Bioenerg. Res. (2013) 6:564–579
DOI 10.1007/s12155-012-9268-1

Composition and Structure of Sugarcane Cell Wall Polysaccharides: Implications for Second-Generation Bioethanol Production

Amanda P. de Souza · Débora C. C. Leite · Sivakumar Pattathil · Michael G. Hahn · Marcos S. Buckeridge

Published online: 6 November 2012

Abstract The structure and fine structure of leaf and culm cell walls of sugarcane plants were analyzed using a combination of microscopic, chemical, biochemical, and immunological approaches. Fluorescence microscopy revealed that leaves and culm display autofluorescence and lignin distributed differently through different cell types, the former resulting from phenylpropanoids associated with vascular bundles and the latter distributed throughout all cell walls in the tissue sections. Polysaccharides in leaf and culm walls are quite similar, but differ in the proportions of xyloglucan and arabinoxylan in some fractions. In both cases, xyloglucan (XG) and arabinoxylan (AX) are closely associated with cellulose, whereas pectins, mixed-linkage-β-glucan (BG), and less branched xylans are strongly bound to cellulose. Accessibility to hydrolases of cell wall fraction increased after fractionation, suggesting that acetyl and phenolic linkages, as well as polysaccharide–polysaccharide interactions, prevented enzyme action when cell walls are assembled in its native architecture. Differently from other hemicelluloses, BG was shown to be readily accessible to lichenase when in intact walls. These results indicate that wall architecture has important implications for the development of more efficient industrial processes for second-generation bioethanol production. Considering that pretreatments such as steam explosion and alkali may lead to loss of more soluble fractions of the cell walls (BG and pectins), second-generation bioethanol, as currently proposed for sugarcane feedstock, might lead to loss of a substantial proportion of the cell wall polysaccharides, therefore decreasing the potential of sugarcane for bioethanol production in the future.

Keywords Bioenergy · Cellulosic ethanol · Hemicelluloses · Cell wall composition · Cell wall structure · Sugarcane

Introduction

One of the main sources of renewable energy for biofuels is the conversion of plant-derived carbohydrates into bioethanol. In this context, industries in the USA and Brazil have developed processes to use corn starch [1] and sugarcane sucrose [2], respectively, to produce bioethanol. As a result, these two countries are currently the top two producers of this biofuel in the world [3]. However, it is becoming increasingly clear that bioethanol produced either from corn starch stored in seeds or from sucrose stored in sugarcane culms, the so-called first-generation (1G) bioethanol, will not be sufficient to meet future demands for biomass-derived transportation fuels. As a result, laboratories around the world are now searching for ways to efficiently hydrolyze cell wall polysaccharides from different

Electronic supplementary material The online version of this article (doi:10.1007/s12155-012-9268-1) contains supplementary material, which is available to authorized users.

A. P. de Souza · D. C. C. Leite · M. S. Buckeridge (✉)
Laboratory of Plant Physiological Ecology (LAFIECO),
Department of Botany, Institute of Biosciences,
University of São Paulo,
Rua do Matão 277,
Sao Paulo, Sao Paulo, Brazil
e-mail: msbuck@usp.br

S. Pattathil · M. G. Hahn
BioEnergy Science Center,
Complex Carbohydrate Research Center,
The University of Georgia,
315 Riverbend Rd.,
Athens, GA 30602, USA

and more complex feedstocks in order to provide the necessary scientific foundation for the development of second-generation (2G) technology for the production of biofuels. Wood chips derived from trees (poplar and eucalyptus) and biomass from grasses (miscanthus, switchgrass, corn stover, and sugarcane) figure prominently among the feedstocks that are being considered as promising for 2G biofuel production [4, 5].

Wood and grass feedstocks differ significantly from each other with respect to their cell walls, the former being composed of Type I or Type III cell walls [6] and the second consisting primarily of Type II cell walls [7]. Cell wall polysaccharide composition has been assessed traditionally through fractionation of the walls with alkali followed by acid hydrolysis and monosaccharide compositional analyses of the resulting wall extracts. This procedure provides partial information about polysaccharides in the wall, but does not provide details of the structure or fine structure of the polymers. Although the differences in monosaccharide composition are usually small in the same cell wall type, the way the polymers are arranged in the walls of each tissue can vary considerably. This arrangement of polymers, called cell wall architecture [7], is thought to be closely associated with the fine structure of these carbohydrates. This brings another dimension of complexity to the goal of designing efficient processes for 2G bioethanol production from biomass.

Brazil currently produces bioethanol intensively from sugarcane feedstock [8]. In spite of the scientific and technological developments related to the sugarcane industry in Brazil, the strategies to produce the fundamental science needed to improve this industry even more have lagged considerably in comparison with other feedstocks like maize [9]. Two thirds of the energy content present in the carbohydrates of sugarcane is stored in the polysaccharides located in the cell walls of culm and leaves [10]. If the energy stored in these cell wall polysaccharides could be easily and efficiently accessed, then this biomass could serve as the raw material for 2G bioethanol production. However, in order to accomplish this, a much more detailed understanding of cell wall polysaccharide structure and a better understanding of the interactions amongst the diverse wall polymers are essential [11, 12].

The production of 2G bioethanol through the biochemical route is based currently on a process that uses optimized pretreatments to "loosen" the walls of different feedstocks, followed by hydrolysis of cell wall polysaccharides using exogenously applied enzymes [10, 13, 14]. The biochemical route has relied on the characterization of enzymes (mainly from microorganisms) that can be produced efficiently in large scale to hydrolyze bioenergy feedstock biomass [4, 10]. In this regard, polysaccharide hydrolytic enzymes from diverse organisms are under investigation by numerous research groups around the world [4, 5]. The strategies adopted differ slightly, but essentially, they concentrate on (1) enzyme characterization, (2) enzyme gene discovery and heterologous expression, (3) enzyme engineering, and (4) development of hydrolytic cocktails containing enzymes from different microorganisms, to name but a few.

A major hurdle that must be overcome in order to use the biochemical route to achieve efficient 2G bioethanol production is to overcome the intrinsic recalcitrance of cell walls to hydrolysis [15] that has been associated with the high level of complexity of the plant cell wall. Most research in this area focuses on the identification of optimal enzymes and enzyme combinations that are capable of fully hydrolyzing cell wall polysaccharides to produce monosaccharides that can then be fermented to produce bioethanol and other biofuels. However, the overall architecture and fine structural details of the polymers extant in sugarcane tissues, which are the potential substrates for these enzymes, have not been studied with the same intensity. This is important because unraveling the structural complexity of cell wall polymers, as well as their interactions in the intact wall, constitutes a major factor in overcoming recalcitrance problems. Thus, the current work aims at achieving a better understanding of the structural basis for recalcitrance associated with the complexity of the cell walls of sugarcane culms and leaves, in order to facilitate the design of enhanced strategies to hydrolyze them efficiently.

Relatively little is known about sugarcane walls, lignin being the most studied component of these cell walls. He and Terashima [16] studied the topology and composition of lignin in different internodes of sugarcane culm. Using a technique of feeding sugarcane tissue with tritiated compounds that became incorporated into coniferyl, coumaryl, and syringyl alcohols in vivo, they produced microautoradiograms that showed lignification that incorporated these three compounds into culm cell walls, the latter two being accumulated mainly in the middle lamella in relation to the fibers surrounding the vascular bundles. These authors also found that young and mature tissues displayed approximately the same proportion of hydroxycinnamic acids, but that ferulic acid decreased in relation to *p*-coumaric acid as tissues aged.

Other studies of wall composition have largely been confined to subfractionating the walls into large classes of compounds such as glucan, hemicellulose, lignin, and extractives [17]. These have been reported for sugarcane as being 38–43 %, 25–32 %, 17–24 %, and 1.6–7.5 %, respectively. Other authors [18] reported a sugarcane cell wall composition of 48.6 % cellulose, 31.1 % hemicelluloses (mainly by xylose and galactose), 19.1 % lignin, and 1.2 % ash. Performing hot water and alkali fractionation of sugarcane bagasse, Peng et al. [19] found glucose, xylose, and galactose (38.3 %, 27.6 %, and 19.2 %, respectively) as being the major monosaccharides present in water-soluble polysaccharides, and xylose (80–90 %) along with some arabinose (3–8 %) and several other monosaccharides in

smaller proportions when 1 % or 3 % (*w*/*v*) NaOH was used to extract the walls. These authors reported that such water–NaOH fractionation of sugarcane cell walls yielded 25.1 % of polysaccharides, which accounted for 74.9 % of the original hemicelluloses. They proposed that the breakage of α-ether bounds between lignin and hemicelluloses from bagasse cell walls promoted solubilization of hemicellulosic polymers as well as lignin macromolecules. Fourier transform infrared (FTIR) spectroscopy and ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectrometry revealed that the hemicellulose fraction of sugarcane walls contains small amounts of lignin, suggesting that these two compounds are closely associated in the walls.

For industrial purposes, sugarcane bagasse has been treated with alkaline hydrogen peroxide, likely to break ester linkages in the walls in order to increase the digestibility of the bagasse in animal rumen [20]. It was reported that this treatment results in the release of twice as much glucose, xylose, and uronic acids in the rumen compared with untreated controls. These authors suggested that the removal of lignin and ferulic acids was key to the increased digestibility.

Although the existing studies in the literature show the average cell wall composition of sugarcane, no fine structure or detailed composition of sugarcane cell wall has been reported. Such detailed knowledge will be essential to design optimal tissue pretreatment and cell wall hydrolysis protocols and, consequently, for the development of 2G bioethanol technology [11, 12]. In the present work, we analyzed cell walls from leaves and culm of sugarcane by sequential alkali fractionation followed by monosaccharide, fine structural (using limit digest oligosaccharides obtained by enzyme hydrolysis) and antibody-based glycome profiling of the polysaccharides present in the various wall extracts. We describe fundamental aspects of the mono- and oligosaccharide compositions as well as fine structural aspects of the cell walls of the two organs of sugarcane—leaf and culm—that are expected to be important feedstocks for second-generation bioethanol production.

Materials and Methods

Plant Material

Leaves and culms of sugarcane (cv. SP80-3280) were harvested from 3-month-old plants, dried at 60 °C until constant weight was achieved, and ground in a ball mill (Tecnal, Brazil).

Light Microscopy

Leaf and culm segments were kept in Karnovsky fixative [21] for 24 h, slowly dehydrated through a graded ethanol series for 2 h each step, and embedded in resin (Historesin, Leica Microsystems, Germany). Sections (12 μm) were cut using a Leica RM2145 microtome; the sections were mounted on glass slides and stained with 1 % (*w*/*v*) Toluidine Blue [22] for 10 min to stain all cell walls or phloroglucinol [22] to stain lignin. Stained sections were observed with a Leica DMLB microscope equipped with epifluorescence optics, and images were captured with a coupled Leica DFC320 camera and the Leica Image Manager 50 software.

Cell Wall Preparations

Five hundred milligrams of each milled sample were subjected to six successive extractions with 25 mL of 80 % (*v*/*v*) ethanol at 80 °C for 20 min. The supernatants were discarded to remove the soluble sugars and other soluble compounds. The residual alcohol-insoluble residue (AIR) of these extractions was washed with distilled water and dried at 60 °C for 24 h.

Cell Wall Fractionation

The cell walls (AIR) were subjected to successive extractions with increasingly harsh reagents to solubilize polysaccharide fractions for further analysis. The AIR was extracted twice with 25 mL of 0.5 M ammonium oxalate (pH 7.0) at 80 °C for 3 h each with continuous stirring. The supernatant was recovered by centrifugation of the suspension at 13,000×*g*. The oxalate-extracted cell wall residues were then extracted with 20 mL of 3 % (*w*/*v*) of sodium chlorite in 0.3 % (*v*/*v*) acetic acid [23], followed in succession by 0.1, 1.0, and 4.0 M NaOH extractions (each containing 3 mg/mL sodium borohydride) at room temperature for 1 h each [24]. After each step, the solubilized material was recovered as before, and the remaining insoluble material was washed with distilled water and dried at 60 °C for 24 h before being subjected to the next extraction. The solubilized fractions were neutralized, dialyzed extensively to remove salts, and freeze-dried. The fraction yields were obtained gravimetrically.

Lignin Quantification

The intact cell walls (AIR) from leaves and culms were homogenized in 50 mM potassium phosphate buffer, pH 7, with a mortar and pestle and transferred into a centrifuge tube. The pellets were centrifuged (1,500×*g*, 5 min) and washed by successive stirring and centrifugation as previously described [25]. Total lignin content of the AIR was estimated using the lignin thioglycolic acid (LTGA) method [26].

Glycome Profiling

Glycome profiling analyses of the cell wall fractions were done as previously described [27]. This technique involves

ELISA screening of the cell wall extracts with a comprehensive library of cell wall glycan-directed monoclonal antibodies [28–31]. The results presented on the heatmaps are the average of four biological replicates of each wall fraction.

Neutral Monosaccharide Analysis

Each cell wall fraction and AIR was hydrolyzed with 72 % (30 min)—4 % (1 h at 121 °C) (*v*/*v*) H_2SO_4 [32]. The monosaccharide solution was taken to a pH between 6 and 8 by addition of 50 % (*w*/*v*) NaOH and subsequently analyzed by HPAEC-PAD on a CarboPac SA10 column (DX-500 system, Dionex®) using a mixture of 99.2 % water and 0.8 % (*v*/*v*) 150 mM NaOH as eluent (1 mL min^{-1}). The monosaccharides were detected with a post-column addition of 500 mM NaOH (1 mL min^{-1}).

Uronic Acids Determination

Uronic acid content of each cell wall fraction and AIR was determined by colorimetric assay using the *m*-hydroxybiphenyl method [33] and galacturonic acid as standard.

Oligosaccharides Analysis

The oligosaccharide profiles were obtained using 1 % (*w*/*v*) of each fraction or AIR in 50 mM sodium acetate buffer, pH 5.0, as substrate. The substrates were incubated with 0.5 U of xylanase (Sigma®), lichenase (Megazyme®), or GH12 xyloglucan endoglucanase (XEG; purified according to [34]) for 24 h at 30 °C to assess the fine structure of arabinoxylan, β-glucan, and xyloglucan, respectively. The oligosaccharides produced were analyzed by HPAEC-PAD on a CarboPac PA-100 (ICS-3000 system, Dionex®) using 88 mM NaOH and 200 mM sodium acetate as eluent (0.9 mL min^{-1}) for 45 min.

Fourier Transform Infrared (FTIR) Spectroscopy

FTIR spectra were obtained with a Perkin-Elmer Spectrum 400, series FT-IR/FT-NIR, operating at 4 cm^{-1} resolution. The analyses of AIR and cell wall extracts were performed using the attenuated total reflectance accessory. The spectral region scanned was between 4,000 and 650 cm^{-1}, and 32 scans were performed for each sample. Only vibrations from 2,000 to 500 cm^{-1} are shown.

Data Analysis

Neutral monosaccharides and uronic acid contents were expressed in relative values considering the total sum of all of these components as 100 %.

The statistical differences between leaf and culm were analyzed by one-way ANOVA test (JMP® Statistical Discovery Software, version 5.0.1, SAS Inc., Cary, NC, USA). A posteriori contrasts were examined by Tukey range test with significance $p \leq 0.05$. Replication, where indicated (e.g., $n=4$), indicates the number of different biological replicates.

Results

Cell Types of Sugarcane Leaves and Culm

The microscopic analyses of leaves and culm of sugarcane (Fig. 1a, b) revealed the cellular complexity of these organs. Leaves contain at least ten distinguishable cell types or tissues (metaxylem vessels, tracheids and vessel elements; phloem, sieve tubes and companion cells; bulliform cell; epidermis; stomata; mesophyll cells; bundle sheath; and sclerenchyma), while the culm contains at least seven distinguishable cell types (metaxylem vessels, tracheids and vessel elements; phloem, sieve tubes and companion cells; parenchyma; sclerenchyma; and epidermis).

Fluorescence microscopy analyses showed that leaf tissue (Fig. 1c) has a stronger autofluorescence than does culm (Fig. 1d). In the leaf, the autofluorescence is concentrated more strongly on the vascular tissues and epidermis. A particularly bright autofluorescence region is on the phloem tissue as well as the bulliform cells. It appears that the fluorescence has different wavelength maxima in these tissues, which might reflect a difference in phenolic compound composition in the walls. In the culm, all cell walls fluoresce as well, with the vascular bundles showing a similar pattern to leaves (Fig. 1d). In the leaf, a higher number of cells display autofluorescence suggesting the presence of greater proportions of phenylpropanoids in the walls of this organ.

Figure 1e and f present sections stained with phloroglucinol to detect lignin. Lignin appears to be concentrated in the fibers of the vascular bundles in leaves (Fig. 1e), whereas it seems to be spread throughout all cell walls in culm (Fig. 1f).

Monosaccharide Analyses and Lignin Content

Table 1 shows the yields, the lignin and uronic acid contents, and neutral monosaccharide composition of the intact cell walls (AIR) and cell wall fractions of sugarcane leaf and culm. Lignin content of AIR was twofold higher in leaves (4.9 %) than in culm (2.5 %; Table 1, B).

The overall monosaccharide compositions of intact cell walls (AIR) isolated from leaf and culm are very similar (Table 1, D). Glucose (Glc) and xylose (Xyl) are the most abundant monosaccharides in AIR, accounting for ~60 %

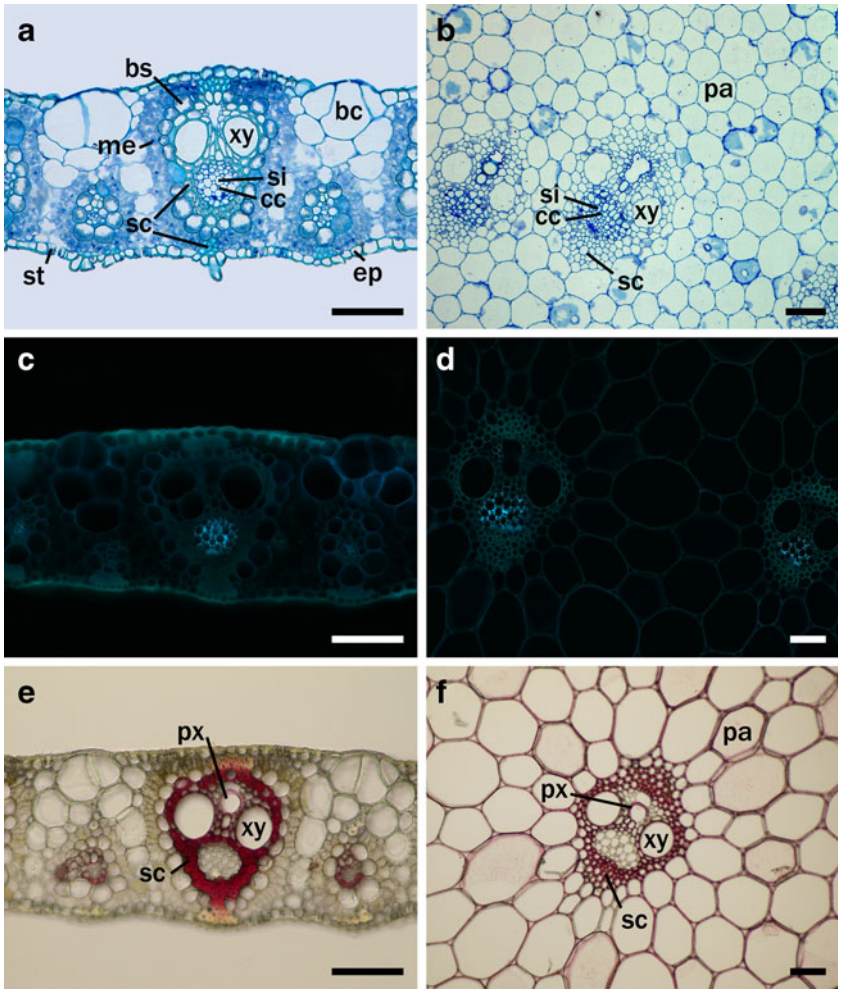

Fig. 1 Anatomical features of sugarcane leaf and culm (first internode) using different microscopic techniques. (**a**) Leaf and (**b**) culm under optical microscopy stained with toluidine blue (1 %) to show the diverse cell types present. (**c**) Leaf and (**d**) culm under fluorescence microscopy to display autofluorescence of phenolic compounds present in virtually all cell walls, but mostly in phloem. (**e**) Leaf and (**f**) culm reacted with phloroglucinol showing lignin in the sclerenchyma, protoxylem, metaxylem in leaf and culm, and also parenchyma in culm. Cell types observed include the following: (*xy*) metaxylem vessel, (*ph*) phloem, (*bc*) bulliform cells, (*ep*) epidermis, (*st*) stomata, (*me*) mesophyll, (*bs*) bundle sheath, (*sc*) sclerenchyma, (*pa*) parenchyma, and (*px*) protoxylem. *Bars* = 100 μm

and ~34 % of the monosaccharides analyzed, respectively. Statistically significant differences were found for arabinose (Ara), which is about half as abundant in culm as it is in leaf, and for galactose (Gal), which is two times more abundant in culm than in leaf. For the other neutral monosaccharides, a trace amount of fucose (Fuc) was detected, but mannose (Man) and rhamnose (Rha) were not detected.

Cell walls were fractionated using sequential extractions with reagents that would first release the more soluble (or less interactive) polymers (ammonium oxalate), followed by disruption of any phenolic-containing compounds (e.g., diferulic acids and lignin) to release materials tied to the wall via these compounds. Finally, the remaining insoluble wall residues were treated with a series of increasing concentrations of NaOH (0.1, 1, and 4 M) in order to extract polysaccharides that are more tightly bound into the wall.

Ammonium oxalate released twice as much material from culm (15 %) as it did from leaf (6 %; Table 1, A). The most abundant monosaccharide in this extract was Glc (approximately 41 % of the total monosaccharides in leaf and 62 % in culm), with Gal (ca. 16 % in leaf and 15 % in culm) and Ara (ca. 11 % in leaf and 9 % in culm) being present in approximately the same relative amounts as Xyl (ca. 10 % in leaf and 9 % in culm), and small amounts of Man (ca. 2.5 % in leaf and 1 % in culm) and traces of Rha (Table 1, D). The majority (~60 %) of the uronic acids, which originate from pectic polymers (homogalacturonan and rhamnogalacturonans) present in the sugarcane samples analyzed, was released by oxalate, and comparable amounts were released from leaf and culm (18.5 % vs. 16.3 %; Table 1, C).

Destruction of phenolic wall components using sodium chlorite released similar amounts of material from leaf and culm. The principal monosaccharides observed in the sodium chlorite extracts were Glc (43 % for leaf and 22 % for culm) and Xyl (ca. 35 % for leaf and 42 % for culm). Monosaccharides likely to be associated with pectins (Ara, Gal, Rha, and uronic acids) were present in similar proportions in the chlorite extracts from the two organs (Ara, 10.5 % and 11.5 %; Gal, 5.7 % and 5.3 %; Rha, 0.1 % and 0.4 %; uronic acids, 3.2 % and 5.1 %), the Glc proportion being similar in sodium chlorite and ammonium oxalate extracts in leaves and about one third in

Table 1 (A) Yields of cell wall extractions of alcohol insoluble residue (AIR) prepared from sugarcane leaf and culm, (B) lignin content of AIR of sugarcane leaf and culm, (C) Uronic acid content of AIR and cell wall extracts of sugarcane leaf and culm, and (D) monosaccharide analyses of AIR and cell wall extracts of sugarcane leaf and culm

		AIR	Ammonium oxalate	Sodium chlorite	0.1 M NaOH	1 M NaOH	4 M NaOH	Residue
A. Yield (%)								
	Leaf	–	**6.3±0.46**	11.0±1.32	**23.2±0.25**	13.9±0.61	**9.5±0.23**	**35.9±0.39**
	Culm	–	**14.9±1.32**	14.1±1.43	**15.9±0.96**	13.0±1.15	**12.9±0.86**	**29.2±1.90**
	p	–	**0.0009**	0.167	**0.0008**	0.518	**0.008**	**0.013**
B. Lignin (%)								
	Leaf	**4.9±0.3**	–	–	–	–	–	–
	Culm	**2.5±0.2**	–	–	–	–	–	–
	p	**<0.001**	–	–	–	–	–	–
C. Uronic acids (%)								
	Leaf	2.6±0.02	18.5±1.5	**3.2±0.13**	**4.0±0.43**	3.7±0.72	1.9±0.25	**0.2±0.01**
	Culm	2.9±0.13	16.3±0.95	**5.1±0.61**	**5.7±0.44**	3.5±0.21	2.0±0.28	**1.0±0.18**
	p	0.091	0.258	**0.047**	**0.027**	0.808	0.860	**0.021**
D. Neutral monosaccharides (%)								
Arabinose	Leaf	**4.6±0.08**	11.2±1.18	10.5±1.47	14.7±0.40	**11.3±0.38**	**5.4±0.36**	0.1±0.01
	Culm	**2.4±0.08**	8.8±0.49	11.5±0.45	13.8±0.27	**7.3±0.28**	**2.0±0.20**	0.1±0.02
	p	**<0.001**	0.125	0.529	0.122	**<0.001**	**<0.001**	0.518
Fucose	Leaf	0.1±0.01	**0.4±0.03**	0.2±0.06	0.1±0.00	n.d.	n.d.	n.d.
	Culm	0.1±0.00	**0.2±0.03**	0.3±0.04	n.d.	n.d.	n.d.	n.d.
	p	0.154	**0.016**	0.676	0.162	–	–	–
Galactose	Leaf	**0.5±0.02**	16.2±2.11	5.7±0.99	0.8±0.04	0.6±0.03	**0.6±0.05**	n.d.
	Culm	**0.9±0.03**	14.8±1.36	5.3±0.13	0.7±0.27	0.7±0.07	**1.2±0.05**	n.d.
	p	**<0.001**	0.593	0.720	0.965	0.128	**<0.001**	–
Glucose	Leaf	58.7±0.46	**41.3±2.02**	**43.0±7.46**	7.7±0.43	13.8±1.01	52.4±2.41	**98.1±0.19**
	Culm	59.8±0.35	**61.9±1.56**	**22.1±4.24**	10.5±1.60	14.3±2.33	63.3±3.38	**97.2±0.18**
	p	0.100	**<0.001**	**0.041**	0.176	0.841	0.043	**0.015**
Mannose	Leaf	n.d.	**2.4±0.22**	2.2±0.50	1.7±0.17	n.d.	3.5±1.24	n.d.
	Culm	n.d.	**1.1±0.19**	2.3±0.25	0.5±0.46	0.4±0.38	5.1±0.81	n.d.
	p	–	**0.004**	0.862	0.069	0.938	0.331	–
Rhamnose	Leaf	n.d.	0.2±0.16	0.1±0.12	0.1±0.05	0.1±0.05	n.d.	n.d.
	Culm	n.d.	n.d.	0.4±0.15	0.1±0.10	n.d.	n.d.	n.d.
	p	–	0.222	0.269	0.954	0.322	–	–
Xylose	Leaf	33.6±0.42	9.7±1.67	35.2±5.58	71.0±0.38	70.1±0.94	**36.1±1.84**	1.6±0.19
	Culm	33.5±0.26	8.2±0.52	41.9±3.63	68.7±1.76	73.7±2.35	**26.3±2.89**	1.7±0.19
	p	0.950	0.432	0.356	0.283	0.226	**0.034**	0.613

Statistically significant differences are shown in bold (n=4)

culm. The amount of Xyl in the sodium chlorite extracts from both organs is higher than that observed in the ammonium oxalate fractions (Table 1, D).

Together, 0.1- and 1-M NaOH extracts account for about 37 % and 29 % of the walls of leaf and culm, respectively (Table 1, A). Even though the Ara content of the leaf 1 M NaOH extract was higher than that of culm, both extracts from both organs are mainly composed of Xyl and Ara (together accounting for ca. 80–85 % of the extracts) followed by Glc (ca. 10–15 % of the extracts). The presence of uronic acids in the 0.1- and 1-M NaOH extracts (ca. 3.5–6 %) was observed in leaves in similar amounts in both fractions, while in culm, about twice the uronic acid content was observed in the 0.1 M NaOH extract relative to the 1 M NaOH extract (Table 1, C).

The 4 M NaOH extract is rich in Glc (ca. 52 % and 63 % for leaf and culm, respectively), followed by Xyl (ca. 36 % and 26 % in leaf and culm, respectively) and Ara (5.5 % and 2 % for leaf and culm, respectively). The Glc content of the 4 M NaOH extract is similar to that of the ammonium oxalate and the sodium chlorite extracts, especially for the leaf (Table 1, D).

The final insoluble wall residue remaining after all extractions consists of ca. 98 % Glc in both organs with a small amount of Xyl (1.6 %) and traces of Ara (0.1 %) and uronic acids (Table 1, D and C).

Glycome Profiling Analyses

All cell wall fractions were subjected to glycome profiling to gain a more complete picture of cell wall composition than the one provided by monosaccharide analysis. The glycan-directed antibodies used for these analyses recognize diverse epitopes (substructures) on most major polysaccharide types present in plant cell walls [28], with the exception of rhamnogalacturonan II and cellulose.

The glycome profiles of the leaf and culm cell wall extracts are remarkably similar overall (Fig. 2) in terms of the glycan epitopes present in these two organs. The profiles of both culm and leaf are particularly rich in xylan epitopes recognized by the Xylan-4, -5, -6, and -7 clades, as would be expected given the monosaccharide composition of sugarcane walls (Table 1, D). Some xyloglucan epitopes, particularly those recognized by the Non-Fuc XG-1, -2, -3, and -4 antibody clades, are also present in both organs, but very little fucosylated XG was detected. Lastly, mixed-linkage-β-glucan (recognized by the BG1 antibody) was present in the ammonium oxalate and alkali extracts of walls from both organs.

However, there are some differences between leaf and culm that were observed in terms of epitope extractability (Fig. 2, red boxes; Supplemental Table S1). Thus, some non-fucosylated xyloglucans (recognized by the Non-Fuc XG-1 and Non-Fuc XG-2 antibodies) were released from the walls by 0.1 M NaOH in culm, but not in leaves. Also, more of the xylan epitopes recognized by the Xylan-6 antibodies were released by oxalate and chlorite extraction from culm walls than from leaf walls. A few instances of single antibody differences between the glycome profiles of culm and leaf were noted. However, the significance of these, if any, cannot be assessed at present.

One prominent feature of the glycome profiles was the presence of significant amounts of homogalacturonan [both unesterified (HG-Backbone-1) and methyl-esterified (HG-Backbone-2)] and arabinogalactan (RG-I/AG, AG-1, -2, -3, and -4) epitopes in the ammonium oxalate fractions from walls of both organs (Fig. 2, blue boxes). Indeed, the bulk of the arabinogalactan epitopes that could be extracted from sugarcane walls were present in these extracts. Relatively little binding of xylan-directed antibodies (primarily Xylan-7 clade) was observed in the chlorite extracts from either organ.

Fine Structure of Cell Wall Hemicelluloses

The fractions obtained from leaves and culms were subjected to hydrolysis with endohydrolases in order to obtain partial access to the fine structure of arabinoxylan (AX), mixed-linkage-β-glucan (BG), and xyloglucan (XG) present in the walls of these organs.

Fractions of leaves treated with endo-β-xylanase, an enzyme that attacks the β-1,4 linkages in the unbranched parts of the AX backbone, yielded xylose from the ammonium oxalate extract and a complex mixture of oligosaccharides from the 0.1-, 1-, and 4-M NaOH extracts (Fig. 3). These mono- and oligosaccharides were characterized as xylose (a), xylobiose (b), xylotriose (c), and arabinosylated xylooligosaccharides (d) (Crivellari, Trifona, Dupree, and Buckeridge, in preparation). The ratio (a+b+c)/d increased in the fractions extracted with higher alkali concentration, suggesting that the less branched xylans were most tightly bound to the walls. Some xylan is also present in the final insoluble residue, suggesting that at least a sub-fraction of AX is strongly bound to cellulose.

It is noteworthy that AIR treated with endo-β-xylanase did not release the same pattern of oligosaccharides observed in the extracted fractions. In particular, the absence of branched oligosaccharides was observed, suggesting that the β-1,4 linkages present in the highly branched portions of arabinoxylan are not accessible to endo-β-xylanase attack in the intact cell walls.

Although BG was hydrolyzed from AIR with lichenase (an endo-enzyme that attacks β-1,4 linkages that are immediately adjacent to β-1,3 linkages in the backbone of this glucan), it seems to be bound in the wall with medium strength, as it was released mainly in the 1-M NaOH extract (Fig. 3). XG is virtually inaccessible to XEG (a GH12 xyloglucanase highly specific towards XG [34]) if AIR is given as substrate. Little XG was released in the ammonium oxalate extract. Most of this polymer was released in 1 and 4 M NaOH extracts. A complex pattern of XG oligosaccharides was observed in the digested extracts, the structure of which will have to be investigated further. Here, though, we considered these oligosaccharides as products of xyloglucan hydrolysis based on the fact that the XEG used has been proven previously to be specific to XG and is not able to attack AX, BG, or cellulose [34]. Similarly to AX, some XG oligosaccharides were released from the final insoluble residue, again suggesting that at least some XG remains strongly bound to cellulose after the alkali extractions. The hydrolysis of culm (Fig. 4) produced similar results as for leaves, with the exception that BG is released in the ammonium oxalate extract instead of the 1 M NaOH extract, and that XG seems to be spread through all extracts, with higher proportions in the 0.1- and 4-M NaOH extracts.

The FTIR spectra (Fig. 5) show that the AIR of leaf and culm exhibited vibrations consistent with the presence of carbohydrates (1045) [35, 36] and also acetyl esters (1240) [35] and lignin (1460) [37]. A comparison of the AIR spectra with spectra of pure lignin from sugarcane (spectra

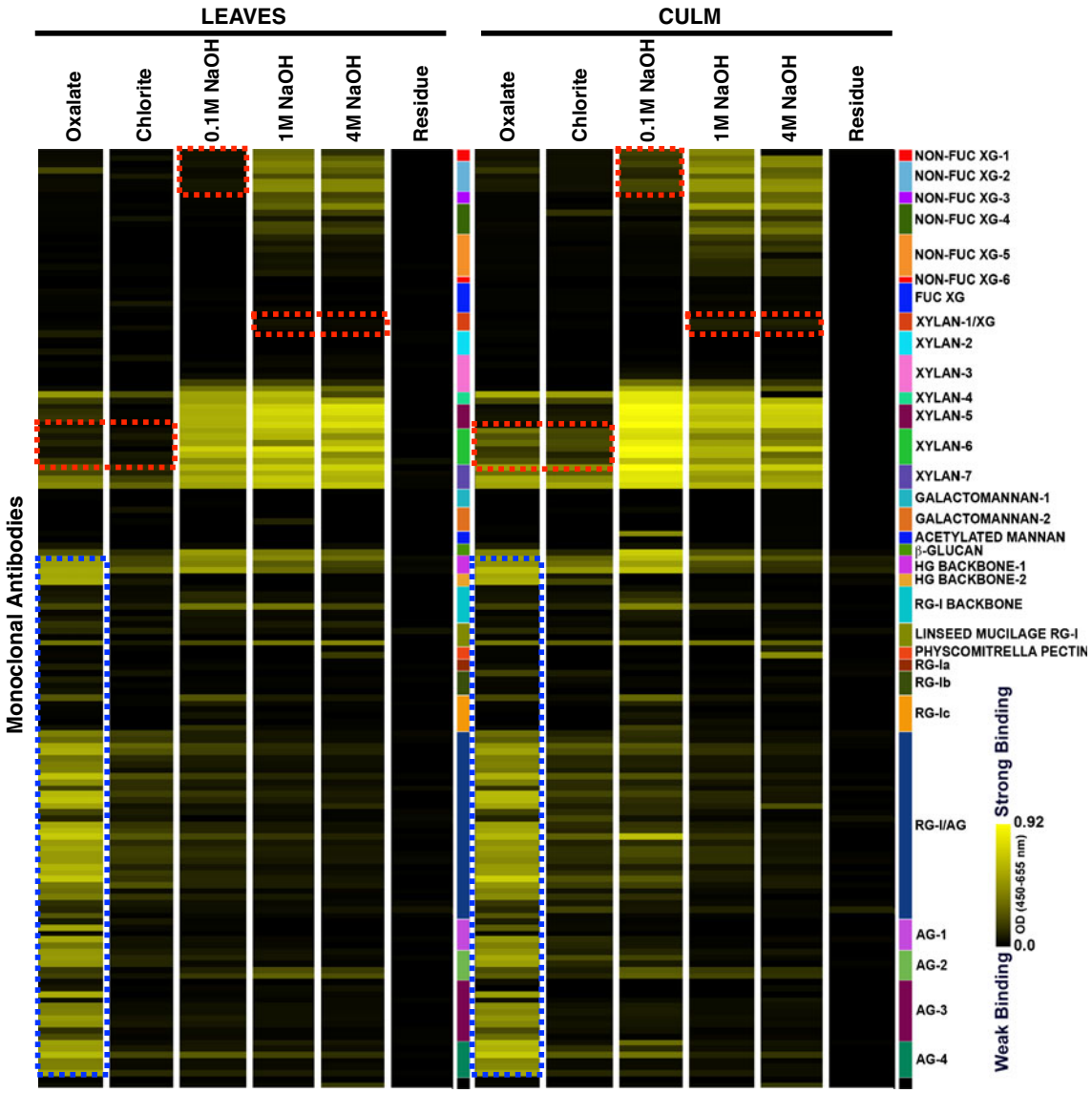

Fig. 2 Glycome profiles of cell wall fractions prepared from sugarcane leaves and culm. The walls were subjected to sequential extraction using oxalate, chlorite, 0.1, 1, and 4 M NaOH as indicated at the *top of the heatmap*. The solubilized extracts were then screened against an array of plant glycan-directed monoclonal antibodies using ELISAs. Antibody binding (average value of four independent replicates) is depicted as *colored heatmaps*, with bright yellow depicting maximal binding and black depicting no binding. The *panel on the right* (*colored boxes)* depicts the groups of antibodies used, identified according to the polysaccharides predominantly recognized by each group (see Supplemental Table S1 for additional details about the individual antibodies used)

supplied by M.T. Borges and K. Marabezi, not shown as they will be published elsewhere by the authors) did not show any coinciding lignin-associated peaks with the spectra obtained in the present work, suggesting that very little lignin is present in the AIR from young plants examined in this study (see confirmation in Table 1, B). After fractionation, several different peaks became apparent, notably those associated with aromatic rings (1607) [38] and carboxylic acids (1425) [39]. A peak that is considered typical of arabinosylated AX (990) [40] was detected in the 0.1- and 1-M NaOH extracts. The 4 M NaOH extract and the final insoluble residue displayed patterns of vibrations consistent with the profiles of samples rich in polysaccharides.

Discussion

The use of biomass for bioethanol production using 2G strategy, i.e., pretreatment followed by enzymatic hydrolysis [10], can be greatly benefitted by increased knowledge about what polysaccharides are present and how they are arranged in the cell walls. In this work, the cell walls of sugarcane leaves and

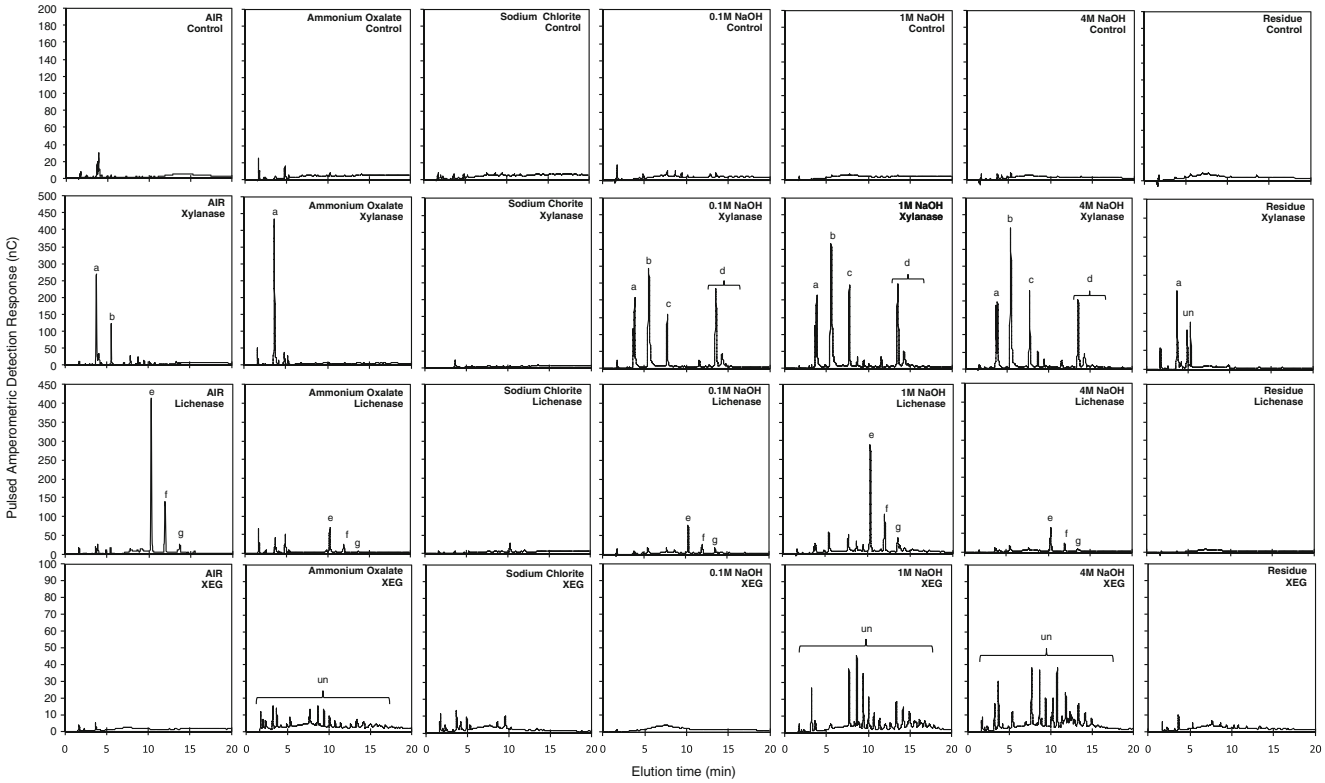

Fig. 3 Oligosaccharide profiles of alcohol-insoluble residue (AIR) and cell wall extracts of sugarcane leaves treated with hemicellulases. Lichenase (for detection of β-glucan, BG), xylanase (for detection of arabinoxylan, AX), and xyloglucan endoglucanase (XEG, for detection of xyloglucan, XG). *a* xylose, *b* xylobiose, *c* xylotriose, *d* arabinosylated oligosaccharides, *e* BG trisaccharide, *f* BG tetrasaccharide, *g* BG pentasaccharide, *un* unknown xyloglucan oligosaccharides

culm were subjected to multiple analytical procedures that helped to reveal not only polysaccharide composition, structure, and fine structure but aimed also at providing information about wall architecture. The data that resulted from this multi-faceted approach provide a consistent framework for sugarcane wall composition and structure. Furthermore, our data also give some insight into how optimized pretreatments might be designed to make sugarcane wall polysaccharides more amenable to hydrolysis through improved access to the key chemical linkages and polymer interactions that need to be broken in order to improve efficiency of 2G technologies for biofuel production from sugarcane feedstocks.

Interactions among Sugarcane Cell Wall Polysaccharides

The plant cell wall is a complex of interwoven polymers that interact in different ways [7]. Covalent and non-covalent links form a supramolecular complex in which polysaccharide structure and fine structure play key roles in cell expansion, plant defense, and biotechnological applications. Polysaccharides can be branched with mono- and/or oligosaccharides whose distribution can affect the mode of action of hydrolases on the polysaccharide, being also important for understanding their biological functions in the wall as well as in their biotechnological applications [41–43]. Polysaccharides can also be branched with non-sugar substituents, such as methyl, acetyl, and phenolic compounds. Acetylation of AX, for example, has been clearly demonstrated to interfere with hydrolysis of xylans by xylanases, as discussed below. Phenolic esterification is also thought to play a key role in interlocking polymers in the wall network. This can be done through simple esterification of ferulic acids to branching monosaccharides such as arabinose in AX [44], galactose, and arabinose in pectins [45, 46] and in XG [47].

A broad examination of the glycome profiling results (Fig. 2) shows that cell wall fractionation appears to partially follow the classical expected results. Pectins and BG are more soluble and are extracted primarily with ammonium oxalate, whereas hemicelluloses (AX and XG, but part of the BG as well) are extracted in the alkali fractions [23]. This suggests that sugarcane walls from cells of the two organs examined display at least two domains, one that is readily accessible (water-soluble) and another that is composed of an entangled polymer network primarily formed by hemicelluloses, but also including pectins and pectic arabinogalactans bound to cellulose.

The data from glycome profiling and the monosaccharide composition of the ammonium oxalate extract confirmed that

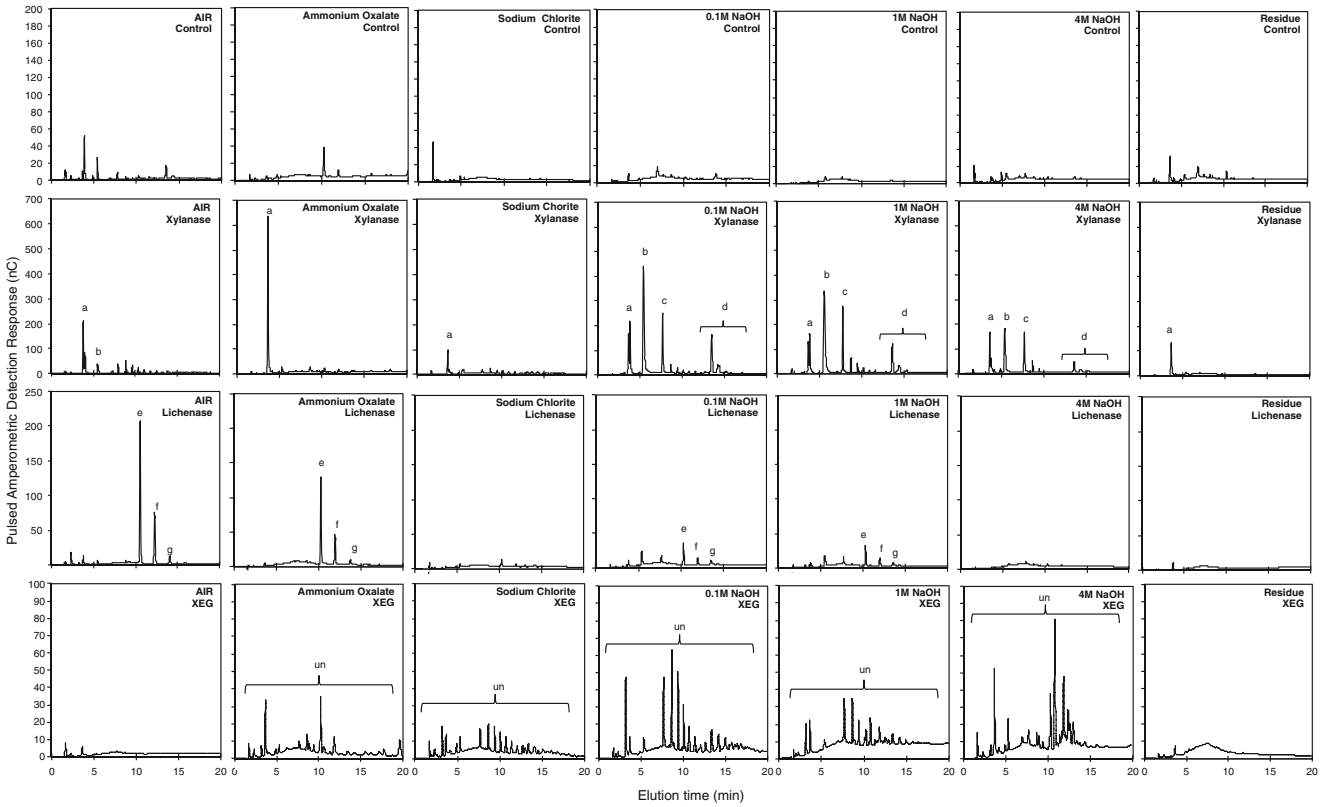

Fig. 4 Oligosaccharide profiles of alcohol-insoluble residue (AIR) and cell wall extracts of sugarcane culm treated with hemicellulases. Lichenase (for detection of β-glucan, BG), xylanase (for detection of arabinoxylan, AX), and xyloglucan endoglucanase (XEG, for detection of xyloglucan, XG). *a* xylose, *b* xylobiose, *c* xylotriose, *d* arabinosylated oligosaccharides, *e* BG trisaccharide, *f* BG tetrasaccharide, *g* BG pentasaccharide, *un* unknown xyloglucan oligosaccharides

a substantial fraction of BG is quite soluble in sugarcane cell walls in general. The fact that this polymer is easily extracted with ammonium oxalate suggests that much of the BG is probably loosely bound to the wall and therefore potentially more accessible to enzyme attack. This is consistent with the results presented in Figs. 3 and 4, in which the ammonium oxalate extract, when hydrolyzed with lichenase, produced BG oligosaccharides. Also, there is evidence of the presence of a less soluble BG sub-fraction that is released in the NaOH extracts, suggesting that some of the BG possibly interacts closely with other wall polysaccharides such as xylans, xyloglucans, and cellulose [48].

In sugarcane cell walls, most of the pectins were observed to be easily extractable polymers. Our results indicate that homogalacturonan (HG), denoted by the presence of uronic acids in the mildest extracts (oxalate and 0.1 M NaOH), as well as by the binding of antibodies that specifically recognize HG (Table 1, C, and Fig. 2), and rhamnogalacturonan I (RGI), probably branched with arabinogalactans (Fig. 2 and Table 1, D), are the principal components of these easily extractable pectins. The presence of some pectic polymers in the sodium chlorite extracts suggests that a sub-population of pectins is linked to cell wall phenolics and/or lignin in the wall, since the role of chlorite in the fractionation procedure is to oxidize and cleave phenolic compounds. Lignin was detected in both leaf and culm tissues (Table 1, B), but is mostly associated with the vascular bundles (Fig. 1e, f). Lignin is thought to be linked mainly to side-chain sugars present in the branched hemicelluloses, AX and XG (arabinose and galactose, respectively). However, some authors have demonstrated lignin cross-links to arabinogalactans, possibly including pectic arabinogalactans [49, 50], while it has been shown that pectins can also be ornamented with phenolic compounds linked to the arabinosyl and galactosyl residues of RGI [45, 46] and that the arabinans in RGI may be linked by feruloyl diesters [51].

On the other hand, autofluorescence, which affords visualization of a greater diversity of phenylpropanoids in the walls of grasses, was detected in all cell walls. Chlorite treatment would also cleave these phenolic linkages, such as ferulic and diferulic acid, thereby releasing polysaccharides cross-linked with these compounds. It is noteworthy that the nature of the autofluorescence differs between different groups of cells. For example, the phloem and bulliform cells (the latter being present in leaves only) appear to contain a different type of phenolic compound in their walls

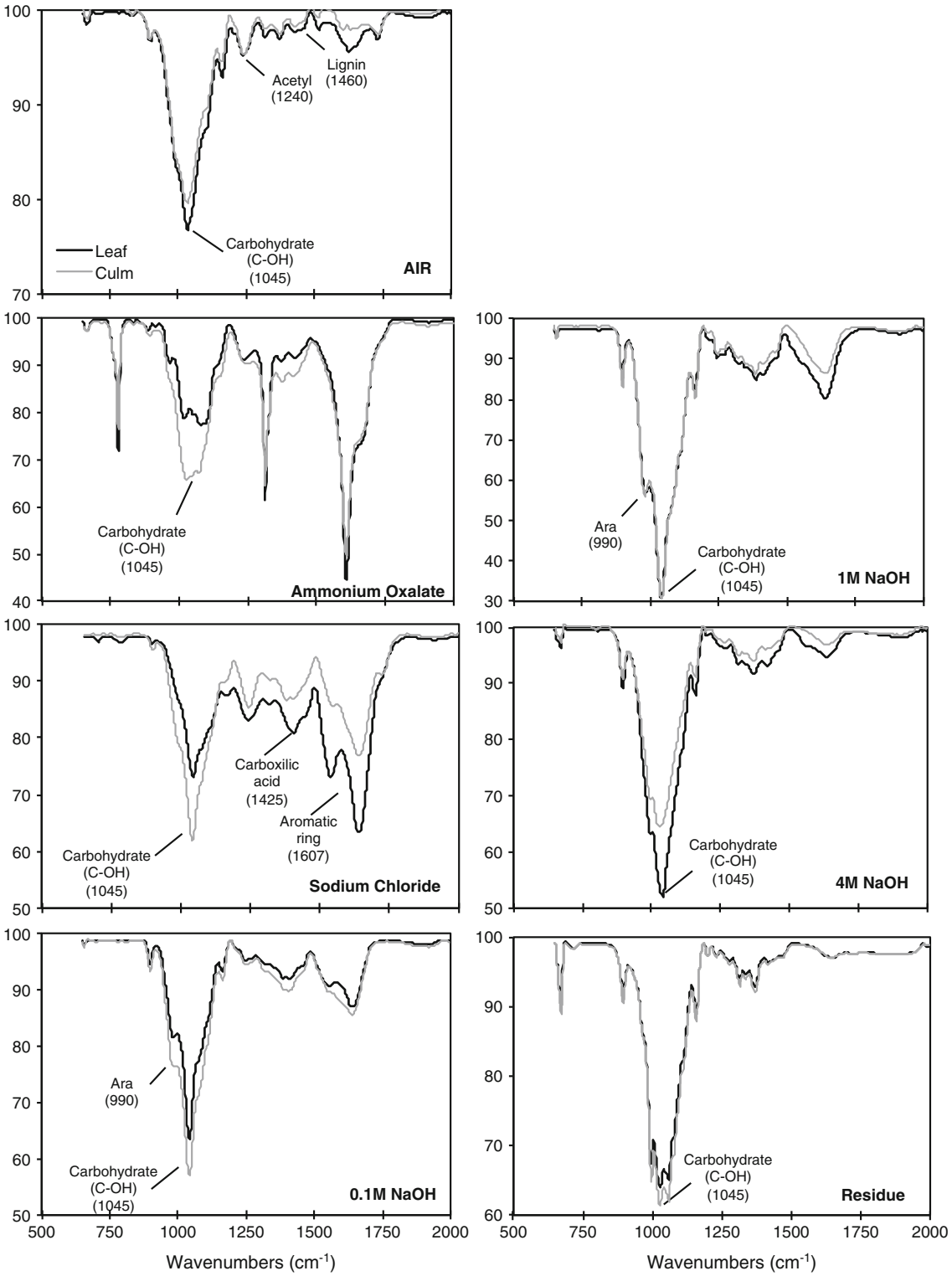

Fig. 5 Fourier Transformed Infra-Red (FTIR) spectroscopy of alcohol-insoluble residue (AIR) and cell wall extracts of leaves (*black lines*) and culm (*grey lines*) of sugarcane

compared with the remaining cells of the organs examined. The characterization of these compounds remains to be studied in the future.

FTIR analyses of the sodium chlorite fraction revealed peaks with vibrations consistent with the presence of carboxylic acid (possibly from galacturonic acid residues present in

pectic polysaccharides; Fig. 5). Additionally, some FTIR vibrations typical of aromatic rings were detected in all cell wall extracts. Binding of antibodies to pectin epitopes was detected in all wall extracts up to and including the 4 M NaOH extract (Fig. 2), which suggests that at least sub-populations of pectin are more tightly associated with cellulose in sugarcane walls. Recent NMR studies also suggest a close association of pectins with cellulose [52, 53]. The manner in which these more tightly bound pectic polysaccharides are bound into the sugarcane wall matrix cannot be ascertained from the studies presented here.

Xylans are present in the 0.1-, 1-, and 4-M NaOH extracts of sugarcane walls (Figs. 2, 3, and 4). The neutral monosaccharide data (Table 1, D) suggest that the xylans present in the cell walls of leaf and culm contain unbranched xylan. The oligosaccharide profiles also suggest the presence of significant stretches of unbranched xylan backbone, leading to the production of xylooligosaccharides after hydrolysis with endo-β-xylanase. The strong binding of the Xylan-5, -6, and -7 clades of antibodies, which are directed at linear, unsubstituted xylan epitopes (unpublished results of the Hahn laboratory), to the alkali extracts in the glycome profiles (Fig. 2) also supports the presence of linear, unbranched xylans sugarcane walls. The monosaccharide composition data also indicate the presence of a considerable amount of arabinosylated xylan backbone (Ara:Xyl ratios of ca. 1:5) in sugarcane walls. With increased alkali concentrations, the ratio of Ara:Xyl is reduced in extracts of both leaf and culm (from ca. 1:5 to 1:7 in leaves and from ca. 1:5 to 1:10 in culm). An Ara:Xyl ratio within this range (1:9) has been observed in run-mill sugarcane bagasse [54]. The degree of AX branching seems to be higher in leaf than in culm (denoted from the peaks marked as "d" in Figs. 3 and 4). This finding might reflect differences in the degree of xylan branching in different cell types [55] present in these organs (Fig. 1).

The monosaccharide composition of the sodium chlorite extract also contains high amounts of Xyl and Ara (Table 1, D) suggesting the presence of AX in this extract from both organs. However, no xylan oligosaccharides are released by endoxylanase digestion of the chlorite extract (Figs. 3 and 4), nor do the glycome profiles of the chlorite extracts show high levels of xylan epitopes, particularly in the leaf chlorite extract (Fig. 2). Xylans are known to be substituted with acetyl esters [56, 57], which would not be cleaved by chlorite treatment [58]. The presence of such acetyl esters would prevent xylan cleavage by the endo-β-xylanase, blocking the formation of xylooligosaccharides. Selig et al. [59] have shown that the hydrolysis of native corn stover xylans by an enzyme mixture containing endoxylanase and β-xylanase is hindered by acetylation as compared with the deacetylated substrate. Acetyl substituents also interfere with the binding of xylan-directed antibodies, particularly of those in the Xylan-5 and -6 clades of antibodies (Avci and Hahn, unpublished results). Exposure to alkaline conditions, such as during the NaOH extractions, will remove these acetyl groups [59]. Hence, endo-β-xylanase treatments of the NaOH extracts result in the release substantial amounts of branched oligosaccharides (Figs. 3 and 4), and these extracts are strongly recognized by xylan-directed antibodies (Fig. 2). Thus, xylan polymers in the two sugarcane organs studied here occur as (1) unbranched xylan, (2) acetylated xylan, and (3) arabinosylated xylan, the latter being likely to be feruloylated on the side-chain arabinosyl residues. The distribution of these xylan domains amongst the various classes of xylans present in sugarcane walls and extractable under different conditions cannot be determined from the data obtained in this study.

Xyloglucans were also detected in lower proportions in sugarcane cell walls of leaves and culm. Diverse non-fucosylated XG epitopes were detected in the glycome profiles of the 1- and 4-M NaOH extracts (Fig. 2), and XG oligosaccharides were released by XEG treatments of these extracts (Fig. 3 and 4). The lower presence of XG compared with xylans in sugarcane walls is very likely due to the limited localization of XG to specific cell types (e.g., phloem) in monocots [60]. The present study was carried out only on above-ground organs of sugarcane. Other work from our labs that focused on sugarcane root tissues demonstrate the presence of XG in all cell types in that organ (to be published elsewhere). Our results do not provide direct evidence of association between AX and XG, but hypothetically, these polysaccharides might be associated through diferulic bridges between arabinosyl and galactosyl branches of AX and XG, respectively.

Relationship Between Cell Wall Complexity and Wall Recalcitrance in Sugarcane

In the present work, we found that enzymatic hydrolysis of AIR with endoglycanases does not occur in the same fashion as does the hydrolysis of isolated fractions of the wall. This suggests that the level of complexity arising out of polymer–polymer interactions within the walls (i.e., the cell wall architecture) of sugarcane leaves and culm has the potential to interfere with the access of endoenzymes (at least of the main endoglycanases, as used in this work) to the wall network. It is noteworthy, for example, that endo-β-xylanase was not able to hydrolyze linkages that release branched oligosaccharides, unless fractionation of the wall has been performed with sodium chlorite and NaOH. However, some xylose and unbranched xylan oligosaccharides were indeed released from AIR after enzyme attack. The explanation for the difficulty to access branched AX when it is part of the wall network is likely to be related to the above-mentioned facts that arabinosyl residues may be esterified with phenolics, forming diferulic linkages and/or lignin.

A similar situation was observed for xyloglucan, which seems to be inaccessible to hydrolysis by XEG (GH12) when walls are intact, but release several different oligosaccharides when wall extracts are treated with this enzyme. Hypothetically, it is possible that AX and XG might be interacting by means of phenolic bridges. This observation is supported by the fact that less branched AX are present in the 4 M NaOH fraction along with XG (Figs. 3 and 4).

On the other hand, BG behaved differently from the other two hemicelluloses (AX and XG). The majority of BG is released from AIR by lichenase and is therefore accessible to the enzyme in the intact wall. However, sub-populations of BG are bound more tightly into the wall networks and are not immediately accessible to lichenase, but are detectable in some base extracts from both leaves and culm.

Our data suggest that polysaccharide fine structure, polysaccharide–polysaccharide interactions, and possibly polysaccharide–lignin/phenolics interactions are important factors that limit complete saccharification of cell wall carbohydrates in sugarcane leaf and culm. Two factors that were not addressed directly in this work appear to play major roles in the recalcitrance of sugarcane walls to hydrolysis. The first is acetylation of wall polysaccharides, xylans being particularly noteworthy in this respect in monocots such as sugarcane. FTIR was readily able to detect the vibration arising from the acetyl ester linkage (1240 in Fig. 5) in AIR, but this peak disappears when the walls are fractionated, particularly with alkali. The second is the presence of aromatics, detected by fluorescence microscopy (for wall-bound phenolics) and phloroglucinol staining (for lignin) in AIR, and also detected by FTIR, especially in the sodium chlorite fraction. Although further experiments are necessary to clarify the acetylation pattern of sugarcane wall polymers, the results obtained in this work allow us to speculate that the polysaccharides present in sugarcane cell walls are likely to be interlocked by phenolic compounds (probably feruloyl esters linked to terminal sugars of pectins such as galactose and arabinose and linked to side-chain glycosyl residues in hemicelluloses, such as arabinose in AX and galactose in XG). Indeed, this argument is strengthened by the findings of Xu et al. [61] that ferulic and *p*-coumaric acids are present in sugarcane bagasse, such that 50 % of the ferulic acid is linked to hemicelluloses, whereas the other 50 % is linked to lignin, and ca. 70 % of *p*-coumaric acid is linked to the cell wall.

The structural features of sugarcane walls described here are likely to have significance to industrial processes, as they have potentially important implications for the use of sugarcane for bioenergy production purposes. Industrial processes impacted by the results presented here include the choice of pretreatment as well as the design of hydrolysis protocols for the breakdown of grass cell walls. Previous studies of the degradation of cell walls during barley malting (reviewed in [62, 63]) yielded a list of enzymes that would effectively degrade the cell walls, which included enzymes related to BG and AX hydrolysis. Jamar et al. [63] mentioned that BG hydrolysis seems to be hindered by the presence of AX, due to the occurrence of acetyl and feruloyl esters on this polysaccharide. In the case of sugarcane walls, the presence of such esters may interfere with AX and XG hydrolysis, but we observed no such effect on BG accessibility to hydrolysis.

Our studies of sugarcane confirm that the structural features of the walls of sugarcane are generally in accordance with what is known for the walls of grasses. Thus, a pretreatment coupled to a hydrolytic protocol to produce sugars for fermentation and bioethanol production would have to take into account that there is a hierarchy of linkages in sugarcane walls that need to be broken. For the walls of sugarcane leaves and culm, most of the pectins and BG appear to be readily accessible to enzymes, whereas the hemicelluloses AX and XG will require a pretreatment to remove acetyl- and/or feruloyl-esters in order to make the backbones and side-chains of these polysaccharides susceptible to hydrolysis by exo- and endoenzymes. Pretreatments normally use water and/or chemical substances such as alkali and ammonia that clearly alter wall structure [10]. For example, the use of water in the form of steam explosion, which is one of the preferred methods suggested for second-generation sugarcane processing protocols, would extract the more soluble polymers, such as BG and pectins, and wash them out of the wall, preventing their utilization for subsequent hydrolysis and sugar utilization. In the case of the alkali pretreatments, there is the possibility that cleavage of ester linkages might also result in the release of even more polysaccharides, which would also be lost in the process. In any case, it must be recognized that pretreatments can lead to significant losses of polysaccharides from the walls, thereby decreasing the yield of sugars available for subsequent fermentation. Thus, procedures may need to be implemented to recover the polysaccharides and/or sugars that are released during pretreatment in order to improve the overall efficiency of the biomass processing.

On the basis of the structural and fine structural results presented here, and the inferences about possible arrangements of polymers into networks (i.e., the wall architecture) in the walls of sugarcane leaves and culm, a hierarchical model for enzymatic hydrolysis of sugarcane biomass can be proposed (Fig. 6). In the absence of a general pretreatment that would break diverse ester linkages (e.g., acetyl esters and phenolic esters) within the cell walls [64], intact walls (AIR) would have to be first treated with pectinases (endopolygalacturonase, pectin-methyl-esterase, α-arabinofuranosidase, and β-galactosidase) together with lichenase to hydrolyze BG. In order to fully release pectins,

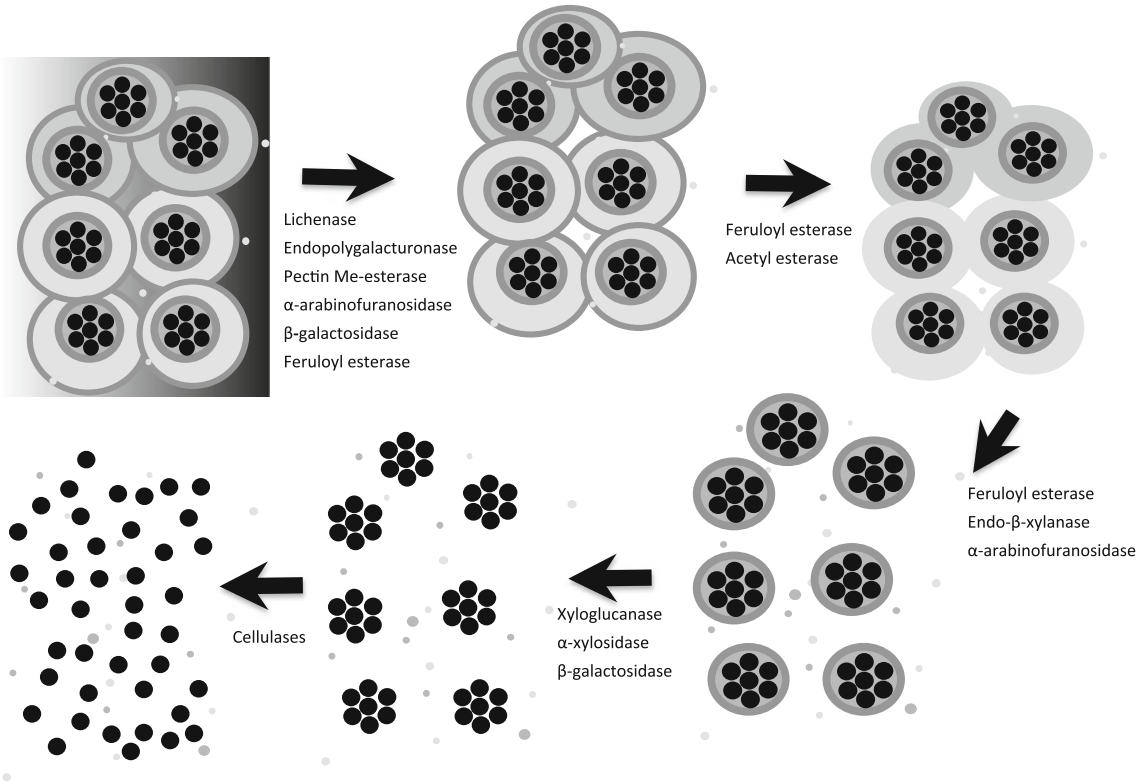

Fig. 6 Schematic hypothetical representation of a hierarchical attack of hydrolytic enzymes on the cell wall networks of sugarcane leaf and culm. The wall is viewed as a transverse slice in which the *black dots* represent sections of cellulose microfibrils that coalesce to form a macrofibril. This would hypothetically be tied by xyloglucan, which is drawn as having a boundary (*darker grey circles*) formed by phenolic compounds. The *lighter grey thick circles* toward the outside surrounding would be formed by arabinoxylan that could be linked to xyloglucan by diferulic bridges. These bundles of cellulose macrofibrils surrounded by hemicelluloses would be embedded into a matrix composed of pectin and β-glucan. In the first step, the enzymes would hydrolyze this matrix, which would need to be attacked in a hierarchical order until naked cellulose would become accessible to cellulases

feruloyl esterase may need to be used as well. The remaining wall architecture, consisting primarily of cellulose, interlocked with AX and XG, would then be treated with a mixture of enzymes capable of hydrolyzing not only the main chains but also the side-chains of both polymers. At the same time, feruloyl- and acetyl-esterases would still have to be used in order to break the ferulic bridges among hemicelluloses and to remove acetyl groups that could interfere with enzymatic cleavage of the polysaccharides. This importance of removal of phenolic substituents is underlined by the discovery of Siqueira et al. [65] that pretreatment of sugarcane tissue (bagasse) with sodium chlorite leads to a higher saccharification level of this biomass. After these two enzymatic pre-treatment steps, the remaining cellulose would likely become available for enzymatic attack and could then be used as a substrate for endo-β-glucanase, cellobiohydrolases, and β-glucosidases.

In conclusion, sugarcane leaf and culm differ little in their wall composition and structure, the differences being mainly in hemicelluloses. Thus, disregarding other logistical problems related to the transport of leaves to the mills, leaves could be included in second-generation biomass processing, and mixtures of leaves and stem could be processed together without the need for complex adjustments to the processing protocols.

The overall sugarcane wall composition in terms of carbohydrates only, based on estimates derived from monosaccharide analysis, as well as on the results of glycome profiling and enzymatically released oligosaccharides, is that cellulose accounts for ~30 % of the wall in both organs, hemicelluloses comprise up to ~50 %, and pectins ~10 %. Knowledge of which polysaccharides are present, their structure and fine-structure, and how they are likely to be arranged in sugarcane cell walls has important implications for 2G-process design. One important issue to be considered for 2G-process design is that targeting of the industrial processes exclusively at cellulose alone as a source of fermentable sugars will waste as much as 70 % of the carbohydrates present in sugarcane walls. These carbohydrates are important as they could be used either for biofuel production and/or as products of the biorefinery processes in which these polymers and/or oligosaccharides derived from them could be used for biotechnological applications (e.g., food and medicine) with higher commercial value.

Acknowledgments The authors thank Maria de Lourdes Polizelli and André Damasio for supplying xyloglucan endoglucanase (XEG, GH12). The authors acknowledge the assistance of Maria Teresa Borges and Karen Marabezi with the FTIR production of data. This work was supported financially and is part of the production of the Instituto Nacional de Ciência e Tecnologia do Bioetanol—INCT do Bioetanol (FAPESP 2008/57908-6 and CNPq 574002/2008-1) and of the Centro de Processos Biológicos e Industriais para Biocombustíveis—CeProBIO (FAPESP 2009/52840-7 and CNPq 490022/2009-0). The glycome profiling was supported by the BioEnergy Science Center administered by Oak Ridge national Laboratory and funded by a grant (DE-AC05-00OR22725) from the Office of Biological and Environmental Research, Office of Science, United States, Department of Energy. Generation of the CCRC series of plant glycan-directed monoclonal antibodies used in this work was supported by the NSF Plant Genome Program (DBI-0421683).

References

1. Pimentel D, Patzek TW (2005) Ethanol production using corn, switchgrass, and wood; biodiesel production using soybean and sunflower. Nat Resour Res 14:65–76
2. Macedo IC, Seabra JEA, Silva JEAR (2008) Green house gases emissions in the production and use of ethanol from sugarcane in Brazil: the 2005/2006 averages and a prediction for 2020. Biomass Bioenergy 32:582–595
3. Renewable Fuels Association (2012) Accelerating industry innovation—2012 ethanol industry outlook. http://www.ethanolrfa.org/pages/annual-industry-outlook. Accessed 14 March 2012
4. Sims REH, Mabee W, Saddler JN, Taylor M (2010) An overview of second generation biofuel technologies. Bioresour Technol 101:1570–1580
5. Buckeridge MS, Goldman GH (2011) Routes to cellulosic ethanol. Springer, New York
6. Silva GB, Ionashiro M, Carrara TB, Crivellari AC, Tiné MAS, Prado J et al (2011) Cell wall polysaccharides from fern leaves: evidence for a mannan-rich Type III cell wall in *Adiantum raddianum*. Phytochemistry 72:2352–2360
7. Carpita NC, Gibeaut DM (1993) Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the cell wall during growth. Plant J 3:1–30
8. Amorim HV, Lopes ML, Oliveira JVCO, Buckeridge MS, Goldman GH (2011) Scientific challenges of bioethanol production in Brazil. Appl Microbiol Biotechnol 91:1267–1275
9. Buckeridge MS, de Souza AP, Arundale RA, Anderson-Teixeira KJ, DeLucia E (2012) Ethanol from sugarcane in Brazil: a midway strategy for increasing ethanol production while maximizing environmental benefits. GCB Bioenergy 4:119–126
10. Soccol CR, Vandenberghe LPS, Medeiros ABP, Karp SG, Buckeridge MS, Ramos LP et al (2010) Bioethanol from lignocelluloses: status and perspectives in Brazil. Bioresour Technol 101:4820–4825
11. Buckeridge MS, Dos Santos WD, de Souza AP (2010) Routes for cellulosic ethanol in Brazil. In: Cortez LAB (ed) Sugarcane bioethanol: R&D for productivity and sustainability. Edgard Blucher, São Paulo, pp 365–380
12. Pauly M, Keegstra K (2010) Plant cell wall polymers as precursors for biofuels. Curr Opin Plant Biol 13:305–312
13. Gomez LD, Steele-King CG, McQueen-Mason SJ (2008) Sustainable liquid biofuels from biomass: the writing's on the walls. New Phytol 178:473–485
14. Dos Santos WD, Gomez EO, Buckeridge MS (2011) Bioenergy and the sustainable revolution. In: Buckeridge MS, Goldman GH (eds) Routes to cellulosic ethanol. Springer, New York, pp 15–26
15. Himmel ME, Ding SY, Johnson DK, Adney WS, Nimlos MR, Brady JW et al (2007) Biomass recalcitrance: engineering plants and enzymes for biofuels production. Science 315:804–807
16. He L, Terashima N (1990) Formation and structure of lignin in monocotyledons. III. Heterogeneity of sugarcane (*Saccharum officinarum* L.) Lignin with respect to the composition of structural units in different morphological regions. J Wood Chem Technol 10:435–459
17. Masarin F, Gurpilhares DB, Bafa DCF, Barbosa MHP, Carvalho W, Ferraz A et al (2011) Chemical composition and enzymatic digestibility of sugarcane clones selected for varied lignin content. Biotechnol Biofuels 4:55
18. Sanjuan R, Anzaldo J, Vargas J, Turrado J, Patt R (2011) Morphological and chemical composition of pith and fibers from Mexican sugarcane bagasse. Holz als Roh- und Werkstoff 59: 447–450
19. Peng F, Ren JL, Xu F, Bian J, Peng P, Sun RC (2009) Comparative study of hemicelluloses obtained by graded ethanol precipitation of sugarcane bagasse. J Agr Food Chem 57:6305–6317
20. Amjed M, Jung HG, Donker JD (1992) Effect of alkaline hydrogen peroxide treatment on cell wall composition and digestion kinetics of sugarcane residues and wheat straw. J Anim Sci 70:2877–2884
21. Karnovsky MJ (1965) A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. J Cell Biol 27:137A–138A
22. Kraus JE, Arduim M (1997) Manual básico de métodos em morfologia vegetal. EDUR, Rio de Janeiro
23. Carpita NC (1984) Cell wall development in maize coleoptiles. Plant Phisiol 76:205–212
24. Gorshokova TA, Wyatt SE, Salnikov VV, Gibeaut DM, Ibragimov MR, Lozovaya VV et al (1996) Cell-wall polysaccharides of developing flax plants. Plant Physiol 110:721–729
25. dos Santos WD, Ferrarese MLL, Nakamura CV, Mourão KSM, Mangolin CA, Ferrarese-Filho O (2008) Soybean (*Glycine max*) root lignification induced by ferulic acid: the possible mode of action. J Chem Ecol 34:1230–1241
26. Bruce RJ, West CA (1989) Elicitation of lignin biosynthesis and isoperoxidase activity by pectic fragments in suspension cultures of castor bean. Plant Physiol 91:889–897
27. Pattathil S, Avci U, Miller JS, Hahn MG (2012) Immunological approaches to plant cell wall and biomass characterization: glycome profiling. In: Himmel M (ed) Biomass conversion: methods and protocols. Methods in molecular biology, vol 908. Humana Press, New York, pp 61–72
28. Pattathil S, Avci U, Baldwin D, Swennes AG, McGill JA, Popper Z et al (2010) A comprehensive toolkit of plant cell wall glycan-directed monoclonal antibodies. Plant Physiol 153:514–525
29. Zhu X, Pattathil S, Mazumder K, Brehm A, Hahn MG, Dinesh-Kumar SP et al (2010) Virus-induced gene silencing offers a functional genomics platform for studying plant cell wall formation. Mol Plant 3:818–833
30. Kong Y, Zhou G, Yin Y, Xu Y, Pattathil S, Hahn M (2011) Molecular analysis of a family of *Arabidopsis* genes related to galacturonosyltranferases. Plant Physiol 155:1791–1805
31. DeMartini JD, Pattathil S, Avci U, Szekalski K, Mazumber K, Hahn MG et al (2011) Application of monoclonal antibodies to investigate plant cell wall deconstruction for biofuels production. Energy Environ Sci 4:4332–4339
32. Saeman JF, Harris EE, Kline AA (1945) Quantitative saccharification of wood and cellulose. Ind Eng Chem Anal 17:35–37
33. Filisetti-Cozzi TMCC, Carpita NC (1991) Measurement of uronic acids without interference from neutral sugars. Anal Biochem 197:157–162

34. Damásio ARL, Ribeiro LFC, Ribeiro LF, Furtado GP, Segato F, Almeida FBR et al (2012) Functional characterization and oligomerization of a recombinant xyloglucan-specific endo-beta-1,4-glucanase (GH12) from *Aspergillus niveus*. Biochem Biophys Acta Proteins Proteomics 1824:461–467
35. Robert P, Marquis M, Barron C, Guillon F, Saulnier L (2005) FT-IR investigation of cell wall polysaccharides from cereal grains. Arabinoxylan infrared assignment. J Agric Food Chem 53: 7014–7018
36. Kacurakova M, Smith A, Gidley MJ, Wilsona RH (2002) Molecular interactions in bacterial cellulose composites studied by 1D FT-IR and dynamic 2D FT-IR spectroscopy. Carbohydr Res 337:1145–1153
37. Gutierrez A, Bocchoni P, Galletti GC, Martinez AT (1996) Analysis of lignin-polysaccharide complexes formed during grass lignin degradation by cultures of *Pleurotus* species. Appl Environ Microbiol 62:1928–1934
38. Trick KA, Saliba TE (1995) Mechanisms of the pyrolysis of phenolic resin in a carbon/phenolic composite. Carbon 33: 1509–1515
39. Marchessault RH (1962) Application of infra-red spectroscopy to cellulose and wood polysaccharides. Pure Appl Chem 5:107–129
40. Kacurakova M, Ebringerova A, Hirsch J, Hromadkova Z (1994) Infrared study of arabinoxylans. J SciFood Agric 66:423–427
41. Cyran M, Courtin C, Delcour JA (2004) Heterogeneity in the fine structure of alcali-extractable arabinoxylans isolated from two rye flours with high and low breadmaking quality and their coexistence with other cell wall components. J Agric Food Chem 52:2671–2680
42. Tiné MAS, Silva CO, Lima DU, Carpita NC, Buckeridge MS (2006) Fine structure of a mixed-oligomer storage xyloglucan from seeds of *Hymenaea courbaril*. Carbohydr Polym 66:444–454
43. Van Gool MP, Schols HA, Toth K, Szakacs G, Gruppen H (2011) Screening for distinct xylan degrading enzymes in complex shake flask fermentation supernatants. Bioresour Technol 102: 6039–6047
44. Geissmann T, Neukom H (1973) A note on ferulic acid as a constituent of the water-insoluble pentosans of wheat flour. Cereal Chem 50:414–416
45. Fry SC (1982) Phenolic components of the primary-cell wall—feruloylated disaccharides of D-galactose and L-arabinose from spinach polysaccharide. Biochem J 203:493–504
46. Fry SC (1983) Feruloylated pectins from the primary-cell wall—their structure and possible functions. Planta 157:111–123
47. Ishii T, Hiroi T, Thomas JR (1990) Feruloylated xyloglucan and *p*-coumaryl arabinoxylan oligosaccharides from bamboo shoot cell-walls. Phytochemistry 29:1999–2003
48. Burton RA, Gidley MJ, Fincher GB (2010) Heterogeneity in the chemistry, structure and function of plant cell walls. Nature Chem Biol 6:724–732
49. Monro JA, Bailey RW, Penny D (1972) Polysaccharide composition in relation to extensibility and possible peptide linked arabinogalactan of lupin hypocotyl cell walls. Phytochemistry 11: 1597–1602
50. Selvendran RR, Davies AMC, Tidder E (1975) Cell wall glycoproteins and polysaccharides of mature runner beans. Phytochemistry 14:2169–2174
51. Jones L, Milne JL, McQueen-Mason S (2003) Cell wall arabinan is essential for guard cell function. Proc Natl Acad Sci USA 100:11783–11788
52. Dick-Perez M, Zhang YA, Hayes J, Salazar A, Zabotina OA, Hong M (2011) Structure and interactions of plant cell-wall polysaccharides by two- and three-dimensional magic-angle-spinning solid-state NMR. Biochemistry 50:989–1000
53. Dick-Perez M, Wang T, Salazar A, Zabotina OA, Hong M (2012) Multidimensional solid-state NMR studies of the structure and dynamics of pectic polysaccharides in uniformly ^{13}C-labeled *Arabidopsis* primary cell walls. Magn Reson Chem 50:539–550
54. Lavarack BP, Griffin GJ, Rodman D (2002) The acid hydrolysis of sugarcane bagasse hemicellulose to produce xylose, arabinose, glucose and other products. Biomass Bioenergy 23:367–380
55. Pauly M, Keegstra K (2008) Cell-wall carbohydrates and their modification as a resource for biofuels. Plant J 54:559–568
56. Chesson A, Gordon AH, Lomax JA (1983) Substituent groups linked by alkali-labile bounds to arabinose and xylose residues of legume, grass, and cereal straw cell walls and their fate during digestion by rumen microorganisms. J Sci Food Agric 34: 1330–1340
57. Carpita NC (1996) Structure and biogenesis of the cell walls of grasses. Annu Rev Plant Physiol Plant Mol Biol 47:445–476
58. Rowell RM, Simonson R, Hess S, Plackett DV, Cronshaw D, Dunningham E (1994) Acetyl distribution in acetylated whole wood and reactivity of isolated wood cell-wall components to acetic anhydride. Wood Fiber Science 26:11–18
59. Selig MJ, Adney WS, Himmel ME, Decker SR (2009) The impact of cell wall acetylation on corn stover hydrolysis by cellulolytic and xylanolytic enzymes. Cellulose 16:711–722
60. Brennan M, Harris PJ (2011) Distribution of fucosylated xyloglucans among the walls of different cell types in monocotyledons determined by immunofluorescence microscopy. Mol Plant 4:144–156
61. Xu F, Sun RC, Sun JX, Liu CF, He BH, Fan JS (2005) Determination of cell wall ferulic and *p*-coumaric acids in sugarcane bagasse. Anal Chim Acta 552:207–217
62. Bamforth CW (2009) Current perspectives on the role of enzymes in brewing. J Cereal Sci 50:353–357
63. Jamar C, Du Jardin P, Fuconnier ML (2011) Cell wall polysaccharides hydrolysis of malting barley (*Hordeum vulgare* L.): a review. Biotechnol Agron Soc Environ 15:301–313
64. Bohn PJ, Fales SL (1991) Effect of two pre-treatments on phenolic acid yield in three forage species. J Sci Food Agric 54:347–354
65. Siqueira G, Milagres AMF, Carvalho W, Koch G, Ferraz A (2011) Topochemical distribution of lignin and hydroxycinnamic acids in sugarcane cell walls and its correlation with the enzymatic hydrolysis of polysaccharides. Biotech Biofuels 2:7