



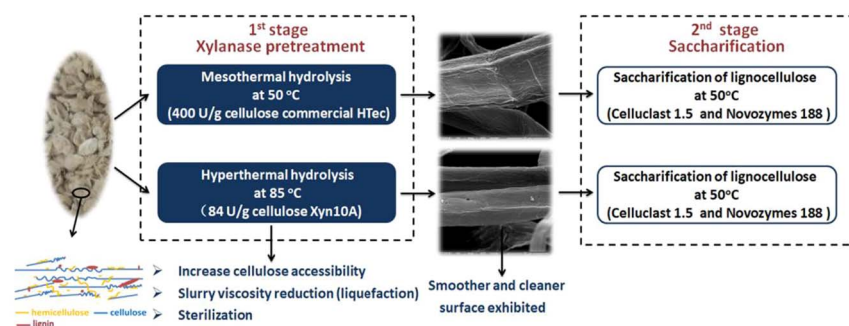
Short Communication

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

Lingfeng Long^{a,b}, Dong Tian^b, Rui Zhai^b, Xun Li^a, Yu Zhang^a, Jinguang Hu^{b,*}, Fei Wang^a, Jack Saddler^b
^a College of Chemical Engineering, Nanjing Forestry University, Jiangsu Key Lab for the Chemistry and Utilization of Agro-forest Biomass, Nanjing 210037, PR China

^b Department of Wood Science, University of British Columbia, Vancouver V6T 1Z4, Canada


GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:

Thermostable enzyme
Xylanase
Bioconversion
Biorefinery
Cellulose hydrolysis

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

* Corresponding author.

E-mail address: jinguang@mail.ubc.ca (J. Hu).

<https://doi.org/10.1016/j.biortech.2018.02.104>

Received 11 January 2018; Received in revised form 21 February 2018; Accepted 22 February 2018

Available online 24 February 2018

0960-8524/ © 2018 Elsevier Ltd. All rights reserved.

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 significantly 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 $\ddot{A}$ KTAFFLC™ (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 5,000g 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 10,000g 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

sigma as described elsewhere (Long et al., 2017). One unit of xylanase activity was defined as the amount of enzyme releasing 1 μmol reducing sugars per minute. All experiments were performed in triplicate.

2.4.2. Water retention value (WRV)

TAPPI Useful Method-256 was used to measure the WRV of xylanases pretreated Kraft pulp. WRV is a measure of water-holding capacity and was calculated as the ratio between the weight of retained water in the fiber after centrifugation and the dried substrate. Two WRV measurements were made for each sample.

2.4.3. Simon's stain (SS)

According to a modified version of the Simon's Stain technique, cellulose accessibility to cellulase enzymes was assessed by using Direct Orange 15 (Pylam products Inc.) (Chandra et al., 2008). Cellulose accessibility increased as the Direct Orange Dye absorption increased. SS (mg/g pulp) is the amount of orange dye absorbed by substrate.

2.4.4. Scanning electron microscopy (SEM) observation

Observations of xylanases pretreated fibers after the 1st stage hydrolysis were performed using a field emission scanning electron microscope (Hitachi S3000N VP-SEM). Before observation, the samples were coated with Pd-Au alloy to prevent charging on the surface.

2.4.5. Yield stress

The slurry yield stress was determined using NDJ-5S digital rotational viscometer (Changji, Shanghai). An automatic switch was set for free selection of proper rotating speed or rotor number, which enables the instrument to measure any viscosity value in the given range. All measurements were made at 25 °C.

3. Results and discussion

To evaluate the effects of xylan removal by thermostable xylanase on cellulose hydrolysis in a relatively simple hydrolysis system, the two-stage hydrolysis was initially conducted at a low consistency (2% w/w) with Kraft pulp as a representative biomass model substrate. In general, the high temperature xylanase treatment considerably enhanced both cellulose hydrolysis rate and extend, and the thermostable Xyn10A also showed considerable advantages over commercial xylanase preparation HTec (Fig. 1). Briefly, the cellulose hydrolysis increased from 45% to 62% at initial 24 h hydrolysis and from 77% to 96% at final 72 h hydrolysis, respectively, after Xyn10A treatment at 85 °C, indicating rather high boosting effect of Xyn10A treatment on the following cellulose hydrolysis (Fig. 1). While the HTec treatment conducted at same temperature (85 °C) showed no effects on the 2nd stage cellulose hydrolysis, which was likely caused by the denaturation of HTec at such high temperature. When the HTec treatment was carried out at its optimized temperature (50 °C), it also improved the 2nd stage cellulose hydrolysis but to a much lower extent as comparing with thermostable Xyn10A treatment, even with 5 times higher of enzyme loadings (Fig. 1). As for the sugar production, 1.2 g of glucose was obtained from 2 g Kraft pulp (dry weight) after the single stage hydrolysis for 72 h, whereas 1.5 g and 1.4 g of glucose was released for Xyn10A and Htec assisted two stages hydrolysis, respectively. Based on the hydrolysis results, it appeared that highly thermostable xylanase treatment had great potential to improve cellulose saccharification with much lower amount of enzyme loading.

The substrate physicochemical characteristics after xylanase treatment were next assessed in order to better understand the reasons of high temperature xylanase treatment boosting cellulose hydrolysis. When the viscosity of these KP fiber suspensions with/without high temperature xylanase treatment were measured on a rotational viscometer, it appeared that the original KP fiber suspension exhibited the highest yield stress of ~889 Pa, followed by HTec (~748 Pa) and Xyn10A (~693 Pa) pre-hydrolyzed KP fibers. Since the yield stress has

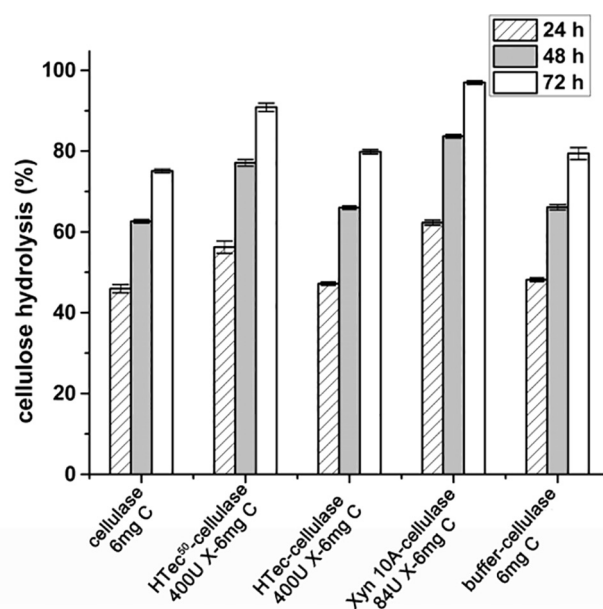


Fig. 1. The effect of different pretreatment methods of 1st stage on cellulose yield and the total enzyme dosage (mg/g cellulose) used in the hydrolysis of Kraft pulp (KP) at 2%. Cellulose yield was monitored after 24 h, 48 h and 72 h of saccharification stage, respectively. Xyn10A/HTec, hydrolysis in the presence of Xyn10A/HTec of xylanase pre-hydrolysis stage at 85 °C for 3 h; HTec⁵⁰, 1st stage hydrolysis with HTec at 50 °C, pH 5 for 3 h; buffer, substrate was incubated at 85 °C for 3 h without xylanase addition; cellulase, substrate was incubated at the same condition without the addition of xylanase. C: cellulase enzyme mixture; X: corresponding xylanases. Two-way ANOVA shows the differences among different xylanase treatment were statistically significant ($p < 0.05$).

Table 1
Characteristics of KP after different pretreatment methods in the 1st stage.

Samples	control	buffer	Xyn10A	HTec ⁵⁰
SS (mg/g pulp)	59.5	61.4	73.5	68.0
WRV %	217.6 ± 0.38	320.7 ± 0.18	387.8 ± 0.04	373.7 ± 0.18
Reducing sugar (μmol/mL)	0	0.5	2.4	5.4

KP, Kraft pulp; SS, Simons' Stain; f, water retention value. buffer, substrate was incubated at 85 °C for 3 h without xylanase addition; Xyn10A, hydrolysis in the presence of Xyn10A of xylanase pre-hydrolysis stage at 85 °C for 3 h; HTec⁵⁰, 1st stage hydrolysis with HTec at 50 °C, pH 5 for 3 h; control, original Kraft pulp. The differences of SS values, WRV and reducing sugar content with/without xylanase treatment and between different xylanase treatment were statistically significant ($p < 0.01$, one-way ANOVA).

Table 2
Chemical composition (dry material basis) of hydrolysis substrates.

Substrate	Sugar and lignin composition of water insoluble component of the substrates						Abbreviation
	Ara	Gal	Glu	Xyl	Man	AIL	
Corn stover	1.4	0.4	49.3	16.7	1.2	23.1	SPCS
Poplar	0	0	58.7	2.2	1.4	33.5	SPP

Ara, Arabinan; Gal, Galactan; Glu, Glucan; Xyl, Xylan; Man, Mannan; AIL, Acid Insoluble Lignin. Values shown were the mean of the average of three experiments.

been recognized as an important parameter for assessing the biomass liquefaction extent for a typical enzymatic hydrolysis process, our results indicated that the high temperature xylanase treatment could significantly reduce slurry viscosity (enhance liquefaction) which could be even more beneficial at high solids loadings. The morphology changes of the gross fiber before and after xylanase treatment were

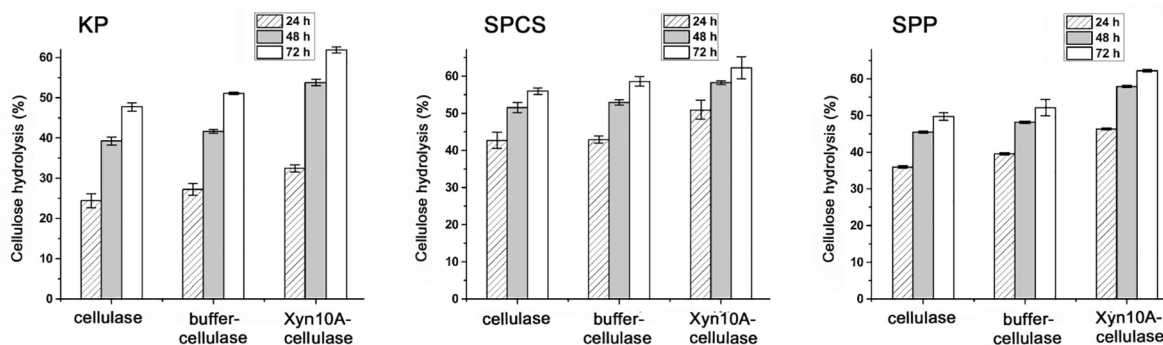


Fig. 2. The extent of cellulose hydrolysis of Kraft pulp (KP), steam pretreated poplar (SPP) and steam pretreated corn stover (SPCS) at 10% solid loading after 24 h, 48 h and 72 h. buffer, substrate was incubated at 85 °C for 3 h without xylanase addition; Xyn10A, hydrolysis in the presence of Xyn10A of 1st stage at 85 °C for 3 h; cellulase, substrate was incubated at 50 °C for 3 h without the addition of xylanase. The statistical analysis showed that the differences (between different hydrolysis time, various substrates, and substrates with/without thermostable xylanase treatment) were all statistically significant (two way ANOVA, $p < 0.05$).

further investigated by using SEM. It seemed that the surface of Xyn10A pre-hydrolyzed KP fiber became smoother and cleaner while the HTec pretreated KP fiber exhibited more fiber collapse as compared with control (KP fiber treated at high temperature without xylanase). The smooth of fiber surface by high temperature Xyn10A treatment might be the reason for the reduced slurry viscosity observed before, due to the rough fiber surface would induce fiber interactions during the hydrolysis (van der Zwan et al., 2017).

Besides cellulose morphology and slurry viscosity, the accessibility of cellulose is another crucial cellulosic fiber characteristic that has already been shown as the major indicator for cellulose hydrolytic potential during biomass deconstruction. The accessibility of the pretreated KP was determined by prevalent Simons' stain (SS) and water retention value (WRV) techniques according to previous reports (Chandra et al., 2008; Chandra et al., 2011). Briefly, the Simons' stain quantitatively tests the available surface area for hydrolytic enzymes while WRV tests the small pores within the substrate that could hold small water molecules. As shown in Table 1, xylanase pretreatment considerably improved the SS values especially for the Xyn10A pretreated fiber (from ~60 to ~74 mg/g substrate, $p < 0.01$, one-way ANOVA). The increased SS values could be explained by the increase surface area. Similar to the trend of SS results, WRV was also increased after xylanase pretreatment (from ~218% to ~389% substrate, $p < 0.01$, one-way ANOVA), indicating more smaller pores generated in the KP substrate after xylanase treatment (Table 1). It was interesting that the sample that was incubated at 85 °C without xylanase also greatly increase the fiber WRV, but not much on cellulose accessibility assessed by SS (from 59.5 to 61.4 mg/g substrate). Combining with the lack of cellulose hydrolysis improvement of the control sample (Fig. 1), it was apparent that the cellulose accessibility could not simply evaluated by only the fiber WRV.

The reducing sugar content after the 1st stage was also measured to gain more insights into the mechanism of xylanase treatment. Results showed that a higher amount of reducing sugar ($5.4 \mu\text{mol mL}^{-1}$) was released after HTec pretreatment at 50 °C, in comparison to that of $2.4 \mu\text{mol mL}^{-1}$ from Xyn10A pretreatment at 85 °C (Table 1, $p < 0.01$, one-way ANOVA). This was probably due to stronger xylosidase activity in the commercial HTec preparation, which could effectively break down xylooligomers to monomers (Long et al., 2017). On the other hand, the thermostable Xyn10A is a pure enzyme (SDS-PAGE Fig. S1), of which mainly exhibited xylanase activity, tended to randomly cleave the xylan backbone into xylooligosaccharides with different chain lengths (Shi et al., 2013; Van Dyk and Pletschke, 2012).

Since high temperature thermostable xylanase treatment showed great advantages on the subsequent cellulose hydrolysis of KP substrate by reducing fiber viscosity and increasing cellulose accessibility (Fig. 1 and Table 1), the potential beneficial effects of such treatment on the

hydrolysis of various industrial relevant pretreated lignocellulosic substrates were also evaluated. Steam pretreated corn stover (SPCS) and poplar (SPP) were selected and their major chemical compositions were shown in Table 2. As expected, the SPCS had much higher xylan content (16.7%) and also more branched xylan (1.4% arabinosyl and 0.4% galactosyl) as compared with SPP. However, SPP has higher cellulose and lignin content than the SPCS (58.7% glucose and 33.5% acid insoluble lignin, Table 2). Since we found that high temperature xylanase treatment could considerably reduce fiber slurry rheology (Fig. 2, Table 1) and previous works have shown that an initial liquefaction step is crucial in enhancing the subsequent cellulose hydrolysis at high solids loading (Skovgaard et al., 2014), the cellulose hydrolysis of pretreated lignocellulosic biomass were performed at relatively high solids loading (10% w/w).

It was apparent that Xyn10A pre-hydrolysis could enhance the cellulose yield on all of the pretreated lignocellulosic substrates, but the extent of improvement is highly substrate dependent (Fig. 2). For example, the thermostable Xyn10A treatment could significantly improve cellulose conversion by 15% (11.0 g/L glucose) and 12% (6.9 g/L glucose) for the KP and SPP substrates respectively, while a less improvement (~7%, 3.3 g/L glucose) was obtained on the SPCS substrate. This was probably due to the highly branched xylan structure of the SPCS restricted the cellulose/xylan accessibility to cellulase/xylanase enzymes (Kumar et al., 2009). It should be noted that although SPP substrate only contained a low amount of xylan (2.2%), a prior thermostable Xyn10A pretreatment still significantly enhance its subsequent cellulose hydrolysis (from 49% to 63% after 72 h hydrolysis). In addition, it appeared that the hydrolysis boosting effect of thermostable xylanase treatment was much higher at the initial cellulose hydrolysis stage (first 24 h), which was likely caused by the increased accessibility of cellulose after the high temperature xylanase treatment. The statistical analysis showed that the differences (between different hydrolysis time, various substrates, and substrates with/without thermostable xylanase treatment) were all statistically significant (two way ANOVA, $p < 0.05$).

4. Conclusion

A new process for saccharification of lignocellulosic biomass by two-stage hydrolysis is introduced in the present research, namely hyperthermal pre-hydrolysis (85 °C) by xylanase of *T. thermarum* DSM 5069 followed with mesothermal enzymolysis (50 °C) by commercial cellulase. It appeared that Xyn10A pre-hydrolysis could greatly improve the subsequent cellulose conversion at both 2% and 10% solid loading by increasing cellulose accessibility and slurry rheology. The Xyn10A-aided two-stage hydrolysis was not dependent on the nature of fiber properties. This work opens the door for formulating more efficient

cocktails containing other thermostable enzymes to better facilitate the deconstruction of lignocellulosic biomass.

Acknowledgments

The National Natural Science Foundation of China (No. 31370572), this work was financially supported by the programs of State Forestry Administration, China (No. 2014-4-37) and Top-notch Academic Programs Project of Jiangsu Higher Education Institutions (TAPP).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.biortech.2018.02.104>.

References

- Adney, B., Baker, J., 1996. Measurement of Cellulase Activities; LAP-006 NREL Analytical Procedure; National Renewable Energy Laboratory: Golden, CO.
- Bayer, E.A., Lamed, R., Himmel, M.E., 2007. The potential of cellulases and cellulosomes for cellulosic waste management. *Curr. Opin. Biotechnol.* 18, 237–245.
- Bondesson, P.-M., Galbe, M., 2016. Process design of SSCF for ethanol production from steam-pretreated, acetic-acid-impregnated wheat straw. *Biotechnol. Biofuels* 9, 222.
- Chandra, R., Ewanick, S., Hsieh, C., Saddler, J.N., 2008. < The characterization of pretreated lignocellulosic substrates prior to enzymatic hydrolysis.pdf > 1178–1185.
- Chandra, R.P., Au-Yeung, K., Chanis, C., Roos, A.A., Mabey, W., Chung, P.A., Ghatora, S., Saddler, J.N., 2011. The influence of pretreatment and enzyme loading on the effectiveness of batch and fed-batch hydrolysis of corn stover. *Biotechnol. Prog.* 27, 77–85.
- Chatterjee, C., Pong, F., Sen, A., 2015. Chemical conversion pathways for carbohydrates. *Green Chem.* 17, 40–71.
- Collins, T., Gerday, C., Feller, G., 2005. Xylanases, xylanase families and extremophilic xylanases. *FEMS Microbiol. Rev.* 29, 3–23.
- Hu, J., Arantes, V., Saddler, J.N., 2011. The enhancement of enzymatic hydrolysis of lignocellulosic substrates by the addition of accessory enzymes such as xylanase: is it an additive or synergistic effect? *Biotechnol. Biofuels* 4, 36.
- Hu, J., Chandra, R., Arantes, V., Gourlay, K., Susan van Dyk, J., Saddler, J.N., 2015. The addition of accessory enzymes enhances the hydrolytic performance of cellulase enzymes at high solid loadings. *Bioresour. Technol.* 186, 149–153.
- Khatri, V., Hébert-Ouellet, Y., Meddeb-Mouelhi, F., Beauregard, M., 2016. Specific tracking of xylan using fluorescent-tagged carbohydrate-binding module 15 as molecular probe. *Biotechnol. Biofuels* 9, 74.
- Kolenová, K., Vršanská, M., Biely, P., 2006. Mode of action of endo- β -1,4-xylanases of families 10 and 11 on acidic xylooligosaccharides. *J. Biotechnol.* 121, 338–345.
- Kumar, R., Mago, G., Balan, V., Wyman, C.E., 2009. Physical and chemical characterizations of corn stover and poplar solids resulting from leading pretreatment technologies. *Bioresour. Technol.* 100, 3948–3962.
- Long, L., Tian, D., Hu, J., Wang, F., Saddler, J., 2017. A xylanase-aided enzymatic pretreatment facilitates cellulose nanofibrillation. *Bioresour. Technol.* 243, 898–904.
- Mok, Y.K., Arantes, V., Saddler, J.N., 2015. A NaBH₄ coupled ninhydrin-based assay for the quantification of protein/enzymes during the enzymatic hydrolysis of pretreated lignocellulosic biomass. *Appl. Biochem. Biotechnol.* 176, 1564–1580.
- Peng, X., Qiao, W., Mi, S., Jia, X., Su, H., Han, Y., 2015. Characterization of hemicellulase and cellulase from the extremely thermophilic bacterium *Caldicellulosiruptor owensensis* and their potential application for bioconversion of lignocellulosic biomass without pretreatment. *Biotechnol. Biofuels* 8, 131.
- Shi, H., Li, X., Gu, H., Zhang, Y., Huang, Y., Wang, L., Wang, F., 2013. Biochemical properties of a novel thermostable and highly xylose-tolerant β -xylosidase/ α -arabinosidase from *Thermotoga thermarum*. *Biotechnol. Biofuels* 6, 27.
- Skovgaard, P.A., Thygesen, L.G., Jørgensen, H., Cardona, M., Tozzi, E., McCarthy, M., Siika-Aho, M., Jeoh, T., 2014. The role of endoglucanase and endoxylanase in liquefaction of hydrothermally pretreated wheat straw. *Biotechnol. Prog.* 1–9.
- Sun, F.F., Hong, J., Hu, J., Saddler, J.N., Fang, X., Zhang, Z., Shen, S., 2015. Accessory enzymes influence cellulase hydrolysis of the model substrate and the realistic lignocellulosic biomass. *Enzyme Microb. Technol.* 79–80, 42–48.
- van der Zwan, T., Hu, J., Saddler, J.N., 2017. Mechanistic insights into the liquefaction stage of enzyme-mediated biomass deconstruction. *Biotechnol. Bioeng.* 114, 2489–2496.
- Van Dyk, J.S., Pletschke, B.I., 2012. A review of lignocellulose bioconversion using enzymatic hydrolysis and synergistic cooperation between enzymes-Factors affecting enzymes, conversion and synergy. *Biotechnol. Adv.* 30, 1458–1480.
- Viiikari, L., Alapuranen, M., Puranen, T., Vehmaanpera, J., Siika-Aho, M., 2007. Thermostable enzymes in lignocellulose hydrolysis. *Adv. Biochem. Eng. Biotechnol.* 108, 121–145.
- Wan Azelee, N.I., Jahim, J.M., Ismail, A.F., Fuzi, S.F.Z.M., Rahman, R.A., Md Illias, R., 2016. High xylooligosaccharides (XOS) production from pretreated kenaf stem by enzyme mixture hydrolysis. *Ind. Crops Prod.* 81, 11–19.
- Wong, K.K.Y., Jong, E., Saddler, J., Allison, R.W., 1996. Mechanisms of xylanase-aided bleaching of kraft pulps. Part II: target substrates. *Appita J.* 50, 509–518.
- Zhai, R., Hu, J., Saddler, J.N., 2016. What are the major components in steam pretreated lignocellulosic biomass that inhibit the efficacy of cellulase enzyme mixtures? *ACS Sustain. Chem. Eng.* 4, 3429–3436.
- Zhang, Y.H.P., Lynd, L.R., 2004. Toward an aggregated understanding of enzymatic hydrolysis of cellulose: noncomplexed cellulase systems. *Biotechnol. Bioeng.* 88, 797–824.