Appl Biochem Biotechnol
DOI 10.1007/s12010-013-0412-y

Enhanced Lignin Biodegradation by a Laccase-Overexpressed White-Rot Fungus *Polyporus brumalis* in the Pretreatment of Wood Chips

Sun-Hwa Ryu · Myung-Kil Cho · Myungkil Kim · Sang-Min Jung · Jin-Ho Seo

Received: 13 March 2013 / Accepted: 19 July 2013

Abstract The laccase gene of *Polyporus brumalis* was genetically transformed to overexpress its laccase. The transformants exhibited increased laccase activity and effective decolorization of the dye Remazol Brilliant Blue R than the wild type. When the transformants were pretreated with wood chips from a red pine (softwood) and a tulip tree (hardwood) for 15 and 45 days, they showed higher lignin-degradation activity as well as higher wood-chip weight loss than the wild type. When the wood chips treated with the transformant were enzymatically saccharified, the highest sugar yields were found to be 32.5 % for the red pine wood and 29.5 % for the tulip tree wood, on the basis of the dried wood weights, which were 1.6-folds higher than those for the wild type. These results suggested that overexpression of the laccase gene from *P. brumalis* significantly contributed to the pretreatment of lignocellulose for increasing sugar yields.

Keywords Lignin biodegradation · Laccase · *Polyporus brumalis* · Pretreatment · White-rot fungi

Introduction

Due to the depletion of petroleum resources and the negative impact of fossil fuels on the environment (i.e., greenhouse gas emissions), there is a growing interest in the development of renewable fuels. The most common renewable fuel today is bioethanol produced mainly from sugar or starch. This resource, which is also an ingredient of animal feed and is used for a variety of human needs, is not sufficient to meet the increasing ethanol fuel demands and, as a result, grain prices have increased [1]. Therefore, lignocellulosic biomass such as that

S.-H. Ryu · M.-K. Cho · M. Kim (✉)
Division of Wood Chemistry and Microbiology, Department of Forest Products,
Korea Forest Research Institute, Seoul 130-712, South Korea
e-mail: mkkim@forest.go.kr

S.-H. Ryu · S.-M. Jung · J.-H. Seo (✉)
Department of Agricultural Biotechnology and Center for Food and Bioconversion,
Seoul National University, Seoul 151-921, South Korea
e-mail: jhseo94@snu.ac.kr

Published online: 23 August 2013

found in forests and agricultural residues may prove to be an alternative substrate for biofuel production. The first step in the conversion of lignocelluloses to biofuel is pretreatment, which is performed by degrading lignin to liberate cellulose and hemicellulose and allows for efficient enzymatic hydrolysis [2]. There has been an increasing interest in the study of microbial pretreatment of lignocelluloses [3]. White-rot fungi are the most effective lignin-degrading microorganisms in nature. Biological pretreatment by white-rot fungi has previously been investigated for enhancing lignocellulosic materials for paper-related applications. Recently, this environment-friendly approach has received renewed attention as a pretreatment method for enhancing enzymatic saccharification of lignocellulosic biomass. Delignification of different lignocellulosic biomasses has been investigated for several white-rot fungi. Pretreatment of *Pleurotus ostreatus* has been shown to increase hydrolysis of biomass feedstocks with a maximum glucose yield of 33 % for rice straw and 38.9 % for rice hull [4, 5]. Corn stover pretreated with *Cyathus stercoreus* showed 36 % conversion of cellulose to glucose [6]. Pretreatment with *Echinodontium taxodii* 2538 has been found to enhance the enzymatic hydrolysis of hardwood by a 4.7-fold (Chinese willow) and softwood (China fir) by a 6.3-fold [7]. The highest glucose yield, 66.6 %, has been obtained from corn stover pretreated with *Ceriporiopsis subvermispora* [8]. However, pretreatment with these fungi took a long time due to their low delignification rates, which is the major barrier for large-scale applications of fungal pretreatment.

White-rot fungi can completely mineralize wood lignin with ligninolytic enzymes such as laccase, lignin peroxidase (LiP), and manganese peroxidase (MnP) [9, 10]. It has been reported that significant lignin degradation occurred after the production of laccase and MnP [11, 12]. Therefore, enzymes that are produced during fungal cultivation are primarily responsible for lignin depolymerization.

The laccase gene was isolated from *Polyporus brumalis* which exhibited a high capacity of survival under toxic conditions and have the ability to degrade recalcitrant chemicals [13, 14]. The recombinant *P. brumalis* was constructed by introducing the laccase gene of this fungus. The recombinant *P. brumalis* strains with enhanced lignin-degradation activity were analyzed for the efficiency of the lignin-degradation activity and of saccharification when using lignocellulosic biomass.

Materials and Methods

Generation of Laccase Overexpression Constructs

A laccase overexpressing vector (pHYlac1) was constructed by using the *Laccase 1* gene (*pblac1*) from *P. brumalis* under the control of the constitutive promoter of the glyceraldehyde-3-phosphate dehydrogenase (*gpd*) gene and a modified pHYgpt plasmid vector containing the hygromycin-resistance gene (*hph*). *P. brumalis* was transformed using by REMI method as previously described [15] with slight modifications. Transformants were selected on minimal medium [16] with hygromycin (100 μg/ml). The genomic DNA from the transformants and the wild-type strain were used as the PCR template, and the *hph* gene was amplified using the following primers: 5′-TGGATATGTCCTGCGGGTAA-3′ as the forward primer and 5′-CGTC AGGACATTGTTGGAGC-3′ as the reverse primer. The laccase activity of the wild type and transformants grown in potato dextrose broth (PDB) were measured using a modified version of the Ross Method [17] with *o*-tolidine as a chromogenic enzyme substrate. Stock solutions of 4.7 mM *o*-tolidine were prepared in 95 % ethanol with 67 mM glycine and 2 mM glacial acetic acid. Assays were carried out in a 96-well multi-micro plate in a total volume of 300 μl at room temperature. The reaction was initiated by adding 7.5 μl of liquid culture medium containing laccase, and then, absorption spectra were taken following 5 min incubation at 590 nm. One

enzyme unit was defined as the amount of enzyme which generated 0.1 OD under the above conditions. The protein concentration was determined by Bradford methods [18]. The transformant strains were maintained on potato dextrose agar (PDA) containing hygromycin.

Remazol Brilliant Blue R Decolorization for Transformants

The assay system (300 μl volume) used contained a filtrate of crude broth, obtained after 6 days incubation of each transformant and wild-type strain in liquid culture in PDB media, 100 μg/mL Remazol Brilliant Blue R (RBBR). After a 5-h incubation at room temperature, the absorption of residual RBBR at 590 nm was determined to examine a capacity for fungal decolorization.

Biological Pretreatment of Wood Chips

Each strain was pregrown on 200 mL PDA medium in a cultivation box for 7 days at 28 °C. For the degradation of lignin in the wood chip, *Pinus densiflora* and *Liriodendron tulipifera* samples were cut to obtain 70×30×4 mm portions and were sterilized at 121 °C for 15 min. Three wood chips were placed onto PDA agar covered with the fungal mycelia and were then incubated for 15 and 45 days. All the cultures were grown in triplicate. A set of sterilized non-inoculated wood chips served as the control wood sample. After incubation, the mycelium attached to wood chips was removed. The wood chips were oven-dried at 50 °C and weighed to determine weight loss. The dried wood chips were milled to a 40-mesh powder for the lignin content analysis. The lignin content was determined using the Klason lignin method (TAPPI Method T249 cm-85) [19]. Comparative statistical analysis was performed with the analysis of variance (ANOVA) test.

Enzymatic Saccharification

For comparison of the sugar yields for the transformant and wild-type strain during the pretreatment process, cellulase from *Trichoderma reesei* ATCC 26921 (Sigma, USA) and cellobiase from *Aspergillus niger* (Sigma) were employed for saccharification. Biologically pretreated woody biomass was ground to pass through a 0.40-mm screen. Then, 0.1 g of ground wood product was transferred to a 100-ml Erlenmeyer flask, and 20 ml of 50 mM sodium acetate buffer (pH 5.0) was added. Next, appropriate amounts of cellulase (80 EGU/g) and β-glucosidase (72 IU/g) were added. The flask was placed in a shaking incubator at 50 °C and 150 rpm and incubated for 24 h. After hydrolysis, 2 ml of the incubation supernatants was centrifuged and filtered through a 0.45-μm filter. Monosaccharides were then isolated using high-performance anionic exchange chromatography (HPAEC) (Dionex Bio-LC50 system; Sunnyvalle, USA). The Carbo Pac PA10 Column (4×250 mm) and ED 50 pulsed amperometric detector (PAD) were used. Chromatography was performed using 3 mM NaOH as the mobile phase at 0.8 ml/min for 45 min. Arabinose, galactose, glucose, xylose, and mannose were used as standard monosaccharides.

Results and Discussion

Enhanced Laccase Activity of Transformants

The genomic integration of the laccase-expressing vector (pHYlac1) was confirmed by *hph*-specific PCR, and the expected amplified band appeared only in the transformants (T21 and T26) (Fig. 1a). The two transformants showed laccase activity approximately 3–4 times higher than that of the wild-type strain in the liquid medium (Fig. 1b). This result seems to be

due to the fact that the glyceraldehydes-3-phosphate dehydrogenase gene (*gpdA*) promoter [20] was used to overexpress the *Laccase 1* gene in the pHYlac vector [21]. The *gpdA* promoter has been widely used for expression of target genes constitutively in fungi. When *Phlebia tremellosa* and *Trametes versicolor* were transformed with the MnP cDNA driven by the *gpdA* promoter, the transformants showed higher MnP activity [16, 22]. Laccases are involved in the degradation of diverse recalcitrant compounds (e.g., dye) [23–25]. In order to investigate the effect of laccase overexpression in *P. brumalis*, the decolorization activity of the transformants was examined using an anthraquinone RBBR dye. As shown in Fig. 1c, the transformants exhibited higher decolorization capabilities than the wild type. The transformants, T21 and T26, decolorized 93–90 % of the dye while the wild type decolorized much less than the transformants (<21 %), coincidently with the laccase activity (Fig. 1). Purified *Laccase 1* decolorized RBBR by 70 % without a mediator [26], which is the same gene used to generate the pHYlac1 construct. Thus, the results in Fig. 1 suggested that the laccase activity of the transformants directly correlates with the decolorization of the dye. Laccases are multicopper blue oxidases and catalyze the oxidation of a wide range of inorganic and aromatic substances by the removal of electrons and the simultaneous reduction of O_2 to water [27]. Due to their oxidation power and relatively low specificity, laccases can oxidize various chemical structures [28]. Several studies reported correlations between the decolorization of dyes and the ligninolytic ability of several fungal strains [29–31]. Therefore, the decolorization of RBBR was used to detect the ligninolytic activity of fungi [32, 33]. The ligninolytic enzymes such as laccase

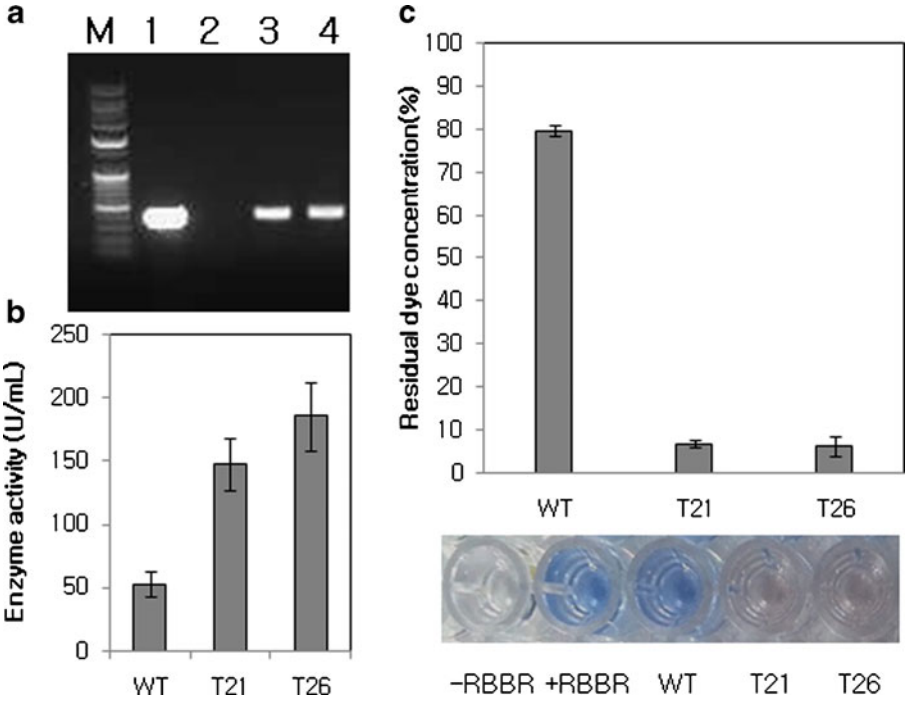

Fig. 1 Analysis of laccase activity and dye decolorization of *P. brumalis* transformants. Confirmation of genomic integration of the expression vector into the host chromosomal DNA by RT-PCR (**a**) (*M* molecular weight maker, *1* vector plasmid, *2* wild-type strain, *3* and *4* transformant). Analysis of laccase activity (**b**) and decolorization of RBBR (**c**) in culture broth of the wild type (WT) and two transformed strains (T21 and T26). The values represent the mean±SE of three measurements, with three replicates each

decolorize RBBR as a result of a redox reaction [21, 34]. Thus, the data in Fig. 1c indicated that the transformants have increased lignin-degradation activity.

Enhanced Wood Chip Lignin Degradation by Transformants

Weight loss of the woody biomass as a result of treatment with the wild type (WT) and transformants is shown in Fig. 2. *P. densiflora* (red pine) and *L. tulipifera* (tulip tree) were used as softwood and hardwood, respectively, for pretreatment. When *P. densiflora* wood chips were treated with the wild-type strain, transformant T21 and transformant T26, the weight losses after 45 days were 2.8, 4.3, and 5.8 %, respectively (Fig. 2a). In the case of *L. tulipifera* wood chips, the weight losses after biodegradation for 45 days were 30 % by WT, 37 % by T21, and 39 % by T26. The hard wood exhibited greater weight loss than the softwood after the treatment with all strains. Although the treatment period and the wood species were different, this phenomenon is common in the pretreatment with white-rot fungi

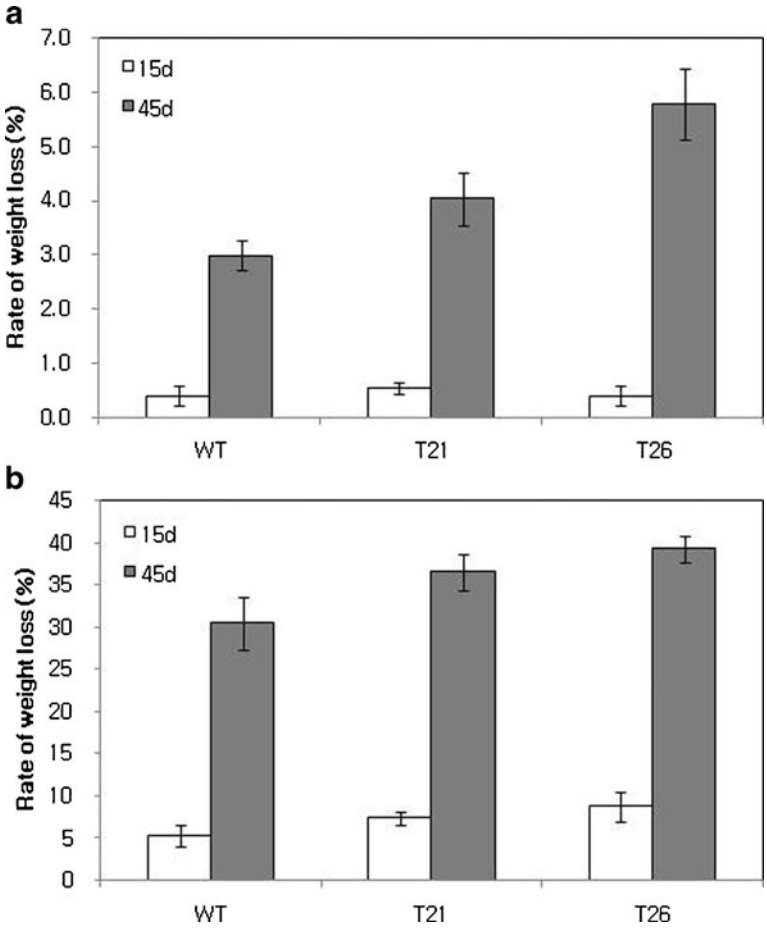

Fig. 2 Weight loss of *P. densiflora* (**a**) and *L. tulipifera* (**b**) after pretreatment with the wild-type strain and transformants for 15 and 45 days. The values represent the mean±SE of three measurements, with three replicates each

and consistent with previous reports using *Pinus strobus* as softwood and *L. tulipifera* as hardwood [35] and with *Cunninghamia lanceolata* as softwood and *Salix babylonica* as hardwood [5]. In this study, the weight losses of two woods by transformants were higher than those by the wild-type strain. Statistical analysis showed that the degrees of weight loss after 15 days were not much different among the strains tested. However, the degrees of lignin degradation by treatment with the transformants were more enhanced (Fig. 3). The highest lignin losses of *P. densiflora* by transformants were 10 % for 15 days and 14 % for 45 days which were two and 1.4-fold higher than those by the wild type. In *L. tulipifera*, the highest lignin losses were 16 % for 15 days and 42 % for 45 days, which were 3.2- and 1.4-fold higher than those for the wild type. In comparison with the previous research using *P. densiflora* [36], lignin loss (14 %) by the transformants after 45 days were greater than the reported case, where 11.6 % lignin loss was obtained after 56 days pretreatment by *P. brumalis*. Lee et al. [36] pretreated *P. densiflora* with three white-rot fungi, *Ceriporia lacerata*, *Stereum hirsutum*, and *P. brumalis*. After 8 weeks of pretreatment, the least lignin

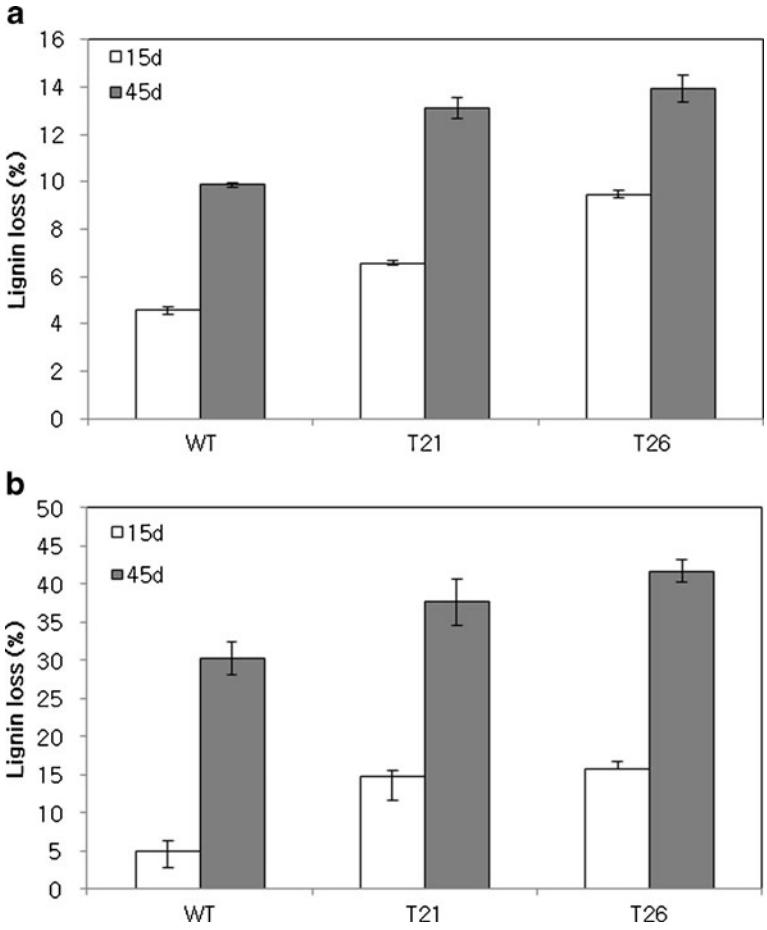

Fig. 3 Lignin loss of *P. densiflora* (**a**) and *L. tulipifera* (**b**) after pretreatment with the wild-type strain and the transformants for 15 and 45 days. The values represent the mean±SE of three measurements, with three replicates each

loss was 11.6 % with *P. brumalis* while the greatest lignin loss was 14.5 % with *S. hirsutum*. This result was due to the fact that *P. brumalis* ligninase activities were less than those of *S. hirsutum* during *P. densiflora* degradation. During *Acacia mangium* wood chip biodegradation with *Phellinus* sp., *Daedalea* sp., *Trametes versicolor*, and *Pycnoporus coccineus*, laccase activities were much greater than those of manganese peroxidase and lignin peroxidase at 10 and 20 days [37]. However, after 30 days, manganese peroxidase activities peaked and laccase activities were very low [37]. Therefore, laccase produced during the initial degradation stage might be primarily responsible for lignin depolymerization. In this study, the transformants enhanced laccase activity by three- to fourfolds compared to that of the wild type (Fig. 1). Thus, the degradation ability of lignin from the transformants were improved in both soft and hard wood (Fig. 2b) because transformants enhanced the activity of laccase. White-rot fungi from the hyphae penetrate adjacent cells through pore holes or pits. Fungal degradation of the cell wall components proceeds from the cell lumen toward the compound middle lamella, with gradual thinning of the cell wall. Softwood is composed of tracheids, parenchyma, and epithelial cells,

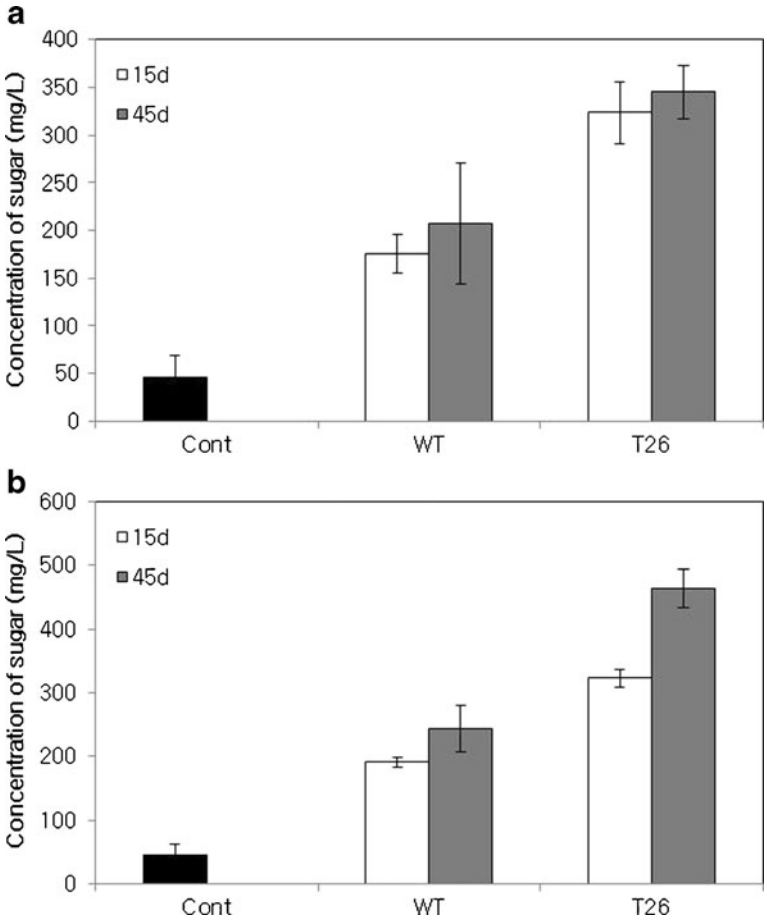

Fig. 4 Amount of total sugar after enzymatic saccharification of *P. densiflora* (**a**) and *L. tulipifera* (**b**) pretreated with the wild-type strain and the transformants for 15 and 45 days. The control had not been pretreated with the fungi. The values represent the mean±SE of three measurements, with three replicates each

whereas hardwood consists of fibers, tracheids, parenchyma, and epithelial cells [38]. The pores that the hyphae are able to penetrate are larger in size than intersectional hardwood fibers. Therefore, the weight loss for tulip tree hardwood was greater than that for red pine softwood. The lignin is uniformly distributed across the middle lamella and the secondary wall of the wood. Degradation of the secondary wall proceeds more rapidly, whereas, at the compound middle lamella, fungal degradation is retarded to a certain extent depending on the wood species. In the case of softwood, the lignin concentration in the middle lamella is higher (0.6–1.0 g/g) than that for hardwood (0.43–0.6) [39]. Thus, the lignin loss rate of the soft wood chips by transformants was lower than those of hard wood, but the increasing rate of lignin loss by transformants was higher in the soft than hard wood chips. The efficiency of loss of lignin tended to be similar to the weight loss fraction of the wood chips.

Sugar Yield of Pretreated Wood Chips

The enzyme saccharification of pretreated wood with fungi gave rise to different yields of total sugars, including glucose, arabinose, xylose, galactose, and mannose (Fig. 4). The pretreatment with the wild type for 15 days resulted in an increase in saccharification yield by a 3.8-fold for *P. densiflora* and a 4.2-fold for *L. tulipifera* as compared to the untreated control. When the *P. densiflora*-pretreated wood chips were treated with commercial cellulase, the highest amount of sugars obtained was 345 mg/L by T26, which was a 1.7-fold higher than that for the wild type (207 mg/L). For *L. tulipifera*, the highest sugar concentration was 464 mg/L by T26, which is a 1.9-fold increase compared to that for the wild type. Lee et al. [36] showed that pretreatment with *S. hirsutum*, which exhibited the highest ligninase activity, increased the available pore size to over 120 nm for *P. densiflora*, which in turn promoted adsorption of hydrolysis enzymes and efficient saccharification. The degradation of lignin by the ligninolytic enzyme from fungi was responsible for the increased available pore size. Thus, a saccharification yield in the transformant-pretreated wood may have been promoted by enhanced ligninolytic activity of laccase in the transformant. Considering the weight loss of the wood chip during pretreatment, the sugar yield was calculated on the basis of the dried wood weight used for pretreatment (Table 1). A sugar yield of T26 for the pretreatment with *P. densiflora* for 15 days were similar to that obtained at 45 days, although a sugar concentration of the 45-day pretreatment was higher than that of 15 days as shown in Fig. 3a. In the case of *L. tulipifera*, sugar yields at 15-day pretreatment were higher than those at 45 days. This is due to the weight loss of wood chips, which also includes the loss of cellulose converted to sugars. Pretreatment for 45 days revealed much higher biomass weight loss than that for 15 days, which caused a decreased total sugar yield based on the oven-dried original wood of *L. tulipifera*. Thus, for high recovery of sugars from lignocellulosic biomass, pretreatment used for fungi should have the ability to degrade lignin with minimal effects on cellulose loss. In a previous study, ligninase activities of *S.*

Table 1 Total sugar yields of non-treated and pretreated woody biomasses by commercial enzymes

Biological pretreatment		*P. densiflora*	*L. tulipifera*
Cont.		4.3±2.2	4.6±1.6
15 days	WT	17.4±2.0	18.2±0.7
	T26	32.2±3.2	29.5±1.3
45 days	WT	20.2±6.1	17.0±2.6
	T26	32.5±2.7	28.2±1.9

Values are expressed as the percent of sugar yield based on the oven-dried weight of original wood

hirsutum were the highest while the activities of the cellulolytic enzymes were similar to those of other fungi [36]. *P. densiflora* pretreated with *S. hirsutum* showed the highest sugar yield at 21.01 % based on dry weight of biomass, which was a 1.4-fold higher than that (14.91 %) of *P. brumalis*. However, the laccase-overexpressed transformant T26 showed 32.2 % sugar yield at 15 days, which was a 1.9-fold higher than that of the wild-type *P. brumalis* (Table 1) and also 1.5-fold higher than that of *S. hirsutum*. Thus, enhanced ligninolytic activity of the transformants contributed to the increase in sugar yield for the conversion of lignocellulosic biomass.

Some transformants with the ligninase gene were used for the degradation of endocrine-disrupting chemicals (EDCs), which mimic and interfere with the mechanisms of action of endogenous gonadal steroid hormones. The laccase activity of the *Irpex lacteus* transformant with the *P. tremellosa* laccase gene was sixfold greater than that of the wild-type strain and exhibited enhanced degradation activity in the case of EDCs [40]. Genetic transformation with genes for laccase and MnP in *P. tremellosa* concurrently augmented laccase and MnP activity and rapidly degraded two EDCs, that is bisphenol A and nonylphenol [40]. To utilize white-rot fungus transformants for the pretreatment of lignocelluloses for biofuel production is more complicated than using them for the degradation of EDCs. The reason is that white-rot fungi consume cellulose as a carbon source along with lignin, resulting in low cellulose recovery from biomass. *T. versicolor* MrP1, able to overexpress the manganese-repressed peroxidase gene, exhibited higher MnP activity and biomass weight loss than the wild type, but the glucose yield is lower than that of the wild type after pretreatment [35]. To develop cost-effective microbial pretreatment, the microbe's ability to delignify lignocellulosic biomass needs to be improved but weight loss of biomass should be minimized during pretreatment. Optimal growth conditions might be also important for achieving high lignin loss and low cellulose loss in parallel.

Conclusions

Genetic transformation of *P. brumalis* by using a laccase overexpression vector generated new strains with increased ligninolytic activity. These transformants exhibit enhanced lignin-degradation rates during the pretreatment of wood chips and sugar yields during saccharification of pretreated wood chips compared to the wild-type strains. Therefore, the overexpression of the laccase gene in *P. brumalis* via genetic transformation is a useful tool for the improvement of the biological conversion of lignocellulosic biomass.

Acknowledgments This study was carried out with the grant funded by Korea Forest Research Institute.

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