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THE STUDY ON INHIBITION AND REGULATION OF SPRUCE MANNAN ON ENZYMATIC HYDROLYSIS OF CELLULOSE

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ABSTRACT

Mannan is the main hemicellulose in coniferous wood. Spruce mannan was extracted and characterized by FT-IR, SEM and specific surface area test. FT-IR showed it was branched galactoglucomannan; SEM revealed its layered surface and rod-like polymerization units with spherical tops at high magnification; it had small specific surface area and large pore size. Hydrolysis experiments showed spruce mannan inhibited commercial second-generation cellulase (max inhibition rate 22.22%), with inhibition related to hydrolysis time, enzyme dosage and mannan amount. Tween 80 alleviated this inhibition (min inhibition rate 8.69%), promoted β-glucosidase at high concentration (max promotion rate 28.57%), and greatly reduced mannan's hydrolysis effect (inhibition rate down to 31.82%). This study provides a basis for improving mannan utilization and biomass energy conversion.

KEYWORDS: Spruce, cellulase, mannan, enzyme hydrolysis, mechanism.

INTRODUCTION

In the process of biomass ethanol production from lignocellulosic raw materials, the cell wall of lignocellulosic raw materials will form a lignin-carbohydrate complex (LCC) structure (Chundawat et al. 2011), that is, lignin, cellulose microfiber and hemicellulose form a mutually entwined, tight, tough, complex composition and insoluble in water. LCC structure can resist various microbial and chemical attacks (Kumar et al. 2008; Mosier et al. 2005; Sánchez 2009; Xin et al. 2015). In general, pretreatment of lignocellulosic materials is required before enzymatic hydrolysis which will improve the conversion rate of lignocellulosic materials into glucose by increasing the attachment of cellulase to lignocellulosic materials. There are generally three pretreatment methods: physical, chemical and biological (Xu and Huang 2014).

Pretreatment of lignocellulosic materials in the production of ethanol can, to a certain extent, remove hemicellulose in the material, remove part of lignin, reduce the crystallinity and degree of polymerization of cellulose in the raw material, and increase the porosity in lignocellulosic materials (Chandra et al. 2007; Mansfield et al. 1999; Pan et al. 2005). Enzymatic hydrolysis is a key step of enzymatic hydrolysis of fibrous raw materials (Modenbach and Nokes 2013). The cellulose and hemicellulose polysaccharides in lignocellulosic materials can be converted into oligosaccharides and monosaccharides by enzymatic hydrolysis technology. Raw material pretreatment is relatively dispersed and structurally destroyed, so as to facilitate the subsequent conversion of sugars into biomass ethanol through fermentation (Hsieh et al. 2014; Inoue et al. 2015).

Cellulose is a class of organic polymers containing more than half of the plant cell wall components (Jørgensen et al. 2007; Xin et al. 2016), and that is an important source of fermentable sugars. Therefore, cellulase is essential in the enzymatic hydrolysis process. Due to the tightly bound natural anti-depolymerization structure in lignofiber materials, a large amount of enzyme preparation is needed in the process of preparing fermentable oligosaccharides and monosaccharides (Maki et al. 2009; Martínez et al. 2009; Viikari et al. 2012; Wang et al. 2015; Xing et al. 2014).

Commercial cellulases mainly include three types of cellulases: β-glucosidase, endoglucanase and exoglucanase (Fig. 1). The beta glucose glycosidase hydrolyze cellobiose and oligosaccharide to glucose which can be used in the fermentation biomass ethanol; endoglucanase cutting inside the main open cellulose internal amorphous region of the beta 1, 4 glycosidic bond, different degree of polymerization can be obtained by depolymerization reducing sugars; fibrobiose can be obtained by exglucanase cut cellulose internal groups (Benoit et al. 2015).

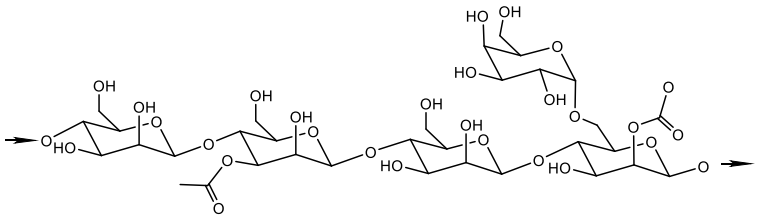


Fig. 1: Chemical structure of O-acetyl-galactoglucomannan.

Since hemicellulose has relatively more components than cellulose, it can form more complex structures and thus requires a greater variety of enzymes for hydrolysis. Similar to the principle of cellulase hydrolysis, in the process of hemicellulose hydrolysis, a single hemicellulase component cannot fully and rapidly hydrolyse the hemicellulose in the raw material. A variety of hemicellulases, especially the interaction of mainchain enzyme and branchchain enzyme, is needed to efficiently depolymerize the complex structure of hemicellulose (Biely et al. 1986; Deshpande et al. 1986; Moreira and Filho 2008). Lignin is an aromatic polymer with complex structure that protects cellulose polymer in plant cell wall from saprophyte and other pathogen microorganisms. The content of lignin in cell wall is only lower than cellulose, which is generally difficult to be biodegraded and has a strong biological resistance. Therefore, due to this property of lignin, which is similar to the hydrolysis of hemicellulose, lignin also needs a number of different enzymes to cooperate with each other

in order to hydrolyse efficiently. During the reaction of lignocellulosic materials in the hydrolysate system, more and more hydrolysates such as cellobiose, oligosaccharide and glucose will be generated in the hydrolysate gradually, and these hydrolysates will more or less have a negative effect on the activity of cellulase. Cellulobiose can inhibit the activity of endoglucanase and exoglucanase, and this inhibition is very obvious (Arnling Bååth et al. 2018; Atreya et al. 2016; Xue et al. 2011).

In the hydrolysis process of lignocellulose, the surface of cellulose will adsorb certain mannan through hydrogen bonds, thus reducing the contact rate of cellulase to cellulose and restricting the efficient production of glucose (Kabel et al. 2007). Before hydrolysis, lignocellulose are usually pretreated, and a part of mannan is dissolved in the pretreatment solution during the pretreatment process (Hsieh et al. 2015; Rémond et al. 2010; Yang and Wyman 2008; Zhang et al. 2012). Studies have pointed out that mannan can significantly reduce the activity of cellulase, the glucose yield reduce hydrolysis of wood fiber. (Kumar and Wyman 2014) in the experiment using beta glucase enzyme and commodity with cellulose enzyme hydrolysis of microcrystalline cellulose, adding different concentration of mannan in the hydrolysis system, can make the glucose yield decline, as the concentration of mannan was 2.5 mg/mL, the yield decreased to 20%.

The objective of this study is to make clear the effect of mannan on cellulase hydrolysis of spruce substrate, and find out the effective measures to alleviate the effect of mannan on cellulase from the perspective of optimizing cellulase hydrolysis of spruce lignocellulose. In addition, it provides the basis for biomass energy conversion of cellulosic lignocellulosic biomass to improve the yield of hydrolysis.

MATERIALS AND METHODS

Materials

Spruce raw material was purchased from California, the United States. After natural air drying, it was shaded into wood dust by double-sided planer and crushed into wood powder by grinder, and then passed through an 80-mesh sieve. 10 g of dry matter raw material was weighed with a solid-liquid ratio of 1:10, and reacted at 170°C for 20 min. The ratio of supernatant to ethanol was 15:85. After precipitation for 24 h, the white precipitates were collected by extraction and filtration. After that, a large amount of anhydrous ethanol, acetone and methyl tert-butyl ether were continuously washed with water.

Fourier transform infrared spectroscopy (FT-IR) and scanning electron microscope (SEM) analysis

The samples for FT-IR spectra analysis were pressed into KBr discs and analyzed using FT-IR spectrophotometer (Nicolet 6700, Thermo Fisher Scientific, USA). The background spectrum of the diamond window without a sample was subtracted from each sample spectrum. 64 scans were collected for each measurement over the wavelength range of 4000–400 cm^{-1}. The surface morphology of spruce mannan was characterized by SEM. Before imaging, the mannan sample was sprayed with gold to improve its surface conductivity. Surface observations were made at a magnification of 500-100 000 times.

Enzymatic hydrolysis

Hydrolysis of the samples with the cellulase preparation was performed in tubes with a 2 mL working volume in 50 mM sodium citrate buffer (pH 5.0) at 50°C. Hydrolysis was conducted in a shaking incubator set to 200 rpm. The substrate dry matter (DM) content was 2% (w/v). NaN_3 0.02% was added to the hydrolysis broth to prevent bacterial infection. Enzyme preparations included second-generation cellulase, β-glucosidase, endoglucanase and exoglucanase at doses of 10, 20 and 30 FPU/g DM (nkat/ml), respectively. Tween 80 at 10, 20, 30 mg/g DM was added to the inhibitory remission test. Samples were withdrawn at 24, 48, 72 h and boiled for 10 min to stop enzymatic hydrolysis. After cooling, the samples were centrifuged at 10 000 × g for 10 min, and the glucose and xylose contents of the supernatants were analysed.

Specific surface area analysis

The specific surface area and pore size and structure were measured by nanometer laser particle size analyser (Zetasizer Nano ZEN3600, Malvern Instruments Ltd., UK). Firstly, the mannan sample was degassed in a vacuum at 30°C for 200 min to remove some impurity gas and water absorbed on the sample. Nitrogen was used as the adsorbent at the liquid nitrogen temperature of 77 K. BET method was used to calculate the specific surface area, pore volume and average porosity of mannan according to the adsorption isotherm.

Analytical methods

The glucose content after cellulase hydrolysis was measured by SBA-40E biosensor analyser. The sample supernatant of 25 μL was accurately absorbed by SBA microinjector and injected into the injector. The reading unit was mg/100 mL, and the glucose content in the hydrolysate to be measured was multiply the reading by dilution. The glucose and xylose yields from the hydrolysis of HP samples were calculated by the following equations:

$$\text{Inhibition rate (\%)} = (G_0 - G_1/ G_0) \times 100 \quad (1)$$

$$\text{Promote rate (\%)} = (G_2 - G_3/ G_3) \times 100 \quad (2)$$

where: G_0 is the glucose content in the hydrolysate without the addition of mannan, and G_1 is the glucose content in the hydrolysate after the addition of mannan, G_2 is the glucose content in the hydrolysate without the addition of Tween 80, and G_3 is the glucose content in the hydrolysate after the addition of Tween 80.

RESULTS AND DISCUSSION

Specific surface area analysis

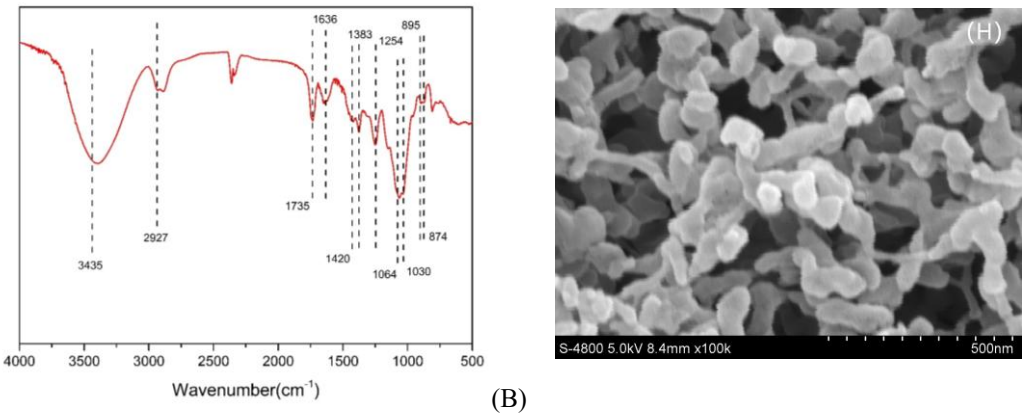
Specific surface area refers to the total area of the material per unit mass, which will affect the reaction rate of mannan participating in the hydrolysis reaction. Tab. 1 shows the specific surface area, pore volume and pore size of spruce mannan.

Tab. 1: Determination of specific surface area of mannan extracted from spruce.

Sample	Specific surface area (m^2/g)	Entrance pore (mL/g)	The aperture (nm)
Spruce mannan	23.05	1.24	19.90

Generally, the larger the specific surface area, the better the surface dispersion, and the higher the dispersion degree, the easier it is to be adsorbed on the cellulase in the hydrolysate. According to the data in the table, compared with other reaction additives, the specific surface area of mannan is relatively small, and the pore volume and aperture are relatively large. However, after the addition of chemical dispersant Tween 80, the inhibition of cellulase was reduced. The reason may be that the stabilization and dispersion effect of Tween 80 on cellulase was stronger than that on mannan, or the adsorption of mannan on cellulase depended on specific sites, so the dispersant could not strengthen the inhibition effect of mannan. The structural properties of hemicelluloses, particularly surface area and pore volume, critically dictate non-productive enzyme binding. Although the extracted spruce mannan has a low specific surface area (23.05 m^2/g), its large pores provide physical pockets that entrap cellulases, consistent with findings that residual mannan structurally traps and competitively inhibits cellobiohydrolase and endoglucanase (Xin et al. 2017). Tween 80 mitigates this by altering interfacial tension and preventing non-productive hydrophobic interactions within the pores.

FT-IR spectrum is shown in Fig. 2A. The wide absorption peak at 3435 cm^{-1} in the figure is the characteristic absorption peak obtained from O-H stretching vibration, which indicates that there is a certain amount of -OH in the main chain of spruce mannan, and there is also intermolecular hydrogen bond in the main chain.



(A) (B)

Fig. 2: FT-IR analysis of mannan (A), the SEM images of manan (magnification 100000 times) (B).

The absorption peak at 2927 cm^{-1} indicates that -CH_2- structure in the mannan, and the C-H bond on the structure induces stretching vibration. The absorption peak of 1735 cm^{-1} caused by C=O stretching vibration on the aldehyde group of mannan, the absorption peak of 1636 cm^{-1} caused by C=O in the hemiacetal structure, the absorption peak of 1420 cm^{-1} caused by the deformation vibration of =CH_2 in mannan, and the absorption peak of 1383 cm^{-1} caused by the bending vibration of C-H in mannan. The absorption peak at 1254 cm^{-1} caused by O-H bending vibration in mannan belongs to the characteristic absorption peak of

carbohydrate. The absorption peaks at 1064 cm^{-1} and 1060 cm^{-1} were caused by C-O stretching vibration, indicating that there may be CH-OH structure in mannan. The symmetric vibration and stretching vibration of epoxy (C-O-C) cause the absorption peak at 895 cm^{-1}. The presence of β-glycosidic bonds leads to the characteristic absorption peak at 874 cm^{-1}. Spruce mannan is a galactomannan containing branched chains. The peak at 1735 cm^{-1} (C=O stretch) confirms heavy acetylation, characteristic of spruce O-acetyl-galactoglucomannan. This high acetylation is a primary recalcitrance factor, as it increases the activation energy for enzymatic cleavage and limits substrate solubility (Xu et al. 2008). Consequently, acetyl groups stiffen the mannan backbone and exacerbate physical hindrance against cellulases, directly contributing to the observed inhibition in our assays.

Scanning electron microscopy (SEM) images of spruce mannan are shown in Fig. 2B. It can be seen that the spruce mannan is formed by the aggregation of rods with spherical tip. Each unit forms a whole through the complex structure polymerization, and there are certain pores among the units. The aggregated rod-like structures with spherical tops observed magnification reveal a dense, polymerized supramolecular network of spruce mannan. This morphology creates a severe steric barrier, restricting cellulase diffusion, similar to nanomechanical hindrances in biomass macromolecular complexes (Zheng et al. 2021). This spatial constraint promotes non-productive adsorption of cellulases onto the hemicellulose surface instead of cellulose fibers, thereby reducing glucose yield.

Effect of mannan of spruce on the hydrolysis of second-generation cellulases during different hydrolysis times

In the experiment of hydrolysing spruce residue with second-generation cellulase at different hydrolysis time (24 h, 48 h and 72 h), the amount of mannan added was 5 mg/mL. When the dosage of cellulase was 30 FPU/ GDM, the glucose content after hydrolysis was shown in Fig. 3A.

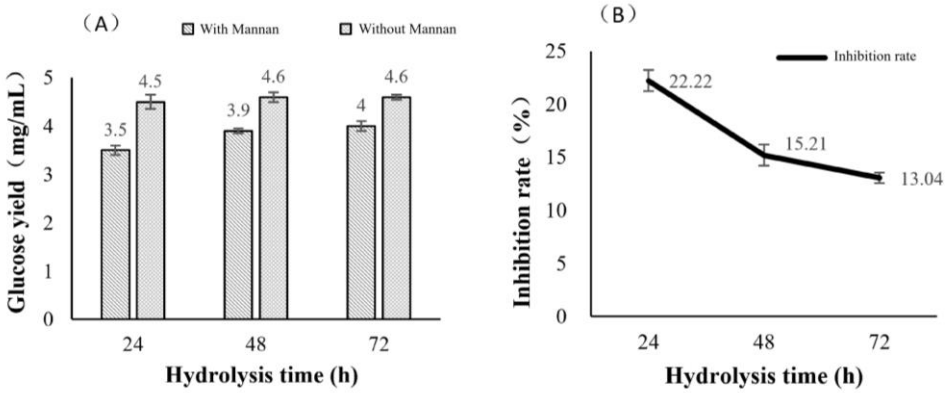


Fig. 3: Glucose content (A) and inhibitory rate of mannan to second-generation cellulase (B) at different hydrolysis time.

With the extension of hydrolysis time, the glucose content after 48 h hydrolysis was the same as that after 72 h hydrolysis, both being 4.6 mg/mL. Compared with 24 h hydrolysis, the glucose content was slightly increased, which was consistent with the results of previous studies. However, after the addition of spruce mannan, the glucose content in the hydrolysate

decreased to a certain extent at three hydrolysis times, and the glucose content in the supernatant decreased the most after 24 h hydrolysis, up to 1 mg/mL, and the glucose content in the hydrolysate decreased slightly at 72 h, up to 0.6 mg/mL. Combined with Fig. 3B, the inhibition rate of mannan on the second-generation cellulase reached the highest of 22.22% at 24 h hydrolysis and then gradually decreased. After 72 h hydrolysis, the inhibition rate decreased to 13.04%. The results indicated that 5 mg/mL mannan had a certain effect on the activity of second-generation cellulase, but this effect was not enough to completely inhibit cellulase from hydrolysing the cellulose in spruce residue, and with the extension of hydrolysis time, the effect of mannan on second-generation cellulase showed a gradually decreasing trend. The peak inhibition at 24 h (22.22%) declining to 13.04% by 72 h aligns with dynamic degradation of mannan inhibitors. Manno-oligosaccharides act as potent signalling and competitive inhibitors of cellulase activity (Hassan et al. 2019). However, over extended hydrolysis, background hemicellulolytic activities in the commercial enzyme blend gradually hydrolyse these oligomers into mannose. As monomers are less inhibitory than longer oligosaccharides, competitive blockage of cellulase active sites is progressively relieved, allowing partial glucose yield recovery by 72 h.

Effect of spruce mannan on different doses of second-generation cellulase during hydrolysis

In different doses of the second generation of cellulose enzyme hydrolysis spruce residue experiments, adding amount of mannan to 5 mg/mL, hydrolysis for 72 h, hydrolysis of glucose levels as shown in Fig. 4A, whether in the hydrolysis system added mannan, glucose content in the hydrolysate were as the second generation of the increase of cellulose enzyme dosage.

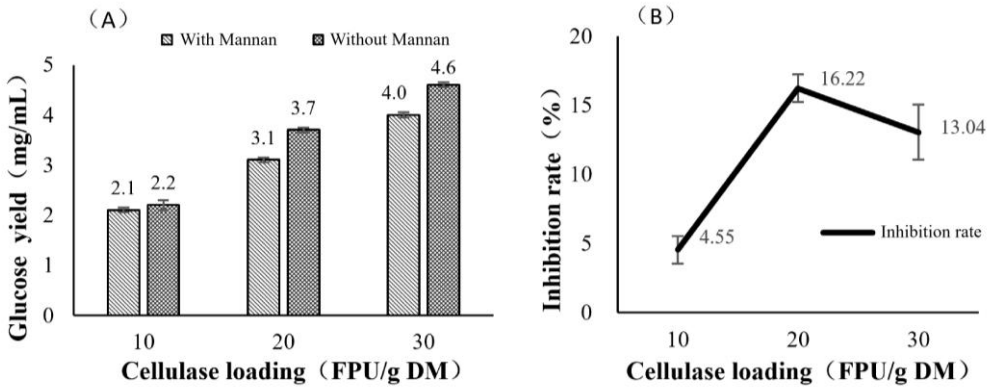


Fig. 4: Glucose content (A) and inhibitory rate of mannan to second generation cellulase (B) with different cellulase loading.

The maximum glucose content in hydrolysate reached 4.6 mg/mL when the enzyme dose was 30 FPU/g DM. However, compared with the glucose content in the hydrolysate without addition of mannan, the glucose content of the three second-generation cellulases at the dosage of mannan was reduced. Combined with Fig. 4B, it can be seen that 5 mg/mL of mannan inhibited the hydrolysis of the second-generation cellulase of 20 FPU/g DM most significantly. The difference of glucose concentration in hydrolysate was 0.6 mg/mL, and

the inhibition rate was 16.22%. The inhibitory effect on 10 FPU/g DM was the least. The difference of glucose concentration in hydrolysate was 0.1 mg/mL and the inhibitory rate was 4.55%. Experiment showed the mannan to the second generation of cellulose enzyme inhibition effect increased with the concentration of enzyme increased first, then showed decrease trend, perhaps because with the improvement of enzyme dosage, added mannan cannot completely with all the cellulose enzyme combined to form three dimensional structure block of cellulose enzyme specificity contact and the decomposition of cellulose in the substrate. The non-linear dose-response indicates saturation of mannan-cellulase binding, consistent with competitive binding to exoglucanase active sites (Xin et al. 2017). At low enzyme dosages (10-20 FPU/g DM), mannan saturates the available cellulases, causing significant inhibition. At 30 FPU/g DM, the higher enzyme-to-inhibitor ratio leaves enough free cellulase active to overcome non-productive binding, reducing the inhibition rate.

Effects of different concentrations of spruce mannan on the hydrolysis of second-generation cellulase

Different concentrations of spruce mannan (0, 0.6, 1, 3 and 5 mg/mL) were added in the hydrolysis of spruce residue using 30 FPU/g DM second-generation cellulase, and the hydrolysis time was 48 h and 72 h. The results were shown in Fig. 5.

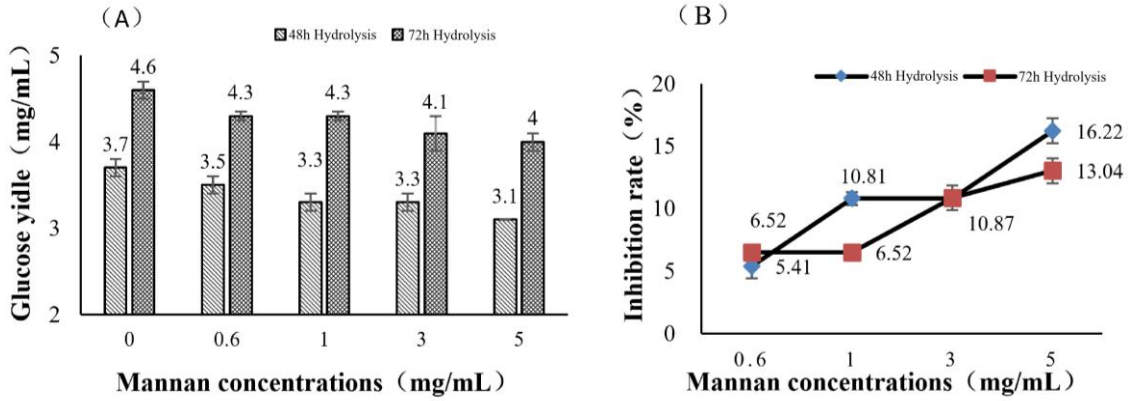


Fig. 5: Glucose content in mannan hydrolysates of different concentrations (A) and inhibitory rate of mannan with different concentration to second-generation cellulase (B).

Under the conditions of hydrolysis for 48 h and 72 h, the glucose content in hydrolysate decreased with the increase of the added concentration of mannan. When the concentration of mannan was 5 mg/mL, the glucose content in hydrolysate reached the lowest, which were 3.1 mg/mL and 4 mg/mL. At this time, the inhibition rate of mannan on second-generation cellulase was 16% and 13%. Moreover, the platform concentration of the effect of mannan on the hydrolysis of second-generation cellulases was found in the hydrolysis at different time. After 48 h hydrolysis, the glucose content in the hydrolysate supplemented with 1 mg/mL mannan and 3 mg/mL mannan was equal to 3.3 mg/mL, and the inhibition rate was 10.81%. After 72 h of hydrolysis, the glucose content in the hydrolysate supplemented with 0.6 mg/mL of mannan and 1 mg/mL of mannan was equal to 4.3 mg/mL, and the inhibition rate was 6.52%. In the experiment, 5 mg/mL mannan hydrolysed for 48 h had the greatest inhibitory effect on the second-generation cellulase, and the inhibitory rate was up to 16.22%.

The experiment showed that the inhibition effect of mannan concentration on the second-generation cellulase showed a trend of first increasing, then gently increasing, and because the spruce mannan has a certain viscosity, its solubility in hydrolysate is limited, and the concentration will not increase infinitely, so it is estimated that its inhibition on the second-generation cellulase is not more than 20% at most. These findings reveal a plateau effect in inhibition, consistent with limits in substrate accessibility and non-productive binding. The polymeric nature of spruce mannan imposes a solubility limit; once saturated, additional mannan cannot effectively interact with the enzymes. This contrasts with the severe, continuous inhibition caused by fully soluble xylans on cellobiohydrolases and endoglucanases (Zhang et al. 2012). Thus, the maximum inhibition observed here (approximately 20%) is fundamentally constrained by the structural and physical properties of spruce mannan.

Effect of different concentrations of Tween 80 on mannan inhibition of second-generation cellulase during hydrolysis

In the experiment of hydrolysis of spruce residue with different dosage of second-generation cellulase and different dosage of Tween 80, the amount of mannan added was 5 mg/mL, and the hydrolysis time was 72 h. After hydrolysis, the glucose content was shown in Fig. 6A.

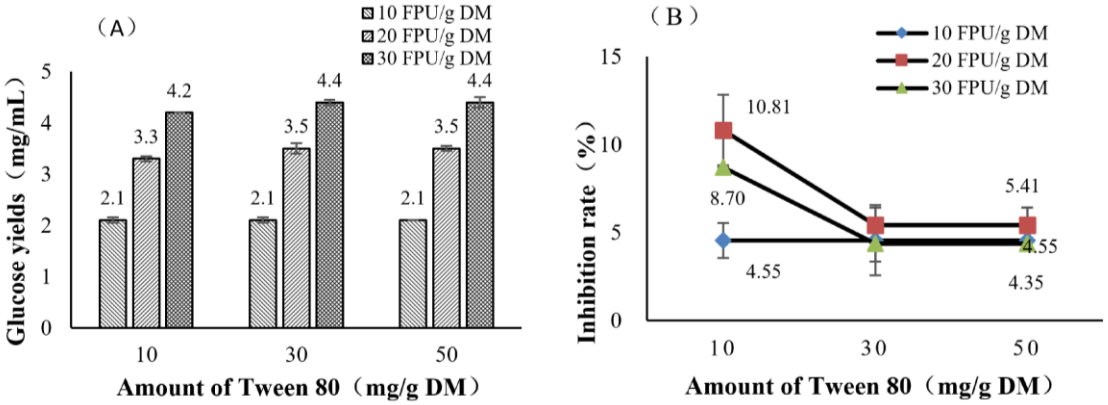


Fig. 6: Glucose content (A) and inhibitory rate of mannan to second-generation cellulase (B) with different cellulase loading and different amount of Tween 80.

With the increase of the dosage of Tween 80, the glucose content in the hydrolysate with the enzyme dosage of 10 FPU/g DM did not increase. Compared with Figs. 4-6 above, it was found that the glucose content was the same as that before the addition of Tween 80, which was 2.1 mg/mL. However, the glucose content in hydrolysates with enzyme doses of 20 and 30 FPU/g DM increased firstly and then remained unchanged with the increase of Tween 80 supplemental level, with the highest values of 3.5 mg/mL and 4.4 mg/mL. Combined with Fig. 6B, it can be seen that Tween 80 can reduce the inhibition of spruce mannan on second-generation cellulase to some extent when the dosage of enzyme reaches 20 FPU/g DM, and the inhibition rate reaches the lowest 4.35% when the dosage of enzyme is 30 FPU/g DM and the dosage of Tween 80 is 30 mg/g DM. Compared with Figs. 3-5, under this condition, the glucose content increased by 0.4 mg/mL, and the inhibition rate decreased by

8.69%. The experiment indicated that Tween 80 could alleviate the inhibition effect of mannan on the second-generation cellulase to some extent, and the alleviating effect increased and then remained unchanged with the increase of enzyme dose. Tween 80 mitigation of mannan inhibition aligns with findings that non-ionic surfactants adsorb onto recalcitrant biomass via hydrophobic interactions, rendering surfaces more hydrophilic (Jiang et al. 2017; Zhan et al. 2025). In our system, Tween 80 competes for hydrophobic binding domains on acetylated mannan, preventing non-productive, irreversible cellulase adsorption and maintaining enzyme activity for cellulose depolymerization.

The amount of mannose and xylose clearly enhanced, which could be resulted from the hydrolysis of mannan both in original-bound form and free form. The degradation of mannan and MOS to end product mannose thus relieved the inhibition of them on cellulase. Inhibition mechanism of mannan on cellulase as shown in Fig. 7.

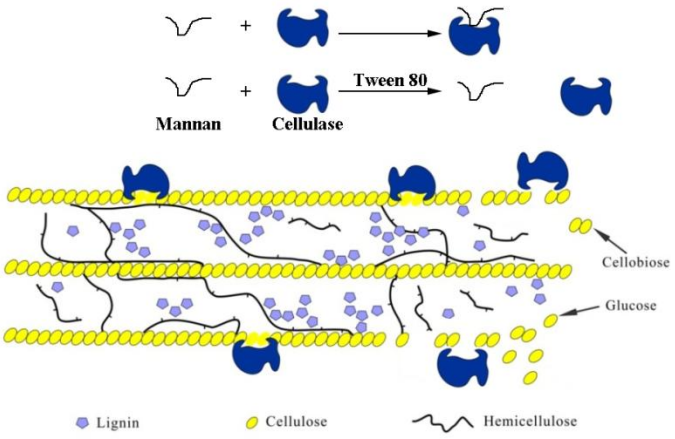


Fig. 7: Inhibition mechanism of mannan on cellulase.

CONCLUSIONS

It was found that spruce mannan was galactomannan with branched chains. At low magnification, the surface of mannan was relatively flat, and a layered structure appeared with the increase of magnification. At high magnification, the polymerization unit of mannan was in the shape of a rod with a ball at the top. The specific surface area of mannan is small, pore volume and pore size are large. The inhibitory effect of spruce mannan on second-generation cellulase was negatively correlated with hydrolysis time and positively correlated with enzyme dosage and mannan dosage. Tween 80 can reduce the inhibitory effect of mannan on the second-generation cellulase to a certain extent, and the relieving effect shows a constant trend with the increase of the dose of the second-generation cellulase. The inhibition and mitigation experiments showed that the chemical dispersant Tween 80 had stronger stabilization and dispersion effect on cellulase than on mannan, or the adsorption of mannan on cellulase depended on specific sites, so Tween 80 could not effectively enhance the inhibition effect of mannan.

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