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Degradation of wood and enzyme production by *Ceriporiopsis subvermispora*

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ABSTRACT

The degradation of the components of Japanese beech and Japanese cedar wood was measured over time in cultures of the white-rot fungus *Ceriporiopsis subvermispora*. Although there was no initial degradation of cedar wood, after 12 weeks the mass loss of both cedar and beech wood was 15–20%. The mass losses of filter paper in beech wood-containing cultures and glucose cultures after 12 weeks were 87% and 70%, respectively. The ratio of lignin loss to mass loss of both beech and cedar wood cultures approached 2.0. Although the cellulose loss in cedar wood was very low throughout the 12-week incubation, *C. subvermispora* degraded the hemicellulose in Japanese cedar much more effectively than that in Japanese beech. These results confirm that *C. subvermispora* is a selective lignin degrader. During the 12-week incubation with Japanese beech wood, *C. subvermispora* continuously produced at least one of three phenol oxidases: laccase was produced initially, followed by Mn-independent peroxidase activity peaking at 6 weeks and Mn-dependent peroxidase activity peaking at 10 weeks. Lignin peroxidase and carboxymethylcellulase activities peaked after 3 weeks of incubation. Avicelase activity was present throughout the incubation period, although the activity was very low. The low-molecular-mass fraction of the extracellular medium, which catalyzes a redox reaction between O_2 and electron donors to produce hydroxyl radical, may act synergistically with the enzymes to degrade wood cell walls.

1. Introduction

Wood is the Earth's major renewable resource and is used primarily as building material and in paper/pulp manufacturing. Wood and other lignocellulosic materials are formed from three main polymeric constituents: cellulose, lignin, and hemicellulose [1]. The processing of these materials by wood-decaying fungi is an important part of the global carbon cycle. There are three main types of wood-decaying fungi: soft rot, brown rot, and white rot. White-rot fungi are particularly useful for biopulping, biodegradation of recalcitrant materials, and other applications [2,3]. There are two types of white-rot decay: the simultaneous degradation of all of the polymers in wood and the selective degradation of the lignin in wood. In simultaneous degradation, carried out by the majority of white-rot fungi, several different fungal enzymes erode the cell walls from the lumen to the middle lamella [4]. In the much rarer process of selective lignin degradation, fungal enzymes remove lignin and non-cellulosic polysaccharides without extensive degradation of cellulose [5–7]. *Ceriporiopsis subvermispora* is one of the best examples of a selective lignin degrader [2,6,8].

C. subvermispora produces oxidative enzymes during wood biodegradation. Mn-dependent peroxidase (MnP) is the predominant enzyme, followed by laccase [9]. However, only low-molecular-mass agents are able to diffuse into the wood cell wall during the early stages of wood degradation by white-rot fungi [6,10,11]. Wood-degrading fungi produce a variety of low-molecular-mass substances that are secreted from the mycelia or derived from wood components [12–18]. *C. subvermispora* has been reported to secrete a number of low-molecular-mass agents, including oxalic and glyoxylic acids in the extracellular fluid [19], oxalic acid during solid-state fermentation of wheat straw [20], and several fatty acids on wood meal cultures [12] and under solid-state fermentation conditions [13]. The MnP-mediated peroxidation of unsaturated fatty acids has been reported [21,22] and the MnP-mediated lipid-peroxidation system cleaves non-phenolic β-*O*-4 lignin model compounds oxidatively [23,24].

The occurrence of Fe^{3+}-reducing compounds in cultures of both brown- and white-rot fungi has been reported [15–17]. Several low-molecular-mass aromatic acids with hydroxyl or hydroxyl and methoxyl groups have been found in decayed wood samples [14,16] and these compounds could reduce Fe^{3+}. Since many wood-degrading fungi produce H_2O_2 [25], Fe^{2+} could generate hydroxyl radical (•OH) through a Fenton reaction [26] in the presence of H_2O_2. The involvement of •OH in cellulose degradation and lignin modification by brown-rot fungi has been reported [17]. In combination with phenol oxidases, •OH is believed to play a role in lignin degradation by the white-rot basidiomycete *Phanerochaete chrysosporium*. During wood decay, *P. chrysosporium*

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secretes low-molecular-mass glycoproteins that catalyze a redox reaction between O_2 and electron donors to produce •OH. This reaction accounts for most of the •OH produced in wood-degrading cultures of *P. chrysosporium* [27]. Thus low-molecular-mass agents may play key roles in the overall wood biodegradation process.

The lack of a complete cellulose-degrading system in cultures of *C. subvermispora* has been reported [28–30]. However most studies of selective lignin degradation have examined decreases in the individual polymers in the wood [8,31]. Guerra et al. [29] have pointed out that these losses represent the transformation of the polymers into carbon dioxide and water or into water-soluble compounds and that the accurate evaluation of degradative selectivity requires measurements of the molecular-weight distribution of the residual polymers. For example, using *in situ* determination of β-*O*-4 linkages in decayed wood samples, Guerra et al. [32] have shown that in the early stages of wood degradation *C. subvermispora* significantly depolymerizes lignin, even though the detectable loss of lignin is quite low. Guerra et al. [29] have further reported decreased yields of α-cellulose in *Pinus taeda* wood chips, while the yields of holocellulose and glucan remained unchanged. Cellulose depolymerization by *C. subvermispora* has also been demonstrated by determination of the α-cellulose content of biotreated samples of *Eucalyptus grandis* [7].

In this study, cellulose and lignin degradation, as well as the production of the hydrolytic enzymes, carboxymethylcellulase (CMCase; endo-1,4-*β*-D-glucanohydrolase, E.C. 3.2.1.4) and Avicelase (exo-1,4-*β*-D-cellobiohydrolase, E.C. 3.2.1.91), and the ligninolytic enzymes, laccase (E.C. 1.10.3.2), MnP (E.C. 1.11.1.13), and lignin peroxidase (LiP; E.C. 1.11.1.14), have been determined during the wood-decay process of *C. subvermispora*. Changes in the composition of decayed wood samples and their potential relationships to the production of specific enzymes are discussed. The participation of a low-molecular-mass fraction reported from the extracellular media of other white-rot fungi, which catalyzes a redox reaction between O_2 and electron donors to produce •OH [33–35], is also discussed.

2. Materials and methods

2.1. Microorganism and culture conditions

C. subvermispora (Pilat) Gilbertson et Ryvarden CBS 347.63 was used throughout this study. Basal agar medium was prepared as described previously [36], except that 0.5% glucose and 10-fold-concentrated trace elements were added. Cultures were inoculated with a small piece of fungal mat and incubated at 28 °C.

2.2. Wood degradation

Sapwoods of Japanese beech (*Fagus crenata* Blume) and Japanese cedar (*Cryptomeria japonica* D. Don) were cut into blocks measuring 2.0 cm × 2.0 cm × 0.5 cm. The blocks were extracted with acetone with refluxing to remove extractives which interfere with *C. subvermispora*, oven-dried at 60 °C, and weighed before use. Three sterilized wood blocks were placed on a mycelial mat on basal agar medium containing 0.5% glucose and incubated at 28 °C. After various periods of incubation, the mycelia covering the blocks were removed carefully and the blocks were dried and weighed.

2.3. Filter paper degradation

Filter paper (No. 2) of 7-cm diameter (ADVANTEC, Tokyo, Japan) was used as a pure cellulose substrate. The papers were extracted first with distilled water, then with acetone, and dried. In one set of experiments, a sterilized filter paper was placed on 50 ml of agar medium containing 0.5% glucose in a 300-ml Erlenmeyer flask. For the second set of experiments, wood meal of Japanese beech was extracted twice with acetone and dried. The dried wood meal (1.25 g) was wetted with 1.25 ml of water, sterilized, and sprinkled evenly over the surface of 50 ml of agar medium containing 0.2% glucose. A sterilized filter paper was placed over the wood meal and 1 g of wood meal, wetted with 1 ml of water and sterilized, was sprinkled evenly over the surface of the filter paper. The cultures were inoculated with two small pieces of fungal mat and incubated at 28 °C. After various incubation periods, the mycelia covering the filter paper were removed carefully and the paper was dried and weighed.

2.4. Chemical analysis of wood samples

Japanese beech and cedar wood samples from the wood degradation cultures described above were milled (Sample Mill TI-100, Heiko Ltd., Tokyo, Japan) for the appropriate time for passage through 80 mesh. The milled wood was extracted with acetone twice for 2 h each in a Soxhlet apparatus to remove extractive compounds. The extracted wood samples (500 mg) were hydrolyzed with 7.5 ml of 72% (v/v) sulfuric acid at 20 °C for 4 h. The acid was diluted to a final concentration of 3% (v/v) by addition of 280 ml of water and the mixture was heated to 121 °C (1 atm) for 1 h according to JIS P8001-1961 and Effland [37]. The residual material was cooled and filtered through a porous glass filter (No. 3). The solids were dried at 105 °C to constant weight and determined as insoluble lignin. Monomeric sugars in the soluble fraction were converted into alditol acetates and the sugar composition was estimated by gas–liquid chromatography according to the method of Sawardeker et al. [38]. Glucose was used as the standard for calculating the amount of cellulose. Xylose and mannose were used as the standards for calculating the amounts of hemicellulose from Japanese beech and cedar, respectively. The soluble lignin concentrations in the filtrates were determined by measuring the absorbance at 205 nm and using the value of 110 L g^{-1} cm^{-1} as the absorptivity of the soluble lignin [39]. All samples were hydrolyzed in duplicate experiments.

2.5. Preparation of culture filtrates

Wood meal of Japanese beech was prepared as described above. The dried wood meal (2.4 g) was wetted with 2.4 ml of water, sterilized, and sprinkled evenly over the surface of 30 ml of basal media in 300-ml Erlenmeyer flasks. At each of the indicated times, media from 5 cultures were acetone-precipitated (70%, v/v), as described previously [34]. The precipitate, dissolved in 10 ml of 50 mM acetate buffer (pH 4.5), was passed through a Sephadex G-50 gel-filtration column equilibrated with 50 mM acetate buffer (pH 4.5) and the UV absorbance of the eluate was measured at 280 nm. Three fractions were collected and designated I, II, and III, in the order of their elution from the column. The fractions were lyophilized, re-dissolved in 10 ml distilled water, and kept at −80 °C until use. The various activities in the fractions were assayed in duplicate.

2.6. Phenol oxidase activity in culture filtrates

Phenol oxidase activity was assayed spectrophotometrically with 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS; $\varepsilon_{420} = 3.6 \times 10^4$ M^{-1} cm^{-1} [40] or $\varepsilon_{414} = 3.6 \times 10^4$ M^{-1} cm^{-1} [41]) or phenol red ($\varepsilon_{610} = 22$ mM^{-1} cm^{-1} [42]) as the substrate. With ABTS as the substrate, the reaction mixture contained 500 μl of 200 mM acetate buffer (pH 4.5), 2 mM ABTS, and 50 μl of sample solution in a total volume of 1 ml. For assaying peroxidase activity, the reaction was initiated by the addition of H_2O_2 to a final concentration of 200 μM. For assaying MnP activity, the reaction mixture also contained 200 μM $MnSO_4$. The mixtures were incubated for 30 min at 30 °C. The A_{416} was measured, since 416 nm was determined by scanning to be the maximum absorbance. With phenol red as the substrate, the reaction mixture contained 690 μl of 20 mM acetate buffer (pH 4.5), 50 μl of 0.2% phenol red, 20 μl of 1 M lactate, 100 μl of 2% egg albumin, and 100 μl of sample solution in a total volume of 1 ml. For assaying peroxidase activity, the reaction was initiated by the addition of H_2O_2 to a final concentration of 200 μM. For assaying MnP activity, the reaction mixture also contained 200 μM $MnSO_4$. The mixtures were incubated at 30 °C for 30 min, the reactions were stopped by the addition of 40 μl of 2 M NaOH, and the A_{610} was measured. MnP activity with each substrate was calculated by subtracting the peroxidase activity in the absence of Mn^{2+} from the activity in presence of Mn^{2+}. One unit of enzyme activity was defined as the amount of enzyme oxidizing 1 μmol of the substrate per min per ml.

2.7. LiP activity in culture filtrates

LiP activity was assayed at 30 °C using veratryl alcohol as described previously [43]. The reaction mixture (3 ml) contained 100 mM tartrate buffer (pH 3.0), veratryl alcohol (500 μM), and sample (200 μl). The reaction was initiated by the addition of H_2O_2 to a final concentration of 170 μM. The A_{310} was measured after 30 min at 30 °C. One unit of enzyme activity was defined as the amount oxidizing 1 μmol of veratryl alcohol to veratraldehyde per min per ml ($\varepsilon_{310} = 9.3 \times 10^3$ M^{-1} cm^{-1}) [43].

2.8. CMCase and Avicelase activities in fraction I

CMCase and Avicelase were assayed by measuring reducing sugars such as glucose using the method of Somogyi-Nelson [44]. CMCase represents endoglucanase (EG) activity and Avicelase represents cellobiohydrolase (CBH) activity. CBH releases cellobiose from the ends of microcrystalline cellulose (Avicel) chains. The reaction mixture (1 ml) contained 800 μl of 100 mM acetate buffer (pH 5.0), 10 mg carboxymethylcellulose sodium salt (CMC; Nacalai Tesque, Japan) or 10 mg of Avicel (Funacel SF, Funakoshi, Japan), and 200 μl of sample. The reaction mixtures were incubated at 30 °C on a shaker for 0, 30, and 60 min for CMCase and 0, 24, and 48 h for Avicelase. The reaction mixture with Avicel was centrifuged to remove non-hydrolyzed Avicel and the supernatant (250 μl) was assayed. One unit of cellulase