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ORIGINAL ARTICLE

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Relationship between production of hydroxyl radicals and degradation of wood, crystalline cellulose, and a lignin-related compound or accumulation of oxalic acid in cultures of brown-rot fungi

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Abstract The degradation of wood, filter paper cellulose, and a lignin-substructure model, was measured in cultures of seven fungi usually regarded as brown-rot fungi. Hydroxyl radical production and the accumulation of oxalic acid in the cultures were also measured. Four of the fungi, *Gloeophyllum trabeum*, *Tyromyces palustris*, *Laetiporus sulphureus*, and *Postia placenta*, were typical brown-rot fungi, in that they preferentially degraded and eliminated the polysaccharides in wood and produced large amounts of hydroxyl radical. The rates of hydroxyl radical generation in cultures of the four fungi were directly proportional to the degradation rates of wood, cellulose, and the lignin-related compound, and inversely proportional to the amount of oxalic acid in the cultures. Two of the fungi, *Daedalea dickinsii* and *Lentinus lepideus*, did not degrade any of the substrates significantly and produced very little hydroxyl radical. *Coniophora puteana* had the highest rate of cellulose degradation, but did not degrade wood or the lignin model significantly and produced only negligible amounts of hydroxyl radical. These results indicate that brown-rot fungi produce large amounts of hydroxyl radical for the degradation of wood and crystalline cellulose.

Key words Brown-rot fungi · Hydroxyl radical production · Oxalic acid · Wood degradation · Crystalline cellulose degradation

Introduction

Brown-rot fungi preferentially degrade the cellulose in wood,[1] although they lack *exo*-1,4-β-glucanase activity for hydrolyzing crystalline or natural cellulose.[2] However, they degrade and metabolize cellulose only when their lignolytic systems are active.[1,3] The one-electron oxidizing activity in cultures of brown-rot fungi is proportional to the degradation of lignin-related model compounds, natural cellulose substrates, and wood in the cultures.[4] These facts suggest that brown-rot fungi generate a unique system that is capable of modifying lignin and fully degrading natural cellulose.

Brown-rot fungi rapidly depolymerize the cellulose in wood before any loss in total wood mass is detectable.[5] Furthermore, fungal enzymes such as cellulases and peroxidases are too large to penetrate cell walls during the early stages of wood degradation by brown-rot fungi.[6,7] This suggests that a component of the brown-rot wood-degrading system is small enough to diffuse into the sound wood cell wall.

The pattern of holocellulose depolymerization in brown-rotted wood is similar to that caused by Fenton reagent,[5,8] and natural cellulose depolymerized by hydroxyl radical (·OH) is similar in chemical structure to brown-rotted cellulose.[9] Furthermore, some brown-rot fungi degrade lignin-related model compounds to yield relatively large amounts of the same products that are obtained via ·OH attack on the lignin model compounds.[10,11] These findings suggest that ·OH may be a component of the brown-rot wood-degrading system.

An extracellular, low molecular weight substance that catalyzes a redox reaction between O_2 and an electron donor to produce ·OH via O_2^- and H_2O_2 has been isolated from wood-degrading cultures of the brown-rot fungus *Tyromyces palustris*.[12] Most of the extracellular ·OH produced in intact cultures of *T. palustris* are generated by this low molecular weight substance.[13] During early stages of wood degradation by *T. palustris*, the low molecular weight substance is found in the fungal cytoplasm and cell wall, in the extracellular sheath surrounding the fungal cell wall, and throughout the wood cell wall, suggesting that it diffuses through the S_3 layer into the S_2 layer and the middle lamella.[14] In the early stages of wood decay by the brown-rot fungi, *T. palustris* and *Coniophora puteana*, H_2O_2 is detected in both the fungal cell wall and the wood cell wall.[15] Thus, there is growing evidence that brown-rot fungi

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produce extracellular ·OH as part of their wood-degrading system.

However, it is not known whether the production of ·OH is a universal characteristic of wood degradation by brown-rot fungi. Some brown-rot fungi exhibit high one-electron oxidation activity in cultures containing wood, but little activity in cultures containing glucose as a carbon source. This one-electron oxidation activity appears to result from ·OH.[4]

Early findings suggest that oxalic acid may be involved in wood decay by brown-rot fungi. Cultures of brown-rot fungi often contain significant amounts of oxalic acid, whereas cultures of white-rot fungi usually have little or no oxalic acid.[16,17] Oxalic acid hydrolyzes the hemicellulose in wood, making the cellulose fibers more accessible to cellulases.[18] It is possible that oxalic acid also initiates the depolymerization of amorphous cellulose directly.[19] *Postia placenta* produces sufficient oxalic acid to decrease the pH of the decaying wood, thereby promoting brown-rot degradation of the cellulose.[20]

Herein we examine the possible roles of ·OH and oxalic acid in the degradation of wood, crystalline cellulose, and a lignin-substructure model by seven fungi that are usually regarded as brown rots.

Materials and methods

Fungal strains

The brown-rot fungi used for this study were *Gloeophyllum trabeum* (Pers. Ex Fr.) Murr. IFO 6268 (LZT), *Tyromyces palustris* (Berk. Et Curt.) Murr. FRI 0507 (TYP), *Laetiporus sulphureus* (Bull. Ex Fr.) Bond. Et Sting. IFO 6497 (LAS), *Postia placenta* (Mad.-698-R) (PPL), *Daedalea dickinsii* Yasuda FRI T 4b (DAD), *Coniophora puteana* (Schumacher ex Fr.) IFO 8764 (COP), and *Lentinus lepideus* (Fr.) IFO 8719 (LEL). Cultures were maintained through periodic transfer on slants as previously described.[21]

Media

Basal agar medium was prepared as previously described,[1] except that tenfold-concentrated trace elements were used. Glucose cultures contained 2% glucose and wood-containing cultures contained 0.2% glucose.

Degradation of wood, cellulose, and a lignin model compound

The degradation of Japanese beech (*Fagus crenata* Blume) by each fungus was determined as previously described,[1] except that the basal agar medium of wood cultures contained 0.2% glucose instead of 1% glucose.

Cellulose degradation in glucose cultures of each fungus was determined as previously described,[1] except that the filter papers were of 5-cm diameter. "Sandwich" cultures were used to measure cellulose degradation in wood-containing cultures. Sawdust of Japanese beech was extracted twice with acetone and dried. The sterilized sawdust (1.5 g), containing 60% distilled water, was sprinkled evenly over the surface of basal agar medium (50 ml) containing 0.2% glucose in 300-ml Erlenmeyer flasks. One filter paper (5 cm) was placed over the sawdust and another 1.5 g of sawdust was spread over the filter. Cultures were inoculated with a small piece of fungal mat and incubated at 28°C in air. After the indicated periods, the mycelia and sawdust were carefully removed from the filter paper with running water. The decayed filter papers were extracted once with acetone, once with distilled water, and again with acetone, and were then dried and weighed.

Degradation of the lignin model compound, 4-ethoxy-3-methoxyphenylglycerol-β-guaiacyl ether, in glucose cultures, was determined as described previously.[1] For determining the degradation of the lignin model compound, 4-ethoxy-3-methoxyphenylglycerol-β-guaiacyl ether in wood cultures, 2 g of sawdust was sprinkled evenly over the surface of basal agar medium (50 ml) containing 0.2% glucose in 300-ml Erlenmeyer flasks. The lignin model compound (2 mg in 200 μl acetone) was added to the surface. After 2 days, cultures were inoculated with a small piece of fungal mat and incubated at 28°C in air. Degradation was determined as previously described.[1] Each measurement was the average of triplicate cultures.

Accumulation of oxalic acid

Basal agar medium (50 ml in 300-ml Erlenmeyer flasks) contained either 2% or 0.2% glucose. Sawdust of Japanese beech (3 g) was sprinkled evenly over the surface of the 0.2% glucose cultures. The cultures were inoculated with a small piece of fungal mat and incubated at 28°C in air. At the incubated intervals, 100 ml of ethyl acetate and 0.2 ml of 1 M HCl were added to each culture. The mixtures were stirred with a glass rod, and shaken for 30 min. Then the mixtures were centrifuged at 10000 *g* for 20 min and filtered through filter paper. Each of the ethyl acetate layers was washed with distilled water (2 × 50 ml). Ethyl acetate solution (50 ml) was taken out of each ethyl acetate layer and evaporated under reduced pressure. Trimethylsilylation of the residues was carried out by adding 1 ml of bis-(*N*,*O*-trimethylsilyl) trifluoracetamide—pyridine (1:1) to each of the dry residues and heating at 80°C for 5 min. Gas chromatography was carried out with a glass column packed with 3% OV-101 on chromsorb WAW DMCS. The oven temperature was programmed from 100°C to 230°C at 10°C/min. Triplicate cultures were measured.

CMCase and Avicelase assays

Basal agar medium (50 ml in 300-ml Erlenmeyer flasks) contained either 2% or 0.2% glucose. Sawdust of Japanese beech (3 g) was sprinkled over the surface of the 0.2% glucose cultures. The cultures were inoculated with a small

piece of fungal mat and incubated at 28°C in air. After 10 to 60 days of incubation, 30 ml of distilled water was added aseptically to ten flasks of each culture. The mixtures were stirred with a glass rod, and centrifuged at 10 000 *g* at 4°C for 20 min. The supernatants collected from each of the ten cultures were combined and lyophilized. The residue was dissolved in 100 ml of distilled water. Cold acetone (−10°C) was added to the solution to 70% (V/V). The mixtures were allowed to stand overnight at 4°C, and were centrifuged. The pellet obtained from each was dissolved in 10 ml distilled water and centrifuged to remove insoluble materials and used for CMCase and Avicelase assays. CMCase and Avicelase were assayed by measurement of reducing sugars by the method of Somogyi-Nelson.[22] Each of the reaction mixtures contained 5 mg of carboxymethylcellulose sodium salt (CMC, Nacalai Tesque) or Avicel (Funacel SF Funakoshi) in 5 ml of Na-acetate buffer (0.1 M, pH 5.0). Two milliliters of each of the solutions was added to each of the reaction mixtures. The reaction mixtures were incubated at 37°C for 0, 0.5, 1, 2, and 3 h without agitation for CMC assay and for 0, 6, 24, and 48 h with agitation for Avicelase assay. The reaction mixtures for Avicelase were centrifuged to remove insoluble Avicel, and the supernatants were assayed.

Hydroxyl radical production

Basal agar medium (50 ml in 300-ml Erlenmeyer flasks) contained either 2% or 0.2% glucose. Sawdust (3 g) was sprinkled evenly over the surface of the 0.2% glucose cultures. The cultures were inoculated with a small piece of fungal mat and incubated at 28°C in air. At the indicated intervals, 20 ml of 10% (v/v) dimethyl sulfoxide (DMSO) was added to each of the cultures. After an additional 24-h incubation at 28°C, 50 ml of distilled water was added and the cultures were shaken vigorously and centrifuged at 10 000 *g*. The supernatants were filtered through filter paper. Fast Yellow salt solution was freshly prepared by dissolving 1.5 g of the salt in 100 ml of distilled water. The salt solution was filtered and 10 ml was added to each of the culture filtrates. The solutions were shaken for 30 min, transferred to separatory funnels, and extracted with ethyl acetate (100 ml). After the ethyl acetate layers were washed three times with distilled water (50 ml), 50 ml was removed and evaporated under reduced pressure. Each residue was dissolved in ethyl acetate (1 ml), filtered through a Millipore filter (pore size 0.2 μm), and analyzed by high performance liquid chromatography as previously described.[23] Triplicate cultures were measured.

Results

Degradation of Japanese beech wood

The weight losses in blocks of Japanese beech caused by seven fungi are shown in Fig. 1. Over the 60-day incubation period, LZT and TYP caused similar weight losses in the wood (17% and 16%, respectively). Although the weight losses of the wood in LAS and PPL cultures over 60 days of incubation (10% and 8%, respectively) were only 47%–63% of the weight losses observed in LZT and TYP cultures, they were still significant. The three other fungi, DAD, COP, and LEL, did not significantly degrade beech wood in culture.

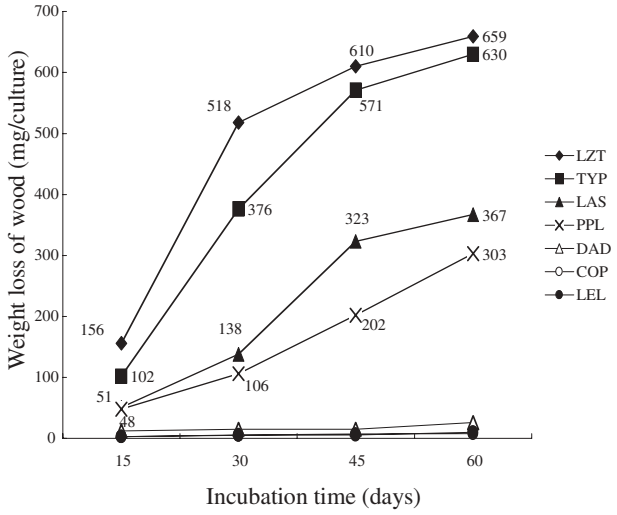

Fig. 1. Weight loss of Japanese beech wood blocks in fungi cultures. LZT, *Gloeophyllum trabeum*; TYP, *Tyromyces palustris*; LAS *Laetiporus sulphureus*; PPL, *Postia placenta*; DAD, *Daedalea dickinsii*; COP, *Coniophora puteana*; LEL, *Lentinus lepideus*

Degradation of cellulose in glucose and wood cultures

The weight losses of filter papers caused in glucose cultures and wood cultures are shown in Fig. 2. Only two of the brown-rot fungi, COP and LZT, significantly degraded cellulose in glucose cultures, with weight losses of 59% and 19%, respectively, over the 60-day incubation period. COP degraded the cellulose at the highest rate in both glucose and wood cultures, with a weight loss of 61% in wood cultures after 30 days of incubation. Because filter papers were seriously destroyed and become very fragile after 45 days, we failed to recover the remaining residues of the filter papers without significant loss of the residues on the 45th or 60th day of incubation. LZT exhibited a filter paper weight loss of 29% during the 60 days of incubation in wood cultures. Wood cultures of TYP exhibited a filter paper weight loss of 22% during the 60 days of incubation in wood cultures. Over the 60-day incubation period in wood cultures, LAS and PPL degraded very similar amounts of filter paper (15% and 14%, respectively). Whereas PPL degraded the filter paper at a steady rate throughout the incubation period, LAS cultures exhibited a 19-mg weight loss during the first 30 days and a 29-mg weight loss during the second 30 days. There was little or no degradation of filter paper cellulose in either glucose or wood cultures of LEL. Filter paper cellulose degradation could not be measured with DAD because the thick mycelia covering the paper

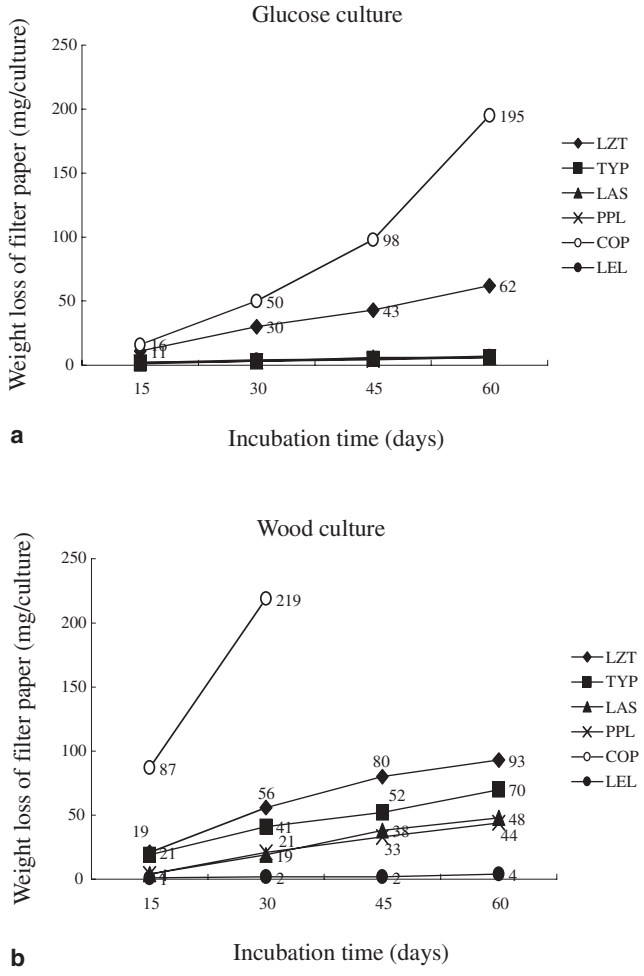

Fig. 2. Degradation of cellulose as filter paper in **a** glucose and **b** wood cultures of six fungi

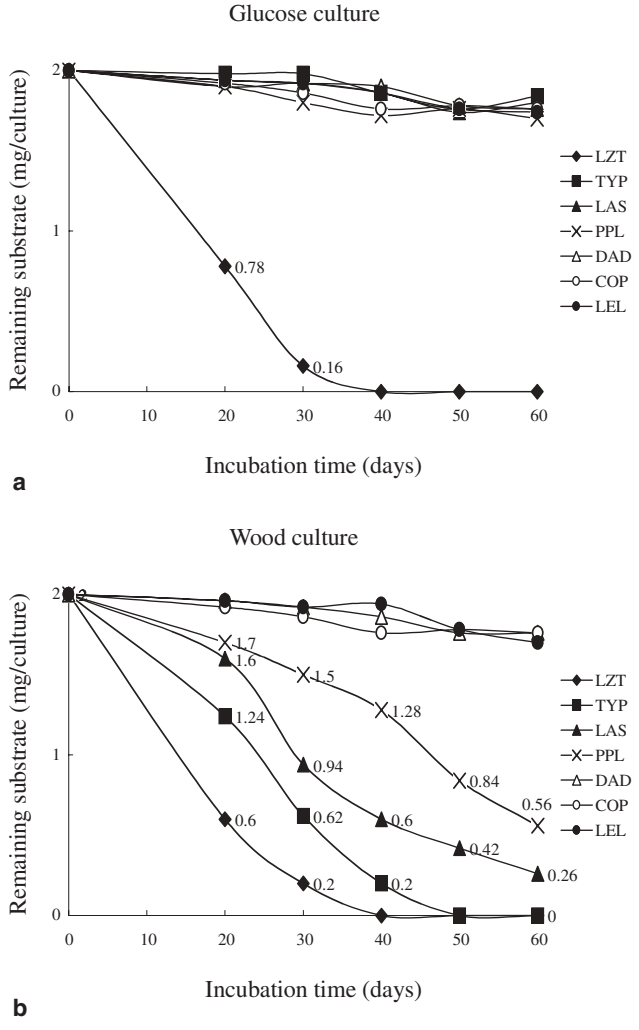

Fig. 3. Degradation of the lignin model compound, 4-ethoxy-3-methoxyphenylglycerol-β-guaiacyl ether, in **a** glucose and **b** wood cultures of seven fungi

could not be completely removed without any significant loss of the substrate. However, the filter papers in both wood and glucose cultures of DAD changed very little in thickness or stiffness during the 60-day incubation, indicating that they were not significantly degraded.

Degradation of a lignin model compound in glucose and wood cultures

As shown in Fig. 3, only LZT degraded 4-ethoxy-3-methoxyphenylglycerol-β-guaiacyl ether in glucose culture. In these cultures, the substrate was undetectable after 40 days incubation, whereas the other six fungi degraded less than 0.3 mg after 60 days in glucose cultures. In wood cultures, LZT degraded all of the substrate (2 mg) within 40 days and TYP degraded it all within 50 days. LAS and PPL degraded 87% and 72% of the substrate, respectively, after 60 days of incubation. The three other fungi showed little or no activity against the lignin model after 60 days in wood culture (Fig. 3).

Oxalic acid concentrations in cultures

As shown in Fig. 4, glucose cultures of TYP, LAS, and PPL accumulated significant amounts of oxalic acid throughout the incubation period. Oxalic acid concentrations peaked in TYP and LAS cultures on day 20 at 3.3×10^{-4} mol and 2.3×10^{-4} mol per culture, respectively. In glucose cultures of COP, some amounts of oxalic acid ($0.1–0.8 \times 10^{-4}$ mol) were detected throughout the incubation period. In LZT, DAD, and LEL, only very small (less than 0.1×10^{-4} mol) of oxalic acid were detected. Much smaller amounts of oxalic acid were detected in wood cultures as compared with the glucose cultures. TYP, LAS, and PPL cultures had small amounts of oxalic acid ($0.3–1.0 \times 10^{-4}$ mol) throughout the incubation period. Wood cultures of the other fungi (Fig. 4) showed amounts of oxalic acid less than 0.1×10^{-4} mol.

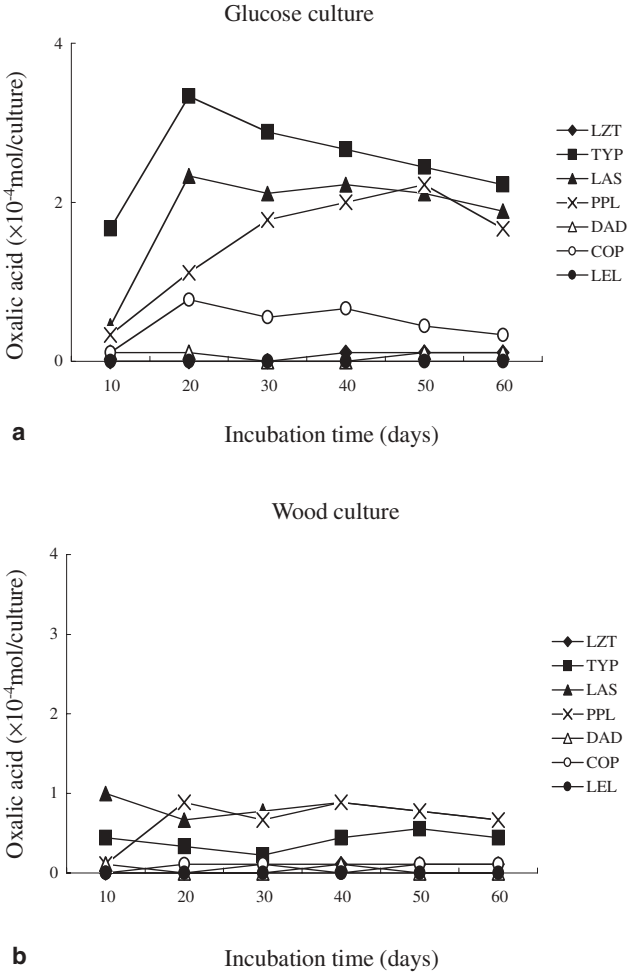

Fig. 4. Oxalic acid concentrations in **a** glucose and **b** wood cultures of seven fungi

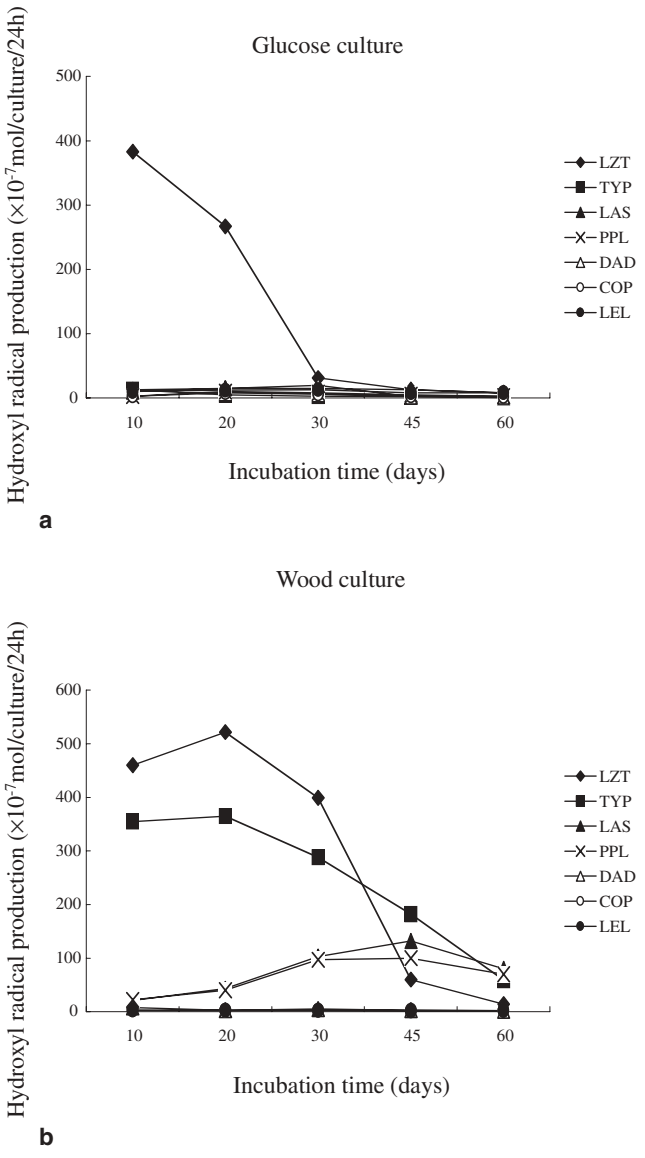

Fig. 5. Hydroxyl radical production in **a** glucose and **b** wood cultures of seven fungi

Hydroxyl radical production in glucose and wood cultures

The assay for ·OH, based on the transformation of dimethyl sulfoxide to methanesulfinic acid, is specific for ·OH.[13] In glucose cultures, only LZT produced significant amounts of ·OH (Fig. 5). Production in LZT glucose cultures was highest on day 10 and dropped by more than 90% by day 30. In wood cultures, only LZT and TYP exhibited high levels of ·OH (Fig. 5). Wood cultures of LAS and PPL exhibited much lower, but significant, ·OH production than LZT and TYP cultures (Fig. 5). There was little or no ·OH production in wood cultures of DAD, COP, and LEL (Fig. 5).

Discussion

White-rot and soft-rot fungi preferentially degrade and eliminate the lignin in wood cell walls via the activities of ·OH and phenol oxidases, including laccase, manganese peroxidase, and lignin peroxidase.[24,25] In addition, white-rot and soft-rot fungi generally produce the full cellulolytic enzyme complement (*endo*-1,4-β-glucanase, *exo*-1,4-β-glucanase, and 1,4-β-glucosidase). Therefore they can hydrolyze highly crystalline cellulose substrates.[2,26] However, a complete cellulolytic system alone is insufficient for degrading the cellulose in wood cell walls because the lignin covering the cellulose prevents the enzymes from reaching the substrate.[26] Some deuteromycetes, such as *Trichoderma* sp. and *Fusarium* sp., produce a complete cellulolytic system and efficiently hydrolyze highly crystalline cellulose to glucose, but can degrade neither wood nor the cellulose component of wood because they have no system for removing the lignin.[26,27]

The brown-rot fungi, LZT, TYP, LAS, and PPL, preferentially degrade and eliminate the polysaccharides in wood, leaving a lignin-rich residue.[1,28] Wood-containing cultures of TYP have high CMCase activity but negligible Avicelase activity. Both activities are negligible in glucose cultures of TYP.[13] Avicelase activity is negligible throughout the

incubation period in both glucose and wood cultures of LZT, LAS, and PPL (data not shown).

Fungal enzymes such as cellulases are too large to diffuse into wood cell walls during the early stages of wood degradation by brown-rot fungi.[6,7] Although the S_2 layer of the wood cell wall is degraded extensively during the early stages of brown-rot degradation, the S_3 layer adjacent to the cell lumen is less affected when it is attacked by fungal hyphae from the lumen.[7,29] However, ·OH can depolymerize crystalline cellulose and destroy its crystalline structure.[9] Furthermore, ·OH can attack the aromatic rings in lignin, causing a variety of reactions including hydroxylation and ring opening.[30] However, ·OH alone does not delignify wood significantly.[31] Endoglucanases isolated from cultures of a brown-rot fungus and a cellulolytic fungus hydrolyze very little crystalline cellulose or the cellulose in wood unless the crystalline cellulose or the wood has been pretreated with ·OH.[32]

The route in the degradation of poly(ethylene oxide) by LZT is the same as that by Fenton reagent.[11] An intermediate product, 4-ethoxy-3-methoxyphenyl glycerol is produced and detected with significant yields during the degradation of 4-ethoxy-3-methoxyphenyl-β-guaiacyl ether by white-rot fungi, but the intermediate product is not produced during the degradation of the lignin model compound by LZT.[33]

These findings suggest that ·OH produced in cultures of brown-rot fungi is primarily responsible for destroying the crystalline structure of the cellulose and attacking the lignin in wood, thereby cutting canals through the S_3 layers to allow *endo*-1,4-β-glucanase diffusion. In the present study, we assessed the possible involvement of ·OH and oxalic acid in the degradation of wood, filter paper cellulose, and a lignin-substructure model by seven fungi usually regarded as brown-rot fungi.

As shown in Fig. 6, which is based on the data in Figs. 1 and 5, the production of ·OH in wood cultures of the seven fungi is directly proportional to the rates of wood degradation. As shown in Fig. 7, which is based on the data in Figs. 2 and 5, the rates of hydroxyl radical generation in wood cultures of LZT, TYP, LAS, PPL, or LEL are related to the rates of filter paper degradation in the cultures. In glucose cultures, only one of the seven fungi tested, LZT, produces significant ·OH and only two of the fungi, COP and LZT, significantly degrade filter paper cellulose. Thus, the rates of hydroxyl radical generation in cultures of the six fungi other than COP are directly proportional to the rates of degradation of filter paper cellulose in the cultures. As shown in Fig. 8, which is based on the data in Figs. 3 and 5, the rates of ·OH generation in glucose or wood cultures of the seven fungi are roughly related to the rates of lignin model degradation in the cultures. Thus, the rates of ·OH generation in cultures of the six fungi other than COP are directly proportional to the degradation rates of wood, cellulose, and the lignin-related compound. These results, together with the facts mentioned above, indicate that ·OH is involved in the degradation of the cellulose and lignin in wood by the six fungi other than COP. Although unable to degrade either wood or the lignin model compound, COP extensively degrades filter paper cellulose in glucose and wood cultures (Fig. 2). In both wood and glucose cultures of COP, the

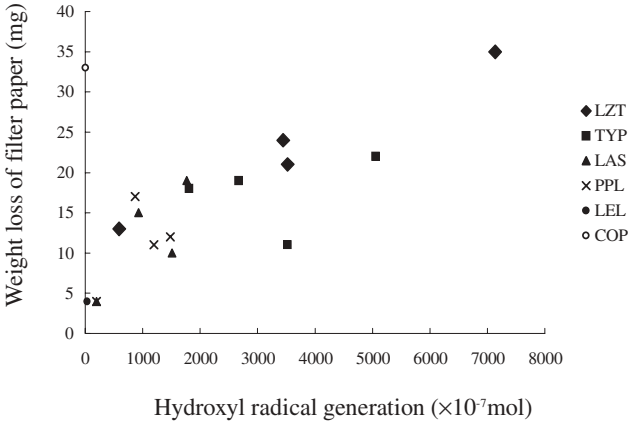

Fig. 7. Correlation between hydroxyl radical generation in wood cultures and the rate of degradation of cellulose as filter paper in wood cultures

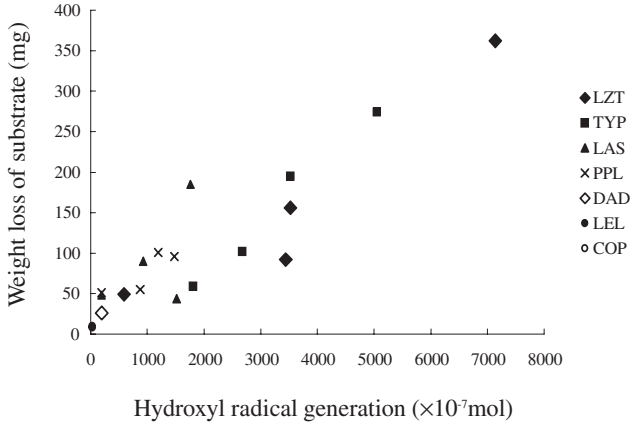

Fig. 6. Correlation between hydroxyl radical generation in wood cultures and the rate of degradation of wood in glucose cultures

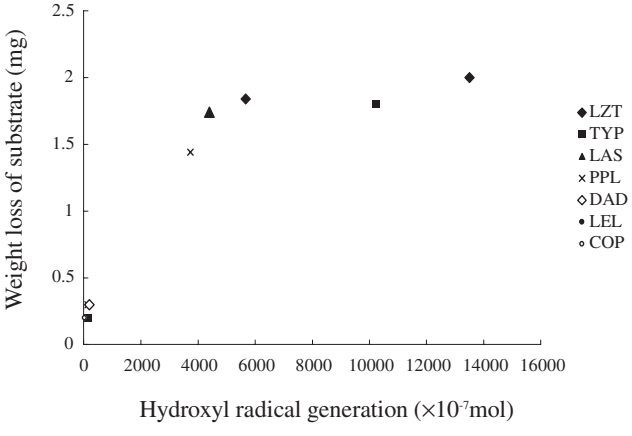

Fig. 8. Correlation between hydroxyl radical generation in wood or glucose cultures and the rate of degradation of a lignin model compound in wood or glucose cultures

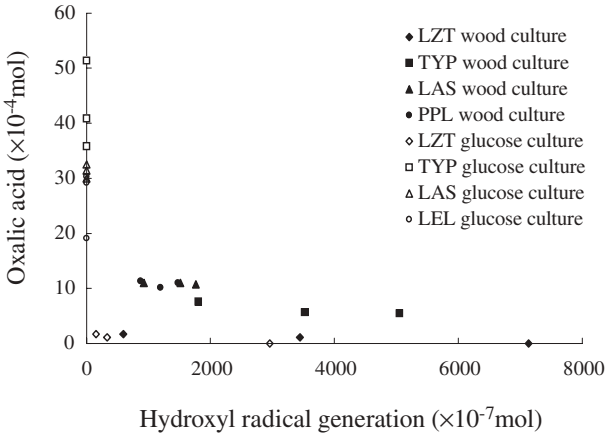

Fig. 9. Correlation between hydroxyl radical generation in wood and glucose cultures of the wood-degrading fungi and the amounts of oxalic acid present in the cultures

activities of Avicelase and CMCase are significant, whereas phenol oxidase activity is negligible (data not shown). In addition, two extracellular *exo*-cellobiohydrolases have been purified to homogeneity from cultures of COP.[34] Thus the COP strain used in the present work has a pattern of wood degradation that is cellulolytic but nonlignolytic, as in fungi such as *Tricoderma* sp. and *Fusarium* sp., rather than a brown-rot pattern of wood degradation.

As shown in Fig. 9, which is based on the data in Figs. 4 and 5, the generation of ·OH in wood or glucose cultures of the wood-degrading fungi, LZT, TYP, LAS, and PPL, is inversely proportional to the amount of oxalic acid present in the cultures. Although little ·OH was produced in glucose or wood cultures of LZT during the final 30 days or the final 15 days of incubation, negligible amounts of oxalic acid were present in the cultures. This could be attributable to the exhaustion of nutrients required for oxalic acid biosynthesis.

These results suggest that ·OH readily decomposes the oxalic acid in cultures. For example, when cultures of LZT are actively depolymerizing holocellulose, exogenously added oxalic acid is rapidly converted to CO_2.[35] LZT significantly degrades wood (Fig. 1). It also significantly degrades filter paper cellulose and a lignin substructure model in both glucose and wood cultures (Figs. 2 and 3). LZT generates large amounts of ·OH in both glucose and wood cultures (Fig. 5) and accumulates only traces of oxalic acid (Fig. 4). TYP, LAS, and PPL degrade wood, filter paper cellulose, and the lignin model compound in wood-containing cultures to a significant extent. They accumulate only very small amounts of oxalic acid in wood cultures. However, in glucose cultures of TYP, LAS, and PPL, degradation of filter paper or the lignin model compound is negligible, as is production of ·OH; whereas large amounts of oxalic acid accumulate. Of all the fungi tested, COP has the highest degradation rate for filter paper cellulose, in both glucose and wood cultures, but does not degrade wood or the lignin model compound (Figs. 1–3). Although oxalic acid is present in glucose cultures of COP throughout the incubation period, only very small amounts are detected in wood cultures (Fig. 4). DAD and LEL do not degrade any of the substrates, produce little or no ·OH, and accumulate only very small amounts of oxalic acid in glucose and wood cultures (Figs. 1–5).

These results suggest that oxalic acid is not directly involved in the degradation of wood, filter paper cellulose, or the lignin model compound by brown-rot fungi. However, oxalic acid may be involved in ·OH production by brown-rot fungi that have substantial wood-degrading activities. In cultures of brown-rot fungi, ·OH is generated by the Fenton reaction:[36]

$$\mathrm{Fe(II)} + \mathrm{H_2O_2} \rightarrow \mathrm{Fe(III)} + \cdot\mathrm{OH} + \mathrm{OH^-}$$

Oxidation of oxalic acid to CO_2 can reduce Fe(III) to Fe(II) in the presence of light.[37] Oxalic acid may enhance the reduction of ferric ions to ferrous ions and the generation of ·OH by increasing the solubility of ferric irons.[38] Some oxidants oxidize oxalic acid to formate anion radical; the formate anion radical immediately reduces O_2 to superoxide anion; superoxide anion reduces Fe(III) to Fe(II) and forms H_2O_2 in a disproportionation reaction.[39,40] In this manner, oxalic acid may be involved in producing both Fe(II) and H_2O_2 for ·OH generation.

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