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# Ethylenediamine pretreatment of corn stover facilitates high gravity fermentation with low enzyme loading

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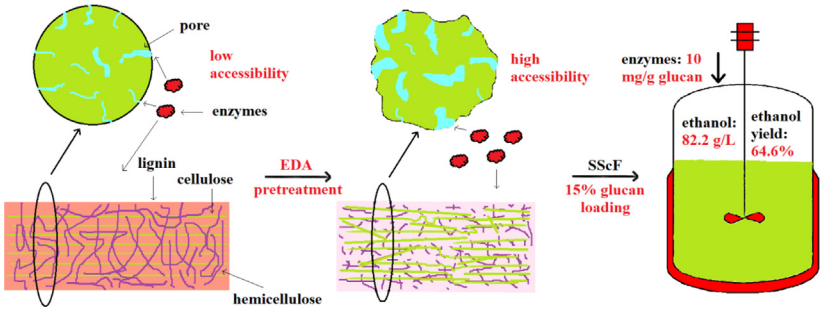
## GRAPHICAL ABSTRACT




## ARTICLE INFO

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Low enzyme loading

## ABSTRACT

This work investigated the effect of ethylenediamine pretreatment on reducing enzyme loading in high gravity fermentation. At optimal conditions of ethylenediamine pretreatment, 85.5% lignin was removed. Enzyme adsorption analysis using a fluorescent cellulose-binding protein showed 35.2% increase of productive adsorption of enzymes to ethylenediamine pretreated biomass, which was caused by high delignification and dramatically increased surface roughness and porosity. In SScF at 15% glucan loading, up to 82.2 g/L ethanol was achieved with a relatively low enzyme loading of 3.6 FPU/g dry matter. It suggested that the remarkably high digestibility of EDA pretreated corn stover could effectively reduce the enzyme loading in the high gravity fermentation of cellulosic ethanol.

## 1. Introduction

Lignocellulosic biomass, such as forest residues, agricultural waste, and agro-industrial residues, is most abundant (worldwide $10^{10}$ MT annually) and widely available resources at relatively low cost (Akhtar et al., 2016). Bioethanol from lignocellulosic biomass, as the promising alternatives to fossil fuels, exhibits outstanding environmental, economic, and strategic benefits (Dale et al., 2014). Process of bioethanol production from lignocellulosic biomass mainly comprises three separate or combined operations: pretreatment, enzymatic saccharification and fermentation.

The accessibility of raw lignocellulosic biomass to the enzymes is

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very low due to the intimate association of the three major components (cellulose, hemicelluloses and lignin). Thus, pretreatment is acclaimed by many researchers as the first prerequisite to overcome the recalcitrance of lignocellulosic biomass (Silveira et al., 2015; Galbe et al., 2012). It renders cellulose and hemicelluloses more vulnerable to enzymes (Yang and Wyman, 2008). Biological, physical and chemical pretreatments or some combinations of these methods have been applied to reduce the recalcitrance of lignocellulosic biomass (Zacchi and Galbe, 2006; Bals et al., 2011; Galbe et al., 2012).

Lignin, one of the major components of the plant cell wall (10%–30%), is derived from hydroxycinnamoyl monomers. These monomers form a highly heterogeneous and cross-linked aromatic macromolecule with undefined molecular mass. First of all, lignin can encapsulate and confine cellulose as a physical barrier. Second, it may cause irreversible adsorption of cellulases because of the high hydrophobicity. Thus, the content and chemical characteristics of lignin are crucial factors in determining the enzymatic hydrolysis efficiency of lignocellulosic biomass (Barcelos et al., 2013; DeMartini et al., 2013). Pretreatments overcome the recalcitrance of lignocellulosic biomass in different ways. Acidic pretreatments remove most of the hemicellulose but lead to the formation of acids and phenolics, which are inhibitors to cellulases and fermentation strains (Wiman et al., 2012; Larsen et al., 2012). Meanwhile, alkali pretreatments increase enzyme accessibility of lignocellulosic biomass mainly through lignin removing and modification (Bali et al., 2015; Chundawat et al., 2011; Kumar et al., 2009; Zhu et al., 2014). Ethylenediamine (EDA) pretreatment has been developed to reduce the recalcitrance of lignocellulosic biomass. It significantly improves the enzymatic hydrolysis through transforming cellulose allomorph and modifying lignin (Qin et al., 2015). However, the delignification ability of this pretreatment has not been intensively explored yet.

It has been demonstrated that a concentration of at least 40 g/L ethanol is a benchmark for an economically feasible bioethanol process (Szijártó et al., 2011). High concentration of ethanol can reduce the energy demand of downstream ethanol recovery and thus significantly save the operating and capital costs (Kang et al., 2015; Zhang et al., 2010). However, there are several technical issues that need to be addressed, including increased mass transfer resistance (Liu and Chen, 2015), sugars and oligomers inhibition to cellulases (Kumar and Wyman, 2014), non-productive binding of cellulases to lignin (Li et al., 2014), high content of inhibitors to cellulases and strains (Zhu et al., 2015, 2016), and large cellulases requirements for high solids operation (Kristensen et al., 2009). Although many pretreatments are very effective to reduce the recalcitrance of lignocellulosic biomass, high enzyme loading (> 10 FPU/g dry matter) is still necessary to achieve high concentration of ethanol, especially at high solids loadings (Öhgren et al., 2006; Qureshi et al., 2015). Thus, an efficient pretreatment process that enables pretreated solids to be more accessible to cellulases at high solid loading with low-cost enzyme loading may be very appealing.

Previous studies focused on establishing optimal EDA pretreatment process and investigating the cellulose allomorph transformation and lignin modification (Qin et al., 2015; Qin et al., 2017; Li et al., 2016). This work extended the investigation of EDA pretreatment to its effect on delignification and accessible structure (pore and surface properties). Most of the lignin (85%) was removed and the surface of the pretreated solid became highly accessible to cellulase after pretreatment. High gravity fermentation of pretreated corn stover with a quite low enzyme loading was also investigated.

## 2. Materials and methods

### 2.1. Materials

Corn stover that harvested from Tianjin of China was used. It was air-dried to constant weight. Then it was milled coarsely and screened. Fractions with particle size between 20 and 100 meshes were collected and then stored in sealed sacks. The composition of raw and pretreated biomass was determined by following the analysis procedure of National Renewable Energy Laboratory (NREL).

Commercial enzymes from Novozymes, Cellic CTec2 (180 mg/mL, 108 FPU/mL) and Cellic HTec2 (198 mg/mL, 120 FPU/mL), were used to conduct enzymatic hydrolysis and SScF experiments in this study. The ratio of CTec2 to HTec2 was 9:1. Sodium citrate buffer with a concentration of 50 mM was used to maintain the pH of enzymatic hydrolysis and SScF. To prevent bacterial contamination, 0.2% $NaN_3$ solution and 50 mg/L ampicillin was used in enzymatic hydrolysis and SScF experiments, respectively.

### 2.2. Pretreatment

The prepared corn stover was put into glass tumbler, and then mixed with four times volumes of EDA. In order to prevent EDA volatilization, the glass tumbler was sealed with aluminum foil. Then the vessel was put into electric oven without agitation. The pretreatment processes were conducted at 130 °C, 150 °C and 180 °C for 20 min, 40 min and 60 min. After pretreatment, the mixtures were washed with ten times volumes of water for two times to remove EDA residues. Composition of the pretreated corn stover was shown in Table 1.

### 2.3. Surface characterization

#### 2.3.1. Scanning electron micrograph (SEM) observation

Samples were sputtered with gold to conduct the SEM observation with field emission scanning electron microscope (S4800, Hitachi, Co., Japan).

#### 2.3.2. Pore analysis

In order to achieve a deeper understanding of the pore distribution of the samples, fully automatic pore analyzer (ASAP 2020, Micromeritics Instrument Crop, USA) was used.

#### 2.3.3. Enzyme adsorption analysis

A fluorescent cellulose-binding protein (GFP-CBM3) was synthesized by following the procedure of previous work (Hong et al., 2007). The adsorption of this protein to samples was used to characterize the accessibility of pretreated corn stover to enzymes. Previous study had described the procedure of protein labeling with and without bovine serum albumin (BSA) blocking in details (He et al., 2013). Multi-mode microplate reader was used to measure the green fluorescence intensity of the marked samples (SpectraMax M2, USA).

### 2.4. FTIR analysis and X-ray diffraction (XRD)

Fourier transform infrared spectrometer was used to conduct FTIR analysis experiments (IS 10, Thermo Fisher Scientific, USA). A total of

**Table 1**
Sugar recovery analysis of the EDA pretreated corn stover.

| | Temperature | Time | Gluan | Xylan | Solid recovery | Glucan recovery | Xylan recovery |
|---|---|---|---|---|---|---|---|
| RCS[a] | – | – | 34.9% | 19.6% | – | – | – |
| 1 | 130 °C | 20 min | 57.6% | 20.0% | 73.2% | 106.0% | 71.9% |
| 2 | 130 °C | 40 min | 62.5% | 13.8% | 58.4% | 102.4% | 40.4% |
| 3 | 130 °C | 60 min | 55.9% | 11.1% | 58.2% | 91.0% | 32.3% |
| 4 | 150 °C | 20 min | 60.3% | 13.9% | 62.7% | 107.1% | 44.0% |
| 5 | 150 °C | 40 min | 55.1% | 14.5% | 56.5% | 89.9% | 42.2% |
| 6 | 150 °C | 60 min | 60.0% | 15.7% | 53.6% | 90.9% | 42.4% |
| 7 | 180 °C | 20 min | 53.0% | 14.6% | 59.2% | 89.6% | 44.0% |
| 8 | 180 °C | 40 min | 56.9% | 16.1% | 55.9% | 91.8% | 46.4% |
| 9 | 180 °C | 60 min | 56.9% | 16.7% | 54.0% | 88.5% | 46.4% |

[a] RCS stands for raw corn stover.