Biological Pretreatment with White Rot Fungi and Their Co-Culture to Overcome Lignocellulosic Recalcitrance for Improved Enzymatic Digestion

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Three white rot fungi (*Lenzites betulinus*, *Trametes orientalis*, and *Trametes velutina*) as well as their respective paired cultures were used to pretreat *Populus tomentosa* for enhanced lignocellulosic degradation and enzymatic hydrolysis. Hemicellulose and cellulose were slightly degraded, while a maximum lignin degradation of 58% was caused by *T. velutina* during the 12-week cultivation. After the pretreated samples were subjected to enzymatic hydrolysis for 96 h, the reducing sugar released by *T. orientalis* at week 12 was as high as 41%, which was in line with the lignin loss at 2.2 times the control sample. Overall, the monocultures of white-rot fungi exhibited better degradation and saccharification of woody biomass than their co-culture. This can be attributed to the partial removal of lignin and hemicellulose, with an associated increase of cellulose accessibility to enzymes.

Keywords: Co-culture; Biological pretreatment; Enzymatic hydrolysis; Ethanol; Woody biomass

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INTRODUCTION

Populus tomentosa, a native poplar wood widely cultivated in China, has a high potential for bioconversion because it can serve as a sustainable alternative for the production of ethanol. Like other lignocellulosic biomass, *P. tomentosa* must undergo pretreatment to break down the lignin structure and disrupt the crystalline structure of cellulose biomass to facilitate the hydrolysis of cellulose and other polymers and provide sugars for ethanol-producing organisms (Moiser *et al.* 2005).

Recently, fungal pretreatment has attracted much attention because it can disrupt the lignin-hemicellulose sheath and requires relatively low energy and mild environmental conditions (Wang *et al.* 2012; Yu *et al.* 2009). Fungal pretreatment shows great potential in converting lignocellulosic materials to ethanol. However, in wood and many other microenvironments, fungi typically live and grow in close proximity to each other. These fungi may form antagonistic interactions, resulting in faster nutrition exploitation or in parasitism, and perhaps may display deadlock interactions in which the hyphae of one species cannot enter the territory occupied by the other. Species can also form synergistic interactions to coordinate the degradation of the same substrate (Boddy 2000). Therefore, it has been hypothesized that the mixed fungal cultures may result in an efficient pretreatment of woody biomass through synergistic interactions. Previous investigations regarding fungal co-cultures have mainly focused on their interactions with each other and the production of enzymes, as well as lignocellulosic degradation (Baldrian 2004; Boddy 2000; Chi *et al.* 2007; Iakovlev and Stenlid 2000; Mata *et al*. 2005; Savoie and Mata 1999;

Score *et al.* 1997). Ma *et al*. (2011) have also reported the influences of a co-fungal culture of *Auricularia polytricha* AP with *Irpex lacteus* CD2 on the pyrolysis characteristics of corn stover. The effects of co-culture pretreatments on the enzymatic hydrolysis of woody biomass have never been extensively investigated.

In this work, monocultures of white-rot fungi (*Lenzites betulinus*, *Trametes orientalis*, or *Trametes velutina*) and their respective paired cultures were employed to pretreat *P. tomentosa*. The influences of both monoculture and co-culture pretreatments on component degradation and enzymatic saccharification were evaluated to exploit the application potential of these fungi.

EXPERIMENTAL

Materials

Fresh poplar wood (*Populus tomentosa*) from Shandong Province of China was chopped into small pieces and air-dried. The samples were ground, and particle sizes of 20 mesh and 80 mesh were prepared for subsequent pretreatment with fungal monoculture and co-culture, respectively.

Methods

Microorganism and inoculum preparation

Three white rot fungi, *Lenzites betulinus* C5617, *Trametes orientalis* C6320, and *Trametes velutina* D10149, were isolated from maple in Liaoning Province, fallen trunk in Hainan Province, and birch in Jilin Province in China, respectively. The organisms were preserved on 2% (w/v) malt-extract agar (MEA) plates at 4 °C at the Institute of Microbiology, Beijing Forestry University. These fungi were activated in 100 mL of basic medium (g/L: glucose, 20; yeast extract, 5; potassium phosphate monobasic, 1; magnesium sulfate, 0.5; Vitamin B_1, 0.01), and cultured on a rotary shaker at 28 °C at a speed of 150 rpm. After 5 days, 100 mL of distilled water was added to the mycelial pellets, and the suspensions were mixed with a laboratory blender for 30 s at 5000 rpm. This homogeneous suspension was the inoculum.

Biological pretreatment of poplar wood

Biological pretreatment was carried out in a 250-mL Erlenmeyer flask with 5 g of air-dried poplar wood and 12.5 mL of distilled water. The wood slurry above were sterilized in an autoclave at 121 °C for 20 min, cooled, and placed in each inoculum; for the monoculture, 5 mL of total inoculum; for the co-culture, 2.5 mL of each inoculum. These cultures were incubated without agitation at 28 °C. After 4, 8, and 12 weeks, the lignocellulosic substrate was thoroughly washed to remove the mycelia and dried at 40 °C in an oven for 24 h. Non-inoculated wood samples served as the untreated controls. All experiments were performed in triplicate.

Enzymatic hydrolysis

The cellulase preparations produced by *Trichoderma reesei* (ATCC 26921) and *β*-glucosidase from almonds were purchased from Sigma-Aldrich (USA). A typical hydrolysis mixture consisted of 0.2 g of the pretreated sample, 10 mL of 50 mM sodium acetate buffer (pH 4.8) supplemented with 40 μL of tetracycline and 20 μL of cycloheximide, 30 FPU/g of cellulose, and 37.5 IU/g of *β*-glucosidase. The mixture was

incubated at 50 °C in a rotary shaker at 150 rpm for 96 h. Samples were taken from the reaction mixture and centrifuged for 10 min at11180 x g, then stored at -20 °C until further use. All experiments were performed in duplicate.

Analytical methods

The chemical composition of the raw material and the pretreated residues was determined according to Sluiter *et al.* (2008) using High Performance Anion Exchange ChromatographyHPAEC. The HPAEC system (Dionex ISC 3000, USA) was equipped with an amperometric detector, AS50 autosampler, a carbopac^{TM} PA-20 column (4×250 mm, Dionex), and a guard PA-20 column (3×30 mm, Dionex). The cellulose contents were calculated based on glucose using the anhydro correction of 0.9, while the hemicellulose contents were calculated based the sum of xylose, galactose, and arabinose using 0.88 as the anhydro correction for xylose and arabinose and 0.9 as that for galactose.

The reducing sugar in the supernatant after enzymatic hydrolysis was measured by the dinitrosalicyclic acid (DNS) method according to Miller (1959). DNS is an aromatic compound that reacts with reducing sugars and other reducing molecules to form 3-amino-5-nitrosalicylic acid, which absorbs light strongly at 540 nm. It was first introduced as a method to detect reducing substances in urine and has since been widely used. The reducing sugar yield was calculated as follows:

$$\text{Reducing sugar yields (\%)} = \frac{\text{amount of reducing sugar in enzyme hydrolysate} \times 0.9 \times 100}{\text{amount of cellulose and hemicellulose}} \quad (1)$$

Statistical analysis

The software SPSS 18.0 (USA) was used for statistical analysis. All degradation data and sugar yields were subjected to analysis of variance (ANOVA) using PROC GLM. Multiple comparisons among different pretreatment methods were performed with Tukey's test with a significance level of 0.05.

RESULTS AND DISCUSSION

Decay of Wood in Mono- and Co-Cultures

The poplar wood was degraded by monocultures and co-cultures of the three white rot fungi. Overall, weight loss (dry mass) increased with culture time, ranging from 8.1% (*L. betulinus* at week 4) to 37.92% (*T. velutina* at week 12) (Fig. 1). The amount of weight loss of the three co-cultures ranged between *T. velutina* and other monocultures.

As shown in Fig. 2, during 12-week cultivation, the three white-rot fungi and their corresponding paired cultures degraded lignin. With respect to the monocultures, the lignin content of pretreated samples decreased with the culture time. A maximum lignin degradation was observed at week 12 by *T. velutina* (58.1% lignin degradation) and by *T. orientalis* (47.3% lignin degradation). In addition, the three monocultures exhibited higher lignin degradation abilities than the paired cultures. At each culture period, lignin losses by monocultures were higher than that of co-cultures.

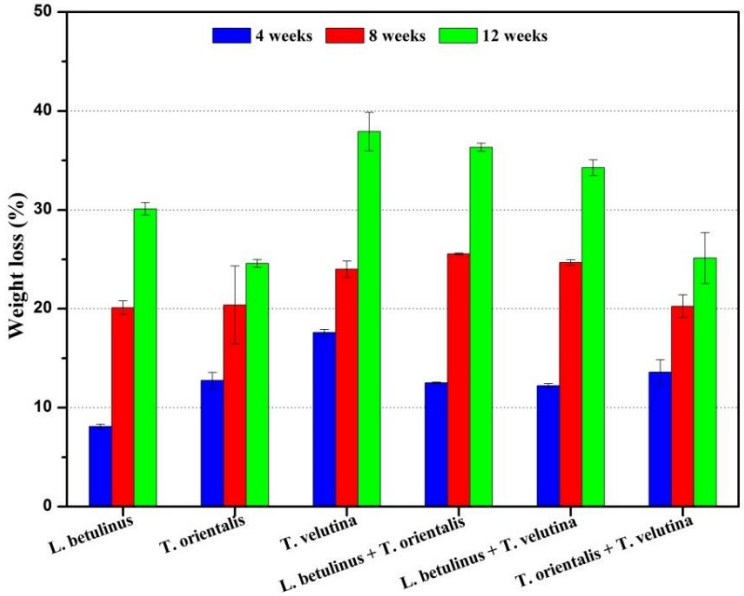

Fig. 1. Weight loss of fungal mono- and co-cultures from 4 weeks to 12 weeks. "%" was defined as dry mass loss based on initial mass. Values were measured in triplicate (n = 3) and are reported as the mean ±SD.

Similar to lignin degradation, both hemicellulose and cellulose decreased with the culture periods, regardless of fungal monocultures or co-cultures (Figs. 3 and 4). Fungal co-cultures consumed less hemicellulose than monocultures (Fig. 3). Up to 17.4% of hemicellulose remained in the co-culture of *T. orientalis* and *T. velutina* after 12 weeks, just a little lower than that in control samples (18.7%). Regarding the cellulose (Fig. 4), it was notable that, after 12 weeks, only 13% and 9.4% of cellulose had been decayed by monoculture *T. orientalis* and co-culture of *T. orientalis* and *T. velutina*, respectively, suggesting that both cultures performed well at preserving cellulose.

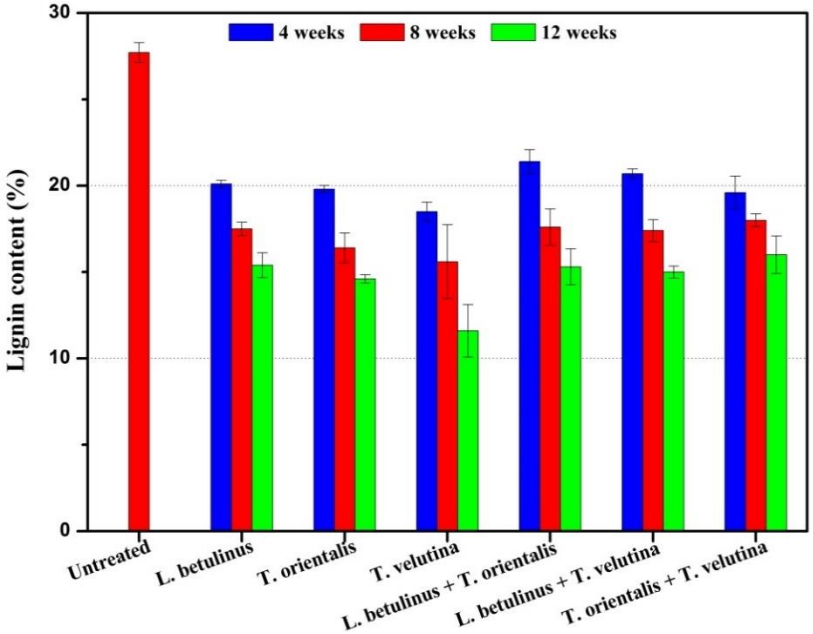

Fig. 2. Lignin content of fungal mono- and co-cultures from 4 weeks to 12 weeks. "%" was defined as lignin percent based on initial mass. Values were measured in triplicate (n = 3) and are reported as the mean±SD

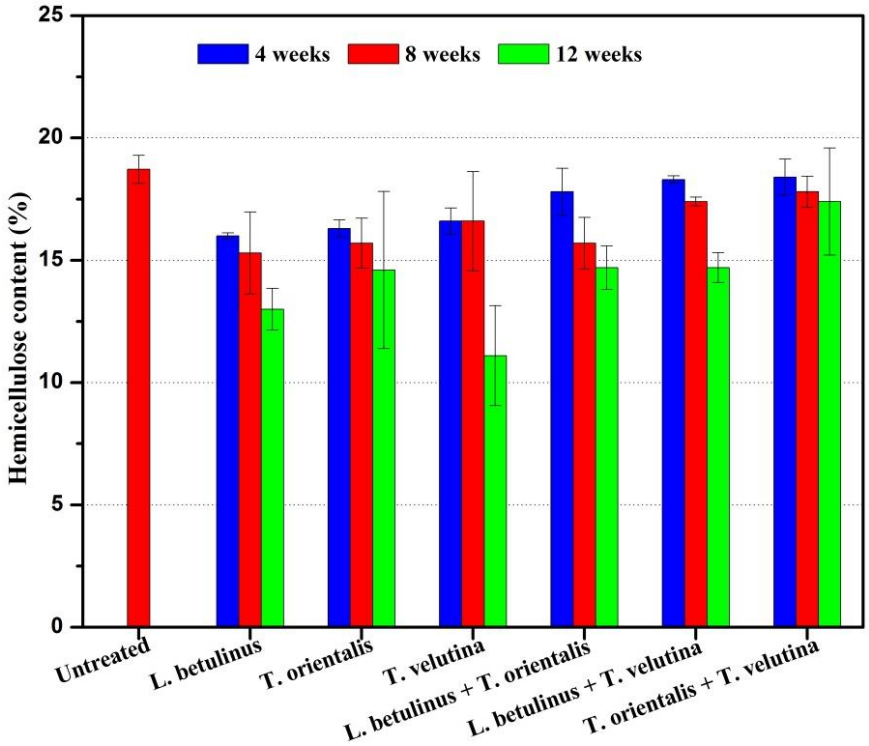

Fig. 3. Hemicellulose content of fungal mono- and co-cultures from 4 weeks to 12 weeks. "%" was defined as hemicellulose percent based on initial mass. Values were measured in triplicate (n = 3) and are reported as the mean±SD

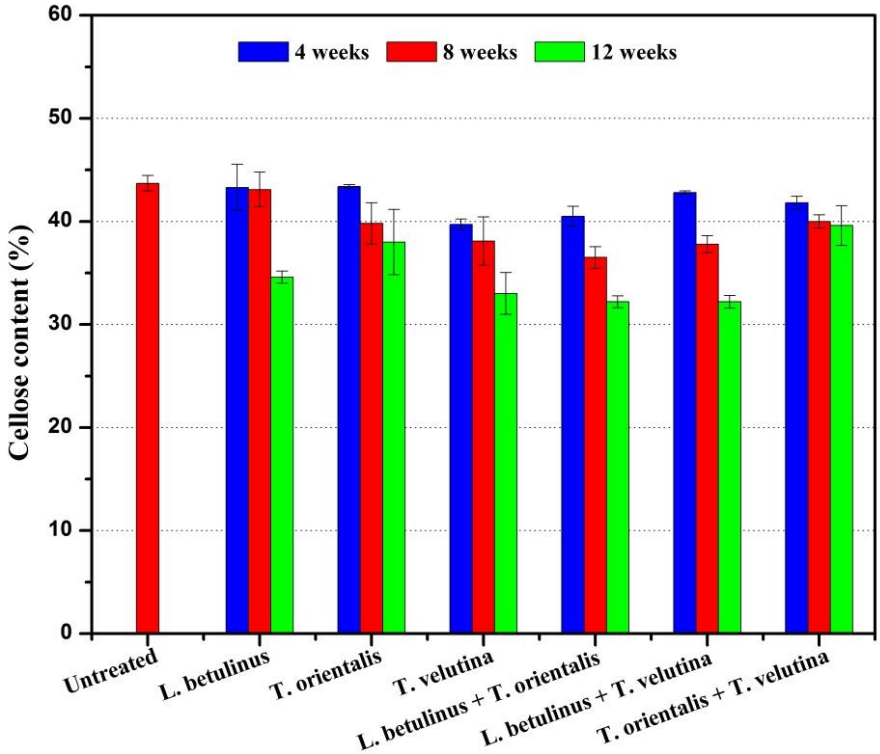

Fig. 4. Cellulose content of fungal mono- and co-cultures from 4 weeks to 12 weeks. "%" was defined as cellulose percent based on initial mass. Values were measured in triplicate (n = 3) and are reported as the mean±SD

Effects of Mono- and Co-Cultures on Enzymatic Hydrolysis

After various fungal pretreatments with a duration from 4 weeks to 12 weeks, the pretreated poplar wood and control samples were exposed to enzymatic hydrolysis for 96 h. The profile of reducing sugar yield is shown in Fig. 5. The fungus-treated samples released much more reducing sugar than the untreated samples ($P<0.05$). The highest reducing sugar yield of 41.3% was obtained by *T. orientalis* at week 12, which was 2.2 times more than the untreated samples. The long pretreatment period resulted in more release of reducing sugar, which is especially obvious in monocultures.

The monocultures *T. orientalis* and *T. velutina* released significantly ($P<0.05$) more reducing sugar than the three co-cultures at each time period. The 8-week monocultures of *T. orientalis* and *T. velutina* released 34.0% and 33.0% of reducing sugar, respectively, which exceeded the best performing paired culture of *T. orientalis* with *T. velutina* (32.2%) at week 12.

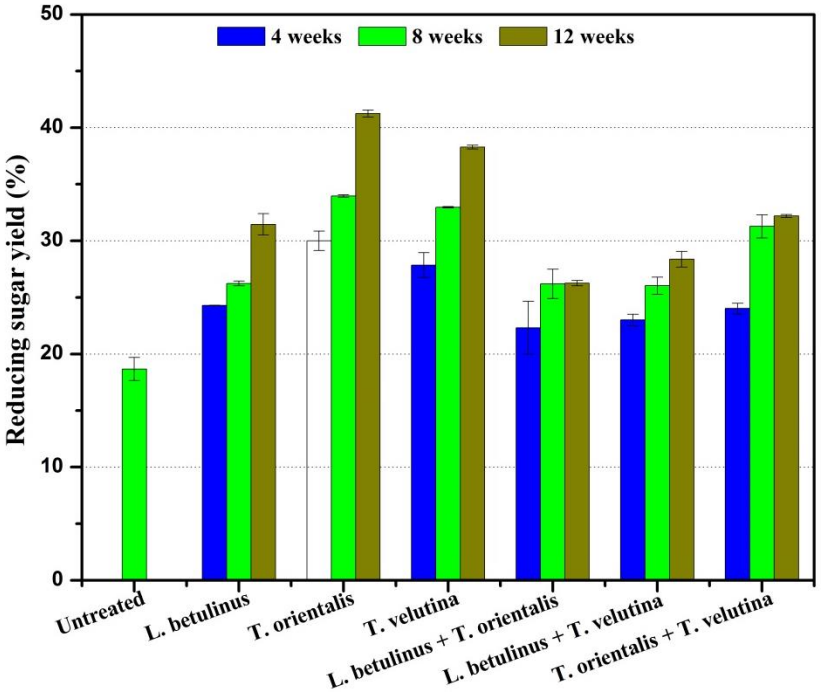

Fig. 5. Reducing sugar yield after 96-h enzymatic hydrolysis of pretreated samples by mono- and co-cultures pretreatment and untreated samples. "%" was defined as the percent of reducing sugar released from cellulose and hemicellulose in enzymatic hydrolysis. Values were measured in duplicate (n = 2) and are reported as the mean±SD

As mentioned previously, fungal pretreatments resulted in high lignin loss but low-level degradation of hemicellulose and cellulose, indicating that these three white rot fungi and their co-cultures performed well in the decay of lignin while preserving carbohydrates, which was in line with the requirements of pretreatment (Sun and Cheng 2002). Meanwhile, it can be concluded that the selective delignification of the white-rot strains *T. orientalis* and *T. velutina* were remarkable, suggesting that these two strains should be further investigated to evaluate their potential in practical application.

It was unexpected that an inhibitory effect of co-cultures on wood degradation occurred during the fungal pretreatments, which was contrary to previous reports (Chi *et al*. 2007; Ma *et al*. 2011; Parani and Eyini 2010). The investigation by Ma *et al*. (2011) showed that a co-culture of *Irpex lacteus* CD2 and *Auricularia polytricha* AP had a

synergetic effect on the degradation of cellulose and hemicelluloses and showed advantages in the degradation of lignocelluloses and destruction of lignocellulose structure. A similar result found that a co-culture of *Pleurotus flabellatus* with *Pleurotus eous* or *Phanerochaete chrysosporium* resulted in an enhancement of holocellulose (cellulose and hemicellulose) degradation (Parani and Eyini 2010). However, as Chi *et al*. (2007) reported, this facilitation was species-specific, so the co-culture also may be slightly stimulating or not stimulatory. In this study, during fungal growth, no hyphae of one species can enter the territory occupied by hyphae of the other, indicating that the three species employed to form the co-culture generate a deadlock interaction. Therefore, low nutrition exploitation occurs, which subsequently causes low degradation of lignocellulose.

The conversion of woody biomass to ethanol is difficult because of the ultrastructural resistance to breakdown and the presence of lignin, which protects carbohydrates from enzymatic attack (Munoz *et al*. 2007). Yu *et al*. (2009) have reported that about 35% and 18% of reducing sugar yield were achieved by the enzymatic hydrolysis of 17 week fungal-treated Chinese willow and China fir, respectively. Only a low 14% glucose yield was obtained when saccharification was performed with 8 week pretreated material by *Stereum hirsutum* (Monrrory *et al*. 2011). In the present work, when poplar wood treated with *T. orientalis* for 12 weeks was subjected to enzymatic hydrolysis, up to 41.3% of reducing sugar was released, implying that *T. orientalis* may prove to be a suitable choice in the pretreatment of lignocellulosic biomass.

Lignin is known to be the primary obstacle to cellulose digestibility. Lignin removal can dramatically increase the pore sizes of biomass, provide more accessible surface area to cellulase and accordingly facilitate the hydrolysis rate (Wang *et al*. 2013; Yu *et al*. 2009). The removal of lignin also reduced the irreversibly binding hydrolytic enzymes and increased the recycling of enzymes (Sun and Chen 2002). Interestingly, the results presented in this study apparently show, in most cases, that more lignin removal resulted in more sugar release. This indicates that sugar yield was inversely proportional to lignin content, which is in good agreement to previous studies (Sun *et al*. 2011; Wan *et al.* 2010).

Mixed fungal cultures can lead to higher enzyme production through synergistic interaction, the final result seems to depend on the particular species combination or on the mode of interaction between species, as well as on the microenvironmental or nutritional conditions in the substrate under colonization (Chi *et al*. 2007; Gutierrez-Correa and Tengerdy 1997). To the best of our knowledge, this is the first report showing the effects of co-cultures of white rot fungi on saccharification of woody biomass. Contrary to expectations, there was no enhancement of enzymatic hydrolysis by fungal co-cultures. This can be attributed to the low lignocellulosic degradation ability of the co-cultures, resulting from the dead-lock interaction between the species used in this work. However, from the negative results in this case study, it is inappropriate to conclude that co-culture pretreatment of white rot fungi would inhibit subsequent enzymatic hydrolysis of lignocellulosic biomass. Since the interaction between fungi is species-specific and depends on the culture conditions, it is still reasonable to hypothesize that co-culture pretreatment may facilitate subsequent saccharification. Screening for the appropriate species to form a paired culture and exploring the optimal conditions for specific co-cultures are currently under investigation.

CONCLUSIONS

1. Three white rot fungi and their respective paired cultures were used to pretreat *Populus tomentosa*. The hemicellulose and cellulose were slightly degraded while a large quantity of lignin was removed during the 12-week cultivation.
2. The reducing sugar yield was in line with the lignin loss, with as high as 41.3% of reducing sugar released by *T. orientalis* at week 12.
3. Monocultures of white-rot fungi performed better at the degradation and saccharification of woody biomass than did their co-cultures.

ACKNOWLEDGMENTS

This research was supported by the Fundamental Research Funds for the Central Universities (Project No. JC2013-1), the Program for New Century Excellent Talents in University (NCET-11-0585), and the Major State Basic Research Projects of China (973-2010CB732204).

REFERENCES CITED

Baldrian, P. (2004). "Increase of laccase activity during interspecific interactions of white-rot fungi," *FEMS Microbiol. Ecol.* 50(3), 245-253.

Boddy, L. (2000). "Interspecific combative interactions between wood-decaying basidiomycetes," *FEMS Microbiol. Ecol*. 31(3), 185-194.

Chi, Y., Hatakka, A., and Maijala, P. (2007). "Can co-culturing of two white-rot fungi increase lignin degradation and the production of lignin-degrading enzymes?" *Int. Biodeter. Biodegr*. 59(1), 32-39.

Gutierrez-Correa, M., and Tengerdy, R. P. (1997). "Production of cellulase on sugar cane bagasse by fungal mixed culture solid substrate fermentation," *Biotechnol. Lett*. 19(7), 665-667.

Iakovlev, A., and Stenlid, J. (2000). "Spatiotemporal patterns of laccase activity in interacting mycelia of wood-decaying basidiomycete fungi," *Microb. Ecol*. 39(3), 236-245.

Ma, F. Y., Wang, J. J., Zeng, Y. L., Yu, H. B., Yang, Y., and Zhang, X. Y. (2011). "Influence of the co-fungal treatment with two white rot fungi on the lignocellulosic degradation and thermogravimetry of corn stover," *Process Biochem*. 46(9), 1767-1773.

Mata, G., Hernández, D., and Andreu, L. (2005). "Changes in lignocellulolytic enzyme activities in six *Pleurotus* spp. strains cultivated on coffee pulp in confrontation with *Trichoderma* spp," *World J. Microb. Biot*. 21(2), 143-150.

Miller, G. L. (1959). "Use of dinitrosalicyclic acid reagent for determination of reducing sugar," *Anal. Chem*. 31(3), 426-428.

Monrroy, M., Ortegab, I., Ramírezb, M., Baezaa, J., and Freer, J. (2011). "Structural change in wood by brown rot fungi and effect on enzymatic hydrolysis," *Enzyme Microb. Tech*. 49(5), 472-477.

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y. Y., Holtzapple, M., and Ladisch, M. (2005). "Features of promising technologies for pretreatment of lignocellulosic biomass," *Bioresour. Technol*. 96(6), 673-686.

Munoz, C., Mendonc, R., Baeza, J., Berlin, A., Saddler, J., and Freer, J. (2007). "Bioethanol production from bioorganosolv pulps of *Pinus radiata* and *Acacia dealbata*," *J. Chem Technol Biot*. 82(8), 767-774.

Parani, K., and Eyini, M. (2010). "Effect of co-fungal treatment on biodegradation of coffee pulp waste in solid state fermentation," *Asian J. Exp. Biol. Sci*. 1(2), 352-359.

Savoie, J. M., and Mata, G. (1999). "The antagonistic action of *Trichoderma* sp. hyphae to *Lentinula edodes* hyphae changes lignocellulotytic activities during cultivation in wheat straw," *World J. Microb. Biot*. 15(6), 369-373.

Score, A. J., Palfreyman, J. W., and White, N. A. (1997). "Extracellular phenoloxidase and peroxidase enzyme production during interspecific fungal interactions," *Int. Biodeter. Biodegr*. 39(3), 225-233.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., and Crocker, D. (2008). "Determination of structural carbohydrates and lignin in biomass. NREL/TP-510-42618 NREL Laboratory Analytical Procedure," National Renewable Energy Laboratory, Golden, CO, USA. http://www.nrel.gov/biomass/analytical_procedures.html www.nrel.gov/biomass/pdfs/42618.pdf

Sun, F. H., Li, J., Yuan, Y. X., Yan, Z. Y., and Liu, X. F. (2011). "Effect of biological pretreatment with *Trametes hirsuta* yj9 on enzymatic hydrolysis of corn stover," *Int. Biodeter. Biodegr*. 65(7), 931-938.

Sun, Y., and Cheng, J. (2002). "Hydrolysis of lignocellulosic materials for ethanol production: A review," *Bioresour. Technol*. 83, 1-11.

Wan, C. X., Li, Y. L., and Li, Y. B. (2010). "Microbial pretreatment of corn stover with *Ceriporiopsis subvermispora* for enzymatic hydrolysis and ethanol production," *Bioresour. Technol*. 101(16), 6398-6403.

Wang, W., Yuan, T. Q., Cui, B. K., and Dai, Y. C. (2013). "Investigating lignin and hemicellulose in white rot fungus-pretreated wood that affect enzymatic hydrolysis," *Bioresour. Technol*. 134, 381-385.

Wang, W., Yuan, T. Q., Wang, K., Cui, B. K., and Dai, Y. C. (2012). "Combination of biological pretreatment with liquid hot water pretreatment to enhance enzymatic hydrolysis of *Populus tomentosa*," *Bioresour. Technol*. 107, 282-286.

Yu, H. B., Guo, G. N., Zhang, X. Y., Yan, K. L., and Xu, C. Y. (2009). "The effect of biological pretreatment with the selective white-rot fungus *Echinodontium taxodii* on enzymatic hydrolysis of softwoods and hardwoods," *Bioresour. Technol*. 100, 5170-5175.

Article submitted: March 5, 2014; Peer review completed: May 7, 2014; Revised version received and accepted: May 8, 2014; Published: May 14, 2014.