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Research Paper

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

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ABSTRACT

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

1. Introduction

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

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

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

To overcome recalcitrance, a dilute acid pretreatment has been

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

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

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

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

2. Material and methods

2.1. Biomass

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

2.2. Sulfuric acid pretreatment

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

2.3. Chemical composition analysis

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

2.4. Crystallinity index of the biomass

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

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

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

2.5. Enzymatic hydrolysis

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

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

Where:

[Glucose]	Concentration of glucose released during enzymatic hydrolysis (g/L)
[Cellobiose]	Concentration of cellobiose released during enzymatic hydrolysis (g/L)
[Biomass]	Dry biomass concentration at the beginning of the enzymatic hydrolysis (g/L)
f	Glucan fraction in dry biomass (g/g)
1.053	Correction factor of cellobiose to glucose equivalents
1.111	Conversion factor of glucan to glucose equivalents

2.6. Heating value

Thermal Degradation (TD) (heating value) was performed in a Calorimeter (Parr Instrument Company, Parr 6400 Calorimeter, Illinois, USA) by burning 300 mg of untreated samples, as well as commercial cellulose and lignin (Sigma Aldrich). The higher heating value is defined as the amount of heat released by a specified quantity of mass (initially at 25 °C) once it is combusted and the products have returned to a temperature of 25 °C, which takes into account the latent heat of vaporization of water in the combustion products.

2.7. Scanning electron microscopy (SEM)

For scanning electron microscopy analysis, untreated and acid pretreated fractions (leaf and stem) were cut into small pieces (5 × 5 mm), washed with deionized water and dried at 45 °C for 24 h. Samples were mounted onto stubs using carbon double-sided tape and examined in a FEI Quanta FEG 450 scanning electron microscope, operating at an accelerating voltage of 1 kV.

3. Results and discussion

3.1. Chemical composition of elephant grass fraction

Acid pretreatment is commonly used for hemicellulose solubilization, improving biomass digestibility. Despite the potential of elephant grass for the use as source for ethanol production, the effectiveness of acid pretreatment of different parts of the plant had not been examined previously. Relatively high concentrations of acid were used to allow us to pretreat samples at a low temperature (Brienzo et al., 2014).

Chemical characterization is the first step in the establishment of energy conversion processes, as it allows the detection of raw material variability, processes of optimization and the establishment of quality parameters (Chies, 2013). In untreated elephant grass samples (i.e., not subjected to acid pretreatment) was observed small differences only in the chemical composition (cellulose/glucan, total hemicelluloses and lignin) between the whole plant and the leaf and stem fractions (Table 1). There were no significant differences in the values of extractives for the whole plant (26.04%) when compared with leaf (25.25%) and stem (25.38%).

The chemical composition data described in this study corroborated previous studies that reported values of 30–37% cellulose, 20–31% hemicellulose, and 8–21% lignin for elephant grass samples (Sladden et al., 1991; Santos et al., 2001; Menegol et al., 2014). The variations observed between studies may be explained by differences in environmental factors – including climatic changes (temperature and humidity), land constitution, infections and pests, planting methods, harvest seasons, among others – which affect the chemical composition of plants, leading to variation between plants of the same species (Menardo et al., 2013).

The breakdown of cellulose into glucose is fundamental for 2G ethanol production, irrespective of the cellulose content of biomass samples. Elephant grass has high cellulose content, which makes this biomass source particularly attractive from the perspective of fermentable sugar production, and consequently ethanol production (Phitsuwan et al., 2016). In addition, the lower lignin content found in elephant grass compared with other lignocellulosic materials likely results in a lower inherent recalcitrance, and hence facilitates cell wall disruption and the efficient release of fermentable monomeric sugars (Brienzo et al., 2017; Wallace et al., 2016).

3.2. Acid pretreatment increases the recovery of water insoluble solids

Pretreatments usually result in a considerable mass loss of plant biomass components, depending on the pretreatment method, the experimental conditions, and the biomass used for conversion (Chatuvedi and Verma, 2013). In the more recalcitrant plant fractions mass recovery after acid pretreatment is high and, thus, lower sugar concentrations are found in liquid fractions (Brodeur et al., 2011). In this work, the solid recovery (water insoluble solids – WIS) from samples pretreated with sulfuric acid varied from 54.36% to 66.37%, with 5% sulfuric acid yielding higher solid recovery than other pretreatments, followed by 10% and 20% sulfuric acid pretreatments (Table 1). The stem fractions showed higher resistance to mass loss (i.e., higher solid recovery) upon pretreatment than leaf and dried whole plant samples (Table 1), indicating reduced solubilization of compounds such as hemicellulose sugars. The decrease in solid recovery observed in this work in the samples pretreated with acid (particularly the leaf and whole plant samples) was likely due both to the solubilization of carbohydrates (mainly hemicellulose) and to the partial removal of lignin

Table 1
Heating value, crystallinity index, solid recovery and chemical composition leaf, stem and whole plant elephant grass samples left untreated or pretreated with 5 (P1), 10 (P2) or 20% (P3) of sulfuric acid (5, 10 and 20% m/m acid at 121 °C/30 min).

Sample	Chemical composition (%, dry mass)					
	Glucan	Hemicellulose total	Lignin total	Solid recovery	Heating value (cal g^{-1})	CrI (%)
Leaf	32.28 ± 0.38	22.41 ± 0.94	14.37 ± 0.51	–	3939.49 ± 6.19	35.13 ± 5.40
Stem	34.10 ± 2.39	24.94 ± 2.54	15.49 ± 1.29	–	3958.45 ± 9.40	40.32 ± 1.45
E.grass	33.85 ± 0.75	23.93 ± 1.75	14.15 ± 0.74	–	–	37.06 ± 1.45
Leaf - P1	41.34 ± 1.68	14.22 ± 1.92 (63.36)	14.87 ± 1.81	59.12 ± 2.78	–	49.03 ± 1.57
Leaf - P2	40.15 ± 1.55	11.47 ± 1.70 (75.70)	26.97 ± 2.32	55.86 ± 0.43	–	54.20 ± 1.41
Leaf - P3	42.70 ± 1.62	9.40 ± 0.25 (85.02)	27.21 ± 3.74	54.56 ± 0.68	–	55.89 ± 4.94
Stem - P1	43.72 ± 2.66	16.39 ± 2.18 (62.63)	19.45 ± 2.45	66.37 ± 3.36	–	52.69 ± 3.19
Stem - P2	44.60 ± 2.89	13.97 ± 0.71 (74.03)	21.02 ± 2.95	60.28 ± 0.56	–	55.53 ± 4.94
Stem - P3	48.87 ± 2.48	12.86 ± 2.86 (76.71)	27.46 ± 3.09	58.34 ± 0.76	–	52.15 ± 0.40
E.grass - P1	36.89 ± 0.87	15.64 ± 0.26 (60.96)	16.56 ± 5.50	59.90 ± 0.94	–	42.14 ± 0.42
E.grass - P2	38.28 ± 2.69	10.80 ± 1.54 (77.02)	22.11 ± 1.89	54.96 ± 1.72	–	47.96 ± 3.36
E.grass - P3	43.13 ± 2.21	10.72 ± 1.14 (74.94)	23.32 ± 4.62	54.36 ± 0.42	–	49.51 ± 1.75

Total hemicellulose: sum of xylose, arabinose and acetyl group content (as anhydromonomers). Values in parentheses represent hemicellulose removal (from the solid fraction) calculated as the percentage in the materials and solid recovery. Total lignin: sum of soluble and insoluble lignin fractions; CrI: biomass crystallinity index. Commercial cellulose and lignin Heating value were 3837.42 ± 11.34 and 4657.25 ± 9.56 cal g^{-1}. (–) not determined.

(Scholl et al., 2015).

The percentage of cellulose and lignin increased in all acid-pretreated samples, when compared with untreated materials (Table 1), which was likely due to the solubilization and removal of the hemicellulosic fraction. Treatment of the stem fraction led to the highest cellulose/glucan percentage compared with other fractions (from 43.72% to 48.87% after exposure to sulfuric acid; Table 1). The reduction in hemicellulose content after pretreatment was due to its relatively amorphous nature, large polidispersity and lower degree of polymerization when compared with native cellulose, making hemicellulose more susceptible to removal/solubilization during acid pretreatment (Bottcher et al., 2013). Among the main biomass components, hemicellulose is removed preferentially after acid pretreatment (Brienzo et al., 2017). The removal of hemicellulose was higher from the leaf fraction (85.02%) than from the stem fraction (76.71%) and from the whole plant (74.94%) (Table 1). The leaf and whole plant samples analyzed in this study had lower lignin content than stem samples, suggesting that the latter were more recalcitrant, since the lignin content is directly linked to the difficulty in disrupting plant cell walls (Jia et al., 2014). In general, leaf samples were more sensitive to acid pretreatment than stem and whole plant samples, as could be observed a higher hemicellulose solubilization. The extension of carbohydrates solubilization probably is not related to the amount of sugars (hemicellulose) or lignin since they are similar (Table 1). Moreover, there are physicochemical properties of the biomass that could have influence in the leaf and stem recalcitrance: accessibility and porosity (Brienzo et al., 2015), lignin distribution (Brienzo et al., 2017). Also, it is clear that a leaf structure result in lower density, influencing the accessibility, compared to a stem.

3.3. Acid pretreatment increases the crystallinity of elephant grass fractions

The crystallinity index (CrI) of lignocellulosic materials is directly influenced by the amount of crystalline cellulose regions within native cellulose (Sant'Anna et al., 2014). Importantly, crystallinity is a key property that affects the enzymatic digestibility of cellulose, with high crystallinity decreasing digestibility (Rg et al., 2012). Acid pretreatment results in the solubilization of sugars, especially amorphous hemicellulose components, increasing the proportion of crystalline components (such as cellulose), which become considerably enriched in the biomass after acid treatment (Corrales et al., 2012). Thus, early studies revealed an increase in the CrI after pretreatment, due to amorphous fraction removal (Corrales et al., 2012; Brienzo et al., 2014).

An increase in the CrI of acid pretreated samples was observed, in relation to untreated materials, for whole plant, leaf and stem samples (Table 1). Also, the stem had higher CrI than both the whole plant and the leaf fraction (Table 1). The crystallinity values reported here were lower than those previously reported by Satyanarayana and Wypych (2007) for elephant grass plant, and by Corrales et al. (2012) for sugarcane bagasse (47 and 48%, respectively, compared with 35.13–40.32% observed here; Table 1). It is possible that the amount of extractives (around 25%) of the samples analyzed here contributed to the observed decrease in the CrI index.

The results indicated that increasing concentrations of acid in the pretreatment led to increased removal of hemicellulose (Table 1). Accordingly, pretreatment with increasing concentrations of acid led to a gradual increase in the CrI, for leaf and whole plant samples. The stem fraction was more resistant to pretreatment than the leaf fraction and the whole plant, as evidenced by the lower sugar solubilization. Moreover, the increase in the CrI value after pretreatment with 20% sulfuric acid (relative to the untreated) was higher for the leaf fraction (by 20.76 percentage points, pp) than for the whole plant (by 12.45 pp) and the stem fraction (by 11.83 pp) (Table 1).

3.4. Both stem and leaf fractions of elephant grass have high heating value

The heating value analysis measures the capacity of fuel energy generation during combustion, in kcal g^{-1} or cal g^{-1} of biomass (Flores et al., 2012b). The heating value was similar for the stem (3958.45 cal g^{-1}) and leaf fraction (3939.49 cal g^{-1}) (Table 1). Nevertheless, the heating values for both fractions are considered high (Sheng and Azevedo, 2005), suggesting that these fractions are suitable for combustion processes. As a comparison, the commercial cellulose and lignin heating values were 3837.42 and 4657.25 cal g^{-1}, respectively (Table 1). This result confirms the influence of the chemical composition on the combustion of lignocellulosic material, as reported previously (Demirbas, 2002). An average heating value of 4096 cal g^{-1} was reported previously for elephant grass stem and leaf (Flores et al., 2012b), while the heating values reported for to whole plant were 3501 cal g^{-1} (Onuegbu et al., 2012) and 4337 cal g^{-1} (Rocha et al., 2017). The elephant grass heating value is close to that of other lignocellulosic materials such as Eucalyptus (4507 cal g^{-1}) (Munalula and Meincken, 2009), and sucrose-free sugarcane fractions (from 3998 to 4236 cal g^{-1}) (Brienzo et al., 2014).

For 2G ethanol production, the selection of varieties with high lignin content is unfavorable due to their high recalcitrance (Benjamin et al., 2013). Similarly, varieties with high recalcitrance are also negative to the conversion process (Brienzo et al., 2015). However, the high heating value of elephant grass favors its use for the generation of thermal and electric energy. Moreover, the higher recalcitrance of the steam, compared to leaf, suggest to be appropriate to use this fraction in the energy co-generation. This energy could supply, for example, part of the heat demand for the transformation of lignocellulosic residues into fuels (Haykiri-Acma et al., 2010).

3.5. The stem fraction of elephant grass has higher recalcitrance to enzymatic hydrolysis

An enzymatic hydrolysis assay was used to investigate the effect of diluted sulfuric acid pretreatment on the recalcitrance of elephant grass plant and its fractions (Fig. 1A–C). Untreated lignocellulosic material typically shows low glucose yield of around 20% (Corrales et al., 2012; Brienzo et al., 2014), due to its intrinsic resistance to enzymatic action (Wang et al., 2009; Brienzo et al., 2015). In this study, the conversion of cellulose into glucose in untreated whole plant, stem or leaf samples was similar, around 20% (Fig. 1). These results suggested that the elephant grass plant and its fractions are recalcitrant, which highlights the need for a pretreatment to overcome this natural resistance to digestion (Sant'Anna et al., 2014).

Indeed, the glucose yield increased in the pretreated samples compared with the untreated ones (Fig. 1). A pretreatment with 5% acid provoked modification in the material digestibility increasing the glucose release compared to untreated material. It was evidenced the lower recalcitrance of the leaf, with higher glucose yield than steam, probably due the physicochemical properties and also lower lignin content (Table 1). The highest glucose yield was obtained in the leaf fraction (89%), followed by the whole plant (76%), and then by the stem fraction (43%), after pretreatment with 20% sulfuric acid (Fig. 1). Pérez-Boada et al. (2014) reported that the response to enzymatic hydrolysis differs between ball-milled elephant grass pith (50%), outer cortex (31%) and whole stem (35%). The enzymatic kinetics was similar for stem, leaf and whole plant samples subjected to pretreatments with 10 and 20% sulfuric acid (Fig. 1). However, pretreatments with 5% sulfuric acid led to reduced glucose yields (Fig. 1).

These enzymatic hydrolysis results also indicated that 10 h (for stem) and 24 h (for leaf and whole plant) are a reliable time to perform the hydrolysis of pretreated material, because there were no relative increase in the glucose yield at longer hydrolysis times (Fig. 1). For leaf, there is an intermediate phase between 10 and 24 h, where after reaching a recalcitrance phase, resulting in low glucose yield increases.

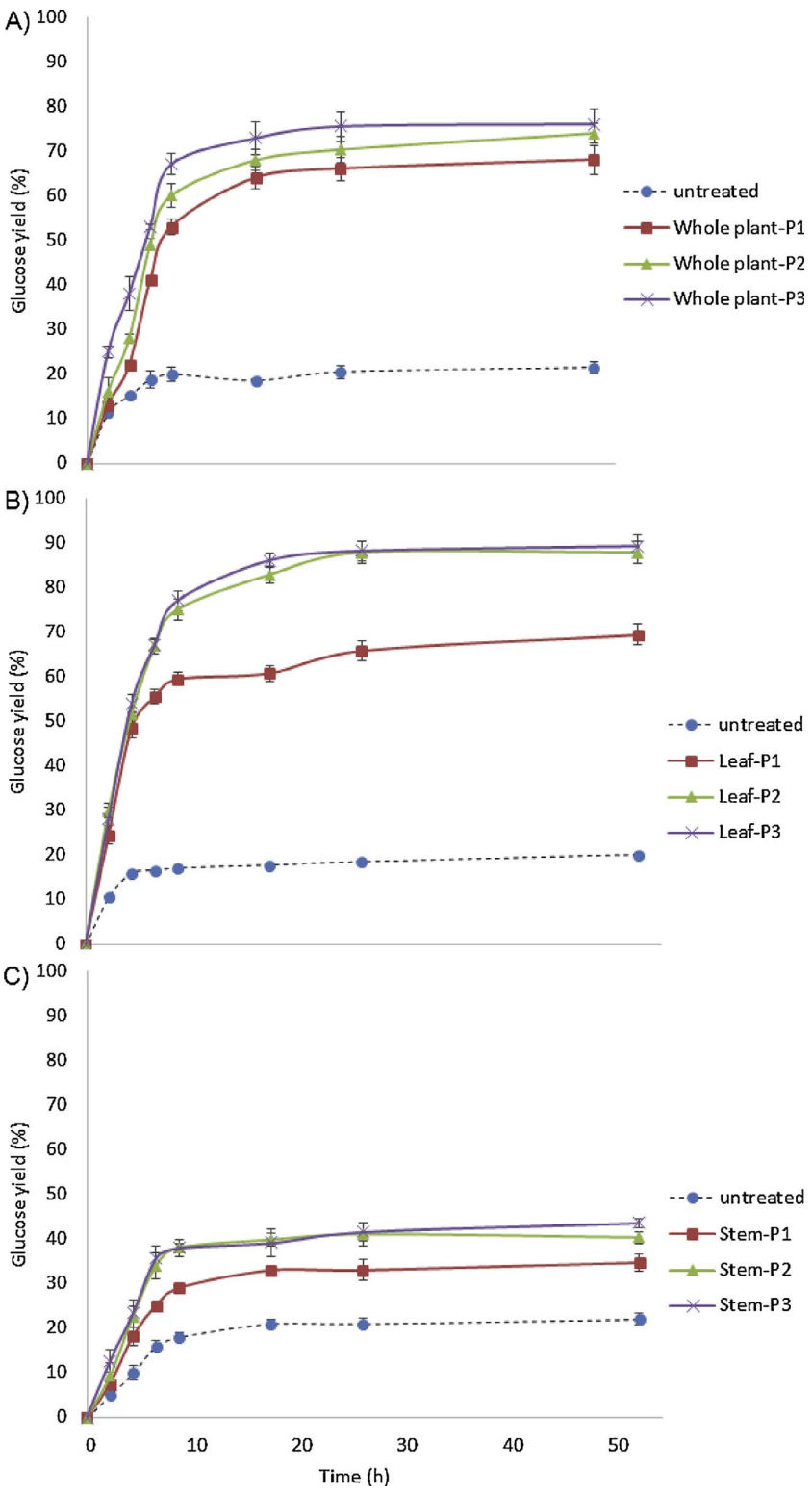

Fig. 1. Glucose yield from enzymatic hydrolysis (using 15 FPU/g and 15 U/g β-glucosidase) of whole plant (A), leaf (B) and stem (C) fractions of elephant grass left untreated or pretreated with 5 P1), 10 (P2) or 20% (P3) of sulfuric acid (5, 10 and 20% m/m, at 121 °C, for 30 min).

During the enzymatic hydrolysis occurs a process slowdown due to cellulose removal and lignin enrichment, which protecs cellulose from enzyme action (Wallace et al., 2016). Elephant grass pretreated with other methods, such as steam explosion and aqueous ammonia soaking, had similar enzymatic hydrolysis kinetics to those reported here, with high glucose yields after 24 h of reaction (Cardona et al., 2014). In contrast, higher yields have been reported using surfactants such as Tween 80, but only after 48 h of reaction (Menegol et al., 2014).

The higher lignin content of the stem fraction is likely to contribute to its higher recalcitrance (compared with the leaf fraction and the whole plant), with lower sensitivity to enzymatic hydrolysis and reduced glucose yield. Lignin forms a physical barrier to the access of enzymes to cellulose (Buranov and Mazza, 2008). The digestibility of the pretreated leaf fraction by enzymatic hydrolysis increased with increasing concentration of sulfuric acid in the pretreatment (Fig. 1). The acid concentration in the pretreatment led to an increase in the hemicellulosic solubilization (removing up to 85% of this fraction) with the most severe condition (Table 1). It is likely that acid pretreatment

also triggered changes in the lignin structure of elephant grass samples, relocating it to the fiber surface, and, in turn, enhancing the yield in the enzymatic hydrolysis step (Domínguez et al., 2008).

Collectively, the data reported here indicate that pretreating the whole elephant grass plant implies in low efficiency of enzymatic hydrolysis, due to the inherent recalcitrance of each fraction that composes the biomass. The leaf fraction showed better results of cellulose conversion into glucose compared with the stem fraction and whole plant (Table 1). The data for the leaf and stem fractions clearly showed that separation of the biomass in more homogeneous fractions improves pretreatment efficacy and enzymatic hydrolysis. This approach favors the design of specific strategies for pretreating each fraction according to its recalcitrance (Sant'Anna et al., 2014). The fractionation of the elephant grass in this work resulted in similar kinetics of enzymatic hydrolysis to those reported for other acid-pretreated improved cultivars (Li et al., 2015).

The results suggested that a higher severity of diluted acid pretreatment should be applied to overcome the high degree of recalcitrance of the stem fraction. Considering the higher recalcitrance of this fraction, it seems that its resistance to digestion relies on the tissue organization, type of cells and/or cell wall structure. These features have been correlated with recalcitrance for other lignocellulosic materials, such as sugarcane bagasse (Sant'Anna et al., 2014; Brienzo et al., 2017).

3.6. Acid pretreatment affected the surface morphology of elephant grass samples

Scanning electron microscopy (SEM) images of untreated leaf and stem revealed a smooth cell wall surface without apparent damage (Figs. 2 A and 3 A). Pretreatment with 5% sulfuric acid caused slight fiber disorganization in both leaf and stem fractions (Figs. 2 B and 3 B), and other morphological alterations were observed with increasing pretreatment severity (Figs. 2 and 3). Leaf samples subjected to 10% sulfuric acid pretreatment had a more disorganized and tumescent surface in comparison with untreated leaf (Fig. 2C). The highest concentration of sulfuric acid (20%) strongly affected leaf surface morphology (Fig. 2D).

Lignin and their fragments migrate to the biomass surface during acid pretreatment, where they are deposited in the form of spheres (Moriya et al., 2007). Also, previous studies demonstrated the presence of pseudo-lignin – a structure composed of lignin and polysaccharide degradation products – deposited on the surface of lignocellulosic materials subjected to sulfuric acid pretreatment (Hu et al., 2012). SEM images of leaf pretreated with 20% sulfuric acid showed the expected modifications for its chemical composition, with the appearance of spherical structures resembling pseudo-lignin (Supplemental Fig. 1) deposited on the cell wall surface.

SEM images showed that the stem fraction suffered less structural damage after acid pretreatment, when compared with the leaf fraction. The higher structural disorganization observed in leaf pretreated with 20% sulfuric acid is in agreement with the higher hemicellulosic fraction removal (85.02%) from this fraction, compared with that observed for other samples, and in other pretreatment conditions (Table 1). The stem fraction has different tissue/cell organization, with higher crystallinity, lignin content and probably higher density than the leaf (Table 1). These factors influence the physico-chemical properties of the material and, thus, its enzymatic digestibility (Brienzo et al., 2015). Moreover, the lignin content influences material recalcitrance, but this influence is highly dependent on the distribution and quantity of lignin in the tissue, and on the type of cells in the fraction (Bond et al., 2008; Sant'Anna et al., 2013; Brienzo et al., 2017). SEM images of the sulfuric acid pretreated leaf and stem fractions are in agreement with the positive effect of different sulfuric acid concentrations on the glucose yield

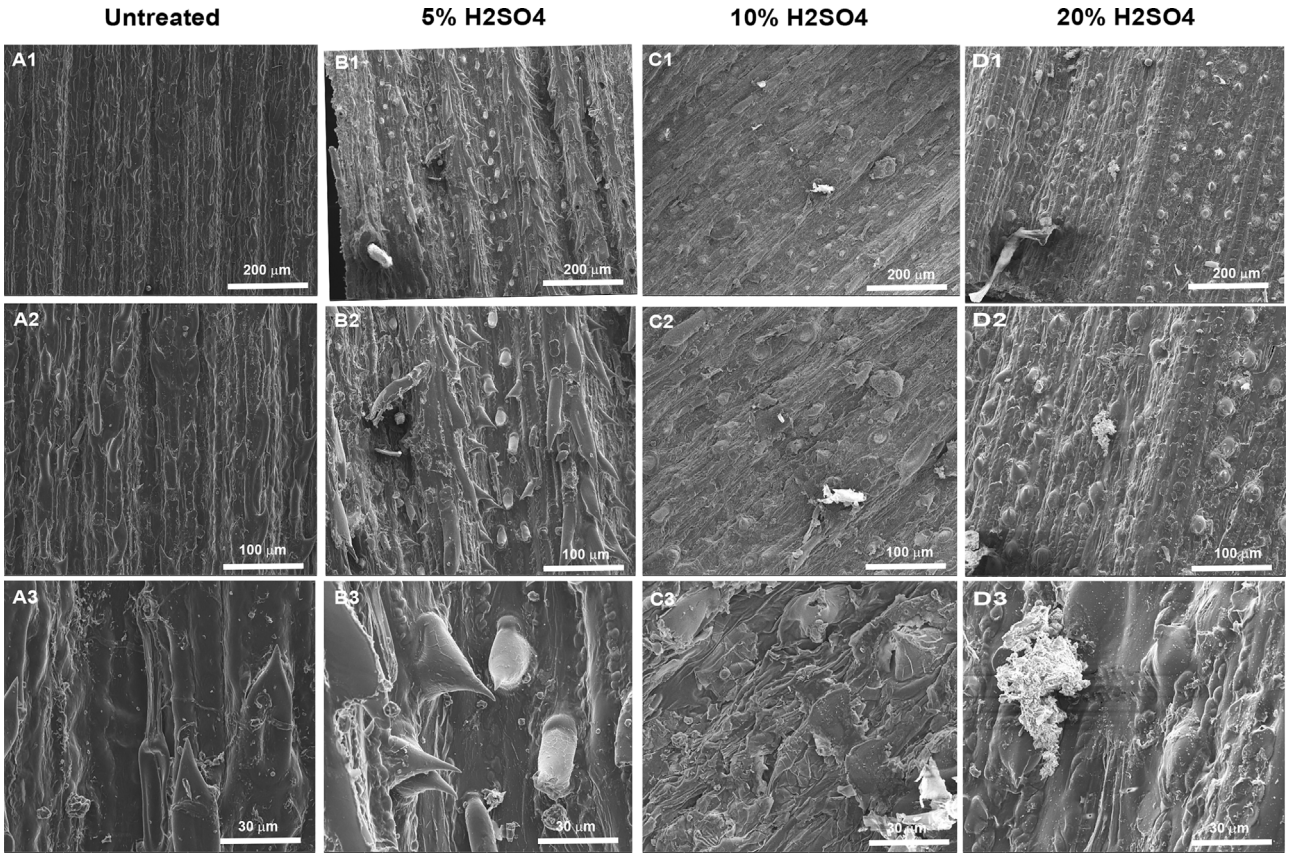

Fig. 2. Scanning electron microscopy images of elephant grass leaf left untreated (A) or pretreated with 5 (B), 10 (C) or 20% (D) sulfuric acid (H_2SO_4) (m/m; at 121 °C, for 30 min). The numbers 1–3 in panel names indicate images of the same area at increasing magnifications.

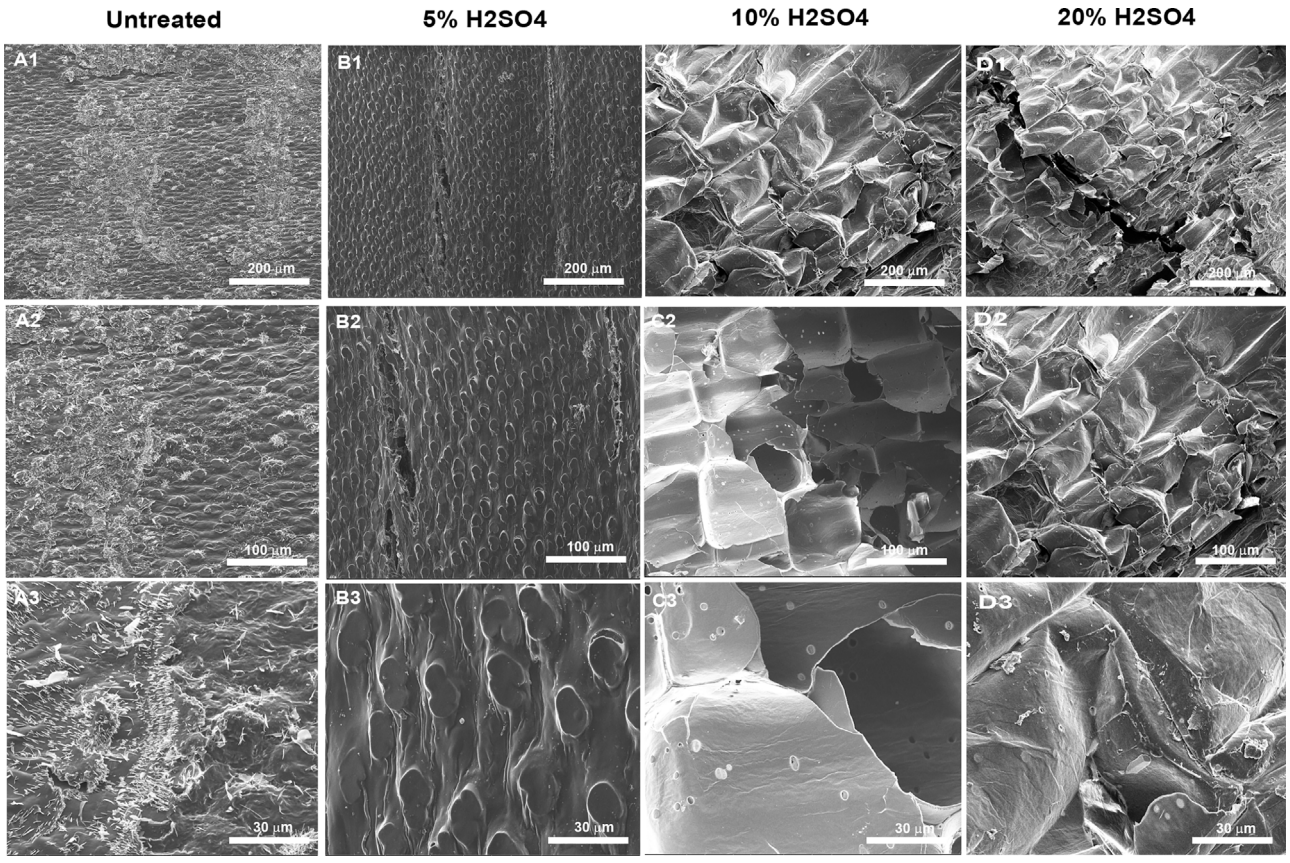

Fig. 3. Scanning electron microscopy images of elephant grass stem left untreated (A) or pretreated with 5 (B), 10 (C) or 20 (D) % (m/m) of sulfuric acid (H_2SO_4, m/m; at 121 °C, for 30 min). The numbers 1–3 in panel names indicate images of the same area at increasing magnifications.

by enzymatic hydrolysis (Fig. 1), showing that pretreatment increases the susceptibility of cellulose to enzyme action.

4. Conclusion

In this study, the potential of elephant grass fractions for ethanol production after acid pretreatment was examined. Acid pretreatment led to increased removal of solids and hemicellulose from elephant grass leaf fraction and whole plant, compared with the stem fraction. Hemicellulose removal was directly proportional to the acid concentration used in the pretreatment. The enzymatic hydrolysis yield after acid pretreatment was higher for the leaf fraction, followed by the whole plant, and the stem fraction. This effect is likely due to the complex stem organization, which is associated with higher lignin content, crystallinity and structural density. Overall, the analysis presented here highlighted the need to take into account the inherent recalcitrance of each plant fraction when evaluating pretreatment conditions, to ensure a high enzymatic hydrolysis yield for biofuel production. Moreover, the fractions could be used separately for different process: the stem, the most recalcitrant fraction, may be more useful for energy co-generation by combustion, while the leaf, which requires less severe pretreatment, is likely to be more useful in the 2G ethanol production.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.indcrop.2017.10.013.

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