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Research Paper

Elephant grass leaves have lower recalcitrance to acid pretreatment than stems, with higher potential for ethanol production

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ABSTRACT

Elephant grass is gaining attention among lignocellulosic materials due to its high growth potential, biomass yield, limited requirement for cultivation land and high rates of carbon dioxide absorption. Here was investigate the effect of pretreatment with different concentrations (5, 10 and 20%, mass acid/mass material) of diluted sulfuric acid on the whole elephant grass plant compared with its leaf and stem fractions. The stem was the most recalcitrant fraction, judging from the high recovery of water insoluble solids (WIS) and lower enzymatic hydrolysis yield, upon acid pretreatment. In enzymatic hydrolysis assays, the glucose yield increased with increasing concentrations of acid, reaching maximum values of 89.20 (leaf), 43.54 (stem) and 76.01% (whole plant). The crystallinity index (CrI) increased in both elephant grass fractions, which correlated with the solubilization of amorphous materials such as hemicellulose. Also, the stem fraction had a slightly higher heating value than the leaf fraction (3958.45 and 3939.49 cal/g, respectively). Scanning electron microscopy (SEM) analysis showed drastic morphological changes in the samples with increasing pretreatment severity, although the stem fraction suffered less structural damage than other materials. Taken together, the results suggest that the separation of elephant grass in different fractions decreases biomass heterogeneity and generates a fraction (leaf) with lower inherent recalcitrance and, thus, higher susceptibility to pretreatment and enzymatic hydrolysis, increasing the efficiency of fermentable sugar release. The results indicate that the leaf fraction of elephant grass has higher potential for use in second-generation ethanol production, while the stem fraction may be more useful for energy co-generation by combustion.

1. Introduction

The increasing demand for energy, the depletion of oil reserves, and the need to preserve and protect the environment have stimulated large interest in alternative fuel sources, which can generate energy with low damage to the environment (Samson et al., 2005). In this context, lignocellulosic biomass emerges as an alternative feedstock resource for second-generation (2G) ethanol production, with economic and environmental advantages (Behera et al., 2014). In the last two decades, numerous studies have been carried out on ethanol production from lignocellulosic biomass (Joshi et al., 2011), which consists mainly of a network of the carbohydrates cellulose and hemicellulose, with 'gaps' filled in by the aromatic macromolecule lignin (Anwar et al., 2014).

Elephant grass (species *Pennisetum purpureum*) is a promising source of lignocellulosic biomass, and represents an alternative renewable material capable of efficient use of solar energy and biomass conversion, as a result of its potent photosynthetic metabolism (Flores et al., 2012a). The cultivation of elephant grass can yield stems with up to 3 m high, with annual production rates of 88 Mg of dry matter per hectare (Pérez-Boada et al., 2014). Fontoura et al. (2015) demonstrated that it is economically feasible to use elephant grass as source of biomass for power plants in biorefinery systems (Fontoura et al., 2015).

Despite their potential industrial uses, lignocellulosic materials have inherent heterogeneity and 'recalcitrance' – the natural resistance of plant cell walls to degradation (Brethauer and Studer, 2015). To convert lignocellulosic materials into ethanol, a pretreatment approach is necessary to overcome biomass 'recalcitrance' and expose lignocellulosic carbohydrates for degradation, by disrupting the cell wall structure and making cellulose more accessible to cellulolytic enzymes that convert carbohydrates into fermentable sugars (Alvira et al., 2010). Several pretreatments techniques (dilute acid and alkaline) are under investigation to improve the digestibility of different biomass sources (Camesasca et al., 2015).

To overcome recalcitrance, a dilute acid pretreatment has been

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widely applied which effectively depolymerizes hemicellulose, with limited generation of toxic degradation products (Alvira et al., 2010). The digestibility of dilute acid-pretreated materials correlates well with the decrease in the hemicellulose fraction, which indicates better enzymatic hydrolysis yield (Öhgren et al., 2007). Moreover, acid pretreatment effectively modifies/disrupts the lignin structure, making cellulose more accessible to enzymatic hydrolysis (Alvira et al., 2010; Brethauer and Studer, 2015).

The macromolecular composition and structural organization differ between plant regions, which generates heterogeneity in lignocellulosic material (Brienzo et al., 2014). The recalcitrant and heterogeneous lignocellulosic biomass responds differently to pretreatments, depending on its chemical and structural properties (Brienzo et al., 2015), and on the plant fractions from which it is originated (Brienzo et al., 2014). This implies that an understanding of the structural properties (such as heterogeneity and morphology) of lignocellulosic materials contributing to recalcitrance– is key to improve the fermentation yield of this promising alternative energy source (Sant'Anna et al., 2014).

The composition, lignin distribution and cell wall thickness in different biomass fractions affect the pretreatment and enzymatic hydrolysis efficiency. Ours and other groups have demonstrated the effect of plant biomass heterogeneity on the recalcitrance of important crops such as sugarcane and corn (Brienzo et al., 2014; Zeng et al., 2012). Sugarcane external fractions, internode and node showed different recalcitrance to acid, alkaline and peroxide pretreatment (Brienzo et al., 2017). On the other hand, Zeng et al. (2012) showed that the corn rind fraction has lower recalcitrance compared with the pith fraction (Zeng et al., 2012).

In this study, it was examined the recalcitrance of different fractions of elephant grass, an industrial crop whose recalcitrance had only been examined previously as a whole plant biomass (Cardona et al., 2014; Menegol et al., 2014). It was examined the heterogeneity of elephant grass after pretreatment with acid, which is widely used and has limited toxic waste generation. A detailed analysis of the individual responses of the leaf and stem fractions of elephant grass to diluted sulfuric acid pretreatment, in comparison with the whole plant was performed. Sugar solubilization, water-insoluble solids (WIS) recovery, crystallinity index and enzymatic hydrolysis of untreated and pretreated materials were performed, and measured the combustion heat of each fraction by calorimetry. Also, scanning electron microscopy (SEM) was used to investigate the changes in fraction surface morphology induced by acid pretreatment.

2. Material and methods

2.1. Biomass

Elephant grass from the species *Pennisetum purpureum* was cultivated for 6 months in an Experimental Field at Embrapa Agrobiology (Rio de Janeiro, Brazil). The elephant grass (10 plants) was used as whole plant samples (including leaf and stem), or separated into leaf and stem fractions. To remove free sugars and extractives, all samples were cut into small pieces of ~5 mm, or milled and selected with a 20 mesh sieve, washed in 95% ethanol for 48 h and then washed in distilled water for a further 48 h, using a Soxhlet extractor system. Samples were dried in an oven at 45 °C for 24 h, and then stored in plastic bottles (at room temperature).

2.2. Sulfuric acid pretreatment

Elephant grass samples (whole plant, and leaf and stem fractions) were left untreated or pretreated with sulfuric acid at 5, 10 or 20% (mass acid/mass material; m/m) in 100 mL glass bottles, by addition of 60 mL sulfuric acid solutions to 3 g of sample (dry material). Pretreatments were performed in an autoclave, at 121 °C, for 30 min. After pretreatment, samples were submerged in a cold-water bath, the slurry was vacuum-filtered, using filter paper, into solid and liquid fractions, and the resulting pretreated solid fraction was washed with distilled water (to neutralize the pH) and dried at 45 °C, for 48 h. Solid fractions were weighed and stored in plastic bottles (at room temperature) until further use, while liquid fractions were filtered using 0.22 μm filters, prior to use in chemical composition analysis.

2.3. Chemical composition analysis

The chemical composition of samples was determined according to the National Renewable Energy Laboratory Analytical Procedures (NREL, USA) (Sluiter et al., 2010). The concentration of monomeric sugars (glucose, xylose and arabinose) in liquid fractions of pretreated samples was analyzed using a high-performance liquid chromatography system (HPLC; Shimadzu Corporation, Japan) equipped with a BIORAD HPX87H column and an RID 10A refractive index detector (Shimadzu). The analysis was performed at 60 °C using 5 mM sulfuric acid as a mobile phase, with a flow rate of 0.6 mL/min and a run-time of 25 min. The following factors were used to convert sugar monomers into anhydromonomers: 0.90 for glucose, 0.88 for xylose and arabinose, and 0.72 for acetyl content. The concentration of each sugar fraction was expressed as the percentage of glucan (anhydroglucose), and 'Total hemicellulose' collectively referred to anhydromonomers of xylose, arabinose and acetic acid. The removal of hemicellulose was calculated relative to its content (g/g) in the untreated and pretreated samples. The initial biomass for the pretreatment was 3 g per sample, and after pretreatment a solid fraction was recovered (water-insoluble solids, WIS).

2.4. Crystallinity index of the biomass

The crystallinity of solid fractions of untreated and pretreated samples was analyzed using an X-ray diffractometer (SuperNova; Oxford Difraction Poland, Wroclaw, Poland) with a Cu tube at an accelerating voltage of 40 kV and a current of 30 mA. Scans were conducted at a 2θ angle, between 8 and 28°, with a step of 0.05°, and at a scan rate of 2°/min. The crystallinity index (CrI) was calculated as the percentage of crystalline material, using the equation 1:

$$CrI\ (\%) = 100*(I_{002} - I_{001})/I_{002} \quad (1)$$

where CrI is the relative degree of crystallinity, I_{002} is the intensity of the diffraction from the 002 plane at 2θ = 22°, and I_{001} is the peak intensity of the amorphous zone at 2θ = 16°, in diffractograms.

2.5. Enzymatic hydrolysis

Enzymatic hydrolysis was performed with untreated and acid pretreated samples, by incubating 0.1 g of samples in 5 mL of 0.05 M citrate buffer (pH 4.8), in 15 mL flasks, at 50 °C, and with constant agitation (in an orbital shaker, at 170 rpm). Reactions mixtures contained 15 FPU/g cellulose (Celluclast 1.5 L, Novozymes) and 15 U/g cellobiase (β-glucosidase, Novozyme 188), to ensure activity and prevent product inhibition, respectively. The enzymatic digestibility of cellulose was calculated from the glucose yield (measured by HPLC as described in the item 2.3) after different reaction times (2, 4, 6, 8, 16, 24 and 48 h). Enzymatic hydrolysis assays were performed in experimental duplicates, and averaged results were reported here. The glucan conversion was calculated according to Eq. (2):

$$\text{Glucan conversion}\ (\%) = 100*(\text{Glucose} + 1.053 \times \text{Cellobiose})/(1.11 \times f \times \text{Biomass}) \quad (2)$$

Where: