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ORIGINAL ARTICLE

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Extracellular peroxidase reaction at hyphal tips of white-rot fungus *Phanerochaete crassa* WD1694 and in fungal slime

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Abstract The distribution of an extracellular peroxidase reaction by white-rot fungus *Phanerochaete crassa* WD1694 was visualized by peroxidase activity staining. The extracellular peroxidase reaction occurred at the hyphal tips and in the fungal slime filling the gaps between the hyphae. We investigated whether the peroxidase reaction occurred from the hyphal tips or in the slime. The hyphal tips were observed by phase-contrast microscopy, which showed that slime did not exist around the hyphal tips. Time-course observation of hyphal tips showed that peroxidase staining became thick and intense at the tips that did not have fungal slime. Daily observation of the peroxidase staining revealed that the staining was first observed at the hyphal tips. Furthermore, strongly stained hyphae were observed in the stained slime. These results suggested that an active species that oxidizes a peroxidase substrate is first produced at the tips of the hyphae, and then occurs in the slime via diffusion when slime exists around the hyphae. Our results show that the extracellular peroxidase reaction that is important to lignin biodegradation by white-rot fungi occurs directly at the tips of the hyphae and in the slime.

Key words Lignin · *Phanerochaete crassa* · Manganese peroxidase · Hyphal tip · Slime

Introduction

After cellulose and hemicellulose, lignin is one of the major components of the cell walls of woody plants.[1] Lignin is a heterogeneous aromatic polymer consisting of random phenylpropanoid units.[2] This structure results in resistance to general microbial attack. White-rot fungi are the only organisms that are capable of efficiently degrading high molecular weight lignin.[3,4]

Because lignin exists as macromolecules in plant cell walls, the initial reaction in the lignin biodegradation process must occur outside the cells of the white-rot fungi. Ligninolytic peroxidases, such as lignin peroxidase (LiP) and manganese peroxidase (MnP), are considered to be responsible for initial attack on high molecular weight lignin, and these are produced extracellularly.[5–9]

The localization of ligninolytic peroxidases has been determined by immunological methods.[10–13] However, to act on substrates, peroxidases require the presence of hydrogen peroxide, so the existence of a peroxidase may not necessarily correspond to the occurrence of a peroxidase reaction.

We previously investigated the extracellular peroxidase reaction of the white-rot fungus *Phanerochaete crassa* WD1694 by peroxidase activity staining.[14] Staining corresponding to peroxidase activity occurred on only the surfaces of mycelial pellets, particularly at the hyphal tips, and no staining was observed in the culture fluids. We also estimated the rate of the peroxidase reaction at the hyphal tips, and showed that MnP catalyzes the reaction in a concentrated condition at the hyphal tips. These results suggested that the extracellular peroxidase reaction is not simply caused by diffusion of peroxidases: there should be mechanisms that regulate the extracellular peroxidase reaction.

In this study, we investigated the distribution of the extracellular peroxidase reaction of *P. crassa* WD1694 as one such system of regulation. It is noteworthy that white-rot and brown-rot fungi have a hyphal sheath or slime outside the hyphae, and it has been suggested that this is the location of a pool of fungal enzymes.[15–17] The localization of MnP and LiP in association with slime materials surrounding hyphae has been confirmed by immunological methods.[11,12] We therefore examined whether fungal slime is involved in the distribution of the extracellular peroxidase reaction of *P. crassa* WD1694.

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Materials and methods

Fungal strain and cultivation

The white-rot fungus *Phanerochaete crassa* WD1694 [MAFF420737, *Phanerochaete crassa* (Lev.) Burdsall] was obtained from the culture collection of the Forestry and Forest Products Research Institute, Tsukuba, Japan. Cultivation was conducted as described previously.[14]

Pulp and reagents

Unbleached kraft pulp (UKP) was obtained from the Yatsushiro mill of Nippon Paper Industries (Yatsushiro, Japan), and was washed with distilled water before use. A solution of 3,3′-5,5′-tetramethylbenzidine (TMBZ), a chromogenic substrate for precipitating immunoassays for peroxidase activity, was purchased from Roche Diagnostics (Mannheim, Germany). Phloxine B was obtained from Nacalai Tesque (Kyoto, Japan).

Staining

Staining for peroxidase activity was carried out by adding TMBZ to mycelial pellets taken from the culture and placed on a plastic plate. The hyphal sheath was stained with 20% Phloxine B solution as described previously.[18]

To stain for peroxidase activity and the hyphal sheath in the same sample, double staining with TMBZ and Phloxine B was conducted. An aliquot containing the mycelial pellets was taken from the culture and TMBZ was added at a concentration of 20%. Immediately after blue staining was observed, the mycelial pellets were washed gently with distilled water and further stained with 20% Phloxine B solution. The mycelial pellets were then washed by repeated removal of the supernatant and addition of distilled water.

Microscopic analysis

Samples were observed by light and phase-contrast microscopy with a Nikon Eclipse TE 2000-U microscope (Nikon, Tochigi, Japan).

Results

Extracellular peroxidase reaction of the white-rot fungus *Phanerochaete crassa* WD1694 was visualized by a peroxidase-active stain TMBZ. Mycelial pellets of *P. crassa* WD1694 cultivated for 3 days were placed on a plastic plate and TMBZ solution was added. Strong blue staining corresponding to the peroxidase reaction occurred on the fungal bodies, especially at the hyphal tips (Fig. 1). Blue deposits were also observed between hyphae.

Previously, we showed that the gaps between hyphae contain hyphal sheath or fungal slime.[18] We therefore investigated whether the peroxidase reaction on the fungal body occurred from the hyphae or slime.

To determine the presence of slime at the tips of the hyphae, we examined the use of phase-contrast microscopy. We showed that slime could be stained with Phloxine B in our previous report;[18] however, staining of thin slime was so weak that thin slime was not observed by conventional microscopy. Phase-contrast microscopy allowed us to validate the presence of slime that was stained but could not be observed under visible light (Fig. 2). In addition, the contour of the slime, which was unclear in the Phloxine B staining, could be clearly observed by phase-contrast microscopy (Fig. 3). These results showed that phase-contrast microscopy is a feasible option for observing slime.

Based on these results, the tips of hyphae were analyzed for the presence of slime by phase-contrast microscopy. Fungal slime was not observed at the hyphal tips, irrespective of whether peroxidase staining had occurred (Fig. 4).

The time-course change in peroxidase staining at the hyphal tips was observed by phase-contrast microscopy (Fig. 5). Fungal slime was not observed at the hyphal tips at the beginning of the experiment. As the reaction time increased, peroxidase staining became thick and intense. The results clearly showed that the peroxidase reaction at hyphal tips could occur without slime.

The peroxidase reaction that occurred in the fungal slime is shown in Fig. 6. The mycelial pellets were placed on a plastic plate, fixed by drying for 1 h, and then stained with TMBZ. The slime, which fills the gaps between the hyphae, was stained blue by TMBZ. However, strongly stained hyphae were also observed amongst the stained slime (Fig. 7). In addition to these results, the staining at the hyphal tips was observed after 1 day of cultivation, although staining of fungal slime was observed after 3 days of cultivation (data not shown). These results suggested that an active species that oxidizes a peroxidase substrate is first produced at the tips of the hyphae, and then occurs in the slime via diffusion when slime exists around the hyphae.

The distribution of fungal slime and peroxidase activity staining was compared by double staining with Phloxine B and TMBZ (Fig. 8). Whole mycelial pellets were stained red with Phloxine B to show distribution of fungal slime, whereas peroxidase activity staining by TMBZ occurred only on part of the surface of the mycelial pellets, particularly at the hyphal tips. The results also support the previous hypothesis that the extracellular peroxidase reaction begins from the hyphal tips.

Discussion

The most important process of lignin biodegradation by white-rot fungi is the initial attack of the lignin polymer in woody cell walls, because only white-rot fungi can accomplish the degradation of the lignin polymer. To clarify the initial process, the distribution of an extracellular peroxidase reaction by a white-rot fungus, *Phanerochaete crassa* WD1694, was visualized by peroxidase activity staining.

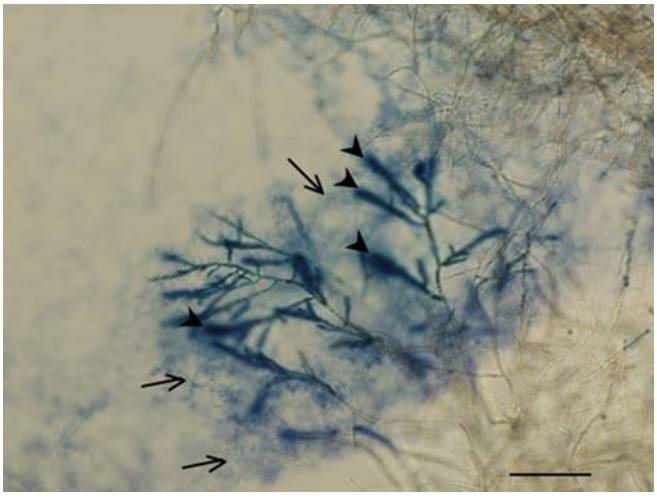


Fig. 1. Staining of peroxidase reaction of *Phanerochaete crassa* WD1694. *Phanerochaete crassa* WD1694 was cultivated for 3 days. Mycelial pellets were placed on a plastic plate and 20% 3,3′-5,5′-tetramethylbenzidine (TMBZ) solution was added. Peroxidase activity staining was observed at the hyphal tips (*arrowheads*) and between hyphae near the hyphal tips (*arrows*). *Bar* 100 µm

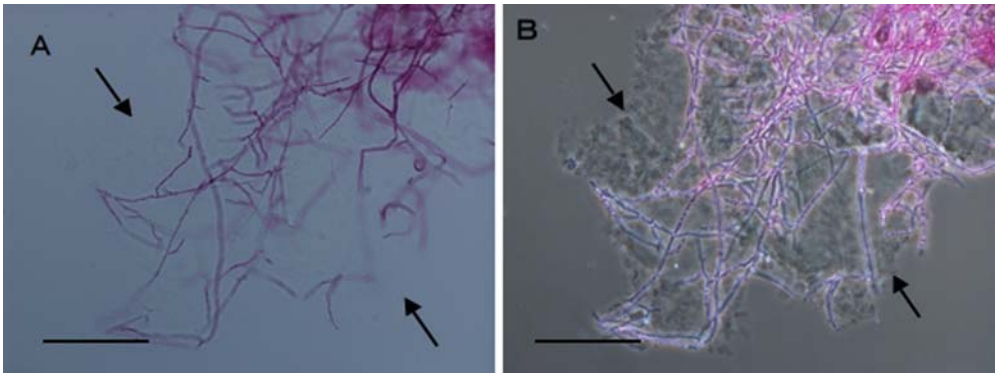


Fig. 2A, B. Comparison of light microscopy and phase-contrast microscopy for observation of slime of *P. crassa* WD1694. *Phanerochaete crassa* WD1694 was cultivated for 3 days and stained with Phloxine B. **A** Light microscopy; thin slime (*arrows*) of *P. crassa* WD1694 invisible. **B** Phase-contrast microscopy; thin slime (*arrows*) clearly visible. *Bars* 100 µm

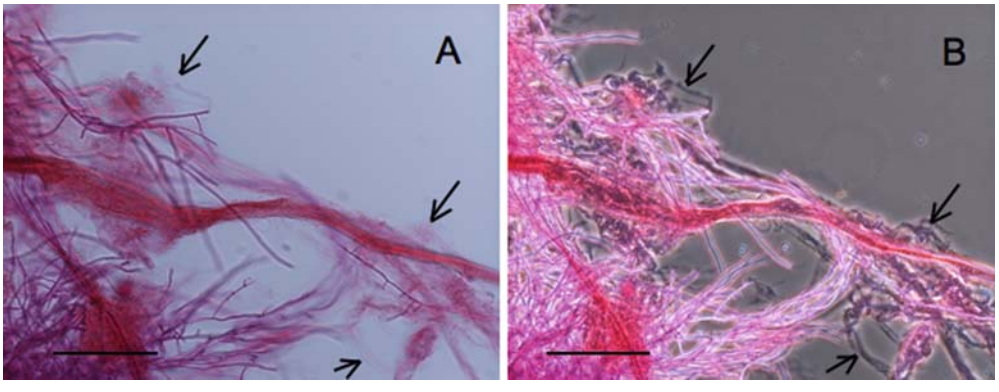


Fig. 3A, B. Comparison of light microscopy and phase-contrast microscopy for observation of contours of slime of *P. crassa* WD1694. *Phanerochaete crassa* WD1694 was cultivated for 3 days and was stained with Phloxine B. **A** Light microscopy; contours of slime (*arrows*) were vague. **B** Phase-contrast microscopy; contours of slime (*arrows*) clearly visible. *Bars* 100 µm

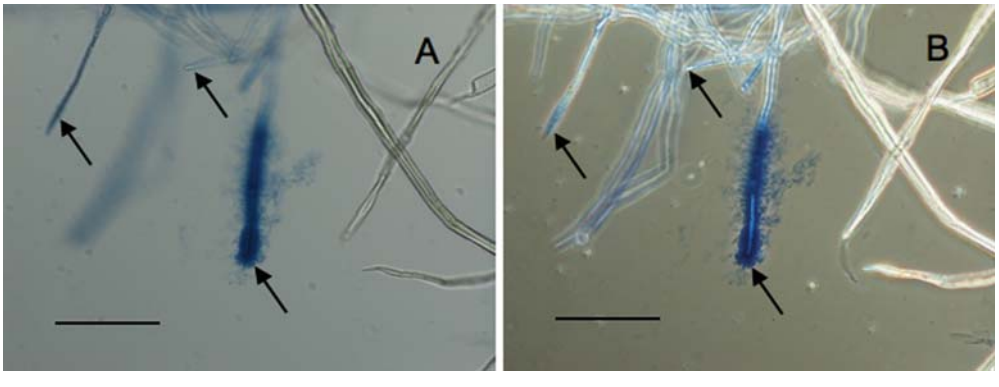


Fig. 4A, B. Absence of slime at the hyphal tips of *P. crassa* WD1694 stained with peroxidase reaction. **A** Hyphal tips observed by light microscopy; **B** hyphal tips observed by phase-contrast microscopy. *Arrows*, fungal slime was not observed at the hyphal tips irrespective of whether peroxidase-active staining occurred. *Bars* 100 µm

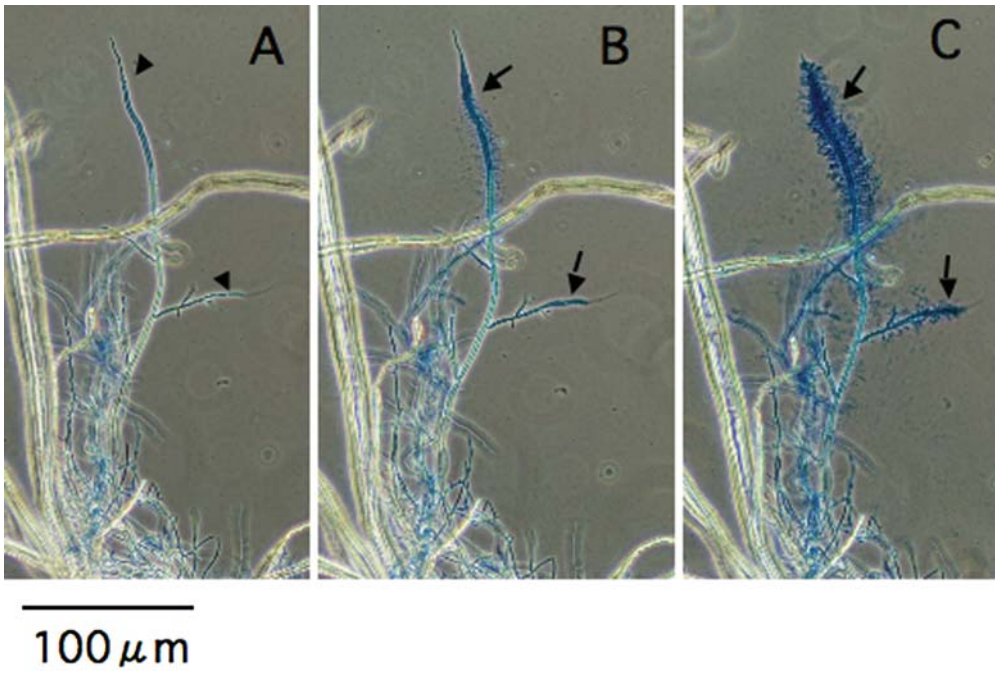


Fig. 5A–C. Accumulation of the staining of peroxidase reaction at the hyphal tips of *P. crassa* WD1694. Staining of peroxidase reaction at the hyphal tips was observed at 1-h intervals by phase-contrast microscopy: **A** 0 h; **B** 1 h; **C** 2 h. *Arrowheads*, slime was completely absent at the hyphal tips at the start of the observation (**A**). *Arrows*, staining of peroxidase reaction at the tips increased with time (**B**, **C**)

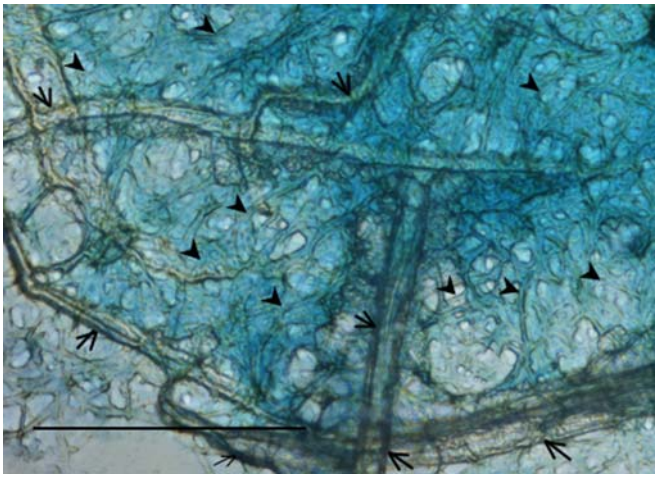


Fig. 6. Staining of peroxidase reaction inside the slime filling the gaps of *P. crassa* WD1694 hyphae. Mycelial pellets of *P. crassa* WD1694 cultivated for 3 days were placed on a plastic plate and dried for 1 h before 20% TMBZ solution was added. Blue staining of peroxidase reaction was observed inside the slime filling the gaps between the hyphae. *Arrows*, unbleached kraft pulp; *arrowheads*, hyphae of *P. crassa* WD1694. *Bar* 100 μm

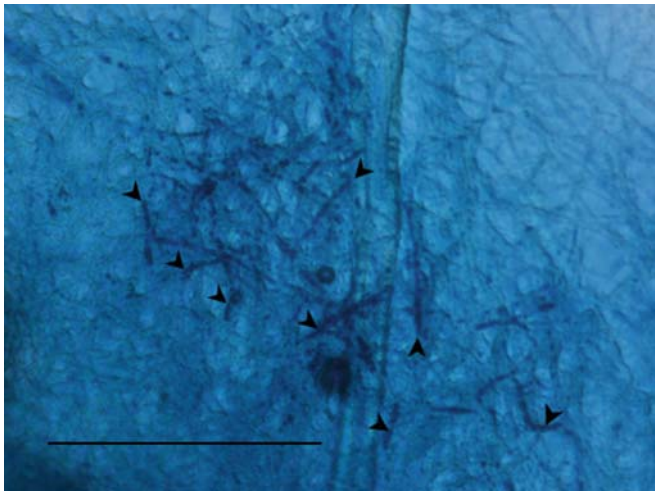
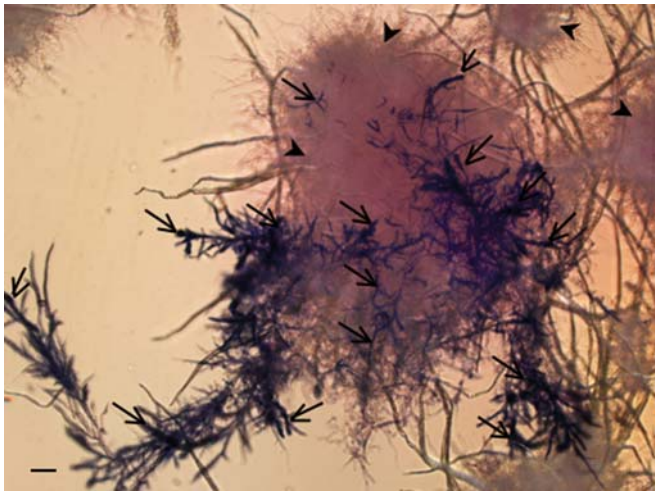


Fig. 7. Staining of peroxidase reaction on the hyphae of *P. crassa* WD1694. Cultivation and staining conditions were the same as those described in Fig. 2. *Arrowheads*, strongly stained hyphae inside the stained fungal slime. *Bar* 100 μm

Fig. 8. Double staining of *P. crassa* WD1694 mycelial pellet with TMBZ and Phloxine B. *Phanerochaete crassa* WD1694 was cultivated for 2 days. Slime was stained red with Phloxine B, and peroxidase reaction was stained blue with TMBZ. *Arrowheads*, entire mycelial pellets stained red with Phloxine B; *arrows*, intense peroxidase reaction staining with TMBZ at the hyphal tips and on the hyphae. *Bar* 100 μm



The results obtained showed that the peroxidase reaction did not occur in the culture solution but occurred in the fungus body (Fig. 1).

Lignin-decomposing peroxidases, such as LiP and MnP, were produced in a culture solution when the fungus was cultivated in a liquid culture medium. It was natural that the culture solution would be stained by peroxidase activity staining due to extracellular diffusion of the enzymes. The results obtained with *P. crassa* WD1694 that the peroxidase reaction was fixed on the fungus body, implies that the fungus has a mechanism of fixing the peroxidase reaction on its body.

We first considered fungal slime as a candidate for the mechanism, because the slime has been considered as a site where the extracellular enzymes of white-rot and brown-rot fungi are concentrated, and MnP and LiP of *Phanerochaete chrysosporium* have been reported to exist in extracellular slime.[11,12,15,16] If the enzymes necessary for a peroxidase reaction are concentrated in slime, the reaction would occur only inside the slime without diffusion and would thus show activity staining on the fungus body.

However, local peroxidase activity staining was caused mainly by peroxidase reaction at hyphal tips, which did not have fungal slime. If slime does not worked at the hyphal tips for keeping the peroxidase reaction, the next possibility is that the enzymes or substrates of the peroxidase reaction would be fixed at the hyphal tips. Because TMBZ – a substrate of peroxidase activity staining – exists in the entire reaction solution, it is not fixed at the hyphal tips. If elements other than TMBZ are fixed at the tips of the hyphae, it indicates that an active species that oxidizes the substrate of the peroxidase reaction is generated at the hyphal tips.

The conclusion drawn from the features of peroxidase activity staining of the WD1694 fungus bodies is as follows. In the extracellular peroxidase reaction of the white-rot fungus *P. crassa* WD1694, an active species that oxidizes a peroxidase substrate is first produced at the tips of the hyphae. If slime exists around the hyphae, the active species passes through the slime via diffusion, and the peroxidase reaction then occurs in the slime.

An active oxidative species considered to be generated at the tips of the hyphae of *P. crassa* WD1694 has not yet been identified. With reference to this point we have reported that the peroxidase reaction at the tips of the hyphae of *P. crassa* WD1694 was mainly caused by MnP.[14] In addition to the enzyme, this reaction needs manganese, hydrogen peroxide, and substrates. In the reported experiment, MnP was detected in the culture solution.[14] Manganese ions necessary for the reaction were present in the culture solution as a constituent of the culture medium. From these facts, we supposed that the reaction that occurs only at the tips of the hyphae is the production of hydrogen peroxide. Further experiments are needed to examine this point.

The experimental results obtained in this study were found under conditions where *P. crassa* WD1694 was grown in shaking culture in a liquid medium. Thus, it should be noted that different phenomena may occur with other strains or culture conditions. However, it is reasonable to assume that the diffusion of enzymes from the hyphae is much easier in a shaking liquid culture than in native conditions, such as in woody cells. Therefore, the same mechanism of initial peroxidase reaction should also occur in native condition of *P. crassa* WD1694.

This study showed that the extracellular peroxidase reaction of the white-rot fungus *P. crassa* WD1694 occurred at the tips of the hyphae and in the fungal slime. It was also shown that the extracellular peroxidase reaction of *P. crassa* WD1694 occurred on the fungus body because an active oxidative species of the peroxidase reaction was produced at the hyphal tips and subsequently diffused through the slime. Our results show temporal changes in the distribution of the extracellular peroxidase reaction of the white-rot fungus and that lignin-decomposing reaction could occur directly at the tips of the hyphae and in the slime. Because the extracellular peroxidase reaction is important to lignin biodegradation by white-rot fungi, these results are extremely important in clarifying the early stages of the lignin biodegradation process.

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