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Lignin Biodegradation by the Ascomycete *Chrysonilia sitophila*

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ABSTRACT

The lignin biodegradation process has an important role in the carbon cycle of the biosphere. The study of this natural process has developed mainly with the use of basidiomycetes in laboratory investigations. This has been a logical approach since most of the microorganisms involved in lignocellulosic degradation belong to this class of fungi. However, other microorganisms such as ascomycetes and also some bacteria, are involved in the lignin decaying process. This work focuses on lignin biodegradation by a microorganism belonging to the ascomycete class, *Chrysonilia sitophila*. Lignin peroxidase production and characterization, mechanisms of lignin degradation (lignin model compounds and lignin in wood matrix) and biosynthesis of veratryl alcohol are outstanding. Applications of *C. sitophila* for effluent treatment, wood biodegradation and single–cell protein production are also discussed.

Index Entries: Lignin biodegradation; *Chrysonilia sitophila*; lignin peroxidase; ascomycete; wood biodegradation; single-cell protein (SCP); *Neurospora sitophila*.

INTRODUCTION

The main constituents of wood are cellulose, hemicellulose and lignin. The first two are carbohydrates and lignin is an aromatic tridimensional polymer, basically consisting of three *p*-hydroxycinnamyl

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alcohols: *p*-coumaryl, coniferyl, and sinapyl alcohols. Lignin, hemicellulose, and pectin surround the spaces between cellulose fibrils in plants, giving the latter resistance to mechanical stress and a protection against microorganism attack *(1)*.

Lignin biodegradation is one of the most important contributions to the carbon cycle of the biosphere. From its study, potential applications employing lignin degrading microorganisms and their enzymes have arisen in the pulp and paper industry and in the utilization of agricultural wastes, production of aromatic chemicals, detoxification of pollutants, and so on *(2–5)*.

Intensive research has been done to elucidate the enzymatic degradation of lignin by the basidiomycete *Phanerochaete chrysosporium (6–10)*. Tien and Kirk *(11)* and Gold et al. *(12)* named the extracellular enzymes that they discovered in the broth of secondary metabolic cultures of *P. chrysosporium*, ligninase, and diarylpropane oxygenase, respectively. Afterwards, fungal peroxidases capable of oxidizing veratryl alcohol were called lignin-peroxidases. Other peroxidases dependent on manganese for activity and capable of oxidizing phenolic lignin model compounds were named Mn-peroxidases. The mechanism of ligninase action has been established for lignin model compounds *(13,14)*.

The enzymatic biodegradation of lignin matrix is a more complex problem and evidences for lignin depolymerization in vitro, using lignin-peroxidase, Mn-peroxidase, or laccase, have been discussed *(15,16)*.

New experiments are aiding the understanding of the mechanism of lignin biodegradation in vivo. Kondo and Imamura *(17)* reported that lignin model compounds, in the presence of carbohydrates, are transformed into their glycosides by the transglycosylation activity of glycosidases excreted from wood-rotting fungi. Based on several experiments, these authors have suggested that the formation of glycosides could prevents lignin repolymerization in vivo *(18)*. The action of cellobiose quinone oxidoreductase in inhibiting lignin polymerization and the participation of iron or manganese chelating agents also cannot be discounted *(19–21)*.

Lignin-peroxidase also has been detected in several other wood decaying basidiomycetes: *Coriolus versicolor*, *Merulius tremellosus*, *Phlebia radiata*, *Phlebia tremellosa*, *Chrysosporium pruinosum*, *Lentinus edodes*, and *Coriolopsis occidentalis*.

The ability to degrade lignin has also been reported for other microorganisms such as ascomycete and some bacteria of the class of actinomycete. Nilsson et al. *(22)* demonstrated that some higher ascomycetes, particularly *Daldinia concentrica*, degraded Aspen wood with the same intensity as *Trametes versicolor*. Some actinomycete were studied in the role of lignin biodegradation by Crawford et al. *(23)*. These microorganisms can degrade low molecular weight lignin fragments *(24)* and transform lignin into a soluble polymer of high molecular weight *(25)*.

The present work focuses on the results of our research on the ligninolytic system of an ascomycete, *C. sitophila*. A critical review of published data and new attempts to elucidate the role of this ascomycete on lignin degradation are presented.

General Aspects of *C. sitophila*

The term *Chrysonilia* was proposed by Von Arx *(26)* to catalog *Monilia sitophila*, known for many years as "red mold of bread" *(27,28)*. *C. sitophila* is the anamorph state and its teleomorph is *Neurospora sitophila*.

Chrysonilia is rarely studied and much of the available literature is about *Neurospora sitophila*. This fungus is used in Indonesia to prepare a fermented peanut cake, for human consumption, named Ontjom *(29,30)*. Other communications about *N. sitophila* report an exuberant growth on burnt trees, after air raids in 1945 in Japan *(31)* and the utilization of hydrolyzate of rice straw and lignocellulosic materials for single cell protein production *(32–37)*.

Cellulolytic ability of *N. sitophila* was described by Tamura and Takai *(38)*. Horowitz and Shen *(39)* detected tyrosinase activity in sulphur-lacking cultures. Berry and Dekker *(40)* reported the fractionation of cellulolytic enzymes from a *Monilia* strain, tentatively classified as *Monilia sitophila*, although present taxonomy favors the term *Neurospora* or *Chrysonilia*.

The *C. sitophila* strain TFB-27441 was isolated by Campos et al. *(41)* from macerated *Tribolium ferrugineum*, an insect inhabiting rice straw in Brazil. Cellulolytic and ligninolytic activity as well as single cell protein production has been reported by our group *(42–47)*.

Lignin-Peroxidase from *C. sitophila*

Three enzymes with ligninolytic activity were purified and further characterized from high ligninase activity cultures of *C. sitophila (42,43,48)*. Purified lignin peroxidase were called LiP-Cs. Table 1 shows the characteristics of the three isolated lignin-peroxidases. First order heat and pH denaturation kinetics were observed for all lignin peroxidases as for crude cultures. Thermal stability of LiP-Cs-I, LiP-Cs-II, and LiP-Cs-III were tested in the range of 28–50°C and LiP-Cs-III was found to be the most stable. All LiP-Cs (I, II, and III) presented an activity decrease at pH values higher than its optimum (Table 1).

Later experiments demonstrating high lignin-peroxidase production, were not reproducible. However, phenol-oxidase activity was always detected when *C. sitophila* was degrading lignin (pine wood) or lignosulfonate. Low reproducibility ligninase production by microorganisms had been reported by other authors *(49–51)*.

Enzyme production in a bioreactor was carried out under different conditions of agitation, air flow, and pH *(52)*. In a typical bioreactor experi-

Table 1
Characteristics of Lignin-Peroxidase from *C. sitophila*[a]

	LiP-Cs I	LiP-Cs II	LiP-Cs III
Proportion[b]	33 %	62 %	5 %
Molecular weight (Da)	68,000	50,200	47,500
Isoelectric point	9.1	6.7	4.5
Fe/Heme	0.8	1.3	1.2
Optimum pH	3.0	5.0	4.0
Specific activity[c]	6.0	4.4	9.1
Km app. V.alcohol mM	0.37	0.30	0.12
V_{max} V. alcohol nmol/min ml	9.09	8.30	2.50
Km app. H_2O_2 μM	50	37	33
V_{max} H_2O_2 nmol/min ml	6.67	7.20	4.80
Optimum H_2O_2 mM	0.1	0.1	0.1
Carbohydrate %	25.7	14.8	17.4

[a]Culture conditions used for ligninase production: fries modified medium containing 1.0% of glucose as carbon source at pH 6.0 and 28°C.
[b]Area below curve at 410 nm in a gel filtration chromatogram.
[c]Specific activity for veratryl alcohol oxidation to veratryl aldehyde nmol/min mg. LiP-Cs = Lignin Peroxidase from *C. sitophila*. V. alcohol = Veratryl alcohol.

ment, glucose and dissolved oxygen decreased in 50 h of culturing followed by an increase of the dissolved oxygen level. The pH decreased from pH 6.0 to 4.5 within 24 h and later increased to 8.8. The ligninase production begin at 48 h, reaching to 250 U/L at 140 h.

When ligninase activity of culture broth produced in bioreactor was measured by the standard method *(11)*, a decrease in the absorbance of the blank (tartrate buffer, culture broth and hydrogen peroxide) at 360 nm was observed *(52)*. This effect was because of to a reaction of extracellular culture broth with H_2O_2. Considering this H_2O_2 consumption, higher hydrogen peroxide concentrations were used to measure ligninase activity. A satisfactory explanation for this H_2O_2 dependence has yet to be achieved, but we are considering reaction of H_2O_2 with Mn^{+3} or Fe^{+3} chelates, since Mn-peroxidase and catalase were not detected in *C. sitophila* cultures *(52,53)*.

Two low molecular weight aromatic compounds were detected by HPLC in a CH_2Cl_2 extract obtained from the extracellular medium of bioreactor cultures. One of them was identified as veratryl alcohol by the retention time, co-injection with standard sample and by its UV spectrum. The other was an undetermined compound with the same retention time as veratryl aldehyde but a different UV spectrum *(52)*. Veratryl

alcohol has an important role in lignin biodegradation, as previously reported *(54–57)*. As veratryl alcohol was also detected in cultures of *C. sitophila*, it could be suggested that this compound has a similar role in the lignin degradation both by basidiomycetes and ascomycetes.

Lignin Biodegradation by *Chrysonilia sitophila*

Nutrient concentration (nitrogen, carbon, and sulfur), agitation and oxygenation are important parameters affecting lignin biodegradation by white-rot fungi. The effect of some of these factors on lignin biodegradation by *C. sitophila* was studied using lignosulfonates as substrate *(58)*. Lignosulfonate biodegradation by *C. sitophila* was stimulated rather than inhibited by an increase in the nitrogen concentration. The change of absorbance at 280 nm and the fluorescence of the culture medium containing 0.1% of lignosulfonate were maximal at the highest nitrogen concentration studied (76 mmol $NH_4^+L^{-1}$). Also the lignosulfonate depolymerization after 12 d of biodegradation was maximal at the highest nitrogen concentration studied. On the contrary, the N-limitation has been used as an effective way to improve lignin degradation and lignin-peroxidase production by *P. chrysosporium* and several other species of white-rot fungi *(13)*. However, Dosoretz et al. *(59)* have reported an overproduction of lignin-peroxidase at nonlimiting nitrogen nutrient condition where the lignin peroxidase production started as a response to depletion of glucose in the culture medium. *Lentinus edodes* also produces a higher rate of lignin degradation at high nitrogen concentrations *(60)*.

A different profile for lignosulfonate depolymerization was also observed in *C. sitophila* cultures with and without oxygenation. No difference was observed between agitated and stationary cultures *(58)*.

To elucidated specific reactions of the lignin metabolism by *C. sitophila*, we studied the biodegradation of different monomeric and dimeric lignin model compounds by intact cells *(61)*. Figure 1 shows a possible pathway for β-O-4 lignin model compound biodegradation by *C. sitophila*. The biodegradation of β-O-4 I dimer (I), gave rise to the formation of C_α oxidation product. In the degradation of β-O-4 II dimer (II), two products were formed, guaiacol and 3,4-dimethoxyacetophenone (IV). These results suggest that the degradation of β-O-4 II takes place in two steps, the initial formation of ketol (III) that through CH_2O loss give rise to 3,4-dimethoxyacetophenone (IV) (Fig. 1). The presence of these products suggests the occurrence of β-aryl-ether cleavage after Cα-oxidation of β-O-4 models, as occurs in biodegradation of similar compounds by *P. chrysosporium (61)*. An alternative possible pathway for β-O-4 II degradation is the presence of oxidoreductase which reduce the ketone to alcohol prior to oxidation that should result in a C_α-C_α cleavage to give compound (V).

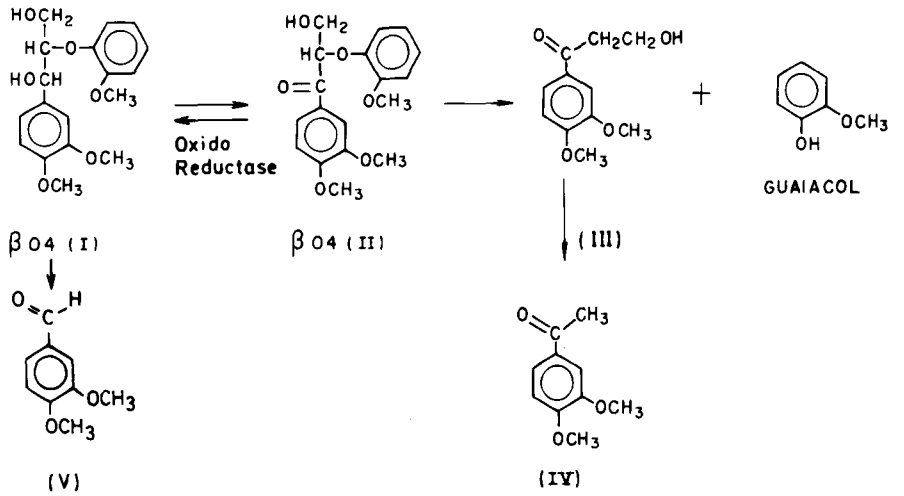

Fig. 1. Suggested pathways for lignin model compounds biodegradation by *C. sitophila* (modified from ref. *61*).

Lignin bioåådegradation by *C. sitophila* during *Pinus radiata* decaying was also evaluated *(62)*. The total weight loss of the wood after 3 mo of decay, was 20% and the carbohydrate and lignin losses were 18 and 25%, respectively. Decayed wood was extracted with solvents of increasing polarity. Methanol and dioxane yielded extracts containing representative low molecular weight degraded lignins. The overall structure of the degraded lignins, as shown by functional groups, molecular weight distribution and spectral characteristics such as UV, IR, ^{1}H-NMR, and ^{13}C-NMR was compared with the structure of milled wood lignin extracted from undecayed *P. radiata*. The compilation of the data allows us to suggest the occurrence of new substructures in degraded lignins such as Ar-OCH_2COOH and Ar-CH_2COOH. Based on these results, oxidative C_α-C_β and β-O-aryl cleavages for the mechanism of lignin degradation by this ascomycete was proposed *(62)*, and corroborate our earlier experiments with lignin model compounds *(61)*.

APPLICATIONS

Single cell protein (SCP) production from *C. sitophila* was evaluated for various carbon sources. The mycelial dry weight yields (g of dry mycelium/g of initial substrate), after 6 d of growth, were: glucose 0.38, cellobiose 0.30, saccharose 0.24, cellulose 0.14, and rice hull 0.05. The chemical composition of mycelium produced in saccharose cultures is shown in Table 2. Amino acid and fatty acid composition were also determined. The presence of the most of essential amino acids and unsaturated fatty acids showed the good quality of this single cell protein. High protein and low nucleic acid content indicates that *C. sitophila* SCP shows

Table 2
Chemical Composition of *C. sitophila* Mycelium[a]

Components	% (w/w)
Protein	39.2
Carbohydrate	29.3
Ether extract	12.1
Fiber	3.3
Ash	5.6
Total nucleic acid	3.3
Ribonucleic acid	2.6
Deoxyribonucleic acid	0.7

[a]Culture conditions for the mycelium production: agar plates cultured for 5 d in Fries modified medium containing 1.5% of saccharose as carbon source at pH 6.0 and 28°C *(47)*.

promise not only as a feed but also as a potential food for humans *(47, 63–66)*. Actually the ontjom food, which is a fermented *N. sitophila* peanut cake, is currently consumed in Indonesia *(30)*.

Application of *C. sitophila* to effluent treatment have also been studied. The high molecular weight chlorinated compounds present in the bleaching effluent from the cellulose industries are recalcitrant to most of the microorganisms and its treatment by conventional aerated lagoon have been unsuccessful. The color removal from this type of effluent has been an additional environmental problem *(67)*. Nylon immobilized mycelium of *C. sitophila* decolorized 85% of E_1 effluent (first alkaline extraction) after 5 d of culturing *(53,68)*. Results with immobilized lignin peroxidase from *C. sitophila* in agarose and silica also have proved to be useful in color removal from Kraft effluents *(69)*.

In order to evaluate the potential of this ascomycete for biological delignification of softwood, the influence of culturing parameters were studied. Optimization studies of the delignification process, in which nitrogen (0–50 mmol NH_4^+ L^{-1}) and glucose (0–2% w/v) concentrations were varied, showed a maximal lignin loss value of 17.8% for sawdust degradation after 15 d in cultures containing 10 mmol NH_4^+ L^{-1} and 1.0% glucose. In these conditions the cellulose and hemicellulose losses were 12% and less than 2%, respectively *(70)*. These results confirmed that *C. sitophila* can degraded considerable amounts of lignin in glucose-supplemented cultures. However, the cellulose degradation could not be suppressed, indicating that this fungus has a limited potential for biological pulping process.

CONCLUSION

Lignin degradation by microorganisms of the ascomycete class have been poorly studied. Our group have employed efforts for understanding several aspects related to lignin biodegradation by *C. sitophila*. This microorganism (or its teleomorph *N. sitophila*) have demonstrated ability to degrade lignocellulosic materials as reported since 1951 by Nakasawa and Yamasaki *(31)*.

Our investigations with *C. sitophila* have shown that this ascomycete produces lignin peroxidase and veratryl alcohol. Also it was possible to demonstrate lignin degradation reactions similar to those produced by basidiomycetes. The ability of this strain to growth in several lignocellulosic substrates have indicated that the use of ascomycetes could have an important role in the biotechnological process related to the SCP production and lignocellulosic bioconversion.

Further research concerning the role of ascomycetes on lignin biodegradation should be carried out using *C. sitophila* and other microorganisms of this class and also by different laboratories, in order to enhance the knowledgment of lignin biodegradation.

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