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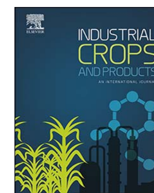

Research paper

Investigation of dynamic changes of substrate features on enzymatic hydrolysis of lignocellulosic biomass

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A B S T R A C T

During enzymatic hydrolysis of lignocellulosic biomass, the retardance of reaction has been widely observed, which is attributed to enzyme activity loss and/or substrate reactivity reduction. A real lignocellulosic biomass, dilute acid pretreated switchgrass instead of pure cellulose was used in this research. A series of hydrolysis of partially hydrolyzed pretreated switchgrass were conducted to study the effect of the dynamic change of substrate properties on the enzymatic hydrolysis. The digestibility, enzyme adsorption characteristics, surface areas of cellulose and lignin in partially hydrolyzed switchgrass were determined and compared. A new method was used to quantify the strong negative correlation between lignin content and hydrolysis rate/yield, which indicates that the increasing lignin content caused significant inhibitory effect. The cellulose decomposition during hydrolysis resulted in decrease of accessible cellulose surface area, but cellulose crystallinity of substrate had only slight change, which coincided with more exposure of lignin and increase of lignin surface area. That led to more nonproductive adsorption of enzyme to lignin and reduced the amount of enzyme available for cellulose hydrolysis.

1. Introduction

Lignocellulose, one of the most abundant raw materials in the biosphere, has presented to be a promising feedstock for production of biofuels and bio-based products to respond to energy security and adverse environmental impact of fossil fuel consumption (Maurya et al., 2015). The production of affordable biofuels and bio-based products from lignocellulose requires cost-efficient conversion of biomass, which still remains a challenging proposition (Menon and Rao, 2012). Among the unit operations of the bioconversion process, enzymatic hydrolysis is usually a rate and efficiency-limiting step (Heiss-Blanquet et al., 2011), as highly recalcitrant lignocellulosic material is difficult to be degraded biologically, leading to the low hydrolysis rate and the need of high enzyme dosage consequently raising the operation cost. As enzymatic hydrolysis proceeded, the reaction rate was observed to decline gradually, which remains a major impediment to efficient saccharification (Eibinger et al., 2014; Shi et al., 2017). Loss of enzyme activity due to adsorption and/or deactivation is an important cause, and change of substrate reactivity can also make a contribution (Wallace et al., 2016). Several parameters, e.g., crystallinity, degree of polymerization (DP), and accessible surface area, have been utilized to estimate cellulose reactivity. During enzymatic hydrolysis, the preferential degradation of amorphous region of cellulose by cellulase would produce a rising crystallinity of substrate, which decreases cellulose accessibility and makes the subsequent hydrolysis more difficult. However, as cellulase breaks microfibrils in cellulose, an increase of surface area of substrate would be expected due to size reduction (Eibinger et al., 2014), which facilitates enzyme adsorption and subsequent hydrolysis as productive adsorption is the first and critical step of enzymatic hydrolysis (Pareek et al., 2013; Qi et al., 2011). With the action of cellulase, more reducing ends would be exposed leading to lower DP. The most significant DP reduction was observed at the very beginning of hydrolysis, and the slowdown of DP reduction suggested the synergistic action of endo-and exoglucanases, which cleaves the internal glycosidic linkages and peels off the newly generated chain ends simultaneously (Meng et al., 2016). These observed phenomena mostly caused contradictory effects on hydrolysis, making it difficult to provide a convincing explanation for the descending rate.

Another issue is the increasing content of lignin, one of main components of lignocellulose, in the substrate with the hydrolysis proceeding, as lignin is immune to the cellulase attack. Many previous studies have proven that lignin could negatively affect enzymatic hydrolysis via non-productive adsorption of cellulase (Huang et al., 2016; Yang and Pan, 2016) and/or steric repulsion to limit cellulose accessibility to cellulase (Kumar et al., 2012; Zheng et al., 2013). Previous research in which lignin was added externally demonstrated that higher

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lignin content caused more severe inhibition on enzymatic hydrolysis of cellulose (Rahikainen et al., 2011; Zhang et al., 2016). In these cases the interactions of externally added lignin with cellulose and cellulase would differ from those between the native lignin and the two aforementioned entities. To study the effect of native lignin content on the hydrolysis of real lignocellulosic biomass, delignification was also used to reduce lignin content of cellulosic materials, and has been proven to improve enzymatic hydrolysis (DeMartini et al., 2013). However, as harsh conditions are generally utilized, other compositions and structures of the biomass can be changed simultaneously, e.g., enlargement of cellulose microfibril dimensions and structure modification of residual lignin (Sun et al., 2014). Such synchronous modifications would confound with lignin effect, which makes it difficult to reveal the roles of native lignin. Due to the complex features, research on enzymatic hydrolysis retardance of lignocellulose is still limited, especially the *in-situ* effect of native lignin in biomass. In the study on steam pretreated sugarcane bagasse, Wallace et al. (2016) analyzed the composition of solid residue sampled from different enzymatic hydrolysis stages, and concluded that the enrichment of lignin was an root cause for hydrolysis slowdown, possibly due to nonproductive adsorption of enzyme. However, such effect was only indirectly supported by the coincidence of increased bound protein and limited glucan conversion, with no evaluation on enzyme adsorption capability of hydrolyzed substrate. The enzyme previously deposited on substrate would interfere the redistribution of released enzyme as hydrolysis progress. In addition, no direct correlation between lignin content and hydrolysis performance was reported.

To better understand the enzymatic hydrolysis of lignocellulosic biomass and identify the factors retarding hydrolysis rate, we investigated the re-hydrolysis behaviors of different progressively hydrolyzed samples from dilute-acid (DA) pretreated switchgrass which had sequential changes of compositions and properties. We determined the composition, cellulose crystallinity, surface area, and enzyme adsorption capability of these substrates and quantitatively revealed the dynamic changes of substrate characteristics during the hydrolysis process. To investigate the correlations among different components, substrate reactivity and overall hydrolysis performance, the initial hydrolysis rate and ultimate yield were fitted with lignin content, cellulose crystallinity and surface area, to illustrate the pattern that these factors affect enzymatic hydrolysis and figure out the most significant one relating to hydrolysis retardance. The knowledge obtained in this research provide a different research method to uncover the mechanisms behind the dwindling enzymatic hydrolysis rate and would be helpful for the development of technologies to mitigate and/or avoid enzymatic hydrolysis retardance.

2. Materials and methods

2.1. Materials

Alamo switchgrass was harvested from the farm at the Pee Dee Research & Education Center, Clemson University in February 2014. It was first air-dried to moisture content (MC) below 10% and cut into small pieces followed by milling through a 40-mesh sieve with a knife mill (Thomas Wiley Mini-Mill 3383-L10, Thomas Scientific, NJ, USA). Cellulase used in this study was Cellic CTec 2 which was provided by Novozyme Inc. (Franklinton, NC, USA) as a gift. The protein content and enzyme activity of the cellulase were 150 mg/mL and 119 filter paper unit (FPU)/mL, respectively.

2.2. Dilute sulfuric acid (DA) pretreatment of switchgrass

DA pretreatment was conducted in a 1-L Parr reactor (Model 4843, Carpenter 20 Cb-3, Parr Co., Moline, IL) equipped with an impeller mixer. A working volume of 700-mL was used to allow space for liquid water expansion at high temperature during pretreatment. Switchgrass particles were pretreated at 160 °C with the agitation speed of 150 rpm for 30 min starting from the time when the mixture of switchgrass water, and sulfuric acid in the reactor reached the desired reaction temperature. The sulfuric acid concentration was 1% (w/w, biomass dry weight) and the grass solid loading was 10% (w/w). The pretreatment was terminated by immersing the reactor into ice water until the internal temperature decreased below 50 °C. The reactor was opened slowly and the contents were recovered. The pretreated switchgrass slurry was thoroughly washed via vacuum filtration using deionized water until the pH of the filtrate reached about 4–5. A portion of the washed pretreated solid was stored at −20 °C for subsequent research on enzymatic hydrolysis and adsorption. The remaining solid was dried at 45 °C in an oven for chemical composition analysis.

2.3. Preparation of partially hydrolyzed switchgrass

DA pretreated switchgrass was used for preparation of partially hydrolyzed substrates. The prehydrolysis experiments were conducted at 50 °C with substrate loading of 20 mg/mL and enzyme loading of 15 FPU/g cellulose, following the NREL procedure (Resch et al., 2015). The total working volume was 250 mL. Sodium azide was added as an antimicrobial agent with a concentration of 0.2 mg/mL and sodium citrate (50 mM) buffer was used for pH control at 4.8. The prehydrolysis was stopped at 0 h, 2 h, 6 h, 12 h, and 24 h, respectively during enzymatic hydrolysis and the partially hydrolyzed samples were recovered for further research. Two methods were used to recover the partially hydrolyzed switchgrass such as proteolysis described by Yang et al. (2006) and alkaline SDS method developed by Hong et al. (2007), which were compared to each other in terms of cellulase protein removal and composition conservation of solid residue. Proteolysis used protease to hydrolyze cellulase. In alkaline SDS method, 10 M NaOH was used to terminate hydrolysis reaction and SDS was then added to denature protein and remove adsorbed enzyme. Nitrogen content was measured by a CHNO elemental analyzer to assess the efficacy of cellulase removal. The recovered solid residues were washed sequentially with deionized water, 1.0 M NaCl solution and deionized water, and the chemical composition was analyzed prior to subsequent hydrolysis.

2.4. Characterization of partially hydrolyzed switchgrass

2.4.1. Cellulose crystallinity

Cellulose crystallinity of partially hydrolyzed switchgrass was analyzed by X-ray diffraction (XRD) to indicate cellulose reactivity. The samples were scanned on a D8 ADVANCE diffractometer equipped with a sealed tube Cu Ka source. Scans were collected from 2θ = 5° to 40° with step size of 0.02 at 1 s per step. The Crystallinity Index (CrI) was calculated according to the method developed by Segal et al. (1959) using Eq. (1):

$$CrI = \frac{(I_{002} - I_{am})}{I_{002}} \times 100\% \tag{1}$$

Where I_{200} is the intensity of the 002 peak (at 2θ = 22.8°), and I_{am} is the intensity of peak at 2θ = 18.9°. The I_{002} peak corresponds to the crystalline fraction and the I_{am} peak corresponds to the amorphous fraction.

2.4.2. Surface areas of cellulose and lignin of switchgrass samples

The surface areas of cellulose and lignin in partially hydrolyzed switchgrass were determined by using dye adsorption methods, i.e., Congo Red and Azure B are specific for dying cellulose and for lignin, respectively (Sipponen et al., 2014; Wiman et al., 2012; Zhang et al., 2017). The solid loading was set as a constant of 1% during dye adsorption for both cellulose and lignin. The Congo Red adsorption was conducted at 60 °C in 0.03 M phosphate buffer (pH = 6) with Congo Red concentrations of 4, 3, 2, 1, 0.5, and 0 mg/mL. All samples were incubated in a shaking incubator at 200 rpm for 24 h to reach the

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