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Improvement of corn stover bioconversion efficiency by using plant glycoside hydrolase

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ABSTRACT

Plant cell wall is the most abundant substrate for bioethanol production, and plants also represent a key resource for glycoside hydrolase (GH). To exploit efficient way for bioethanol production with lower cellulase loading, the potential of plant GH for lignocellulose bioconversion was evaluated. The GH activity for cell wall proteins (CWPs) was detected from fresh corn stover (FCS), and the synergism of which with *Trichoderma reesei* cellulase was also observed. The properties for the GH of FCS make it a promising enzyme additive for lignocellulose biodegradation. To make use of the plant GH, novel technology for hydrolysis and ethanol fermentation was developed with corn stover as substrate. Taking steam-exploded corn stover as substrate for hydrolysis and ethanol fermentation, compared with *T. reesei* cellulase loaded alone, the final glucose and ethanol accumulation increased by 60% and 63% respectively with GH of FCS as an addition.

1. Introduction

Plant cell wall is the most abundant source of organic carbon on the earth, utilization of which for bioethanol production has received increasing interest over the past few decades (Arantes and Saddler, 2010; Gilbert, 2010). The basic structure of plant cell wall is a scaffold of polysaccharide cellulose, and crosslinking glycans hemicellulose, pectin, lignin, and aromatic polymer (Gilbert, 2010). The rigid structure of plant cell wall makes it recalcitrant to biological depolymerization, the accessibility of glycoside hydrolase (GH) to substrates was decreased accordingly.

Enzymatic conversion of the polysaccharides to fermentable monosaccharide is a key step in production of bioethanol from lignocellulose. An increasing number of GH and noncatalytic carbohydrate-binding modules (http://www.cazy.org/) have been characterized in the last decade. The complete degradation of polysaccharide to monosaccharide was carried out by the synergism of GH, such as exo-glucanase, endo-glucanase, β-glucosidase, endo-xylanase, and β-xylosidase (Woodward, 1991). To overcome drawback such as end-product inhibition and lower synergism in enzymatic hydrolysis, cellulase complex from fungi and bacteria was therefore developed (Gnansounou and Dauriat, 2010). Effective pretreatment followed by excessive enzyme loading is usually adopted for converting lignocellulose to bioethanol (Merino and Cherry, 2007). In the overall process of bioethanol production, the cost of enzyme accounts for around 50–60%. Although extensively effort has been made over the last few decades, the high enzyme cost is still a major obstacle for the commercial development of bioethanol from lignocellulose (Hahn-Hägerdal et al., 2006; Himmel et al., 2007; Wyman, 2007).

Glycoside hydrolase is present extensively in nature, and a large number of which has been found in bacteria, fungi, and plants. However, the majority of research is directed at GH from bacteria and fungi, the attention on properties and application of plant GH for biofuel production is just beginning (Sticklen, 2008). In recent publications, the plant GH and biofuel production have been speculated as a natural marriage (Lopez-Casado et al., 2008), the promotion of the GH for cellulose hydrolysis has also been found (Cosgrove, 2000; Sticklen, 2008).

Corn stover (CS) is one of the important feedstock for biofuel production. In view of botanic physiology, the harvested fresh corn stover (FCS) is in the senescent stage, GH is therefore speculated to be present in the FCS. To exploit the potential of plant GH for biofuel production, the present project was initiated to address the

Abbreviations: SSF, simultaneous saccharification and fermentation; GH, glycoside hydrolase; FCS, fresh corn stover; FCS-3, fresh corn stover stored for 3 months; FCS-6, fresh corn stover stored for 6 months; FCS-9, fresh corn stover stored for 9 months; DM, dry mass; CS, corn stover; SECS, Steam-exploded corn stover; FPU, filter paper unit; IU, international unit; CMC-NA, sodium carboxymethyl cellulose; CWP, cell wall protein; FPA, filter paper activity; DNS, dinitrosalicylic acid; *p*-NPC, *p*-nitrophenyl-cellobiose; *p*-NPX, *p*-nitrophenyl-xyloside; DS, degree of synergism; SP, soluble protein; HPLC, high-performance liquid chromatography.

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characterization and application of plant GH. To date, the synergism between proteins of FCS and microbial GH was observed (Han and Chen, 2007; Han and Chen, 2010a, b), the β-glucosidase and β-xylosidase have also been purified and characterized from FCS (Han and Chen, 2008; Han and Chen, 2010a, b). The properties of the plant GH make it a promising enzyme candidate for lignocellulose biodegradation. To understand the property and diversity of plant proteins deeply, the GH activity of FCS was studied systematically in the present study. The efficient and economical method for bioethanol production from corn stover based on plant GH application was also explored.

2. Methods

2.1. Materials and enzymes

The maize (Zea mays NongDa 108, Beijing, China) stover without corn cob was harvested at maturity in autumn of 2007. The stem and green leaves of FCS without disease and insect pest injury were collected, and enclosed in plastic bags after washing thoroughly with tap water, then stored at −20 °C for GH activity and biodegradation assay. Partial harvested FCS was stored at ambient temperature, in dry and ventilated place for different period (3, 6, and 9 months), then moved to −20 °C until use. The water content for FCS, FCS stored for 3 months (FCS-3), 6 months (FCS-6), and 9 months (FCS-9) was determined to be 70.0%, 2.2%, 2.0%, and 2.0%, respectively. According to the method of Van Soest (Goering and Van Soest, 1970), the composition of FCS-9 was found to be cellulose 35.8%, hemicellulose 26.1%, lignin 18.3%, protein 6.5% and others 13.3% by dry mass (DM).

Corn stover (FCS-9) pretreated with steam-explosion (SECS) was used as substrate for the following enzymatic hydrolysis and ethanol simultaneous saccharification and fermentation (SSF). The steam explosion pretreatment was conducted with pressure of 1.50 Mpa for 10 min as described previously (Jin and Chen, 2006). The steam-exploded corn stover (SECS) was washed with two volumes of tap water to remove soluble material, milled and passed through a 60-mesh screen, then dried at ambient temperature. The composition of SECS was found to be cellulose 50.5%, lignin 30.6%, hemicellulose 4.2% and others 14.7% by DM. The FCS was also milled to pass through a 60-mesh screen for enzymatic hydrolysis and SSF, the composition of the FCS was found to be cellulose 21.5%, hemicelluloses 32.5%, lignin 16.5%, protein 11.2% and others 18.3% by DM.

Commercial cellulase (84.0 FPU/mL, 42.2 IU/mL β-glucosidase, 5465.8 IU/mL β-endo-glucanase and 10.1 IU/mL β-exo glucanase, Ningxia xiasheng Co. Ltd, China) produced by *Trichoderma reesei* was used for hydrolysis and SSF. The total protein content of different materials (FCS, FCS-3, FCS-6, and FCS-9) was assayed with the method of Kjeldahl Nitrogen (Scheiner, 1976). Sodium carboxymethyirch wood xylan were purchased from Sigma (St Louis, MO, USA). Biochemical reagents of analytical grade, were obtained from Beijing chemical Co. Ltd, China. Aminex Hpx 87H column (300 mm × 7.8 mm) was purchased from Bio-Rad Laboratories (Hercules, CA, USA).

2.2. Extraction of cell wall proteins from corn stover

The cell wall proteins (CWPs) of FCS, FCS-3, FCS-6, and FCS-9 were extracted with the method described previously (Han and Chen, 2007). Briefly, the corn stover was cut into 2–3 cm, and grinded with acetate buffer (20 mM, 1 mM EDTA, pH 4.5), then filtered with a 0.47 μm^2 nylon mesh, the insoluble material was then washed extensively with the same buffer. The recovered insoluble material was then mixed with extracting buffer (20 mM Hepes, 2 mM EDTA, 3 mM $Na_2S_2O_5$, 1 mM NaCl, 5 mM PMSF, pH 6.8) with solid to liquid ratio of 1:15 (w/v), and filtered through a 0.47 μm^2 screen after homogenizing for 12 h. The filtrate was used as the crude CWPs for the following GH activity assay. The protein concentration was measured with the method of Coomassie Brilliant Blue using bovine serum albumin as a standard (Bradford, 1976).

To analyze the effect of pretreatment on the content and GH activity of CWPs, the FCS was pretreated with steam explosion as described in Section 2.1. The CWPs of steam exploded FCS were subjected to GH and synergetic activity assay. Unless otherwise specified, the experiments in present study were performed in triplicate, and the data are reported as means ± standard errors.

2.3. Glycoside hydrolase activity assay for CWPs of corn stover

The extracted CWPs from corn stover were subjected to the following GH activity assay: filter paper activity (FPA), endo-glucanase, β-exo glucanase, β-glucosidase, xylanase and β-xylosidase. The GH activity assay was conducted in sodium acetate buffer (100 mM, pH 4.5) at 37 °C. The activity of FPA, endo-glucanase, xylanase, and β-glucosidase was measured versus Whatman filter paper (1.0%), CMC-Na (1.0%), birch wood xylan (1.0%), and salicin (1.0%), respectively. The reducing sugar released was measured with dinitrosalicylic acid (DNS) method. The activity of β-exo glucanase and β-xylosidase was measured against *p*-nitrophenyl-cellobiose (*p*-NPC, 5 mM) and *p*-Nitrophenyl-xyloside (*p*-NPX, 5 mM), respectively. The reaction was terminated by adding Na_2CO_3 (1.0 M) to a final concentration of 60 mM, and the released *p*-nitrophenol was measured at 400 nm with a spectrophotometer. To get the initial reaction rate, CWPs (0.5 mg) were incubated with different substrates separately, the time course of hydrolysis was determined firstly. For endo-glucanase, β-exo glucanase, β-glucosidase, xylanase and β-xylosidase, the hydrolysis samples were taken at regular time intervals (0, 5, 10, 15, 20, and 30 min). A linear hydrolysis curve was obtained, and the reaction time of 10 min was therefore taken for enzymatic activity assay. In FPA determination, Whatman filter paper (1.0%) was incubated with CWPs (0.5 mg), a linear hydrolysis curve between reducing sugar released and reaction time (0, 30, 60, 90, 120, and 180 min) was obtained, and reaction time of 60 min was used for FPA assay. The enzyme activity was expressed in international unit (IU), which is defined as the amount of enzyme that produces the equivalence of 1 μmol of product (reducing sugar or *p*-nitrophenol) per minute.

2.4. Degree of synergism between CWPs of corn stover and T. reesei cellulase

The synergism for CWPs of corn stover and *T. reesei* cellulase was assayed using filter paper as substrate. Briefly, filter paper was hydrolyzed at 50 °C for 60 min in acetate buffer (pH 4.5, 100 mM) in volume of 1 mL, different enzyme combinations were applied separately: (1) CWPs (0.5 mg) of FCS, FCS-3, FCS-6, and FCS-9; (2) *T. reesei* cellulase (0.042 FPU); (3) CWPs (0.5 mg) of FCS, FCS-3, FCS-6, and FCS-9 and *T. reesei* cellulase (0.042 FPU). The FPA was measured as described in Section 2.3., and a linear curve for hydrolysis was obtained at different reaction time (0, 30, 60, 90, 120, and 180 min). The degree of synergism (DS) was calculated using the Eq. (1) (Woodward et al., 1988).

$$DS = \frac{FPA_{\text{cellulase \& CWPs}}}{FPA_{\text{cellulase}} + FPA_{\text{CWPs}}} \quad (1)$$

2.5. Enzymatic hydrolysis of fresh corn stover and steam-exploded corn stover

For hydrolysis, the substrates of FCS and SECS were first smashed and passed through a 60-mesh filter. The hydrolysis