Lignin-degrading fungi as a biotechnological tool for biomass conversion

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Abstract
To avoid serious global warming by the emission of carbon dioxide from fossil fuels, there is a growing demand to produce energy and chemicals from carbon-neutral renewable resources, known as biomass. To convert wood biomass by enzymatic saccharification and fermentation, degradation of the lignin network is necessary because cell wall polysaccharides are covered with lignin in lignified plant cell walls. One potential approach to degrade lignin prior to saccharification and fermentation is to use the ligninolytic systems of white rot fungi. In the JSPS project "Production of cellulosic materials and biomass chemicals from unutilized plant resources", we studied conversion of lignified plant resources to energy, chemicals and feedstuff using the ligninolytic systems of white rot fungi. Among the numerous fungi so far isolated, a white rot fungus, *Ceriporiopsis subvermispora*, is characterized as one of the best biopulping fungi that degrade lignin without intensive damage of cellulose. Previous studies revealed that the selective ligninolysis by this fungus is catalyzed by low molecular mass compounds at a site far from extracellular enzymes and fungal hyphae. As a possible ligninolytic system by this fungus we focused on a mechanism involving *in situ* lipid peroxidation and suppression of the Fenton reaction by fungal metabolites. In this chapter, we review the lipid-related extracellular metabolites of the selective white rot fungus, *C. subvermispora*, and conversion of unutilized biomass including hardwood, softwood and bagasse into ethanol, methane and feedstuff using biological functions of white rot fungi.
Key words: *Biomass, White rot fungi, Lignin, Bioethanol, Feedstuff, Bagasse.*

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INTRODUCTION

Continued usage of fossil fuels has caused a serious environmental problem, emission of carbon dioxide. Therefore, utilization of biomass as a chemical and energy resource in harmony with environmental safeguards is an urgent task for ensuring human activities for the next generation. In particular, the production of ethanol from lignocellulosics has received much attention due to their immense potential as biofuels. Since lignin makes the access of cellulolytic enzymes to cellulose difficult, it is necessary to decompose the network of lignin prior to enzymatic hydrolysis. To this end, biological treatments with lignin-degrading fungi have great potential if the fungal treatment decomposes the network of lignin with minimum loss of polysaccharides. A biopulping fungus, *C. subvermispora*, is able to degrade lignin in wood without intensive damage of cellulose (Messner *et al.*, 1994). Due to its wood decay pattern, we applied the fungus to pretreatment of wood and non-wood fibers including bagasse. Bagasse is one of the potential bioresources for biofuel production. However, in Indonesia, utilization of bagasse in industrial fields is still limited, except for some applications such as feedstuff and energy production for steam. Utilization of bagasse for particle board has been industrialized but still faces several problems such as insufficient mechanical strength. Use of bagasse for paper making has also been done in East

Java, however, in other districts such as Sumatra (Lampung) and Sulawesi, bagasse is not effectively utilized (Samsuri *et al.*, 2004). Therefore, we studied production of ethanol from bagasse using pretreatment with white rot fungi.

Control of active oxygen species by extracellular lipid-related metabolites produced by a selective white rot fungus, *C. subvermispora*: The biodegradation of lignin is an extracellular free radical-mediated event that proceeds in concert with the activation of molecular oxygen and redox cycling of transition metals. When wood degrading fungi colonize wood, their extracellular enzymes are not able to diffuse into intact wood cell walls because the enzymes are too large to penetrate the pores of the wood cell walls (Messner *et al*., 1994). Hydroxyl radicals (•OH), a radical species highly destructive for cellulose and lignin, are proposed as a principal low molecular mass oxidant that erodes wood cell walls to enhance the accessibility of the extracellular enzymes of wood rot fungi to wood cell wall components (Wood, 1994).

Hydroxyl radicals are produced by the reaction of Fe^{2+} with H_2O_2 (Fenton reaction: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \bullet OH$), although other transition metals like Cu^+ are able to participate in me production of •OH. In the Fenton system, catalysts for the reductive half cycle ($Fe^{3+} \rightarrow Fe^{2+}$) accelerate hydroxyl radical formation. Wood rot fungi have versatile enzymatic and nonenzymatic systems to accelerate the reductive half cycle.

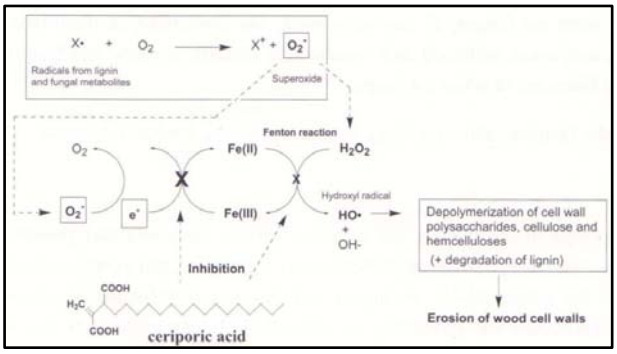

Figure 1. Inhibition of the production of hydroxyl radicals by ceriporic acid.

In contrast to brown rot and nonselective white rot fungi, selective lignin-degrading fungi like lignin-degrading fungi *C. subvermispora* are able to decompose lignin in wood cell walls without intensive damage to cellulose.

Wood decay by the biopulping fungus proceeds without the penetration of extracellular biopulping fungus into the wood cell wall regions. Lipid peroxidation has been proposed as a major pathway for the ligninolysis of this fungus at an incipient stage of wood decay (Jensen *et al.*, 1996; Enoki *et al.*, 1999; Watanabe *et al.*, 2000; Watanabe *et al.*, 2001). Lignin biodegradation proceeds by a free radical process in the presence of molecular oxygen and iron. Reductive radicals such as semiquinone radical reduce molecular oxygen to produce superoxide, which in turn reduces Fe^{3+} or disproportionates into H_2O_2. Fe^{3+} is directly reduced by lignin-derived phenols such as guaiacol and catechol (Pracht *et al.*, 2001). Thus, production of the cellulolytic oxidant, hydroxyl radical, would be inevitable if some inhibition system for the iron redox reactions were not involved in wood decaying systems. This indicates that the selective white rot fungus possesses unknown extracellular systems that attenuate the production of hydroxyl radicals. We first isolated a series of novel itaconic acid derivatives having a lone, alkyl side chain at position C-3 of their core (ceriporic acids) from cultures of *C. subvermispora* (Enoki *et al.*, 2002; Amirta *et al.*, 2003). We also reported that alkylitaconic acid strongly suppresses the Fenton reactions even in the presence of Fe^{3+} reductants such as cysteine and hydroquinone (Watanabe *et al.*, 2002). The inhibition of •OH production by a diffusible fungal metabolite accounts for the extracellular system of the fungus that attenuates the formation of •OH in the presence of iron, molecular oxygen and free radicals produced during lignin biodegradation (Fig. 1). Recently, we reported that 1-nonadecene-2,3-dicarboxylic acid (ceriporic acid B), an extracellular metabolite of *C. subvermispora*, strongly inhibits •OH production and the depolymerization of cellulose by the Fenton reaction in the presence of iron ions, cellulose, H_2O_2, and a reductant for Fe^{3+}, hydroquinone (HQ), at the physiological pH of the fungus (Fig. 2) (Rahmawati *et al.*, 2005). The extracellular fungal system to control free radicals, active oxygen species and the peroxo-complex of transition metals can be applied to biomimetic lignin degradation and pulp bleaching (Rahmawati *et al.*, 2005).

The reactions were carried out by mixing 0.5mM $FeC1_3$, 0.25mM HQ, 100mM H_2O_2 and 0.1g cellulose in the presence and absence of 2.5mM ceriporic acid B. Control contained cellulose and H_2O_2.

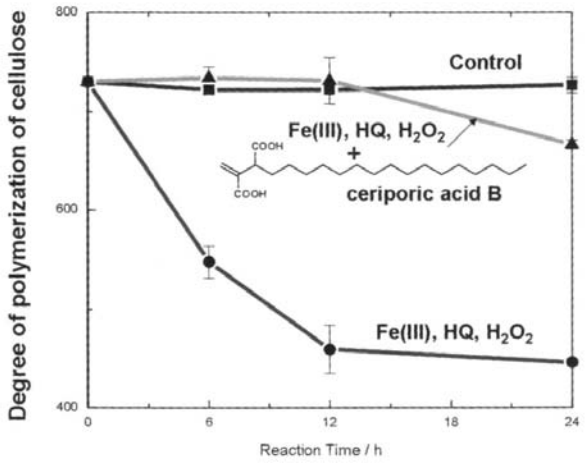

Figure 2. Ceriporic acid B, an extracellular metabolite of *C. subvemispora*, suppresses the depolymerization of cellulose by the Fenton reaction (Rahmawati *et al.*, 2005).

Production of bioethanol, methane and feedstuff from lignocellulosics using white rot fungi: Development of conversion systems from lignocellulosics into biofuels and chemicals has received much attention due to immense potential for the utilization of renewable bioresources. For example, ethanol production from lignocellulosics has been examined by saccharification with acids or cellulolytic enzymes and subsequent ethanol fermentation with yeast or genetically engineered bacteria. Since lignin makes the access of cellulolytic enzymes to cellulose difficult, it is necessary to decompose the network of lignin prior to enzymatic hydrolysis. Thus, effective pretreatments are needed for enzymatic saccharification and ethanol production from lignocellulosics. Biological pretreatment with lignin-degrading fungi is one possible approach. We reported that pretreatment with white rot fungi increases yield of ethanol by simultaneous saccharification and fermentation (SSF) with Saccharomyces cerevisiae from wood (Itoh *et al.*, 2003) and sugarcane bagasse (Samsuri *et al.*, 2004) (Fig. 3).

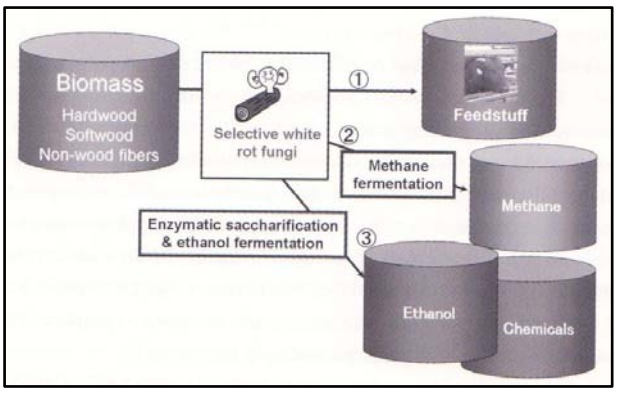

Figure 3. Production of ethanol, methane, feedstuff and other useful chemicals from biomass using pretreatment with selective white rot fungi.

Ethanol was produced by SSF of Indonesian bagasse using *S. cerevisiae* AM12 and Meicellase after pretreatment with steam and the white rot fungi *Pleurotus ostreatus* ATCC 66376, *C. subvermispora* ATCC 90467, *Lentinus edodes* IFO 6654, *C. subvermispora* ATCC 90467 and a new isolate of Indonesian white rot fungus, PSMOL Pretreatment with several white rot fungi increased ethanol production from the bagasse about 1.6 to 2 times (Figs. 4, 5). Ethanol was also produced from residual bagasse after cultivation of mushrooms of *P. eryngii*. The combination of mushroom cultivation and ethanol fermentation is a practical approach to utilize bagasse.

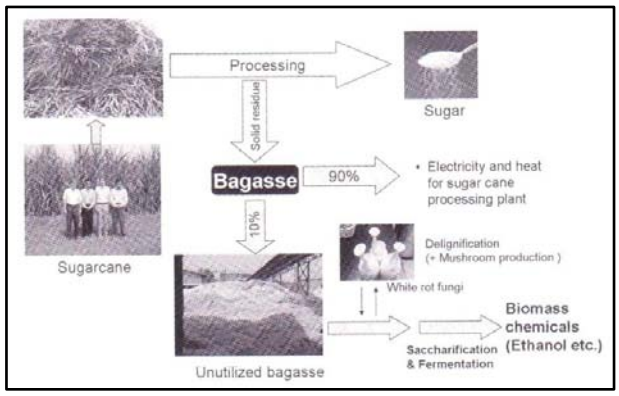

Figure 4. Production of ethanol from unutilized bagasse

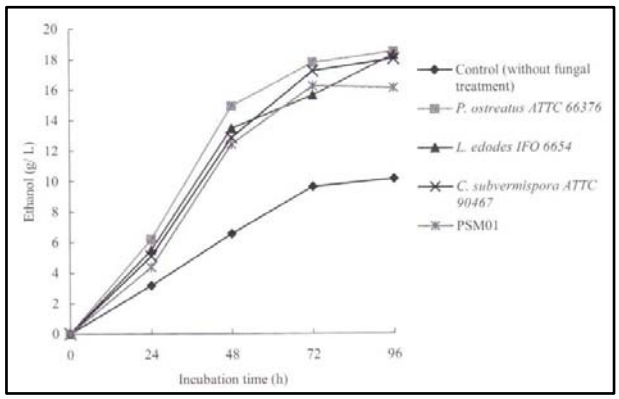

Figure 5. Ethanol production from Indonesian bagasse by SSF with *S. cerevisiae* AM12 and Meicclase after treatment with several white rot fungi for 8 weeks and subsequent steaming at 180°C for 1 h (Samsuri *et al.*, 2004). Initial substrate concentration was 50g/l. PSMO1 is a white rot fungus isolated in Indonesia.

Pretreatment with the white rot fungi *Lentinula edodes, Pleurotus eryngii, P. sahmoneostramineus,* and *C. subvermispora* (ATCC 90467, CZ-3) was applied to feedstuff production from bagasse (Okano *et al.*, 2005). The capability of four white-rot fungi to improve the digestibility of sugarcane bagasse for ruminants was determined in terms of *in vitro* organic matter (OM) digestibility (IVOMD), neutral detergent fiber digestibility (IVNDFD) and *in vitro* gas

production (IVGP). These analyses demonstrated that *L. edodes* and two strains of *C. subvermispora* increased digestibility while no accelerating effects were found in the treatments with *P. eryngii* and *P. salmoneostramineus*. The highest digestibility of bagasse was obtained with an edible mushroom, *L. edodes*.

Fungal treatments using white rot fungi were also applied to the production of feed for ruminants from Japanese cedar wood (Okano *et al.*, 2005). Japanese cedar wood chips were treated with the white rot fund *P. ostreatus, L. edodes, Pholiota natneko, Dichomitus squalens* and *C. subvermispora*. IVOMD in Japanese cedar wood without fugnal treatment was between 0.047 and 0.068, while it was elevated to 0.046 by culturing with *C. subvermispora* for 20 weeks. The in vitro gas production (IVGP) in Japanese cedar wood cultured with *C. subvermispora* for 20 weeks increased to 107m1/g OM, while IVGP for *P. ostreatus, P nameko* or *D. squalens* was 37 ml/g OM or lower. These results demonstrate that *C. subvermispora* has the highest potential to convert Japanese cedar wood into a feed for ruminants.

Pretreatment with white rot fungi was applied to methane fermentation from Japanese cedar wood (Amirta *et al.*, 2005). Methane fermentation is advantageous for on-site energy supply. Methane gas can be converted to electricity using fuel cells or turbine systems, or combusted directly. Development of a bioconversion system from wood to methane should accelerate the establishment of bioenergy-based societies that use wood and forestry wastes to make electricity, heat, and fuels. We studied fungal pretreatment with several strains of *C. subvermispora* and *Pleurocybella porrigens* for methane fermentation of Japanese cedar wood. Methane fermentation of Japanese cedar wood was carried out after pretreatment with four strains of white rot fungi, *C. subvermispora* ATCC 90467, CZ-3, CBS 347.63 or *P. porrigens* K-2855. These fungi were cultivated on wood chip media with and without wheat bran for 4-8 weeks. The pretreated wood chips were fermented anaerobically with sludge from a sewage treatment plant. Pretreatment with *C. subvermispora* ATCC 90467, CZ-3 and CBS 347.63 in the presence of wheat bran for 8 weeks decreased 74-76% of β-*O*-4 aryl ether linkages in the lignin to accelerate production of methane. After fungal pretreatment with *C. subvermispora* ATCC 90467 and subsequent 30-day methane fermentation, the methane yield reached 35% and 25% of the theoretical yield based on the holocellulose contents of the decayed and original wood respectively. In contrast, treatment with the three strains of *C. subvermispora* without wheat bran cleaved 15-26% of the linkages and produced 6-9% methane. There were no significant accelerating effects in wood chips treated with *P. porrigen*, which has a lower ability to decompose lignin. Thus, *C. subvermispora*, with a high ability to decompose aryl ether bonds of lignin, promoted methane fermentation of softwood in the presence of wheat bran.

Studies on lignin biodegradation by selective white rot fungi will give us new insight into the development of environmentally friendly processes for the production of chemicals, fuels and ecomaterials from wood and non-wood lignocellulosics.

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