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Short Communication

Thermostable xylanase-aided two-stage hydrolysis approach enhances sugar release of pretreated lignocellulosic biomass

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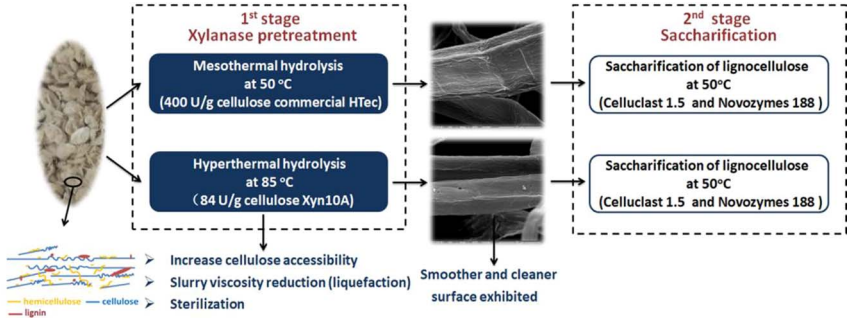
GRAPHICAL ABSTRACT



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ABSTRACT

One of the challenges in biorefinery is the still too much enzyme involved in the saccharification of the cellulosic component. High-temperature hydrolysis with thermostable enzyme showed promise. In this study, a temperature-elevated two-stage hydrolysis, including xylan "coat" removal at high-temperature by thermostable xylanase (Xyn10A) from *Thermotoga thermarum* DSM 5069 followed with saccharification step by commercial cellulase, was introduced to improve biomass deconstruction. Results showed that high-temperature xylanase treatment considerably increased cellulose accessibility/hydrolyzability towards cellulases, with smoothed fiber surface morphology. Comparing with commercial xylanase (HTec) treatment at 50 °C, thermostable Xyn10A pre-hydrolysis at 85 °C was able to achieve a slightly better improvement of cellulose hydrolysis with much lower xylanase loading (about 5 times lower than HTec). It appeared that the increased temperature during thermostable xylanase treatment facilitated biomass slurry viscosity reduction, which exhibited more benefits during hydrolysis of various steam pretreated substrates at increased solid content (up to 10% w/w).

1. Introduction

The nascent lignocellulosic biomass based integrated biorefinery involves an essential enzymatic hydrolysis step, converting polysaccharides (mainly cellulose) into fermentable sugars by using engineered enzyme cocktail (Bayer et al., 2007; Van Dyk and Pletschke, 2012). To compromise the properties of most commercial cellulases and hemicellulases, the existing enzymatic hydrolysis often carries out at around 50 °C (Wan Azelee et al., 2016; Sun et al., 2015). A reasonable biomass hydrolysis yield (> 80% cellulose hydrolysis) usually requires

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either a large amount of enzymes or much longer hydrolysis time, which considerably limits the economic feasibility of this biorefinery concept (Hu et al., 2015; Khatri et al., 2016). In order to further improve hydrolysis efficacy, a promising approach is to carry out the enzymatic hydrolysis at higher temperature (80–90 °C) to accelerate catalytic reaction, improve mass transfer, reduce slurry viscosity and avoid microbial contamination (Chatterjee et al., 2015; Zhang and Lynd, 2004). For example, the results by Viikari et al. (2007) indicated that the enzymatic hydrolysis at higher temperatures with thermostable cellulases potentially reduced the reaction time and enzyme loading. In addition, a recent study by Peng et al, (2015) showed that the synergistic interactions between thermophilic enzymes from *C. owensensis* and commercial enzyme cocktail CTec2 (Novozymes) resulted in an efficient deconstruction of native lignocellulosic biomass even without pretreatment. Aside from improved enzymatic hydrolysis, hyperthermal enzymolysis step could also act as biomass sterilization to benefit the downstream fermentation process. Therefore, higher temperature hydrolysis and the corresponding thermostable enzymes have attracted high interests in the biorefinery process.

Pretreatment is firstly required in any of lignocellulosic bioconversion process to fractionate, at least partially, the hemicellulose and lignin while improving cellulose accessibility to cellulase enzymes (Bondesson and Galbe, 2016). Although high severity pretreatment can improve the hydrolyzability of pretreated substrates, it generates high concentration of inhibitory component and also causes significant sugar loss (Zhai et al., 2016). Therefore, the compromised mild-severity pretreatment conditions are often used. Under these milder pretreatment conditions, some of the hemicellulose, mostly xylan in agricultural residues and hardwood, has remained with the cellulosic-rich water insoluble fraction. This residual hemicellulose is known to significant limit the effectiveness of enzymatic hydrolysis of cellulose (Hu et al., 2011). Recent works have shown that xylanases, even though do not directly hydrolyze cellulose, could greatly improve the hydrolytic efficiency by increasing cellulose accessibility, fiber porosity, and fiber swelling, and thus assist in the hydrolysis of cellulose to boost a release of fermentable sugars from lignocellulosic biomass (Wong et al., 1996; Long et al., 2017). This xylanase-boosting effect has been observed on a range of pretreated lignocellulosic materials (Hu et al., 2011).

We have recently identified a thermostable glycoside hydrolase (GH) family 10 xylanase (Xyn10A) from Thermotoga thermarum DSM 5069, which exhibited higher optimal temperature (95 °C) and thermostability than xylanases from other species such as Talaromyces thermophilus, Paecilomyces thermophila, Cellulomonas flavigena etc. (Shi et al., 2013). Comparing with other family xylanases, GH10 xylanases are also more capable of cleaving glycosidic linkages in the highly substituted xylan backbone within biomass (Collins et al., 2005; Kolenová et al., 2006). Therefore, we hypothesized that this highly thermostable GH10 xylanase (Xyn10A) holds great potential to enhance the hydrolysis of pretreated lignocellulosic substrates.

Being inspired by the industrial production of starch-to-sugar process (a two-stage enzyme-mediated hydrolysis), in the work reported here, we introduced a temperature-elevated two-stage hydrolysis of pretreated biomass. The first pre-hydrolyzing the pretreated lignocellulosic biomass was conducted by using thermostable Xyn10A at relatively high temperature (85 °C), which was then followed with the common biomass hydrolysis process by using commercial cellulase preparations at 50 °C. After evaluating the potential effect of thermostable xylanase (Xyn10A) pre-hydrolysis at 85 °C on the subsequent biomass deconstruction and the major changes of cellulosic fiber physicochemical characteristics, our results indicated that the two-stage hydrolysis with thermostable enzymes could greatly improve biomass deconstruction especially at relative high biomass loading.

2. Material and methods

2.1. Materials

Commercial xylanase enzyme mixture HTec, Celluclast 1.5 L (cellulase mixture), and Novozyme 188 (β-glucosidase) were generous gifts from Novozymes. Cellulase activity was determined using the filter paper assay recommended by International Union of Pure and Applied Chemistry (Adney and Baker, 1996). β-glucosidase activity was measured using substrate *p*-nitrophenyl-β-D-glucoside purchased from Sigma. The total protein content of commercial Celluclast 1.5 L (50 FPU, 129.2 mg/mL), Novozyme 188 (239 CBU, 233.4 mg/mL), and HTec (5223.7 U/mL, 35.2 mg protein/mL) were determined according to the Ninhydrin assay using bovine serum albumin (BSA) as the protein standard (Mok et al., 2015). Kraft pulp (KP, 77.6% cellulose, 17.9% xylan, and 0.4% lignin) used in this study was in lab store. Corn stover and poplar were steam pretreated according to previously described procedures (Hu et al., 2011; Zhai et al., 2016).

2.2. Purification of the recombinant xylanase Xyn10A

Thermostable GH10 xylanase from *Thermotoga thermarum* DSM 5069 (Xyn10A) was expressed in the recombinant *E. coli* BL21 (DE3) carrying pET-20b-*xyn10A* (lab store). The obtained protein was purified through a heat treatment at 60 °C for 30 min followed by Ni affinity chromatography on a Ä KTA*FPLC*™ (GE Healthcare Life Sciences) system with a HisTrap column (GE, Shanghai) (Shi et al., 2013). SDS-PAGE was employed to verify the purity of the target protein, and the protein bands were analyzed using an image analysis system (Bio-Rad, USA). Since Xyn10A activity was greatly stimulated by Ca^{2+}, 5 mM Ca^{2+} was added in the Xyn10A hydrolysis solution.

2.3. Enzymatic hydrolysis

The hydrolysis experiment was carried out by hydrolyzing Kraft pulp (KP), steam pretreated corn stover (SPCS) and steam pretreated poplar (SPP) at 2% (w/w) and 10% (w/w) solids loading accordingly. The reaction mixtures were shaken in a horizontal shaker, stirring speed 150 rpm. The two-stage hydrolysis was conducted as following: (1) xylanase pre-hydrolysis stage (the first stage), Xyn10A and HTec were added to the substrates at different conditions (85 °C and pH 7 for Xyn10A, 50 °C and pH5 for HTec) respectively. After 3 h of incubation, fibers were separated by centrifugation at 5,000*g* for 10 min and washed three times. The xylanase pretreated substrates were then used as substrates of the second step. (2) saccharification stage (the second stage), Celluclast 1.5 L (6 mg/g cellulose, commercial cellulase enzyme mixture) with Novozyme 188 (β-glucosidase) supplementation in activity ratio of 1 FPU to 2 CBU was added to the pre-hydrolyzed insoluble fraction and incubated at 50 °C, pH 5 for 72 h. Samples for sugar analysis were collected after 24, 48 and 72 h of hydrolysis, respectively, followed by boiling the reaction mixture at 100 °C for 20 min to inactivate the enzymes. Supernatant was removed for glucose analysis by centrifugation at 10,000*g* for 5 min. All hydrolysis experiments were performed in duplicate and standard deviations were presented.

2.4. Analytic methods

2.4.1. Sugar and xylanase activity assay

The modified Klason lignin method (the TAPPI standard methods T222 om-88) was used to examine the chemical composition of pretreated substrates. Quantification of chemical compositions was performed by high performance anion exchange chromatography (Dionex DX-3000, Sunnyvale, CA). Glucose concentration after hydrolysis was analyzed by a YSI-2700 glucose analyzer (Yellow Springs Instruments, US). As for xylanase activity assay, the 3,5-dinitrosalicylic acid (DNS) method was conducted at 50 °C with substrate birchwood xylan from