WOOD RESEARCH
60 (1): 2015
25-32

ENHANCING ENZYMATIC DIGESTIBILITY OF BAMBOO BY FUNGAL PRETREATMENT

Daochun Qin, Hongling Ren, Lusheng Zhang, Zhiqiang Li
International Centre For Bamboo and Rattan
Beijing, P.R. China

(Received May 2014)

ABSTRACT

Bamboo, as one of the most abundant cellulosic materials in China, has a great potential to be used for bioenergy production. However, with a high content of free sugars and starch, it is susceptible to be attacked by termites and decay fungi. In this research, four bamboo species were biopretreated with six species of fungi. The relationship between cellulose-to-glucose conversion yield (CGCY) of the pretreated substrates and fungi species, pretreatment time, and culture medium were tested. The results indicated that some tested fungi could significantly improve the CGCY after pretreatment. The CGCY increased from 2.4 (raw untreated bamboo) to 26.0 %. The enzymatic digestibility of bamboo pretreated by fungi that were cultured on sand & sawdust medium were better than that cultured on potato dextrose agar medium. It was feasible to treat bamboo species with fungi for the saccharification.

KEYWORDS: Bamboo, bioenergy, fungal pretreatment, enzymatic hydrolysis, glucose yield.

INTRODUCTION

In order to mitigate the growing energy and environment crisis, bioenergy production from lignocellulosic biomass materials has been received considerable attention. Bamboo, as one of the most abundant cellulosic materials in China, has great potential to be used for birefinery production such as bioethanol. However, the complex structure of bamboo is highly resistant to enzymatic hydrolysis, resulting in low cellulose-to-glucose conversion yield (CGCY). There have been some reports on pretreatment processes for bamboo. Dilute acid pretreatment and alkaline pretreatment can significantly improve the enzymatic digestibility of bamboo, but the CGCY was limited (Leenakul and Tippayawong 2010). Sulfite pretreatment to overcome the recalcitrance of lignocellulose (SPORL) pretreatment seems ineffective on bamboo, as the CGCY was much lower than for some woods that were pretreated under the same conditions (Li et al. 2012a). Organosolv pretreatment of bamboo can significantly increase the CGCY, which is normally over 80 %. However, a considerable part of the cellulose and the hemicelluloses are degraded

into fermentation inhibitor compounds, like furfural and hydroxymethylfurfural (HMF) (Li et al. 2012b).

Recently, because of the environment pollution, the high cost and high energy devotion in the pretreatment process, fungal pretreatment as a more environmentally friendly compatible approach has received renewed attention as a pretreatment method for enhancing enzymatic saccharification of lignocellulosic biomass in the ethanol production processes. Fungal pretreatment of agricultural residuals and hardwood can also achieve 15 % to 5 folds increase of methane yield (Zheng et al. 2014). Biological pretreatments employ microorganisms, primarily mainly brown-, white- and soft-rot fungi, which degrade lignin (minimal with brown rot fungi), hemicelluloses and/or cellulose, and very little of cellulose, which is more resistant to breakdown, intact than the other components (Sánchez 2009). Fungus pretreatment with *G. trabeum* proved to be an effective way of increasing enzymatic hydrolysis of corn stover for bioethanol production (Gao et al. 2012). Bamboo, with its high content of free sugars and starch, is susceptible to be attacked by termites and decay fungi (Jiang 2007). It was feasible to treat moso bamboo residues with *Coriolus versicolor* B1 for the saccharification has been demonstrated. Zhang et al. (2007) examined that moso bamboo was pretreated with this fungus *Coriolus versicolor* B1 under certain conditons caused a significant enhancement of the saccharification rate and the maximum saccharification rate was 37.0 %. They defined the saccharification rate as the percentage of holocellulose (cellulose and hemicelluloses) in raw material converted to reduce sugar by considering the weight loss during the pretreatment. Indeed, the hemicelluloses are more easily saccharificated than cellulose. The main goal of pretreatment is to improve the cellulose saccharification rate. Otherwise, the free sugars in the raw bamboo were not considered. Bamboos easily get mildewed, one of the reasons is the high free sugars and starch contents in bamboo.

This study focused on the fungal pretreatment for bamboos to enhance the cellulose-to-glucose conversion yield of enzymatic hydrolysis. And the free sugars include glucose in the raw bamboo are considered. Four common species of Chinese bamboo (excluding moso bamboo, which is a good construction material), six fungi species, and two kinds of medium were investigated in this study of the enzymatic digestibility of bamboo.

MATERIAL AND METHODS

Material

Four species of bamboo, *Neosinocalamus affinis, Bambusa rigida* Keng et Keng f., *Dendrocalamus yunnanicus*, and *Bambusa pervaniabilis*, were acquired from Chishui, Guizhou province, China. After being air-dried, the culms of the bamboo were prepared as specimens for fungal pretreatment with dimensions of 20 mm longitudinal (L) by 20 mm tangential (T) by 3 to 5 mm radial (R). Feeding strips were *Populus tomentosa* Carr wood, with dimensions of 20 mm longitudinal (L) by 20 mm tangential (T) by 5 mm radial (R).

Six species of fungi were purchase from the China Forestry Culture Collection Center (CFCC). There were two brown-rot fungi: *Gloeophyllum trabeum* (Pers.) Murrill and *Postia placenta*; one soft-rot fungus: *Cheatomium globosum*; and three white-rot fungi: *Coriolus versicolor* (CFCC No. 5336), *Trametes versicolor* (CFCC No. 6282), and *Phanerochaete chrysosphorium.*

Cellulase was purchased from Shanghai Eysin Biotechnology Co., Ltd., China. All chemical reagents used in this research were purchased from Sinopharm Chemical Reagent Beijing Co., Ltd., China.

Pretreatment in potato dextrose agar (PDA) Medium

The medium was brewed with 300 g of potato, 20 g of dextrose and 15 to 20 g of agar. The medium was sterilized at 121°C for 30 min, and then poured into 90-mm- diameter culture dishes. Then, the test fungal strains were introduced to the sterilized solid medium. Culture dishes were then placed in a 28°C, 80 % relative humidity conditioning room for 7 to 10 days. Bamboo specimens were sterilized by gamma ray and introduced into the culture dishes on a completely mycelium-covered surface. Glass rods were placed between the medium and specimens to avoid direct contact. Six replicates were performed for each decay fungus and treatment.

Pretreatment in sand and sawdust medium

Fungal cultures were prepared by mixing the following in a glass jar: 75 g of clean sand, 7.5 g of wood sawdust, 4.3 g of corn flour, and 0.5 g of brown sugar. After being uniformly stirred, two separately feed strips were placed on the surface of the mixture, and then filled with 50 mL of maltose solution. Glass jars were sterilized at 121°C for 60 minutes. The fungal strains were introduced to the sterilized medium. Glass jars were then placed into a 28°C, 80 % relative humidity conditioning room for about 10 days. Bamboo specimens were sterilized by gamma ray radiation and introduced into the culture dishes on a completely mycelium-covered surface. Glass rods were placed between the medium and specimens to avoid direct contact. Six replicates were performed for each decay fungus and treatment.

At set pretreatment times, the bamboo specimens were sampled and hyp has were removed from the surface of the specimens. Afterwards, these pretreated specimens were milled into a powder, which was passed through a 40- to 60-mesh sieve for further analysis.

Enzymatic hydrolysis

Enzymatic hydrolysis was carried out in a 150-mL flask at 50°C, which was placed into a shaking incubator (KYC-100C, Shanghai Fuma Laboratory Equipment Co. Ltd., China) at 260 rpm. Pretreated bamboo substrate equivalent to 0.8 g of glucan was loaded with 40 mL of a 0.05 M sodium acetate buffer (pH 4.8). Approximately 1.5 mg of tetracycline chloride was added to control the growth of the microorganisms and to prevent the consumption of liberated sugars. Two enzymes, cellulase (20 filter paper units (FPU) per gram glucan) and β-glucosidase (40 international units (IU) per gram glucan), were loaded. Hydrolysates were sampled to analyze glucose concentration. The hydrolysis was conducted in duplicate for each substrate; the averages of the duplicates are reported here.

Analytical methods

The free sugars and starch in raw bamboos and pretreated bamboo substrates were determined by analyzed water/ethanol extractives. The raw bamboo and fungal pretreated bamboo substrates were extracted with water in soxhlet extractor for 12 to 16 h, and then with ethanol extraction for 6 to 8 h. The solution with extractives was hydrolyzed to monomeric sugars under a 3 % sulfuric acid in a steam sterilizer at 121°C. The monomeric sugars were determined by HPLC.

The carbohydrates of the untreated and all pretreated bamboo substrates were analyzed in accordance with the National Renewable Energy Laboratory (NREL) analytical procedure "Determination of structural carbohydrates and lignin in biomass" (NREL/TP-510-42618, 2008), with some modification. This method is based on the degradation of carbohydrates (cellulose and hemicellulose) into monomeric sugars by a two-stage sulfuric acid hydrolysis, from which the sugars are analyzed and quantified with HPLC.

The enzymatic hydrolysis reactions from each of the pretreated substrates were monitored by measuring the glucose in the reaction solutions. For fast analysis, the glucose in the solutions was determined by a commercial Biosensor Analyzer (SBA-40E, Shandong Academy of Science, Shandong Province, China). The instrument precision was about 2 %, based on the manufacturer specifications. The average of duplicate runs is reported here. The cellulose-to-glucose conversion yield (CGCY) was calculated as:

$$CGCY = (c \times V \times 0.9)/m \times 100 \quad (\%)$$

where: c - the concentration of glucose in hydrolyzate, $g.L^{-1}$;
V - the volume of hydrolyzate, L
m - the weight of cellulose in the substrate (g).

According to the filter paper assay recommended by the International Union of Pure and Applied Chemists, the cellulase activity was determined and its expression is filter paper units (FPUs). β-glucosidase activity was determined through p-nitrophenyl-b-D-glucoside as the substrate and its expression is International Units (IUs).

RESULTS AND DISCUSSION

Chemical composition and properties of bamboo

Similar to other lignocellulosic biomass, bamboo consist of cellulose, hemicelluloses, and lignin. The chemical compositions and physical properties of the four bamboo species are listed in Tab. 1. Compared to wood, bamboo has higher cellulose content, over 45 %. The cellulose content of *B. rigida* bamboo reached as high as 48.7 %. The lignin content of bamboo is similar to that of softwoods (Li et al. 2008). However, the extractives of bamboo are much higher than that of wood, and bamboo has more free sugars and starch. Thus, bamboo is susceptible to attack by termites and decay fungi due to free sugars and starch as nutrients (Ma et al. 2011). The density of the tested bamboos ranged from 0.58 to 0.60 $g.cm^{-3}$.

Tab. 1: Chemical constituents and physical properties of bamboo species (%).

Bamboos	Density ($g.cm^{-3}$)	Cellulose	Hemicellulose	Lignin	Ash	Hot water extractives	Free sugars and starch
N. affinis	0.601±0.012	46.8±2.3	15.7±0.8	23.4±0.4	3.7±0.1	10.9±0.9	6.7±0.3
D. yunnanicus	0.591±0.010	47.5±1.8	20.1±0.6	24.1±0.3	1.3±0.0	9.5±0.8	8.5±0.1
B. rigida	0.599±0.008	48.7±2.0	16.1±0.9	22.3±0.3	2.7±0.1	9.3±0.8	7.4±0.2
B. pervaniabilis	0.594±0.011	45.1±1.9	19.3±1.0	20.4±0.2	2.8±0.1	8.9±0.9	8.4±0.2

The cross-sections of the four species bamboo tested are shown in Fig. 1. The magnification in Fig. 1 is 45 times. Vascular bundles with dark color and arranged in rings on the cross-sections of the bamboos. The vascular bundles rings were isolated by parenchyma. The distributions of vascular bundles were shown in Fig. 1. It indicated that the vascular bundles of *B. rigida* Keng et Keng f. was slightly large and dense distribution (a); the vascular bundles of *N. affinis* was large and dense distribution (b); the vascular bundles of *B. pervaniabilis* was slightly small but dense distribution (c); and, the vascular bundles of *D. yunnanicus* was slightly large and slightly dispersed distribution.

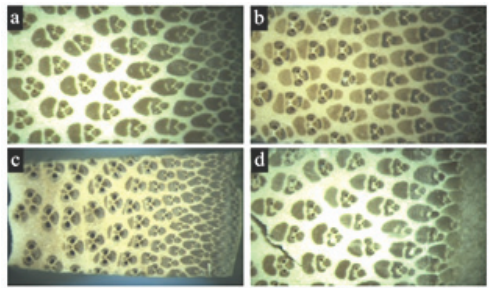

Fig. 1: Cross sections of bamboo species (at 45x magnification): a) B. rigida; b) N. affinis; c) B. pervaniabilis; d) D. yunnanicus.

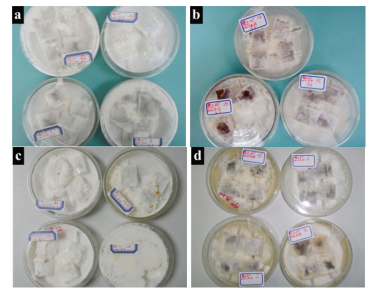

Fig. 2: The growth of fungi on PDA medium: a) T. versicolor, 10 days; b) G. trabeum, 10 days; c) T. versicolor, 30 days; d) G. trabeum, 30 days.

The vascular bundles distribution of bamboo is one of the methods to evaluate the economic utilization of bamboo. It closely related with the physical properties of the bamboo, such as density, strength and bamboo pulping (Huang et al. 2012). Meanwhile, bamboo is a kind of woody grass, and its maturity usually within 3-5 years. The composition and convertibility of bamboo may be largely determined by its maturity. Less mature grass resulted in higher cellulose convertibility due to the lower lignin content (Ambye-Jensen et al. 2013).

Fungal pretreatment of bamboo

The cellulose and lignin contents of *N. affinis* during the course of fungal pretreatment are shown in Tab. 2. Both the cellulose and lignin contents declined with increasing pretreatment time. The cellulose content decreased from 46.8 to 36.5 % for *T. versicolor* and to 35.2 for *G. trabeum*. The lignin content declined from 23.4 to 17.4 % for *T. versicolor*) and to 20.2 % for *G. trabeum*. Lignin content and its distribution are critical factors that affect the enzymatic hydrolysis of the lignocelluloses (Siqueira et al. 2011). The lignin was degraded during the fungal pretreatment; however, considerable cellulose was also degraded. Therefore, further research may focus on how to minimize the loss of cellulose and hemicelluloses while significantly improving the enzymatic hydrolysis of the substrates.

Tab. 2: The cellulose and lignin contents of N. affinis pretreated with T. versicolor and G. trabeum.

Pretreated time (d)	Cellulose content (%)		Lignin content (%)	
	T. versicolor	*G. trabeum*	*T. versicolor*	*G. trabeum*
0	46.8±2.3	46.8±2.3	23.4±0.4	23.4±0.4
7	44.0±1.8	43.8±2.0	21.9±0.3	22.7±0.9
14	42.5±2.2	42.6±1.3	19.9±0.5	21.9±0.4
21	40.7±1.0	40.8±0.9	18.9±0.1	21.1±0.6
28	38.4±1.2	37.9±2.1	18.2±0.8	20.8 ±0.3
35	37.5±1.9	36.7±1.7	17.8±0.4	20.5 ±0.7
42	36.5±2.1	35.2±2.0	17.4±0.7	20.2±0.3

The growth conditions of the fungi on sand & sawdust medium and PDA medium are presented in Fig. 2. The fungal hypha of the sand & sawdust medium grew faster than the hypha on the PDA medium. It took about 7 days for fungal hypha to cover the bamboo specimens with

the sand & sawdust medium; and the hypha tightly wrapped the specimens with very a thick layer after 30 days of growth. However, for the PDA medium, it took about 10 days for the fungal hypha to cover the bamboo specimens, while the hypha layer was also thinner after 30 days of growth. The growth of *T. versicolor* was better than that of *G. trabeum*, which was consistent with the effect of enzymatic hydrolysis of the fungal pretreated bamboo. The CGCY of *T. versicolor*- pretreated bamboo substrates was higher than that of *G. trabeum*-pretreated bamboo substrates. Song et al. (2013) reported that manganese could greatly improve the effects of fungal pretreatment and enzymatic hydrolysis of corn stover. Biological pretreatment with *I. lacteus* enhanced the enzymatic hydrolysis of biomass and the yield of glucose reached to 191.45 $mg.g^{-1}$ corn stover. It is consistent with bamboo biological pretreatment. A rapidly increase of glucose yield was obtained with manganese added in corn stover. In the future research, manganese maybe also an option for promoting effect of biological pretreatment of bamboo.

Effect of fungus species on enzymatic hydrolysis of bamboo

N. affinis was chosen as an example to illustrate the effect of fungus species pretreatment on bamboo enzymatic hydrolysis. For both pretreatment in both potato dextrose agar and sand & sawdust medium, the pretreated time was 30 days. Afterwards, the pretreated bamboo substrates were milled into a powder and the powder was subjected to enzymatic hydrolysis. And the free sugars and starch content in the bamboo substrates were analyzed. They were negligible. It indicates that free sugars and starch were prior to be consumed by fungi. The CGCYs of pretreated bamboo substrates after enzymatic hydrolysis are shown in Fig. 3. The CGCY of untreated bamboo was only 2.5 %. Fungus species pretreatment affected the CGCY of the bamboo substrates. Bamboo pretreated with *G. trabeum* (Pers.) Murrill and *T. versicolor* had higher CGCYs (12.7 to 17.5 %). For every tested fungus species, the CGCY of sand & sawdust medium was higher than the PDA medium. However, the effect of fungal pretreatment on enzymatic hydrolysis was very limited.

Fig. 4 shows the CGCY of the four tested species bamboo utilizing *G. trabeum* (Pers.) Murrill and *T. versicolor* fungal pretreatments. The pretreatment time was also 30 days. The CGCY of the sand & sawdust medium-pretreated bamboo was higher than that of the PDA medium-pretreated bamboo, which ranged from 2 to 8 %. The CGCYs of *D. yunnanicus, B. pervaniabilis*, and *B. rigida* after pretreatment with sand & sawdust medium and *T. versicolor* were 25.6, 24.6 and 22.5 %, respectively. The bamboo components degraded by fungi excretive enzyme. In different pretreated medium, the fungi enzyme would excretived in different kinds and quantity.

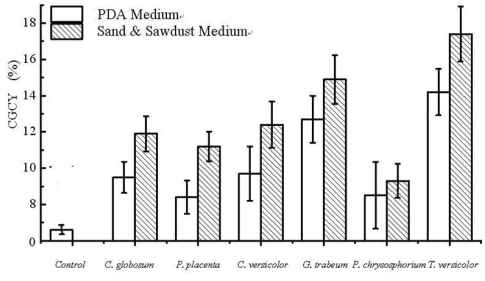

Fig. 3: The CGCYs of N. affinis pretreated with six fungi in various medium. CGCY: cellulose-to-glucose conversion yield.

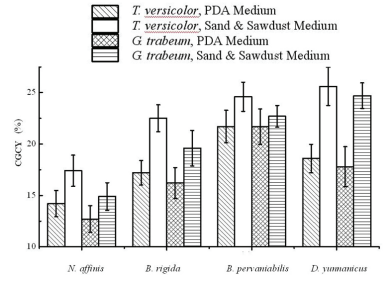

Fig. 4: The CGCY of various bamboo species pretreated with G. trabeum or T. versicolor in various medium.

So it causes the bamboo substrates in different hydrolyability. Based on the biological mechanisms of lignocelluloses degradation, the search for new enzymes or interesting enzyme complexes is valuable. For further study, we should focuse on fungal secretomes for enhancing the enzymatic hydrolysis of lignocelluloses (Salvachúa et al. 2013).

CONCLUSIONS

In summary, the cellulose-to-glucose conversion yield (CGCY) of pretreated bamboo substrates was below 30 %. The effect of fungal pretreatment on enzymatic hydrolysis of bamboo was very limited. The fungal pretreatment effect of the sand & sawdust medium was better than that of the PDA medium. *G. trabeum*- and *T. versicolor*-pretreated bamboo substrates had higher CGCYs than bamboo treated with other fungus species. Both fungus species *G. trabeum*- and *T. versicolor* degraded both cellulose and lignin of the bamboo.

ACKNOWLEDGEMENT

The authors are grateful for the support from the National Project of Scientific and Technical Supporting Program (No. 2012BAD54G01), the Fundamental Research Funds for the International Centre for Bamboo and Rattan (No. 1632012001), and the Beijing Key Lab Co-Bulit Projects.

REFERENCES

1. Ambye-Jensen, M., Johansen, K.S., Didion, T., Kádár, Z., Schmidt, J.E., Meyer, A.S., 2013: Ensiling as biological pretreatment of grass (*Festulolium* Hykor): The effect of composition, dry matter, and inocula on cellulose convertibility. Biomass and Bioenergy 58(2): 303-312.
2. Gao, Z., Mori, T., Kondo, R., 2012: The pretreatment of corn stover with *Gloeophyllum trabeum* KU-41 for enzymatic hydrolysis. Biotechnology for Biofuels 5(28): 1-11.
3. Huang, D., Zhou, A., Li, H., Su, Y., Chen, G., 2012: Experimental study on the tensile properties of bamboo related to its distribution of vascular bundles. Key Engineering Materials 517: 112-117.
4. Jiang, Z.H., 2007: Bamboo and rattan in the world. Chinese Forestry Publishing House. Beijing, China. Pp 144-147 (in Chinese with English summary).
5. Huang, D., Zhou, A., Li, H., Su, Y., Chen, G., 2012: Experimental study on the tensile properties of bamboo related to its distribution of vascular bundles. Key Engineering Materials 517: 112-117.
6. Li, K., Jin, Y., Gan, M., Liu, X., Zhao, H., 2008: Progress in research of key techniques for ethanol production from lignocellulos. Chinese Journal of Applied Environment and Biology 14(6): 877-884.
7. Li, Z., Jiang, Z., Fei, B., Pan, X., Cai, Z., Liu, X., Yu, Y., 2012b: Ethanol organosolv pretreatment of bamboo for efficient enzymatic saccharification. BioResources 7(3): 3452-3462.

8. Li, Z., Yang, Q., Jiang, Z., Fei, B., Cai, Z., Pan, X., 2012a: Comparative study of sulfite (SPORL), dilute-acid and NaOH pretreatments of bamboo for enzymatic saccharification. Journal of Biobased Materials and Bioenergy 6(5): 544-551.
9. Ma, X., Jiang, M., Li, Z., 2011: Wood biodegradation and protection. Chinese Forestry Publishing house, Beijing, China. Pp 134-139.
10. NREL/TP-510-42618, 2008: Laboratory analytical procedure (LAP): Determination of structural carbohydrates and lignin in biomass.
11. Salvachúa, D., Martínez, A.T., Tien, M., López-Lucendo, M.F., García, F., Ríos, V., Martínez, M.J., Prieto, A., 2013: Differential proteomic analysis of the secretome of *Irpex lacteus* and other white-rot fungi during wheat straw pretreatment. Biotechnology for Biofuels 6(1):115.
12. Sánchez, C., 2009: Lignocellulosic residues: Biodegradation and bioconversion by fungi. Biotechnology Advices 27(2): 185-194.
13. Siqueira, G., Milagres, A.M., Carvalho, W., Gerald Koch, G., Ferraz, A., 2011: Topochemical distribution of lignin and hydroxycinnamic acids in sugar-cane cell walls and its correlation with the enzymatic hydrolysis of polysaccharides. Biotechnology for Biofuels 4(3): 7.
14. Song, L., Ma, F., Zeng, Y., Zhang, X., Yu, H., 2013: The promoting effects of manganese on biological pretreatment with *Irpex lacteus* and enzymatic hydrolysis of corn stover. Bioresource Technology 135: 89-92.
15. Zhang, X., Xu, C., Wang, H., 2007: Pretreatment of bamboo residues with *Coriolus versicolor* for enzymatic hydrolysis. Journal of Bioscience and Bioengineering 104(2): 149-151.
16. Zheng, Y., Zhao, J., Xu, F., Li, Y., 2014: Pretreatment of lignocellulosic biomass for enhanced biogas production. Progress in Energy and Combustion Science 42(1): 35-53.

Daochun Qin, Hongling Ren, Lusheng Zhang, Zhiqiang Li*
International Centre For Bamboo and Rattan
Beijing 100102
China
Corresponding author: lizq@icbr.ac.cn