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Biodegradation and Saccharification of Wood Chips of *Pinus strobus* and *Liriodendron tulipifera* by White Rot Fungi

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Degradation and glucose production from wood chips of white pine (*Pinus strobus*) and tulip tree (*Liriodendron tulipifera*) by several white rot fungi were investigated. The highest weight losses from 4 g of wood chips of *P. strobus* and *L. tulipifera* by the fungal degradation on yeast extract-malt extract-glucose agar medium were 38% of *Irpex lacteus* and 93.7% of *Trametes versicolor* MrP 1 after 90 days, respectively. When 4 g of wood chips of *P. strobus* and *L. tulipifera* biodegraded for 30 days were treated with cellulase, glucose was recovered at the highest values of 106 mg/g degraded wood by *I. lacteus* and 450 mg/g degraded wood by *T. versicolor*. The weight loss of 10 g of wood chip of *L. tulipifera* by *T. versicolor* on the nutrient non-added agar under the nonsterile conditions was 35% during 7 weeks of incubation, and the cumulative amount of glucose produced during this period was 239 mg without cellulase treatment. The activities of ligninolytic enzymes (lignin peroxidase, manganese peroxidase, and laccase) of fungi tested did not show a high correlation with degradation of the wood chips and subsequent glucose formation. These results suggest that the selection of proper wood species and fungal strain and optimization of glucose recovery are all necessary for the fungal pretreatment of woody biomass as a carbon substrate.

Keywords: White rot fungi, wood chip, degradation, saccharification, ligninolytic enzymes

Owing to the depletion of petroleum resources, oil price is rising, and the interests for the development of alternative secure and renewable energy are increasing at the same time. Recently, various new or renewable energy sources have actively been investigated, and the bioenergy among them is regarded as the most useful energy source [1]. Currently, more than 80% of bioenergy is made from solid biomass through combustion, but its energy yield is somewhat low [9]. This solid biomass can be alternatively used for the production of liquid fuel. Biodiesel is a pioneer in the commercialization of liquid biofuel; however, the raw material for the extraction of biodiesel is restricted, and pollutants are produced during its combustion. Therefore, bioethanol seems to be a better alternative liquid fuel than biodiesel. Traditionally, bioethanol has been produced from sugary and starchy biomass, but nevertheless, woody biomass can also be an alternative substrate for bioethanol production, since a large amount of wood including tree pruning and waste wood can be provided at a lower cost than the grains, the price of which is going up sharply in recent days [17].

Methods for ethanol production from lignocellulose, a main component of wood, have variously been tested [12, 14, 18, 21]. The biological process for the conversion of lignocellulose to bioethanol is performed by delignification to liberate cellulose and hemicellulose, depolymerization of these carbohydrates to produce reducing sugars such as hexose or pentose sugars, and ethanol fermentation from those sugars. Lignin combines with cellulose and hemicellulose in the plant, forming the net-shaped lignocellulose, which interferes with the enzymatic hydrolysis and production of reducing sugars. To elevate the production of reducing sugars from lignocellulosic biomass, pretreatments such as softening and disruption of lignocellulose structure are necessary; however, lignin itself is difficult to degrade. Usually, acid hydrolysis, steam explosion and enzyme treatment have been commonly used for the pretreatment of woody biomass [2, 3]. Although microbial pretreatment takes longer time than those methods, it costs less and is environmentally friendly. Among wood degrading microbes, white rot fungi have been tested in the pretreatment process for bioethanol production [12]. These fungi can degrade not only lignin but also cellulose as their carbon and energy source, using several extracellular enzymes including ligninolytic enzymes [5, 6].

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In this study, several white rot fungi were tested as the biological pretreatment method for bioethanol production from two kinds of wood chips. In addition, the activities of the lignin degrading enzymes and cellulase of white rot fungi were examined during the wood biodegradation.

Materials and Methods

Fungal Strains and Wood Chips

White rot fungi used in the biodegradation of wood chips were *Irpex lacteus*, *Trametes versicolor*, and *Merulius tremellosus*, which had been isolated in Korea, and *T. versicolor* MrP 1, which is a transformant of *T. versicolor* overexpressing a manganese repressed peroxidase gene, *mrp*. These fungal strains have been shown in the previous experiments to have a high degrading capability for various recalcitrant organics [8, 19]. Wood chips of white pine (*Pinus strobus*) and tulip tree (*Liriodendron tulipifera*) were selected for this study. Since they are fast-growing trees and can be used as wood resource, the Korean government recommends the planting of these trees. The wood chips were applied to the biodegradation experiment after steam sterilization at 121°C for 15 min and drying for 72 h at 80°C. Dried wood chips were processed to 4 g (width 2.5–3 cm, length 2.5–3 cm, height 1 cm) and 10 g pieces (diam. 4–5 cm, height 1 cm) by a scroll saw.

Preparation of Fungal Inocula

Ten agar plugs (5×5 mm) of fungi grown on potato dextrose agar (PDA) plate were added to 100 ml of YMG medium (yeast extract 4 g/L, malt extract 10 g/L, glucose 4 g/L) and incubated at 30°C and 130 rpm for 10 days. This fungal culture was homogenized with a homogenizer (X120, CAT, Germany) for 30 sec, and centrifuged at 6,140×*g* for 30 min. Sterile distilled water (300 ml) was added to the fungal pellet, and the mycelial suspension was extracted by a reciprocal shaker (300 rpm) for 20 min. To remove residual YMG medium, the mycelial suspension was centrifuged again and the fungal pellet was resuspended with sterile distilled water. This process was repeated five times for complete removal of medium. After washing of mycelia, 40% (wet w/v) inocula were prepared by mixing the centrifuged mycelia with sterile distilled water, followed by homogenization for 30 sec.

Biodegradation of Wood Chips by White Rot Fungi

Only 1 agar plug of white rot fungi grown on a PDA plate for 10 days was reversely placed to the surface of 100 ml of YMG agar medium in a 250-ml beaker and incubated at 30°C for 7 days. Wood chips of 4 g were placed on the surface of YMG agar covered with the mycelia, and were incubated for 90 days. Weight loss of wood chips, enzyme activities, and released glucose were analyzed every 30 days.

Nutrient non-added agar (NNA) (100 ml) was prepared by the addition of 100 ml of 1.5% agar solution to a 250-ml beaker. Wood chips of 10 g were soaked in 40% (wet w/v) fungal inocula for 12 h at 4°C and placed on the surface of 100 ml of NNA. Weight loss of biodegraded wood chips, enzyme activities, and released glucose were measured every week during the incubation period of 7 weeks at 30°C.

Measurements of Weight Loss and Enzyme Activity in the Biodegraded Wood Chips

Fungal mycelia were removed from the biodegraded wood chips collected from the 100 ml of YMG agar and NNA, and then wood chips were soaked in 50 ml of sodium acetate buffer (50 mM, pH 5.0) at 4°C. After 24 h, sodium acetate buffer was decanted, filtered with filter paper, and centrifuged at 6,140×*g* for 40 min. Supernatants were used for the measurement of enzyme activities. The residual wood chips were soaked twice in 50 ml of distilled water for 12 h. Washed biodegraded wood chips were weighed after drying at 80°C for 24 h.

For the measurement of laccase activity in the degraded wood chips, 500 µl of the extracted supernatant was mixed and reacted with 500 µl of *o*-tolidine (2 mM, pH 3.5) in an 1-ml cuvette for 10 min. Increased absorbance by laccase was measured at 590 nm using a spectrophotometer (UV 1700; Shimadzu, Japan). Lignin peroxidase (LiP) activity was measured by oxidation of veratryl alcohol (molar extinction coefficient: $\varepsilon 477=9800\ M^{-1}\ cm^{-1}$) to veratryl aldehyde. One unit of LiP activity was defined as the transformation of 1 µmole of veratryl alcohol to 1 µmole of veratryl aldehyde per min. The activity of manganese peroxidase (MnP) was determined by monitoring 3,3',5,5'-tetramethoxydiphenoquinone ($\varepsilon 477=14800\ M^{-1}\ cm^{-1}$) production from 2,6-dimethoxyphenol at 477 nm. One unit of MnP activity was defined as the oxidation of 1 µmole of substrate for 1 min. Cellulase activity was determined by the method of filter paper cellulase using a filter paper strip (Whatman No. 1, width 1 cm, length 6 cm). One unit of cellulase activity was defined as the amount of enzyme producing 1 µmole of glucose.

Glucose Production from Degraded by Fung of Wood Chips

After fungal mycelia were removed by forceps from the biodegraded wood chips, glucose from 4 to 400 g wood chips was extracted with 50 mM sodium acetate buffer (pH 5.0) of 25 and 2,000 ml, respectively, at 4°C for 24 h. Recovered buffer was centrifuged at 6,140×*g* for 40 min, followed by dinitrosalicylic acid (DNS) assay to measure the glucose concentration of extract. One ml of the extracted sample was mixed with 3 ml of DNS solution followed by heating in boiling water for 15 min. The heated sample was immediately cooled and the absorbance of the cooled sample was measured at 550 nm.

Additional glucose production from the degraded wood chips was examined after treatment with 1.9 unit/mg of cellulase from *Aspergillus niger* (Sigma Chemical Co., U.S.A.). Residual wood chips after measurement of enzyme activity were dried at 80°C for 12 h, followed by cutting into 4 pieces. These wood chips were treated with 100 ml of cellulase solution (10 unit/ml in 50 mM sodium acetate buffer, pH 5.0) and reacted for 48 h at 30°C and 200 rpm. Reaction sample (2 ml) was taken after 2, 4, 6, 8, 10, 24, and 48 h followed by centrifugation at 2,490×*g* for 15 min. Collected supernatant was filtered with a membrane filter of 0.45 µm pore size, and released glucose was analyzed by high performance liquid chromatography (Breeze Model; Waters Co., U.S.A.). Analytical conditions were as follows: column, OP NH_2 (250×46 mm) (RS Tech, Korea); column temperature, 40°C; mobile phase, 75:25 (acetonitrile:water); flow rate, 1.2 ml/min; detector, RI.

All the experiments were carried out in triplicate, and mean values and the standard deviations are presented.

Results

Fungal Degradation of Wood Chips on YMG Agar and Activities of Ligninolytic Enzymes

When 4 g of wood chips of *P. strobus* were treated with *I. lacteus*, *T. versicolor*, *T. versicolor* MrP 1, and *M. tremellosus*,

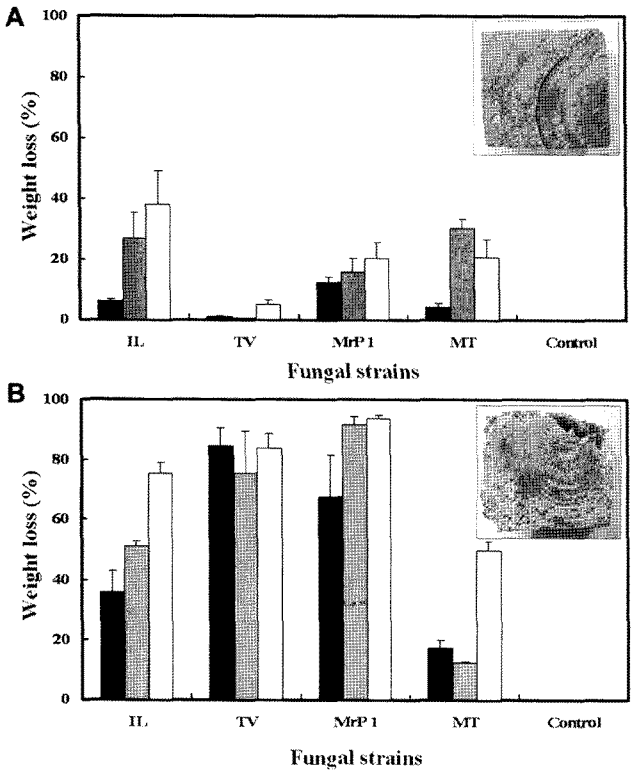

Fig. 1. Weight loss of of 4 g dry wood chips of *Pinus strobus* (**A**) and *Liriodendron tulipifera* (**B**) after degradation by white rot fungi *Irpex lacteus* (IL), *Trametes versicolor* (TV), *T. versicolor* MrP 1 (MrP 1), and *Merulius tremellosus* (MT) on YMG agar for 30 (■), 60 (■) and 90 days (□).
Control was not treated with white rot fungus.

the weight losses after 90 days were 38.0%, 5.1%, 20.5%, and 20.8%, respectively (Fig. 1A). In the case of the wood chips of *L. tulipifera*, the weight losses after biodegradation by *I. lacteus*, *T. versicolor*, *T. versicolor* MrP 1, and *M. tremellosus* were 75.6%, 83.8%, 93.7% and 50%, respectively (Fig. 1B).

During the fungal degradation of 4 g of wood chip of *P. strobus*, laccase activities of *I. lacteus* were very low, but the other fungi showed higher activities until 60 days, with the highest activity of 102.3 unit/ml by *T. versicolor* MrP 1 (Fig. 2A). The activities of MnP in the wood chips degraded by *M. tremellosus* were higher than other fungi (Fig. 2B). The highest activity of LiP (207.5 unit/ml) was shown by *I. lacteus* at 30 days (Fig. 2C).

When 4 g of wood chips of *L. tulipifera* were treated with *I. lacteus*, *T. versicolor*, *T. versicolor* MrP 1, and *M. tremellosus*, very low activity of laccase of *I. lacteus* was detected during the entire period, and the other fungi showed their highest activities at 60 days, with the highest activity of 67.1 unit/ml of *M. tremellosus* (Fig 2a). The activities of MnP in the degraded wood chips were lower than those in the wood chips of *P. strobus* (Fig. 2b). Furthermore, the coefficients of correlation between weight loss and enzyme activities of all fungi showed negative values (−0.2~−1.0). The highest LiP activity in the degraded wood chips of *L. tulipifera*, 32 unit/ml, was shown by *M. tremellosus* at 60 days (Fig. 2c).

Glucose Production from Biodegraded Wood Chips by Cellulase Treatment

When the biodegraded wood chips of *P. strobus* were treated with commercial cellulase, the highest amount of glucose released was 106 mg/g degraded wood by *I. lacteus*, which was lower than that (148 mg/g dry wood chip) of control (Fig. 3A). In the case of the wood chips of *L. tulipifera*, a large amount of glucose (450 mg/g) was released from the wood chips degraded by *T. versicolor* (Fig. 3B).

Fungal Degradation of Wood Chips on Nutrient Non-Added Agar

When 10 g of wood chips on NNA was inoculated with *T. versicolor*, the weight loss of *L. tulipifera* wood chips was about 35% during 4–7 weeks (Fig. 4A). Control, not treated with fungi, showed the weight loss of about 8% during 3–7 weeks. The cumulative glucose that was liberated from the wood chip of *L. tulipifera* degraded by *T. versicolor* without cellulase treatment was 239 mg, which is 2.2 times higher than that of the control that had not been inoculated but degraded by some contaminated fungi under nonsterile conditions (Fig. 4B).

Enzyme activities in the wood chips of *L. tulipifera* degraded by *T. versicolor* on NNA showed some variations (Fig. 5). Most enzyme activities in the wood chips degraded by *T. versicolor* were higher; however, the cellulase activity in the uninoculated control was higher than that of the fungal degraded wood chips in the later stage.

DISCUSSION

The weight losses of wood chips of *P. strobus* and *L. tulipifera* on YMG agar (Fig. 1) were much higher than the reported 11.7% weight loss of *Eucalyptus glandis* wood in 90 days by white rot fungus *Ceriporiopsis subvermispora* [6], 1.56–5.30% of *E. glandis* wood in 60 days by *Phellinus flavomarginatus* [5], and 7.58–26.75% of wood chips of eucalyptus, poplar, and larch in 60 days by *Pseudomonas* sp. [23]. This result might be due to the continuous supply of water or nutrients to the fungi on wood chips through contact with YMG agar. In addition, the inhibition of water evaporation by a thick mycelial mat tightly covered on the wood chips may also be helpful for the fungal degradation of wood. Under similar experimental conditions to this study, the highest weight loss of *E. grandis* by *T. versicolor* was similar to our results [15]. However, robust decrease of cellulose in the wood chip and transformation into glucose by cellulase could have induced the high weight loss in their study.

In the case of *L. tulipifera* wood chips; *T. versicolor* showed the highest degradation rate in the initial stage, however, the

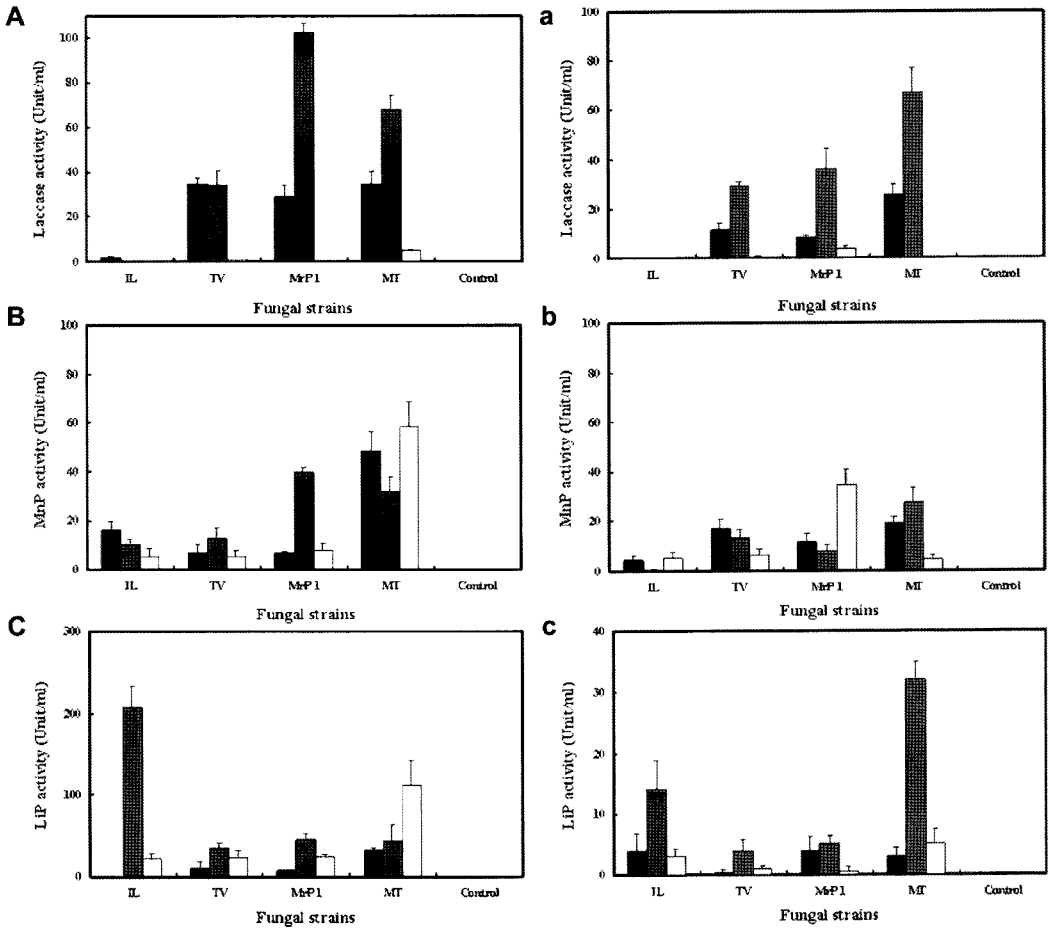

Fig. 2. Enzyme activities during the biodegradation of 4 g of wood chips of *Pinus strobus* (**A**: laccase; **B**: MnP; **C**: LiP) and *Liriodendron tulipifera* (**a**: laccase; **b**: MnP; **c**: LiP) by white rot fungi *Irpex lacteus* (IL), *Trametes versicolor* (TV), *T. versicolor* MrP 1 (MrP 1), and *Merulius tremellosus* (MT) on YMG agar for 30 (■), 60 (■), and 90 days (□).
Control was not treated with white rot fungus.

final weight loss by *T. versicolor* MrP 1 was higher than those of the others (Fig. 1B). *T. versicolor* showed a higher growth rate than *T. versicolor* MrP 1 (data not shown), but more ligninolytic enzymes or other enzymes were secreted from *T. versicolor* MrP 1 during 30–60 days (Fig. 2), which might support the higher degradation rate. *I. lacteus* showed the highest rates of growth (data not shown) and degradation of *P. strobus* wood chips among the fungi tested, but slightly lower degradation of *L. tulipifera* wood chips than *T. versicolor* strains. Although the activities of laccase, MnP, and LiP of *M. tremellosus* were higher than those of the other fungi, the degradation rate of wood chip of *L. tulipifera* was lower than those of the other fungi. It is quite likely that some other enzymes such as xylanase, cellulase, versatile peroxidase, and mycelia-associated dehydrogenases may be involved in the degradation of wood [13, 16]. In fact, most correlation coefficients between the weight loss of both wood chips and activities of LiP, MnP, and laccase showed negative values. The involvement and relative contribution of degrading enzymes should be examined to elucidate the mechanism of wood biodegradation. The wood chips of *L. tulipifera* degraded by *T. versicolor* and *T. versicolor* MrP 1 with high weight loss were decolorized to white color, physically softened, and contained more cracks and pores on the surface than wood chips of *P. strobus* (inset of Fig. 1B).

The fungal strain is critical in the degradation of woody biomass, but plant species may be more important. Although the activities of ligninolytic enzymes in *P. strobus* wood chips were not lower than those of *L. tulipifera* wood chips (Fig. 2), the biodegradation rates of *P. strobus* wood chips were much lower than those of *L. tulipifera* wood chips by all the fungi tested (Fig. 1). The hardwood such as *L. tulipifera* contains less amount of recalcitrant lignin and has a structure more suitable for degradation than softwood like *P. strobus* [10]. In addition, *P. strobus* produces antifungal substances including phytoalexins, which seemed to inhibit the fungal degradation of wood chips [22]. Therefore, the appropriate woody biomass should be selected based on its biodegradability for the utilization as a substrate for any biotechnological purposes.

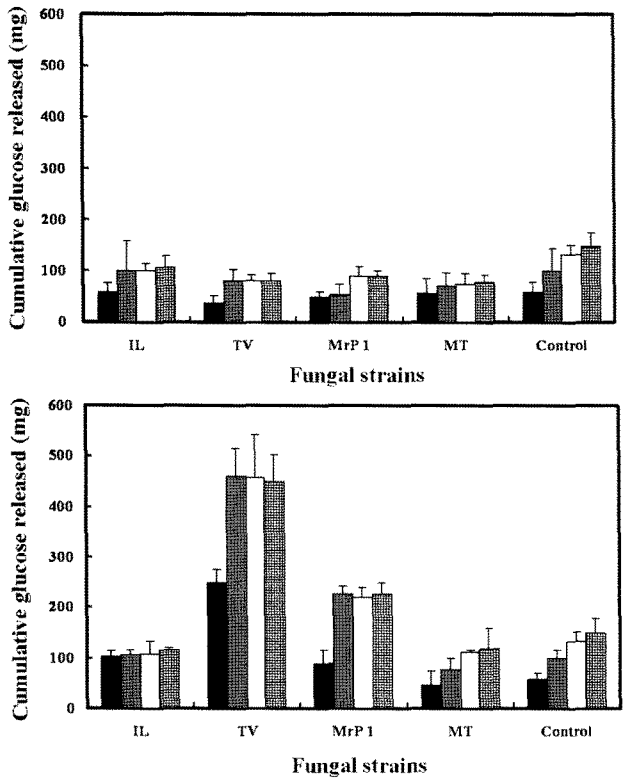

Fig. 3. Amount of cumulative glucose released from the wood chips (**A**: *Pinus strobus*; **B**: *Liriodendron tulipifera*), which had been degraded for 30 days by white rot fungi (IL: *I. lacteus*; TV: *T. versicolor*; MrP 1: *T. versicolor* MrP 1; MT: *M. tremellosus*) and treated with cellulase for 2 (■), 10 (▦), 24 (□), and 48 h (▦). Control had not been degraded by the white rot fungi.

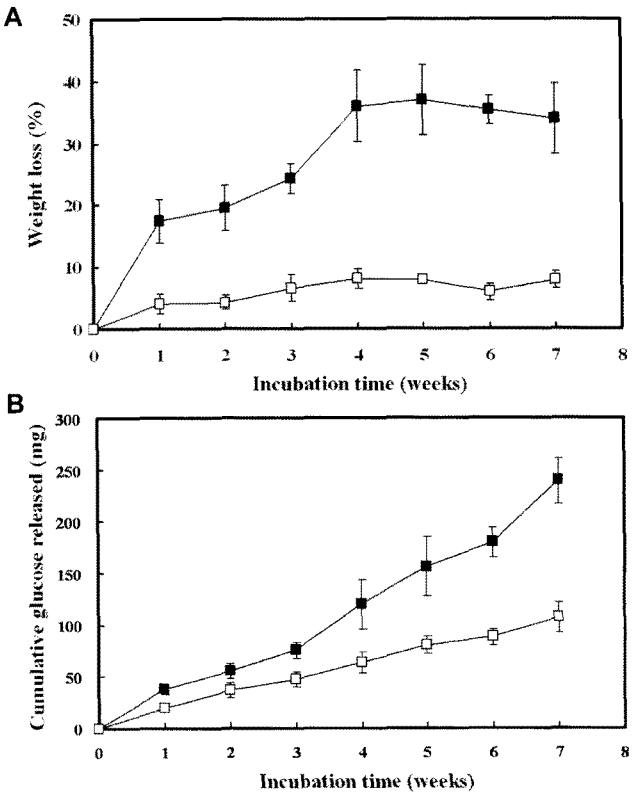

Fig. 4. Weight loss (**A**) and amount of cumulative glucose (**B**) released from 10 g of dry wood chips of *L. tulipifera* treated with *T. versicolor* on nutrient non-added agar. All samples were manipulated and incubated under nonsterile conditions for 7 weeks at 30°C. Symbols: ■, treated with *T. versicolor*; □, uninoculated control.

I. lacteus could degrade both types of wood chips, but laccase activities were very low or even could not be detected in both wood chips (Fig. 2). Guerra *et al.* [7] also could not detect laccase activity in the biodegradation of *Pinus taeda* by *Ceriporiopsis subvermispora*. They even suggested that lignin depolymerization and mineralization may occur when *C. subvermispora* cultures have no laccase activity. Although the activity of extracellular laccase was very low in both wood chips degraded *by I. lacteus*, some laccase associated with the cell membrane could be involved in the fungal degradation, as reported by Svobodová *et al.* [20]. Dark blue spots on the surface of mycelial mat were produced when the mycelia of *I. lacteus* was reacted with *o*-tolidine, indicating the presence of laccase activity. The involvement and relative contribution of this kind of laccase to fungal degradation should be investigated to understand the precise mechanism of wood biodegradation.

The total amounts of glucose liberated from the degraded *L. tulipifera* wood chips were higher than those from the degraded *P. strobus* wood chips (Fig. 3). This result was similar to the result of weight loss (Fig. 1), and it may be caused by the same mechanisms described above. The highest glucose recovery (450 mg/g biodegraded wood chip) was shown by *T. versicolor* with *L. tulipifera* wood chips. This glucose yield was 4 times higher than that of 100 g of animal manure by an optimized hydrolysis process using sulfuric acid and cellulolytic enzymes [21]. It was also higher than the yield of 36.3 g sugar/100 g olive tree biomass with pretreatment at 180°C and 1% sulfuric acid followed by enzymatic saccharification [3], but similar to the maximal glucose recovery (47.8%) from steam explosion [4]. Except for the cases of *T. versicolor* and *T. versicolor* MrP 1 on *L. tulipifera*, all the other glucose recoveries were not higher than those of control, which was not treated with white rot fungi but treated with commercial cellulase. This low recovery of glucose is most likely due to the utilization of liberated glucose by the fungi themselves. The weight loss of wood chips of *L. tulipifera* was slightly higher by *T. versicolor* MrP 1 than *T. versicolor*, but glucose recovery was much higher by *T. versicolor*.

The weight loss of *L. tulipifera* wood chips by *T. versicolor* on NNA in 4 weeks (35%) was much lower than the 83.8% on YMG agar in 30 days (Fig. 4), but still higher than those with different woods and fungi [5, 6, 23], indicating that the fungal degradation could occur without continuous supply of nutrients. The amount of recovered glucose (450 mg/g

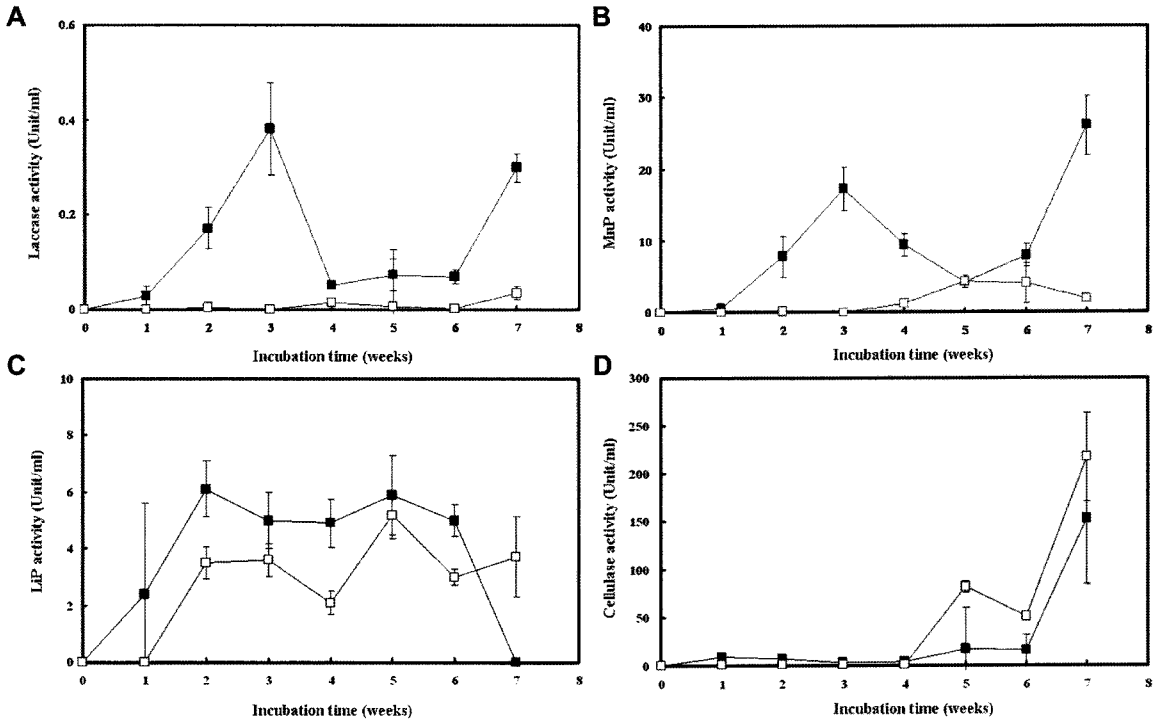

Fig. 5. Activities of laccase (**A**), manganese peroxidase (**B**), lignin peroxidase (**C**), and cellulase (**D**) during the biodegradation of wood chips of *L. tulipifera* on nutrient non-added agar. Symbols: ■, treated with *T. versicolor*; □, uninoculated control.

biodegraded wood chip) from *L. tulipifera* wood chips was much higher than that (9.68–13.56% of yield) from softwood of Japanese red pine treated by cellulase after pretreatment with white rot fungi, *Ceriporia lacerata*, *Stereum hirsutum*, and *Polyporus brumalis* [12], and the latter was similar to the glucose recovery of 10.6% from the wood chips of *P. strobus* pretreated with *I. lacteus* in this study. The glucose recovery in this study was slightly lower than that of 526 mg/g treated wood chip of olive tree by enzymatic hydrolysis after the pretreatment of steam explosion and alkaline peroxide; however, it was the total sugar value, including various monosaccharides [2]. Because the biodegradation of 10 g of wood chips was not aseptically treated, uninoculated control was contaminated by some fungi, and a small amount of glucose was released. The activities of all lignin-degrading enzymes were increased in the initial stage (Fig. 5), similar to solid state fermentation of *Achras zapota* by *Phanerochaete chrysosporium* [11]. After active degradation during initial 2–4 weeks of incubation, the activities of ligninolytic enzymes showed some fluctuations, but the cellulase activity increased. The activities of ligninolytic enzymes, especially laccase of *T. versicolor*, during degradation of *L. tulipifera* wood chips on NNA were lower than those on YMG agar, which indicated that nutrients may affect the enzyme activity as well as the weight loss of wood chips. Therefore, the selection of proper wood species and fungal strain and optimization of glucose recovery are all necessary for the fungal pretreatment of woody biomass as a carbon substrate. Simultaneous saccharification and fermentation technology [18] should be further investigated for bioethanol production using these white rot fungi.

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