate is able to chelate Mn(III) generated by MnP and this stable complex can act as a diffusible mediator in the degradation of lignin and other aromatic compounds (16). Secondly, the presence of organic acids is necessary to strip Mn(III) from MnP (15, 16). Finally, it has been shown, that anion radicals were formed by Mn mediated oxidation of oxalate by MnP. Those radicals may participate in lignin degradation either directly or indirectly by generating active oxygen species (32).

Aside from their role in catalysis, organic acids also seems to participate in the regulation of MnP production. In a previous study with *Phlebia radiata*, the production of MnP only increased in response to high Mn content if malonate was added to the cultures (26). In this study, we evaluated the effect of various organic acids on MnP production by *Bjerkandera* sp. strain BOS55. Those organic acids were chosen, which are either the precursors of oxalate or were found in kraft pulp containing cultures of *Trametes versicolor* (5, 31, 32). Not only did *Bjerkandera* sp. strain BOS55 produce more MnP in response to the increasing Mn concentration, but even higher MnP titres were obtained by adding various organic acids. Glycolate was shown to stimulate the production of all the MnP isoenzymes. Surprisingly, oxalate did not enhance the MnP level, although it is a major organic acid metabolite. Only biosynthetic precursors of oxalate such as glycolate gave the stimulatory results. A possible explanation for this phenomenon is that the addition of oxalate induces the production of oxalate decarboxylase responsible for oxalate catabolism in fungi (5). This enzyme was shown to be produced by white rot fungi (5). Therefore, the early addition of oxalate could have resulted in a negative effect on the oxalate concentration in the later periods of the fungal cultures.

The mechanism of organic acid stimulation on MnP production has not yet been clarified. Possibly the organic acids induce the MnP production at the molecular level. The *mnp* gene of *P. chrysosporium* contains a heat shock element in the promoter region (3). Aside from heat, the gene transcription also responded to various chemicals such as hydrogen peroxide, ethanol, 2,4-dichlorophenol, peracetic acid, sodium arsenite and N,N-dimethylformamide in the presence of Mn (18). Future research should determine if the *mnp* genes of *Bjerkandera* sp. strain BOS55 contain organic acid response elements. Likewise, the regulating factor responsible for MnP production in the absence of Mn should be elucidated.

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6. Characterization of a novel manganese peroxidase - lignin peroxidase hybrid isozyme produced by *Bjerkandera* sp. strain BOS55 in the absence of manganese

Tünde Mester and Jim A. Field

SUMMARY

A novel manganese-dependent peroxidase (MnP) isozyme produced in manganese-free cultures of *Bjerkandera* sp. strain BOS55 was purified and characterized. The production of the enzyme was greatly stimulated by the exogenous addition of various physiological organic acids such as glycolate, glyoxylate and oxalate. The physical properties of the enzyme are similar to those of MnP isozymes from different white rot fungi (M_r = 43,000, pI 3.88 and $\varepsilon_{407\ nm}$ = 123 $mM^{-1}\ cm^{-1}$). The Bjerkandera MnP was efficient in the oxidation of Mn(II), as indicated by the kinetic constants (low K_m of 51 μM, turnover number of 59 s^{-1}). Furthermore, the isozyme was able to oxidize various substrates in the absence of manganese such as 2,6 dimethoxyphenol (DMP), guaiacol, 2,2'azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS), 3-hydroxyanthranilic acid, *o*- and *p*-anisidine. An interesting characteristic of the isozyme was its ability to oxidize nonphenolic substrates, veratryl alcohol and 1,4-dimethoxybenzene without manganese addition. The affinity towards veratryl alcohol (K_m 116 μM) and its turnover number (2.8 s^{-1}) are comparable to those of lignin peroxidase (LiP) isozymes from other white rot fungi. Manganese at concentrations greater than 0.1 mM severely inhibited the oxidation of veratryl alcohol. The results suggest that this single isozyme is a hybrid between MnP and LiP found in other white rot fungi. The N-terminal amino acid sequence showed a very high homology to those of both MnP and LiP isozymes of *Trametes versicolor*.

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INTRODUCTION

Lignin is an abundant natural aromatic polymer occurring in the woody tissue of higher plants. Due to its hydrophobicity and complex random structure lacking regular hydrolyzable bonds, lignin is poorly biodegraded by most microorganisms. The best degraders of lignin are white rot fungi that produce extracellular peroxidases (22). These enzymes are involved in the initial attack of the lignin polymer (16). Manganese dependent peroxidase (MnP) is one of the most frequently encountered peroxidases among white rot fungi (7).

MnP is a glycosylated heme protein, occurring mostly as a number of isozymes (22). MnP has the same catalytic cycle as other peroxidase, involving a 2-electron oxidation of the heme by H_2O_2 and subsequent reduction of compound I via compound II in two 1-electron steps to the native enzyme (11, 51). A distinctive characteristic of this enzyme is that the best reducing substrate for compound I and II is Mn(II), a metal naturally present in wood. The Mn(III) formed oxidizes other substrates (52). Crystallographic and site directed mutant studies confirmed the presence of a unique manganese binding site in MnP from the best studied white rot fungus, *Phanerochaete chrysosporium* (45, 46).

Organic acids such as oxalate, glyoxylate, lactate were shown to have an important role in the mechanism of MnP and lignin degradation (26, 44, 52). Mn(III) is stripped from the enzyme by organic acids and the formed Mn(III)-organic acid complex acts as a diffusible mediator in the oxidation of lignin by MnP (26, 52). Mn(III) can also oxidize organic acids yielding radicals (23). There are two possible sources of organic acids during lignin degradation. The fungi are able to produce de novo aliphatic organic acids, mainly oxalate (8). Moreover, organic acids are formed from the degradation of lignin (17, 40).

The ligninolytic enzymes are produced during the secondary metabolism triggered by nutrient limitation (13, 22). Manganese is an absolute requirement for expression of *mnp* genes in several white rot fungi (4, 37). Consistent with manganese requirement, putative metal response elements were found in the promoter regions (1, 13). Furthermore, enhanced MnP production is associated with increased manganese concentrations in many white rot fungi (2, 32, 37).

Bjerkandera sp. strain BOS55 is a good MnP producer (32). Previous studies have shown that this fungus is nitrogen-unregulated, MnP production is greatly increased by nutrient nitrogen sufficiency and excess (33). MnP production is also enhanced by supplementing the culture medium with simple organic acids (32). Following the general trend of most white rot fungi, manganese is stimulatory for MnP in this fungus (32). However, *Bjerkandera* sp. strain BOS55 is atypical since it produces considerable MnP in manganese-free media (32, 35). In this study we characterized a MnP isozyme produced in the absence of manganese. Furthermore, the manganese dependency for the oxidation of various substrates was tested.

MATERIALS AND METHODS

Microorganism. *Bjerkandera* sp. strain BOS55 (ATCC 90940) was maintained in glucose/peptone/yeast extract slants as described earlier (31). Prior to experiment, the fungus was transferred to glucose/malt extract plates (32) which were incubated for 4 to 6 days at 30°C. As inocula, 6 mm diameter agar plugs from the leading mycelial edge were used in the experiments.

Media. The standard basal medium used contained 2.2 mM N as diammonium tartrate, 56 mM glucose, 2 mg liter^{-1} thiamine, Mn-free BIII mineral medium (pH 4.5) and the standard buffer, 2,2 dimethylsuccinate was omitted (48). All glassware was previously washed with 5 M HNO_3 to remove contaminating Mn. The measured concentration in manganese free medium was always < 0.01 μM. In some experiments, the basal medium was also supplemented with manganese nutrients providing a final concentration of 0.033 mM. Glycolic acid, glyoxylic acid and oxalic acids (5 mM) were individually added (neutralized to pH 4.5) to the cultures at the time of incubation in order to study their stimulatory effect on MnP production.

Culture conditions. Filter sterilized 25 ml aliquots were placed in 250 ml cylindrical flasks which were previously autoclaved with a drop of demineralized water (35). Each flask was inoculated with 3 agar plugs. Cultures were incubated statically, under an air atmosphere at 27°C.

Enzyme purification with fast protein liquid chromatography (FPLC). Collected supernatant was separated from the mycelial mat by centrifugation (20,000 × *g*). The extracellular fluid was washed with 10 mM sodium acetate (pH 6.0) and concentrated by ultrafiltration with a PM-10 membrane (10 kDa; Amicon, Rotterdam, The Netherlands). The concentrated supernatants were loaded in 10 mM sodium acetate to a MonoQ anion exchange column (Pharmacia, Uppsala, Sweden) and heme-containing proteins were monitored with a wavelength of 405 nm. Proteins were eluted with a linear gradient of sodium acetate up to 450 mM (pH 6.0) in 45 min and 1 ml fractions were collected (1 ml min^{-1}) for 45 min. The fractions, 33-35 that contained the most MnP activity were collected, then washed and concentrated by ultrafiltration. The concentrate was eluted by creating a decreasing pH gradient in the range of pH values of 4.5 -2.7 on a MonoP chromatofocussing column (Pharmacia) as described earlier (19) and 1 ml fractions were collected (1 ml min^{-1}) for 40 min. The MnP containing fraction was washed with demineralized water and concentrated by ultrafiltration. The concentrate was used for enzyme characterization, kinetic and substrate oxidation studies.

The MonoP enzyme concentrate was brought through two additional FPLC steps to confirm its purity. First gel filtration was carried out using a Superdex 75 HR 10/30 (10 × 300 mm, volume 24 ml) (Pharmacia, Uppsala, Sweden). The enzyme was eluted in a 10 mM sodium acetate buffer (pH 6.0) with a flow rate of 0.5 ml min^{-1} for 50 min., and 0.5-ml fractions were collected. A single symmetrical peak was observed, and the enzyme-containing fractions 22 and 23 were pooled and concentrated by ultrafiltration. The enzyme was washed with 10 mM potassium phosphate buffer (pH 6.0). Diammonium sulfate was added to the protein reaching the final concentration of 1 M and loaded onto a 1 ml hydrophobic interaction chromatography column, Phenyl Sepharose HP (Pharmacia, Uppsala, Sweden). Proteins were eluted in a decreasing linear gradient of 1 M diammonium sulfate in 10 mM potassium phosphate buffer (pH 6.0) in 45 min (1 ml min^{-1}). Fractions (1 ml) were also collected every minute. Again a single symmetrical peak was observed eluting from 19 to 22 min.

Enzyme characterization. Protein concentration was determined according to Bradford (3) using bovine serum albumin (BSA) as a standard. The concentration of purified MnP was also measured at wavelength 406 nm and calculated using $\varepsilon_{406\ nm}$ = 129 mM^{-1} cm^{-1} (52) which gave approximately the same result. The molecular weight of the enzyme was determined by SDS-PAGE (12% polyacrylamide gel) in a Phastsystem (Pharmacia, Uppsala, Sweden), using marker proteins (low molecular mass calibration kit from Pharmacia) as standards. Isoelectric

point was estimated by chromatofocussing by measuring the pH of the collected fraction containing MnP isozyme. Enzyme absorbance spectrum was determined with 86 mg liter^{-1} MnP in the presence of 50 mM malonate buffer (pH 4.5) by scanning the absorbance of the enzyme at the wavelength range from 350 to 700 nm using a Perkin-Elmer Lambda 1 UV/VIS spectrophotometer. The oxidized enzyme was scanned after addition of 0.2 mM H_2O_2.

Kinetic study. Kinetic constants of MnP activities for H_2O_2 and Mn(II) were calculated by the formation of Mn(III) malonate complex at 270 nm (ε = 11590 M^{-1} cm^{-1}) (52). The oxidation of 2,6-dimethoxyphenol to coerulignone (ε = 49600 M^{-1} cm^{-1}), ABTS to $ABTS^{\cdot+}$ (ε = 36000 M^{-1} cm^{-1}) and veratryl alcohol to veratraldehyde (ε = 9300 M^{-1} cm^{-1}) was measured at the wavelength of 469 nm, 420 nm, and 310 nm, respectively (48, 52, 53).

H_2O_2 inactivation. MnP (4 µg) were incubated in 0.2 ml 50 mM succinate buffer (pH 4) at 25°C with or without 0.4 mM H_2O_2 to study the effect of either 0 or 0.1 mM veratryl alcohol on the inactivation rate. Sample collected at selected time intervals were diluted 50 times into cuvettes to measure residual activity with Mn(II) in 50 mM malonate (pH 4.5). The incubations were carried out in triplicate.

Determination of substrate oxidation by high performance liquid chromatography (HPLC). To test the oxidation of benzyl alcohol, veratryl alcohol, 1,4-dimethoxybenzene, and 2-chloro-1,4-dimethoxybenzene, of *Bjerkandera* MnP was incubated with 0.3 mM compound, in 50 mM succinate buffer (pH 3.0). The reaction was initiated with 0.1 mM H_2O_2. H_2O_2 was added once every hour reaching the final concentration of 0.4 mM. After 4 hours incubation time the reaction was stopped with 1 equivalent volume of acetonitrile. The compound oxidation was detected by HPLC. All the incubation was carried out in triplicate. 50 µL samples were analyzed by a Pascal series HPLC, ChemStation (Hewlett-Packard, Waldbronn, Germany), equipped with an HP1040 series diode array detector. The column (200 by 3 mm) was filled with ChromoSpher C-18-PAH (5 µm particles) (Chrompack, Middelburg, The Netherlands). The following gradient (0.4 ml min^{-1}, 30°C) was used: 90:10, 0:100, and 0:100 ratios of H_20 to CH_3CN at 0, 15, and 20 min, respectively. Compound identifications were based on matching retention times and UV spectra with those of standards.

Manganese inhibition of veratryl alcohol oxidation. In some experiments the effect of Mn(II) on veratryl alcohol oxidation was elucidated. The oxidation of 0.1 mM veratryl alcohol was tested during a 10 min. incubation period with 0.4 mM H_2O_2 in a 50 mM sodium succinate buffer (pH 3.8) with 2 mM sodium pyrophosphate and varying Mn(II) concentrations. For comparison, a similar experiment was conducted with purified LiP-5 isozyme from *Bjerkandera* sp. strain BOS55 (47) supplied at the same units in terms of veratryl alcohol oxidation. Formation of veratraldehyde was analyzed by HPLC.

Determination of background manganese content in culture media. Manganese content of the culture media was measured by a Inductivity Coupled Plasma Mass Spectrometer (ICP-MS) by the Department of Soil Science and Plant Nutrition, Wageningen Agricultural University (Wageningen, The Netherlands). The detection limit for manganese is 0.002 µM.

N-terminal sequence determination. The N-terminal amino acid sequence determination was carried out at the Rijks Universiteit Leiden, Faculteit der Geneeskunde, Vakgroep Medische Biochemie, Leiden (The Netherlands).

Chemicals. All chemicals were commercially available and used without further purification.

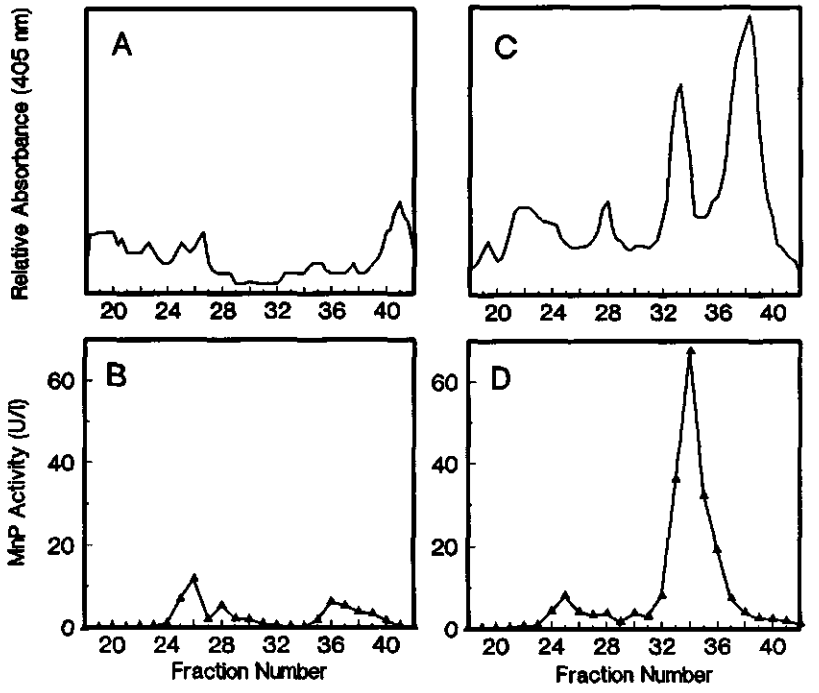

Figure 1. FPLC protein profile of 7-day-old extracellular fluid of *Bjerkandera* sp. strain BOS55 grown in manganese-free medium supplemented without (A and B) or with (C and D) 5 mM of glycolic acid. A and C, relative absorbance at 405 nm wavelength; B and D, MnP activity measured in the collected fractions.

RESULTS

MnP production in the cultures of *Bjerkandera* sp. strain BOS55 and enzyme purification. *Bjerkandera* sp. strain BOS55 was cultivated in manganese-free nitrogen-limited glucose medium in the presence and absence of simple organic acids. The FPLC studies using the MonoQ column demonstrated the increased production of heme-protein with MnP activity as a result of organic acid addition to the cultures. As an example, FPLC profiles and enzyme activities measured in the collected fractions of extracellular fluid from a 7-day old culture grown in the absence or presence of 5 mM glycolate are shown in Figure 1. Similar results were obtained with oxalate and glyoxylate. In the cultures without the organic acids, very little heme-protein and limited MnP activity was detected. The addition of glycolate stimulated the production of several heme peaks. However, most of the MnP activity was attributed to one peak (fraction 33-35). In other studies, with glycolate and with other organic acids (data not shown), the MnP activity was sometimes distributed between two major peaks: fractions 33-35 and 38-39, respectively. Since MnP activity was consistently present in fractions 33 to 35, this

isozyme was isolated and used for further characterization. The purification is summarized in Table 1.

TABLE 1. Purification of MnP isozyme from Bjerkandera sp. strain BOS55

Step	Protein (mg)	Activity[a]		Purification factor
		Total (U)	Specific ($U\ mg^{-1}$)	
EF[b]	97	97	1.0	1.0
Concentrated EF	85	128	1.5	1.5
MonoQ	1.6	85	53	53
MonoP	0.87	70	80	80

[a] MnP activity was determined by monitoring the Mn(III) malonate formation as described under "Materials and Methods".
[b] EF, extracellular fluid

Physical properties and N-terminal amino acid sequence. As the first step in the enzyme characterization, molecular weight was measured by SDS-PAGE method and was found to be 43,000 Da (Fig. 2). This gel electrophoresis resulted in only one band indicating the successful purification. The pI was 3.88 as estimated by the chromatofocussing method. The spectrum of the enzyme showed a typical peak for native enzyme at 407 nm with a molar extinction coefficient of 123 $mM^{-1}\ cm^{-1}$ and the addition of excess hydrogen peroxide resulted in a shift to 420 nm (Fig. 3). The N-terminal amino acid sequence of the protein was also determined up to 25 amino acids: VACPDGVNTATNAACCALFAVRDDI.

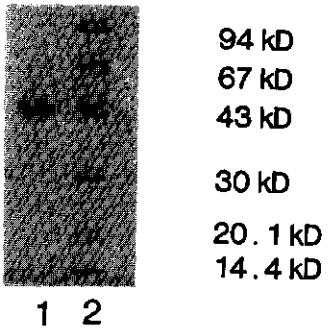

Figure 2. SDS polyacrylamide gel of the purified MnP isozyme. Lane 1, *Bjerkandera* MnP; lane 2, molecular mass protein markers.

Catalytic properties and Mn dependency of substrate oxidation. Different substrates were used to evaluate the manganese dependency of their oxidation at pH 4.5 in 50 mM malonate buffer. The results are summarized in Table 2. The substrate 2,6-dimethoxyphenol (DMP) was oxidized in the presence and absence of manganese. The oxidation rate of DMP without manganese was 18% of that in the presence of manganese. This trend was observed in the case of other substrates such as guaiacol, ABTS, 3-hydroxyanthranilate, *o*-anisidine, and *p*-

anisidine. The enzyme was also able to directly oxidize veratryl alcohol at pH 4.5 without any manganese (Table 3).

TABLE 2. Mn dependency of substrate oxidation by MnP isozyme. The activity of the enzyme was tested in the absence and presence of 1 mM of Mn at pH 4.5 in 50 mM malonate buffer. The concentration of the enzyme was 4 mg liter^{-1}.

Substrate (1mM)	Wavelength (nm)	ΔA (per min^{-1} μg protein^{-1})		Mn-independent activity (%)[a]
		+Mn	-Mn	
DMP	469	0.414	0.076	18
guaiacol	465	0.259	0.026	10
ABTS	420	0.369	0.072	19
3-hydroxyanthranilate	469	0.025	0.005	14
o-anisidine	460	0.025	0.003	11
p-anisidine	460	0.049	0.012	24

[a] (ΔABS min^{-1} without Mn(II))/(ΔABS min^{-1} with Mn(II)) × 100.

TABLE 3. Kinetic constants of MnP isozyme from *Bjerkandera* sp. strain BOS55

Substrate	pH[a]	K_m (μM)	V_{max} (U mg^{-1})	Turnover Number (s^{-1})
H_2O_2 (0.1 mM Mn(II))	4.5	31	104	75
Mn(II) (0.1 mM H_2O_2)	4.5	51	81	59
DMP (0.1 mM H_2O_2)[b]	4.5	41	3.1[c]	2.3
ABTS (0.1 mM H_2O_2)[b]	4.5	37	1.8	1.3
Veratryl alcohol (0.1 mM H_2O_2)[b]	4.5	534	1.9	1.4
Veratryl alcohol (0.1 mM H_2O_2)[b]	3.0	116	3.9	2.8

[a] pH 4.5 in 50 mM malonate buffer; pH 3.0 in 50 mM succinate buffer

[b] Kinetic data for DMP, ABTS, veratryl alcohol determined in the absence of Mn

[c] an extinction coefficient of 24800 M^{-1} cm^{-1} was used since each coerulignone (ε = 49600 M^{-1} cm^{-1}) refers to two DMP molecules oxidized.

The kinetics of the oxidizing substrate (H_2O_2) and of various direct reducing substrates of the enzyme were compared in terms of K_m and turnover number (Table 3). At a physiological pH of 4.5, Mn(II) was clearly the best reducing substrate with 59 turnover s^{-1}. Nonetheless, veratryl alcohol, DMP and ABTS were directly oxidized by the enzyme at this pH with 2.3 to 1.3 turnover s^{-1} in the absence of Mn (II). The enzyme had a high affinity for Mn(II), DMP, ABTS; whereas, the affinity for veratryl alcohol at pH 4.5 was one order of magnitude less.

The pH dependency of the oxidation of several substrate was also studied. Figure 4 shows the pH dependency of Mn(II) and DMP oxidation. The highest rate of Mn(II) oxidation was observed at pH value 4.5. At pH 3.0, the Mn(II) oxidizing activity was negligible. The direct oxidation of DMP was the highest at pH 3.0 and at this pH, the rate was comparable in the presence and absence of manganese. Likewise, the oxidation of ABTS and veratryl alcohol in the absence of manganese was 2-3-fold higher at pH 3.0 compared to pH 4.5. At pH 3.0, the affinity of the *Bjerkandera* MnP for veratryl alcohol was approximately 5-fold greater than at pH 4.5 (Table 3).

To verify that the manganese and veratryl alcohol oxidation activities were due to one isozyme, the enzyme was purified further by two additional FPLC step: gel filtration and hydrophobic interaction. After each step only one symmetrical peak was observed and the molar ratio of 0.1 mM Mn(II) oxidation rate to 0.1 mM of veratryl alcohol oxidation rate at pH 4.0 remained constant at 23 (data not shown).

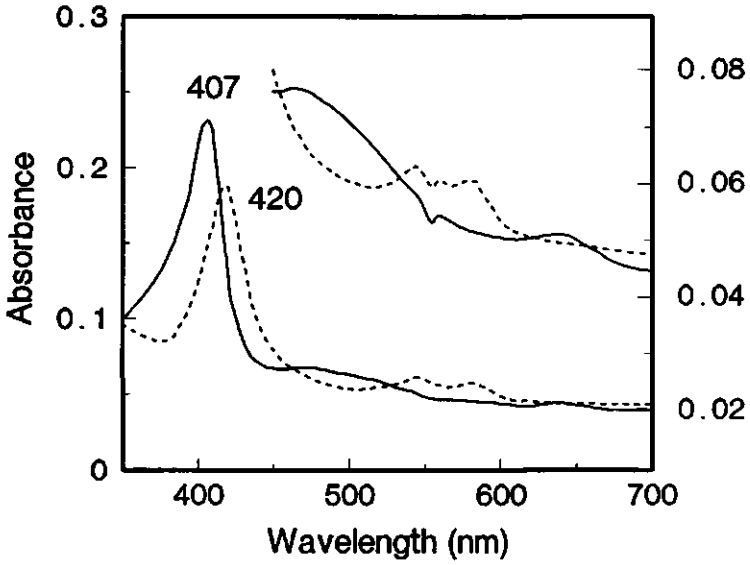

Figure 3. Absorption spectra of the native and the hydrogen peroxide-oxidized *Bjerkandera* MnP isozyme. Solid line, native enzyme; dotted line, hydrogen peroxide-oxidized enzyme.

Product identification of nonphenolic compound oxidation. Several nonphenolic compounds were incubated in the absence of manganese for four hours with the *Bjerkandera* MnP at pH 3.0 in 50 mM succinate buffer. The products of the oxidation were identified by HPLC-DAD. The results are summarized in Table 4. The main product of veratryl alcohol oxidation was veratraldehyde recovered with the molar yield of 46 %. 1,4-Benzoquinone was also identified as product of 1,4-dimethoxybenzene oxidation with a molar yield of 41 %. Even 2-chloro-1,4-dimethoxybenzene was oxidized to a small extent by the isozyme. Benzyl alcohol was not significantly oxidized by this enzyme.

Effect of manganese on veratryl alcohol oxidation. The veratryl alcohol oxidation to veratraldehyde of *Bjerkandera* MnP and LiP was compared in the presence of Mn (Table 5). LiP was unaffected by the presence either of 0.3 mM Mn(II) or Mn(III). However, *Bjerkandera* MnP behaved differently. Mn(II) in the concentration range of 0.1-1 mM inhibited the oxidation of veratryl alcohol. Interestingly, Mn(II) at a low concentration (0.033 mM) stimulated the veratryl alcohol oxidation to veratraldehyde.

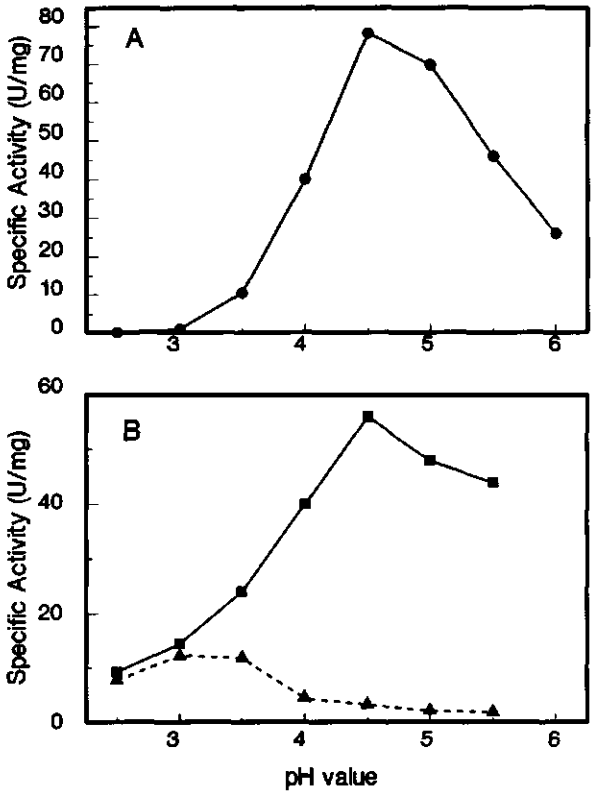

Figure 4. Effect of pH on the oxidation of 0.5 mM Mn(II) (A) and of 1 mM of DMP (B) by the purified MnP isozyme. The oxidation of DMP was tested in the presence (■) and absence (▲) of 1 mM Mn(II).

DISCUSSION

Bjerkandera sp. strain BOS55 produces MnP in high quantities under different culture conditions. An interesting characteristic of this fungus, is the ability to produce MnP in manganese-free media (32, 35). Recently, the production of MnP under manganese deficiency was reported for two *Pleurotus* species, *P. eryngii* and *P. ostreatus* and the isozymes were characterized (28, 42). In the *Pleurotus* strains, the MnP production was inhibited by even trace amounts of manganese. In contrast, *Bjerkandera* sp. strain BOS55 MnP production is stimulated by manganese nutrients. The same MnP isozymes produced under manganese deficiency, were also detected in manganese-sufficient cultures (data not shown). The previous study showed that the addition of certain organic acids enhanced the MnP production under manganese excess conditions (32). In this study, glycolate, glyoxylate, and oxalate highly stimulated the MnP production under manganese deficient conditions. These acids are physiological metabolites of white rot fungi (8, 21, 40, 52). The role of the organic acids on MnP production still requires further studies.

TABLE 4. Oxidation of various nonphenolic compounds by the purified *Bjerkandera* MnP. The reaction mixture contained 4 mg liter $^{-1}$ MnP, 0.3 mM substrate, 0.4 mM H_2O_2 in 50 mM succinate buffer (pH 3.0).

Compound	Identified product	% of Oxidation[a]	Molar product yield[b]
benzyl alcohol	none	0	n.a.[c]
veratryl alcohol	veratraldehyde	34±5[d]	46±4
1,4-dimethoxybenzene	1,4-benzoquinone	35±7	41±8
2-chloro-1,4-dimethoxybenzene	2-chloro-1,4-benzoquinone	10±1	23±3

[a] % of oxidation: 100 × (($[substrate]_{initial}$ - $[substrate]_{final}$)/ $[substrate]_{initial}$).
[b] molar product yield = 100 × ([product] / ($[substrate]_{initial}$ - $[substrate]_{final}$)).
[c] na, not applicable
[d] Results are the means ± SD of three parallel incubations.

TABLE 5. Effect of manganese on the oxidation of veratryl alcohol to veratraldehyde by the purified *Bjerkandera* MnP in comparison with *Bjerkandera* LiP-5 isozyme. The reaction mixture contained 10 mg liter $^{-1}$ MnP, 0.1 mM veratryl alcohol, 0.4 mM H_2O_2, in 50 mM succinate buffer with 2 mM pyrophosphate (pH 3.8). The incubation time was 10 min. The LiP-5 isozyme was added at equivalent veratryl alcohol oxidizing units as MnP.

Mn conc	Veratraldehyde (μM) formed by	
	MnP	LiP
0.000 mM Mn(II)	38.6±0.4[a]	54.5±3.3
0.033 mM Mn(II)	63.6±1.7	nt[b]
0.100 mM Mn(II)	24.2±0.5	nt
0.300 mM Mn(II)	8.6±0.3	51.4±7.8
1.000 mM Mn(II)	4.4±0.3	nt
0.300 mM Mn(III)	nt	57.6±6.1

[a] Values are presented as the means ± SD of three parallel incubations.
[b] nt, not tested.

The physical characteristics in terms of molecular weight, isoelectric point and heme absorbance of the purified MnP does not differ from that of other MnP isozymes described from other white rot fungi (12, 38, 41, 50). The turnover number of Mn(II) oxidation by MnP from *Bjerkandera* sp. strain BOS55 shows that this isozyme is efficient and comparable to other MnP isozymes described in the literature (25, 29, 28, 47, 30). However, the *Bjerkandera* MnP appears to be unique since it has a broader substrate specificity compared to most MnP isozymes reported. This isozyme is able to directly oxidize various aromatic amines as well as guaiacol and DMP in the absence of manganese. Although, the oxidation rate was lower in the absence of manganese compared to in the presence of manganese at pH 4.5, the oxidation of these compounds was completely independent of manganese at pH 3.0.

Remarkable was the observation that *Bjerkandera* MnP was even able to oxidize veratryl alcohol and 1,4-dimethoxybenzene in the absence of manganese which are traditional LiP substrates. Since veratryl alcohol was oxidized but not benzyl alcohol, the oxidation of veratryl alcohol most likely proceeds via a cationic radical mechanism rather than a benzylic radical mechanism. Also the observed oxidation of 1,4-dimethoxybenzene to 1,4-benzoquinone supports a cation radical mechanism. The K_m and V_{max} values for veratryl alcohol oxidation (Table 3) are comparable to the results reported for LiP isozymes from other white rot fungi (9,

49). However, unlike classic LiP isozymes (15, 18), Mn(II) concentrations greater than 0.1 mM were found to severely inhibit the oxidation of veratryl alcohol by the *Bjerkandera* MnP. This observation would be expected since both compounds being substrates of the enzyme would have to compete for the same oxidized heme group. The fact that a low rate limiting (non-competitive) concentration (0.033 mM) of Mn(II) stimulated the veratryl alcohol oxidation, indicates that Mn(II) helped to protect the enzyme from H_2O_2 inactivation probably by completing the catalytic cycle. Also it was observed that veratryl alcohol (0.1 mM) incubated with the enzyme without Mn(II), could delay the H_2O_2 inactivation of the enzyme as measured by Mn(II) oxidizing ability (data not shown). These phenomena can only be explained by a single enzyme utilizing both compounds as a substrate.

Veratryl alcohol is a de novo secondary metabolite produced by many white rot fungi including *Bjerkandera* sp. strain BOS55 (7). Concentrations ranging from 0.01 to 0.80 mM occur in liquid cultures as well as during solid state fermentation of wood (31, 34). The oxidation of veratryl alcohol has generally been attributed to LiP as opposed to MnP due to the high ionization potential of the compound which is not directly oxidized by Mn(III). Veratryl alcohol has been implicated in several roles in the catalysis of LiP. This metabolite mediates the oxidation of aromatic compounds with lower redox potential than that of veratryl alcohol cation radical (14, 43). As the best reducing substrate for LiP compound II, veratryl alcohol can reduce the enzyme to the native state completing the catalytic cycle (24). Consequently, the presence of veratryl alcohol prevents the inactivation of LiP by H_2O_2 (5). The fact that the *Bjerkandera* MnP isozyme oxidizes veratryl alcohol, may indicate that this metabolite can have a role in the catalysis of MnP under manganese-free conditions.

Previously only a few MnP isozymes have been shown to carry out manganese independent oxidation. Several aromatic amines were directly oxidized by MnP from *Ceriporiopsis subvermispora* (50); however, manganese was essential for the oxidation of guaiacol. The MnP of *Pleurotus* strains was shown to oxidize DMP in the absence of manganese (28, 42). The MnP from *Lentinus edodes* is reported to oxidize veratryl alcohol; however unlike the *Bjerkandera* MnP, manganese was an essential requirement (6). Although it is known that purified MnP of *P. eryngii* and *P. ostreatus* directly oxidize veratryl alcohol without manganese (28, 42), the K_m value of the *Bjerkandera* MnP was ~25-35-fold lower. Thus only the *Bjerkandera* MnP would truly be functional under physiological conditions.

All the kinetic data suggest that the *Bjerkandera* MnP isozyme described here has characteristics of both MnP and LiP. The enzyme with the most comparable substrate spectrum reported in the literature to that of *Bjerkandera* MnP is the *Pleurotus* MnP (28). However, the N-terminal amino acid sequences of the *Pleurotus* MnP isozymes have 8 to 9 amino acids which do not match in the first 20 amino acids, indicating that the *Bjerkandera* MnP is a distinct gene product. The N terminal amino acid sequences of MnP from other white rot fungi such as *P. chrysosporium*, *C. subvermispora*, *P. sordida*, *L. edodes*, and *Dichomitus squalens* were also not very homologous to that of *Bjerkandera* MnP (10, 27, 36, 38, 39, 41). The highest homology of the N-terminal amino acid sequence of *Bjerkandera* MnP is with that of both MnP2 and LiP7 from *Trametes versicolor* (20) having only 1 and 2 non-matching amino acids in the first 25 residues, respectively. However, the manganese independent substrate specifity of *Trametes* MnP isozymes has yet to be studied.

In conclusion, the MnP of *Bjerkandera* sp. strain BOS55 is a unique enzyme which can best be described as a hybrid between manganese peroxidase - lignin peroxidase. The catalytic

efficiencies for Mn(II) and veratryl alcohol oxidation by this single isozyme are comparable to MnP and LiP respectively of other white rot fungi.

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7. Concluding Remarks

Bjerkandera sp. strain BOS55 is an outstanding white rot fungus which can efficiently degrade polycyclic aromatic hydrocarbons (PAH) and bleach kraft pulp (13, 17, 28). This fungus produces extracellular peroxidases as the main components of its ligninolytic system; manganese dependent peroxidase (MnP) and lignin peroxidase (LiP) and a third type of peroxidases denominated as manganese independent peroxidase (MIP) (Chapter 2, Chapter 4, Chapter 5, 9, 10, 25). Moreover, it is also a good producer of secondary aryl metabolites, veratryl alcohol (3,4-dimethoxybenzyl alcohol) is produced in the highest amount along with some chloroanisyl metabolites (Chapter 3, Chapter 4, 9).

Low molecular weight cofactors are important components of the ligninolytic system for the proper catalytic function of the extracellular peroxidases. These compounds fulfil roles as redox mediators or cooxidants enabling the proper turnover of the catalytic cycle. Veratryl alcohol is the most important cofactor of LiP; whereas, the naturally occurring metal in wood, manganese, is the most important cofactor of MnP (Chapter 1).

Table 1. Components influenced by manganese in the ligninolytic system of white rot fungi.

Component	Effect of Mn	Possible mechanism	References
MnP	+[a]	a) gene induction b) prevent H_2O_2 inactivation of the enzyme	3, 31 Chapter 6, 38
LiP	-	a) indirect via inhibition of veratryl alcohol biosynthesis b) lowering O_2 stress by Mn-SOD	Chapter 2 32
Veratryl alcohol	-	lowering the concentration of phenolic precursors	Chapter 3
(Chloro)anisyl metabolites	-	lowering the concentration of phenolic precursors	Chapter 3
Oxalate	+	via Mn-dependent oxaloacetase	11, 27

[a] +: stimulation; -: inhibition

The objectives of this thesis were to study the effect of these cofactors on the physiological regulation of *Bjerkandera* sp. strain BOS55 and to determine the interrelationship between the cofactors and the extracellular peroxidases. Based on previous studies and the results of this thesis, it can be concluded that manganese plays a central role in the regulation of the production of various ligninolytic components. Table 1 summarizes the effect of manganese on regulating the production of secondary metabolites and extracellular peroxidases. The mechanisms of this regulation will be discussed in this Chapter. Moreover, this thesis demonstrates that *Bjerkandera* sp. strain BOS55 adapts to manganese deficient conditions. The mechanism and the physiological relevance of this adaptation will also be discussed.

VERATRYL ALCOHOL AND OTHER SECONDARY METABOLITES

In Chapter 2, this thesis demonstrated for the first time that manganese inhibits the production of veratryl alcohol in white rot fungi (*Bjerkandera* sp. strain BOS55 and the well-known *Phanerochaete chrysosporium*). By adding potential precursors to bypass the manganese inhibited step in the biosynthesis (Chapter 3), it was observed that only fully methylated precursors (e.g., veratrate) could bypass the inhibition. The addition of all biosynthetic precursors improved the veratryl alcohol production versus the complete de novo production; however, the extent of the production was always significantly lower in the presence of manganese except for veratrate and veratraldehyde. From these observations, it was concluded that manganese lowers the concentration of phenolic intermediates in the biosynthesis to veratryl alcohol rather than inhibiting any given enzymatic step as is shown in Figure 1 (Chapter 3). The fact that adding phenolic precursors to the manganese containing cultures increased the production of veratryl alcohol supported this hypothesis (Chapter 3).

The mechanism how manganese can affect the concentration of phenolic precursors inside the fungal cells has not yet been studied. Manganese peroxidase would be an obvious candidate because it oxidizes phenolic compounds very efficiently and it is induced by the presence of manganese (16, 34). Although, this enzyme can be found in the intracellular matrix (8), it is considered to be an extracellular enzyme. Therefore, other intracellular enzymes such as vanillate hydroxylase which occurs in *P. chrysosporium* can be a potential candidate (4). Phenolic acids (e.g., 4-hydroxybenzoate, protocatechuate, vanillate) are good substrates of the enzyme while the methylated forms of these compounds such as veratrate are poorly oxidized (4). Consequently the role of manganese in the regulation or the enzymatic mechanism of vanillate hydroxylase or of a similar enzyme should be elucidated.

Manganese is also inhibited the production of (chlorinated) anisyl metabolites (e.g., p-anisaldehyde, 3-chloro-*p*-anisaldehyde), however, to a lesser extent (Chapter 3). Again it was observed that the inhibition of manganese most probably occurred by decreasing the concentration of phenolic precursors. This study also demonstrated that there are common precursors in the biosynthesis of veratryl alcohol and (chlorinated) anisyl metabolites. The addition of phenylalanine, tyrosine, cinnamate, benzoate were able to stimulate the production of all aryl metabolites.

High concentrations of veratryl alcohol and other aryl metabolites (e.g., 3-chloro-*p*-anisaldehyde) were observed during incubations on natural woody substrates which contain high levels of manganese (Chapter 4). The most probable explanation is that phenolic compounds such as 4-hydroxybenzoate, protocatechuate and vanillate derived from lignin degradation continuously feed into the biosynthetic pathway of aryl metabolites resulting in high concentrations of these metabolites. This hypothesis was confirmed in Chapter 3 by adding deuterium labelled 4-hydroxybenzoate which resulted in deuterated veratryl alcohol, p-anisyl alcohol, 3-chloro-p-anisaldehyde and 3,5-dichloro-p-anisaldehyde. Figure 1 shows a review of the possible pathways leading to the production of veratryl alcohol during wood decay, also shown the interaction of biodegradation with biosynthesis.

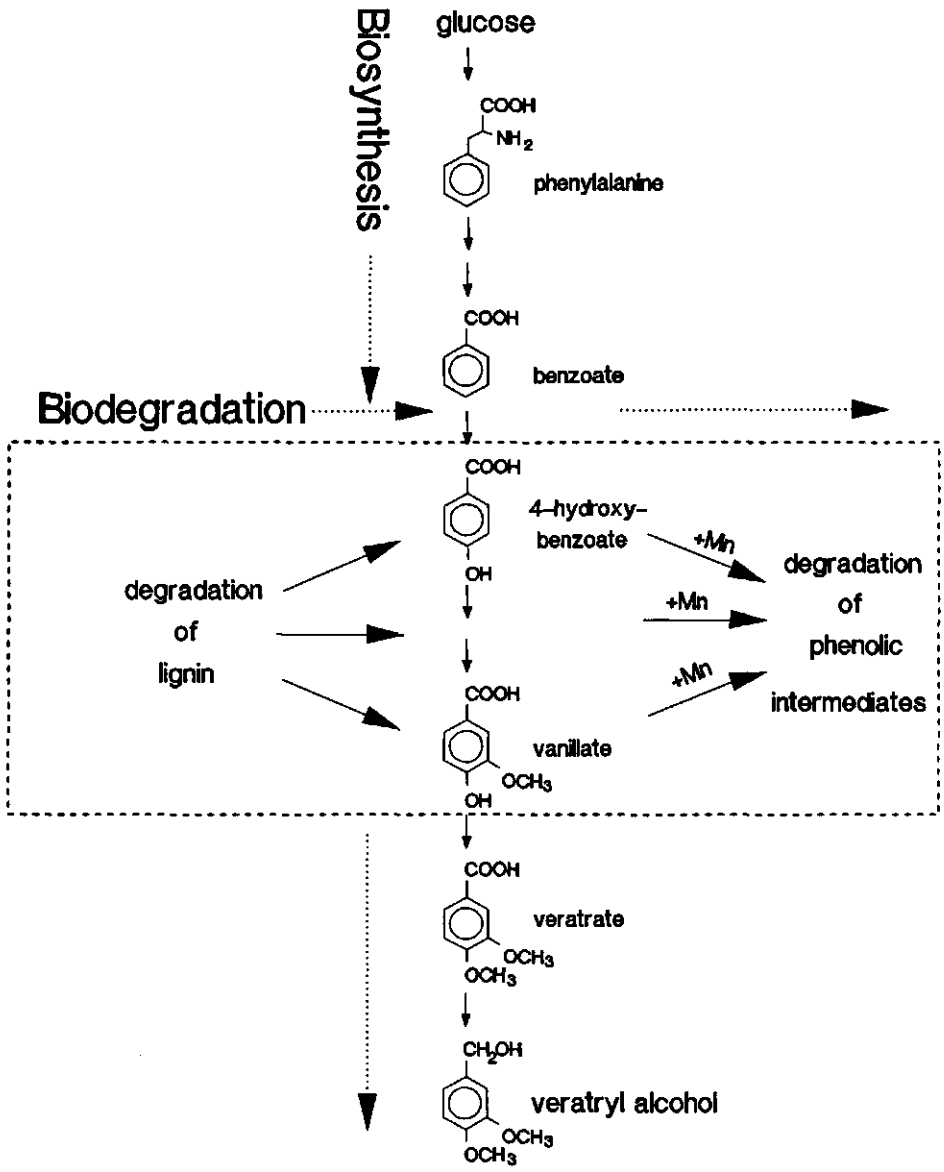

Figure 1. Pathways leading to veratryl alcohol production during wood decay. White rot fungi synthesize veratryl alcohol from glucose during the secondary metabolism. Moreover, monoaromatic lignin degradation products are also able to serve as precursors. In the presence of manganese, however, the amount of phenolic precursors are lowered.

Not all the secondary metabolite were inhibited by manganese in *Bjerkandera* sp. strain BOS55. Manganese had a stimulatory effect on the production of simple organic acids which are essential components of the ligninolytic system of white rot fungi (11, 35). The presence of manganese was shown to increase the production of oxalate in biobleaching cultures of the fungus compared to that under manganese deficiency (27). Most likely this is the consequence that one of the major biosynthetic enzyme, oxaloacetase, is dependent on manganese (11).

LIGNIN PEROXIDASE

Low LiP titres are known to occur in the manganese containing cultures of several white rot fungi which have high LiP activities in manganese deficient cultures (2). *Bjerkandera* sp. strain BOS55 was also observed to follow this trend having high LiP titres in the complete absence of manganese and approximately ten-fold lower activities in the presence of manganese (Chapter 2). This trend is remarkable since it is known that manganese itself does not repress *lip* gene transcription (20). In Chapter 2, a probable explanation for the effect of manganese on lowering LiP is revealed by relating the enzyme activities to the production of veratryl alcohol. The addition of veratryl alcohol to the cultures of several white rot fungi has repeatedly been found to be beneficial for the LiP titres (6, 12, 21, 29); although, veratryl alcohol itself does not induce the *lip* gene transcription in *P. chrysosporium* (5). The stimulatory effect of veratryl alcohol can be explained by the fact that veratryl alcohol is able to protect LiP from inactivation by physiological concentrations of H_2O_2 which was demonstrated by incubating LiP isozymes with H_2O_2 in the presence and absence of veratryl alcohol (5, 6, 37). Inactivation of LiP by H_2O_2 is caused by the formation of a relatively non-reactive oxidation state of LiP, compound III. In support of this hypothesis, a good correlation was found between the level of veratryl alcohol and LiP titres in *Bjerkandera* sp. strain BOS55 irrespective of whether veratryl alcohol was produced due to the enhanced biosynthesis by manganese deficiency or was exogenously added to manganese sufficient cultures (Chapter 2). These findings combined together the fact that manganese inhibits the production of veratryl alcohol suggested that the decrease in LiP titres under manganese sufficiency is due to an indirect effect of manganese via decreased production of veratryl alcohol.

Rotschild *et al.* (32) have offered an alternative explanation for the effect of manganese on LiP activity in *P. chrysosporium*. According to their hypothesis, *lip* gene expression is induced by reduced oxygen radicals. Manganese reduces the oxygen stress by acting as a radical scavenger and by inducing the formation of manganese-superoxide dismutase (Mn-SOD). If the oxygen stress was restored using pure oxygen atmosphere, high LiP titres were found even in the presence of high manganese concentrations. However, the manganese concentrations required (> 236 μM) to lower the oxygen stress and repress LiP production which are much higher than the manganese concentration (33 μM) which was found to severely repress veratryl alcohol biosynthesis in both *P. chrysosporium* and *Bjerkandera* sp. strain BOS55. Consequently at the low manganese concentration, the incubation of *Bjerkandera* sp. strain BOS55 under an oxygen atmosphere had no positive effect on LiP production compared to an air atmosphere (18, 25).

Surprisingly, high LiP activity was detected in cultures grown on natural woody substrates during solid state fermentation (Chapter 4). This observation apparently contradicts the fact that the woody substrates contained soluble manganese in relatively high concentrations (110 and 450 μM). Most probably the high LiP titres were due to the enhanced production of veratryl alcohol by lignin degradation intermediates as was proposed in Chapters 3. Also the oxidation of phenols in the natural substrates probably contributed to an enhanced production of reduced oxygen radicals compared to synthetic culture media.

MANGANESE PEROXIDASE

Manganese is known to stimulate the production of MnP in many white rot fungi (2). Putative metal response elements were found in the promoter region of the genes of various MnP isozymes, which can explain the inductive effect of Mn on *mnp* gene transcription (16, 22). The addition of manganese was also beneficial for the MnP production in *Bjerkandera* sp. strain BOS55 (Chapter 2, Chapter 5) by inducing the *mnp* gene transcription (unpublished data). The optimal concentration of manganese in liquid cultures was found to be similar to that measured in wood during solid state fermentation (Chapter 4, Chapter 5). In both cases, the level of MnP activity was very similar.

ADAPTATION TO MANGANESE DEFICIENCY

Manganese deficient conditions are clearly favorable for LiP production in *Bjerkandera* sp. strain BOS55. However, a remarkable finding of this thesis was the production of MnP occurring in the complete absence of manganese under various culture conditions (Chapter 5, Chapter 6, 27, 28). The MnP isozymes produced under manganese deficiency were found to increase in response to manganese, indicating that manganese still plays a regulatory role in *mnp* gene transcription of those isozymes. Aside from manganese, simple organic acids such as oxalate, glycolate or glyoxylate were able to directly induce MnP protein production (Chapter 6) and *mnp* gene transcription in the complete absence of manganese (unpublished data).

The major MnP isozyme produced in the manganese deficient and glycolate containing cultures of *Bjerkandera* sp. strain BOS55 was purified and characterized (Chapter 6). It was found that this remarkable MnP is able to directly oxidize Mn(II) like many other MnP isozymes as well as directly oxidize veratryl alcohol in the absence of manganese as efficiently as LiP isozymes from other white rot fungi. Therefore, the *Bjerkandera* MnP can be best described as a hybrid between manganese peroxidase and lignin peroxidase. Previously only MnP isozymes isolated from *Pleurotus* spp. have been shown to be capable of the manganese independent oxidation of veratryl alcohol (23, 33); however, the Km values for veratryl alcohol (3.0-3.5 mM) were too high to be realistic under physiological conditions.

Wood species contain considerable manganese which would apparently bring into question the relevance of a MnP - LiP hybrid enzyme under natural conditions. However, it is well known that the oxidation state and the localization of manganese changes during white rot decay resulting in manganese deficient regions (1, 30). From the results of this

thesis and the study of Moreira *et al.* (27), it can be concluded that *Bjerkandera* sp. strain BOS55 is capable of degrading lignin in the absence of manganese. The dominating peroxidases and the used cofactors as the result of the adaptation mechanism to manganese deficiency by *Bjerkandera* sp. strain BOS55 is illustrated in Figure 2 and compared to manganese sufficiency. Manganese deficiency is favorable for veratryl alcohol production. Both *Bjerkandera* MnP and LiP can utilize veratryl alcohol as cofactor in manganese-deficient sections of the wood. On the other hand, where manganese is available, MnP production is stimulated and the preferred cofactor for MnP is definitely Mn(II) since Mn(II) is the best substrate for the hybrid enzyme. Due to the continuous production of veratryl alcohol derived from phenolic intermediates of lignin degradation, LiP is also functional in the presence of manganese.

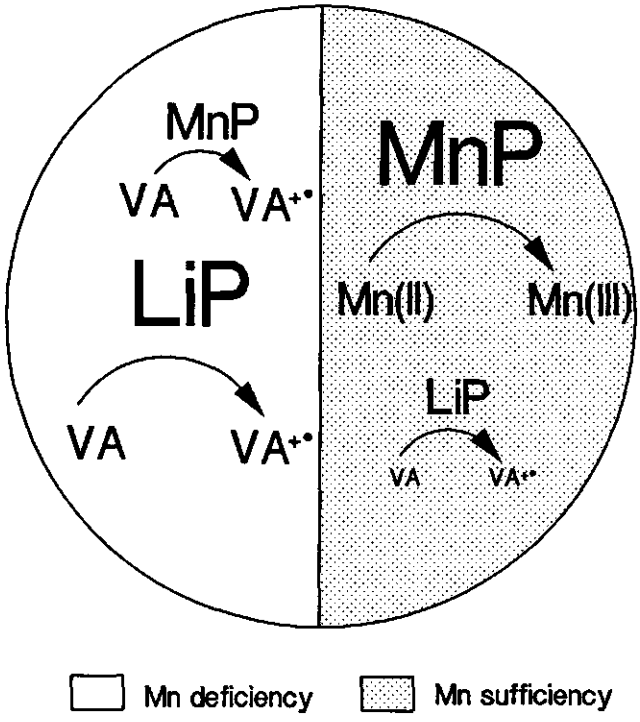

Figure 2. Adaptation mechanism of the ligninolytic system of *Bjerkandera* sp. strain BOS55 to manganese deficiency and sufficiency (the letter size indicates the relative amounts produced from peroxidases and veratryl alcohol). Under Mn deficiency, the dominating enzyme is LiP and veratryl alcohol is present in the highest amount. However, MnP is also produced which is also able to use veratryl alcohol as cofactor. In the presence of Mn, more MnP is present and its cofactor is Mn(II). LiP also can be found due to the relatively high veratryl alcohol production. Veratryl alcohol production in the presence of manganese is mostly due to lignin degradation products serving as precursors.

POSSIBLE ROLE OF ORGANIC ACIDS

The role of organic acids in ligninolysis is a very fascinating aspect which should be elucidated in future research. On the one hand, organic acids are able to replace manganese by inducing *mnp* gene transcription in *Bjerkandera* sp. strain BOS55 when manganese is absent. Reactive oxygen species such as superoxide anion radicals generated by peroxidases from oxalate and dioxygen could be an obvious candidate in inducing *mnp* gene transcription. Previously, regulation of gene transcription responding to the presence of H_2O_2 was observed in *P. chrysosporium* (19). However, MnP proteins were never detected in *P. chrysosporium* when manganese was absent (14, 16).

On the other hand, there is increasing evidence that the organic acids are an important source of the reduced oxygen radicals which may have a role in enhancing the lignin degradation (15, 27, 35) and the pathogen defence system of white rot fungi (7, 24). Additionally, the presence of organic acids may serve as an early warning for the upcoming presence of bioavailable manganese. The fungal organic acid metabolites are well known for their ability to solubilize insoluble MnO_2 deposits in fungal attacked wood (26, 35, 36).

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Summary

INTRODUCTION

Lignin is a three dimensional hydrophobic plant polymer derived from the random coupling of phenylpropanoid precursors. The chemical and physical characteristics of lignin require a nonspecific, extracellular oxidative process for biodegradation. White rot basidiomycetes are the only group of organisms having an efficient extracellular ligninolytic system. These fungi produce peroxidases and laccases that are involved in the initial attack of lignin. The peroxidases work with H_2O_2 which is also enzymatically produced by the fungi by different H_2O_2-producing oxidases. For their proper operation, peroxidases require appropriate cofactors which are the best substrates of the enzymes. Lignin peroxidase (LiP) uses the de novo produced secondary metabolite, veratryl alcohol (3,4-dimethoxybenzyl alcohol) as cofactor; whereas, the cofactor of manganese peroxidase (MnP) is the metal Mn(II) which occurs naturally in wood. The in vitro oxidation of lignin preparations by LiP or MnP is only feasible in the presence of veratryl alcohol or manganese, respectively. Additionally, simple aliphatic organic acid metabolites such as oxalate are involved in lignin degradation. On the one hand, oxalate is a good chelator of Mn(III) generated by MnP. This complex is relatively stable forming a low molecular weight diffusible oxidant capable of oxidizing phenolic lignin. Oxalate is also oxidized by ligninolytic enzymes in the presence of their cofactors generating reactive oxygen species that possibly participate in the oxidation of lignin.

The low molecular weight cofactors also influence the physiological regulation of white rot fungi. Veratryl alcohol is known to increase LiP activities in white rot fungal cultures, although, veratryl alcohol itself does not induce *lip* gene transcription. Manganese is essential for the induction of *mnp* gene expression and MnP activity in various white rot fungi. In contrast, manganese lowers LiP production so that the highest LiP activities are measured under manganese deficiency. Nonetheless manganese has no direct repressive effect on *lip* gene transcription.

Despite the great research efforts conducted so far on various white rot fungi, the true mechanism of lignin degradation is still not fully understood. However, it is becoming clear that low molecular weight cofactors are important catalytic and physiological regulating components of the ligninolytic system.

This thesis was dedicated to study the interrelationship between low molecular weight cofactors and ligninolytic enzymes. *Bjerkandera* sp. strain BOS55 was used as the fungus of study, since it was found to be an outstanding producer of ligninolytic enzymes and a great variety of secondary aryl metabolites. Moreover, this strain was observed to be a good degrader of polycyclic aromatic hydrocarbons (PAH) and biobleacher of kraft pulp in various screenings. The main objective of the thesis was to gain insight into the role of veratryl alcohol and manganese in regulating the physiology and participating in the ligninolytic system of *Bjerkandera* sp. strain BOS55.

CHAPTER 2

In Chapter 2, it was demonstrated for the first time that manganese inhibits the biosynthesis of veratryl alcohol in white rot fungi. This explains at least in part the general observation that LiP production is lowered in the presence of manganese although manganese itself does not repress the transcription of *lip* genes. The ten-fold increase in veratryl alcohol biosynthesis caused by manganese deficiency stimulated the LiP titres by protecting the enzyme from inactivation by physiological levels of H_2O_2. Adding veratryl alcohol to manganese containing cultures of the fungus sustained high LiP titres similar to that found under manganese deficient conditions. Moreover, a good correlation was observed between the LiP titres and veratryl alcohol concentrations irrespective of whether veratryl alcohol was produced by the fungus or added exogenously.

CHAPTER 3

In Chapter 3, the mechanism resulting in manganese inhibition of veratryl alcohol biosynthesis was studied. Potential biosynthetic precursors of veratryl alcohol were added to manganese deficient and sufficient cultures of *Bjerkandera* sp. strain BOS55 in order to bypass the inhibited step. The addition of fully methylated precursors (veratrate, veratraldehyde) equally increased the production of veratryl alcohol irrespective of the manganese concentration. This observation indicated that the reduction of the benzylic acid to benzyl alcohol group is not inhibited by manganese. All the other known precursors such as phenylalanine, cinnamate, benzoate/benzaldehyde as well as the partially hydroxylated benzylic compounds (e.g. 3-hydroxybenzoate, 4-hydroxybenzoate, protocatechuate, vanillate, isovanillate) did increase the veratryl alcohol production in the presence of manganese but never as much as that under manganese deficiency. From these observations it was concluded that no single step along the biosynthetic pathway was inhibited by the presence of manganese. Instead, the availability of phenolic precursors is limited when manganese was added. From this study, we also learned that there are several alternative precursors resulting in increased veratryl alcohol production which can potentially originate from lignin degradation. In addition, it was demonstrated that many of the veratryl alcohol precursors (phenylalanine, cinnamate, benzoate, 4-hydroxybenzoate) enhanced the production of anisyl and chloroanisyl metabolites indicating the existence of common precursors in these biosynthetic pathways. Deuterium labelled benzoate and 4-hydroxybenzoate were converted to a broad spectrum of labelled aryl metabolites.

CHAPTER 4

In Chapter 4, the interrelationship between cofactors and peroxidases in cultures grown on natural substrates (beech wood and hemp stem wood sawdust) was studied. Beech wood and hemp stem wood substrates, which contain 6 and 25 mg kg^{-1} dry wood of soluble manganese, respectively were favourable for MnP production. Many studies in the past have failed to demonstrate the presence of LiP on natural substrates even in fungi having *lip* genes. Surprisingly, *Bjerkandera* sp. strain BOS55 produced very high LiP titres on the wood substrates. The high LiP activity observed suggested that this enzyme

may have an important role during wood decay. The significant LiP production in spite of the presence of manganese can be explained by the very high production of veratryl alcohol throughout the incubation. The fact that veratryl alcohol was produced in high amounts although soluble manganese was always present apparently contradicts the findings presented in Chapter 2 . However, the high veratryl alcohol production can be explained by the presence of lignin degradation products such as 4-hydroxybenzoate, protocatechuate, vanillate, isovanillate entering the biosynthetic pathway of veratryl alcohol as was suggested in Chapter 3.

CHAPTER 5

Bjerkandera sp. strain BOS55 is a good MnP producer. In Chapter 5, the conditions for optimal MnP production were examined. The highest production was observed in nitrogen rich medium with 0.2 to 1 mM manganese at a pH value of 5.2 and at a temperature of 30°C. Two interesting phenomena were discovered while studying the physiology of MnP production. Firstly, significant MnP production was also observed in the absence of manganese which previously only has been shown to be the case for *Pleurotus* spp. However, unlike *Pleurotus* spp., MnP production in cultures of *Bjerkandera* sp. strain BOS55 was enhanced in response to increasing Mn levels. Secondly, it was demonstrated that the addition of various organic acid metabolites significantly increased the MnP titres under manganese sufficient conditions. The best results were obtained with glycolate.

CHAPTER 6

In Chapter 6, the study on the MnP production was continued in order to better understand why MnP is produced in the absence of manganese and to elucidate the induction mechanism under manganese deficient conditions. In the absence of manganese, oxalate and related organic acids, such as glycolate or glyoxylate were found to induce MnP production. The stimulatory effect of organic acids on MnP production was demonstrated to be due to the increased production of MnP proteins. Additionally, it was shown that the acids induced *mnp* gene transcription (unpublished data).

The major MnP isozyme produced in the absence of Mn and in the presence of glycolate was purified and characterized. Like other MnP isozymes, this enzyme was able to efficiently oxidize Mn. However, unlike other MnP isozymes, it was also able to directly oxidize veratryl alcohol and 1,4-dimethoxybenzene with a very high affinity in the absence of manganese. These nonphenolic substrates are typical substrates of LiP. Methoxyphenols and aromatic amines could also be oxidized in the absence of manganese. The optimal pH for the manganese independent oxidation of all the substrates tested was 3.0 similar to that observed for LiP isozymes. On the other hand, the oxidation of Mn(II) and consequently the manganese dependent oxidation of phenolic substrate reached the highest rate at pH 4.5 as described for many MnP isozymes. The kinetic values in terms of turnover number and affinity for Mn(II) and veratryl alcohol oxidation were similar to those found for other MnP and LiP isozymes. Therefore, the *Bjerkandera* MnP could be best described as a hybrid enzyme between MnP and LiP, having a binding site for Mn(II) as well as for

methoxy aromatics/phenols. This conclusion is supported by the finding that Mn(II) at concentrations greater than 0.1 mM severely inhibited veratryl alcohol oxidation by the enzyme; whereas Mn(II) has no effect on LiP. The fact that this enzyme can oxidize Mn(II) as well as directly oxidize veratryl alcohol and other aromatic amines and phenols clarifies the physiological relevance of the occurrence of this MnP under Mn deficient and sufficient conditions.

CONCLUSIONS

In conclusion, this thesis has resulted in new insights into the key regulatory role of manganese in lignin degradation such as the repressive effect of manganese on the production of aryl metabolites. The addition of manganese at concentrations as low as 33 μM completely changed the pattern of peroxidases and aryl metabolites production compared to that under manganese deficiency. Additionally, it was observed that MnP is purposefully produced and regulated by organic acids in the total absence of manganese. Under these conditions, a novel type of MnP-LiP hybrid isozyme was isolated which was functional under any manganese regime using either veratryl alcohol or Mn(II) as a cofactor. Under natural conditions, such an enzyme would have considerable physiological significance since soluble manganese is known to be leached out or become oxidized to insoluble MnO_2 during fungal attack, possibly resulting in manganese deficient areas in wood. In manganese deficient areas, veratryl alcohol biosynthesis is stimulated and the enzyme can use this secondary metabolite as an alternative to manganese.

Future research should elucidate the newly discovered role of organic acids in regulating MnP in white rot fungi. The physiological significance of this regulation may be due to the role of organic acids as an important source of reduced oxygen radicals. The oxidative stress resulting from radicals may be the signal for MnP gene expression as has been shown to be the case with H_2O_2. The radicals are also required for extensive degradation of lignin. Additionally, the presence of organic acids may serve as an early warning for the upcoming presence of bioavailable manganese. The fungal organic acid metabolites are well known for their ability to solubilize insoluble MnO_2 deposits in fungal attacked wood.

Samenvatting

INLEIDING

Lignine is een driedimensionale hydrofobe plantpolymeer afgeleid van de willekeurige koppeling van fenylpropanoide precursors. De chemische en fysische eigenschappen van lignine vereisen een niet-specifiek, extracellulair oxidatief proces voor diens afbraak. Wit rot schimmels zijn de enige groep organismen die een efficiënt extracellulair ligninolytisch systeem bezitten. Deze schimmels produceren peroxidases en laccases die zijn betrokken bij de initiële aanval op lignine. De peroxidases hebben H_2O_2 nodig, dat eveneens enzymatisch wordt geproduceerd door verschillende H_2O_2-producerende oxidases van de schimmels. Voor een goede werking hebben peroxidases geschikte cofactoren nodig, die de beste substraten van het enzym zijn. Lignine peroxidase (LiP) gebruikt de de novo geproduceerde secundaire metaboliet veratryl alcohol (3,4-dimethoxybenzyl alcohol) als cofactor, terwijl de cofactor van mangaan peroxidase (MnP) het metaal Mn(II) is, dat van nature in hout voorkomt. De in vitro oxidatie van lignine structuren door LiP of MnP is uitsluitend mogelijk in de aanwezigheid van respectievelijk veratryl alcohol of mangaan. Bovendien zijn eenvoudige alifatische organische zuren zoals oxalaat betrokken bij de lignine afbraak. Aan de ene kant is oxalaat een goede chelator van Mn(III), dat wordt aangemaakt door MnP. Dit complex is relatief stabiel en vormt een laagmoleculaire oxidant, die in staat is fenolisch lignine te oxideren. Aan de andere kant wordt oxalaat zelf ook geoxideerd door ligninolytische enzymen in aanwezigheid van hun cofactoren, waarbij reactieve zuurstof deeltjes worden gegenereerd, die mogelijk deelnemen aan de oxidatie van lignine.

De laagmoleculaire cofactoren beïnvloeden tevens de fysiologische regulering van wit rot schimmels. Van veratryl alcohol is bekend dat het LiP activiteiten verhoogt in cultures van wit rot schimmels, alhoewel veratryl alcohol zelf de transcriptie van het *lip* gen niet induceert. Mangaan is essentieel voor zowel de inductie van de *mnp* gen expressie als MnP activiteit in verscheidene wit rot schimmels. Hiertegenover staat dat mangaan de productie van LiP verlaagt, zodat de hoogste LiP activiteiten worden gemeten onder mangaan gebrek. Desalniettemin heeft mangaan geen direct onderdrukkend effect op de *lip* gen transcriptie.

Ondanks de aanzienlijke onderzoeksinspanningen tot nu toe met verscheidene wit rot schimmels wordt het ware mechanisme van de lignine afbraak nog steeds niet volledig begrepen. Hoewel, het wordt steeds duidelijker dat laagmoleculaire cofactoren belangrijke katalytisch en fysiologisch regulerende componenten vormen in het ligninolytische systeem.

Dit proefschrift heeft het doel om het onderlinge verband tussen laagmoleculaire cofactoren en ligninolytische enzymen te bestuderen. In dit onderzoek werd de schimmel *Bjerkandera* sp. stam BOS55 gebruikt als organisme, omdat werd aangetoond dat het een uitstekende producent van ligninolytische enzymen en van een grote variëteit aan secundaire aryl metabolieten is. Bovendien werd aangetoond dat deze schimmel een goede afbreker van polycyclische aromatische koolwaterstoffen (PAK) is en in verscheidene screenings werd aangetoond dat het een goede biobleacher is van kraft pulp. Het belangrijkste doel van het onderzoek was om inzicht te krijgen in de rol van veratryl alcohol en mangaan in de regulering

van de fysiologie van de schimmel en in hun deelname aan het ligninolytische systeem van *Bjerkandera* sp. stam BOS55.

HOOFDSTUK 2

In hoofdstuk 2 wordt voor de eerste keer aangetoond dat mangaan de biosynthese van veratryl alcohol in wit rot schimmels inhibeert. Dit verklaart op zijn minst ten dele de algemene waarneming dat de LiP productie lager wordt in aanwezigheid van mangaan, alhoewel mangaan zelf de transcriptie van de *lip* genen niet onderdrukt. De tienvoudige toename in de biosynthese van veratryl alcohol, die werd veroorzaakt door mangaan gebrek, stimuleerde de LiP titers door het enzym te beschermen tegen inactivatie door fysiologische concentraties aan H_2O_2. Het toevoegen van veratryl alcohol aan mangaan-bevattende schimmelcultures leverde hoge LiP titers op, die vergelijkbaar waren met die onder mangaan deficiënte omstandigheden. Bovendien werd een goede correlatie gevonden tussen de LiP titers en veratryl alcohol concentraties ongeacht het feit of veratryl alcohol werd geproduceerd door de schimmel zelf of van buitenaf werd toegevoegd.

HOOFDSTUK 3

In hoofdstuk 3 werd het mechanisme bestudeerd dat leidde tot de inhibitie van de biosynthese van veratryl alcohol door mangaan. Potentiële biosynthetische precursors van veratryl alcohol werden toegevoegd aan mangaan-deficiënte en mangaan-verrijkte cultures van *Bjerkandera* sp. stam BOS55 om de geïnhibeerde stap in de veratryl alcohol biosynthese te overbruggen. Het toevoegen van volledig gemethyleerde precursors (veratraat, veratraldehyde) bevorderden de productie van veratryl alcohol in gelijke mate, ongeacht de mangaan concentratie. Deze waarneming duidde erop dat de reductie van de benzylische groep tot een benzyl alcohol groep niet werd geïnhibeerd door mangaan. Alle andere bekende precursors zoals fenylalanine, cinnamaat, benzoaat/benzaldehyde en gedeeltelijk gehydroxyleerde benzylische verbindingen (b.v. 3-hydroxybenzoaat, 4-hydroxybenzoaat, protocatechuaat, vanillaat, isovanillaat) bevorderden de productie van veratryl alcohol in aanwezigheid van mangaan, maar nooit zoveel als bij mangaan gebrek. Uit deze waarnemingen werd geconcludeerd dat geen enkele stap in de biosynthese route werd geïnhibeerd door de aanwezigheid van mangaan. Daarentegen, de beschikbaarheid van fenolische precursors bleek gelimiteerd als mangaan werd toegevoegd. Uit deze experimenten leerden we tevens dat er verscheidene alternatieve precursors zijn, die leiden tot een verhoogde productie van veratryl alcohol en die mogelijk afkomstig zijn van de afbraak van lignine. Daarenboven werd aangetoond dat vele van de veratryl alcohol precursors (fenylalanine, cinnamaat, benzoaat, 4-hydroxybenzoaat) de productie van anisyl en chloro-anisyl metabolieten stimuleerden, hetgeen duidde op het bestaan van gemeenschappelijke precursors in de biosynthese routes. Benzoaat en 4-hydroxybenzoaat met daaraan deuterium labels werden omgezet in een breed scala aan gelabelde aryl metabolieten.

HOOFDSTUK 4

In hoofdstuk 4 werd de onderlinge relatie tussen cofactoren en peroxidases in cultures op natuurlijk substraten (beukenhout- en hennep houtpijp zaagsel) bestudeerd. Beukenhout en hennep houtpijp als substraat, die respectievelijk 6 en 25 mg oplosbaar mangaan per kg drooggewicht bevatten, bleken geschikt voor de productie van MnP. In vele onderzoeken in het verleden werd de aanwezigheid van LiP op natuurlijke substraten niet aangetoond, zelfs niet bij schimmels met *lip* genen. Echter, *Bjerkandera* sp. BOS55 produceerde verrassend hoge LiP titers op houtachtige substraten. De waargenomen hoge LiP activiteit suggereerde dat dit enzym een belangrijke rol kan spelen tijdens de afbraak van hout. De significante LiP productie ondanks de aanwezigheid van mangaan kan worden verklaard door de zeer hoge productie van veratryl alcohol tijdens de gehele incubatie. Het feit dat veratryl alcohol werd geproduceerd in grote hoeveelheden ondanks de aanwezigheid van oplosbaar mangaan spreekt blijkbaar de resultaten gepresenteerd in Hoofdstuk 2 tegen. Echter, de hoge veratryl alcohol productie kan worden verklaard door de aanwezigheid van lignine afbraakproducten zoals 4-hydroxybenzoaat, protocatechuaat, vanillaat en isovanillaat, die voorkomen in de biosynthese route van veratryl alcohol zoals was gesuggereerd in Hoofdstuk 3.

HOOFDSTUK 5

Bjerkandera sp. stam BOS55 is een goede MnP producent. In Hoofdstuk 5 werden de omstandigheden voor een optimale MnP productie onderzocht. De hoogste productie werd waargenomen in een stikstofrijk medium met 0.2 tot 1 mM mangaan bij een pH van 5.2 en een temperatuur van 30°C. Twee interessante fenomenen werden ontdekt tijdens het bestuderen van de fysiologie van de MnP productie. Als eerste, een significante productie van MnP werd ook waargenomen in de afwezigheid van mangaan, hetgeen voorheen was aangetoond uitsluitend in het geval van *Pleurotus* spp. Echter, in tegenstelling tot *Pleurotus* spp. werd de MnP productie in cultures van *Bjerkandera* sp. stam BOS55 verhoogd als reactie op toenemende Mn niveaus. Ten tweede, het werd aangetoond dat de toevoeging van verscheidene organische zuur metabolieten de MnP titers significant verhoogden onder omstandigheden met voldoende mangaan. Het beste resultaat werd verkregen met glycolaat.

HOOFDSTUK 6

In Hoofdstuk 6 werd het bestuderen van de MnP productie voortgezet met het doel om beter te begrijpen waarom MnP wordt geproduceerd in afwezigheid van mangaan en om het inductie mechanisme onder mangaan deficiënte omstandigheden op te helderen. In afwezigheid van mangaan bleken oxalaat en gerelateerde organische zuren zoals glycolaat of glyoxylaat de MnP productie te induceren. Het werd aangetoond dat het stimulerend effect van organische zuren op de MnP productie werd veroorzaakt door de toegenomen productie van MnP eiwitten. Bovendien werd aangetoond dat deze zuren de transcriptie van *mnp* genen induceerden (niet gepubliceerde gegevens).

Het belangrijkste MnP iso-enzym geproduceerd in afwezigheid van Mn maar in aanwezigheid van glycolaat werd gezuiverd en gekarakteriseerd. Net zoals andere MnP iso-enzymen was dit enzym in staat om Mn efficiënt te oxideren. Echter, in tegenstelling tot andere

MnP iso-enzymen was het tevens in staat om veratryl alcohol en 1,4-dimethoxybenzeen direct en met een zeer hoge affiniteit te oxideren in de afwezigheid van mangaan. Deze niet-fenolische substraten zijn typische substraten voor LiP. Ook methoxyfenolen en aromatische aminen konden in afwezigheid van mangaan worden geoxideerd. De optimale pH voor deze mangaan-onafhankelijke oxidatie van alle geteste substraten was 3.0, vergelijkbaar met die werd waargenomen voor LiP iso-enzymen. Echter, de oxidatie van Mn(II) en bij gevolg de mangaan-afhankelijke oxidatie van fenolische substraten bereikte de hoogste snelheid bij pH 4.5, zoals werd beschreven voor vele MnP iso-enzymen. De kinetische waarden in termen als turnover number, affiniteit voor Mn(II) en veratryl alcohol oxidatie waren gelijk aan die gevonden voor andere MnP en LiP iso-enzymen. Daarom kan het MnP van *Bjerkandera* het best worden beschreven als een hybride enzym tussen MnP en LiP, dat in het bezit is van een bindingsplaats voor zowel Mn(II) als voor methoxy-aromaten/fenolen. Deze conclusie wordt ondersteund door de bevinding dat Mn(II) boven 0.1 mM de veratryl alcohol oxidatie door het enzym sterk inhibeerde, terwijl Mn(II) geen effect heeft op LiP. Het feit dat dit enzym Mn(II) kan oxideren en zowel veratryl alcohol alsook andere aromatische aminen en fenolen direct kan oxideren verklaart het fysiologische belang van het voorkomen van dit MnP onder zowel Mn-deficiënte als Mn-rijke omstandigheden.

CONCLUSIES

Concluderend, dit proefschrift heeft geleid tot nieuwe inzichten in de belangrijkste regulerende rol van mangaan in de afbraak van lignine zoals het onderdrukkend effect van mangaan op de productie van aryl metabolieten. Het toevoegen van mangaan in concentraties zo laag als 33 mM veranderde het patroon van de peroxidases en aryl metaboliet productie volkomen in vergelijking met deze bij mangaan deficiëntie. Bovendien werd waargenomen dat MnP doelbewust werd geproduceerd en gereguleerd door organische zuren in absolute afwezigheid van mangaan. Onder deze omstandigheden werd een nieuw type MnP-LiP hybride iso-enzym geïsoleerd, dat functioneel was bij elk mangaan regiem waarbij of veratryl alcohol of Mn(II) as cofactor werd gebruikt. Onder natuurlijke omstandigheden zou zo'n enzym een aanzienlijk voordeel hebben, aangezien van oplosbaar mangaan bekend is dat het kan uitspoelen of kan worden geoxideerd tot onoplosbaar MnO_2 tijdens schimmelgroei, waardoor delen van het hout mogelijk mangaan deficiënt kunnen worden. In deze mangaan deficiënte delen wordt de veratryl alcohol biosynthese gestimuleerd en het enzym kan deze secundaire metaboliet gebruiken als een alternatief voor mangaan.

Vervolgonderzoek zou de rol van de onlangs ontdekte organische zuren in de regulatie van MnP in wit rot schimmels moeten ophelderen. De fysiologische betekenis van deze regulatie kan het gevolg zijn van de rol van organische zuren als een belangrijke bron van gereduceerde zuurstof radicalen. De oxidatieve stress als gevolg van radicalen kan het signaal zijn voor MnP gen expressie, zoals werd aangetoond in het geval van H_2O_2. De radicalen zijn tevens vereist voor een verregaande afbraak van lignine. Bovendien kan de aanwezigheid van organische zuren dienen als een eerste waarschuwing voor de naderende aanwezigheid van biobeschikbaar mangaan. De organische zuur metabolieten van schimmels zijn bekend om hun vermogen om onoplosbaar MnO_2 afzettingen op te lossen in door schimmels gekoloniseerd hout.

Összefoglaló

BEVEZETÉS

Lignin egy három dimenziós, hidrofób növényi polimer mely fenilpropanoid egységek random reakciójából keletkezik. A fehér korhadást okozó gombák az egyetlen élölény csoport mely hatékonyan képes lignint bontani. Ezek a gombák egy extracelluláris, komplex ligninbontó rendszerrel rendelkeznek, mely fö komponensei az oxidative enzimek: peroxidázok, lakkázok. A peroxidázok hidrogénperoxidot igényelnek müködésükhöz, melyet szintén a ligninbontó gombák választanak ki hidrogén peroxidot termelö oxidázaikkal. Hidrogénperoxid mellett a peroxidázok müködéséhez különbözö kofaktorok szükségesek, melyek egyben a peroxidázok legjobb szubsztrátjai is. A Lignin peroxidáz (LiP) kofaktorja egy de novo termelt szekunder metabolit, veratrilalkohol (3,4-dimethoxibenzilalkohol), míg a másik enzim, mangán dependens peroxidáz (MnP) kofaktorja a növényi szövetekben is elöforduló mangán. Mangánon és veratrilalkoholon kívül, a gombák által termelt egyszerü alifás szerves savak mint például oxálsav szintén szerepet játszanak a lignin bontásában. Egyrészt ezek a savak stabil komplexet képeznek az MnP által képzett Mn(III)-mal. Ez a komplex képes oxidálni egyszerü fenolokat és fenolos lignint. Másrészt, a ligninbontó peroxidázok magát az oxálsavat is oxidálják, mely reakció oxigén jelenlétében reaktiv szuperoxid anion gyököket erdményez. Ezek a reaktiv gyökök valószínüleg szintén részt vesznek a lignin bontásában.

A fenti kofaktorok szabályozzák a ligninbontás fiziologiájának is. Veratrilalkohol növeli a LiP mennyiségét a gomba kultúrákban, habár nem indukálja a *lip* gén expresszióját. Mangán pedig elengedhetetlen induktora a *mnp* gén transzkripciójának. Ezzel ellentétben, a mangán jelenléte jelentösen csökkenti a LiP enzim szintjét, holott a mangánnak nincs hatása a *lip* génre.

Annak ellenére, hogy a kutatók húsz éve próbálják megfejteni hogyan bontják a fehér korhadást okozó gombák a lignint, a ligninolízis mechanizmusát még nem értjük tökéletesen. Az azonban egyre világosabb számunkra, hogy a kofaktoroknak igen fontos szerepük van az egyes ligninbontó elemek gombák által való termelésében, nemcsak mint enzim kofaktorok és oxidációs mediátorok, de mint regulátorok is.

Ez a doktori értekezés a lignin bontásban szerepet játszó kofaktorok és peroxidázok egymásra gyakorolt hatásának vizsgálatát tüzte ki céljául. Egy kevésbé tanulmányozott ligninbontó bazidiumos gombát, *Bjerkandera* sp. BOS55 törzset használtam erre a célra, mivel ez egy kiváló enzim termelö törzs, valamint számos szekunder metabolitot is szintetizál. Korábbi munkák során az is bebizonyosodott, hogy a *Bjerkandera* sp. BOS55 törzs kiválóan oxidál poliaromás szénhidrogéneket és hatékonyan fementesíti a papírt a papírgyártás folyamatában.

MÁSODIK FEJEZET

Ebben a fejezetben elsőként mutattam be, hogy a mangán jelenléte gátolja a veratrilalkohol bioszintézisét. Ez a felfedezés magyarázatot adott arra, miért csökkenti a mangán a LiP mennyiségét, holott mangán nem hat a *lip* gén expressziójára. Mangánmentes kultúrákban tízszeres veratrilalkohol termelést figyeltem meg. Ez a mennyiségü veratrilalkohol képes volt megvédeni a termelt LiP-et a gomba által termelt hidrogénperoxid okozta inaktivációtól. Szintén magas LiP aktivitásokat kaptam, ha veratrilalkoholt adtam a mangánt tartalmazó *Bjerkandera* kultúrákhoz. Ezek a kisérletek azt mutatták hogy a LiP aktivitás mértéke korellációban van a veratrilalkohol koncentrációjával függetlenül attól, hogy a gomba termelte vagy kívülről adtam a kultúrához. Valamint azt is világossá vált, hogy a mangán a veratrilalkohol szintézisének gátlásán keresztül csökkenti a LiP szintjét.

HARMADIK FEJEZET

A továbbiakban folytattam a mangán veratrilalkohol és más aril metabolitok bioszintézisére gyakorolt gátló hatásának tanulmányozását. Ismert bioszintetikus prekurzorokat és lehetséges prekurzorokat adtam mangánmentes és mangánt tartalmazó *Bjerkandera* kultúrákhoz. Ezzel célom az volt, hogy megvizsgáljam melyik prekurzor hozzáadásával kapok azonos mennyiségü veratrilalkoholt mindkét mangán szinten. Az azonos mennyiségü veratrilalkohol azt jelzi, hogy a gátolt lépes a bioszintézisben éppen az illető prekurzor előtt található. Ha teljesen metilált prekurzorokat, veratrátot vagy veratraldehidet adtam, azonos mennyiségü veratrilalkoholt termelt a gomba mind a mangán mentes és a mangánt tartalmazó tápoldatokban. Ez az eredmény azt jelezte, hogy valoszínüleg a metilálást gátolja a mangán. Azonban azt is meg kell említenem, hogy ha a metilálási lépés előtti prekurzorokat pl. 4-hidroxibenzoátot, 3,4-dihidroxibenzoátot vagy vaniliasavat adtam a gomba kultúrához, a veratrilalkohol mennyisége mindig növekedett mangán jelenlétében is, de soha nem volt olyan magas mint a mangánmentes kultúrákban. Ebből arra következtettem, hogy a mangán nem gátolhat egy bizonyos bioszintetikus lépést, hanem valamilyen módon csökkenti a fenolos prekurzorok mennyiségét. Ebben a fejezetben szintén rámutattam arra, hogy számos alternatív precurzor is lehetséges a veratrilalkohol bioszintézisében, mely potenciálisan a lignin molekulából is eredhet. Továbbá, azt is kimutattam, hogy sok veratrilalkohol prekurzor (fenilalanin, fahéjsav, benzoát, 4-hidroxibenzoát) szintén növelte az ánizs (4-metoxibenzil) és klórozott ánizs (3-kloro-4-metoxibenzil) metabolitok termelését. Deuteriummal jelölt benzoát és 4-hidroxibenzoát hozzáadása a gomba kultúrákhoz számos fajta jelölt aril metabolit termelését eredményezte.

NEGYEDIK FEJEZET

A *Bjerkandera* törzs peroxidáz és aril metabolit termelését szintén megvizsgáltam természetes tápanyagforrásokon, bükk- és kenderfürészporon. Legnagyobb mennyiségben MnP termelödött mindkét fajta fürészporon, melyeknek viszonylag magas a mangán tartalmuk (kenderé 6 mg Mn kg^{-1} és bükké 25 mg Mn kg^{-1} szárazanyag tartalomra

vonatkoztatva). Igen sok gomba esetében LiP termelés nem kimutatható természetes tápközegben, habár a legtöbb gomba rendelkezik *lip* génnel. Ezért ezek a tanulmányok arra a következtetésre jutottak, hogy LiP nem játszik fontos szerepet a lignin bontásában. Ezzel ellentétben, a *Bjerkandera* törzs jelentös mennyiségü LiP-et termelt a vizsgált természetes tápanyagforrásokon is, mely azt jelzi, hogy ennek az enzimnek is van szerepe a lignin bontásakor. A magas LiP szint valószínü a nagy mennyiség veratrilalkohol termelésnek volt köszönhetö. Az a tény hogy magas veratrilalkohol termelést kaptam mangán jelenlétében látszólag ellent mond a Második fejezetben leirtakkal. Azonban, ahogy a Harmadik fejezetben is jeleztem, lignin fragmentumok, mint például 4-hidroxibenzoát, 3,4-dihidroxibenzoát, vaniliasav beléphetnek a bioszintézisbe kompenzálva a mangán negativ hatását.

ÖTÖDIK FEJEZET

Bjerkandera sp. BOS55 törzs igen hatékony MnP termelö. Optimalizáltam a gomba növekedési körülményeit a maximális MnP termelés érdekében. A legmagasabb MnP termelést szerves nitrogénben gazdag tápoldatban kaptam 0.2-1 mM mangán koncentrációnál, 5,2-es pH-nál, 30°C-on. Mialatt a MnP termelés fiziologiáját tanulmányoztam, két érdekes megfigyelést is tettem. Elöször, MnP termelést mangán mentes kultúrákban is mértem, melyet eddig csak a *Pleurotus* genus eseténben irtak le. Ellentétben azonban a *Pleurotus* genus-szal, az MnP termelés a *Bjerkandera* törzsben növekedett amikor emeltem a mangán koncentrációját. Másodszor, ha a gombák által is termelt egyszerü szerves savakat adtam a *Bjerkandera* kultúrához, növekedett a MnP termelés. A legmagasabb MnP termelést glikolát hozzáadásával értem el.

HATODIK FEJEZET

Folytattam a MnP termelés fiziológiájának tanulmányozását. Mangán mentes kultúrákban, oxálsav és az oxálsav bioszintetikus prekurzorai, glikolsav és glioxilsav stimulálta a MnP produkciót. A szerves savakak növelték az MnP fehérje termelést valamint az *mnp* gén transzkripcióját (nem publikált adat).

A fö MnP izoenzimet, mely mangánmentes körülmények között, glikolsav jelenlétében termelödött izoláltam és karakterizáltam. Mint más MnP izoenzimek képes volt oxidálni Mn(II)-t. Habár, ellentétben más MnP izoenzimekkel a *Bjerkandera* MnP közvetlenül oxidált veratrilalkoholt 1,4-dimetoxibenzolt mangán hiányában is. Ezek a metilált szubsztrátok tipikusan LiP szubsztrátok. Veratrilalkohol és 1,4-dimetoxibenzol oxidálásának pH optimum is azonos volt a LiP pH optimumával. Másrészt, Mn(II) oxidálásának és a Mn(II) által közvetve oxidált fenolok oxidálásának pH optimuma 4.5 volt, mely azonos más MnP izoenzimekével. A veratrilalkohol és Mn(II) oxidálásának enzim kinetikai adatai (K_m, turnover number) hasonlóak voltak más LiP és MnP enzimek esetében mértekéhez. Ezért a *Bjerkandera* MnP-t egy MnP - LiP hibrid enzimnek tekinthetjük. Ezt a következtetést az is alátámasztotta hogy a veratrilalkohol *Bjerkandera* MnP által történö oxidálását mangán hozzáadása (> 0.1 mM koncentrációban) jelentösen gátolta, melyet igazi LiP esetében nem figyeltek meg. Az a tény, hogy ez az enzim képes Mn(II)-t is oxidálni valamint mangán nélkül, közvetlenül veratrilalkoholt és más aromás

aminokat, fenolokat magyarázatot ad arra, hogy miért termeli a gomba ezt az enzimet mangán hiányában és mangán jelenlétében is.

KONKLUZIÓK

Ez a doktori disszertáció új betekintést eredményezett a mangán lignin bontásában játszott szabályozó szerepébe. A mangán jelenléte megváltoztatja a peroxidázok termelésének arányát, valamint gátolja a szekunder aril metabolitok bioszintézisét. Ellentmondva az általanosan elfogadott nézetnek, miszerint mangán elengedethetetlen MnP induktor, MnP termelést figyeltünk meg szerves savak indukáló hatására, mangánmentes kultúrákban. Ez az enzim nem véletlenül termelödött mangán hiányában is, hiszen magában egyesíti mindkét fajta peroxidáz (LiP és MnP) katalitikus tulajdonságait. Természetes körülmények között, a faanyagok dekompoziciójánál, egy ilyen enzim fiziológiai jelentöséggel bír, hiszen fehér korhadás folyamata alatt a mangán kilúgozódik a faanyagból vagy kicsapódik oldhatatlan MnO_2 formában, mely mangánmentes területeket eredményezhet. Mangán hiánya kedvez a veratrilalkohol bioszintézisnek, melyet LiP és a *Bjerkandera* MnP is képes hasznosítani mint kofaktor.

Az elkövetkezö kutatásoknak tisztáznia kell a szerves savak szerepét az MnP termelés szabályozásában. Ezek a szerves savak fontos forrásai a reaktiv oxigén gyököknek, melyek részt vesznek a lignin lebontásában. Ezek a szerves savak feltehetöen a megnövelt oxigén stresszen keresztül szabályozzák a MnP termelést. Ezek a szerves savak szintén képesek redukálni és oldatba vinni a kicsapódott MnO_2-ot, ezért megjelenésük jelzi a hamarosan rendelkezésre álló mangánt.

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Curriculum Vitae

The author of this dissertation was born on December 16th, 1961 in Pécs (Hungary). She received her high school diploma in 1980 from Vajda Péter High School in Szarvas (Hungary). In 1985, she obtained her master of science degree from biology in Kossuth Lajos University (Debrecen, Hungary). Between 1985 and 1991, she was employed as lecturer in the Debrecen Agricultural University (Szarvas, Hungary). From 1991 to 1992, she participated a post-graduate course on Advanced Environmental Sanitation at the International Institute for Infrastructure, Hydraulic and Environmental Engineering (Delft, The Netherlands). In 1993, she started her PhD studies at the Division of Industrial Microbiology of the Wageningen Agricultural University. In October, the author will go to Department of Biochemistry and Molecular Biology in the Penn State University (USA) to conduct post-doctoral research on biochemistry of ligninolysis.

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