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IMPROVEMENT OF BIOCONVERSION SUITABILITY OF JAPANESE CYPRESS WOOD BY COMBINATION OF UV RADIATION, OZONIZATION AND DECAY TREATMENT WITH WHITE-ROT AND BROWN-ROT FUNGI

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ABSTRACT

The present study is the first report for decay treatment of Japanses cypress wood by white-rot and brown-rot fungi. Japanese cypress is well-known as a refractory wood. Investigation of pretreatments to increase the fragility of Japanese cypress wood should aid with development of techniques to overcome the durability of any wood material. In the present study, the effects of ultraviolet (UV)/ozone exposure and decay treatment using the white-rot fungus *Ceriporiopsis subvermispora* and brown-rot fungus *Wolfiporia cocos* with or without addition of linoleic acid were investigated. The proportion of the 'available component' (potential substrate for bioconversion) of Japanese cypress wood particles increased from 9% to 27% in the decay treatment using *W. cocos* for 2 months after UV/ozone exposure. UV/ozone exposure and addition of linoleic acid in the decay treatment were both effective for enhancing its suitability for bioconversion. Further optimization of a method combining *C. subvermispora* and *W. cocos* treatment has great potential for shortening the treatment period and/or improvement of the suitability of Japanese cypress wood for bioconversion.

Keywords: *Ceriporiopsis subvermispora*, decay pretreatment, Japanese cypress, linoleic acid, UV/ozone exposure, *Wolfiporia cocos*.

Abbreviations: ABTS, 2,2-azobis(3-ethylbenzthiazoline-6-sulfonate); CMC, carboxymethyl cellulose sodium salt; 2,6-DMP, 2,6-dimethoxyphenol; DRIFTS, diffuse reflectance infrared Fourier transform spectroscopy; JC, Japanese cypress; LA, linoleic acid; Lac, laccase; LiP, lignin peroxidase; MnP, manganese peroxidase; PDA, potato dextrose agar; pNB, *p*-nitrophenyl butyrate; UV, ultraviolet.

INTRODUCTION

Japanese cypress (JC; *Chamaecyparis obtusa*), called Hinoki in Japan, is well known as a refractory wood. In Japan, JC has been used as a long-lasting wood for building materials for construction of palaces, shrines and temples. Annual production of JC in 2007 was 65,000 m^3 but, surprisingly, 70% of the JC material was treated as waste excluding the fine wood proportion (Forestry Agency, 2008; Ikami and Murata, 2003). In another side, the durability of JC makes it unsuitable for utilization as a biomass resource. The durability of JC is reported to depend on the high content of oily components, such as 2-hydroxy-4-isopropyl-2,4,6-cyclo-heptatrien-1-one (β-thujaplicin or hinokitiol) and 4,4-dimethoxy-6-methylene-2-cyclo-hexen-1-one (yoshixol), which have antimicrobial activities (Hong *et al*., 2004; Koyama *et al*., 1997). In addition, the superior durability of JC is likely to reflect the complex structure of its wood components, and is illustrated by Horyuji Temple in Japan, which is the oldest (1,300 years old) wooden structure in the world. Therefore, very few reports on the decomposition and utilization of JC have been published. In the present study, we investigated the pretreatment of JC powder to increase its fragility because reducing the durability of JC wood should contribute to advances in the application of such techniques to any wood material. The fragility and potential suitability of JC wood for bioconversion was evaluated by enzymatic digestibility and determination of the decrease in holocellulose content because we postulated that enzymatic saccharification or direct fermentation such as production of methane and hydrogen would be applied after the pretreatment of JC wood.

In many studies of utilization of wood materials, lignin is a major obstacle because of its resistance to chemical and biological degradation (Baldrian and Valáskova's, 2008; Lundell *et al*., 2010; Sánchez, 2009). Recent trends concerning environmental problems demand the development of a pretreatment technique for wood decomposition and utilization with a reduced environmental impact. We considered that natural phenomena would provide a key to overcome the presence of lignin because wood lignocelluloses are

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completely degraded in natural forests. It is known that the initial degradation of forest wood is progressed by synergistic action between white-rot and brown-rot fungi (Martínez *et al*., 2005), nevertheless to our knowledge few studies on the degradation of wood using a combination of these fungi have been conducted. Schilling *et al*. (2009) studied the effect of pretreatment of woods with brown-rot fungi on subsequent enzymatic saccharification, but the effect of culturing the two types of fungi in combination has not been examined previously.

In the process of wood decay, fungal delignification generally depends on the peroxidation system, including manganese peroxidases (MnP), lignin peroxidases (LiP) and laccases (Lac). Many kinds of peroxidation enzymes are reported, and it is suggested that the presence of unsaturated fatty acids during the decay process accelerates degradation of lignin via induction of peroxidation enzymes or acts as a mediator of reactive oxygen species (Bermek *et al*., 2002; Enoki *et al*., 1999; Jensen *et al*., 1996; Kapich *et al*., 1999; Watanabe *et al*., 2000). Thus, we investigated whether addition of linoleic acid (LA) to the culture was effective for delignification by white-rot and/or brown-rot fungi.

Another clue to support delignification of lignocelluloses is found in traditional Japanese techniques. In regions of Japan receiving snow in winter, craftsmen of traditional wood-based paper and hemp cloth use the technique called Yuki-sarashi, which involves bleaching with UV radiation with sunlight reflected by snow and simultaneous ozonization. We also tested the effect of Yuki-sarashi (i.e. UV radiation and ozonization) to enhance fungal decay of JC powder.

In the present study, we defined the potential substrate for bioconversion in JC wood as ‘available component’ and evaluated by amount of decreased holocellulose. An increase of ‘available component’ means ‘suitability for bioconversion’ of JC wood. Overcoming the durability of JC wood would contribute to the utilization of all types of wood resources more efficiently.

MATERIALS AND METHODS

Microorganisms and inocula

Cultures of the white-rot fungus *Ceriporiopsis subvermispora* ATCC96608 and brown-rot fungus *Wolfiporia cocos* NBRC30268 were maintained on potato dextrose agar (PDA) plates at 4°C. In the experiments, a 4 mm plug of PDA-grown mycelia was transferred to fresh PDA medium and incubated at 25°C for 5 d. The mycelia were harvested with 3ml of Basal III medium (Tien and Kirk, 1988). The mycelial cells were washed and dispersed in carbon source-free Basal III medium by passage through a stainless-steel screen with 0.8mm pores. One milliliter of the cell suspension (1mg dry cell weight/ml) was added to 200ml of Basal III medium containing 1% glucose (pH 5.0), and shaken at 25°C at 150rpm for 2 weeks. The cultured mycelial cells were harvested, washed and dispersed with carbon source-free Basal III medium using the stainless-steel screen, and the resulting cell suspension was used as an inoculum.

Treatment of Japanese cypress wood

JC wood was ground and sieved using screens with 100μm and 300μm pores. The particles ranging in size from 100 to 300μm were dried at 105°C for 6h and were used for the following experiments.

The effects of exposure to UV/ozone, decay by white-rot and/or brown-rot fungi in the presence or absence of LA, and combination of these treatments on the digestibility of JC wood were investigated (see Table 1). For exposure to UV/ozone, we constructed a special apparatus to expose JC wood to conditions mimicking Yuki-sarashi (Fig. 1). Sieved JC powder (300g) was evenly spread on the bottom of a stainless container. The powder was moistened with 300ml deionized water and continuously stirred at 10rpm for 24h under UV light (180μW/cm^2 at 254nm) and in ozone-containing atmosphere (100ppm ozone). In the fungal decay treatment, fungal inocula (15mg or 5 mg dry cell weight-equivalent/g JC for *C. subvermispora* and *W. cocos*, respectively) were added to 200 ml Basal III medium containing 1% (w/v) JC powder and cultured at 25°C for 1 or 2 months with shaking at 200rpm. For combined treatment with both fungi, the 1-month culture with *C. subvermispora* was autoclaved, followed by inoculation and 1-month culture with *W. cocos*, and vice versa. With regard to LA treatment, 0.1% final concentration of filter-sterilized LA was added with the inoculum.

Determination of enzyme activities

The activities of enzymes isolated from the wood-rot fungi were assayed after 1 and 2 months of decay treatment. Homogeneously mixed cultures were centrifuged at 8,000 ×*g* for 5 min, and the supernatants were used for the enzyme assays. For assays of cellulases and xylanases, the pellets were resuspended in deionized water and also used for the assays.

To determine activities of cellulases and xylanases, carboxymethyl cellulose sodium salt (CMC), Avicel, oat spelt xylan or beechwood xylan were used as substrates. Reactions were conducted in 100mM sodium tartrate buffer (pH 5.0) containing 1% substrate at 25°C for 1 h. The quantity of released sugar in the supernatant of reaction mixtures was determined by the method of Imoto and Yagishita (1971). Total activities of cellulases and xylanases were calculated as the sum of activities in the supernatant and pellet.

In this study, it was impossible to measure activities of MnP and LiP separately because abundant manganese sufficient to activate MnP was supplied by addition of JC powder to cultures. Therefore, total peroxidase (MnP/LiP) activities were monitored by oxidation of 2,6-dimethoxyphenol (2,6-DMP). The reaction was conducted in 100 mM sodium tartrate buffer (pH 5.0) containing 1 mM 2,6-DMP, 1mM $MnSO_4$, 0.4mM H_2O_2 at 25°C for 30min (Wariishi *et al.*, 1992). Activity of Lac was also tested by oxidation of 2,2-azobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS). The reaction was conducted in 100mM sodium tartrate buffer (pH 5.0) containing 1 mM ABTS at 25°C for 30min (Mester and Field, 1998).

Activities of miscellaneous esterases were determined in 100mM sodium tartrate buffer (pH 5.0) containing 0.5 mM *p*-nitrophenyl butyrate (pNB) at 25°C for 1h (Horsted *et al*., 1998).

Chemical analysis of Japanese cypress wood components

The α-celluloses and hemicelluloses in treated JC wood

Table 1. Summary of treatments of Japanese cypress.

Treatment No.	Treatment							
	UV/O_3	AC	First 1 month			Next 1 month		
			LA	Inoculum	AC	LA	Inoculum	AC
1	-	+	-	-	+	N	N	N
2	-	+	-	-	-	-	-	+
3	-	+	+	-	+	N	N	N
4	-	+	+	-	-	-	-	+
5	-	+	-	-	+	-	-	+
6	-	+	+	-	+	+	-	+
7	-	+	-	CS	+	N	N	N
8	-	+	-	CS	-	-	-	+
9	-	+	+	CS	+	N	N	N
10	-	+	+	CS	-	-	-	+
11	-	+	-	WC	+	N	N	N
12	-	+	-	WC	-	-	-	+
13	-	+	+	WC	+	N	N	N
14	-	+	+	WC	-	-	-	+
15	-	+	-	CS	+	-	WC	+
16	-	+	+	CS	+	+	WC	+
17	-	+	-	WC	+	-	CS	+
18	-	+	+	WC	+	+	CS	+
19	+	+	-	-	+	N	N	N
20	+	+	-	-	-	-	-	+
21	+	+	+	-	+	N	N	N
22	+	+	+	-	-	-	-	+
23	+	+	-	-	+	-	-	+
24	+	+	+	-	+	+	-	+
25	+	+	-	CS	+	N	N	N
26	+	+	-	CS	-	-	-	+
27	+	+	+	CS	+	N	N	N
28	+	+	+	CS	-	-	-	+
29	+	+	-	WC	+	N	N	N
30	+	+	-	WC	-	-	-	+
31	+	+	+	WC	+	N	N	N
32	+	+	+	WC	-	-	-	+
33	+	+	-	CS	+	-	WC	+
34	+	+	+	CS	+	+	WC	+
35	+	+	-	WC	+	-	CS	+
36	+	+	+	WC	+	+	CS	+

Abbreviations: AC, autoclaved; LA, addition of linoleic acid; N, not examined; CS, *C. subvermispora* inoculated; WC, *W. cocos* inoculated; +, treated; -, not treated.

were analyzed according to the method of Yokoyama *et al.* (2002). Change in lignin content was determined by the Klason lignin method (Browning, 1967). Fungal biomasses contained in treated JC wood were estimated by determination of the glucosamine content as described by Blix (1948).

Diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS)

DRIFTS was performed using a FTIR-8700 Fourier transform infrared spectrophotometer equipped with a DRS-8000 diffuse reflectance unit (Shimadzu Corporation, Kyoto, Japan). Samples were diluted to 0.33% (w/w) in infrared-grade KBr (Wako Pure Chemical Industries, Osaka, Japan). The diluted samples were ground to a fine powder using an agate mortar and pestle. Samples were packed into a ø4 mm × 1.5mm deep aluminum sample cup. Spectra were collected in the range of 4,000 to 400 cm^{-1} at a resolution of 4 cm^{-1} with 50 scans. The spectra were then transformed with Kubelka-Munk's function, ratioed against the background spectrum of infrared grade KBr, and displayed in the transmission mode (%T).

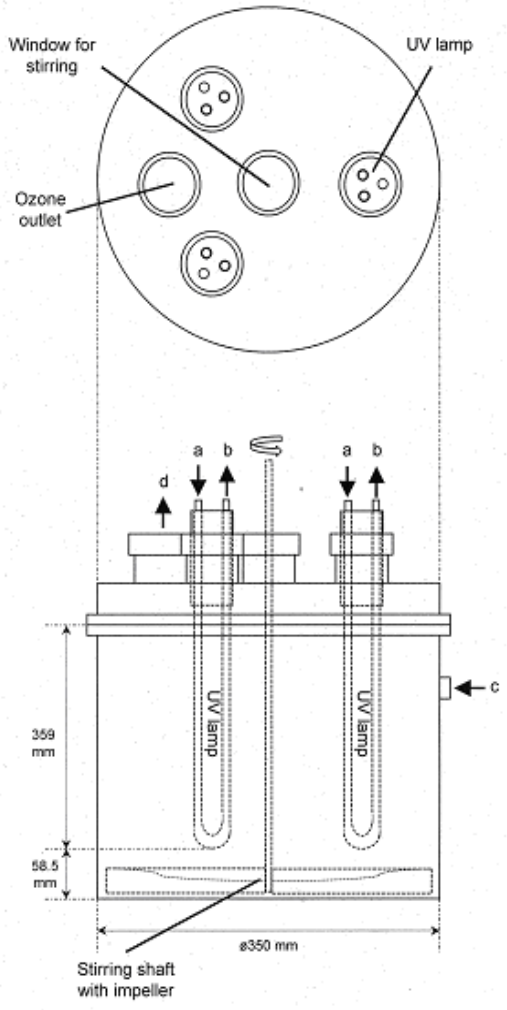

Fig. 1. Schematic representation of the special apparatus for UV/ozone exposure. The UV lamps were jacketed by a quartz tube, and the compressed air flowed from (a) to (b) at 2.6 l/min thorough the space between the jacket and lamp. Ozone gases generated around the three UV lamps were injected from inlet (c) and exhausted from outlet (d). The windows were sealed with silicone rubber.

Enzymatic digestibility test

The JC wood treated with combinations of UV/ozone and fungal degradation were subjected to enzymatic digestion and the quantity of sugar released was determined. The enzymatic digestibility test was carried out in a 10-ml volume with 2% substrate concentration in 50mM sodium acetate buffer (pH 4.5) using Meiselase (250 FPU/g substrate) at 45°C. Meiselase was obtained from Meiji Seika, Japan. The reaction mixtures were collected (0.5 ml each) every 2 h, and were centrifuged at 8,000 ×*g* for 5 min. The released reducing sugars in the supernatants were determined by the dinitrosalicylic acid method (Mohun and Cook, 1962). The amount of reducing sugars was calculated as glucose-equivalents.

RESULTS AND DISCUSSION

Fungal enzyme activities during decay treatment

The changes in pH and enzyme activities during fungal degradation are shown in figure 2. The pH values of all cultures decreased over the course of the 2-month treatment. In particular, the pH of cultures containing LA declined below 4 after 2-months culture, suggesting enhanced production of a variety of organic acids.

The enzyme activities with CMC and Avicel as the substrate reflect activities of cellulases mainly hydrolyzing amorphous and crystalline celluloses, respectively. At least 2–5 mU/ml of cellulase activities were obtained in all cultures, showing that both *C. subvermispora* and *W. cocos* produced cellulases during culture. In particular, the activities obtained in CMC were higher (5–18 mU/ml) in the presence of *C. subvermispora* (Fig. 2). Heidorne *et al.* (2006) reported that *C. subvermispora* grown on woods for pulping (*Pinus taeda* and *Eucalyptus grandis*) produced cellulases hydrolyzing CMC but not Avicel. Our results supported the production of endoglucanases hydrolyzing CMC, but hydrolysis of Avicel was also detected. The different responses of JC and pulp woods should be fundamental to understanding the decay mechanism of *C. subvermispora*. In xylanase assays of cultures lacking LA, activities with oat spelt xylan of *C. subvermispora* grown on JC wood without UV/ozone exposure were five-fold higher than activities of *C. subvermispora* grown on UV/ozone-treated JC wood; in contrast, activities with beechwood xylan of *W.*

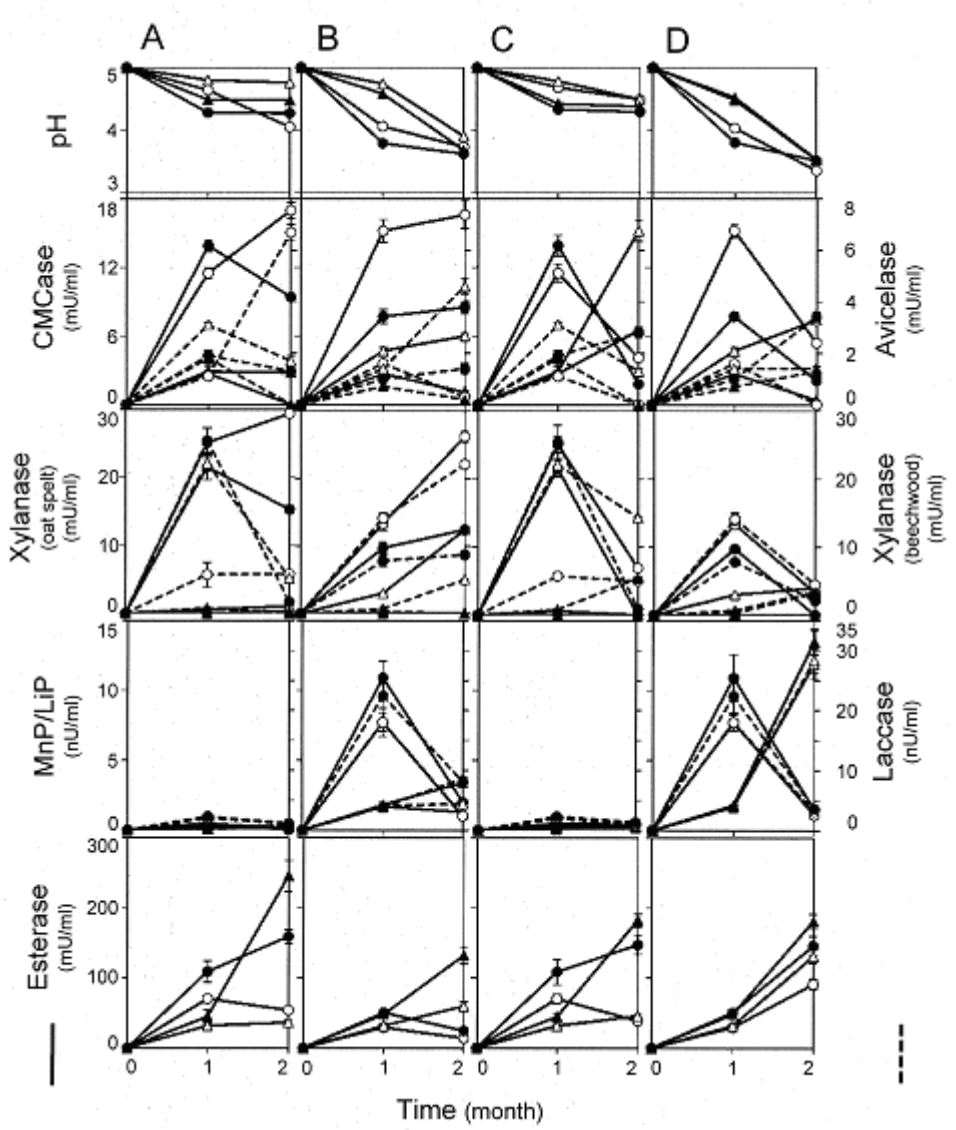

Fig. 2. Changes in pH and enzyme activities during decay treatments. Values of parameters on the left and right vertical axes are indicated by solid lines and dashed lines, respectively. Data from experiments without addition of LA are presented in panels A (treatment no. 8, 12, 26, and 30) and C (treatment no. 15, 17, 33, and 35), and data from experiments with addition of LA are presented in panels B (treatment no. 10, 14, 28, and 32) and D (treatment no. 16, 18, 34, and 36). Symbols in panels A and B: open circle, decayed by CS without UV/ozone exposure (treatment no. 8 and 10); open triangle, decayed by WC without UV/ozone exposure (treatment no. 12 and 14); closed circle, decayed by CS after UV/ozone exposure (treatment no. 26 and 28); closed triangle, decayed by WC after UV/ozone exposure (treatment no. 30 and 32). Symbols in panels C and D: open circle, decayed by CS followed by WC without UV/ozone exposure (treatment no. 15 and 16); open triangle, decayed by WC followed by CS without UV/ozone exposure (treatment no. 17 and 18); closed circle, decayed by CS followed by WC after UV/ozone exposure (treatment no. 33 and 34); closed triangle, decayed by WC followed by CS after UV/ozone exposure (treatment no. 35 and 36). All data presented are the means of three separate experiments; error bars represent the standard deviation.

cocos grown on JC wood without UV/ozone exposure were twenty-fold higher than activities of *W. cocos* grown on UV/ozone-treated JC wood (Fig. 2A and C). No substance showing xylanase expression-inducing activity has been reported in JC, and our results indicated that some component affecting xylanase expression by both fungal strains was eliminated by UV/ozone exposure. Considering that the activities determined with oat spelt xylan mainly represent xylanases hydrolyzing arabinoxylan, our results are very interesting and a study

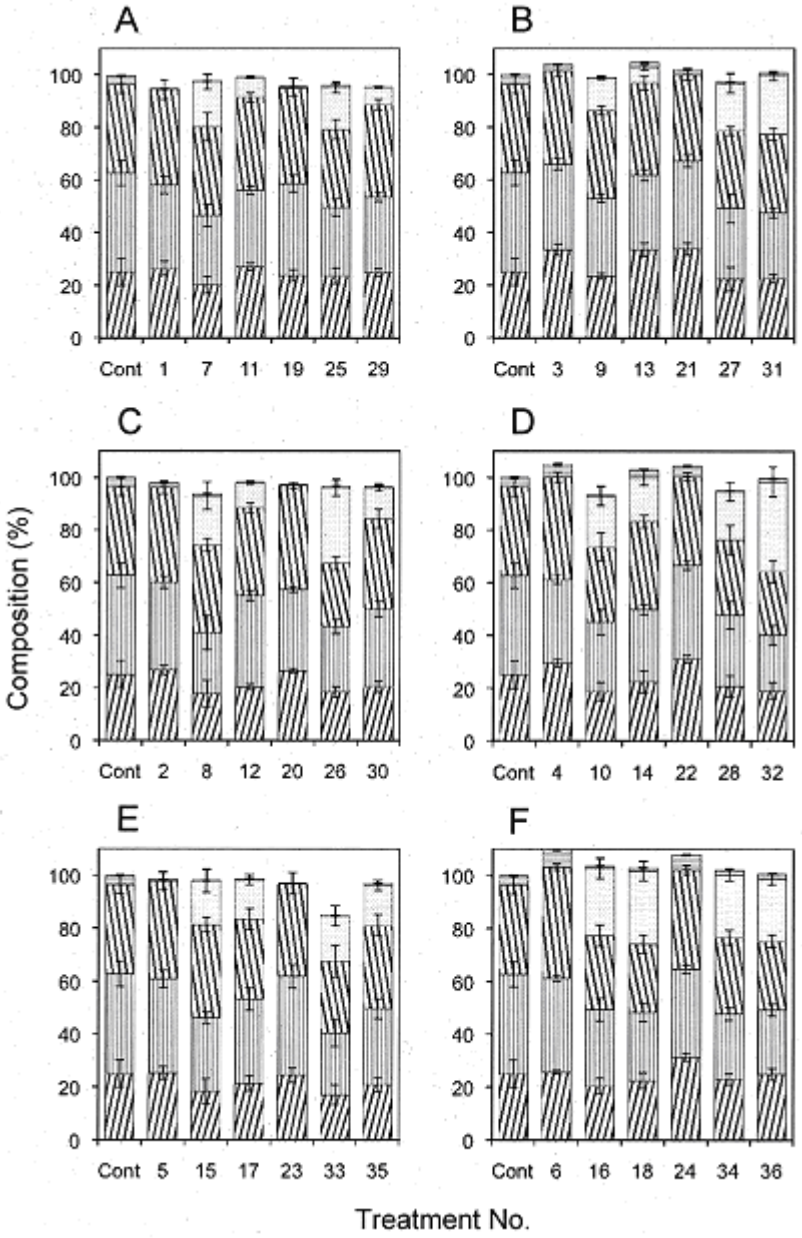

Fig. 3. Effects of decay treatments on the composition proportion of JC. Control JC (Cont) is the original JC without any treatment and is presented as 100% of the total sum of components. The value of each decay-treated JC is presented relative to the control JC. The treatment numbers listed in Table 1 are specified on the x-axis. Panels: A, Decay treatment for 1 month in the absence of LA; B, Decay treatment for 1 month in the presence of LA; C, Decay treatment for 2 months in the absence of LA; D, Decay treatment for 2 months in the presence of LA; E, Decay treatment for 1 + 1 months by both fungal strains in the absence of LA; F, Decay treatment for 1 + 1 months by both fungal strains in the presence of LA. Bar patterns: upward diagonal, hemicelluloses; vertical, α-cellulose; downward diagonal, lignin; dotted, fungal biomass; horizontal, other components. All data presented are the means of three separate experiments; error bars represent the standard deviation.

of the mechanism of cellulase/xylanase regulation in *C. subvermispora* and *W. cocos* is in progress.

Esterase activities were detected in all cultures. Both fungal strains showed higher esterase activities when grown on JC powder with UV/ozone exposure, compared with culture on JC without UV/ozone exposure (Fig. 2A). Esterase activities were indicated to be lower in cultures containing LA (Fig. 2B).

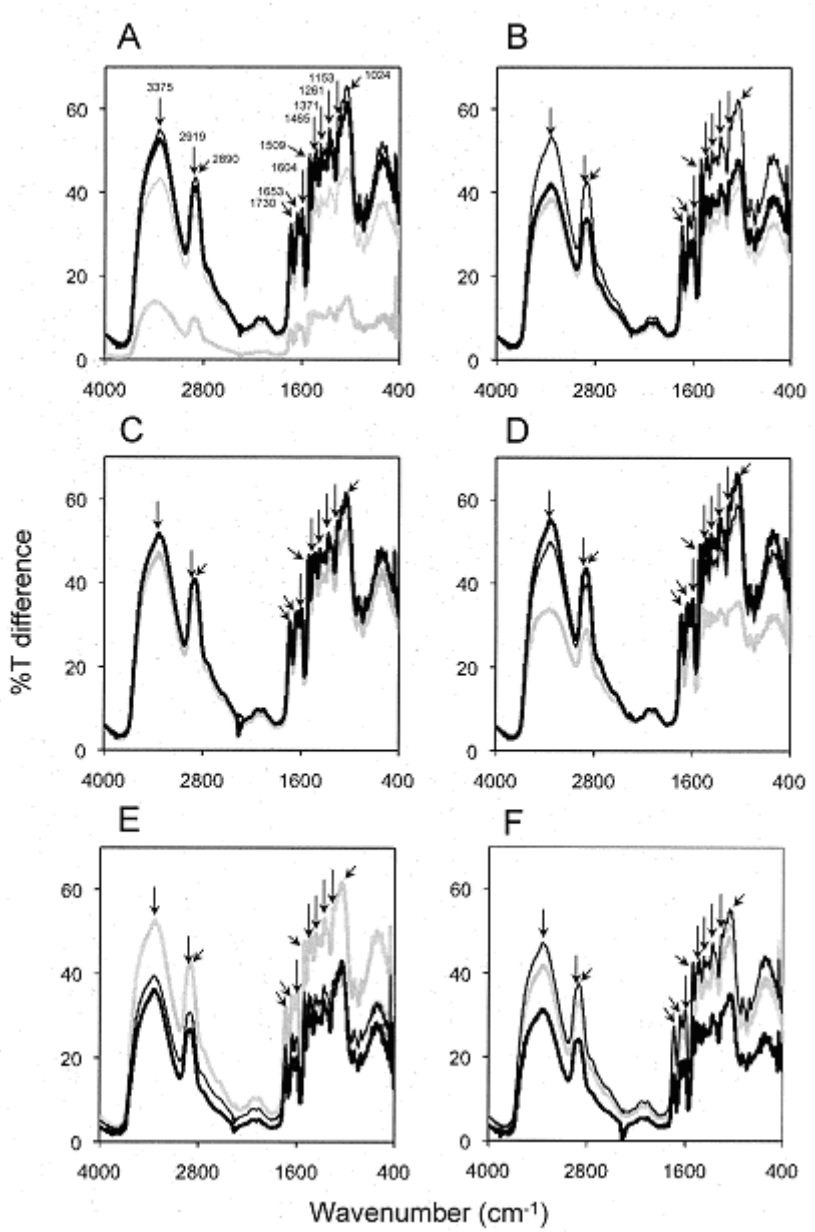

Fig. 4. DRIFTS spectra differences of decay-treated JC. The %T differences indicate the values calculated by subtraction of spectra of control JC from those of decay-treated JC. Panels: A, Decay treatment for 1 month in the absence of LA; B, Decay treatment for 1 month in the presence of LA; C, Decay treatment for 2 months in the absence of LA; D, Decay treatment for 2 months in the presence of LA; E, Decay treatment for 1 + 1 months by both fungal strains in the absence of LA; F, Decay treatment for 1 + 1 months by both fungal strains in the presence of LA. Thin gray line, thick gray line, thin black line and thick black line indicate treatment no. 7, 11, 25, and 29 in panel A; no. 9, 13, 27, and 31 in panel B; no. 8, 12, 26, and 30 in panel C; no. 10, 14, 28, and 32 in panel D; no. 15, 17, 33, and 35 in panel E; no. 16, 18, 34, and 36 in panel F, respectively. Lignin-associated peaks are indicated by arrows and their wavenumber positions are indicated in panel A.

Extraordinary changes were observed in MnP/LiP and Lac activities. These activities in both fungal strains increased by 10- to 45-fold in the presence of LA (Fig. 2B and D). A previous report showed that LA plays a role as an oxidation mediator for MnP via formation of acyl radicals (Watanabe *et al*., 2000). To confirm the mediator effect, we demonstrated the enzyme reaction using supernatants of cultures lacking LA (treatment no. 8, 12, 26, and 30) but the reactions were conducted in the presence of LA. Increases in activities were at most two-fold higher (data not shown). Therefore, it was strongly indicated that addition of LA in the fungal degradation treatment would have an effect on induction of MnP/Lac expression and/or activation of MnP/Lac enzymes.

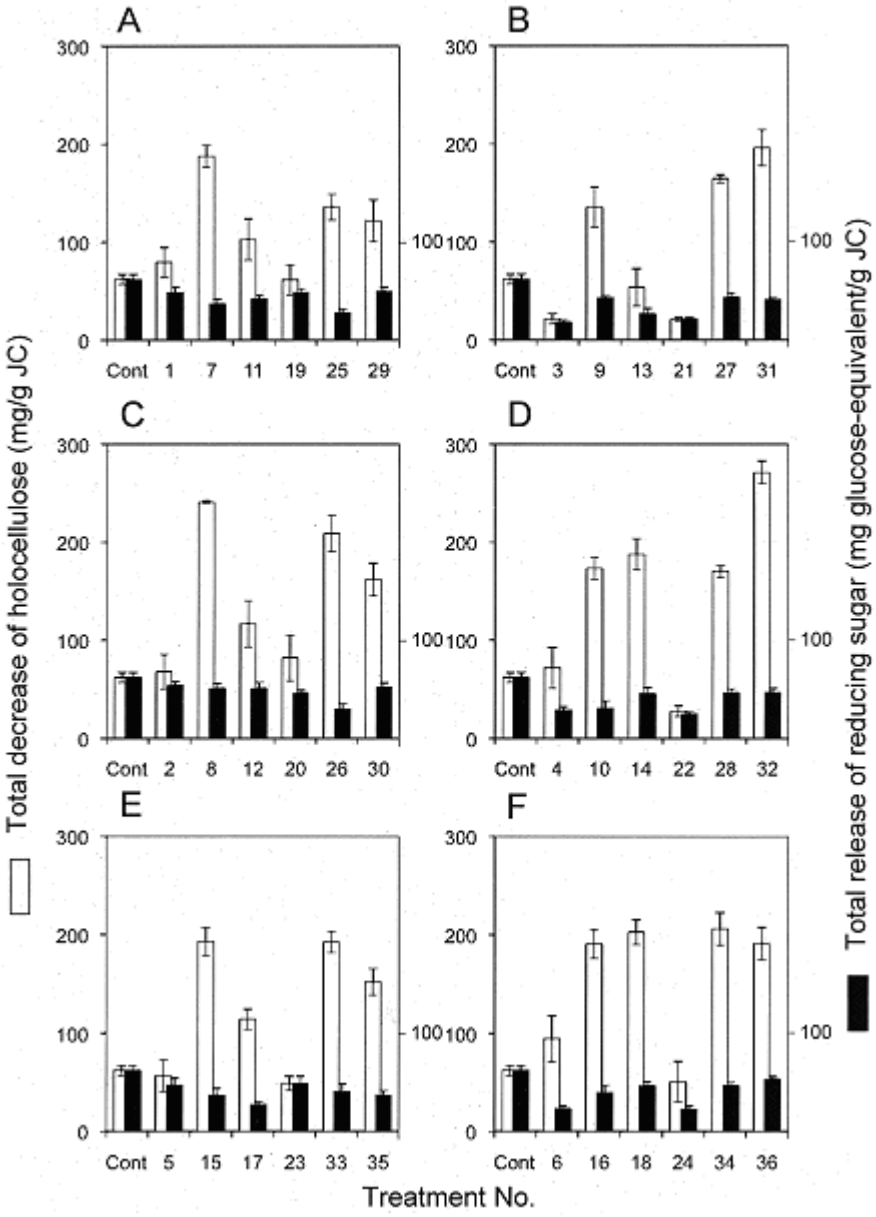

Fig. 5. Amounts of reducing sugars released in the enzyme digestibility test and total decrease of holocellulose content in JC after the enzyme digestibility test. Control JC (Cont) is the original JC without any treatment. Amounts of reducing sugars released by the enzyme digestibility test are indicated by closed bars, whereas total decrease of holocellulose content in JC through the whole experiment including UV/ozone exposure, decay treatment and the enzyme digestibility test are indicated as sums of the decrease of hemicellulose and α–cellulose contents by open bars. Panels: A, Decay treatment for 1 month in the absence of LA; B, Decay treatment for 1 month in the presence of LA; C, Decaying treatment for 2 months in the absence of LA; D, Decay treatment for 2 months in the presence of LA; E, Decay treatment for 1 + 1 months by both fungal strains in the absence of LA; F, Decay treatment for 1 + 1 months by both fungal strains in the presence of LA. All data represent the means of three separate experiments; error bars represent the standard deviation.

Experiments to investigate gene expression patterns are currently in progress.

Overall, it was shown that addition of LA in the fungal decay treatments slightly decreased production and/or activities of cellulases and xylanases, and drastically increased production and/or activities of peroxidation enzymes. Thus, it was expected that selective degradation of lignin would be enhanced by the addition of LA.

Composition of JC powder

Figure 3 shows the results of chemical analysis of decay-treated JC components. In comparison with the untreated control, the total dry weight of JC without inocula decreased 2–5% (treatment no. 1, 2, 5, 19, 20, and 23 in Fig. 3). These losses would be caused by washout of soluble components during autoclaving and submerged culture in the experiments. In contrast, in experiments with addition of LA, the total dry weight of JC without inocula increased 2–10% (treatment no. 3, 4, 6, 21, 22, and 24 in Fig. 3). These gains would be due to LA adsorbed by JC powders.

Apparent delignification effects were observed in cultures inoculated with *C. subvermispora*, as reported previously (Guerra *et al*., 2004; Mendonça *et al*., 2008), in contrast with the unchanged lignin content of JC cultures inoculated with *W. cocos*. In JC cultures without UV/ozone exposure inoculated with *C. subvermispora*, LA enhanced delignification as shown in treatment no. 10, 16 and 18 (4.5–7.3% increase compared to cultures without LA). UV/ozone exposure was also effectively induced delignification even in the absence of LA, as shown in treatment no. 26 and 33 (9.2% and 8.1% increases compared to cultures without UV/ozone exposure). In addition, delignification in treatment no. 27 was enhanced by 4.2% only after 1-month culture, similar to the level after 2-months culture, in the presence of LA, demonstrating that a combination of UV/ozone exposure and addition of LA accelerates delignification (Fig. 3). Disappointingly, a significant contribution of *W. cocos* to delignification was not detected in the chemical analyses.

Diffuse reflection infrared Fourier transform spectra

DRIFTS was utilized to estimate the status of lignin in decay-treated JC. The %T differences between decay-treated and control JC are shown in figure 4. In the %T differences, decreased bands are detected as specific peaks, and many peaks considered to be associated with lignin structure were obtained. In the detected peaks, the wavenumbers (cm^{-1}) for which band assignments have been reported are as follows: 3373, O-H stretching (hydrogen-bonded); 2890 and 2919, C-H stretching vibration in methyl and methylene groups; 1730, carbonyl stretching vibration in non-conjugated ketenes and in free aldehyde present in lignin and hemicelluloses; 1653, C=O stretching of conjugated or aromatic ketones; 1604, C=C unsaturated linkages, aromatic rings present in lignin; 1509, C=C stretching vibration in aromatic structure of lignin; 1465, C-H deformations, asymmetric bending vibration of $–CH_3$ and $–CH_2$- groups from lignin; 1371, CH deformation vibration, O-H bending vibrations in lignin and phenols; 1261, C-O of guaiacyl unit in lignin; 1153, asymmetric bridge stretching vibration of C-O-C group in the structure of cellulose; 1024, aromatic C-H in plane deformation, symmetrical C-O stretching (Rosu *et al*., 2010). However, the strengths of these peaks were not strictly quantitative because baseline spectra of glucans including α–cellulose and xylan were overlaid on lignin spectra and decreases in the amount and crystallinity of glucans would diminish the peak strengths of lignin (Åkerholm *et al*., 2004).

In our experiments, JC decay-treated for 1 month showed low %T differences, especially when inoculated with *W. cocos* (treatment no. 7 and 11 in Fig. 4A). The 2-month decay treatment gave 40–60%T differences, indicating that delignification occurred (Fig. 4C). In cultures containing LA, the %T differences increased to a similar level to the difference obtained after 2-months culture (Fig. 4B and C), suggesting that higher activities of peroxidation enzymes (Fig. 2) accelerate delignification. It is noteworthy that UV/ozone exposure effectively destroyed lignin structures (Fig. 4). In contrast, it was unexpected that lower %T differences were obtained in the decay treatments by combination of both fungal strains, even in comparison with the results obtained from decay treatment for 1 month (Fig. 4A, B, E and F). In Fig. 4E and F, lower %T differences were observed for decay-treated JC after UV/ozone exposure (treatment no. 33, 35 and 36) and JC decay-treated by combination of prior *C. subvermispora* and posterior *W. cocos* in the absence of LA (treatment no. 15). These decreases in %T could not be explained only by the simultaneous decrease of glucan content, therefore other analytic approaches should be considered.

Evaluation of suitability of decay-treated JC for bioconversion

The amount of potential substrate for bioconversion (termed the ‘available component’ in this study) of decay-treated JC was evaluated by enzyme digestibilities and total decreases in holocellulose content (Fig. 5). Considering that >85% dry weight of the original JC remained after decay treatment (Fig. 3), almost all of the initially ‘available’ holocelluloses in JC were converted to fungal biomass (also available for further conversion) during decay treatment. Thus, we considered that the decrease in holocellulose content indirectly represented the ‘available’ components in JC. The reducing sugars released in the enzyme digestibility assay could be considered to represent the amounts of holocelluloses becoming ‘newly available’ following decay treatment.

The amount of reducing sugars released from decay-treated JC in the enzyme digestibility assay reached a similar level to that of the untreated control (60 mg glucose-equivalent/g JC) except in the presence of LA without inoculation (treatment no. 3 and 21 in Fig. 5B, 4 and 22 in Fig. 5D, 6 and 24 in Fig. 5F). These findings indicated that the presence of LA without inoculation results in absorption of LA to JC powders and inhibits degradation of holocellulose by carbohydrate hydrolases in JC powders, and that concealed holocellulose

components were freshly exposed following delignification by fungi.

To estimate the 'total available component', the total decrease in holocellulose content in JC was determined (Fig. 5). Without inoculation, the highest decrease in holocellulose content (mainly elution into the culture supernatant by shaking for a long time) was 94.2 mg holocellulose/g JC (9.4% of the original JC, treatment no. 6 in Fig. 5F). In contrast, the highest value with inoculation was 270.9 mg holocellulose/g JC (27% of original JC, treatment no. 32 in Fig. 5D), which was 2.9-fold higher than that of JC without decay treatment. Considering the results of all experiments, the selective white-rot fungus *C. subvermispora* could make JC available without the support of UV/ozone exposure and LA addition (treatment no. 7 in Fig. 5A and no. 8 in Fig. 5C), whereas the weakly delignifying brown-rot fungus *W. cocos* needed the support of both UV/ozone exposure and LA addition (treatment no. 31 in Fig. 5B and no. 32 in Fig. 5D). In this respect, prior inoculation of *C. subvermispora* was important to destroy lignin structure to make JC more available prior to inoculation with *W. cocos* in combination experiments without addition of LA (treatment no. 15 and 33 in Fig. 5E). Nevertheless, the results of combination experiments with UV/ozone exposure and LA addition did not achieve the sum of values obtained in single-fungus experiments (treatment no. 27 and 31 in Fig. 5B versus 34 or 36 in Fig. 5F), suggesting that optimization of the combination method, e.g. the duration of each culture period, should be investigated further.

The results obtained in this study are important for development of a novel system for utilization of lignocellulogic materials. For example, we have maintained a microbial community for methane fermentation of wood. Our microbial community can produce methane directly from a variety of lignocellulogics including rice straw, beechwood and Japanese cedar wood. However, this microbial community cannot utilize JC without pretreatment (Ohtsuki *et al*., unpublished data). Although methane fermentation of Japanese cedar wood pretreated with *C. subvermispora* has been reported previously (Amirta *et al*., 2006), no-one has succeeded in producing methane from JC. Taken collectively, the improved suitability of JC for fungal degradation achieved in this study will help to achieve effective methane fermentation from all types of lignocellulogics, including JC.

CONCLUSION

We succeeded in increasing the 'available component' proportion of JC wood powder from 9% to 27%. UV/ozone exposure and addition of LA in the decay treatment were both effective for enhancing the suitability of JC wood by fungal decay. Further optimization of the method for combining *C. subvermispora* and *W. cocos* has great potential for increasing the 'available component' and/or shortening of the treatment period.

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