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Laccase Production and One-electron Oxidation Activity in Wood Degradation by the Soft-rot Deuteromycete *Graphium* sp.

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The mechanism of wood degradation by the soft-rot deuteromycete, *Graphium* sp., was investigated. One-electron oxidation activity, assayed by ethylene generation from 2-keto-4-thiomethylbutyric acid (KTBA), and phenol oxidase activity were measured in intact cultures and in Sephadex G-50 fractions from the acetone-precipitated extracellular media, with glucose or wood as the carbon source for the cultures. Both the one-electron oxidation activity and the phenol oxidase activity were higher in wood cultures than in glucose cultures. Most of the phenol oxidase activity in wood cultures appeared to derive from laccase. Most of the ethylene generation from KTBA was attributed to hydroxyl radicals, produced by a low-molecular-weight substance in the extracellular media. This substance was composed of peptides, carbohydrates, and Fe(II), and catalyzed redox reactions between O₂ and unidentified electron donors, to produce hydroxyl radicals via H₂O₂. It is suggested that hydroxyl radicals may produce new phenolic substructures on the lignin polymer, making it susceptible to attack by laccase. Thus, both one-electron oxidation activity and laccase activity are important in wood degradation by *Graphium* sp.

Key words : Soft-rot decay/*Graphium* sp. /Laccase/One-electron oxidation.

INTRODUCTION

Graphium sp. is the asexual form of the ascomycete fungus *Ceratocystis* sp., a well-known blue-stainer of softwood. *Graphium* sp. has been isolated from imported North American timber such as Douglas fir (*Pseudotsuga menziesii* Franco) and western hemlock (*Tsuga heterophylla* Sarg.) that has been treated for the prevention of sap-stain (Kumagai and Tsunoda, 1999). Since these two softwood species account for about 40% of the total wood supply in Japan, sap-stain treatments are very important for reducing economic losses. *Graphium* sp. also causes significant weight loss in hardwood and therefore is considered to be a soft-rot fungus (Eslyn et al., 1975; Tanaka et al., 1992). Thus, the control of *Graphium*

sp. is of economic importance.

There are three types of fungal wood decay. White-rot, produced by some basidiomycetes, results from the removal of the cell wall components of wood (Enoki et al., 1988). In contrast, brown-rot, which also is produced by members of the basidiomycetes, results from the preferential degradation of wood polysaccharides (Enoki et al., 1988). Soft-rot is caused by members of the ascomycetes and deuteromycetes (Duncan, 1960; Eslyn et al., 1975; Savory, 1954). The typical soft-rot decay symptoms are cavity formation in the S₂ layer of wood cell walls, erosion of the luminal surfaces of the cell walls, and penetration by fine hyphae through cell walls (Zabel and Morrell, 1992). During soft-rot decay, the cellulose and hemicellulose in wood are depleted at similar rates; whereas the lignin in wood is depleted much more slowly (Eslyn et al., 1975; Tanaka et al., 1992). However the mechanisms of wood degradation by

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soft-rot fungi are not well understood.

Some deuteromycetes produce a complete system of cellulases and hydrolyze highly-crystalline cellulose to glucose efficiently; however, they cannot degrade the cellulose component in wood, because lignin prevents the cellulase from accessing its substrate and these fungi lack a system for attacking the lignin (Highley, 1973; Tanaka et al., 1985). Since direct contact between the cellulases and the substrate is essential, wood-degrading fungi must possess a pre-cellulolytic system for opening up the complex cell wall structure of wood in order to hydrolyze the cellulose (Bailey et al., 1968). A number of studies have investigated the pre-cellulolytic systems of white-rot and brown-rot fungi.

White-rot fungi degrade the lignin in wood, in preference to the cellulose, only in cultures with high levels of phenol oxidase activity (Tanaka et al., 1985, 1986). In white-rot fungi, at least one phenol-oxidizing enzyme is involved in and/or is essential for the preferential degradation of lignin in wood, in concert with a system that produces hydroxyl radicals (Tanaka et al., 1999b). During brown-rot decay, characterized by the preferential elimination of the polysaccharides in wood, significant amounts of the hydroxyl radical are produced in the wood cell walls (Enoki et al., 1989; Itakura et al., 1994). Hydroxyl radicals can depolymerize crystalline cellulose and destroy the crystalline structure (Kirk et al., 1991; Koenigs, 1974). Hydroxyl radicals also can attack the aromatic rings in lignin, causing a variety of reactions including hydroxylation and ring-opening (Gierer et al., 1992), but they cannot remove the lignin from wood to a significant degree (Chirat and Lachenal, 1997; Enoki et al., 1997). Therefore brown-rot fungi, which lack both phenol oxidase activity and exo-1,4- β -glucanase activity, remove the polysaccharides in wood in preference to the lignin (Enoki et al., 1988; Highley, 1987).

In order to better understand the mechanism of wood degradation by the soft-rot fungus *Graphium* sp., we have isolated and identified phenol oxidases from this fungus and determined the agent responsible for the one-electron oxidation in cultures of the fungus.

MATERIALS AND METHODS

Organism

The soft-rot deuteromycete, *Graphium* sp. M-1-9, was isolated from untreated sapwood stakes (2 x 2 x 20 cm) of Japanese beech (*Fagus crenata* Blume) and Japanese cedar (*Cryptomeria japonica* D. Don) (Tanaka and Fuse, 1981 and 1983). Stock cultures of the fungus were maintained on slants of potato

sucrose agar.

Culture conditions

Duncan's medium contained KH_2PO_4 (5.0g), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (4.0g), K_2HPO_4 (4.0g), NH_4NO_3 (6.0g), and agar (20g), per liter of distilled water. For "glucose cultures", 10ml of Duncan's medium contained 2% (w/v) glucose. For "wood particle cultures", particles (about 5mm³, 1.2 g) of Japanese beech or Japanese cedar were sterilized and sprinkled evenly over the surface of 10ml of Duncan's medium containing 0.25% (w/v) glucose. For "wood meal cultures", wood meal (10-80 mesh) of Japanese beech (1.2g) replaced the wood particles. The wood particles and wood meal were extracted twice with acetone while being refluxed, and then dried before use. The cultures, in 50-ml Erlenmeyer flasks, were inoculated with a small piece of fungal mat and incubated at 28 °C.

Ethylene generation from 2-keto-4-thiomethylbutyric acid (KTBA) in cultures

At the indicated times, KTBA (3ml of 10mM) was added to the glucose, wood particle, and wood meal cultures. The cotton plugs of the flasks were replaced with rubber septa and the flasks were purged with 100% (v/v) O_2 for 10min. After 24h at 28°C, 1ml of gas was removed from the head space of each flask, and the ethylene concentration was measured directly by gas chromatography as previously described (Enoki et al., 1989).

Phenol oxidase activity in cultures

Phenol oxidase activity was assayed spectrophotometrically, using 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) or guaiacol as the substrate. At the indicated times, 2ml of 10mM ABTS or 20mM guaiacol and 18ml of 50mM acetate buffer (pH 4.5) were added to the glucose, wood particle, and wood meal cultures. After shaking for 1 h at 30°C, the cultures were passed through filter paper to remove the fungal mat, agar, and wood. The absorbance of the filtrate was measured at 416 nm for ABTS or 436 nm for guaiacol. One unit of enzyme activity was defined as an increase of one unit of absorbance per 1 h per flask. The absorbance due to fungal pigments in the filtrates, as determined in control filtrates which lacked substrate, were subtracted from the total absorbance to obtain the absorbance due to phenol oxidase activity.

Phenol oxidase activity was also assayed by a Bavendamm reaction in glucose and wood particle cultures. Guaiacol, used as the substrate, was dissolved in ethanol and added to the medium, which

had been autoclaved and cooled to 50–60°C. The final concentration of guaiacol in the medium was 0.02 and 0.2% (w/v). The cultures were incubated at 28°C in the dark. The color of the cultures was checked by visual inspection from d 7 to d 30 after inoculation.

Preparation of culture filtrates

At each of the indicated times, the extracellular medium from the 10 glucose and Japanese beech wood particle cultures was acetone-precipitated (70%, v/v), as described previously (Tanaka et al., 1999a). The precipitate was passed through a Sephadex G-50 gel filtration column and the UV absorbances of the fractions were measured at 280nm. Three fractions were collected and designated as fractions I, II, and III, in the order of their elution from the column. The fractions were lyophilized, redissolved in 10ml of distilled water, and kept at –80°C until use. The activities in each of the fractions were measured in duplicate.

Ethylene generation from KTBA in culture filtrate fractions

The reaction mixture (1ml) contained 300 µl of 160mM acetate buffer (pH 4.5), 300 µl of 10mM KTBA, and 100 µl of sample. The reaction tube was purged through a rubber septum with 100% (v/v) O₂ for 5min, followed by the addition of 10mM NADH (300 µl). After 24 h at 30°C, 1ml of gas was removed from the head space and the ethylene concentration was measured directly by gas chromatography, as previously described (Enoki et al., 1989).

Phenol oxidase activity in culture filtrate fractions

Phenol oxidase activity was assayed spectrophotometrically, using ABTS or guaiacol as the substrate. With ABTS as the substrate, the reaction mixture (1ml) contained 100 mM acetate buffer (pH 4.5), ABTS (2mM), and the sample (100 µl). For peroxidase activity, the reaction was initiated by the addition of H₂O₂ to a final concentration of 200 µM. For manganese peroxidase (MnP) activity, the reaction mixture also contained MnSO₄ (200 µM). The absorbance at 416nm (A_{416}) was measured after 10min at 30°C. With guaiacol as the substrate, the reaction mixture (1ml) contained 100mM acetate buffer (pH 4.5), guaiacol (400 µM), and the sample (150 µl). For peroxidase activity, the reaction was initiated by the addition of H₂O₂ to a final concn of 200 µM. For MnP activity, the reaction mixture also contained MnSO₄ (200 µM). The A_{436} was measured after 24 h at 30°C. Non-MnP peroxidase activity was calculated by subtracting the laccase activity measured in the

absence of H₂O₂, from the total activity measured in the presence of H₂O₂. MnP activity was calculated by subtracting the total activity measured in the presence of H₂O₂ and the absence of Mn²⁺, from the total activity measured in the presence of Mn²⁺. One unit of enzyme activity was defined as an increase of one unit of absorbance per min (ABTS) or per 24 h (guaiacol) per culture.

Preparation of the low-molecular-weight fraction

Cultures were grown in 30ml Duncan's medium containing 0.25% (w/v) glucose, Japanese beech wood particles (2.4 g), prepared as described above, and 0.5 g agar, in 300-ml Erlenmeyer flasks. After 63 d of incubation, the extracellular medium from 60 cultures was acetone-precipitated (70%, v/v), as described previously (Tanaka et al., 1999a). Fraction III was obtained as described above, lyophilized, weighed, and redissolved in an appropriate amount of distilled water.

The protein content of the low-molecular-weight fraction was assayed by the method of Lowry et al. (1951) with bovine serum albumin as the standard. The neutral carbohydrate content was measured by the phenol-sulfuric acid method with glucose as the standard (Dubois et al., 1956). The ferrous content was measured with ferrozine, a colorimetric reagent that forms a stable magenta complex with Fe(II), by the procedure of Sørensen (1982). A 20-fold diluted sample (1ml) was reacted with 500 µl of 0.1% ferrozine solution in 50mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer (pH 7.0) at 28 °C for 24h. The absorbance was measured over the range of 700nm to 400nm.

RESULTS

The one-electron oxidation activities, as determined by ethylene generation from KTBA, in intact glucose, wood particle, and wood meal cultures of *Graphium* sp. are shown in Fig. 1. The highest activity was observed in wood meal cultures after one wk of incubation. The activity in wood meal cultures remained high after 2 wk of incubation, then decreased rapidly, and was negligible after 6 wk. The activity in wood particle cultures also was highest after one wk of incubation. It then decreased, remaining at a constant level between 5 and 8 wk of incubation. The activity in glucose cultures was highest after wk 2, and then decreased until wk 4, fluctuating within a narrow range between 4 and 8 wk of incubation. The integral of the ethylene generation was 16.5 × 10⁻⁸ mol for glucose cultures, 21.9 × 10⁻⁸ mol for wood meal cultures, and 34.2 × 10⁻⁸ mol for wood particle cultures.

FIG. 1. Ethylene generation from KTBA in cultures of *Graphium* sp. containing glucose (□), or Japanese beech wood particles (○) or wood meal (●). Assays were carried out as described in the text. Each value is the mean of the three replicate cultures.

The phenol oxidase activities in intact glucose, Japanese beech wood particle or wood meal, and Japanese cedar wood particle cultures of *Graphium* sp., with ABTS or guaiacol as the substrate, are shown in Fig. 2. With ABTS as the substrate, the activities in Japanese beech wood particle and wood meal cultures were high after one wk of incubation, peaking after wk 2, and then gradually decreasing. With guaiacol as the substrate, the activity in Japanese beech wood particle cultures was much higher than that in wood meal cultures after wk 1. In Japanese cedar wood particle cultures, the time

course of the activity was very similar to that in beech wood particle cultures with ABTS or guaiacol as the substrate. However the activity in the cedar particle cultures was much lower than that in the beech particle cultures. In both beech and cedar wood particle and beech wood meal cultures, the activity peaked after 2 wk and then declined. No phenol oxidase activity was detectable in glucose cultures with either substrate.

The phenol oxidase reaction by *Graphium* sp. in cultures containing glucose or Japanese beech wood particles is shown in Table 1. The *Graphium* sp. had no phenol oxidase activity at either concn of guaiacol in glucose cultures, but it had phenol oxidase activity at 0.2% (w/v) of guaiacol in wood particle cultures.

The one-electron oxidation activities in culture filtrate fractions I and III from Japanese beech wood particle cultures, as measured by ethylene generation from KTBA in the presence of NADH under O_2 , are shown in Fig. 3. The highest activity was detected in fraction III from 10-d-old cultures. The activity in fraction III was higher than that in fraction I for cultures at each stage of incubation. The activity in fraction I from 16- and 63-d-old cultures was very low.

TABLE 1. Phenol oxidase reaction by *Graphium* sp. in cultures containing glucose or Japanese beech wood particles.

Culture	Concn of guaiacol (% w/v)	
	0.02	0.2
Glucose	— ^a	—
Wood particle	—	+ ^b

^a Negative reaction.

^b Positive reaction.

FIG. 2. Phenol oxidase activity, with ABTS (A) or guaiacol (B) as the substrate, in cultures of *Graphium* sp. containing glucose (□), Japanese beech wood particles (○) or wood meal (●), and Japanese cedar wood particles (△). Assays were carried out as described in the text. Each value is the mean of the three replicate cultures.

FIG. 3. Ethylene generation from KTBA with fractions I (■) and III (▨) of the extracellular media from Japanese beech wood particle cultures of *Graphium* sp. Ethylene generation in the presence of NADH, under O_2 , was assayed as described in the text.

The activities of laccase and non-MnP peroxidase in culture filtrate fraction I from glucose and Japanese beech wood particle cultures, measured using ABTS or guaiacol as the substrate, are shown in Fig. 4. With ABTS as the substrate, non-MnP peroxidase was the predominant phenol oxidase activity in fraction I from the extracellular media of glucose cultures after 10 and 16 d of incubation (Fig. 4A). With guaiacol as the substrate, activities were very low and negligible (Fig. 4B). With ABTS as the substrate, laccase activity in fraction I from the extracellular media of wood particle cultures was much higher than peroxidase activity (Fig. 4C) and much higher than the laccase activity in fraction I from glucose cultures (Figs. 4A and 4C). With guaiacol as the substrate, only laccase activity was detected (Fig. 4D). No MnP activity was detected in fraction I from either the glucose or wood particle cultures, with either substrate. Only extremely low levels of enzyme activity were detected in fractions II and III from both the glucose or wood particle cultures (data not shown).

Table 2 shows the dry weight, protein content, neutral carbohydrate content, Fe(II) content, and the one-electron oxidation activity of the low-molecular-weight fraction (fraction III) from the extracellular media of 60-d-old Japanese beech wood particle cultures. The ethylene generation from KTBA by the substance contained in fraction III under various conditions is shown in Table 3. A small amount of ethylene was generated from KTBA under 100% (v/v)

FIG. 4. Phenol oxidase activities in fraction I of the extracellular media from glucose (A and B) and Japanese beech wood particle (C and D) cultures of *Graphium* sp. The activities of laccase (▨) and non-MnP peroxidase (■) were determined as described in the text, with ABTS (A and C) or guaiacol (B and D) as the substrate.

O_2 and under N_2 . In the presence of H_2O_2 , some ethylene was generated under anaerobic conditions. In the presence of NADH under O_2 , ethylene generation from KTBA was 30-fold higher than in the absence of NADH. The presence of catalase or the hydroxyl radical scavenger, *N,N'*-dimethyl-4-nitrosoaniline, greatly reduced ethylene generation from KTBA in the presence of NADH (Table 3).

DISCUSSION

Recently, we demonstrated that one-electron oxidation activity and phenol oxidase activity of four soft-rot deuteromycetes, including *Graphium* sp. M-1-9, are

TABLE 2. Analysis of the low-molecular-weight fraction (Fraction III) from the extracellular media of 63-d-old Japanese beech wood particle cultures of *Graphium* sp.

Item	Value
Dry wt. (mg)	84.2
Protein (mg)	10.5 (12.5) ^a
Neutral carbohydrate (mg)	47.2 (56.0)
Fe (II) (μg)	5.1 (0.01)
Ethylene generation (nmol mg dry wt. ⁻¹ h ⁻¹)	0.05

^a Percent of mg dry wt.

TABLE 3. Effect of O₂ and N₂ atmospheres, and of H₂O₂, electron donors, and a hydroxyl radical scavenger on the generation of ethylene from KTBA by an extracellular one-electron oxidant isolated from *Graphium* sp.

Addition to reaction mixture ^a	Atmosphere	Ethylene generation	
		nmol h ⁻¹	% ^b
None	O ₂	0.5	3
None	N ₂	0.3	2
H ₂ O ₂ (3 × 10 ⁻⁶ mol)	N ₂	2.6	18
NADH (3 × 10 ⁻⁶ mol)	O ₂	14.9	100
NADH (3 × 10 ⁻⁶ mol), catalase ^c (0.3mg)	O ₂	4.4	29
NADH (3 × 10 ⁻⁶ mol), DMNA ^d (6 × 10 ⁻⁶ mol)	O ₂	0.9	6

^aReaction mixtures (2ml in 7-ml test tube) contained 1mg of the low-molecular-weight fraction, prepared as described in the text from 63-d-old wood particle cultures, KTBA (3 × 10⁻⁶ mol), and acetate bufer (pH4.5, 160mM). The tubes were purged with 100% O₂ for min or 100% N₂ for 30 min.

^bPercent of ethylene generation with NADH.

^c41,000 Units/mg.

^d*N,N'*-Dimethyl-4-nitrosoaniline.

stimulated by the presence of Japanese beech wood (Tanaka et al., 1999c). *Fusarium* sp. and *Trichoderma* sp., which are strong cellulolytic fungi but which cannot degrade wood, have a lower one-electron oxidation activity in wood particle cultures than in glucose cultures. No phenol oxidase activity is detected in these two fungi when guaiacol is used as the substrate. Therefore, we have suggested that one-electron oxidation activity and phenol oxidase activity play important roles in wood degradation by soft-rot fungi, as they do in wood degradation by white-rot fungi (Tanaka et al., 1999a and 1999b). However, since ethylene is generated from KTBA by one-electron oxidants such as the hydroxyl radical, peroxidase/H₂O₂ systems, and ceric ammonium sulfate (Enoki et al., 1989), it is not clear what produces the one-electron oxidation activity in these soft-rot fungi. In order to better understand the mechanism of wood decay by the soft-rot fungus *Graphium* sp., we have attempted to isolate and identify its phenol oxidases and determine the agent that is responsible for one-electron oxidation in wood cultures of this fungus.

No phenol oxidase activity was detected in intact glucose cultures of *Graphium* sp. (Fig. 2), although peroxidase activity was detectable in fraction I of the extracellular media from glucose cultures (Fig. 4). Although under natural conditions, soft-rot is detected in softwood poles in the ground-line zones (Eaton and Hale, 1993), under laboratory conditions soft-rot fungi cannot degrade softwood as rapidly or extensively as it degrades hardwood (Duncan, 1960; Eslyn et al., 1975; Nilsson et al., 1989; Savory, 1954), and *Graphium* sp. cannot degrade Japanese cedar efficiently (data not shown). When *Graphium* sp. was grown with particles of Japanese cedar instead of

Japanese beech, phenol oxidase activity was much lower, with either ABTS or guaiacol as the substrate. No phenol oxidase activity was detected in cultures containing 0.25% glucose as the sole carbon source (data not shown). These results indicate that *Graphium* sp. exhibits phenol oxidase activity only in wood-degrading cultures. Laccase activity was much higher in fraction I of the extracellular media from wood cultures as compared with glucose cultures. With guaiacol as the substrate, laccase activity was the only phenol oxidase activity detected in wood cultures (Fig. 4). Thus, most of the phenol oxidase activity in wood cultures of *Graphium* sp. appears to derive from laccase.

Most white-rot fungi exhibit a positive Bavendamm reaction for phenol oxidase activity in both glucose and wood-containing cultures when guaiacol is added to a final concentration of 0.02% to agar medium (Käärik, 1965; Tanaka et al., 1996). Under these conditions *Graphium* sp. does not exhibit a positive Bavendamm reaction. However at a final guaiacol concentration of 0.2%, wood-containing cultures of *Graphium* sp. in fact exhibited a positive reaction (Table 1). This result may reflect a difference in the diffusability of phenol oxidases from white-rot and soft-rot fungi.

The maximum level of ethylene generation in intact cultures of *Graphium* sp. (Fig. 1) is about 3% of that in cultures of the brown-rot fungus *Tyromyces palustris* (Hirano et al., 1997) and about 5% of that in cultures of the white-rot fungi *Phanerochaete chrysosporium* (Tanaka et al., 1999a) and *Trametes versicolor* (Tanaka et al., 1999b). However, the ethylene generated in Japanese beech wood particle cultures of *Graphium* sp. remained at a fairly constant level in the later stages of incubation (Fig. 1), and

ethylene generation was much higher in fraction III of the extracellular media than in fraction I (Fig. 3). Fraction III contained a low-molecular-weight substance consisting of peptides, carbohydrates, and Fe(II) (Table 2). This low-molecular-weight substance was very similar to that obtained from brown-rot and white-rot fungi (Hirano et al., 1997; Tanaka et al., 1991 and 1996).

Ethylene generation from KTBA by fraction III was inhibited by the addition of catalase or a hydroxyl radical scavenger (Table 3). This suggests that the low-molecular-weight fraction, isolated from the extracellular media of wood cultures of *Graphium* sp., catalyzes a redox reaction between an electron donor, such as NADH, and O₂ to produce hydroxyl radical via H₂O₂, as previously described for brown-rot and white-rot fungi (Hirano et al., 1997; Tanaka et al., 1991 and 1996). Although laccase cannot modify or degrade non-phenolic lignins, the main component of wood cell wall lignin, if the hydroxyl radical produces terminal phenolics on the polymer, laccase then can degrade lignin in wood. The fairly constant level of one-electron oxidation activity in intact wood cultures and in fraction III from the extracellular media of wood cultures (Figs. 1 and 3) indicates that the hydroxyl radical is produced throughout the course of incubation of *Graphium* sp. In this study, the low-molecular-weight fraction (fraction III) was prepared from the extracellular medium of 63-d-old cultures in order to maximize the amount of acetone-precipitable material. However, since the one-electron oxidation activity in fraction III was highest on d 10, future experiments will utilize 10-d-old cultures.

The addition of lignocellulosic substrates to cultures of *Thermoascus aurantiacus*, a thermophilic fungus, induces phenol oxidases. The type of phenol oxidase activity depends on the type of lignocellulosic substrate and the highest laccase activity is obtained with sawdust (Machuca and Durán, 1993). Recently, it has been reported that hydroxyl radicals and laccase are produced in cultures of the soft-rot fungus *Fusarium proliferatum*, and that the generation of the hydroxyl radical, but not laccase activity, is related to lignin mineralization by the cultures (Regalado et al., 1999). Likewise with *Graphium* sp., one-electron oxidation activity and laccase activity are stimulated by the presence of wood particles or wood meal of Japanese beech (Figs. 1 and 2). The integrals of both activities are higher in wood particle cultures than in wood meal cultures. The cellulose in wood meal is probably more exposed. Therefore, less lignin degradation is required for the cellulolytic enzymes to access the cellulose, resulting in lower one-electron oxidation and laccase activities.

Because of the vast anatomical, microstructural, and chemical variabilities among hardwoods, it is difficult to perform the efficient application and fixation of wood preservatives (Ryan and Drysdale, 1988; Srinivasan et al., 1998). In contrast to white-rot and brown-rot fungi, whose hyphae develop inside the cell lumen, soft-rot fungi grow preferentially within the S₂ layer of the cell wall, making effective treatment difficult. Fewer hydroxyl radicals and low diffusability of phenol oxidases of *Graphium* sp. may cause localized decay around hyphae. Transverse sections of copper-chromium-arsenic (CCA) preservative-treated *Eucalyptus deglupta* infected with the soft-rot fungus *Lecythophora* (*Phialophora*) *mutabilis* reveals heavy granular deposits of the preservative on cell wall lumen surfaces, but only low levels of CCA in the S₂ layers of the cell walls that are vulnerable to soft-rot cavity attack (Eaton and Hale, 1993). Once soft-rot fungi that are present in the cell lumina are able to breach the CCA deposits on the cell wall surface, hyphae can grow within the colonized cell walls and into adjacent cell walls without re-encountering the thick CCA deposits. Thus, protection from soft-rot decay requires highly-permeable preservatives or treatments which can be efficiently applied and fixed to prevent and control fungal penetration into cell wall lumina.

In conclusion, one-electron oxidation activity, primarily due to hydroxyl radical, and at least one phenol oxidase activity are involved in wood degradation by the soft-rot deuteromycete *Graphium* sp. The laccase from this fungus is being purified in our laboratory and compared with laccases from white-rot fungi. This work should lead to more effective preventative treatments against soft-rot decay.

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