

THE ROLE OF MANGANESE IN ENHANCED LIGNIN DEGRADATION BY *PLEUROTUS OSTREATUS*

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ABSTRACT

P. ostreatus belongs to a group of basidiomycetes, degrading preferentially the lignin moiety in woody tissues. A system enabling the conduction of solid state fermentation in chemically defined medium using inert solid support was developed. Under these conditions we could study fungal growth and activities in the absence of manganese. Mn amendments were found to enhance preferential lignin degradation by *P. ostreatus* as shown by ¹⁴C-lignin mineralization versus ¹⁴C-Cellulose mineralization as well as the production of Mn-peroxidase. Plant extractives of peeled cotton branches were also found to enhance lignin degradation by the fungus. Cotton stalks amended with MnSO₄ were shown to lose 56% of their lignin fraction. Thus showing the potential of *Pleurotus* in biopulping of non-woody material. The role of Mn(III) in lignin degradation is studied using EPR spectroscopy and spin-traps. It was shown that Mn(III) can lead to the formation of reactive peroxy radicals after one-electron abstraction from keto-acids. This is supported by the obligatory need for oxygen in the reaction. These radicals, can serve as diffusible oxidants. A complete system, performing the first step of ligninolysis could then be the production of Peroxy-radicals in the reaction of Mn(III) and plant extractives in the presence of oxygen.

INTRODUCTION

Nonwood lignocellulose represents huge amounts of unutilized renewable resource. As nonwood fiber pulping capacity has increased globally, so has interest in research on pulping operations (1). White rot fungi consist of a group of basidiomycetes capable of efficiently and selectively degrading lignin, thus these fungi can act as ligninolytic agents themselves or produce ligninolytic enzymes to be used in the emerging technology of biopulping (2, 3). The focus of this work is on the effects of manganese ions (Mn) levels in the medium on the preferential lignin degradation and the lignin degrading system of the white-rot fungus and the edible mushroom *Pleurotus ostreatus*. Cut cotton plants (stalks) served as the nonwood fibrous substrate for the fermentation by *P. ostreatus*. This material poses agrotechnical problems since its fibrous structure is similar to that of hardwood consisting of 46% cellulose and 22% lignin (4). Its worldwide importance is illustrated by the 10 million tons of cotton stalks reported for India yearly (5), and it was already found suitable for the manufacture of paper (1, 5). Analyses of the fungus and the role of Mn in ligninolytic activity on other wood substrate can be considered.

RESULTS AND DISCUSSION

Development of a solid state Fermentation System

Aeration and consequent oxygen availability are extremely important in studying the physiology of fungal growth, in particular lignin biodegradation by white-rot fungi. Research over the last decade has generally concentrated on *P. chrysosporium* grown in non-agitated liquid cultures under an atmosphere of 100% oxygen to overcome oxygen limitation (due to slow diffusion and low solubility in water) (6, 7). Some publications have shown that lignin degradation by *P. chrysosporium* does not require 100% oxygen once aeration conditions are suitable (8, 9). Michel *et al.* (7) modeled the respiration kinetics of *P.*

chrysosporium, concluding that under Solid State Fermentation (SSF), the oxygen effectiveness factor reaches its maximal value of 1.

In the case of ligninolytic fungi, SSF can be conducted on the indigenous substrate itself, i.e. woody tissues, and the physiology of the fungus can be studied by following the fate of the native substrate during fermentation. Indeed wheat straw, flax shive, cotton stalks, and other substrates have been used for SSF of several *Pleurotus* strains, for both mushroom production and physiological studies (4). However, an inert system would be advantageous, since fungal products and metabolites interact with the natural substrate, and can be released only by harsh treatments (6, 10).

An experimental system that mimics the natural growth conditions of the fungus was developed (11). Perlite was found to be the most suitable solid support for growth on a chemically defined synthetic liquid medium. The growth medium consisted of 10% (v/v) solids, 40% solution and 50% air. The fungus was exposed to atmospheric air conditions, as opposed to the dissolved oxygen present in liquid cultures. Vigorously growing aerial hyphae of *P. ostreatus* were observed concomitant to glucose utilization. Uniform growth over the solid particle surface resulted in small variance in both mineralization rates of radiolabeled glucose and lignin, and physiological parameters. The system also allows the use of a defined growth medium, the complete extraction of medium ingredients and conservation of enzymatic activities through extraction procedures. The patterns of mineralization of radiolabeled substrates and CO₂ evolution in cultures were similar to those observed during SSF on natural lignocellulose. This model system enabled the study of the biochemical processes occurring during SSF, and the control of the chemical composition of the medium (11).

Many white-rot fungi oxidize Mn(II) during delignification (12-15). The chemical nature and abundance of manganese constitute the basis for its involvement in lignin biodegradation. Manganese peroxidases, are the most abundant group of extracellular ligninolytic enzymes in white-rot fungi (16, 17). Their production by the fungi is affected by the levels of Mn(II), and their primary product is Mn(III). Mn(III) can diffuse and penetrate the lignocellulosic tissues, and then serve as a mediator for free radical reactions (15), or as an indigenous oxidant. However the effects of high Mn levels in the medium on white-rot fungal growth during SSF and on the lignocellulose degradation-process were hardly studied. Earlier studies (18) have also shown that Mn(III) is an important ligninolytic agent, but requires a suitable chelator. Thus, the effects of adding malonic acid to the medium were also studied in this work (19).

The chemically defined SSF system was used to study the effect of Mn levels in the medium on the growth extracellular ligninolytic enzymes profile and lignocellulose degradation of *P. ostreatus*. The system enabled the study of a Mn deficient medium as well as high concentrations of Mn(II), added as MnSO₄, up to 4.5 mM in the growth medium. MnO₂ was found to precipitate rapidly in most of the manganese-containing media indicating an extracellular oxidizing activity specific to manganese, or high extracellular oxidizing conditions (19). The precipitation of Mn(II) from the growth medium of *P. ostreatus* probably occurred via its oxidation to higher oxidative forms. This is in agreement with previous reports, suggesting the oxidation of Mn(II) to MnO₂ by *P. chrysosporium* (13, 20).

It was questioned whether manganese has a general effect on fungal physiology. A growth inhibition was observed in the presence of 4.5 mM Mn and was reversed by the addition of malonic acid. This could have been the result of either stabilization of the oxidized form of manganese in the medium until its deposition on nuclei, or reduction of excess Mn(III) by the organic acid itself, thereby protecting the fungus (19).

Ligninolytic Enzymes of *P. ostreatus*.

The ligninolytic enzyme-system of *P. ostreatus* was investigated in the chemically defined SSF system. Ligninolytic systems of many white rot fungi involve the synergistic action of several extracellular enzymes (14, 17, 20-22). The study of three groups of extracellular oxidizing enzymes - Laccases, Mn-peroxidases and hydrogen-peroxide-producing oxidase is described here.

Laccase activity on day 4 under Mn deficiency was unusually high (23). Levels of laccase activity during the secondary growth phase correlated with Mn-supplementation, in agreement with results shown by Ruttimattm *et al.* (14) for *P. brevispora* and *C. subvermispora*. Induction of MnP by Mn(II) in the medium has been reported for some white rot fungi (14, 20). High MnP activity was detected in an extract of enriched oak sawdust, fermented for 30 days with *Pleurotus sapidus*, *P. eryngii* or *P. pulmonarius* (21). These authors suggested the ubiquity of MnP in lignin degradation by white rot fungi. However, they reported that neither laccase nor glyoxal oxidase (Glox) are found under these conditions. In *P. ostreatus* grown under chemically defined SSF extracellular MnP activity was detected in the presence of high Mn concentrations in the medium (23). The proteins included in the MnP fraction were eluted from an HPLC ion exchange column by relatively high salt concentrations. The identification of MnP from *P. ostreatus* was confirmed using several assays. Mn(II) oxidation by this enzyme was measured and verified as well its capability of oxidizing 2,6 dimethoxyphenol and phenol red following the addition of H₂O₂. Other extracellular activities that may interfere with the common measurement of phenol-red oxidation by crude extracellular media were subtracted. Fungal hyphae of *P. ostreatus* growing in high Mn media were blackened, but the brown Mn-oxide deposits were not observed on the glassware. This may have resulted from the presence of a cell-wall-bound enzyme. If MnP in *P. ostreatus* is indeed hyphae-bound it may also account for the low levels of soluble extracellular MnP.

The coupling of enzymatic production of hydrogen peroxide by glucose oxidase (22) or Glox (24) with peroxidase activity has been suggested to be involved in an efficient degradation system. In the present work *P. ostreatus* was found to produce an extracellular oxidase under the above experimental conditions. This enzyme is capable of oxidizing glyoxal and other small compounds such as formaldehyde or oxalate and produces H₂O₂, thus it may be characterized as Glox (23).

Selective Delignification by *P. ostreatus*.

The effects of Mn supplementation on lignin-cellulose degradation by *P. ostreatus* were studied. ¹⁴C-lignin-lignocellulose and ¹⁴C-cellulose-lignocellulose were prepared by the method of Crawford (25) by using ¹⁴C-phenylalanine and ¹⁴C-glucose, respectively, as precursors for polymer synthesis in cut cotton stems. The degradation and mineralization of ¹⁴C-lignin in this system were enhanced when MnSO₄ was added at concentrations ranging from 0-730 μM (Table 1), mainly from day 7 on (19). Increases in both MnP and Glox activities were temporally correlated with lignin degradation and with the secondary growth phase in this experimental system.

Mineralization and modification of native [¹⁴C]lignin by *P. ostreatus* occurred in synthetic media with no added manganese. However, the addition of 730 μM Mn(II) to the growth medium resulted in the highest mineralization as well as the highest accumulation of water-soluble ¹⁴C-products (19). Total lignin modification in this treatment summed to 57% of the labeled lignin. Enhanced degradation of ¹⁴C-labeled lignin-lignocellulose was observed in this work with the addition of malonate to medium containing a low concentration of manganese (73 μM). However, this effect was not apparent when malonate was added to a medium containing 730 μM Mn(II). This suggests the possibility of intrinsic production of a Mn chelator by *P. ostreatus*, which may be induced by the presence of manganese ions in the medium. This may also be the cause of the increased lignin mineralization rate at the high manganese concentration from day 13 to day 26 (Table 1).

The effect of Mn on cellulose biodegradation may lead to a better definition of the unique role of Mn in lignin degradation by *P. ostreatus*. Cellulose degradation in nature has been suggested to require the activity of small agents that can attack the cellulose at the beginning of lignocellulose decay, thereby enabling the relatively large cellulases to penetrate the plant tissue (26). Mn, in combination with the extracellular production of hydrogen peroxide, could suit this process' demands. However, Halliwell (26) examined a large group of transition metals (among them Mn-sulfate) in different oxidation states and combined with various "biological" concentrations of hydrogen peroxide. He found only ferrous or ferric salts to be effective in promoting the degradation of cellulose under experimental conditions.

The degradation of ¹⁴C-labeled cellulose-lignocellulose, as well as total recovery of ¹⁴C by dissolving the residue in 72% sulfuric acid, was studied in the chemically defined SSF (27). Mineralization began when glucose was completely consumed from the medium at Mn concentrations of 0 to 2.7 mM. No differences were found in cellulose mineralization patterns and levels during the first 18 days. Mineralization rates at 46- and 480-μM Mn treatments remained constant thereafter, whereas rates at 0 and 2.7 mM Mn treatments decreased. A comparison of these results with our previous lignin mineralization studies under the same experimental conditions emphasizes the finding that the addition of Mn(II) enhances preferential lignin degradation by *P. ostreatus*. Recovery of ¹⁴C reached 98-100% in all treatments, using concentrated sulfuric acid.

Cotton stalks were used to study lignin and cellulose degradation by *P. ostreatus* during SSF on lignocellulose (27). The natural amount of Mn in this substrate was 30 μg/gram dry weight. preferential degradation of lignin by *P. ostreatus* was enhanced by the addition of Mn(II) to the fermentation medium and was most apparent in the mineralization of [¹⁴C]cellulose vs. [¹⁴C]lignin to ¹⁴CO₂ (Table 2; (27)). An increase of 25% was observed in the mineralization of cellulose in a 150-μg Mn treatment over that in the control treatment (without the addition of Mn to the cotton stalks), but no increase was observed in a 620-μg Mn treatment. In contrast lignin mineralization increased by 50% following the addition of either level of Mn to the cotton stalks.

Enhancement of selectivity was also demonstrated by chemical analyses of the residual material at the end of the fermentation period using both Goering-Van-Soest(ADL) (28) and Klason (29) methods. The Klason lignin has previously been shown to be a better estimate of the total lignin concentration in forages than ADL, especially for grass species (30). It was found that for cotton stalks (fermented and non-inoculated), Klason lignin was indeed higher by 4% of the dry matter than ADL (27). The most intensive degradation of both lignin and cellulose was achieved in the 150-μg Mn treatment whereas the least degradation was observed in the control treatment (Table 3). Highest lignin degradation was achieved with the addition of 620 μg/g MnSO₄ to the cotton stalks showing the degradation of 50% of the lignin with a small increase in cellulose degradation. Preferential degradation values were similar in terms of Klason lignin degradation vs. weight loss or vs. cellulose, or in terms of ADL vs. cellulose. The cellulose (acid detergent) fraction in the uninoculated stalks was 2.3 times higher than the Klason lignin. This ratio (cellulose:lignin) increased during the 32 days of SSF, from 2.5 in the control to 3.3, with the addition of 620 μg/g Mn to the medium.

Preferential lignin degradation was also observed by dissolving the substrate at the end of the experiment in 72% sulfuric acid. Using [¹⁴C]cellulose, this procedure reflects residual cellulose and using [¹⁴C]lignin it reflects fragmented lignin structures. While there was no quantitative difference in the labeled lignin dissolved by this method at the end of the experiment, 40% of the [¹⁴C]cellulose was extracted from the stalks of the 150-μg Mn treatment compared to only 33% in the other treatments, (adding up the ¹⁴C-CO₂ and the extracellular and soluble compounds). A complete recovery of ¹⁴C was achieved in this treatment, whereas in the other treatment the total was only 84-85%. The high recovery in the 150-μg Mn treatment could be a consequence of

rapid degradation and modification of lignin and cellulose to stable products which are readily dissolved. One should bear in mind that conducting SSF on cotton stalks results in a high dilution of the radioisotope marker in the natural substrate (10-20 mg in 1 g d.w.). This may result from condensation reactions which occur during biological delignification and production of reactive products (free radicals, semiquinones quinones), causing a strong association of the labeled isotopes with the surrounding matter. Higher and more rapid degradation of the whole material reduces the possibility of such reactions taking place (150 µg Mn vs. control treatments). Higher overall degradation was indeed visualized by comparing respiration patterns and residual matter in both Mn treatments to the control treatment. The different degradation patterns also resulted in increased *in vitro* digestibility of the organic portion of the substrate, reaching higher values than wheat straw, showing a clear relationship between higher lignin degradation and increased digestibility values (27).

Free Oxygen Radicals in the Extracellular Medium of *P. ostreatus*.

An enhancement of preferential lignin degradation by *P. ostreatus* following the addition of Mn(II) to the fermentation substrate could be the result of either increasing the activities of ligninolytic enzymes or the production of Mn(III), which might preferentially degrade aromatic structures in the lignocellulosic complex. Enzymes can not penetrate the condensed structure of the lignocellulosic complex (31). Thus, small agents such as free radicals, aryl-alcohols and Metallic complexes were suggested as potential diffusible oxidants, starting the breakdown of lignin at a distance from the fungal hyphae.

The measurement of free radicals in biological-aqueous media is problematic and becomes even more complex *in vivo*. The thioether substance 2-keto-4-thiomethyl-butyric-acid (KTBA) provides an easy to apply and detection assay for the presence of oxidants in aqueous media. The addition of KTBA to ligninolytic cultures of *P. ostreatus* and *P. chrysosporium* results in cleavage of this substrate and release of ethylene to the headspace atmosphere (10, 32). Ethylene formation was found to be time correlated with the onset of ligninolysis by white-rot fungi (10, 32-34). Cleavage of KTBA to ethylene has been recently shown to be a suitable detector of the presence of hydroxyl radical ($\cdot\text{OH}$), although cleavage could result from metallic free radicals as well (35). In this work, similar amounts of ethylene were detected in the absence and presence of manganese in the medium. At the highest manganese concentration (4.5 mM), without exogenous addition of malonate, ethylene production decreased.

A possible mechanism of oxidation of KTBA by white-rot fungi was investigated, suggesting that ethylene formation *in vitro* could result from the oxidation of KTBA by Mn(III) complexed with organic acids. It was found that the oxidation of KTBA and the formation of ethylene requires molecular oxygen. Using EPR spectroscopy and spin traps, it was found that a free peroxy-radical is formed during the cleavage of KTBA. Both phenomena are closely related to the process of lignin degradation by white-rot fungi. Since it is water soluble and its degradation product is easily detected, KTBA may serve for the investigation of oxidation mechanisms in aquatic and *in-vivo* processes (34,36). More research is needed to verify the different kinetics and yields of the oxidation of KTBA.

SUMMARY

P. ostreatus belongs to a group of basidiomycetes, degrading preferentially the lignin moiety in woody tissues. A system enabling solid state fermentation in chemically defined medium using inert solid support was developed. Under these conditions we could study fungal growth and activities in the absence of manganese. It was found that significant lignin degradation and mineralization by *P. ostreatus* occur under manganese deficiency.

Amending the medium with Mn(II) was shown to enhance lignin degradation and to induce MnP activity in *P. ostreatus*. These results suggest that manganese-dependent system may exist in *P. ostreatus* and be responsible for the enhancement of the macromolecule's breakdown to water-soluble oligomers. Cotton stalks amended with MnSO₄ were shown to lose 56% of their lignin fraction. Mn(II) amendments were found to enhance preferential lignin degradation by *P. ostreatus* as shown by ¹⁴C-lignin mineralization versus ¹⁴C-cellulose mineralization as well as the production of Mn-peroxidase. Laccase and MnP are both secreted by most white-rot fungi and are believed to oxidize the same residues in the lignin molecule (phenolic and substituted phenolic end-groups). Thus, studying the role of Mn(II) in expanding the degradative abilities of fungi is essential in the understanding of lignin biodegradation. KTBA cleavage is a useful assay for the detection of oxidizing agents of different nature such as $\cdot\text{OH}$ or Mn(III) as demonstrated in this work. It was shown that Mn(III) can lead to the formation of reactive peroxy radicals after one-electron abstraction from keto-acids.

A complete system, performing the first step of ligninolysis could then be suggested on the basis of this work findings starting with the production of H₂O₂ by glyoxal oxidase and the oxidation of Mn(II) to Mn(III) by manganese peroxidase and production of peroxy-radicals through the reaction of Mn(III) and plant extractives in the presence of oxygen, which can cleave non-phenolic structures in the lignin forming new phenolic and non-phenolic sites for further degradation.

Manganese amendment to the raw substrate may improve the preferential degradation of lignin, thus improving the process of biopulping by white-rot fungi. However, the effect of Mn amendment on cellulose biodegradation maybe crucial for its potential use in biopulping processes. Ethylene production from KTBA, which appears simultaneously with the onset of lignin breakdown can save as a measure and an indicator for the beginning of the ligninolytic phase. The results of this work suggest that *P. ostreatus* maybe an efficient biopulping agent for the use of non-woody agricultural wastes such as cotton stalks.

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Table 1: The effect of Mn on mineralization of ¹⁴C labeled lignin and cellulose by *P. ostreatus*, grown on chemically defined SSF medium.

Mn level in the medium	Lignin mineralization (%)		Cellulose mineralization (%)	
	10 days	26 days	10 days	26 days
Mn deficiency	2.6	10.7	7.3	19.5
46-73 μM	3.7	14.0	5.8	22.3
580-730 μM	4.4	16.1	6.9	22.6
2.7-4.5 mM	2.4	12.8	7.6	25.6

Table 2: The effect of Mn on mineralization of ¹⁴C labeled lignin and cellulose by *P. ostreatus*, grown on cotton stalks for 32 days.

Mn level in the medium	Lignin mineralization (%)	Cellulose mineralization (%)
30 μg/g (control)	18.6	29.3
150 μg/g	28.7	36.5
630 μg/g	27.0	29.5

Table 3: Acid detergent cellulose and lignin, and ash levels (% of dry matter) in cotton stalks before and after 32 days of SSF by *P. ostreatus*.

Preparation	Cellulose		Lignin		Ash
	% of d.m. ^a	% degraded ^b	% of d.m.	% degraded	% of d.m.
Cotton stalks	52.2		18.1		6.2
Fermented cotton stalks, 30 μg/g Mn	47.9	22	14.8	30	7.4
Fermented cotton stalks, 150 μg/g Mn	45.5	34	11.9	50	8.4
Fermented cotton stalks, 630 μg/g Mn	46.6	31	10.4	56	8.9

^aDry matter.

^bCalculated as follows: [(actual percentage of dry matter * initial percentage of ash/actual percentage of ash)/initial percentage of dry matter] * 100.

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