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Comparison of pretreatment protocols for cellulase-mediated saccharification of wood derived from transgenic low-xylan lines of cottonwood (*P. trichocarpa*)

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ABSTRACT

The novel low xylan content transgenic cottonwood (P. trichocarpa) was used to elucidate recalcitrance of enzymatic saccharification with or without four different pretreatment methods. The xylan contents of two transgenic samples (8Di3 and 8Di5) were 11.4% and 11.7%, respectively, as compared with the wild type (16.0%). Contrarily, the lignin contents of two transgenic samples were 23.1% and 24.5%, respectively, as compared with the wild type (20.8%). The four pretreatments were dilute acid (0.1% sulfuric acid, 185 °C, 30 min), green liquor (6% total titratable alkali (TTA), 25% sulfidity based on TTA, 185 °C and 15 min), auto hydrolysis (185 °C, 30 min) and ozone delignification (25 °C, 30 min). Following the pretreatment, enzymatic saccharification was carried out using three enzyme charges of 10, 20 and 30 FPU per gram of substrate. The removal of lignin and hemicelluloses varied with the type of pretreatment and with the lignin content of the transgenic trees. High lignin content implied low enzymatic saccharification. Low xylan content native substrates lead to high enzymatic saccharification. High S to V (sryingaldehyde to vanillin) ratio substrates had high delignification during pretreatment. Compared to the wild type, the transgenics were better choice as feed stocks due to higher enzymatic saccharification without pretreatment which mean low the cost of bio-ethanol. Compared to three pretreatment methods, the green liquor pretreatment greatly improves the conversion of polysaccharides in general.

1. Introduction

Due to the increasing price of gasoline and emission of greenhouse gas, bio-energy such as bio-ethanol and bio-diesel converted from biomass is an attractive forefront to the field of alternative energy. Biomass is a naturally sustainable resource which includes forest and mill residues, agricultural crops and waste, wood and wood wastes, animal wastes, livestock operation residues, aquatic plants, fast-growing trees and plants, and municipal and industrial wastes. It has been estimated that bio-ethanol produced from biomass resources could decrease greenhouse gas emission by 86% [1]. Now, bio-ethanol is the most important renewable fuel in terms of volume and market value [2], however most of it is produced from sugar-or starch-based materials such as sugarcane and corn. As a result, the cost of this type of

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bio-ethanol is very high and it also causes social problems; for instance, it is competing with food supply. Fortunately, the second generation bio-ethanol derived from lignocellulosics is a promising alternative and is already being tested in pilot plants [3,4]. Lignocellulosic biomass is the most abundant type on earth, which can be converted into fuels by enzymatic hydrolysis and (or) microbial fermentation. Lignocellulosics are composed of cellulose, hemicelluloses, lignin, extractives, and several inorganic materials [5]. Lignin is the most recalcitrant component of the plant cell wall; and the higher content of lignin, the higher the resistance to chemical and enzymatic degradation. The inherent properties of native lignocellulosics materials make them resistant to enzymatic attack. Thus, certain kinds of pretreatments make carbohydrates accessible to enzymes. Several methods have been introduced for the pretreatment of lignocellulosics which are classified into *Physical*, *Physico-chemical*, *Chemical*, and *Biological* pretreatment. There were many hypotheses tested to elucidate the correlation between the pretreatments and enzyme saccharification improvement [6–11]. Various studies have reported that cellulose digestibility improves with increasing lignin removal, although differences have been reported in the degree of necessary lignin removal. On the other side, many investigations have shown a direct relationship between cellulose digestibility and hemicelluloses and (or) xylan removal, where the lignin removal is not necessary for good cellulose digestibility.

In this article, the effect of xylan content and lignin content on enzymatic saccharification was evaluated with or without pretreatment, using two low xylan transgenic cottonwood samples and a wild type as control. Four pretreatments, diluted sulfuric acid, ozone delignification, auto hydrolysis and green liquor were applied as pretreatments on all substrates. Thus we can figure out the correlation between lignin content and enzymatic saccharification. We also can elucidate the relationship between hemicelluloses (xylan) content and enzymatic saccharification.

2. Materials and methods

2.1. Raw materials

Wood of two low xylan transgenic cottonwood (*Populus trichocarpa*) and wild type (WT) were collected from trees grown in a green house. All of these three samples are about one and a half years old. Two transgenics ptrGT8D-RNAi-2 and ptrGT8D-RNAi-5 (8Di3 and 8Di5), the gene expression of GT8D (glycosyltransferase) were silenced.

All air-dried and debarked samples were ground to pass 40 mesh sieves using a Wiley mill. The fraction between 40 and 60 mesh was collected and used as raw materials. Raw materials were extracted by 2:1 (volume) benzene: ethanol for 8 h to remove the extractives.

Green liquor was prepared by dissolving 0.375 g of Na_2S and 1.125 g of Na_2CO_3 in 100 mL of de-ion water.

Enzymes used for saccharification were kindly provided by Novozyme North America, Inc. (Franklinton, NC). A cocktail of enzymes were prepared by mixing cellulase (NS50013), hemicellulase (NS500014) and β-glucosidase (NS50010) in a ratio of 1 FPU: 1.2 FXU: 1 CBU.

2.2. Pretreatments on substrates

For acid hydrolysis (AH) and auto hydrolysis (Auto), sawdust of each substrate (0.75 g) was reacted with 3 ml of reagents (0.1% (weight) sulfuric acid and De-ion water, respectively) at 185 °C for 30 min sealed in a stainless steel reactor. Green liquor pretreatment (GL) was carried out as the same as AH and Auto except the reaction time was 15 min. For ozone delignification (Ozone), 0.75 g of dried sawdust was suspended in 15 ml of aqueous acetic acid solution (pH = 4.0) and oxygen containing 3% ozone was bubbled through the slurry at 25 °C for 30 min with a flow rate of 1L per min. After reaction, the mixture was filtrated under vacuum. About 0.1g of air-dried pretreated substrates was used for the chemical composition analysis. About 0.5g pretreated substrates went to enzymatic saccharification. 0.1g of air-dried residue was used for the chemical composition change analysis after pretreatment.

2.3. Composition analysis of substrates

The lignin content of all samples before and after pretreatment was determined according to the TAPPI Standard Method T222 om-98. Briefly, 0.1 g dried sawdust reacted with 1.5 ml 72% (weight) H_2SO_4 at room temperature for 2 h with stirring every 15 min. Then the reaction was diluted with 56 ml D.I. water (the concentration of H_2SO_4 was 3%) and transferred to a serum bottle. The mixture was subjected to autoclaving at 122 °C for 1.5 h. The concentration of sugars (arabinose, rhaminose, galactose, glucose, xylose and mannose) in the filtrate of Klason lignin determination was quantified by Dionex-IC (Dionex IC-3000; Dionex, USA). The Dionex-IC system was equipped with an ion-exchange PA1 (Dionex) column, a pulsed amperometric detector with a gold electrode, and a Spectra AS 300 autosampler. Prior to injection, samples were filtered through 0.2 um Nylon filters (Millipore) and a volume of 5 uL was loaded.

Table 1 – Composition of original wood (mean ± SD) (%).

Species	Glucan	Xylan	TC*	TL*	Extractive	Balance	S/V ratio
8Di3	45.1 ± 0.25	11.4 ± 0.08	62.2 ± 0.42	23.1 ± 0.07	3.5 ± 0.05	85.3	2.2 ± 0.08
8Di5	45.1 ± 0.37	11.7 ± 0.02	62.2 ± 0.38	24.5 ± 0.01	3.6 ± 0.03	86.7	2.5 ± 0.10
WT	45.4 ± 0.12	16.0 ± 0.02	64.6 ± 0.10	20.8 ± 0.16	2.7 ± 0.02	86.4	2.7 ± 0.07

Note: Sugars are expressed as % (w/w) of original extracted free substrate and S/V ratio. TC*: Total carbohydrates including arabinan, rhamnan, galactan, glucan, xylan and mannan. TL*: Total lignin including Klason lignin and acid soluble lignin.