Lignin degradation and roles of white rot fungi: Study on an efficient symbiotic system in fungus-growing termites and its application to bioremediation

Moriya Ohkuma,[*1] Yoshimasa Maeda,[*1] Toru Johjima,[*2] and Toshiaki Kudo[*1]
[*1]*Microbiology Laboratory, RIKEN*
[*2]*JST-ICORP*

White rot fungi produce extracellular phenoloxidases and can decompose lignin efficiently. *Elfvingia applanata* has been successfully applied for the bioconversion of bisphenol A, suggesting the usefulness of white rot fungi for bioremediation. In order to attain real bioremediation, the recycling of ecomolecules in the ecosystem is important. The sophisticated symbiotic system of white rot fungi of the genera *Termitomyces* with fungus-growing termites is an attractive example of an efficient natural system to be studied, in which cooperation between termites and fungi accomplishes efficient decomposition of lignin and complete biorecycling of plant litter.

Lignocellulose is the predominant component of woody plant and dead plant materials, and the most abundant biomass on earth. Lignin is a heterogenous and irregular arrangement of phenylpropanoid polymer that resists chemical or enzymatic degradation to protect cellulose. It has been thought that lignin degradation is a rate-limiting step of carbon recycling. Microbial activity of metabolizing lignin and its components is one of the plausible evolutional origins for the degrading pathway of aromatic xenobiotics and/or environmental pollutants, such as PCB and dioxin. For applied and environmental science, it is important to understand the nature of the decomposition and biorecycling of lignocellulose by responsible organisms.

Basidiomycetes, which cause white rot decay, are able to degrade lignin in wood. These fungi are called white rot fungi. Lignin degradation by white rot fungi has been extensively studied, and results revealed that three kinds of extracellular phenoloxidases, namely, lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Lac), are responsible for initiating the depolymerization of lignin.[1] The expression pattern of these enzymes depends on the organisms: some secrete LiP and MnP (no Lac), whereas others secrete MnP and Lac (no LiP). In addition to lignin, white rot fungi are able to degrade a variety of environmentally persistent pollutants, such as chlorinated aromatic compounds, heterocyclic aromatic hydrocarbons, various dyes and synthetic high polymers.[2] Probably, this degradability of white rot fungi is due to the strong oxidative activity and the low substrate specificity of their ligninolytic enzymes. Thus, white rot fungi and their enzymes are thought to be useful not only in some industrial processes like biopulping and biobreaching but also in bioremediation.

We investigated the degradation of an endocrine disruptor, bisphenol A (BPA), by a white rot fungus, *Elfvingia applanata* (Fig. 1), which is known in Japan as "kofuki-saru-no-koshikake" mushroom. Decolorization of lignin and degradation of aromatic rings in lignin were observed in the culture (Fig. 2), indicating that *E. applanata* is a strong lignin-

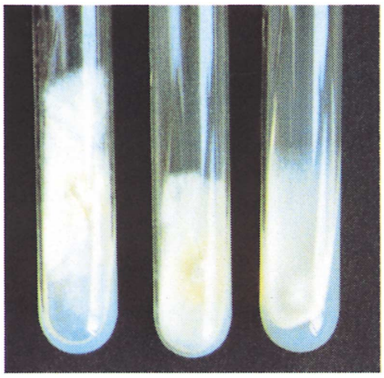

Fig. 1. Slant culture of *Elfvingia applanata*.

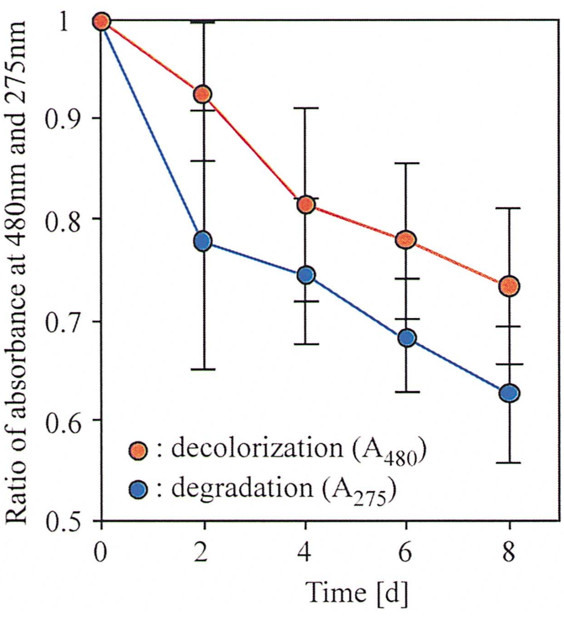

Fig. 2. Lignin decomposition in the culture of *Elfvingia applanata*.

Table 1. Ligninolytic activity of enzymes produced by *Elfvingia applanata*.

Enzyme	Method	Rate ($\Delta A\ min^{-1}\ ml^{-1}$)
Laccase	2,6-Dimethoxyphenol oxidation monitored at 469 nm	0.09
LiP	Veratryl alcohol oxidation to veratraldehyde monitored at 310 nm	ND
MnP	Mn^{3+}-malonate complex monitored at 270 nm	0.435

The enzyme activities in cultures were measured after four days of culture. ND, not detected.

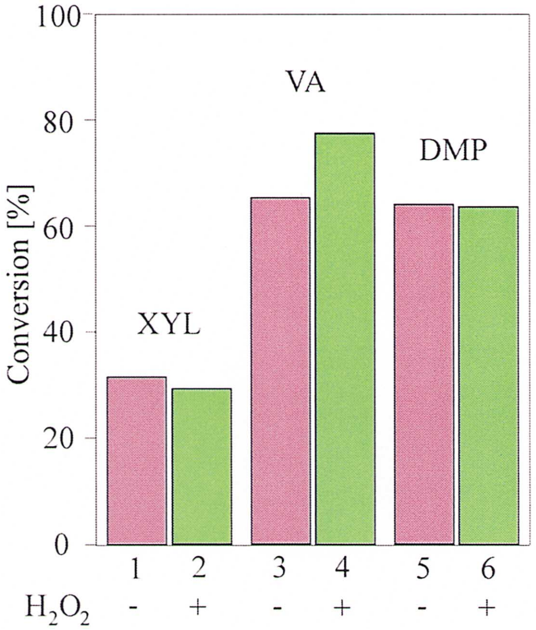

Fig. 3. Conversion of bisphenol A by the culture supernatant of *Elfvingia applanata*.

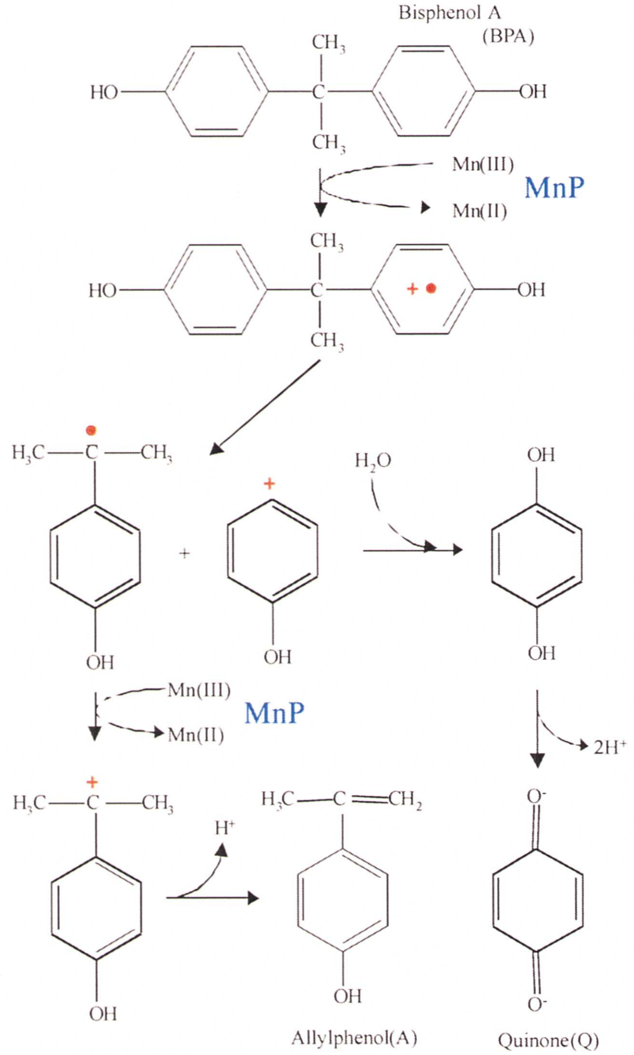

Fig. 4. Proposed pathway for the conversion of bisphenol A by *Elfvingia applanata* MnP.

degrading white rot fungus. Under ligininolytic condition, *E. applanata* secreted MnP and Lac but not LiP (Table 1). The molecular cloning of the gene encoding MnP of *E. applanata* has been reported and the production of MnP was induced at the transcriptional level by the presence of Mn^{II} and aromatic compounds like 2,5-xylidine.[3] When the culture supernatant was incubated with BPA, the conversion of BPA was observed, showing the usefulness of *E. applanata* for bioremediation. The presence of either veratryl alcohol or 2,6-dimethoxyphenol in the culture stimulated the conversion of BPA (Fig. 3). The addition of hydrogen peroxide (1 mM) in the reaction mixture stimulated the conversion only slightly, suggesting a sufficient endogenous supply of hydrogen peroxide in the culture. Higher concentrations of hydrogen peroxide inhibited the conversion. The putative pathway of the conversion of BPA by MnP was shown in Fig. 4.

In order to attain real bioremediation, the degradation products should be effectively mineralized and reutilized or recycled in the ecosystem. As shown in the case of *E. applanata*, white rot fungi and the enzymes produced by them are useful as primary agents in bioremediation. It is considered beneficial to learn from the most efficient natural systems in which white rot fungi are involved in recycling lignocellulose. A symbiotic relationship between termites and fungi is such an attractive example of an efficient natural system. Termites (Isoptera) are extremely abundant in tropical terrestrial ecosystems, and play important roles in biorecycling of plant litter. The so-called fungus-growing termites belong to an evolutionary related group of termites (Termitidae, Macrotermitinae) and are abundant in Asian and African tropics.[4] They consume more than 90% of dry wood in some arid tropical areas and directly mineralize up to 20%

of the net primary production in wetter savannas. This group of termites has an interesting symbiotic relationship with basidiomycete fungi of the genus *Termitomyces*, such that they cultivate the symbiotic fungi within their nests. In some types of fungus-growing termites, the "termite mushroom", the fruiting body of *Termitomyces*, blooms seasonally from termite nests (Fig. 5). The termite mushroom is unique in nature, growing from only the termite nests.

There have been several suggestions for the roles of the symbiotic fungi in termite nutrition: (1) decomposition of lignin; (2) supply of cellulase and xylanase to work synergistically with the enzymes produced by the termite; and (3) concentration of nutrients such as nitrogen for the termite. Many works are involved in studies of the second suggestion, also known as the "acquired enzyme hypothesis",[5] but some researchers questioned a part of this hypothesis. Since an endogenous cellulase, an enzyme produced by the termite, has recently been recognized in wood- and litter-feeding termites,[6] it is difficult to make generalizations on the significance of the "acquired" fungal cellulase in cellulose digestion in fungus-growing termites.

Fig. 5. Mushroom blooming fungus comb of termite. The photograph is courtesy of Drs. Moriya and Inoue.

The sophisticated and well-coordinated cooperation between the termites and the fungi enables efficient utilization of lignocellulose (Fig. 6).[4] The so-called old workers forage outside the nest and collect plant litter. In the nest, young workers masticate and ingest the collected plant litter which passes rapidly through the termite gut without digestion. The resulting fecal pellets (primary feces) are pressed together to form a sponge-like structure (called fungus comb). The symbiotic fungi grow on the comb-like matrices of the fungus comb. They form mycelia and white round and asexual conidial structures called fungus nodules. It has been reported that the lignin content progressively decreased as the fungus comb matured.[7] It has also been shown that *in vitro* digestibility of cellulose in a matured fungus comb was approximately 3-fold higher than that in a newly formed one. The fungus nodules are usually consumed by young workers, whereas the old senescent combs are consumed by old workers to produce final feces. However, final feces are rarely found in the nest of fungus-growing termites, suggesting the highly efficient decomposition and the complete biorecycling of plant litter. These observations support the finding that symbiotic fungi have the ability to degrade lignin, which makes cellulose more easily degraded by the cellulase produced by the termite.

We are now studying the extracellular phenoloxidases produced by a symbiotic fungus, *Termitomyces albuminosus*. Two genes encoding MnP (*tam1* and *2*) were identified, both of which have essential amino acid residues for peroxidase activity and Mn^{II} ion binding whereas the residue for veratryl alcohol oxidation was not observed. These features suggest

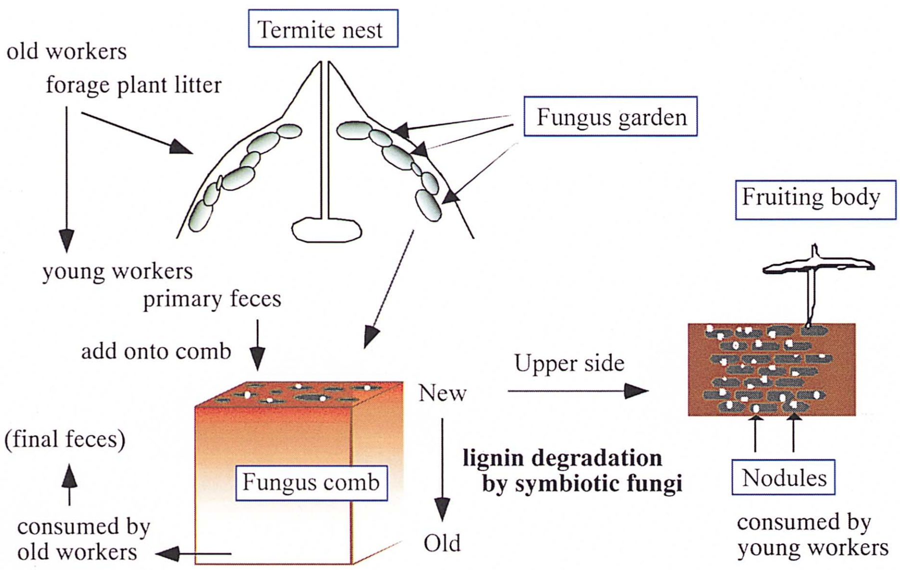

Fig. 6. Symbiotic relationship between fungus-growing termites and *Termitomyces* fungi.

that both genes encode typical MnP. The mRNA level of the *tam2* was higher than that of *tam1*, and interestingly, the expression of *tam2* was not affected by the presence of Mn^{II} in the culture medium in spite of the induction of MnP by Mn^{II} in general. Moreover, we have found a novel peroxidase (designated as TAP) in the culture supernatant. TAP was able to oxidize phenolic compounds in the presence of hydrogen peroxide. However, Mn^{II} was not required in the reaction and veratryl alcohol was not oxidized. TAP has some interesting characteristics with respect to the optimum pH and the optimum concentration of hydrogen peroxide, and we are now characterizing them in detail.

We have cultivated a number of the symbiotic fungi from the fungus nodules of various termite species. Molecular phylogenetic analysis of the cultivated fungi is in progress. Identification of fungal species in the combs without cultivation is probably advantageous to avoid some biases introduced during their cultivation. These phylogenetic analyses are beneficial for understanding the selection and evolution of the associations of symbiotic fungi with fungus-growing termites. Moreover, we are now studying the lignin-degrading enzymes of the cultivates, particularly laccase, because laccase is the major enzyme exhibiting ligninolytic activity in the fungus combs. The characterization of ligninolytic activity of the symbiotic fungi is important for understanding the nature of this symbiotic relationship, which is most successful in biorecycling the naturally occurring ecomolecule, lignocellulose.

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