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ARTICLE

Enhanced saccharification of reed and rice straws by the addition of β-1,3-1,4-glucanase with broad substrate specificity and calcium ion

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Abstract The possibility of using additive enzymes to improve the saccharification of lignocellulosic substrates with commercial cellulolytic enzymes was studied. Reed (*Phragmites communis*) and rice (*Oryza sativa*) straw powders were pretreated with NaOH/steam via a high-temperature explosion system. The saccharification of untreated reed and rice straw powders by commercial enzymes (Celluclast 1.5 L + Novozym 188) was not significantly increased by the addition of xylanases (Xyn10J, XynX), a cellulase (Cel6H), and a β-1,3-1,4-glucanase (BGlc8H) with broad substrate specificity. The saccharification of the pretreated reed and rice straw powders by the commercial enzymes was increased by 10.4 and 4.8 %, respectively, by the addition of BGlc8H. In the presence of Ca^{2+} and BGlc8H, the saccharification of the pretreated reed and rice straw powders by the commercial enzymes was increased by 18.5 and 11.7 %, respectively. No such effect of Ca^{2+} was observed with Xyn10J, XynX, or Cel6H. The results suggest that the enzymatic conversion of lignocellulosic biomass to reducing sugars could be enhanced by certain additive enzymes such as β-1,3-1,4-glucanase, and that the enhancement could further be increased by Ca^{2+}.

Keywords β-1,3-1,4-Glucanase · Calcium ion · Pretreatment · Reed straw powder · Rice straw powder · Saccharification

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Introduction

Lignocellulosic biomass is the most abundantly available biomass on Earth, and has long been studied as a renewable fuel source via bioconversion (Limayem and Ricke 2012). Lignocellulosic biomass is composed of cellulose, hemicellulose, lignin, and other complex materials, and must be hydrolyzed to obtain fermentable sugars for the production of biofuels and other biochemicals. Many structural and compositional factors hinder the hydrolysis of cellulose present in lignocellulosic biomass. Pretreatment of lignocellulosic biomass is an essential step in hydrolysis and makes the cellulose more accessible to chemicals or enzymes (Himmel et al. 2007; Yang and Wyman 2008; Hendriks and Zeeman 2009). Various pretreatment techniques have been developed, including the diluted alkali treatment, ammonia explosion, and others (Sills and Gossett 2011; Vancov and McIntosh 2011; Barman et al. 2012; Chen et al. 2013; Haque et al. 2013; Liu et al. 2013). Pretreated biomass such as rice and reed straws can be hydrolyzed to fermentable sugars by either chemical or enzymatic treatment. With increasing interest in the conservation of environment, intensive studies are being

carried out to find more efficient ways to use enzymes in the production of biofuels and other chemicals from rice straw (Binod et al. 2010).

In the present study, the possibility of using additive enzymes to improve the enzymatic hydrolysis of lignocellulosic biomass was investigated. The effects of β-1,3-1,4-glucanase with broad substrate specificity and other enzymes on the production of fermentable sugars by commercial cellulolytic enzymes were studied using readily available substrates, reed (*Phragmites communis*) and rice (*Oryza sativa*) straws.

Materials and methods

Biomass, chemicals, and enzymes

Reed (*P. communis*) and rice (*O. sativa*, Hopum) straws were collected at Muan, Korea (34°58′7.59″N and 126°27′18.66″E) in the winter and fall of 2009, respectively. Reed and rice straws were briefly washed with water to remove dusts, dried for 24 h at 50 °C, ground using a two-stage cutting mill (Green Bank, Daegu, Korea), and sieved through a 100-mesh pan sieve. The Celluclast 1.5 L and Novozym 188 commercial enzymes were obtained from Novozymes (Bagsvaerd, Denmark). Lichenan was purchased from Megazyme (Wicklow, Ireland). Xylan, carboxymethylcellulose (CMC), and other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Pretreatment of biomass

Ground reed and rice straw powders were pretreated using a newly designed high-temperature explosion system (Cha et al. 2013). The system consisted of an 800-mL reactor, a cyclone-type separation jar, and a control box. The powder was suspended in 1 M NaOH (sample: NaOH = 14:1, w/v), mixed with CO_2 at 3 MPa, and incubated for 30 min at 150 °C with agitation at 120 rpm. After increasing the CO_2 pressure to 6 MPa, the mixture was exploded into the separation jar under the reactor. The exploded mixture was washed and neutralized with tap water to a pH of 6 to 7, and the solid portion was collected using a 10-μm filter.

Analysis of biomass chemical compositions

The biomass chemical compositions were analyzed using standard National Renewable Energy Laboratory (NREL) methods. Shortly, a 0.3-g dried sample was suspended in 3 mL 72 % H_2SO_4 and incubated for 1 h at 30 °C. After adding 84 mL distilled water and reacting for 1 h at 121 °C, the mixture was filtered through a crucible filter. The solid portion was ashed for more than 3 h at 575 °C and used for the analysis of insoluble lignin. Soluble lignin in the filtrate was determined by measuring the absorbance at 205 nm. A portion of the filtrate was neutralized with $CaCO_3$ and used for the analysis of glucose, xylose, and arabinose. Sugars were analyzed at 65 °C using a HPLC (HPLC-RID, Agilent 1260 system, Santa Clara, CA, USA) equipped with an Aminex HPX 87H column (BioRad, Hercules, CA, USA). The mobile phase was 5 mM H_2SO_4 and the flow rate was 0.5 mL/min.

Preparation of additive enzymes

A xylanase (Xyn10 J), a β-1,3-1,4-glucanase (BGlc8H), and a cellulase (Cel6H) were prepared from *Escherichia coli* transformants harboring the corresponding genes. Xyn10 J was originated from a compost metagenomic library (Jeong et al. 2012). The BGlc8H was originated from *Paenibacillus* sp. X4 isolated from the soil of the North American alpine region (Na et al. 2014). Cel6H (accession number HQ143745) (Na 2012) was originated from a compost metagenomic library (Kwon et al. 2010). Transformants were grown for 12 h at 37 °C with rotary agitation at 200 rpm in 200 mL of LB broth containing 100 μg/mL ampicillin. Harvested cells were resuspended in a 5-mL sodium citrate buffer (pH 5.5), sonicated, and dialyzed against a 50-mM Tris–HCl buffer (pH 8.0) as described previously (Shin et al. 2002). Partially purified Xyn10J, BGlc8H, and Cel6H were prepared as described previously (Jeong et al. 2012; Na 2012; Na et al. 2014). Crude enzyme preparations were loaded onto a High-Q column (5 ml, BIO-RAD, Hercules, CA, USA), eluted with a linear gradient of 0–1.0 M NaCl in a 50-mM Tris–HCl buffer (pH 8.0) at a flow rate of 1.0 mL/min; the active fractions were then collected. Another xylanase, XynX, originated from *Thermoanaerobacter* sp. (formerly *Clostridium thermocellum*), was produced from *Bacillus subtilis* WB800 (pJX33) carrying the gene (Nguyen et al. 2012a, b). Cells were grown for 12 h at 37 °C with rotary agitation at 200 rpm in 200 mL of LB broth containing 10 μg/mL kanamycin. The culture broth was centrifuged and the supernatant was used as an enzyme preparation after concentration by ultrafiltration. Protein concentration was determined by the Lowry method (Lowry et al. 1951). SDS-PAGE of the proteins was carried out on 11.5 % polyacrylamide gels (Laemmli 1970).

Determination of enzyme activity

The xylanase activity was assayed using 0.5 % (w/v) birch wood xylan in a 50-mM sodium citrate buffer (pH 5.5) as the substrate, as described previously (Shin et al. 2002). The β-1,3-1,4-glucanase and CMCase activities were determined using lichenan and CMC, respectively. The

amount of reducing sugar released after 15 min reaction at 50 °C was determined using the dinitrosalicylic acid (DNS) method (Miller 1959). One unit of enzyme activity was defined as the amount of enzyme that liberated 1 μmol of reducing sugar per min under the conditions.

Enzymatic saccharification

Enzymatic hydrolysis was routinely performed with 3 mL of 6 % reed powder or rice straw powder suspended in a 50-mM sodium citrate buffer (pH 5.5) in a 50-mL Falcon tube. The concentrations of enzymes used were 0.2 U/mL of Celluclast 1.5 L; 0.33 U/mL of Novozym 188; 0.2 U/mL of BGlc8H; and 0.33 U/mL of Xyn10 J, XynX, or Cel6H unless otherwise mentioned. Reactions were carried out for up to 72 h at 50 °C with shaking at 150 rpm. Aliquots of the reaction mixture were withdrawn at 0, 6, 12, 24, 48, and 72 h and centrifuged for 10 min at 12,000 rpm; the amount of released soluble sugar was measured by the DNS method using D-glucose as a standard (Miller 1959). All experiments were performed in triplicate.

Results and discussion

Pretreatment of reed and rice straw powders

The pretreatment of the reed and rice straw powders using the high-temperature explosion system was efficient in recovering cellulose and removing ash, but less efficient in removing lignin and hemicellulose (Table 1). The cellulose contents of the rice and reed straw powders were increased by 54.6 and 36.1 %, respectively, by the pretreatment. The ash content of the rice straw powder was decreased from 12.3 to 5.2 % by the pretreatment, and that of the reed straw powder from 4.6 to 0.9 %.

Hydrolysis of the biomass

The production of reducing sugar from the untreated (UReP) and pretreated (PReP) reed straw powders by hydrolysis with commercial enzymes (Celluclast 1.5 L, Novozym 188, Celluclast 1.5 L + Novozym 188) reached its plateau after 24 h reaction and increased only slightly thereafter (Fig. 1). Similar hydrolysis patterns were observed with the untreated (URiP) and pretreated (PRiP) rice straw powders (Fig. 2). The hydrolysis of 6 % UReP and 6 % PReP with Celluclast 1.5 L + Novozym 188 yielded 15.9 and 39.5 g/L reducing sugar, respectively, (Fig. 1A, B). With 6 % URiP and PRiP, the yields were 19.2 and 33.4 g/L reducing sugar, respectively, (Fig. 2A, B). The results indicate that the pretreatment was efficient in improving the enzymatic hydrolysis of the biomass. Doubling the concentrations of the commercial enzymes resulted in less than 10 % increase in the production of reducing sugar (data not shown).

The influence of the additive enzymes, Xyn10J, XynX, Cel6H, and BGlc8H, on the saccharification was determined using Celluclast 1.5 L + Novozym 188 as the control. With the untreated biomass, UReP and URiP, the increase in the degree of saccharification due to the use of the additive enzymes was not significant (Figs. 1A, 2B). The addition of BGlc8H to the commercial enzyme preparation increased the saccharification of the pretreated biomass, PReP and PRiP, by 10.4 and 4.8 %, respectively, yielding 43.6 (39.5 without BGlc8H) and 35.0 (33.4 without BGlc8H) g/L reducing sugar, respectively, (Figs. 1A, 2B). The addition of Xyn10J, XynX, or Cel6H resulted in no or a negligible increase in the production of reducing sugar from the untreated biomass or the pretreated biomass by the commercial enzymes (Figs. 1A, B, 2A, B).

The effect of Ca^{2+} on the enhancement of saccharification by BGlc8H was investigated, since calcium is known to increase the activity of BGlc8H (Na et al. 2014). With the UReP, the addition of Ca^{2+} increased the production of reducing sugar with BGlc8H + Celluclast 1.5 L + Novozym 188 by 17.0 %, from 15.9 to 18.6 g/L (Fig. 1A). With the PReP, the addition of Ca^{2+} increased the production of reducing sugar by 18.5 %, from 39.5 to 46.8 g/L (Fig. 1B). A similar influence of Ca^{2+} was observed with the URiP and PRiP. The addition of Ca^{2+} increased the saccharification of URiP and PRiP with

Table 1 Chemical composition and moisture content of reed and rice straw powders after high-temperature extrusion pretreatment

Samples	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Ash (%)	Moisture content (%)
Reed straw					
Untreated	40.2	20.8	22.9	4.6	2.53
Pretreated	54.7	19.8	20.2	0.9	74.4
Rice straw					
Untreated	36.8	21.7	18.9	12.3	2.13
Pretreated	56.9	23.8	10.8	5.2	74.7

Pretreatment was carried out as described in the "materials and methods" section

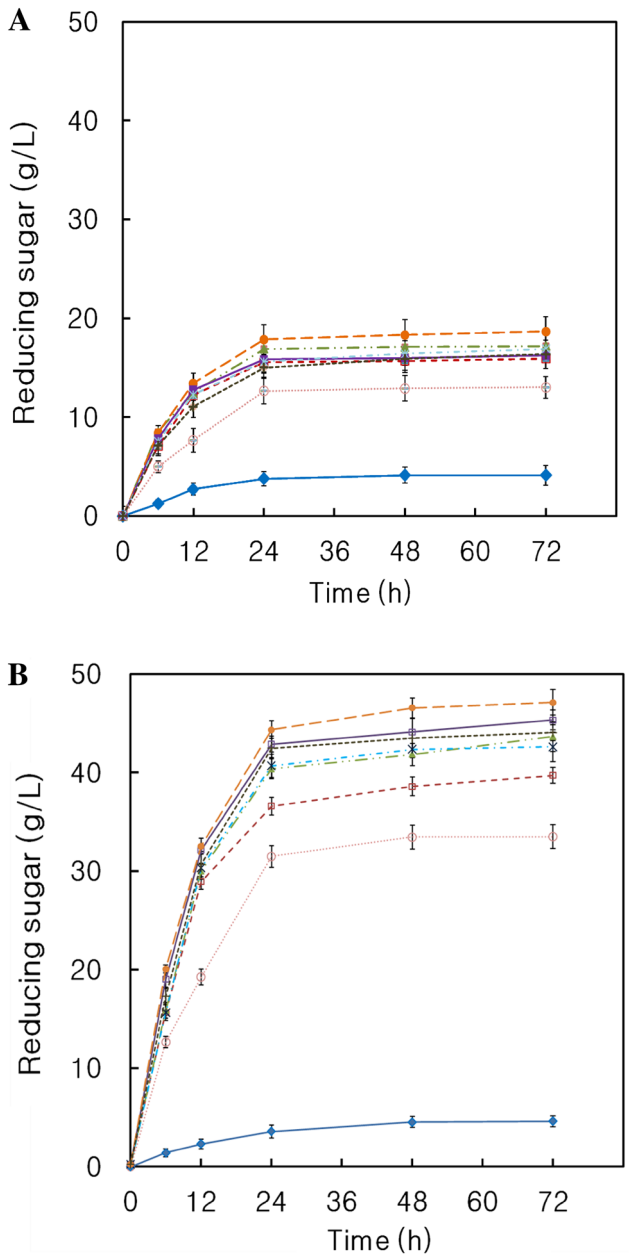

Fig. 1 Enzymatic hydrolysis of untreated (**A**) and pretreated (**B**) reed straw powders. Reactions were carried out with 3 mL of 6 % reed straw powder in a 50-mM sodium citrate buffer (pH 5.5) in 50-mL-Falcon tubes at 50 °C with shaking at 150 rpm. The enzyme concentration of Celluclast 1.5 L or BGlc8H was 0.2 U/mL, and that of Novozym 188, Xyn10J, XynX, and Cel6H was 0.33 U/mL. Symbols: —◆—, Novozym 188; ···○···, Celluclast 1.5 L; --□--, Novozym 188 + Celluclast 1.5 L; — · — + — · —, Celluclast 1.5 L + Novozym 188 + Cel6H; —■—, Celluclast 1.5 L + Novozym 188 + Xyn10J; --×--, Celluclast 1.5L + Novozym 188 + XynX; — ·· — ▲ — ·· —, Celluclast 1.5 L + Novozym 188 + BGlc8H; — — ● — —, Celluclast 1.5 L + Novozym 188 + BGlc8H + Ca^{2+}

BGlc8H + Celluclast 1.5 L + Novozym 188 by 16.7 and 11.7 %, respectively, yielding 22.4 and 37.3 g/L reducing sugar, from 19.2 and 33.4 g/L, respectively, (Fig. 2A, B).

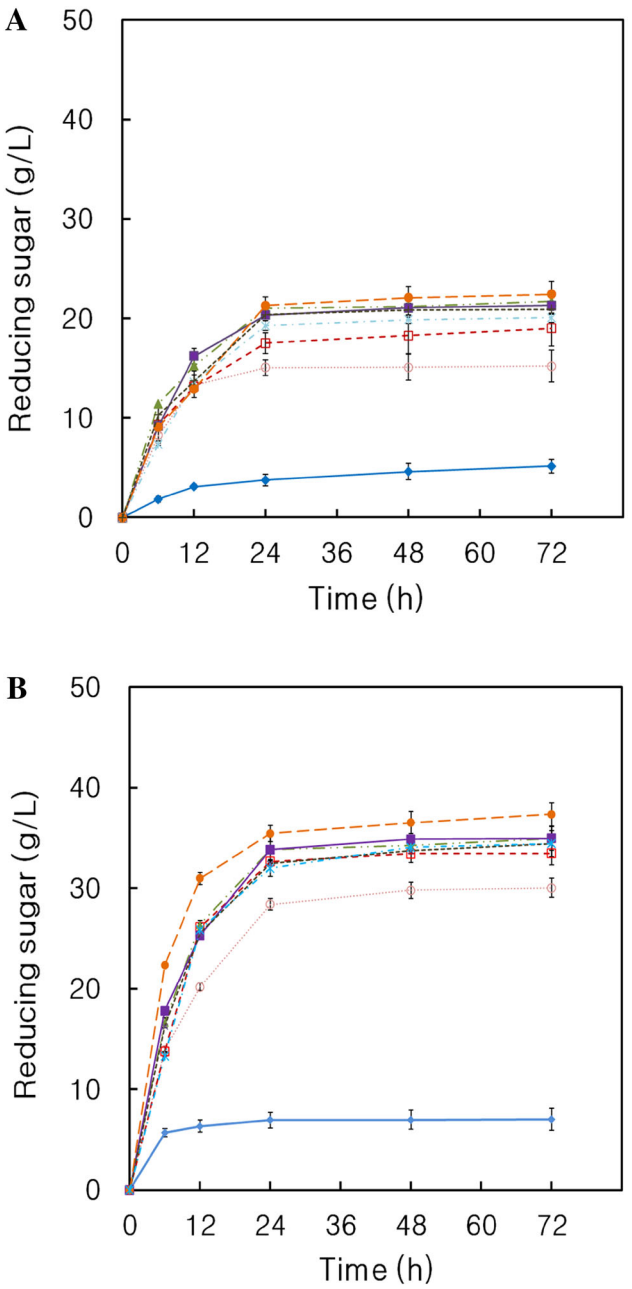

Fig. 2 Enzymatic hydrolysis of untreated (**A**) and pretreated (**B**) rice straw powders. Conditions and symbols were the same as described in Fig. 1

No such influence of Ca^{2+} was observed with the other additive enzymes, Xyn10J, XynX, and Cel6H.

It has been reported that nonenzymatic protein such as bovine serum albumin (BSA) enhanced enzyme activity by 32.5 % in filter paper hydrolysis (Wang et al. 2015), and saccharification efficiency was increased in enzymatic hydrolysis of pretreated rice straw using optimized crude enzyme cocktail (Singh and Bishnoi 2012). In this study, the use of an additive enzyme, BGlc8H, was found to increase the saccharification of pretreated biomass by the commercial

enzymes, Celluclast 1.5 L and Novozym 188, and the saccharification was further enhanced by Ca^{2+}. The increase in the saccharification with BGlc8H + Ca^{2+} was greater than that could be obtained by doubling the concentration of the commercial enzymes. The effect of BGlc8H + Ca^{2+} was also observed when the concentration of the substrate, PReP, was increased up to 15 % (data not shown). BGlc8H is a β-1,3-1,4-glucanase produced by *Paenibacillus* sp. X4 and belongs to glycoside hydrolase family 8 (GH8). Unlike other GH8 members that hydrolyze β-1,4-glycosidic bonds adjacent to β-1,4-glycosidic bonds to produce 3-O-cellobiosyl-D-glucose but cannot hydrolyze β-1,4-glycosidic bonds in CMC (Bielecki and Galas 1991; Planas 2000), BGlc8H was found to be able to hydrolyze pure β-1,4-glucans such as CMC, as well as β-1,4 linkages of mixed-linked β-1,3-1,4-glucans (Na 2012). However, it is not clear at this time why BGlc8H in the presence of Ca^{2+} increased the degree of saccharification beyond the extent that could be obtained by doubling the concentration of the commercial enzymes, even though it is reasonable to assume that the commercial enzymes would be more efficient in hydrolyzing pure β-1,4-glucans. The results of this study suggest the possibility of using additive enzymes, such as β-1,3-1,4-glucanases, to improve the saccharification of lignocellulosic substrates by cellulolytic enzymes.

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