Delignification overmatches hemicellulose removal for improving hydrolysis of wheat straw using the enzyme cocktail from *Aspergillus niger*

Jiahong Wang, Xuntong Chen, Chonlong Chio, Chun Yang, Erzheng Su, Yongcan Jin, Fuliang Cao, Wensheng Qin

PII: S0960-8524(18)31673-0
DOI: https://doi.org/10.1016/j.biortech.2018.12.029
Reference: BITE 20786

To appear in: *Bioresource Technology*

Received Date: 15 October 2018
Revised Date: 7 December 2018
Accepted Date: 9 December 2018

Please cite this article as: Wang, J., Chen, X., Chio, C., Yang, C., Su, E., Jin, Y., Cao, F., Qin, W., Delignification overmatches hemicellulose removal for improving hydrolysis of wheat straw using the enzyme cocktail from *Aspergillus niger*, *Bioresource Technology* (2018), doi: https://doi.org/10.1016/j.biortech.2018.12.029

Delignification overmatches hemicellulose removal for improving hydrolysis of wheat straw using the enzyme cocktail from *Aspergillus niger*

Jiahong Wang [a, b,c], Xuntong Chen [b], Chonlong Chio [b], Chun Yang [b,d], Erzheng Su [a], Yongcan Jin[a], Fuliang Cao[c], Wensheng Qin [b*]

[a] College of Light Industry and Food Engineering, Nanjing Forestry University, Nanjing, 210037, China.

[b] Department of Biology, Lakehead University, Ontario, P7B 5E1, Canada

[c] Co-innovation Center for the Sustainable Forestry in Southern China, College of Forestry, Nanjing Forestry University, Nanjing, 210037, China

[d] Institute of Agricultural Products Processing, Shanxi Academy of Agricultural Science, Tianyuan, 030031, China

*Corresponding author：

Wensheng Qin: Tel.: +1 807 3438467 E-mail: wqin@lakeheadu.ca

Address: Lakehead University, Thunder Bay, P7B 5E1, Ontario, Canada

Abstract: Based on a general understanding that hemicellulose removal is more efficient than delignification for biomass deconstruction, an *Aspergillus niger* strain producing high xylanase activity was screened out from seventeen strains by clear halo experiments. Low-cost enzyme cocktail with high xylanase activity was produced from wheat straw medium fermented by the Gyx086 strain. The enzyme cocktail with high xylanase activity could more effectively hydrolyze wheat straw than other biomasses. However, only 30 % of total carbohydrates could be hydrolyzed to reducing sugar in untreated wheat straw. Further enzymatic hydrolysis and pretreated trials were carried out, the results indicated that hemicellulose removal was less effective than delignification for de-recalcitrance of wheat straw and the crystallinity is little interference with the hydrolysis process. Delignified wheat straw was near-completely hydrolyzed by the enzyme cocktail in 60 h. This study advanced the knowledge in promoting wheat straw as feedstock for bio-based industry.

Keywords: Wheat straw, *Aspergillus niger*, Xylanase, Enzymatic hydrolysis, Delignification, Hemicellulose removal

1. Introduction

To cut down global warming, environmental pollution, and combat high oil price, bio-based industries have been rapidly developing. Potential bio-based products include biofuels, biochemicals, and biomaterials, especially bioenergy and biofuels have received greater attention (Hassan et al., 2018; Ravindran & Jaiswal, 2016), due to the depletion of petroleum resources. Lignocelluloses derived from agriculture

residues are drop-in renewable feedstock for producing the second-generation biofuels – bioethanol, compared with the first-generation facilities using feedstocks such as sugar, starch, oils and fat (Kawaguchi et al., 2016; Mosier et al., 2005). However, lignocellulosic biomass is a complex and recalcitrant structure, and a pretreatment step is needed for improving sugar release (Caffall & Mohnen, 2009; Zhao et al., 2012b).

Biomass recalcitrance of enzymatic hydrolysis is mainly derived from the complex matrix structure of cellulose, hemicelluloses, lignin, and pectin in plant cell walls (Himmel et al., 2007; Xu et al., 2012). Cellulose is a tough, insoluble, crystalline glucose polymer that plants use for defense, structure and scaffolding, which causes cellulose water-insoluble and tolerant to decomposition (Beckham et al., 2011; Keiluweit et al., 2010). Unlike cellulose, hemicelluloses are copolymers composed of different amounts of several saccharide molecules in the main chain and side chains. The main components of hemicelluloses are xylan, xyloglucan, glucomannan, mannan, and galactomannan (Chen, 2014; Yin & Fan, 1999). Combining hemicelluloses with lignin, cellulose, and pectin via both non-covalent and covalent bonds increases the resistance to enzymatic degradation of the cell wall (Abasolo et al., 2009; Chen, 2014). Lignin is a complex racemic aromatic heteropolymer derived from three precursors: coniferyl alcohol, sinapyl alcohol and *p*-coumaryl alcohol (Boerjan et al., 2003; Miyamoto et al., 2018). These precursors are linked by the ether linkages and condensed linkages which cause the lignin structure to become complicated and difficult to be modified. It is an insoluble, low

reactivity layer around the hemi/cellulose through ether and ester bonds to further preventing the cellulose saccharification (Studer et al., 2011). Pectin is also a major component which is located between the cellulose microfilaments of the plant cell wall, mainly condensed by galacturonic acid and its methyl ester. Abasolo et al. (2009) have shown a negative correlation between the level of de-methyl-esterified homogalacturonan regions and cellulose degradability. In addition, the pore size, pore volume, particle size, and specific surface area are also the relevant factors to the enzyme accessibility of cellulose (Alvira et al., 2010; Zhao et al., 2012a).

To expose the fiber to cellulase enzymes, mechanical, physicochemical, chemical or biological pretreatment method is an essential step to increase cellulose accessibility (Kristensen et al., 2008). The mechanical comminution enhances the surface area of feedstock for the enzyme, such operation is a prerequisite step for further chemical or biochemical processing (Zhao et al., 2012b; Zhu, 2011). However, the recalcitrance which to be relieved is limited due to limited destructure can be achieved on cellulose which is embedded by hemicelluloses and lignin. So other physicochemical or chemical methods were carried out to improve the digestibility of biomass based on via cellulose decrystallization, hemicellulose removal or delignification from biomass (Guo et al., 2017; Yang & Wyman, 2008; Yoon et al., 1995). Although the digestion rate can be significantly improved, lots of drawbacks, such as high energy requirement, high production cost, special equipment requirements, inhibitors generation, and environmental problems, gave rise to them

not being economically viable (Lee et al., 1995; Mosier et al., 2005; Saha et al., 2017; Zhao et al., 2012b).

Biological pretreatment has been proposed as an economical, eco-friendly technology to improve biomass digestibility based on the fact that it can be performed at the mild condition in a simple equipment and is less harmful to the environment (Chen et al., 2010; Rabemanolontsoa & Saka, 2016). Cellulose hydrolyzing species are widely distributed among fungi but much fewer in bacteria (Rabemanolontsoa & Saka, 2016). Brown, white and soft rot fungi were widely investigated for delignification of the lignocellulosic biomass (Sindhu et al., 2016). However, the process is too slow, typically greater than two weeks, and is not recommended for industrial purposes (Chaturvedi & Verma, 2013). Moreover, cellulosic saccharification is influenced more by the removal of xylan than lignin because xylan removal directly affects glucan chain accessibility (Sindhu et al., 2016). *Aspergillus niger* (*A. niger*) is well known as a safety microbe widely used for food and feed, with high production of xylanase activity. It has a higher xylanase productivity and is an ecological friendly microbe for industrial application (Wang et al., 2018a).In this paper, a lignocellulosic enzyme cocktail from elaborated *A. niger* was investigated, the effectiveness of enzymatic pretreatment or direct saccharification of biomass was evaluated.

2. Materials and methods

2.1. Materials

The mature green-yellow wheat straws (WS), collected in October 2017 from Shanxi Province, China, were air-dried for one week outdoor. The dried WS was commercially ground, further ground through 20 – 100 mesh sieves when arrived the lab and stored in a plastic bag until use. Carbohydrates, hemicelluloses, and lignin in the WS is 50.07±0.18 %, 18.33 ±0.13%, and 16.17±0.44%, respectively. Seventeen of *A. niger* and two *Penicillium* spp. fungi were used for this research. Gyx086 and Gyx017 were isolated from ancient ginkgo tree soil in Anlu city, China (Wang et al., 2013). Other strains were stored in the lab which was isolated from the forestry soil in Thunder Bay, ON, Canada. The *Penicillium ramulosum* N1 was reported to produce xylanase (Lin et al., 2015), was used as a positive control and *Penicillium* LN not producing xylanase in potato agar medium was used as a negative control.

2.2 Evaluation of xylanase enzymatic activity

Congo red stain was used for evaluating high xylanase activity *A. niger* strain as described previously by Guo et al. (2017) with some modification. In brief, 2 μl of spore liquids was inoculated on the center of the plate with 9.0 cm diameter containing 12 mL of potato agar containing 0.1 % (m/v) of xylan from corn core (Tokyo chemical industry Co., LTD, Japan). After 48 h incubation at 30 ℃, all of the plates flooded with 10 mL of 0.1 % Congo red solution in each agar plate and executed a dye–polysaccharide interaction for 10 min, then poured out the dye liquid and washed the plates three times with 10 mL distilled water each time. The diameters

of the fungal colony (d) and halo region (D) were then measured on a millimeter scale. The hydrolysis activity was calculated as $(D/d)^2$.

2.3 Production of lignocellulosic enzymes

Fifty milliliters of various liquid medium with 5 % of different solid biomasses without other additive was used as a medium in 250 mL Erlenmeyer flasks for production of lignocellulosic enzymes. These flasks were covered with aluminum foil and autoclaved at 121 ℃ for 20 min. After cooled to room temperature, the spores of *A. niger*, 3 loops with diameter 0.5 cm of the agar plate, were inoculated into each flask. The flasks were incubated in the incubator at 30 ℃ for 8 days with a rotate speed of 150 rpm. Two milliliters of each sample was taken for enzymatic activity analysis every 24 h. In the single factor and optimized experiment, the same rotate speed was set.

2.4 Analysis of enzymatic activity and reducing sugar determined

Enzymatic activities of xylanase, polygalacturonase (PG), CMCase and filter paper activity (FPA) were measured refer to our lab method (Guo et al., 2017) with Beechwood xylan (Megazyme, Ireland), polygalacturonic acid (Sigma, USA), carboxymethyl cellulose (Acros organic,USA) and No. $1^{\#}$ filter paper (Whatman, England) as substrate, respectively. Briefly, 10 μl of diluted crude enzyme liquid was mixed with 20 μl 1% of various substance solution (pH 5.0) in each pores of microplate, placing in the water bath at 50 ℃ for 10 min (FPase for 20 min), then instantly cooled and added 60 μl of the 3,5-dinitrosalicylic acid (DNS) reagent and heated in boiling water for 5 min. The absorbance was determined at 540 nm and the

release amount of reducing sugar was calculated by the relevant standard curve. Beta-glucosidase activity was determined according to a scribe by Shrestha et al. (2015) with minor modification. Thirty microliter reaction system including 10 μl crude enzyme and 20 μl 500 μm 4-nitrophenyl β-D-glucopyranoside (pNPG) (Sigma, USA) was incubated in a water bath at 50 ℃ for 20 min. Then mixed 70 μl distill water following 100 μl of 100 mM sodium bicarbonate, absorbance was measured at 400 nm. Enzyme activities were expressed in International Units (IU), as the amount of enzyme which releases 1 μmol of glucose, xylose, and *p*-nitrophenol in 1 min. The reducing sugars were determined using the DNS method (Miller, 1959).

2. 5 Hydrolysis of biomasses

Hydrolysis of biomass was conducted in 10 mL volume system. The enzyme cocktails were adjusted to pH 5.0 ± 0.1 using 20 % of sodium hydroxide solution, then 5 mL enzyme cocktail and 5 mL citrate buffer solution (0.1 M, pH 5.0) were mixed and pour into the 10 mL tubes including 0.1 g WS or other biomass feedstock, 10 μl 50 mg/ml kanamycin sulfate and 10 μl 10 % sodium azide solution. Four commercial enzymes were also used for hydrolyzing WS: E1: Glucosidase 49291-1G from *A. niger* (Sigma, USA); E2: Cellulase "Onozuka R-10" derived from *Trichoderma viride* (PhytoTechnology Laboratories, USA); E3: Pectinase "Guojiaomei" (Hengsheng Bio-Tech. Co, China); E4: Cellulase "Celluclast 1.5L" from *Trichoderma reesei* ATCC 26921 (Sigma, USA). Hydrolysis was carried out at 50 ℃ for 3 days and 200 μl sample was taken every 12 h. Reducing sugars were determined by DNS method and structural monosaccharide was measured

by an ion chromatography, Dionex, ICS 5000, Thermofisher Scientific, equipped with CarboPacTM SA10 column and an electrochemical detector (ED) (Dionex-300, Dionex Corporation, Canada). Microbial contaminate was surveyed by potato - LB agar plate at 30 ℃ for 48 h. The releasing reducing sugar value was counted as the differential of reducing sugar of the hydrolysis mediums to the controls (no enzymes added).

2. 6 Pretreatment and Composition analysis of wheat straw

Wheat straw with 0 - 20 meshes size was used as pretreated feedstock, the pretreatment using 80 % of *p*-toluenesulfonic acid (TsOH) was implemented at 80 ℃ for 20 min refer to the description by Chen et al. (2017). 1.5 % of NaOH and 1 % of H_2SO_4 was also used as a dilute base and dilute acid to treat WS, respectively. The wetted WS has executed in an autoclave at 121 ℃ for 1h. All of the samples treated by the above chemicals were washed to neutral and then air-dried at 40 ℃ for a week and used for further testing.

The cell wall composition was measured according to the description of NREL (Sluiter et al., 2010) with some minor modifications. Briefly, about 0.10 g pretreated and untreated dry biomasses were extracted six times for 30 min each: one time in 1.5 mL and twice in 1.0 mL hot water at 65 ℃, twice in 1.0 mL absolute ethanol and once in 1.5 mL acetone. The extractive-free residue was dried in a chemical hood for 2 days and weighted to calculate the content of extractives. Then the dry samples were incubated with 1 mL of 72 % sulfuric acid at 30 ℃ with vortexing every 15 min. After 1 h, the hydrolysate was diluted to 3 % sulfuric acid by adding deionized water

and autoclaved at 121 ℃ for 1 h before storing at 4 ℃ overnight to allow the solids to settle. Finally, the supernatant and solid residues were separated by centrifugation at 12,000 g for 3 min. The supernatant was carefully removed and used to determine the content of hexose and pentose using the anthrone-sulfuric acid method and orcinol-hydrochloric acid method, respectively. The solid residues were used to measure the content of Klason lignin by a weight method.

2.7 Box-Behnken design (BBD) and statistical analysis

Response surface methodology (RSM) was used to optimize the production of xylanase. Three factors and three levels were selected for BBD based on the results of the single factor experiments; the ranges and levels of the independent variables are shown in Table 1. SYSTAT 12 (Systat Software, Inc., USA) was used to generate the BBD matrix and to analyze the experiment data, describing the response surface, and drawing the contour maps. The goodness of fit of the second-order polynomial model equation, the determination coefficient R^2 and the lack of fit were indicated by an F test at the 5 % level of significance. The most optimized conditions were also decided by the "Response Surface Method – Optimize – Ridge max analysis" program of SYSTAT 12. The statistical analysis of data was carried out in the one-way ANOVA program of the SAS system for Windows 8.02 (SAS Institute Inc., USA). Duncan's multiple-range test was selected as the comparative method in the program and the significance level was set at 0.05.

3. Results and discussion

3.1 Screening *A. niger* strain of higher production of xylanase activity

For the demonstration of extracellular xylanase activity, 17 *A. nigers* were preliminary compared by clear zones using Congo red staining on agar plates containing 0.1% xylan. Congo red, which has been used for decades, is more reliable than Gram's iodine as the chromogenic dye which may lead to the identification of false positives (Meddeb-Mouelhi et al., 2014). Usually, a big colony suggests a faster growth of strain and a bigger halo suggests a more secretion of extracellular enzyme. The negative control LN which do not secret xylanase has the biggest colony but without a halo. The positive control previously reported with high xylanase activity (Lin et al., 2015) only possessed a small halo in the potato agar medium. All of *A. niger* have obvious halos of 2.83 - 4.63 cm around the fungi colonies with diameter from 1.37 cm to 2.45 cm. Among these strains, the Gyx086 was with a bigger and clearer transparent zone than other strain, which suggested that Gyx086 could secrete higher xylanase activity. The square of the halo diameter/colony diameter ratio was the biggest 6.87 in Gyx086, which could further verify its biggest enzymatic production ability. Moreover, the fungus with high cellulase activity has been found in our previous study (Wang et al., 2013), which suggested that this strain has a high ability of lignocellulosic degradation. Therefore, the *A. niger* strain of Gyx086 was preliminarily selected for the next experiment.

3.2 Substance selection of lignocellulosic production

The biomass medium can cause a dramatic effect on the enzymatic activity of *Aspergillus* strains (Kang et al., 2004). Thus, to induce the secretion of xylanase, three different kinds of biomasses, namely hardwood (wood dust), grass (barley straw,

wheat straw, agave), tree leaf (ginkgo leaf) were used as the medium due to *A. niger* Gyx086 could not grow well in the wood dust suspension medium but could grow well in the medium with 5 % of barley straw, agave, corn stover, miscanthus or gingko leaves, without additional additives. Additive-free is a benefit to simplify the production process and reduces the cost of production (Wang et al., 2018a). Extracellular xylanase activity produced during 8 days of fermentation was shown in Figure 1a, the result indicated that wheat straw (WS) is the most suitable medium for producing significantly higher xylanase activity ($p < 0.01$), accordingly wheat straws were used for further research. Other few strains were again used to investigate the production of xylanase. Gyx086, LKW17, LKW18, and LKW05 produced higher xylanase activities than 8UV15 and 19EMS20. The highest values of xylanase activity were presented in the second day during fermentation. However, the activities of PG, CMCase, and β-glucosaccharase were not significantly different. The results again suggested that the Gyx086 is suitable for further research and wheat straw (WS) may be an excellent biomass as hydrolysis substance.

3.3 Effect of temperature, pH and solid concentration on xylanase activities

Gyx086 strain was inoculated in 5 % of wheat straw medium at three different temperatures. As being seen in Figure 1b, the lower temperature at 27 ℃ delayed the enzyme secretion due to the delayed fermentation and with a relatively lower xylanase activity during the later fermentation. The xylanase activities were not significantly different at 33 and 39 ℃ but were significantly higher than at 27 ℃. As a result, the mean of xylanase activity has no correlation with the temperature during fermentation

(Prob > F = 0.326). Interestingly, the curves of xylanase activity appeared as a V-shape change during fermentation when over 33 ℃, while gradually rose when below 30 ℃. The initial partial acid condition was more suitable for the *A. niger* growth as shown in Figure 1c. *A. niger* grew well from pH 3 to pH 7 and a distinct suppression raised in pH 9.0, in addition, a higher xylanase activity could be obtained via 2 d fermentation. Therefore, pH 5-7 was planned to further experiment. Fermentation temperature also significantly affected cellulase activity; especially *β*-glucosaccharase was barely secreted in early fermentation (Data not shown).

3.4 Optimization of xylanase enzyme production

Optimized trials were carried out according to the BBD design and the results were shown in Table 1. Fermentation temperature (T,℃), pH value, and fermentation time (t, h) were independent variables and xylanase activity was the response variable. The linear relationship between response and independent variables can be built by the following quadratic equation:

$$XA\ (\mathrm{U.ml^{-1}}) = 5.589+0.48\ T-1.176pH+0{,}053t-0.162\ T^2-1.276\ pH^2+0.482t^2+0.589\ T*pH-0.188pH*t-0.094\ T*t.$$

As the results shown in Table 2, the squared multiple R is 0.961, which indicated that 96.1 % of response values can be well explained by the independent values (Açıkel et al., 2010). The *p*-values of "Regression" and "Lack of Fit" by variance analysis were 0.005 and 0.115, respectively, which indicated that the built quadratic equation is relatively credible for assessment of xylanase activity (Wang et al., 2018b). According to the 2-D contour plots shown in Figure 2, the xylanase activity was

interactively affected by the temperature, pH, and fermenting time. Due to that, all of the contour plots appeared as ellipses (Wu et al., 2009). By the ridge analysis for maximization program of SASTAT 12 (Systat Software, Inc., USA), the optimal forecasted response is 6.503 (95.00% Confidence Interval from 5.788 to 7.218) which appears in the edge with 32.496 ℃, pH 5.642, and 96.783 h for temperature, pH, and time, respectively. To verify the model, five batches of the experiment were performed with the fermentation condition at 32.5 ℃ for 96 h with the initial pH 5.6. The average value of xylanase activity was 6.19 ± 0.27 U/ml with no significant difference compared with the run 11. Thus fermentation at 32 - 32.5 ℃ for 96 h with the initial pH 5.0-5.6 was applicable for the production of high activity xylanase enzyme.

3.5 Characterization of enzyme cocktail

The lignocellulosic enzymes in the optimized enzyme cocktail were analyzed, in addition to 6.19 ± 0.27 U/ml of xylanase activity, there is 0.24 ± 0.03 U/ml of *β*-glucosidase and 0.54 ± 0.02 U/ml of CMCase activities. The total cellulase activities can be expressed by FPA and the activity of the FPA is 0.11 U/ml. The xylanase has relatively higher activity at 50 - 60 ℃ and pH 5.0 - 6.0 as shown in Figure 3a,b. CMCase obtained the most value of enzymatic activity at 60℃ and the acidic reaction condition is very beneficial to its hydrolysis reaction. *β*-glucosidase appeared significantly high enzymatic activity at 40-50℃ and pH 4.0-5.0. Thus the reaction condition of 50-60℃ and pH 5.0 is more suitable by combining these results. Furthermore, thermotolerance of the enzyme is another important factor for a long-

time enzyme hydrolysis process. As shown in Figure 3c, all of the lignocellulosic enzymes held most of the enzymatic activity for 72 h. Xylanase and CMCase lost 24.2 % and 23.8 % of enzymatic activities at 72 h, respectively, and glucosidase activity is not significantly lost. However, when the reaction temperature is 60℃ (Figure 3d), 54.2 % of xylanase activity and 48.1 % of CMCase activity was lost only after 6 h compared with the origin activity value. Due to that, most of the lignocellulosic enzyme activities are lost after 6h at 60℃, as a result, the hydrolysis temperature should be decided at 50℃.

3.6 Enzymatic hydrolysis of lignocellulosic biomass

To investigate the efficiency of biomass hydrolysis, a few of the common lignocellulosic biomass was used as feedstock hydrolyzed at 50 ℃ with pH 5.0 base on the above mentioned enzymatic character. As shown in Figure 4a, the enzyme cocktail has a higher efficiency of hydrolysis to corn stover (CS) and wheat straw (WS) with relatively higher released reducing sugar than other biomasses. However, the released reducing sugar fast reduced on CS after 90 min, probably due to the inhibitor generated from CS which decreased the activity of the lignocellulosic enzyme in the enzyme cocktail. Thus, WS was selected as the experiment feedstock due to its hydrolysis efficient by the enzyme cocktail.

A series of different size WS fragment from 20 to 100 meshes were hydrolyzed using this enzyme cocktail and the results were shown in Figure 4b. The reducing sugar of 17.9 % of WS biomass was released from the > 100 mesh WS at 72 h, which is significantly higher than other size samples (α =0.05). The 80 -100 mesh WS

obtained the second high yield of reducing sugar but there was no significant difference between the 20 - 40 and 40 - 80 mesh samples. The result indicated that the reduced WS size is advantageous for improving hydrolysis of WS, due to the fact that size reduction is associated with an increase of the specific surface area (Zhao et al., 2012b). Moreover, the results suggested that the depth of enzyme invading into biomass was more important to improve sugar releasing. Thus, it can be explained why there was no significant difference of released sugar from 20 to 80 meshes WS during the earlier stage of enzymatic hydrolysis. As a result, a mixed index of the surface area combine with invading depth is a more suitable index for assessing the effect of feedstock size, although, it is difficult to measure such variable. The sugar concentration was paid more attention during practical production due to the fact that a higher concentration of sugar could harvest higher concentration of bioethanol or another chemical (Koppram et al., 2014; Modenbach & Nokes, 2012). For 72 h hydrolysis of WS, a 4.09 $\pm$ 0.23 mg/ml of reducing sugar hydrolysate was obtained with 8 % of WS (20 - 40 mesh) solid concentration which was 2.04 and 3.64 times higher than the 4 % and 2 % of WS solid concentration, respectively (Figure 4c). While high solid concentration resulted in lower lignocellulose conversion into fermentable sugar because the high solid concentration could increase the inhibitors (Liu et al., 2014). However, the phenomenon has not been observed in this test. Moreover, hemicellulose removal is usually considered a more effective method of de-recalcitrance to improve cellulose hydrolysis than delignification (Guo et al., 2017; Sindhu et al., 2016). As a result, the released reducing sugar from WS should fast

increase until polysaccharides are near completely converted when using the enzyme cocktail with high xylanase activity due to its deconstructed action. While after only 12 h of hydrolysis, the released sugar was greatly decreased (Figure 4b,c) and the total released sugar for 72 h was less than 33.6 % of total carbohydrate which is 50.07± 0.18 % of total WS weight by a two-step acid hydrolysis process (Sluiter et al., 2008). Therefore, the reason for recalcitrant needs further investigation.

3.7 Advance cognition of enzymatic hydrolysis of WS biomass

To advance enzymatic hydrolysis cognition of WS, a few commercial enzymes with different kind of enzyme activities were used for hydrolysis of WS, the results were shown in Table 3. Correlation Matrix was built via factor analysis program by SASTAT 12 statistical software (SASTAT, USA). The correlation values R were 0.844, 0.840, 0.704, 0.527, and -0.642 for CMCase, FPase, Xylanase, Glucosidase, and Polygalacturonase, respectively. The results suggested that high xylanase activity is beneficial to improve sugar releasing from WS during enzymatic hydrolysis. However, the polygalacturonase was very little effective for improving WS hydrolysis, which is different from our previous study on agave biomass (Wang et al., 2019). Xylanase degrades xylan and de-embedded cellulose from hemicelluloses (Liu et al., 2005; Saha et al., 2017; Volynets & Dahman, 2011). As a result, this enzyme cocktail from *A. niger* Gyx086 with high xylanase activity is very beneficial for enzymatic hydrolysis of WS into reducing sugar. Moreover, as mentioned above, there is less than 33.6 % of total carbohydrate converted into reducing sugar when includes lignin in the biomass. Thus delignification is probably necessary due to lignin is another

structural component embedded with cellulose (Liu et al., 2005; Zhao et al., 2012b). To prove this, different chemical pretreatments were used to remove lignin and/or hemicelluloses. Hemicelluloses and lignin removed from WS were 96.4 % and 91.7 % pretreated by 80 % of *p*-toluenesulfonic acid (TsOH), 4.63 % and 24.5 % by 1.5 % of NaOH, and 99.2 % and 0 % by 1 % of H_2SO_4, respectively. Moreover, 5.49 % of biomass weight of cellulose was also hydrolyzed when using 1% of H_2SO_4. These pretreated WS were hydrolyzed using the enzyme cocktail and the results were shown in Figure 4d. The pretreated WS using TsOH removed most of the lignin and hemicelluloses, which could be completely hydrolyzed by the enzyme cocktail with 122.4 % of released reducing sugar compared with total carbohydrate weight. Actually, this cellulose has very high crystallinity *CrI* (41.19 %) in our previous study (Wang et al., 2019), which suggested that WS crystallinity has a little hamper for the cellulose hydrolysis using the enzyme cocktail. NaOH pretreatment removed part of hemicelluloses and lignin, also harvested a more efficient conversation than the control with releasing 72.3% of reducing sugar of total carbohydrates weight. Lignin removal increases the effectiveness of hydrolysis by reducing non-productive adsorption sites for enzymes and by increasing cellulose accessibility (Kim & Holtzapple, 2006). However, the pretreated WS via 1 % of H_2SO_4 pretreatment got a lesser released reducing sugar than the CK, probably because the easiest hydrolyzed hemicelluloses have been removed and the raised lignin fraction increased embedding cellulose, which resulted in a slow releasing sugar than CK. The results indicated that: 1) delignification was more efficient than hemicellulose removal for enzymatic

hydrolysis of WS; 2) the enzyme cocktail from *A. niger* Gyx086 was very efficient for enzymatic hydrolysis of WS especially combine with delignification pretreatment; 3) Crystallinity is little hamper cellulose hydrolysis using the enzyme cocktail of Gyx086.

Conclusion

Delignification was more important than hemicellulose removal for improving enzymatic hydrolysis of WS, and high crystallinity was little-impeded WS hydrolysis. A low-cost lignocellulosic enzyme cocktail with high xylanase activity can be produced on wheat straw medium fermented by *A. niger*. The enzyme cocktail can hydrolyze unpretreated WS releasing reducing sugar amount to 33 % of total carbohydrate and completely hydrolyze delignification of WS total carbohydrate. The low-cost and efficient saccharification system is promising to promote wheat straw bio-based industry and the work provides a valued knowledge for some kinds of biomass saccharification similar to WS.

E-supplementary data of this work can be found in the online version of the paper.

Acknowledgments

This work was supported by National Natural Science Foundation of China (31730106); the national key research and development projects (2017YFD0600701); The Natural Sciences and Engineering Research Council of Canada (RGPIN-2017-05366); Jiangsu postdoctoral fund (1601082C) and Priority Academic Program Development of Jiangsu Higher Education Institution (PAPD).

Notes

Declarations of interest: none.

References

Açıkel, Ü., Erşan, M., Açıkel, Y.S., 2010. Optimization of critical medium components using response surface methodology for lipase production by Rhizopus delemar. Food Bioprod. Process. 88, 31-39.

Abasolo, W., Eder, M., Yamauchi, K., Obel, N., Reinecke, A., Neumetzler, L., Dunlop, J.W., Mouille, G., Pauly, M., Höfte, H., 2009. Pectin may hinder the unfolding of xyloglucan chains during cell deformation: implications of the mechanical performance of Arabidopsis hypocotyls with pectin alterations. Mol. Plant 2, 990-999.

Alvira, P., Tomás-Pejó, E., Ballesteros, M., Negro, M., 2010. Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: a review. Bioresour. Technol. 101, 4851-4861.

Beckham, G.T., Matthews, J.F., Peters, B., Bomble, Y.J., Himmel, M.E., Crowley, M.F., 2011. Molecular-level origins of biomass recalcitrance: decrystallization free energies for four common cellulose polymorphs. J. Phys. Chem. B 115, 4118-4127.

Boerjan, W., Ralph, J., Baucher, M., 2003. Lignin biosynthesis. Annu. Rev. Plant Biol. 54, 519-546.

Caffall, K.H., Mohnen, D., 2009. The structure, function, and biosynthesis of plant cell wall pectic polysaccharides. Carbohydr. Res. 344, 1879-1900.

Chaturvedi, V., Verma, P., 2013. An overview of key pretreatment processes employed for bioconversion of lignocellulosic biomass into biofuels and value added products. 3 Biotech 3, 415-431.

Chen, H., 2014. Chemical composition and structure of natural lignocellulose, Biotechnology of lignocellulose. Springer, pp. 25-71.

Chen, L., Dou, J., Ma, Q., Li, N., Wu, R., Bian, H., Yelle, D.J., Vuorinen, T., Fu, S., Pan, X., 2017. Rapid and near-complete dissolution of wood lignin at≤ 80℃ by a recyclable acid hydrotrope. Sci. adv. 3 (9) :e1701735.

Chen, S., Zhang, X., Singh, D., Yu, H., Yang, X., 2010. Biological pretreatment of lignocellulosics: potential, progress and challenges. Biofuels 1, 177-199.

Guo, H., Wu, Y., Hong, C., Chen, H., Chen, X., Zheng, B., Jiang, D., Qin, W., 2017. Enhancing digestibility of Miscanthus using lignocellulolytic enzyme produced by Bacillus. Bioresour. Technol. 245, 1008-1015.

Hassan, S.S., Williams, G.A., Jaiswal, A.K., 2018. Lignocellulosic Biorefineries in Europe: Current State and Prospects. Trends Biotechnol, DOI: https://doi.org/10.1016/j.tibtech.

Himmel, M.E., Ding, S.-Y., Johnson, D.K., Adney, W.S., Nimlos, M.R., Brady, J.W., Foust, T.D., 2007. Biomass recalcitrance: engineering plants and enzymes for biofuels production. Sci. 315, 804-807.

Kang, S., Park, Y., Lee, J., Hong, S., Kim, S., 2004. Production of cellulases and hemicellulases by Aspergillus niger KK2 from lignocellulosic biomass. Bioresour. Technol. 91, 153-156.

Kawaguchi, H., Hasunuma, T., Ogino, C., Kondo, A., 2016. Bioprocessing of bio-based chemicals produced from lignocellulosic feedstocks. Curr. Opin. Biotechnol. 42, 30-39.

Keiluweit, M., Nico, P.S., Johnson, M.G., Kleber, M., 2010. Dynamic molecular structure of plant biomass-derived black carbon (biochar). Environ. Sci. Technol. 44, 1247-1253.

Kim, S., Holtzapple, M.T., 2006. Delignification kinetics of corn stover in lime pretreatment. Bioresour. Technol. 97, 778-785.

Koppram, R., Tomás-Pejó, E., Xiros, C., Olsson, L., 2014. Lignocellulosic ethanol production at high-gravity: challenges and perspectives. Trends Biotechnol. 32, 46-53.

Kristensen, J.B., Thygesen, L.G., Felby, C., Jørgensen, H., Elder, T., 2008. Cell-wall structural changes in wheat straw pretreated for bioethanol production. Biotechnol. Biofuels. 1, 5.

Lee, D., Yu, A.H., Saddler, J.N., 1995. Evaluation of cellulase recycling strategies for the hydrolysis of lignocellulosic substrates. Biotechnol. Bioeng. 45, 328-336.

Lin, C., Shen, Z., Zhu, T., Qin, W., 2015. Newly isolated Penicillium ramulosum N1 is excellent for producing protease-resistant acidophilic xylanase. J. Mol. Microbiol. Biotechnol. 25, 320-326.

Liu, R., Yu, H., Huang, Y., 2005. Structure and morphology of cellulose in wheat straw. Cellulose 12, 25-34.

Liu, Z.-H., Qin, L., Zhu, J.-Q., Li, B.-Z., Yuan, Y.-J., 2014. Simultaneous saccharification and fermentation of steam-exploded corn stover at high glucan loading and high temperature. Biotechnol. Biofuels. 7, 167.

Meddeb-Mouelhi, F., Moisan, J.K., Beauregard, M., 2014. A comparison of plate assay methods for detecting extracellular cellulase and xylanase activity. Enzyme Microb. Tech. 66, 16-19.

Miller, G., 1959. Modified DNS method for reducing sugars. Anal. Chem 31, 426-428.

Modenbach, A.A., Nokes, S.E., 2012. The use of high‐solids loadings in biomass pretreatment—a review. Biotechnol. Bioeng. 109, 1430-1442.

Miyamoto, T., Mihashi, A., Yamamura, M., Tobimatsu, Y., Suzuki, S., Takada, R., Kobayashi, Y., Umezawa, T. 2018. Comparative analysis of lignin chemical structures of sugarcane bagasse pretreated by alkaline, hydrothermal, and dilute sulfuric acid methods. Industrial Crops And Products, 121, 124-131.

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y., Holtzapple, M., Ladisch, M., 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresour. Technol. 96, 673-686.

Rabemanolontsoa, H., Saka, S., 2016. Various pretreatments of lignocellulosics. Bioresour. Technol. 199, 83-91.

Ravindran, R., Jaiswal, A.K., 2016. Exploitation of food industry waste for high-value products. Trends Biotechnol. 34, 58-69.

Saha, B.C., Kennedy, G.J., Qureshi, N., Cotta, M.A., 2017. Biological pretreatment of corn stover with Phlebia brevispora NRRL‐13108 for enhanced enzymatic hydrolysis and efficient ethanol production. Biotechnol. Prog. 33, 365-374.

Shrestha, P., Ibáñez, A.B., Bauer, S., Glassman, S.I., Szaro, T.M., Bruns, T.D., Taylor, J.W., 2015. Fungi isolated from Miscanthus and sugarcane: biomass conversion, fungal enzymes, and hydrolysis of plant cell wall polymers. Biotechnol. Biofuels. 8, 38.

Sindhu, R., Binod, P., Pandey, A., 2016. Biological pretreatment of lignocellulosic biomass–An overview. Bioresour. Technol. 199, 76-82.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Crocker, D., 2008. Determination of structural carbohydrates and lignin in biomass. Laboratory analytical procedure 1617, 1-16.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Crocker, D., 2010. Determination of structural carbohydrates and lignin in biomass. Laboratory analytical procedure.

Studer, M.H., DeMartini, J.D., Davis, M.F., Sykes, R.W., Davison, B., Keller, M., Tuskan, G.A., Wyman, C.E., 2011. Lignin content in natural Populus variants affects sugar release. P. Natl. Acad. Sci. USA 108, 6300-6305.

Volynets, B., Dahman, Y., 2011. Assessment of pretreatments and enzymatic hydrolysis of wheat straw as a sugar source for bioprocess industry. Int. J. Energy Environ. 2, 427-446.

Wang, J., Cao, F., Su, E., Wu, C., Zhao, L., Ying, R., 2013. Improving flavonoid extraction from Ginkgo biloba leaves by prefermentation processing. J. Agric. Food Chem. 61, 5783-5791.

Wang, J., Cao, F., Su, E., Zhao, L., Qin, W., 2018a. Improvement of Animal Feed Additives of Ginkgo Leaves through Solid-state Fermentation using Aspergillus niger. Int. J. Biol. Sci. 14, 736-747.

Wang, J., Cao, F., Zhu, Z., Zhang, X., Sheng, Q., Qin, W., 2018b. Improvement of Quality and Digestibility of Moringa Oleifera Leaves Feed via Solid-State Fermentation by Aspergillus Niger. Int. J. Chem. React. Eng., DOI: 10.1515/ijcre-2018-0094.

Wang, J., Chio, C., Chen, X., Su, E., Cao, F., Jin, Y., Qin, W., 2019. Efficient Saccharification of Agave Biomass Using *Aspergillus niger* Produced Low-cost Enzyme Cocktail with Hyperactive Pectinase Activity, Bioresour. Technol., 272, 26-33.

Wu, S., Yu, X., Hu, Z., Zhang, L., Chen, J., 2009. Optimizing aerobic biodegradation of dichloromethane using response surface methodology. J. Environ. Sci. 21, 1276-1283.

Xu, N., Zhang, W., Ren, S., Liu, F., Zhao, C., Liao, H., Xu, Z., Huang, J., Li, Q., Tu, Y., 2012. Hemicelluloses negatively affect lignocellulose crystallinity for high biomass digestibility under NaOH and H 2 SO 4 pretreatments in Miscanthus. Biotechnol. Biofuels. 5, 58.

Yang, B., Wyman, C.E., 2008. Pretreatment: the key to unlocking low‐cost cellulosic ethanol. Biofuels. Bioprod. Biorefin. 2, 26-40.

Yin, Z., Fan, R., 1999. The research progress of plant cell wall. Bull Bot Res 19, 407-414.

Yoon, H., Wu, Z., Lee, Y., 1995. Ammonia-recycled percolation process for pretreatment of biomass feedstock. Appl. Biochem. Biotechnol. 51, 5-19.

Zhao, X., Zhang, L., Liu, D., 2012a. Biomass recalcitrance. Part I: the chemical compositions and physical structures affecting the enzymatic hydrolysis of lignocellulose. Biofuels. Bioprod. Biorefin. 6, 465-482.

Zhao, X., Zhang, L., Liu, D., 2012b. Biomass recalcitrance. Part II: Fundamentals of different pre‐treatments to increase the enzymatic digestibility of lignocellulose. Biofuels. Bioprod. Biorefin. 6, 561-579.

Zhu, J., 2011. Physical Pretreatment− Woody Biomass Size Reduction− for Forest Biorefinery, Sustainable production of fuels, chemicals, and fibers from forest biomass. ACS Publications, pp. 89-107.

Figure 1 Characterization of xylanase activities from Gyx086. (a) Xylanase activities from different biomass medium; (b) different temperature levels with 5% of wheat straw at nature pH (4.7 ± 0.2); (c) different pH with 5% of wheat straw at 30℃; (d) different substance content levels with native pH at 30℃. All of the experiments, three loops with 0.5 diameters of spores were inoculated in each flask bottle of 250ml with 50ml liquid.

Figure 2 2-D contour plots on interactive effects among temperature, moisture content, and fermentation time on xylanase activity in the wheat straw medium by *A. niger* Gyx086

Figure 3 Character of lignocellulosic enzymes on temperature, pH, and thermotolerance. (a) Relative activities from 30℃ to 70℃ with pH 5.0; (b) Relative activities with pH from 2 to 8 at 50℃; (c) Thermostability at 50℃ with pH 5.0; (d) Thermostability at 50℃ with pH 5.0.

Figure 4 Enzymatic hydrolysis of un-pretreated and pretreated biomass by the enzyme cocktail of the *A. niger* Gyx086 in 10 mL of reaction volume with pH 5.0 at 50℃ for 72 h. (a) Different biomasses of less than 20 mesh were hydrolyzed using equivalent volume of enzyme broth and 0.05 M citrate buffer solution; (b) Different size of WS were hydrolyzed using enzyme broth with 1 % of solid concentration; (c) 20-40 mesh WS was hydrolyzed using equivalent volume of enzyme broth and 0.05 M citrate buffer solution with 2 % - 8 % of solid concentration. (d) Un-pretreated or pretreated thick WS were hydrolyzed using enzyme broth with 1 % of solid concentration.

Table 1 Box–Behnken design matrix for optimization of the PG activity

Run	X_1 Temperature(℃)	X_2 pH value	X_3 Time (h)	Xylanase activities (U/ml)
1	-1 (28)	-1 (5)	0 (72)	5.09±0.61
2	1 (36)	-1	0	5.50±0.11
3	-1	1 (7)	0	1.62±0.13
4	1	1	0	4.39±0.29
5	-1	0 (6)	-1 (48)	5.63±0.59
6	1	0	-1	6.15±0.32
7	-1	0	1 (96)	5.85±0.21
8	1	0	1	5.99±0.17
9	0 (32)	-1	-1	5.72±0.56
10	0	1	-1	3.69±0.13
11	0	-1	1	6.28±0.16
12	0	1	1	3.49±0.09
13	0	0	0	5.38±0.41
14	0	0	0	5.72±0.23
15	0	0	0	5.67±0.24

Table 2 The variance analysis ANOVA and the lack of a fit test for the response surface quadratic model

Source	**df**	**SS**	**Mean Squares**	**F-ratio**	***p*-value**
Regression	9	21.807	2.423	13.742	0.005
Linear	3	12.940	4.313	24.463	0.002
Quadratic	3	7.304	2.435	13.809	0.007
Interaction	3	1.563	0.521	2.954	0.137
Residual Error	5	0.882	0.176		
Total Error	14	22.689			
Squared Multiple R	0.961				
Adjusted Squared	0.891				
Lack of Fit	3	0.813	0.271	7.891	0.115
Pure Error	2	0.069	0.034		
Residual Error	5	0.882	0.176		

Table 3 Enzymatic dosages for hydrolysis and sugars released from WS biomass using these enzyme cocktails

	E1+E2	E1+E3	E1+E4	E2	E3	E_{A1}**	E_{A2}**
FPase (U/g dw)	36.5±1.8[a]	22.9±1.1[b]	37.1±1.8[a]	25.6±2.3[b]	14.3±2.5[c]	4.77±0.14	6.77±0.29
CMCase (U/g dw)	62.4±1.1[b]	69.7 ±1.3[a]	44.3±5.3[d]	52.1±0.1[c]	66.3±4.7[ab]	14.31±0.29	13.82±0.38
β - Glucosidase (U/g dw)	31.3±7.6[a]	38.5±0.47[a]	31.8±4.9[a]	2.1±0.3[c]	5.2±0.11[b]	2.51±0.18	2.96±0.37
Xylanase (U/g dw)	302.4±5.2[a]	135.9±2.22[b]	143.1±8.5[b]	273.3±21.6[a]	55.8±0.7[c]	89.23±2.15	122.5±4.57
Polygalacturonase (U/g dw)	-	51.4±7.5[a]	-	-	61.5±4.9[a]	105.92±4.62	6.60±1.59
Released RS (mg/g)*	132.79±1.45[a]	100.51±1.03[a]	94.58±0.92[b]	116.57±0.19[c]	91.96±1.32[a]	23.21±0.74	51.65±1.08

* Released reducing sugar from WS in a 10 mL reaction volume with 1% of solid concentration for 12 h at 50 ℃, pH 5.0.

** E_{A1} and E_{A2} were the enzyme cocktails fermented WS by *A. niger* Gyx086 at 28℃ for 3d and at 39℃ for 12 d, respectively.

Figure 1

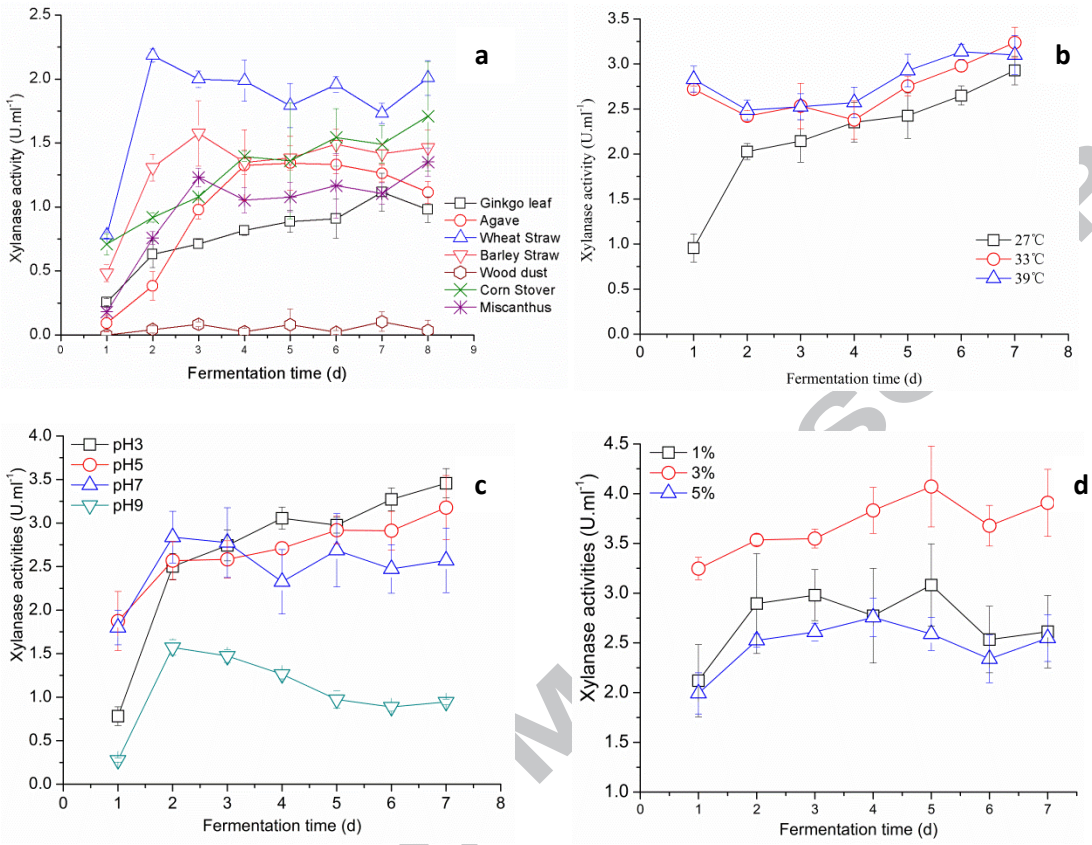


Figure 2

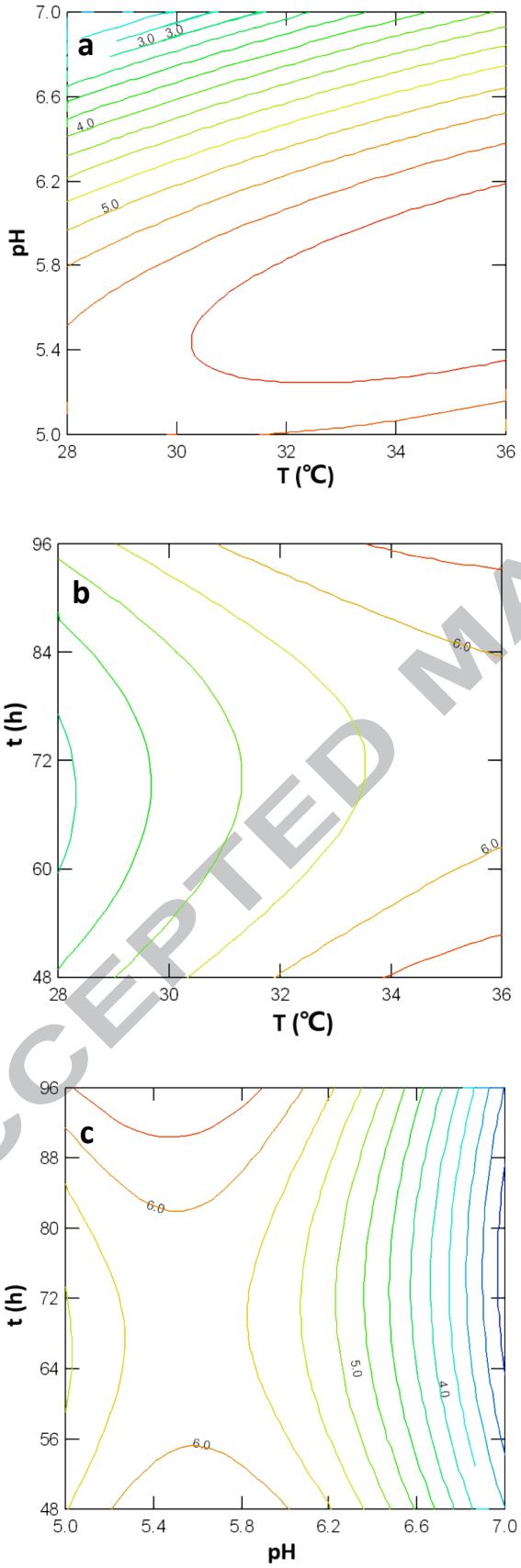


Figure 3

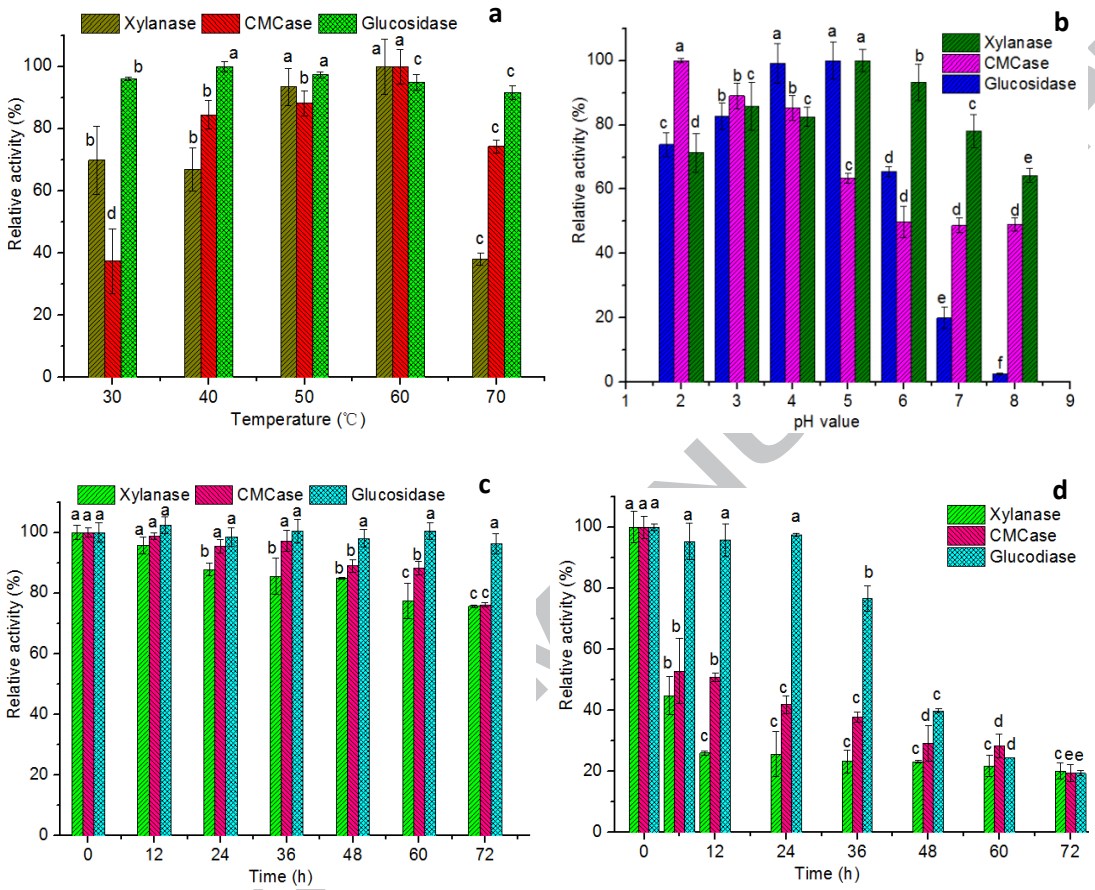
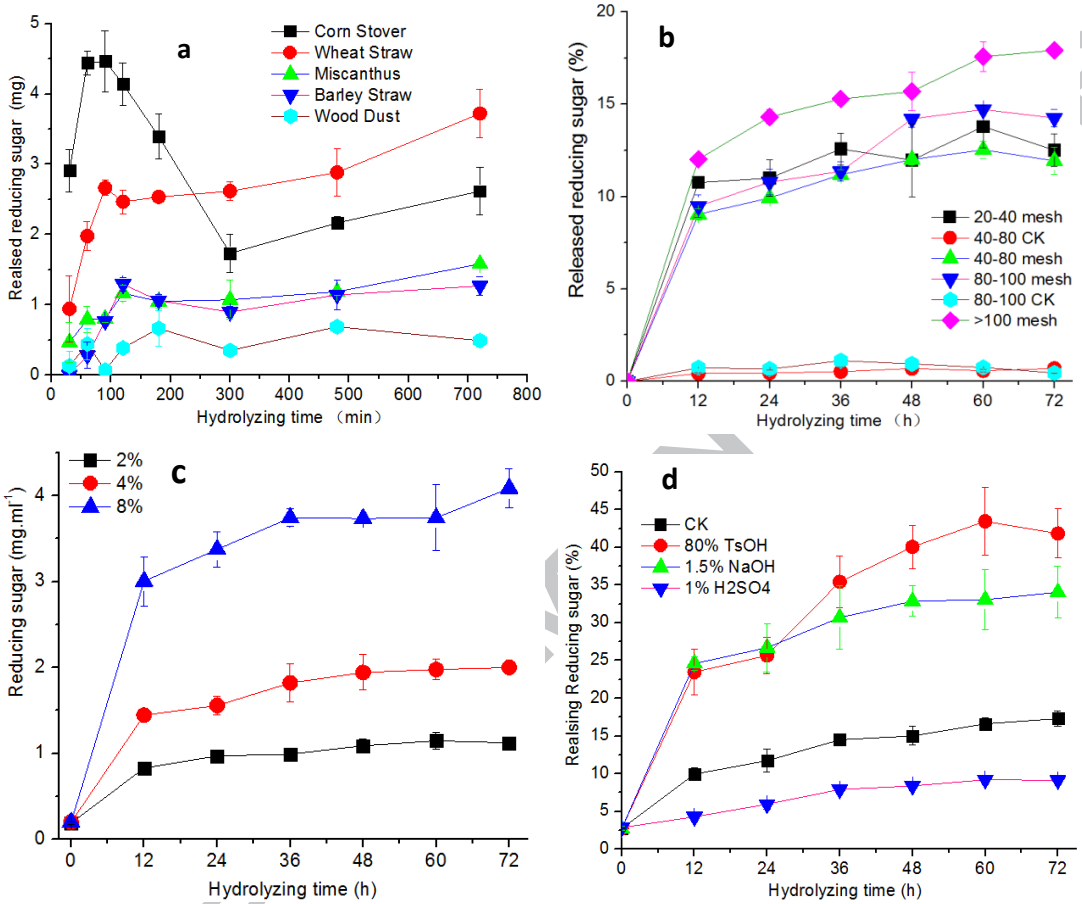


Figure 4



Highlights

1. Low-cost enzyme cocktail with high xylanase activity was produced by *A. niger*;
2. Wheat straw was hydrolyzed more effectively than other biomass by the enzyme cocktail;
3. Xylanase combined with cellulase significantly enhanced the hydrolysis of wheat straw;
4. Delignification was more efficient than de-hemicellulose for wheat straw hydrolysis;
5. Crystallinity is little interference with the hydrolysis process of wheat straw.