Cellulose (2020) 27:3143–3152
https://doi.org/10.1007/s10570-020-02996-z

ORIGINAL RESEARCH

Factors affecting the hydrolytic action of xylanase during pennisetum saccharification: role of lignin

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Received: 7 August 2019 / Accepted: 10 January 2020 / Published online: 21 January 2020

Abstract Inhibition of cellulose hydrolysis has been reported extensively, however, there is a paucity of information describing the effect of lignin on xylan hydrolysis by endo-xylanase and β-xylosidase. In this report, the effects of two different lignins on enzymatic hydrolysis of isolated xylan and NaOH-pretreated pennisetum by endo-xylanase and β-xylosidase were assessed. Both acid insoluble lignin (AIL) and enzymatic hydrolysis lignin (EHL) were found to inhibit hydrolysis of endo-xylanase and β-xylosidase, and AIL had a stronger negative effect on enzymatic hydrolysis when compared with that of EHL. Results from inhibitory kinetics experiments showed that the inhibition of AIL and EHL on xylanase did not follow Michaelis–Menten kinetics. The higher adsorption capacity of AIL toward xylanase arose from its higher hydrophobicity and lower absolute zeta potential, and this likely explains the higher inhibitory effect of AIL than that of EHL. The results aid our understanding of the role of lignin in xylan hydrolysis by xylanolytic enzymes.

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Keywords Xylanase · Xylosidase · Lignin · Inhibition · Adsorption · Hydrophobicity

Abberviations

NACP	Chinese pennisetum with NaOH pretreatment
AIL	Acid insoluble lignin
EHL	Enzymatic hydrolysis lignin
XYL	Endo-xylanase from *Trichoderma longibrachiatum*
βX	β-Xylosidase from *Selenomonas ruminantium*
XOS	Xylo-oligosaccharides
DM	Dry matter

Introduction

In lignocellulosic materials, lignin is one of the main components of lignocellulose that forms complex structures with cellulose and hemicellulose, which can hinder the saccharification of cellulose for fermentation (Chen et al. 2013; Silveira et al. 2015). Polysaccharides such as cellulose and hemicellulose found in the cell wall of lignocellulose plants form three-dimensional networks with embedded colloidal lignin (Ko et al. 2015; Xin et al. 2016a). This complex structure is dense and difficult to breakdown for enzymatic hydrolysis (Brigitte et al. 2018, Yang et al.

2013). The removal of lignin and hemicelluloses improves the enzymatic hydrolysis of lignocelluloses (Huang et al. 2019; Jiang et al. 2018; Liu et al. 2019, Tang et al. 2018). Therefore, in order to produce sugars and biofuels from lignocelluloses effectively, high-efficiency pretreatment and optimization of enzymatic hydrolysis have attracted significant attention (Alvira et al. 2010; Lai et al. 2019; Xin et al. 2016b; Zhang and Wu 2015). Enzymatic digestibility of biomasses after pretreatment is higher when compared with that of raw materials as pretreatment leads to the exposure of lignin, and increases the accessibility of cellulase to cellulose, decreases the amount of cellulase used for enzymatic hydrolysis of lignocelluloses (Financie et al. 2016; Mhlongo et al. 2015; Zhang and Wu 2014). Hence, it can be concluded that lignin inhibits cellulase hydrolysis of cellulose to monosaccharide, and the action mechanism of lignin in the process of hydrolysis has attracted much attention (Ghorbani et al. 2015; Nakagame et al. 2010; García-Torreiro et al. 2018).

The primary reason for lignin-induced inhibition is that lignin acts as a steric hindrance, which hampers enzyme activity substantially (Wen et al. 2013). In addition, lignin contains many groups that can bind to the carbohydrate binding zone of hydrolytic enzymes through hydrophobic and ionic interactions, resulting in the adsorption of enzymes to lignin and the denaturation of enzymes, which restricts enzymatic hydrolysis of substrate (Berlin et al. 2006; Pan 2008; Zhao et al. 2012; Zilliox and Debeire 1998). Lignin binding to enzymes through hydrophobic forces involves irreversible adsorption, thereby affecting the efficiency of enzymatic hydrolysis (Nakagame et al. 2011). Various groups of lignin have a decisive effect on its hydrophilicity and hydrophobicity. The hydrophilic and hydrophobic properties of lignin can be distinguished by measuring the zeta potential, and lignin with greater hydrophobicity exhibits a stronger inhibitory effect (Li et al. 2016b; Pareek et al. 2013). Thus, the determination of zeta potential of lignin can provide insights into the enzyme adsorption on lignin and inhibitory effect of lignin on enzymatic hydrolysis.

The adsorption of lignin by different enzymes has been studied extensively, and other studies have examined the effects of different lignins extracted from raw materials by different pretreatment methods on hydrolytic enzymes (Berlin et al. 2006; Pareek et al. 2013; Rahikainen et al. 2013; Tu et al. 2009; Yu et al. 2019). Studies have shown that lignin significantly affects the cellulase system and xylanase, with beta-glucosidase the least affected (Berlin et al. 2006). Pan (2008) also indicated that inhibition of hydrolysis was linked to the sources and structural characteristics of lignin, such as the importance of lignin functional groups to affect enzyme binding to substrates or interfere with enzyme activity. Zilliox and Debeire (1998) reported that xylanase is irreversibly adsorbed onto the surface of lignin and loses activity by denaturation. Berlin et al. (2006) reported that lignin from *Douglas-fir* had a stronger inhibitory effect on cellulase than the recombinant xylanase from *Penicillium* sp. The results of Zhang et al. (2013) showed that the adsorption of xylanase from *Nonomuraea flexuosa* on wheat straw and lignin fitted Langmuir isotherm and the presence of the carbohydrate-binding module in the xylanase showed positive and negative effects on its adsorption on insoluble xylan and lignin, respectively. Guo et al. (2014) reported that lignin affected xylanase adsorption, and lignin from various sources had a stronger adsorption capacity to endoglucanase when compared to with that of β-glucosidase. In summary, lignin negatively effects enzymatic hydrolysis of biomasses by nonspecific adsorption or steric hindrance or denaturation of enzymes, and the action of lignin on xylan hydrolysis by xylanase remains unresolved.

In this work, the behavior of lignin preparations (acid-insoluble and enzymatic hydrolysis lignins) on the digestibility of insoluble xylan was investigated. Furthermore, structural characteristics, including hydrophobicity and zeta potential, of these two lignins were analyzed, and the relationship between the structural characteristics and inhibition was evaluated. The effects of these lignin preparations on xylanase activity and adsorption onto insoluble xylan were also determined to elucidate the inhibition mechanism of lignin on xylanase hydrolysis.

Materials and methods

Materials

The preparation of Chinese pennisetum with NaOH pretreatment (NACP) was based on previous studies (Chen et al. 2019).Acid insoluble lignin (AIL) was

prepared from Chinese pennisetum by a two-step acid hydrolysis process using the NERL method (Sluiter et al. 2012). Enzymatic hydrolysis lignin (EHL) was provided by Shandong Longli Co., Ltd (China). Insoluble xylan was prepared from oat spelt xylan, as described by Ryan et al. (2003). The prepared xylo-oligosaccharides (XOS) by the method of Zhang and Viikari (2012) contained 32.2% xylobiose, 43.6% xylotriose and a low amount of xylose or residual xylan. The commercial endo-1,4-β-xylanase (XYL) and β-xylosidase (βX) were purchased from Sigma Chemical Co. (USA) and Megazyme (Ireland), respectively. Detailed information of the two enzyme preparations is provided by Chen et al. (2019).

Endo-xylanase and βX activity determination

Different concentrations of beechwood xylan and *p*-nitrophenyl-β-D-xylopyranoside were used as substrates to determine the activity of XYL and βX, respectively. XYL and βX were added to the reaction system and incubated for different times at 50 °C in the absence or presence of different lignins. After the reaction, the activities of XYL and βX were determined by the method of Bailey and Biely (1992) and Poutanen and Puls (1988).

Enzymatic hydrolysis

Enzymatic hydrolysis of insoluble xylan and xylan-containing NACP by XYL with/without βX was performed as reported by Chen et al. (2019). The two xylanase loadings were 8335 nkat XYL per gram dry matter (DM) and 500 nkat βX per gram DM. After the addition of substrates and buffer, AIL and EHL (2, 10, 20 and 50 mg/mL, w/v) were added, and then XYL and/or βX were added. Samples were taken after 6, 24 and 48 h, and enzymatic hydrolysis was stopped by boiling the samples for 10 min. Experiments examining the effect of lignin on βX (500 nkat/g DM) hydrolysis of XOS were performed as described above. AIL and EHL (10 mg/mL, w/v) were added after XOS and buffer. Samples were withdrawn after 6 h and enzymatic hydrolysis was stopped by boiling the samples for 10 min. After cooling, the supernatants of all samples were centrifuged (10,000 × *g*, 10 min) to measure the amount of xylose in the hydrolysate by HPLC. The data points in all hydrolysis experiments were the average of three replicate tests.

Enzyme adsorption experiments

The adsorption capacity of XYL on lignin was researched in 50 mM sodium citrate buffer (pH 5.0) by a magnetic stirring apparatus at 4 °C. AIL and EHL (0.5%, 1%, 2% and 5%, w/v) were used as adsorption substrates, and XYL at the dose of 8335 nkat/g DM was added to the reaction system finally. The experiments without adding lignin were used as blank control. The effects of different lignins on XYL (8335 nkat/g DM) adsorption of insoluble xylan (1%, w/v) were performed by the same method as described previously. AIL and EHL (1%, w/v) were added before the beginning of the adsorption experiment. The experimental samples without lignin were used as blank control. After 1 h, the supernatants of all samples were collected by centrifugation at 10, 000 × *g* for 10 min, then the residual activity of XYL in the supernatants was measured by the method of Bailey and Biely (1992). The adsorption (%) of xylanase was the ratio of adsorbed xylanase to the added xylanase and the adsorbed xylanase was calculated by subtracting the activity of xylanase remaining in the supernatant from the amount of total enzyme activity added. All experiments were performed in triplicate and averaged.

Hydrophobicity analysis of lignin

The hydrophobicity of lignin was determined and calculated by the methods reported in Gessner et al. (2000) with Rose Bengal solution. Different concentrations of AIL and EHL were incubated in 50 mM citrate buffer (pH 5.0) for 2 h with the Rose Bengal solution at 50 °C and 200 rpm. After the reaction, the absorbance of the supernatant was measured at 543 nm. All hydrophobicity experiments were performed in triplicate.

Zeta potential analysis of lignin

A Zetasizer (Malvern Instruments Ltd, UK) with laser Doppler micro electrophoresis was used to determine the zeta potentials of different lignin samples. AIL and EHL were dispersed in sodium citrate buffer before carrying out measurements. Three replicates were measured for each lignin sample.

Analytical method and calculations

The soluble monosaccharide content in hydrolysates was analyzed by HPLC system and the hydrolysis yield of glucose and xylose were calculated according to a previous report (Chen et al. 2019).

Degree of inhibition was evaluated by the following equation:

$$\text{Degree of inhibition } (\%) = \frac{Y_0 - Y_{lignin}}{Y_0} \times 100 \quad (1)$$

where Y_0 and Y_{lignin} are the xylose yield (%) without and with the addition of lignin preparations (AIL and EHL).

Results and discussion

Inhibition of XYL and βX by AIL and EHL

The efficient hydrolysis of xylan-containing biomass requires the synergistic action of endo-xylanase and β-xylosidase (Xin et al. 2015) and thus commercial xylanase preparations usually contain both endo-xylanase and βX. In this work, the impacts of lignin on the hydrolysis of isolated xylan and Chinese pennisetum after NaOH pretreatment (NACP) by XYL and βX were researched (Fig. 1). For the isolated xylan hydrolysis reaction, the addition of 10 mg/mL AIL or EHL decreased the xylose yield from 72.8% to 56.8% and 60.5%, respectively (Fig. 1A). The degree of inhibition of AIL and EHL in the hydrolysis of isolated xylan was 22.0% and 16.8%, respectively, indicating that inhibition by AIL was stronger when compared with that of EHL. To verify the above results, NACP was used as a real substrate for enzymatic hydrolysis experiments. For the hydrolysis of xylan in NACP by XYL and βX, the addition of AIL and EHL (10 mg/mL) caused both glucose and xylose release from NACP (Fig. 1B, C) but the 48 h glucose yield decreased from 20.5% to 8.4% and 13.6%, respectively. Thus, AIL and EHL decreased the hydrolysis of xylan in NACP and the inhibition ratio was 59.5% and 33.2%, respectively (data not shown). Clearly, the results showed that AIL exhibited stronger inhibition of xylanase hydrolysis of xylan in NACP than EHL. Such strong inhibition has also been reported by Kellock et al. (2017) in the hydrolysis of birchwood xylan by purified xylanase from

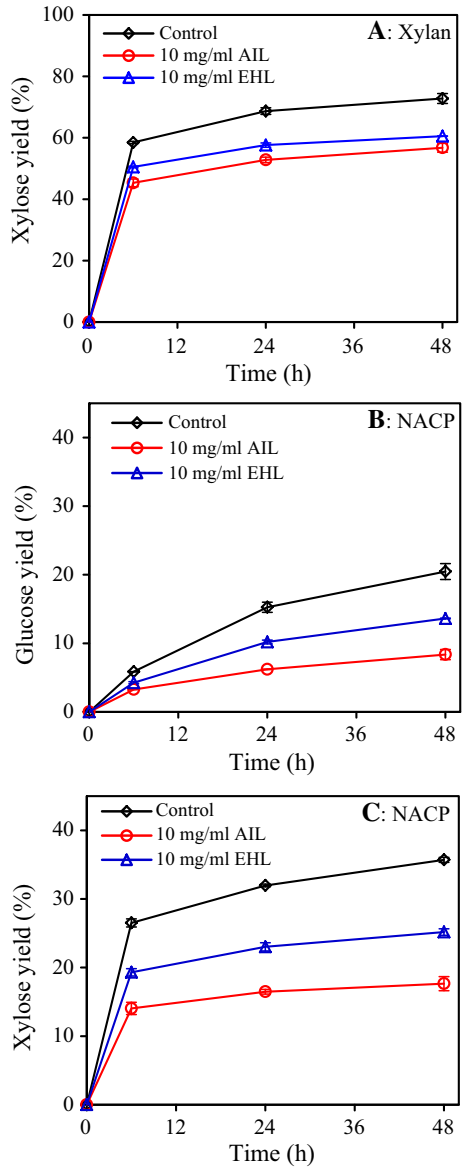

Fig. 1 Hydrolysis of 2% (w/v) insoluble xylan (**A**) and NACP (**B** and **C**) of 2% (w/v) by XYL (8335 nkat/g DM) and βX (500 nkat/g DM) with added AIL and EHL (10 mg/mL) at pH 5.0 and 50 °C. The error bars represent the standard errors of three independent experiments

Trichoderma reesei with 10 mg/mL enzymatic hydrolysis lignin from spruce or wheat straw. The main inhibitory mechanism of AIL and EHL on xylanase may arise from a physical barrier that limits the accessibility of xylanase to substrates. In addition, xylanase may be adsorbed onto the surface of lignins,

which reduce contacts between xylanase and substrate (Berlin et al. 2006; Pan 2008; Zhao et al. 2012).

Inhibition of XYL by AIL and EHL

In order to further verify the results of Fig. 1, the effects of AIL and EHL at different concentrations on the hydrolysis of isolated insoluble xylan by XYL were studied (Fig. 2). Although the main products of endo-xylanase are XOS, long time hydrolysis by endo-xylanase would convert XOS into xylose. Additionally, according to the information provided by the supplier, XYL as the commercial endo-xylanase contains βX, so that the content of xylose can be detected to reflect the difference in inhibition. A large decrease in xylose yields was observed with the addition of 2 mg/mL AIL and EHL, and the degrees of inhibition by AIL and EHL reached 28.8% and 19.4%, respectively (data not shown). Hydrolysis yields of isolated xylan decreased with the increasing doses of AIL and EHL. AIL was a stronger inhibitor of xylan hydrolysis when compared with EHL. The degree of inhibition may differ because of the different chemical structures and properties of AIL and EHL (Guo et al. 2014; Nakagame et al. 2010; Zhang et al. 2017).

Effects of AIL and EHL on the hydrolysis of a real substrate (NACP) by XYL were researched to verify the above experimental results. The results showed that at 48 h the xylose yields of xylan in NACP decreased from 31.8% to 13.5% and 22.9% when incubates AIL and EHL (Fig. 3B), respectively, corresponding to degrees of inhibition of 56.6% and 26.6%. The inhibitory effect of XYL on glucose yields from NACP was also examined (Fig. 3A), which confirmed that the commercial endo-xylanase preparation was not very pure and contained some cellulolytic activity. The results of the experiments indicated that the inhibitory effects of lignins on xylan hydrolysis were not affected by different substrate species. The effect of lignin on xylan hydrolysis has been reported in many studies and the degree of inhibition of xylanase on lignin prepared using different methods varies (Guo et al. 2014; Kellock et al. 2017; Olivataravilla et al. 2016).

Effect of AIL and EHL on hydrolysis of XOS by βX

AIL and EHL inhibited the hydrolysis of isolated xylan and xylan in NACP by the combination of XYL and βX (Fig. 1), and inhibitory effects of AIL and EHL on xylan hydrolysis by XYL was confirmed (Figs. 2 and 3). However, the function of lignins in the hydrolytic capacity of βX is unknown and the impact of AIL and EHL on XOS hydrolysis by βX was investigated (Fig. 4). Endo-xylanase breaks the main chain of xylan and hydrolyzes xylan into XOS, XOS are then hydrolyzed to xylose from the non-reducing ends by βX. Thus, βX plays a key role in xylan degradation (Knob et al. 2010; Li et al. 2016a). In this work, the βX used had been reported to hydrolyze xylan poorly (Xin et al. 2015). A xylose yield of 2.4 mg/mL was obtained from 5 mg/mL XOS by βX. After the addition of AIL and EHL (10 mg/mL), the xylose yield decreased to 1.6 and 1.9 mg/mL, respectively. The results indicated that both AIL and EHL inhibited enzymatic hydrolysis of XOS by βX and the inhibitory effect of AIL on βX was stronger than that of EHL.

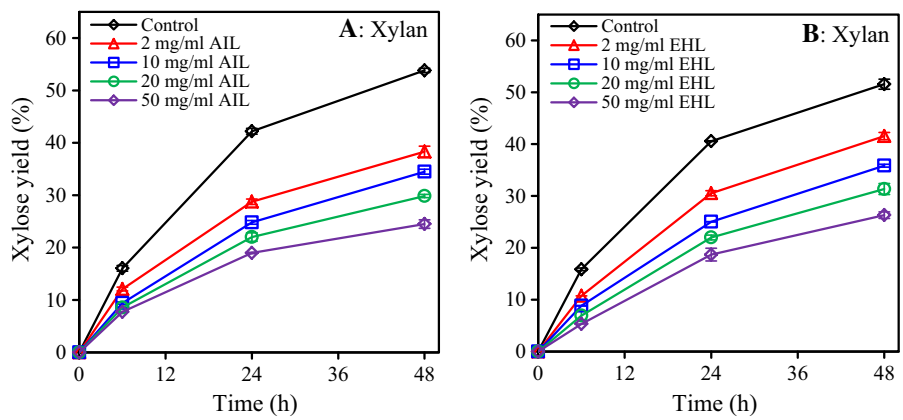

Fig. 2 Hydrolysis of 2% (w/v) insoluble xylan by XYL (8335 nkat/g DM) with added AIL (2, 10, 20, 50 mg/mL) (**A**) and EHL (2, 10, 20, 50 mg/mL) (**B**) at 50 °C and pH 5.0 for 6, 24, 48 h. The error bars represent the standard errors of three independent experiments

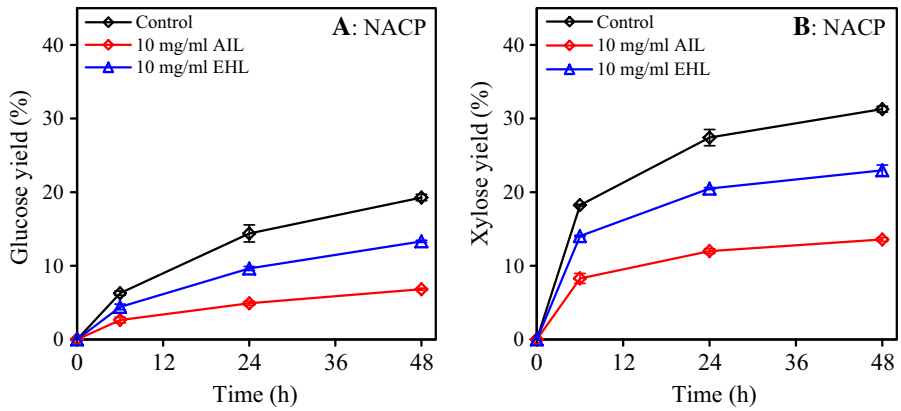

Fig. 3 Hydrolysis of 2% (w/v) NACP by XYL (8335 nkat/g DM) with added AIL (10 mg/mL) and EHL (10 mg/mL) at 50 °C and pH 5.0 for 6, 24, 48 h. The error bars represent the standard errors of three independent experiments

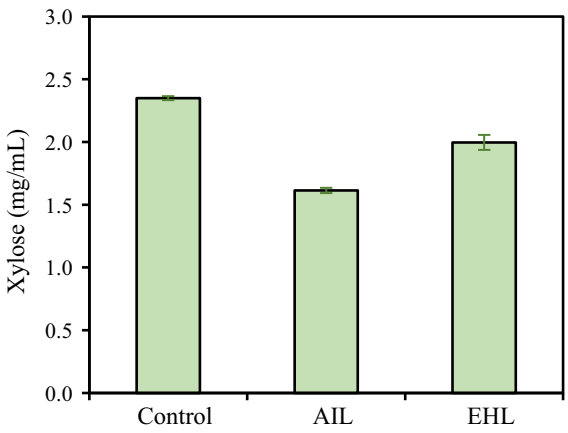

Fig. 4 Effect of AIL and EHL (10 mg/mL) on the hydrolysis of XOS (5 mg/mL) by βX (500 nkat/g DM) at 50 °C and pH 5.0 for 6 h. The error bars represent the standard errors of three independent experiments

Adsorption of xylanase on lignin

Steric hindrance of lignin and nonspecific adsorption of hydrolytic enzymes on lignin are the main mechanisms of lignin inhibiting the hydrolysis of lignocelluloses (Guo et al. 2014; Lu et al. 2016; Nakagame et al. 2010; Rahikainen et al. 2013), and thus the adsorption of XYL on AIL and EHL should be a key inhibitory factor. Increasing the lignin consistency from 0.5% to 5% caused an increase in the adsorption of XYL onto AIL and EHL from 36.2% and 19.3% to 89.5% and 79.4%, respectively (Fig. 5A), indicating that AIL absorbed more XYL than EHL. Zilliox and Debeire (1998) reported that the adsorption of xylanase on dioxane lignin is irreversible and the bound xylanase has no hydrolytic activity. Our previous work reported that acid insoluble lignin adsorbs xylanase and desorbed xylanase still exhibits hydrolytic activity (Li et al. 2015). The results here showed that the strong adsorption capacity of XYL on AIL and EHL was consistent with these previous reports.

Adsorption experiments of XYL on insoluble xylan (1%) at 4 °C and pH 5.0 were performed and the results revealed that the adsorption of XYL onto xylan was 38.3% (Fig. 5B). Tenkanen et al. (1995) reported that xylanase binds to xylan when the pH is below the isoelectric point of the xylanase. After the addition of AIL and EHL (10 mg/mL) to the reaction mixture, the adsorption of XYL increased from 38.3% to 67.4% and 56.0%, respectively, indicating that XYL adsorbs strongly onto lignins. The XYL adsorption on lignins may reduce the capacity of xylanase to hydrolyze xylan (Li et al. 2015), which could explain the reduced hydrolysis yield observed for isolated xylan and xylan in NACP (Figs. 2 and 3). Additionally, lignin might precipitate on the surface of insoluble xylan and decrease the contact area between XYL and insoluble xylan, which would also contribute to the negative effect of lignins in xylan hydrolysis by XYL (Figs. 2 and 3).

Effects of lignin on the activity of XYL and βX

The effects of AIL and EHL on the endo-xylanase activities of XYL as a function of the different amounts of beechwood xylan as the substrate were measured and results showed that the inhibition of AIL and EHL on XYL were not competitive, non-competitive or uncompetitive, and the loss of activity was greater when AIL was present (Fig. 6A and B). Berlin et al. (2006) reported that enzymatic hydrolysis lignin

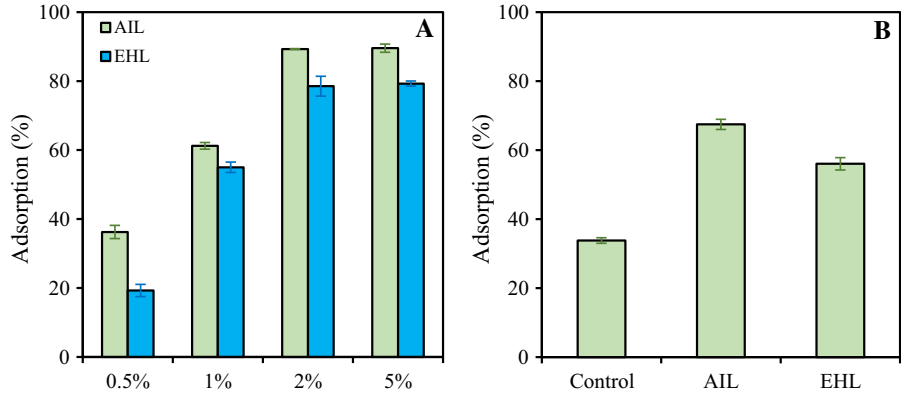

Fig. 5 Adsorption of XYL (8335 nkat/g DM) on AIL and EHL (0.5%, 1%, 2%, 5%) at 4 °C and pH 5.0 for 1 h (**A**). Effect of AIL and EHL (10 mg/mL) on the adsorption of XYL (8335 nkat/g DM) on insoluble xylan (1%, w/v) at 4 °C and pH 5.0 for 1 h (**B**). The error bars represent the standard errors of three independent experiments

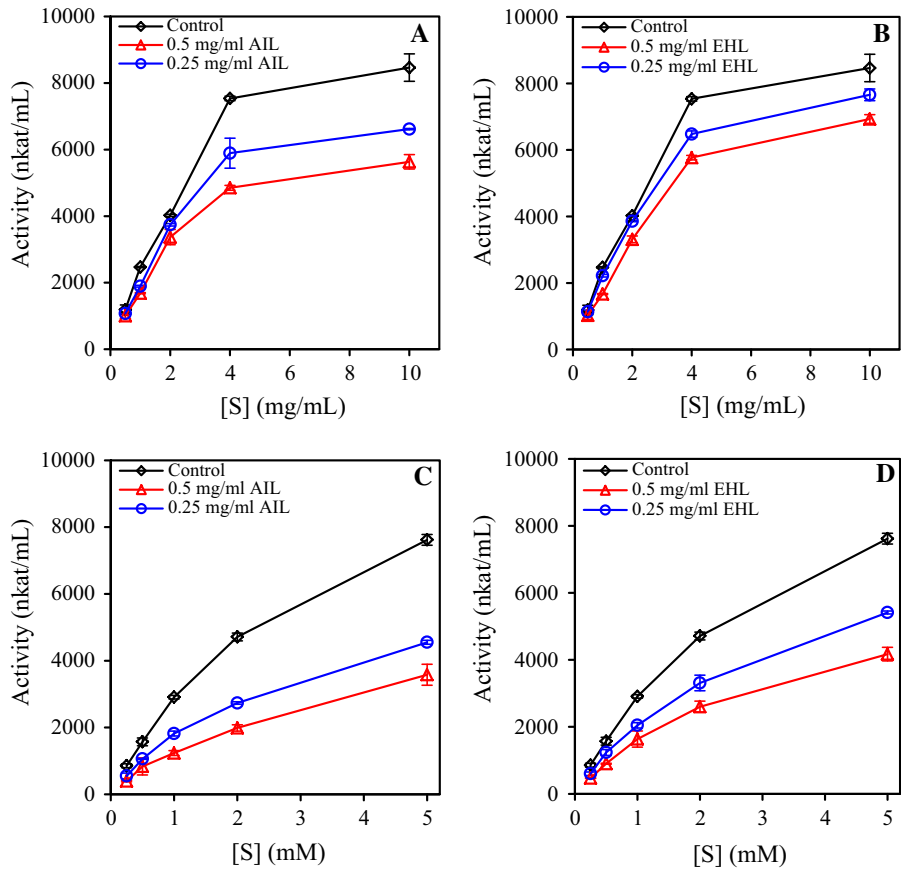

Fig. 6 Effect of AIL and EHL on the activity of XYL at 50 °C and pH 5.0 using different concentrations of beechwood xylan as substrate (**A** and B). Effect of AIL and EHL on the activity of βX at 50 °C and pH 5.0 using different concentrations of *p*NPX as substrate (C and D). The error bars represent the standard errors of three independent experiments

had inhibitory effect on xylanase activity, which is consistent with the results herein. Similarly, the effect of AIL and EHL on βX activity was determined by using *p*NPX as a substrate and the results showed that

βX inhibition by AIL and EHL was not competitive, non-competitive or uncompetitive, and the loss of activity was greater when AIL was present (Fig. 6C and D). Thus, the effects of AIL and EHL on the activities of βX were consistent with the results of enzymatic hydrolysis of XOS by βX (Fig. 4).

The results here were in accordance with the above results (Figs. 1, 2 and 3) showing that AIL had a stronger negative effect on the XYL and βX activities, which contributed to the lower hydrolysis yield of substrates by XYL and/or βX. Thus, the negative effect of isolated lignin on hydrolysis of xylan-containing substrates by XYL and βX may be due to precipitation of lignins onto xylan, which formed a barrier and physically impeded these enzymes. Additionally, lignin might also adsorb endo-xylanase and β-xylosidase and bind into the active sites of these xylanolytic enzymes, which reduced their activities.

Hydrophobicity and zeta potential analysis of lignin

The hydrophobicity of lignin is very important for enzymatic non-productive adsorption on lignin, and lignin with greater hydrophobicity exhibits a stronger inhibitory effect (Li et al. 2016b; Pareek et al. 2013; Rakotoarivonina et al. 2015). Xylanase and cellulase display hydrophobicity because they contain tryptophan, phenylalanine, and tyrosine residues (Berlin et al. 2006; Shuai et al. 2010). The hydrophobicity of AIL and EHL was 1.632 and 1.189, respectively, indicating that the hydrophobicity of AIL was stronger than that of EHL, which supported the observed stronger adsorption capacities of XYL and βX to AIL (Figs. 4 and 5).

Zeta potential analysis showed that AIL and EHL were negatively charged in the water, which was because of the negatively charged groups, such as − OH and − COOH. The zeta potential of AIL was − 4.4 mV, while the absolute zeta potential of EHL (− 30.2 mV) was higher than that of AIL. Lan et al. (2013) and Lou et al. (2013) showed that higher absolute values of the zeta potential indicated higher hydrophilicity of the lignin and reduced nonspecific binding of enzymes on lignin samples. Therefore, these results here supported that the hydrophobicity and the inhibitory effect of EHL were weaker than those of AIL, as confirmed in adsorption and hydrolysis experiments (Figs. 2, 3 and 4).

In this work, the impact of acid-insoluble lignin and enzymatic hydrolysis lignin on the hydrolysis of insoluble xylan and the NaOH-pretreated pennisetum were investigated and the results confirmed the strong inhibitory effect of the two lignin preparations on endo-xylanase and βX. The inhibition mechanism of lignins on endo-xylanase and βX were investigated from the perspective of non-productive adsorption and inhibition kinetics. However, in the enzymatic hydrolysis of actual substrates, the amount of lignin in substrates and on the surface of substrates would gradually increase, which differs from the conditions used herein. Furthermore, during the hydrolysis of actual substrates by cellulose hydrolytic enzymes and xylan hydrolytic enzymes, the competitive adsorption of enzymes on lignin would be different from the situation in our work. Therefore, the results herein might be different from the results of enzymatic hydrolysis of real substrates. In addition, different lignin structures and properties would affect the adsorption of xylan hydrolytic enzymes on lignin and the hydrolysis activity of xylanolytic enzymes. Therefore, further studied are required to characterize the effect of different lignin preparations on xylanase hydrolysis of raw materials with different pretreatment methods.

Conclusions

In this work, we characterized the effect of AIL and EHL on the hydrolysis of insoluble xylan and xylan in NACP by xylanase. We found that AIL and EHL inhibited endo-xylanase and/or βX, and the inhibitory capacity of AIL was stronger than that of EHL. The degrees of inhibition of 10 mg/mL AIL and EHL on the hydrolysis of NACP by XYL and βX were 50.6% and 29.5%, respectively. The xylanase was more readily adsorbed on AIL when compared with that of EHL, which may arise from EHL being more hydrophilic and having a higher absolute zeta potential than AIL. These findings aid our understanding of how lignin affects the activity of xylanolytic enzymes in the saccharification of biomasses.

Funding This work was supported by the National Natural Science Foundation of China (No. 31670598 and No. 31270622) and the Science Foundation for Distinguished Young Scholars of Northwest A&F University, China (No. 2452015098).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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