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Evaluation of a simple alkaline pretreatment for screening of sugarcane hybrids according to their *in vitro* digestibility

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ABSTRACT

Lignin depletion in new cultivars or transgenic plants has been identified as a way to diminish the recalcitrance of lignocellulosic materials. Direct enzymatic digestion of these materials provides some discrimination concerning their digestibility, but the cellulose conversion to glucose is usually lower than 30% in the case of sugarcane bagasse. This study evaluates the alkaline pretreatment of eleven sugarcane hybrids and one reference sample as a method for enhancing sample digestibility with the aim of better discrimination among cultivars. The treatment of the bagasse samples with 0.1% NaOH solutions resulted in an average of 10% of dry mass solubilization, with significant variation among the samples. The mass balances for these treatments showed that the cellulose fraction remained almost unchanged, whereas the hemicellulose decreased in most of the samples. The lignin fraction decreased only in the plants that contained the highest initial lignin contents. Alkali-treated samples provided enhanced cellulose conversion levels, varying from 23% to 48% after 72 h of enzymatic digestion. After pretreatment, the samples were better distinguished according to their digestibility by enzymes. In general, the experimental hybrids with originally low lignin contents presented the highest digestibility.

1. Introduction

Sugar cane bagasse is an abundant agricultural residue and a promising substrate for second-generation ethanol production (Goldemberg, 2008). Although sugarcane bagasse contains enough cellulose to be an excellent source of sugars for ethanol production, it is a recalcitrant lignocellulosic material that requires efficient pretreatment to afford the cellulose conversion to glucose by enzymes (Mendes et al., 2011; Wyman et al., 2005). The recalcitrance of lignocellulosic materials is related to several factors, including the close association of cellulose with hemicellulose and lignin in the cell walls, which hinders the enzyme infiltration and action (Himmel et al., 2007).

Recent work has focused on lignin depletion in new cultivars or transgenic plants as a way to diminish lignocellulose recalcitrance (Chen and Dixon, 2007; Fu et al., 2011; Li et al., 2011; Lorenz et al., 2009; Mendes et al., 2011; Sticklen, 2008; Wang et al., 2012). In grasses, part of the recalcitrance has been associated with the presence of hydroxycinnamic acids linked to the hemicellulose (Lam et al., 2003; Grabber et al., 2009; Masarin et al., 2011; Siqueira et al., 2011). In all cases, cell wall digestibility varies within plant varieties (Lam et al., 2003; Lorenz et al., 2009) and within different cell types (Akin, 2008; Jung and Casler, 2006; Siqueira et al., 2011). Part of this variation is associated with the lignin and hydroxycinnamic acid concentrations in the cell walls (Akin, 2008; Lam et al., 2003; Lorenz et al., 2009; Siqueira et al., 2011).

Plant breeding is a common technique used to select appropriate cultivars (Jensen et al., 2011; Loureiro et al., 2011; Wu et al., 2006). In the case of plant breeding focused on the selection of highly digestible cultivars, a limitation in the evaluation of experimental plants is the low *in vitro* digestibility of untreated samples. Usually, the enzymatic conversion of the polysaccharides into monomeric sugars in untreated lignocellulosic materials is limited to a range of 10–35%, depending on the material (Lam et al., 2003; Masarin et al., 2011). In contrast, the pretreatment processes that increase the digestibility of lignocellulosic materials usually involve complex procedures performed at high temperatures in specialized reactors (Wyman et al., 2005). These complexities impede the evaluation of a large number of experimental plants in breeding programs intended to select highly digestible materials. In this context, one of the simplest pretreatments involves the partial dissolution of cell wall components with alkaline solutions in mild conditions (Sun et al., 1995; Wu et al., 2011; Yang et al., 2013). Under these conditions, the lignocellulosic materials undergo the dissolution of extractives, saponification of ester side groups in

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hemicellulose, dissolution of low molar mass hemicelluloses and lignin, and partial lignin degradation. This type of pretreatment can cause a swelled state in the biomass, making it more accessible to enzymes (Hendriks and Zeeman, 2009; Wu et al., 2011). At high alkali loads and temperatures, peeling of polysaccharide end-groups or even alkaline hydrolysis can occur, giving low solid yields after pretreatment (Gellerstedt, 2009).

The aim of this work was to develop a simple alkaline pretreatment procedure that could reduce the sugar cane bagasse recalcitrance and permit good discrimination among experimental hybrids according to their *in vitro* digestibility. Eleven experimental sugar cane hybrids plus a reference cultivar were evaluated according to their chemical composition before and after mild-alkaline treatment. Enzymatic digestion of the alkaline-pretreated materials was compared with that obtained without pretreatment and used to anticipate the performance of the plant materials in subsequent studies of second-generation ethanol production from sugar cane bagasse.

2. Materials and methods

2.1. Raw materials and biomass preparation

Eleven experimental sugarcane hybrids were selected from the breeding program developed by the "Academic Network for the Development of Sugar-Alcohol Sector" (www.ridesa.com.br) associated with the Federal University of Viçosa, Viçosa, MG, Brazil (Masarin et al., 2011; Loureiro et al., 2011). The hybrid plants and the reference cultivar were obtained as previously described (Masarin et al., 2011). Approximately 15 stalks of the harvested hybrids and the reference cultivar were cut in a reaper machine that released 5- to 10-mm long fragments. The cut material was blended in water and washed to remove sucrose. To avoid fines (particles shorter than 0.2 mm) loss, washing of the material was performed inside a 1-m long, 150-mm diameter PVC column set with a 200-mesh screen at the bottom. Fines passing through the screen were pumped back to the column top. Filtrate recirculation permitted the formation of a fiber mat at the column base that retained fines. Water recirculation was stopped when the wash water was free of turbidity. After this point, additional blended biomass was applied to the column, and fresh water was passed through the column until the wash reached negative color results in the phenol–sulfuric acid assay. The obtained biomass material was stored at −18 °C until use.

2.2. Pretreatment with alkaline solutions and characterization of the soluble and insoluble fractions

The pretreatment with 0.1% (w/v) NaOH solutions was performed with samples previously milled to pass through a 0.84 mm screen. Milled samples (300 mg) were treated inside screw-capped assay tubes with 15 mL of NaOH solution for 1 h at 98–100 °C. After treatment, the mixture was cooled to room temperature and filtered through a porous glass filter number 3. Aliquots of the alkaline filtrate were diluted (1:1) with 0.5 mol L^{-1} NaOH and analyzed by HPLC (AKTA 10 GE chromatograph). For this procedure, 1 mL of the diluted solution was injected into a 30 mm × 500 mm Sephadex G-50 column previously equilibrated with 0.5 mol L^{-1} NaOH. The column was eluted with 0.5 mol L^{-1} NaOH at 1.5 mL min^{-1}. The eluted solution was detected in line at 280 nm using the chromatograph UV detector. The concentration of aromatic compounds in this solution was estimated based on an average absorptivity of 20 L g^{-1} cm^{-1} (Guerra et al., 2000). The eluted solution was automatically collected in 5 mL fractions. An aliquot of 0.5 mL of each fraction was reacted with 1.25 mL of concentrated sulfuric acid and 25 μL of 40% (w/v) phenol. After cooling, the reaction mixture was analyzed at 490 nm in a GBC spectrophotometer to detect total carbohydrates. The carbohydrate concentration in these samples was estimated based on external calibration using pure sucrose as the standard (Dubois et al., 1976).

The pretreated solids were successively washed with 50 mL of 0.1% (w/v) NaOH solution and 70 mL of water. Washed solids were dried to a constant weight at 105 °C. The yield of pretreated solids was calculated based on the final dry weight divided by the initial dry weight. The yields and standard deviation values reported in the text are based on experiments performed in triplicate.

For the chemical characterization of the pretreated solids and the evaluation of their enzymatic digestibility, the pretreatment experiments were scaled up to 5 g. In this case, 5 g (dry basis) of each sample was treated with 250 mL of 0.1% NaOH solution in 500 mL Erlenmeyer flasks and washed as previously described.

For the chemical characterization, approximately 3 g of each sample was extracted with 95% (v/v) ethanol for 6 h in a Soxhlet apparatus. The amount of extractives was determined on the basis of the dry weights of extracted and non-extracted samples. This procedure was conducted in triplicate. The average result values and the corresponding standard deviations are reported in the text. Extracted samples were hydrolyzed with 72% (w/w) sulfuric acid at 30 °C for l h (300 mg of sample and 3 mL of acid), as previously (Ferraz et al., 2000). The acid hydrolysate was diluted with the addition of 79 mL of water, and the mixture was autoclaved at 121 °C for 1 h. The residual material was cooled and filtered through porous glass filter number 3. Solids were dried to a constant weight at 105 °C and determined to be insoluble lignin. The soluble lignin concentration in the aqueous fraction was determined by measuring the absorbance at 205 nm and using the value of 105 L cm^{-1} g^{-1} as the absorptivity of soluble lignin (Dence, 1992). The concentrations of monomeric sugars in the soluble fraction were determined by HPLC using a BIORAD HPX87H column at 45 °C, eluted at 0.6 mL min^{-1} with 5 mmol L^{-1} sulfuric acid. Sugars were detected in a 35 °C temperature-controlled RI detector. In these conditions, xylose plus mannose and galactose (when present) were eluted at the same retention time and were integrated as a single peak. Glucose, xylose, arabinose and acetic acid were used as external calibration standards. No corrections were performed because of sugar degradation reactions during acid hydrolysis. The factors used to convert sugar monomers to anhydromonomers were 0.90 for glucose and 0.88 for xylose and arabinose. Acetyl content was calculated as the acetic acid content multiplied by 0.72. This procedure was conducted in triplicate, and the average results, followed by their standard deviations, are reported in the text. Glucose was reported as glucan after correction by the hydrolysis factor, whereas the other sugars and acetic acid were used to calculate the hemicellulose contents.

2.3. Enzymatic hydrolysis of the samples

Enzymatic hydrolysis experiments were performed using a mixture of commercial enzyme preparations (Celluclast and Novozym 188; Novozymes, Denmark) at a dosage of 20 FPU plus 40 IU of β-glucosidase/g bagasse (oven dry basis). Each hydrolysis experiment was conducted in 125-mL Erlenmeyer flasks containing 1 g of milled sample (oven dry basis) and 10 mL of 50 mM sodium-acetate buffer at pH 4.8 containing 0.01% sodium azide in addition to the enzyme solution (final consistency of 10%). The flasks were incubated at 45 °C under rotary agitation at 120 rpm. The reaction was stopped at defined periods from 4 to 72 h by heating the flask to 100 °C for 5 min, followed by centrifugation of the material at 7800 × *g* for 15 min. The soluble fractions were assayed for glucose and xylose content using the previously described HPLC procedure. Cellulose and xylan conversion levels reported in the text refer