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The enzymatic recalcitrance of internodes of sugar cane hybrids with contrasting lignin contents

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ABSTRACT

The recalcitrance in grasses varies according to cell type and maturation. The origin of the recalcitrance in different regions from sugar cane internodes with varied lignin contents was evaluated. The efficiency of enzymatic hydrolysis was correlated with the chemical, micromorphological and microspectrophotometric characteristics of the samples. The internodes of three sugar cane hybrids were dissected into four different fractions. The outermost fraction and the rind were the most recalcitrant regions, whereas the pith–rind interface and the pith were less recalcitrant. Cellulose conversion reached 86% after 72 h of enzymatic digestion of the pith from the hybrid with the lowest lignin content. There was an inversely proportional correlation between the area occupied by vascular bundles and the efficiency of cellulose hydrolysis. High cellulose and low lignin or hemicellulose contents enhanced the efficiency of enzymatic hydrolysis of the polysaccharides. The critical evaluation of the results permitted to propose an empirical parameter for predicting cellulose conversion levels that accounts for the positive effect of high cellulose and low lignin plus hemicellulose and the detrimental effect of abundant vascular bundles. The cellulose conversion levels fit well to this calculated parameter, following a second order polynomial with an r^2 value of 0.96.

1. Introduction

The original recalcitrance of lignocellulosic materials to enzymatic digestion is due their non-porous cell walls, which hinder enzyme infiltration (Flournoy et al., 1991; Himmel et al., 2007). Therefore, enzymatic digestion of these materials is restricted to the freshly exposed surfaces of the lumen or the external surface of the cells. The topochemical distribution of lignin and hemicellulose is also relevant, as these components encapsulate and restrict access to cellulose in microfibrils (Ding et al., 2013; Siqueira et al., 2011). Lignin in cell walls also inhibits the action of the cellulases because the binding domain of the enzymes can join with lignin in an unproductive way (Berlin et al., 2005; Várnai et al., 2010).

In grasses, the recalcitrance varies according to the cell type and maturation stage (Grabber et al., 2002; Jung and Casler, 2006; Siqueira et al., 2011). In sugar cane stalks, the vascular bundles are surrounded by an abundant parenchyma. The vascular bundles contain lignified vessels and fibers and a small number of non-lignified phloem cells, whereas the parenchyma is poorly lignified (He and Terashima, 1990; Moore, 1987). UV-microspectrophotometry was previously used to correlate the digestion of various sugar cane internode regions with the lignin and hydroxycinnamic acid content of the cell walls (Siqueira et al., 2011). The pith region was promptly hydrolyzed by commercial cellulases; this region contained parenchyma cells that were not extensively lignified and presented limited amounts of hydroxycinnamic acids. In contrast, the rind region contained highly lignified vessels and fibers and was very recalcitrant to enzymatic hydrolysis. When lignin and hydroxycinnamic acids were removed from the rind via selective chlorite delignification, the digestibility of the material was significantly increased.

To overcome the challenge of high recalcitrance, a variety of chemical and/or mechanical processes have been used to pretreat lignocellulosic materials (Sun et al., 1995; Yang et al., 2013). Selective lignin removal from lignocellulosic materials significantly improves cell wall digestibility (Lee et al., 2009; Siqueira et al., 2013). Modified plants with lower lignin levels or modified lignin structures are also more amenable to pretreatment and subsequent enzymatic hydrolysis (Himmel et al., 2007; Jung et al., 2012; Wang et al., 2012).

The present study evaluates the recalcitrance of internodes from mature sugar cane plants previously selected for their varied lignin contents (Masarin et al., 2011). To characterize recalcitrance in

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each plant, the untreated internodes were dissected in four different fractions from the outermost region to the pith and digested with commercial cellulases. The same samples were characterized according to the chemical composition, micromorphological and microspectrophotometric characteristics. A new parameter to predict cellulose conversion in untreated sugar cane was proposed with basis on the sample characteristics.

2. Materials and methods

2.1. Raw material and biomass preparation

Experimental sugarcane hybrids were selected from the breeding program developed by RIDESA, which is associated with the Federal University of Viçosa, Viçosa, MG, Brazil (Loureiro et al., 2011). Each studied hybrid originated from sugar cane seeds obtained after hybridization as described before (Loureiro et al., 2011). The seeds were initially planted in trays and maintained in a greenhouse for 30 days. The seedlings were first transferred to tubs and then to the field to generate initiating plants for subsequent vegetative propagation. The first clone plantation was set in May 2007 using rows 5 m in length in an experimental field in Oratórios, MG, Brazil (20°25′50″ latitude south, 42°48′20″ longitude west). The first clonal crop was obtained in July 2008. The plant material that regrew after the first cut (second clonal crop) was harvested in July 2009 and used for the productivity studies and chemical characterizations reported in a previous paper (Masarin et al., 2011). The plant material that regrew after the second cut (third clonal crop) was harvested in December 2009 and used as starting material for vegetative propagation of selected hybrids in an experimental field in Lorena, SP, Brazil (22°43′51″ S, 45°07′29″ W). This experimental field was set up to generate sugar cane stalks for the microscopic, chemical characterization and digestibility studies reported in the present paper. Each hybrid was planted in January 2010 in 0.60 m × 1.0 m rows with 3.0 m × 2.0 m spacing between hybrids. Each row received 100 g of 4:14:8 NPK (nitrogen, phosphorous and potassium) fertilizer and 100 g of calcium carbonate in August 2010. Subsequent fertilizations were performed with 100 g of 4:14:8 NPK in September 2010 and March 2011. In July 2011, each row produced from 16 to 22 stalks of 16-month-old mature sugar cane plants that were harvest and stored at −18 °C. Internodes 3 to 7 (from the plant base) were separated from all stalks. These internodes were cut into 25-mm circular pieces following the longitudinal axis of the stalks. The 25-mm pieces were then cut from the periphery to the center as follows: (a) the first external 2 mm containing epidermis, cortical cells and part of the outermost rind (outermost fraction); (b) the remaining material was measured and divided into 3 equal segments, which were rind, pith–rind interface and pith fractions (from exterior to interior). The volume occupied by each of these fractions was calculated assuming a cylindrical shape for the internode piece. Four concentrically cylinder volumes were calculated with basis on the total volume of the internode piece subtracted from the sum of the volumes for the tree remaining fractions. All samples were extracted with water in a Soxleth apparatus in 8-h cycles to remove sucrose. After each extraction cycle, total sugars in the extract were measured by a phenol-sulfuric acid assay (Dubois et al., 1956). Extraction cycles were performed with fresh water until the extract was free of soluble sugars (on average, 5 cycles). For micromorphological and microspectrophotometric studies, 5 pieces of each fraction were randomly selected from each hybrid and cut into small blocks (approximately 1 mm× 1 mm× 5 mm), which were stored at 4 °C in water containing 0.01% (w/v) sodium azide. The remaining of the extracted samples were air-dried and stored in dry conditions until use.

To determine the mass proportion of each fraction, a group of samples was oven-dried to a constant weight at 105 °C. This determination was performed in triplicate. The mean values and standard deviations are reported in the text. The volume ratio of each fraction was calculated assuming a cylindrical format for the internode.

Air-dried samples were also used for enzymatic digestion and chemical composition assays.

2.2. Enzymatic hydrolysis of the sugarcane samples

Each air-dried sample was milled to pass through a 0.84-mm screen. The enzymatic hydrolysis experiments were performed using a mixture of commercial enzyme preparations (Cellubrix and Novozym 188, both trademarks of Novozymes A/S, Denmark) at the dosage of 10 FPU/g of substrate and 20 IU β-glucosidase/g of substrate in all experiments. The hydrolysis reaction was performed in 50 mL centrifuge tubes containing 200 mg of substrate (oven-dry weight), the enzyme mixture and 10 mL of 50 mmol/L sodium acetate buffer, pH 4.8, containing 0.01% (w/v) sodium azide. The flasks were incubated at 45 °C with reciprocal agitation at 120 cycles per min. The reaction was stopped at defined periods from 4 to 72 h by heating the flask to 100 °C for 5 min; the material was then centrifuged at 7800 × *g* for 15 min. The soluble fractions were assayed for glucose and xylose by HPLC (Waters Corporation, Millford, USA) using an HPX87H column (Bio-Rad, Hercules, CA, USA) at 45 °C, with an elution rate of 0.6 mL/min with 5 mmol/L sulfuric acid. Sugars were detected with a temperature-controlled refractive index detector (model 2414, Waters Corporation, Millford, USA) set at 35 °C. The cellulose and xylan conversion levels reported in the text refer to the average conversion of the polysaccharides to their monomers and their standard deviations estimated from triplicate runs.

2.3. Microscopic evaluation of the samples

For microscopy, small blocks (approximately 1 mm × 1 mm × 5 mm) were dissected from the outermost, rind, pith–rind interface and pith regions of each sample. The specimens were dehydrated in a graded series of acetone and embedded in Spurr's epoxy resin (Spurr, 1969) formulated to the standard hardness of the blocks (Spurr Low-Viscosity Embedding Kit, EM0300, SIGMA-ALDRICH, St. Loius, MO, USA). Sections (1 μm) were prepared from these samples with a LEICA EM-UC7 ultramicrotome fitted with a diamond knife (4 mm-Histo, DIATOME, Switzerland). The sections were transferred to microscope slides, stained with 1% (w/v) toluidine blue for 30 min, washed with water and visualized with a ZEISS Axio Imager/J&M microscope (ZEISS, Germany). Pictures were recorded at 50× magnification. For determination of the area occupied by vascular bundles, at least 5 different cuts from each sample were evaluated. Micrographs were printed and the area of each vascular bundle was calculated by approximation of their shape to a circle. The calculated circle area was divided by the total area of the viewing filed in the printed micrograph. The data reported in the text correspond to the average values and their standard deviations.

For the UV spectroscopy analyses, similar cuts were prepared and transferred to quartz microscope slides without staining. The topochemical analysis was performed with a ZEISS Axio Imager/J&M microspectrophotometer (ZEISS/J&M, Germany) equipped with a scanning stage enabling the determination of image profiles at defined wavelengths using the scan program 4-D Map (J&M, Germany). UV spectra were recorded from 1 μm^2 areas focused in the secondary wall of each sample. At least 15 spectra were recorded from each individual cell type (vessel, fiber and parenchyma). The average spectra are presented throughout the paper. Standard deviations, calculated from the absorbance