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Improving ethanol production from alfalfa stems *via* ambient-temperature acid pretreatment and washing

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HIGHLIGHTS

- On-farm ensiling of alfalfa stems is a strategy for storage prior to biorefining.
- Pretreatment with dilute sulfuric acid prior to ensiling improves subsequent SSF.
- Washing after ensiling to remove ash further improves ethanol production by SSF.
- Acid pretreatment alters plant cell wall structure, and washing removes inhibitors.

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ABSTRACT

The concept of co-production of liquid fuel (ethanol) along with animal feed on farm was proposed, and the strategy of using ambient-temperature acid pretreatment, ensiling and washing to improve ethanol production from alfalfa stems was investigated. Alfalfa stems were separated and pretreated with sulfuric acid at ambient-temperature after harvest, and following ensiling, after which the ensiled stems were subjected to simultaneous saccharification and fermentation (SSF) for ethanol production. Ethanol yield was improved by ambient-temperature sulfuric acid pretreatment before ensiling, and by washing before SSF. It was theorized that the acid pretreatment at ambient temperature partially degraded hemicellulose, and altered cell wall structure, resulted in improved cellulose accessibility, whereas washing removed soluble ash in substrates which could inhibit the SSF. The pH of stored alfalfa stems can be used to predict the ethanol yield, with a correlation coefficient of +0.83 for washed alfalfa stems.

1. Introduction

Ethanol production from lignocellulosic biomass has become increasingly vital in this century due to the increasing fuel demand, decreasing fossil fuel reserves, environmental concerns and national energy security (Roman-Leshkov et al., 2007). Currently, ethanol produced from lignocellulosic biomass is considered as the most feasible replacement of fossil fuels for transportation (Kuhad et al., 2011). Because maize and sugar cane have been primary feedstock for ethanol production in the United States and Brazil, respectively, there is some concern of a negative impact on food supply, especially when there is limited fertile land and water available to meet the demands for food, feed and energy production (Valentine et al., 2012).

Alfalfa, with high protein content in the leaves and high carbohydrates content in the stems, can be used both for food (animal feed) and fuel production. Alfalfa leaves can produce protein concentrate products useful as animal feed; Alfalfa stems, with high carbohydrates content (Dien et al., 2011), can be used as resource for fiber, energy, and liquid fuel (ethanol) production. Cultivating alfalfa has agronomic advantages such as soil, water and nutrient retention; reduced nitrogen fertilizer input; and increased subsequent crop yield when used in rotation with corn (Bolton et al., 1976; Rasse and Smucker, 1999; Dien et al., 2011). Additionally, alfalfa cultivation is beneficial to the environment *via* lower N_2O emission relative to corn cultivation and reduced nitrate-N leaching that contributes to air and water pollution (MacKenzie et al., 1997; Digman et al., 2013).

Traditionally, alfalfa has been used for forage, resulting in three or four harvests per season to obtain high forage quality and yield

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in the North Central region of U.S. (Sheaffer et al., 2000). With fractionation into leaves and stems, fewer harvests of alfalfa per season could potentially reduce the seasonal costs for fuel and labor and improve harvest flexibility (Sheaffer et al., 2000). When the alfalfa stems recently have been used as primary biofuel source (Sheaffer et al., 2000), it became important to maximize the stem yield and quality. Recent studies found that two harvests per season did not decrease total seasonal alfalfa yield and stem quality (Sheaffer et al., 2000). However, the optimal harvest schedule of alfalfa for biofuel system is unknown, because it depends on the relative value of alfalfa leaves and stems (Sheaffer et al., 2000). The added value of alfalfa leaves for animal feed is relatively clear, while the added value of alfalfa stems for power or liquid fuel production has been less studied.

Pretreatment is regarded as an essential step for maximizing ethanol production from lignocellulosic biomass (Alvira et al., 2010; Chiaramonti et al., 2012), because it can reduce the recalcitrance of lignocellulosic biomass, which prevents the complete hydrolysis of cellulose and lowers the yield of ethanol. Pretreatment can be classified as physical, chemical, biological or a combination of these, which can reduce particle size, disrupt lignin structure, remove hemicellulose, decrease crystallinity of cellulose, and increase pore size and/or volume of biomass (Chiaramonti et al., 2012). Dilute acid pretreatment at elevated temperature has been widely studied, and is considered as promising pretreatment for industrial application (Alvira et al., 2010). However, the feasibility of ambient-temperature dilute acid for pretreatment has been less studied. At ambient temperature, acid corrosion of process equipment and energy input can be potentially reduced, thus the cost for ambient-temperature dilute acid pretreatment might be less than hot dilute acid pretreatment. However, it may need longer time to be effective (Chiaramonti et al., 2012). Fortunately, prolonged time is not a problem for on-farm storage since it will likely be stored for several months with or without pretreatment. Hence, ambient-temperature dilute acid pretreatment could be an attractive pretreatment for farm-stored biomass prior to biorefining. Though on-farm storage still requires shipping to the process site, it relieves to need for large on-site storage areas for the bulky biomass.

Forages are biomasses that are already stored on farm using different methods depending on the feed type, harvest facility and technology, silo type, area and weather. Ensiling, which can retain the highest quality and most dry matter (Cherney and Cherney, 2011), is an optimal storage method. However, ensiling of biomass prior to biorefining has been less studied. Researchers have proposed that this storage method for biomass can reduce harvesting costs, increase product uniformity, and reduce risk of fire (Digman et al., 2010).

The inhibitory effect of acid hydrolysis on ethanol production *via* enzymatic hydrolysis is widely known (Alvira et al., 2010). Hot dilute acid pretreatment can produce furan derivatives, short-chain organic acids and phenolic compounds, which all have inhibitory effect on enzymatic hydrolysis or ethanol production at certain concentrations (Alvira et al., 2010). It is known that some short-chain organic acids are produced during ensiling, which could potentially inhibit ethanol production. In addition, alfalfa can have high ash content. The effects of these factors on ethanol production are also of interest.

Thus, several questions may be raised: (1) What is the effect of ambient-temperature dilute acid pretreatment on biomass properties and ethanol production? (2) What is the effect of ensiling on biomass properties and ethanol production? and (3) Can post-pretreatment washing improve ethanol yield? In our work, alfalfa stems were separated from freshly harvested alfalfa and pretreated with dilute sulfuric acid at ambient temperature. Then, alfalfa stems were inoculated with lactic acid bacteria (LAB), and ensiled in sealed jars at room temperature for 10 months. After ensiling, the silage properties including ash, water soluble carbohydrates (WSCs), lactic acid, volatile fatty acids (VFAs), ethanol, crude protein (CP), neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) were analyzed. Simultaneous saccharification and fermentation (SSF) was carried out on unwashed and washed alfalfa stem powder to produce ethanol. The effects of ambient-temperature dilute acid pretreatment, ensiling and washing on ethanol production from alfalfa stems are described in this paper.

2. Methods

2.1. Materials and chemicals

Alfalfa was harvested at Arlington Agricultural Research Station (Arlington, WI), University of Wisconsin, on June 4 (cutting 1) and July 3, 2012 (cutting 2). Alfalfa leaves were harvested with Oxbo snap-bean harvester. The alfalfa stems were windrowed with a self-propelled windrower, gathered by hand, transported to the laboratory and partially dried on tarps.

Sulfuric acid and sodium hydroxide were purchased from Fisher Scientific (Fair Lawn, NJ, US). BactoTM yeast extract (extract of autolysed yeast cells) was purchased from Becton, Dickinson and Company (Sparks, MD, USA). Sigmacell 50 microcrystalline cellulose (type 50, 50 μm), D-(+)-glucose (⩾99%), tetracycline and peptone (from soybean, type III) were purchased from Sigma Aldrich (St. Louis, MO, USA). Distilled water was used for SSF.

Cellic® CTec3 enzyme, with a filter paper activity of 217 FPU/mL measured at the USDA Forest Service, Forest Products Laboratory (FPL, Madison, WI) according to literature (Wood and Bhat, 1988), was from Novozymes (Denmark). Yeast *Saccharomyces cerevisiae* D5a was used for fermentation.

2.2. Acid pretreatment and ensiling

Dilute acid pretreatment was carried out similar to that described by Digman et al. (2010). Briefly, substrate was rehydrated to meet the moisture content (MC) targets of 35%, 50% or 65% (w.b.) prior to pretreatment. Sulfuric acid was applied as a 9 M solution at ambient (room) temperature by manually mixing for individual samples to reach 35 or 75 g acid/kg DM, resulting in an acid concentration of 1.8–14.9% in water of samples by weight. Next, alfalfa stems were inoculated with lactic acid bacteria inoculant (Ecosyl MTD/1, Ecosyl Products Ltd., Stokesley, UK) at a rate of 10^5 colony-forming units/g alfalfa, according to manufacturer's specification. The inoculated material was ensiled by sealing it in a 1 liter Tulpen-form borosilicate glass canning jars (Weck-Rundrandglas 100, J. Weck GmbH and Co., Öflingen, Germany). After ensiling for about 10 months, samples were taken from each jar, and the remaining silages were frozen at −20 °C.

2.3. Silage analysis

Ensiled alfalfa stem samples (33–40 g, w.b.) were removed from jars, mixed with 150 mL D.I. water, and liquefied by a Büchi Mixer B-400 in a 1 L glass beaker. The homogenates were transferred into a 200 mL centrifuge bottle and the beaker was carefully washed with 50 mL D.I. water. The pH of the homogenates was determined before neutralizing with 4 M NaOH to pH ~7. Liquid sample (1 mL) was taken for analyzing water soluble carbohydrates (WSCs), lactic acid, volatile fatty acids (VFAs) and ethanol.

The slurry was freeze-dried and milled on a Wiley mill with a 1 mm screen. The dried and ground biomass was used for property analysis and SSF. Ash, crude protein (CP), neutral detergent fiber