

**Structural analysis of the cell wall and its modification for efficient
production of wood degrading enzymes
in the white-rot fungus, *Pleurotus ostreatus***

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White-rot fungi, which belong to the phylum Basidiomycota, are the only organisms capable of growing on and degrading wood completely. Some white-rot fungi have been cultivated and consumed worldwide as edible mushrooms for a long time. In recent years, there has been increasing attention in various aspects on the use of white-rot fungi for purposes other than food. For example, white-rot fungi secrete unique enzymes to degrade plant cell wall components. These enzymes have the potential to improve the effective utilization of lignocellulosic biomass in a bio-based society. Additionally, white-rot fungi have gained attention for their role in sustainable material production. Some mycelium-based materials like mushroom leather serve as eco-friendly alternatives to traditional animal leather and plastics, helping to reduce carbon emissions and reliance on unsustainable resources.

The objective of this study is to reveal the important and unexplained structure of the cell wall in *P. ostreatus* by visualizing the polysaccharides in living vegetative mycelia. In addition, the visualization technique was used to evaluate cell wall modifications for the efficient production of the unique wood-degrading enzymes in a genetically modified strain.

In chapter 1, a triple staining method was established to visualize major polysaccharides in the cell wall of the model wood-rot fungus *Pleurotus ostreatus*. This

method employs two fluorescent probes for distinct glucans and a fluorescent dye for chitin. The method employed fluorescent probes targeting for α -1,3-glucan and β -1,3-glucan, in addition to Calcofluor White for chitin staining, enabling multicolor imaging of the fungal cell wall surface. Furthermore, the implementation of a hydrolytic enzymatic treatment to peel off the outer cell wall layers enabled the staining of polysaccharides in the inner layer. This approach enabled the visualization of a triple-layered structure of *P. ostreatus* cell wall, comprising a β -1,3-glucan-rich layer, an α -1,3-glucan-rich layer, and a chitin-rich layer for the first time. Furthermore, the presence of β -1,4-glucan was detected on the branching part of mycelia in *P. ostreatus* using a fluorescent protein fused with the cellulose-binding domain CBM3 of the cellulosome-integrating protein CipA from *Clostridium thermocellum*. A comparison was made between the triple staining and analysis of cell wall components of *P. ostreatus* and those of an ascomycete fungus, *Aspergillus nidulans*. The results of this study revealed that the outer cell wall layer of *P. ostreatus* is predominantly composed of β -1,3-glucan, serving as the principal component of the cell wall in this fungus. In contrast, mycelia in *A. nidulans* were found to be covered by a rich α -1,3-glucan layer. This chapter provides a comprehensive survey of the cell wall analyses of *P. ostreatus* and the potential for substantial disparities in cell wall structure between ascomycete and basidiomycete.

In chapter 2, a mycelial dispersion strain was developed by disrupting the *pkac2* gene in the white-rot fungus *Pleurotus ostreatus*. *pkac2* is a catalytic subunit gene of protein kinase A, which regulates several transcription factors related to cell wall synthesis. Liquid cultures of the Δ *pkac2* strains showed very high mycelial dispersibility and were visibly different from the wild-type strain (WT). Although growth on agar medium was slower than that of WT, Δ *pkac2* strains grew faster in liquid culture and had

approximately twice the mycelial dry weight of WT after 5 d of culture. The triple staining method developed in chapter 1 was used to label the cell wall polysaccharides of $\Delta pkac2$. And it was demonstrated that the cell wall surface β -glucan reduced in the $\Delta pkac2$ strains. Microscopic observations showed that the cell walls of the $\Delta pkac2$ strains were thinner compared to WT. The β -glucan content in the cell walls decreased in the $pkac2$ disruptants, although the transcription levels of β -glucan synthase genes increased. Furthermore, the $\Delta pkac2$ strains showed decreased hydrophobicity and stress tolerance compared to WT. These results indicate that disruption of the $pkac2$ gene in *P. ostreatus* alters the structure of the cell wall surface layer, resulting in high-density cultures due to mycelial dispersion.

In chapter 3, I investigated the productivity and transcriptional profiles of wood-degrading enzymes of the $\Delta pkac2$ strain in the high-density liquid cultivation. Cellulase and xylanase activities in the culture filtrate of $\Delta pkac2$ strains in liquid shaking culture were 7.8- and 49-fold higher than those of the wild-type (WT) strain, respectively. In this condition, the mycelial dry weight of $\Delta pkac2$ strains was two-fold higher than that of the WT, showing their greater efficiencies for cellulase and xylanase production than the WT. RNA-seq analysis indicated that nine of 35 predicted cellulase genes and two of five predicted xylanase genes were significantly upregulated in the $\Delta pkac2$ strains. On the contrary, the transcript levels of laccase-encoding genes were significantly decreased, indicating that $pkac2$ is involved in their transcriptional regulation. These results indicate that the $\Delta pkac2$ strains have high potential to produce cellulase and xylanase in the high-density liquid cultivation by increasing mycelial growth as well as upregulating the expression of relevant genes.

This thesis revealed the cell wall structure of white-rot fungus *P. ostreatus*. In addition, alterations in the structure of the cell surface layer have enabled dispersed

culture, and high-density culture using this strain enables high expression of enzymes in *P. ostreatus*. In conclusion, this study is the beginning of cell wall engineering of white-rot fungi, which will be useful for the development of mushroom materials and enzyme production with genetically engineered fungi having special properties that are not found in nature.