Lignocellulosic Film as a Valid Tool to Investigate Inhibitions to Cellulose Enzymatic Hydrolysis

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The complex supramolecular structure of lignocellulose makes it difficult to clarify the recalcitrance to enzymatic digestibility. As an extensively used model mimicking the plant cell wall, assembled lignocellulosic film was applied to investigate inhibition factors of enzymatic hydrolysis for the first time. Xylan was deposited and then a model lignin compound was synthesized to equip bacterial cellulose films. Scanning electron microscopy and surface contact angle analyses revealed that lignin inhibited enzymatic hydrolysis by reducing cellulase accessibility to the cellulose surface. Moreover, xylan showed a good correlation (R^2 = 0.969) with the decreased cellulose conversion, although xylan itself did not display inhibitions. The component and surface analyses revealed that xylan enhanced lignin formation on the cellulose surface, and thus increased lignin inhibition. These results highlighted the validity of lignocellulosic films in elucidating inhibitions of cellulose enzymatic hydrolysis. The research could thus open a new avenue for the mechanistic study of the recalcitrance of lignocellulose to enzymatic hydrolysis.

Keywords: Lignocellulosic film; Lignin; Xylan; Enzymatic hydrolysis; Inhibition

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INTRODUCTION

Biofuels produced from lignocellulosic biorefineries have been sought after in recent decades for its renewability and as a potential alternative to fossil fuels, yet the recalcitrance of lignocellulose to enzymatic hydrolysis is still challenging. The cell wall of plant biomass is mainly composed of cellulose, hemicellulose, and lignin (Higuchi 1990; Plomion *et al.* 2001). These three polymers are intimately connected with each other and in turn form a highly complex ultrastructure in cell walls (Salmén and Olsson 1998; Ruel *et al.* 2002; Lawoko *et al.* 2005; Terashima *et al.* 2009; Du *et al.* 2014). Lignin, as a major cell wall component, inhibits the enzymatic hydrolysis of cellulose, although the mechanism of the inhibition is still controversial (Zhu *et al.* 2008; Li *et al.* 2014). This inhibition may be caused by enzyme inactivation, the unproductive adsorption of cellulase on lignin, or limited substrate accessibility to cellulase due to the ether and ester linkages between lignin and polysaccharides (Kumar *et al.* 2009; Rahikainen *et al.* 2013; Zeng *et al.* 2014; Fritz *et al.* 2015; Liu *et al.* 2016; Ma *et al.* 2017). Ding *et al.* (2012) quantitatively studied the key factor of enzymatic hydrolysis in plant cell wall nanostructure and found that lignin was the dominant factor that affects hydrolysis, which was mainly due to the steric exclusion effect of the lignin network. In general, traditional strategies to investigate the inhibition effect of lignocellulosic components on enzymatic hydrolysis involve the removal of a specific component (Huang *et al.* 2010; DeMartini *et al.* 2013; Pihlajaniemi *et al.* 2016). However, no method can remove one cell wall component while leaving another

component completely, due to the intimate polysaccharides-lignin complex. Thus, the mechanism of lignin inhibition of cellulose enzymatic hydrolysis is unclear. The complex cell wall structures make *in vivo* investigation of lignin inhibition highly challenging (Taherzadeh and Karimi 2008). Thereby, developing new technologies toward simplified models of plant cell wall structure could elucidate lignin inhibition of cellulose enzymatic hydrolysis.

A type of model study, termed the artificial plant cell wall, has been widely used in elucidating plant cell wall formation (Whitney *et al.* 1999; Chanliaud *et al.* 2002; Touzel *et al.* 2003; Dammström *et al.* 2008; Cybulska *et al.* 2010; Uraki *et al.* 2011; Li *et al.* 2015). The formation of plant biomass cell wall includes three major steps: cellulose biosynthesis to form a cell wall scaffold, hemicellulose biosynthesis in the cellulose scaffold to form a polysaccharides matrix, and finally lignin polymerization and deposition in the polysaccharides matrix to form a whole cell wall (Higuchi 1990; Plomion *et al.* 2001; Li *et al.* 2015). The high complexity of the plant cell wall lies in its supramolecular architecture, in which cellulose microfibrils of natural lignocellulose are tightly embedded in a matrix that is made up of lignin and hemicellulose through covalent cross-linkages (Lawoko *et al.* 2005; Du *et al.* 2014; Li *et al.* 2015). *In vitro* plant cell wall models have clarified the interactions of cellulose, hemicellulose, and lignin in the cell wall (Touzel *et al.* 2003; Teeri *et al.* 2007; Li *et al.* 2015). First, self-organized bacterial cellulose film has been extensively used as the cellulose scaffold due to the same polymorphism (cellulose I) with native cellulose (Teeri *et al.* 2007; Uraki *et al.* 2007; Li *et al.* 2015). Second, hemicellulose was deposited onto the cellulose scaffold to mimic the formation process of the polysaccharides matrix (Touzel *et al.* 2003; Li *et al.* 2015). Third, dehydrogenative polymer (DHP) has been widely used as a model lignin to study lignin biosynthesis; in particular, DHP results from endwise polymerization, which has a high *β*-aryl ether (*β*-*O*-4) content and is one of the best models of lignin (Grabber *et al.* 1996; Eggert *et al.* 1997; Steffen *et al.* 2000; Touzel *et al.* 2003; Nakamura *et al.* 2006; Barakat *et al.* 2007; Li *et al.* 2015). Inspired by the above model studies, the *in vitro* plant cell wall model is a potential tool to investigate the inhibition of cellulose enzymatic hydrolysis. Basically, each cell wall component is assembled into a cellulose scaffold, and its possible inhibition to cellulose enzymatic hydrolysis can be evaluated. This model could clarify the recalcitrance factors to enzymatic digestibility.

In this research, the inhibition of lignin and hemicellulose to cellulose enzymatic hydrolysis was addressed by using a novel plant cell wall *in vitro* mimicking model. Lignocellulosic films were assembled by depositing xylan and then synthesizing DHP on a bacterial cellulose scaffold. This assembly process mimics the formation of natural plant cell walls. To validate the lignocellulosic films in elucidating inhibitions to cellulose hydrolysis, enzymatic hydrolysis of the synthesized lignocellulosic films were conducted, and the impacts of xylan and lignin on cellulose enzymatic hydrolysis were evaluated. Using this model, the inhibition of both lignin and xylan in cellulose enzymatic hydrolysis were investigated.

EXPERIMENTAL

Assembly of Lignocellulosic Films

The lignocellulosic film consisted of bacterial cellulose, xylan, and lignin, which was assembled as reported (Li *et al.* 2015). Briefly, a bacterial cellulose film scaffold was fabricated by incubating *Gluconacetobacter xylinus* (ATCC53582) in Hestrin-Schramm medium for 12 h. Xylan adsorption onto the films was carried out by immersing the films into xylan solutions (0.1 to 1.0 mg/mL) for 24 h at room temperature. Finally, the *in vitro* model lignin (DHP) was

synthesized onto the xylan deposited cellulose film by the Zutropfverfahren method (endwise polymerization) (Cathala *et al.* 1998). At first, the xylan-deposited cellulose film was immersed in a horseradish peroxidase solution (5 mg in 50 mL PBS buffer, pH 6.1, 0.01 mol/L), and then a coniferyl alcohol solution (100 mg in 2 mL acetone and 48 mL PBS buffer) and hydrogen peroxide (30%, 0.25 mL in 50 mL PBS buffer) was separately added into the horseradish peroxidase solution by two micro-pumps with a feeding rate of 5 mL/h. The reaction was carried out at room temperature, and lasted for more than 16 h after the addition. Xylan, coniferyl alcohol, and horseradish peroxidase were all purchased from Sigma-Aldrich (Shanghai, China).

Characterizations of Assembled Lignocellulosic Films

The adsorption of xylan onto cellulose was measured using high-performance liquid chromatography (HPLC) and DHP content was measured by the acetyl bromide method as described before (Li *et al.* 2015). The contact angle of cellulosic films against deionized water was measured by using JC2000C8 contact angle instrument (POWEREACH, Shanghai, China). The morphologies of the films were analyzed by a field emission scanning electron microscopy (FE-SEM, FEI Company). The accelerating voltage applied was 5 kV and the working distance was 10 mm.

Enzymatic Hydrolysis of Lignocellulosic Films

Cellulase used in this research was purchased from Sigma-Aldrich (*Aspergillus niger*). The activity of the enzyme was analyzed using the DNS (3,5-dinitrosalicylic acid) method (Mata and Savoie 1998).

The self-assembled lignocellulosic films were cut into pieces, and 0.65 mg of cut films was placed in a 2 mL Eppendorf tube. One milliliter of sodium acetate buffer (0.05 M, pH 4.8) with cellulase loading of 50 FPU/g cellulose was then added into the tube. Enzymatic hydrolysis reactions were carried out at 48 °C for 10 h. Next, 5 μL of reactant was taken at the time frame of 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, and 10 h. Cellulose conversion was measured with a glucose oxidase method kit (Applygen Technologies Inc., Beijing, China). The cellulase was purchased from Sigma-Aldrich (Shanghai, China).

RESULTS AND DISCUSSION

Four kinds of lignocellulosic films were fabricated using self-assembled bacterial cellulose as the scaffolds, namely bacterial cellulose film (BC), xylan-adsorbed bacterial cellulose film (Xyl-BC), DHP-deposited bacterial cellulose film (DHP-BC), and xylan-adsorbed and DHP-deposited bacterial cellulose film (DHP-Xyl-BC). The xylan adsorption isotherm was previously reported by Li *et al.* (2015). In this research, the maximum adsorption of xylan on BC was 0.26 mg/mg cellulose when 1 g/mL of xylan solution was used. The content of the deposited DHP in the DHP-Xyl-BC film was 0.58 mg/mg cellulose, while DHP content was 0.22 mg/mg cellulose in DHP-BC. These films were then hydrolyzed by cellulase to validate their application in elucidating the inhibition of lignin and xylan, respectively, to the enzymatic hydrolysis of cellulose.

The results showed that lignin deposited on the cellulose film inhibited the enzymatic hydrolysis of cellulose. As shown in Fig. 1a, DHP-BC showed remarkably lower cellulose conversion than BC during the entire course of time (0 to 10 h). In particular, cellulose conversion of DHP-BC (35.6%) was 12.2% lower than that of BC (47.8%) after 5 h hydrolysis.

Moreover, DHP-Xyl-BC had much lower cellulose conversion than Xyl-BC, which further displayed the lignin inhibition on cellulase hydrolysis. The mechanism of the lignin inhibition to enzymatic hydrolysis could result from less accessibility of cellulase to cellulose and more hydrophobic BC film surface given by lignin deposition. First, morphological analysis of the DHP-deposited BC films suggested that less cellulose microfibrils were exposed outside after the deposition of DHP on the BC film. SEM analysis was carried out to measure the BC film morphology.

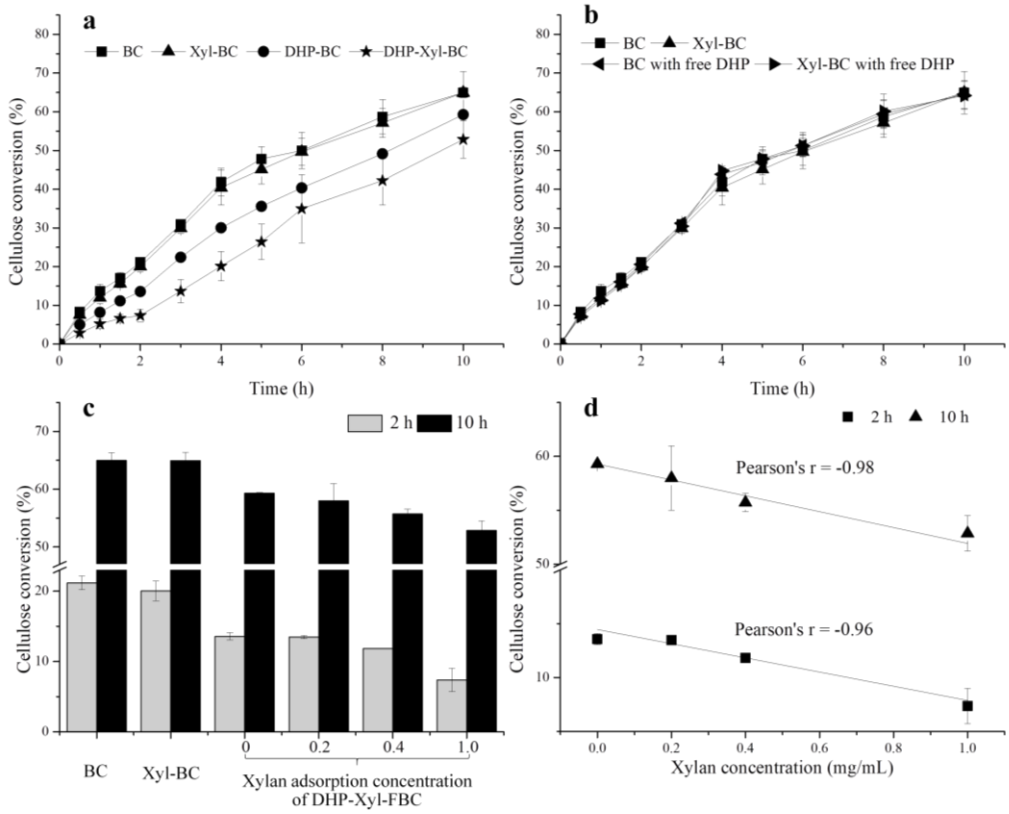

Fig. 1. Enzymatic hydrolysis of lignocellulosic films. 1a, time courses of lignocellulosic films hydrolysis; 1b, impact of xylan adsorption and free DHP on cellulose hydrolysis; 1c, impact of xylan concentrations on cellulose hydrolysis; 1d, the correlation of the cellulose conversion with xylan concentrations. BC, bacterial cellulose film; Xyl-BC, xylan-deposited bacterial cellulose film; DHP-BC, xylan-deposited bacterial cellulose film; DHP-Xyl-BC, xylan- and DHP-deposited bacterial cellulose film; BC with free DHP, synthesized DHP powder added into BC hydrolysis medium; Xyl-BC with free DHP, synthesized DHP powder added into Xyl-BC hydrolysis medium.

As shown in Fig. 2a, the white aggregates were deposited uniformly on the surface of BC films. Li *et al.* (2015) demonstrated that these white parts were the synthesized DHP using SEM-EDXA technique. DHP was obvious on the surface of BC films, and some cellulose microfibrils were embedded in the DHP aggregates (Fig. 2a). The embedded cellulose microfibrils would be difficult to be exposed to enzyme, and thus the accessibility of cellulose fibrils to cellulase was reduced.

The wettability of the films was analyzed, as shown in Fig. 3. The water contact angle of BC was 68.4°, while the water contact angle of the DHP-BC was increased to 74.3° when DHP was deposited. The contact angle was further increased to the highest value of 77.9° for DHP-Xyl-BC when DHP had the highest content as shown in Fig. 4 (1.0 mg/mL xylan). These data suggest that the hydrophilicity of BC films was decreased after DHP deposition on their surfaces.

Lignin is much more hydrophobic than cellulose (Doherty et al. 2011). The decreased wettability of DHP-deposited BC films (DHP-Xyl-BC) indicated that hydrophobic lignin covered on the cellulose surface could make the enzyme in buffer solution difficult to diffuse into cellulose substrate. Thus, the decreased wettability of DHP-deposited BC films could reduce the accessibility of cellulase to cellulose microfibrils in/on BC.

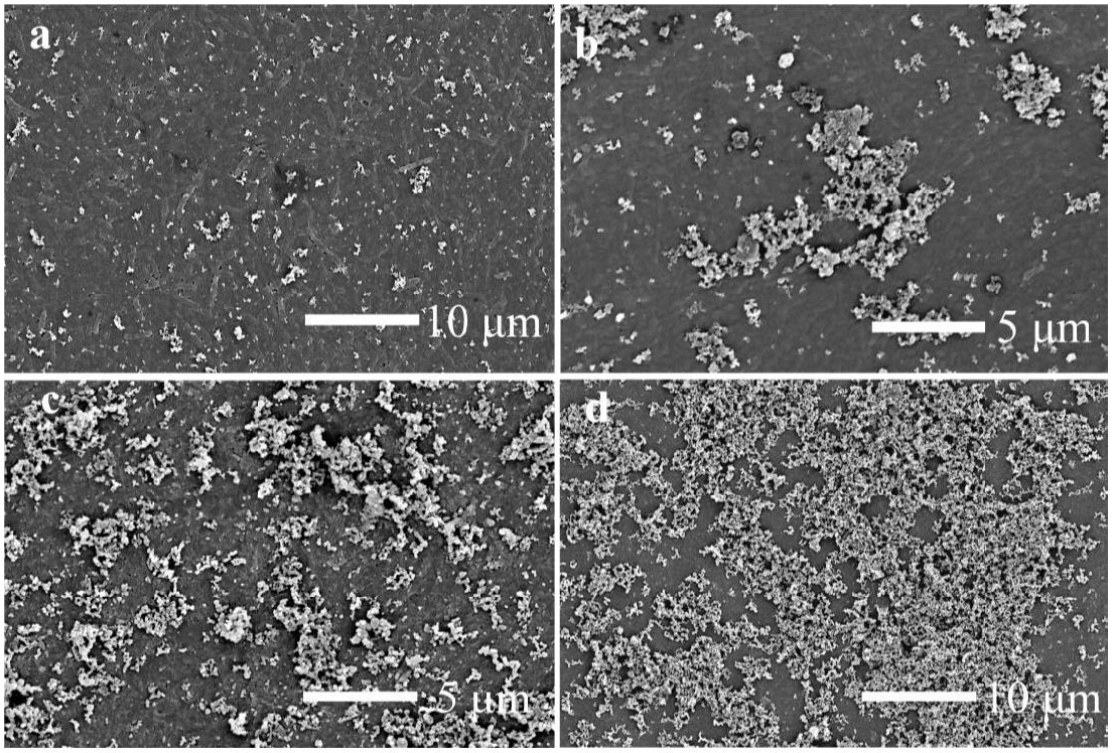

Fig. 2. SEM images of DHP-Xyl-BC films prepared at different xylan concentrations. a) 0 mg/mL; b) 0.2 mg/mL; c) 0.4 mg/mL; d) 1.0 mg/mL.

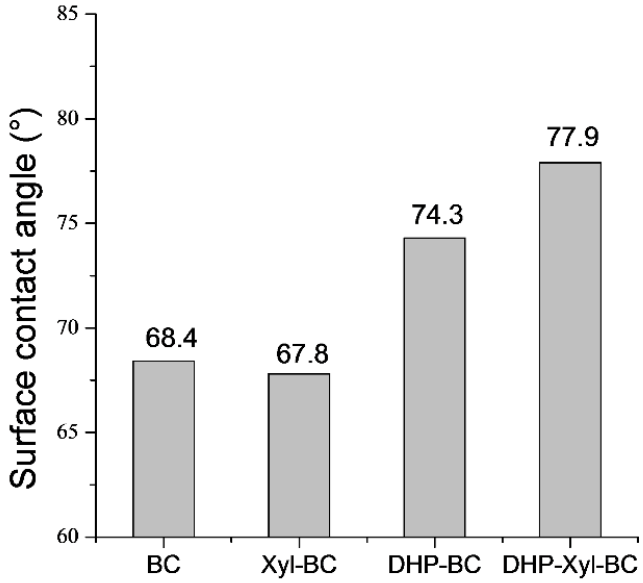

Fig. 3. Surface contact angle of lignocellulosic films. DHP-Xyl-BC was prepared with xylan concentration of 1.0 mg/mL

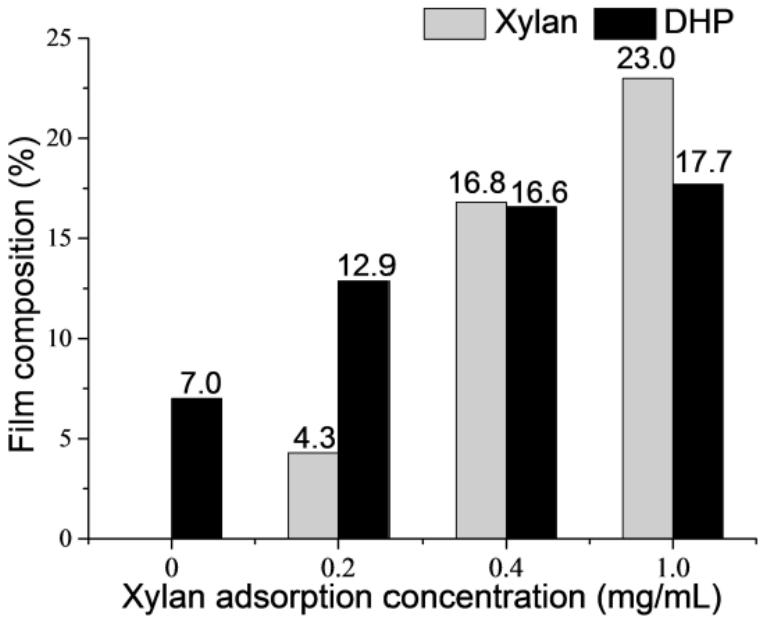

Fig. 4. Composition analysis of DHP-Xyl-BC films. The contents of both xylan and DHP (%) were calculated based on the weight of cellulose.

DHP suspended in the hydrolysis medium did not inhibit the hydrolysis through competitive and unproductive adsorption of cellulase, which further supported the conclusion that DHP deposited on BC films reduced enzymatic hydrolysis. To evaluate the inhibition effects of DHP suspended in the hydrolysis medium, DHP was also synthesized from coniferyl alcohol without the presence of BC films using endwise polymerization. This synthesized DHP powder was then added into the BC enzymatic hydrolysis medium (BC with free DHP and Xyl-BC with free DHP, in Fig. 1b). The added DHP was suspended in the medium but not on the BC films. As shown in Fig. 1b, the cellulose conversion of BC exhibited no remarkable changes when the synthesized DHP was added into the hydrolysis medium, indicating that the non-productive adsorption of cellulase on the suspended DHP had no inhibition on the cellulase hydrolysis. We further evaluated the adsorption of cellulase to suspended DHP. The synthesized DHP was added into cellulase solution. As shown in Fig. S1, free protein concentration was not significantly changed as the increasing in DHP concentration ($P < 0.01$), which further indicated that competitive binding of cellulase to DHP could be negligible. In fact, previous studies have showed that the inhibitory effect of unproductive adsorption resulted from drastic alteration of lignin structure during harsh thermochemical pretreatment, while non-pretreated lignin has very low ability of cellulase adsorption under saccharification condition (Ding *et al.* 2012; Zeng *et al.* 2014). Thereby, the non-productive adsorption of cellulase on the suspended DHP did not inhibit cellulase hydrolysis in the artificial cell wall system. Overall, the results highlighted that the DHP deposited on the BC films decreased the accessibility of cellulase to cellulose films and in turn inhibited the enzymatic hydrolysis of cellulose by physically impeding it. The results validated the lignocellulosic films in elucidating lignin inhibition to cellulose enzymatic hydrolysis.

Besides lignin, hemicellulose could be involved in the inhibitions of cellulose enzymatic hydrolysis. Many research studies on acid pretreatment have reported that xylan removal can

remarkably improve the digestibility of cellulose (Li *et al.* 2012; Zhang *et al.* 2012, 2013), and the negative impacts of hemicellulose on enzymatic hydrolysis could depend on the inhibition of lignin (Chen *et al.* 2016). Lignocellulosic films, in particular the xylan deposited BC films, could be a potential tool to evaluate the effects of xylan on enzymatic hydrolysis. As shown in Fig. 1a, cellulose conversion did not show a noticeable difference between Xyl-BC and BC, indicating that xylan itself does not inhibit cellulase hydrolysis. In addition, when DHP was synthesized and deposited onto a xylan-deposited polysaccharide scaffold (DHP-Xyl-BC), cellulose conversion was decreased markedly as compared to DHP-BC film without xylan. Based on this result, it was hypothesized that the presence of xylan in the polysaccharide matrix could increase the inhibition of lignin on cellulase hydrolysis.

To further evaluate this hypothesis, different DHP-Xyl-BC films with different xylan concentrations (0, 0.2, 0.4, 1.0 g/mol) were prepared. As shown in Fig. 1c, cellulose conversion of DHP-Xyl-BC film was decreased as the concentration of xylan in the matrix was increased. Moreover, all DHP-Xyl-BC films had lower cellulose conversion than both BC and Xyl-BC (Fig. 1c). To further quantify the impact of xylan on lignin inhibition, the scatter plot of cellulose conversion *vs.* xylan concentration after 2 and 10 h of enzymatic hydrolysis was generated (Fig. 1d), and the data was fitted into linear regression. In fact, the linear regression of cellulose conversion *vs.* xylan concentration resulted in the coefficient of determination (R^2) at 0.966 after 2 h hydrolysis and at 0.969 after 10 h hydrolysis (Fig. 1d). The results highlighted that the presence of xylan in polysaccharide matrix can increase the inhibition of lignin on cellulose enzymatic hydrolysis, and such inhibition is increased when more xylan is presented.

The mechanism that allows xylan to increase lignin inhibition could be in the increased DHP content of DHP-Xyl-BC. As shown in Fig. 4, the DHP content of DHP-Xyl-BC was increased when the xylan concentration was increased, suggesting that xylan could induce more DHP to deposit in the xylan-BC polysaccharide matrix. SEM analysis showed that the density of DHP deposited on BC surface was increased significantly when more xylan was present in the BC matrix (Fig. 2a-d). These data suggested that xylan rendered more lignin deposited on the cellulose surface and decreased the accessibility of cellulase to cellulose microfibrils. The role of xylan in lignin formation was thought of as the scaffold to induce lignin deposition onto BC films (Li *et al.* 2015). Interestingly, when the xylan concentration was increased from 0.4 g/mol to 1.0 g/mol, the deposited DHP in DHP-Xyl-BC had only minor increments from 0.166 g/g of cellulose (16.6%) to 0.177 g/g of cellulose (17.7%) (Fig. 4), but cellulose conversion had more significant decrements from 11.8% to 7.4% ($P < 0.05$) after 2 h of hydrolysis and from 55.7% to 52.9% ($P < 0.01$) after 10 h of hydrolysis (Fig. 1c). In fact, xylan could form covalent cross-linking (lignin-carbohydrate complex, LCC) with DHP during DHP formation (Lawoko *et al.* 2005; Miyagawa *et al.* 2013; Du *et al.* 2014), in particular, the covalent linkages of benzyl ester bond could be formed between the uronic acid moieties in xylan and lignin hydroxyl groups (Grabber 2005; Lawoko *et al.* 2005; Achyuthan *et al.* 2010). The xylan-bonded lignin may have higher *β-O*-4 linkages and bounded localization in plant cell wall (Reis and Vian 2004; Lawoko *et al.* 2005; Terashima *et al.* 2009; Du *et al.* 2014). These data may indicate that xylan-lignin cross-linking formed during lignin formation could contribute to the lignin inhibition of cellulase hydrolysis (Ding *et al.* 2012). However, it is impractical to determine the LCC structure in the DHP-Xyl-BC films due to the small amount of the samples, and direct evidence for the impact of xylan-lignin linkage on hydrolysis in turn is still lacking. Overall, the results highlighted that xylan increases the lignin inhibition of cellulase hydrolysis by enhancing the deposition of lignin onto cellulose. Such results validated the xylan-equipped lignocellulosic films in investigating how xylan could impact lignin inhibition.

In summary, by using lignocellulosic films composed of bacterial cellulose, hemicellulose (xylan), and synthesized lignin (DHP), it was found that lignin inhibited cellulose enzymatic hydrolysis and xylan increased this inhibition. Lignocellulosic films assembled with cell wall components can thus be used as a valid tool to investigate the recalcitrance of plant biomass to enzymatic hydrolysis. The results obtained from the enzymatic hydrolysis of lignocellulosic films may help the mechanistic understanding of inhibition factors to cellulase hydrolysis. The research presented a valid model, the *in-vitro* mimicking model of the plant cell wall, to investigate how lignin and xylan could inhibit enzymatic hydrolysis. This study could guide the fundamental studies in the recalcitrance of lignocellulose to enzymatic hydrolysis.

CONCLUSIONS

1. A lignocellulosic film, with the template of self-assembled bacterial cellulose and deposited xylan and synthesized lignin (DHP), was utilized to study the inhibitions of lignin and xylan on cellulose enzymatic hydrolysis. Lignin inhibited cellulose enzymatic hydrolysis by reducing cellulase accessibility to the cellulose surface.
2. Xylan did not inhibit enzymatic hydrolysis, but xylan increased lignin inhibition by enhancing lignin formation on cellulose.

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