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Research paper

Heterologous expression of thermoregulated xylanases in switchgrass reduces the amount of exogenous enzyme required for saccharification

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ABSTRACT

The expression of cell wall degrading enzymes in bioenergy crops has been proposed as a cost-effective method to produce fuel and chemicals. In this study, the thermoregulated intein-modified endo-β-1,4-xylanases GH10 and GH11 (iXyn GH10 and iXyn GH11) were expressed in switchgrass (*Panicum virgatum*). Two xylanase-expressing lines (Alamo background genetics) were compared to the wild type (Alamo genetics) to determine their composition, pretreatment, enzymatic hydrolysis, and ethanol fermentation. Three switchgrass samples with similar carbohydrate contents performed differently under four selected pretreatment conditions utilizing ammonia, oxalic acid/ferric chloride, sodium carbonate, or sodium hydroxide. Pretreated iXyn samples had higher glucan and lower xylan concentrations compared to the pretreated Alamo. An increased rate of glucose production and a higher glucan conversion ratio were observed in both iXyn lines. Pretreated iXyn samples required less exogenous enzyme to achieve similar levels of saccharification. Ethanol production increased from the glucan portion of the pretreated iXyn lines. However, the overall yield may vary depending upon the characteristics of the yeast and its conversion processes.

1. Introduction

Overcoming biomass recalcitrance is a key step to produce biofuels and manufacture renewable chemicals. The efficient saccharification of plant polysaccharides remains a major technical challenge. To reach an efficient enzymatic hydrolysis, a combination of cellulases, hemicellulases and lignin degrading enzymes is used to depolymerize the main components of cellulosic materials [1]. Currently, the high unit operation capital and enzyme costs required to convert cellulosic biomass into fermentable sugars impedes the progress of cellulosic fuel and chemical production [2].

Endo-β-1,4-xylanases are a group of glycosidases that catalyze the β-1,4-xylosidic bonds of xylan. Xylanases are classified into glycoside hydrolase families 5, 7, 8, 10, 11 and 43 based on their catalytic domain sequence, reaction mechanism, substrate specificity and physiological characteristics. Among the six xylanase families, families 10 (GH10) and 11 (GH11) have been studied extensively. The members of the GH10 family have an $(\alpha/\beta)_8$ barrel-like structure with a high molecular weight and a low isoelectric point (pI) value, while members of the GH11 family typically have a β-jelly roll structure with a low molecular weight and a high pI value [3]. GH10 xylanases can cleave the xylan backbone adjacent to substituted xylose residues [4] that is essential for maize cell wall degradation, since the xylose residues in maize xylan contain arabinose and glucuronic acid substitutions that limit the depolymerization of xylan by the GH11 xylanases. In addition to their uses in cellulosic biomass conversion, xylanases are also utilized in the cereal processing, baking, animal feed, and fiber industries [5].

The heterologous expression of cell wall degrading (CWD) enzymes in bioenergy crops has been proposed to reduce the production cost of cellulosic biofuels [6]. Expressed CWD enzymes accumulate in the biomass, enabling a reduction in the amount of resources necessary for enzyme preparation and improving the apparent accessibility of cell wall substrates [7]. While the plant-expressed enzymes improve the economics of cellulosic biorefineries, the expression in plants and the subsequent accumulation of certain CWD enzymes have been reported to cause plant damage, infertility and seed shriveling [8]. Inteins, a group of self-splicing peptides, were used to create fusions within CWD enzymes to develop thermoregulated intein-modified enzymes. An intein inserted into the target CWD enzyme disrupts its function during bioenergy crop planting, harvesting and storage. However, the enzyme activity could be restored after the induction of intein splicing that excises the intein using temperature stimuli ranging from 50 °C to 75 °C [9].

Pretreatment is an essential step in cellulosic fuel conversion to

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break down the linkages within the cell wall components and open the cellulose crystalline structure. Pretreatment methods, including biological, chemical, physical, and physio-chemical methods, have been studied on many types of feedstocks. Alkali such as sodium carbonate, ammonia, or sodium hydroxide lead to the removal of lignin by disconnecting its ether bonds [10]. Dicarboxylic organic acids, such as oxalic acid, chelate with the magnesium or ferric ion to hydrolyze lignin and depolymerize hemicellulose [11]. Inorganic salts, such as ferric chloride, are used as metal catalysts to increase the release of sugar [12]. Currently, studies on the pretreatment of biomass expressing CWD enzymes are limited.

The objective of this study was to investigate the pretreatment methods, hydrolysis kinetics and fuel ethanol conversion of the iXyn10 and iXyn11 endo-β-1,4-xylanases expressed in switchgrass in comparison with the wild type.

2. Materials and methods

2.1. Raw materials, enzymes and microbial strains

Switchgrass, including the wild type (Alamo) and two lines expressing intein-modified xylanases (iXyn GH10, iXyn GH11), was harvested in 2012. The switchgrass lines were generated using *Agrobacterium* transformation of embryogenic calli derived from switchgrass seeds as described previously with minor modifications [9,13]. Samples were air dried, cut into one-inch pieces and ground in a hammer mill (model MHM4, Glen Mills, Clifton, NJ) to pass through a 250 μm screen. The milled biomass was sealed in plastic bags and stored in a 4 °C refrigerator until further analysis. Cellic® CTec2 cellulosic enzyme (140 FPU mL^{-1}) was obtained from Novozymes Inc. (Franklinton, NC). Commercial Ethanol Red® yeast was a gift from the Lesaffre Yeast Corporation (Milwaukee, WI). All of the chemicals and reagents, unless stated otherwise, were of analytical grade and purchased from Fisher Scientific (Springfield, NJ).

2.2. Biomass composition analysis

The composition of the raw and pretreated switchgrass samples was analyzed following the National Renewable Laboratory (NREL) standard procedures: "Determination of extractives in biomass (TP- 510-42619)," "Determination of structural carbohydrates and lignin in biomass (TP-510-42618)," and "Determination of ash in biomass (NREL/TP-510-42622)." Briefly, non-structural materials were first removed by utilizing sequential hot water and alcohol extraction; the samples were sealed in filter bags (XT4, Ankom Technology, Macedon, NY) and extracted by deionized (DI) water and 190 proof ethanol in Soxhlet extractors for 24 and 16 h, respectively. Weight losses during the two-stage extraction were determined as extractives. The carbohydrates, acid soluble lignins, and acid insoluble lignins in the dried extractive free samples were analyzed utilizing sulfuric acid hydrolysis. Samples were incubated with 72% (w/w) sulfuric acid at 30 °C for 2 h, diluted to 4% sulfuric acid using DI water, and then autoclaved at 121 °C for 1 h. The switchgrass hydrolysates were then filtered through crucibles. An aliquot of the filtrate was utilized for biomass sugar determination by HPLC. A second aliquot of the filtrate was utilized to quantify the acid-soluble lignin by spectrophotometry (Evolution Array, Thermo Scientific, Waltham, MA) at OD240. Crucibles with biomass residues were dried in a 105 °C oven for 12 h, and the ash contents were determined by ashing at 575 °C for 4 h.

2.3. Switchgrass pretreatments

Four pretreatment methods were selected and applied to the switchgrass samples. Pretreatments were performed at a solid:liquid ratio of 1:9 (10% solid loading) with 20 g of biomass mixed with 180 mL solution. After pretreatment, the pretreated solids were separated from the liquid portion utilizing centrifugation at 3000 rpm for 5 min, and the solids were washed with 200 mL DI water three times. Residual solid ratios were calculated by recovered wet solids, moisture and divided by the initial dry solid content (Equation (1)). Pretreatment conditions were selected from the following: (1) incubation in 15% ammonia solution (pH 12.8) at 75 °C for 48 h [14]; (2) incubation in 3% (w/v) oxalic acid and 1% (w/v) $FeCl_3$ solution (pH 0.70) at 75 °C for 4 h; (3) incubation in 2.5% (w/v) sodium carbonate solution (pH 11.4) at 75 °C for 4 h; and (4) incubation in 1% (w/v) sodium hydroxide solution (pH 13.0) at 50 °C for 12 h [15].

$$Residual\ solid\ (\%) = \left(\frac{Recovered\ pretreated\ solids\ \times \left(1 - \frac{moisture}{100}\right)}{Initial\ dry\ solid\ content} \right) \times 100 \quad (1)$$

2.4. Scanning electron microscopy (SEM) analysis of raw and pretreated samples

Pretreated switchgrass samples were steeped in a fixative solution containing 2.5% glutaraldehyde and 0.5% paraformaldehyde overnight at 4 °C. Fixed switchgrass samples were dried in a vacuum environment and placed on a cylinder holder with a carbon gel. Before imaging, samples were coated with Au/Pd using a turbo sputter coater operating at 12 V, 20 mA for 60 s (Emitech K575, Ashford, United Kingdom). SEM was performed using a Hitachi S4700 field emission SEM with accelerating voltage set at 10 kV, magnitude set at 130.

2.5. Enzymatic hydrolysis

The maximum extent of the sugar yield of pretreated switchgrass was determined by cellulase hydrolysis following the NREL standard protocol "Enzymatic saccharification of lignocellulosic biomass (TP-510-42629)" with modifications. The reaction mixture was composed of 10% (w/w) pretreated solids with 0.05 M sodium citrate buffer at pH 5.0, and the total reaction mixture was 20 g. The enzymatic reaction was performed at 50 °C for 72 h with Cellic® CTec2 loading at 20% (v/w, 28 FPU g^{-1} of pretreated biomass). At the end of the reaction, an aliquot was removed, and the sugars released were analyzed by HPLC.

To further elucidate the hydrolysis kinetics at the beginning of the reaction, glucose production was monitored to examine the cellulase loading and type of switchgrass. Sodium hydroxide-treated switchgrass samples were mixed with 0.05 M sodium citrate buffer at pH 5.0; cellulase additions (CTec2) were lowered to 10 and 20 FPU g^{-1} pretreated biomass, and the total reaction mixtures were 40 g with the solid content adjusted to 5% (w/w). The enzymatic reaction was performed at 50 °C for 12 h. An aliquot of the samples was taken at 0, 3, 6, 9 and 12 h, and the sugars released were analyzed by HPLC.

2.6. Cellulosic ethanol fermentation

Ethanol yields from switchgrass hydrolysates were evaluated using separate hydrolysis and fermentation (SHF). After 72 h of enzymatic hydrolysis at 50 °C, reaction tubes were transferred to a 32 °C water bath; yeast extract (YE) solution was added at a concentration of 0.2 mL 20 g^{-1}, and the yeast seed culture was inoculated in 0.2 mL 20 g^{-1}. YE solution was used as the nitrogen source and prepared by dissolving 4 g YE in 5 mL DI water. Ethanol Red® yeast culture was prepared by incubating 5 g of yeast with 25 mL DI water in a 32 °C water bath shaking at 120 rpm for 25 min. Fermentation was carried out for 72 h, and the final ethanol concentrations were monitored by HPLC.

Ethanol production from the sodium hydroxide-treated switchgrass samples was investigated further utilizing simultaneous saccharification and fermentation (SSF) and a commercial xylose-utilizing yeast. Xylose utilizing yeast strain (M11205) was acquired from Lallemand