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Short Communication

# Pretreatment of *Agave americana* stalk for enzymatic saccharification

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## HIGHLIGHTS

- ▶ *Agave americana* stalk was pretreated with dilute acid, sulfite (SPORL) and alkali.
- ▶ The enzymatic hydrolysability of the pretreated agave stalk was evaluated.
- ▶ SPORL pretreatment gave the highest substrate and sugar recovery yield.
- ▶ NaOH pretreated stalk had low lignin and hemicellulose and high cellulase affinity.
- ▶ NaOH pretreated stalk had better hydrolysability under the conditions investigated.

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## ABSTRACT

*Agave americana* is one of commonly grown agave species but currently less valuable because its large flower stalk cannot be used for producing alcoholic beverage. In the present study, the stalk was pretreated with dilute acid (DA), sulfite (SPORL), and sodium hydroxide (NaOH) to preliminarily assess its potential as feedstock for bioethanol production. The changes of cell wall components during the pretreatments, enzymatic digestibility of the pretreated stalks, and the adsorption of cellulases on the substrates were investigated. Results indicated that the pretreatments significantly improved the enzymatic digestibility of the agave stalk. SPORL pretreatment gave higher substrate and sugar yields, while NaOH pretreated stalk had better digestibility under the investigated conditions. The better hydrolysability of NaOH-pretreated stalk was attributed to low lignin and hemicellulose content and high affinity to cellulases.

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## 1. Introduction

Agave is usually thrived in semi-arid regions such as Mexico, Australia, and Africa. Commonly grown species include *Agave americana*, *Agave attenuate*, and *Agave tequilana*. Attractively, the annual biomass productivity of agave is 10–34 Mg/ha, which is much higher than that of switchgrass (∼15 Mg/ha) and poplar (∼11 Mg/ha) (Garcia-Moya et al., 2011). Agave is currently known for its applications in the production of alcoholic beverages such as tequila and mescal from the fructose-rich sugars extracted from either the agave piñas (stem and basal of the leaves) or leaves (Botello-Alvarez et al., 2011). However, these fermentable sugars only represent approximately 24–27% of the total carbohydrates available in the agave (Martinez-Torres et al., 2011). The residual parts of the agave plant, such as roots, stalks, and the bagasse from beverages production, are currently not used in the industry. These parts of the plant represent almost 50% of the plant weight, and are basically lignocellulosic biomass with high carbohydrates but low lignin content (Nuñez et al., 2011), which could be a good feedstock for biofuel production.

Different from other agave species, *A. americana* has a large asparagus-like flower stalk, but no piñas. Because of no piñas (a reservoir of fructans), the *A. americana* is commercially less valuable for the production of alcoholic beverages, compared to other agave species such as *A. tequilana* and *A. attenuate*, although its leaves can be used for pulque (a beer-like drink) production. From this point of view, *A. americana* is more suitable as the feedstock for biofuel production.

To the best of our knowledge, there is no report of using *A. americana* flower stalk for bioethanol production. Therefore, the objective of this study is to preliminarily assess the viability of *A. americana* stalk for bioethanol production. To this end, three representative pretreatment technologies including dilute acid (DA), sulfite (SPORL) (Wang et al., 2009), and alkali (NaOH) methods were compared for the pretreatment of the stalk of *A. americana*. The pretreated stalk was evaluated in terms of enzymatic saccharification. In addition, the interactions (adsorption) between the pretreated agave stalks and cellulases were investigated.

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## 2. Experimental

### 2.1. Materials

The *A. americana* used in this study was planted and harvested in San Jose, California. After being air-dried, the agave stalks were ground to pass a 40-mesh screen using a Wiley mill and stored in a plastic bag at room temperature for analysis and pretreatment. Cellulase with activity of 70 FPU (filter paper unit)/g and $\beta$-glucosidase with activity of 250 cellobiohydrase unit (CBU)/g were generously provided by Novozymes (Franklinton, NC).

### 2.2. Pretreatments

All pretreatments were conducted at 180 °C for 30 min in 100-mL Teflon vessels on a microwave reactor (Mars, CEM Corporation, Matthews, NC). Pretreatment liquors were prepared by dissolving required chemicals ($H_2SO_4$, $Na_2SO_3$, or NaOH) in water. Charges of the chemicals were based on oven dried agave stalk (%, w/w). The ratio of pretreatment liquor to the stalk (10 g, oven dried) was 5.0 (v/w). For DA pretreatment, the charge of sulfuric acid was 2% (pretreatment liquor pH 1.4). For SPORL pretreatment, in addition to 2% sulfuric acid, 4% sodium sulfite was charged (pretreatment liquor pH 3.2). For alkali pretreatment, the charge of sodium hydroxide on the agave stalk was 8%.

### 2.3. Enzymatic hydrolysis

Enzymatic hydrolysis was carried out in a 100-mL flask at 1% consistency at 50 °C on a shaking incubator (Thermo Fisher Scientific, Model 4450, Waltham, MA) at 160 rpm in 80 mL sodium acetate buffer (50 mM, pH 4.8) containing 2 mg tetracycline chloride as microorganism controller. The cellulase loading was 15 FPU/g cellulose, and the $\beta$-glucosidase loading was 30 CBU/g cellulose. Aliquot (0.5 mL) was taken periodically for glucose and xylose analysis, and 0.5 mL sodium acetate buffer (50 mM, pH 4.8) was added back to the hydrolysis flask to maintain the total volume of the hydrolysate.

### 2.4. Adsorption parameters

To determine adsorption isotherm, cellulase solutions at varied protein concentrations (0.05, 0.1, 0.2, 0.3, and 0.4 mg/mL) were incubated with 40 mg pretreated substrate sample in 5 mL acetate buffer (50 mM, pH 5.0) for 3 h at 8 °C to reach equilibrium. All adsorption experiments were conducted in duplicate. Protein content in supernatant was determined using ninhydrin assay with BSA as a standard. Adsorbed protein on the substrates was calculated from the difference between the initial cellulase loaded and the free cellulase left in the supernatant.

To calculate adsorption parameters, maximal adsorption capacity ($\sigma$, mg/g substrate) and affinity constant ($A$, L/g protein) were estimated by nonlinear regressions of the adsorption data (free protein amount, $C$, mg/mL and corresponding adsorbed protein amount by substrate, $\Gamma$, mg/g substrate) using Polymath software according to the Langmuir adsorption isotherm (Tu et al., 2009): $\Gamma = \sigma AC/(1 + AC)$. The strength of binding (mL/g substrate) was the combination of maximal adsorption capacity and affinity constant.

### 2.5. Analytic methods

Ash, extractives, and acid-insoluble lignin were analyzed according to NERL Laboratory Analytical Procedure with modifications. Monomeric sugars were measured using a High Performance Ion Chromatograph (HPIC) system (Dionex ICS-3000, Sunnyvale, CA) equipped with an integrated amperometric detector and Carbopac™ PA1 guard and analytic columns. Soluble lignin was determined on a UV–vis spectrophotometer (Cary 50 Bio, Varian) at 205 nm with extinction coefficient of 110 L $g^{-1}cm^{-1}$.

## 3. Results and discussion

Agave stalk was pretreated with three methods (2% DA, 4% SPORL and 8% NaOH), and the changes of cell wall component, yields of solid substrate and sugar, and mass balances were investigated. Compositions of the untreated and pretreated stalks are summarized and compared in Table 1. Sugars are expressed in terms of monosaccharides rather than polysaccharides.

The untreated agave stalk basically contained 1.0% arabinose, 2.4% galactose, 37.5% glucose, 13.6% xylose, and 18.0% klason lignin. Water/ethanol extractives of the stalk were about 21.8%, while only 1.3% of the extractives were identified as monosaccharides, including 0.1% arabinose, 0.1% galactose, and 1.1% glucose. Solubilization of biomass components (hemicelluloses, cellulose and lignin) was inevitable during the pretreatments, dependent on the pretreatment methods. The solid yields from the SPORL, DA and NaOH pretreatments of agave stalk were 65.1%, 61.8%, and 52.2%, respectively. The reason why the SPORL pretreatment gave higher yield than DA pretreatment was that at the same acid loading, addition of sodium sulfite elevated the pH value of the pretreatment liquor slightly, and thereby alleviated hydrolysis and further degradation of carbohydrates, in particular hemicelluloses, as discussed below. Lower solid yield of NaOH pretreatment was primarily caused by extensive alkaline delignification. Because of the removals of hemicelluloses and lignin from the agave stalk during the pretreatments, the pretreated stalk was enriched in cellulose. Specifically, cellulose contents in the SPORL, DA and NaOH pretreated stalks were 54.5%, 51.2% and 58.8%, respectively.

Solubilized sugars from the carbohydrates hydrolysis presented in the spent pretreatment liquors, and part of the sugars likely further degraded into other substances. The sugars in the spent liquors of SPORL, DA and NaOH pretreatments are presented in Table 1. It was apparent there were significantly lower sugars in the SPORL spent liquor than those in the DA spent liquor. This was because sodium sulfite brought the pH value of SPORL pretreatment liquor up and protected carbohydrates from extensive hydrolysis (Shuai et al., 2010). Only trace monosaccharides were detected in the NaOH spent liquor because the behaviors of cellulose and hemicellulose in alkaline pretreatment were different from those in acidic pretreatments, and the removed monosaccharides from the reducing end of polysaccharides through peeling reaction were further degraded to varied hydroxy carboxylic acids in alkaline pretreatment at high temperature. However, NaOH was the most effective chemical for delignifying the agave stalk, compared to sulfuric acid and sulfite. Approximately 37.0% of lignin was removed during the NaOH pretreatment, determined as soluble lignin in spent liquor in percentage of the original lignin content in agave. At the same loading of sulfuric acid, SPORL performed better than DA pretreatment in terms of delignification (15.9% vs. 12.0%). Sulfonation of lignin in SPORL pretreatment should account for their difference in delignification (Zhu et al., 2012).

Mass balance was created in order to gain insights into the changes of cell wall components during the pretreatments. As shown in Fig. 1, the SPORL pretreatment kept 95.3% cellulose in solid substrate, and only 4.7% cellulose was dissolved, of which approximately 0.4% was detected as glucose, suggesting that the rest be undesirably dehydrated into HMF, levulinic acid, and formic acid. Differently, the cellulose yield in the DA pretreatment was

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