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Combined approaches to obtain cellulose nanocrystals, nanofibrils and fermentable sugars from elephant grass

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ABSTRACT

This work is focused in methodologies to obtain cellulose nanocrystals, cellulose nanofibrils, soluble lignin and fermentable sugars from elephant grass, aiming a more integral use of this biomass. To improve hydrolysis, three different pretreatments for biomass delignification and bleaching were compared: 1. Concentrated alkaline peroxide (CP); 2. Diluted alkaline peroxide (DP); and 3. Acid-alkali (AA) pretreatment. Cellulose nanocrystals were obtained from elephant grass leaves by acid hydrolysis, with a 12–16% w/w yield considering the initial biomass weight, and presented high crystallinity index (CI = 72–77%) and aspect ratios (30–44), depending on the pretreatment approach. Together with the cellulose nanocrystals, other useful by-products were obtained, such as cellulose nanofibrils (3.8%–9.7% w/w yield), extractives (12.3%), liquors rich in hydrolysed lignin (16.0–21.1%) and sugars (22.1–25.2%). The results presented herewith should contribute to the economic viability and the sustainability of this biomass fractionation process.

1. Introduction

The recent interest in obtaining nanoparticles from cellulose, such as nanocrystals and nanofibrils, has increased due to the their wide range of applications (Abdul Khalil, Bhat, & Ireana Yusra, 2012; Charreau, Foresti, & Vazquez, 2013). Cellulose nanoparticles have unique and useful properties, including low density, large surface area, high stiffness and crystallinity, high aspect ratio, biocompatibility, biodegradability, and renewable origin (Siqueira, Bras, & Dufresne, 2010). Cellulose nanofibrils consist of fine and flexible fibrils (4–20 nm wide and 500–2000 nm length) produced by mechanical refining of wood or plant fibers through fibrillation techniques. They are pure cellulose structures, containing both crystalline and amorphous regions, with target applications in aerogels, thickeners and also nanocomposites (Moon, Martini, Nairn, Simonsen, & Youngblood, 2011; Abdul Khalil et al., 2012). In turn, cellulose nanocrystals are needle-shaped or whisker-shaped particles, commonly obtained from partial hydrolysis of cellulose fibers, using sulfuric acid in concentrations from 60 to 65% (w/w) and under mild heating (45–60 °C) (Siqueira, Bras et al., 2010; Moon et al., 2011). This procedure results in single and well-defined crystals dispersed in a colloidal suspension, stabilized by sulphate groups distributed on the surface (Dufresne, 2006; Teixeira et al., 2011). Prior to hydrolysis, biomass is normally pretreated to remove hemicellulose and lignin and also bleached. The pretreated cellulose matrix is formed by microfibrils containing highly crystalline domains intercepted by amorphous regions corresponding to the areas where polymer chains have spaces and kinks (Sheltami, Abdullah, Ahmad, Dufresne, & Kargarzadeh, 2012). Since the kinetics of hydrolysis is favoured in these amorphous domains, the microfibrils can be longitudinally cleaved during hydrolysis, producing nanocrystals with high crystallinity (54–84% according to X-ray diffraction data) and a Young modulus in the 50–143 GPa range, which are very suitable as reinforcing agents in polymers. In terms of dimensions, their diameter can vary from 3 to 70 nm and they are normally shorter than nanofibrils (length from 50 to 500 nm) (Abdul Khalil et al., 2014; Moon et al., 2011), though both the dimension and the crystallinity will depend on the cellulose source and on the preparation conditions, mainly on the hydrolysis time (Abdul Khalil et al., 2014; Azizi Samir, Alloin, & Dufresne, 2005). Longer hydrolysis times normally result in shorter and thinner nanocrystals.

Cellulose nanocrystals have been isolated from many kinds of plant biomasses, such as sugarcane (Teixeira et al., 2011), corn (Silvério, Flauzino Neto, Dantas, & Pasquini, 2013), rice husks (Johar, Ahmad, & Dufresne, 2012), golden grass (Siqueira, Abdillahi, Bras, & Dufresne, 2010), sisal (Teodoro et al., 2011) and cotton fibers (Morais et al., 2013). Different pretreatment technologies can be used, resulting in different morphologies and yields of nanocrystals from different biomasses.

In the case of corn cob and golden grass, a pretreatment methodology consisting of a first step with NaOH solutions 2 or 4% (w/w) at

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80 °C for 2–6 h, followed by bleaching with aqueous chlorite ($NaClO_2$ 1.7% w/w) in acetate buffer was used (Silvério et al., 2013; Siqueira, Abdillahi et al., 2010). Nanocrystals were also obtained from sugarcane bagasse after bleaching with alkaline peroxide (NaOH 5% w/w + H_2O_2 11% v/v) (Teixeira et al., 2011) at 55 °C for 90 min. Mass yields of nanocrystals obtained by these methods are typically low (lower than 15% from the raw biomass and around 50% with delignified biomasses as starting material), which reinforces the importance of using as many by-products obtained as possible.

It is also important to diversify the sources of cellulosic fibers to produce nanocrystals, because the properties of both the particles and the materials prepared with them will depend on the cellulose source. Besides this, the diversification will allow a broader range of possibilities to obtain high performance materials and value-added products from agricultural sources, thus contributing to the sustainability of this sector (Abdul Khalil et al., 2012; Silvério et al., 2013).

In this work, cellulose nanocrystals were isolated from elephant grass (*Pennisetum purpureum*), which is a perennial grass widely used as pasture in Brazil. Elephant grass is interesting for its high biomass productivity per hectare (*ca.* 35 tons/ha) and by its ability to sequester large amounts of carbon from the environment (*ca.* 15 ton of carbon/year/ha), contributing to long term storage of atmospheric carbon dioxide. In addition, it is a culture of high adaptability, with low demand for nutrients and resistance to harsh climates (Lima et al., 2014).

To the best of our knowledge, this is first work dealing with isolation of cellulose nanocrystals from elephant grass. But more than the comparison among different methods to extract nanocrystals from a neglected plant biomass, this work is focused in the identification and quantification of the useful by-products formed during process steps, including fermentable sugars, hydrolysed lignin, and solid products, such as nanofibrils. This is important to achieve an integral use of the biomass, so that none of its fractions is underutilized, and thus contributing to the environmental and economical sustainability of the biomass utilization.

The solids resulting from the various process steps were characterized as to their morphology, by field emission scanning electron microscopy (FESEM), crystallinity by x-ray diffraction (XRD) and composition by high liquid performance chromatography (HPLC) and UV–vis spectroscopy. Cellulose nanocrystals had their morphology characterized by transmission electron microscopy (TEM) and also their zeta potential determined in dispersion, which is an important parameter considering their stability and shelf life.

2. Methods

2.1. Materials

Elephant grass leaves were kindly provided by the Instituto de Zootecnia (Nova Odessa-SP, Brazil) from plants harvested with 12 months. Plant biomass was dried in a convection oven (Tecnal TE-394/3, Piracicaba-SP, Brazil) at 60 °C for 24 h, knife milled (SOLAB – SL 31, Piracicaba-SP, Brazil) until passing through a 2 mm sieve and stored in plastic bags. The reactants used in pretreatments and nanocrystal isolation (concentrated H_2SO_4 grade 98%, H_2O_2 29% (v/v) and NaOH) were all purchased from Synth (Diadema-SP, Brazil) and used as received.

2.2. Biomass pretreatments

Three pretreatment methodologies were applied to the dried and milled elephant grass leaves:

2.2.1. Concentrated alkaline peroxide (CP) in three stages

(1) Alkali treatment, (2) bleaching and (3) bleaching repetition. CP was based on previous works (Teodoro et al., 2011; Teixeira et al., 2011) with modifications, and consists of a first alkali step, where samples were treated with an aqueous solution (NaOH 5% w/v), using a 1:10 (g/mL) fiber to solution ratio, for 2 h at 70 °C, under mechanical stirring. Then, the suspension was filtered and the solid fraction was rinsed with water until reaching neutral pH, and then dried at 60 °C for 6 h. The solid was then bleached with a 1:1 solution of 4% (w/v) NaOH and 7% (v/v) H_2O_2, under manual stirring for 2 h at 60 °C and using a 1:20 (g/mL) fiber to solution ratio. Then the solids were filtered, and still wet, subjected to the bleaching step in the same conditions more one time. The solid resulting from the final bleaching step was rinsed, dried at 60 °C for 6 h and knife milled (Thomas Scientific, Swedesboro-NJ, USA) until passing through a 0.5 mm sieve.

2.2.2. Diluted alkaline peroxide (DP) in two stages

(1) Alkali treatment and (2) bleaching. DP was based on the previously described CP, but using reduced time and lower reactant concentrations. Elephant grass leaves were treated with a 2% (w/v) NaOH aqueous solution, using a 1:20 (g/mL) fiber to solution ratio, during 1 h at 121 °C and 1.05 bar in autoclave. Then, the suspension was filtered and the solid fraction was rinsed with water until neutral pH. The solid was dried at 60 °C for 6 h and knife milled until passing through a 0.5 mm sieve. In step 2, the pretreated solid was bleached with a 1:1 solution of 2% (w/v) NaOH and 2.6% (v/v) H_2O_2, using a 1:20 (g/mL) fiber to solution ratio for 2 h at 70 °C, under mechanical stirring. At the end of the process, the fibers were rinsed, dried and milled as in the first step.

2.2.3. Acid-alkali (AA) in two stages

(1) Diluted acid hydrolysis and (2) delignification. Firstly, biomass samples were treated with aqueous 1% (v/v) H_2SO_4 in autoclave at 121 °C and 1.05 bar for 1 h, using a 1:10 (g/mL) fiber to solution ratio. Then, the solid was filtered, rinsed until neutral pH and oven dried at 60 °C for 6 h. The dried fibers were then treated with 2% (w/v) NaOH in autoclave at 121 °C and 1.05 bar for 40 min, using a 1:20 (g/mL) solid to solution ratio. At the end of this step, solid samples were rinsed, dried and milled until passing through a 0.5 mm sieve. AA was based on a method previously applied in this research group to obtain cellulose enriched substrates for the production of 2G ethanol (Rezende et al., 2011).

2.3. Isolation and mass yield of cellulose nanocrystals and nanofibrils

To obtain nanocrystals, 10 g of the milled pretreated solids were hydrolysed with a 60% (w/w) H_2SO_4 solution at 45 ± 3 °C, for 20, 40 or 60 min, using a 1:30 (g/mL) solid to solution ratio. The reaction mixture was manually stirred with a glass rod for 1 min every 10 min, and was stopped by adding 400 mL of cold distilled water. The acid excess was removed from the reaction medium by a sequence of steps consisting of three successive rinsing with water and centrifugation, followed by the addition of a 2% w/v NaOH solution, until reaching pH = 5, and another two rinsing and centrifugation steps, until dispersed cellulose nanocrystals were obtained at pH 6–7. The formation of the nanocrystal dispersion can be clearly noticed by a visual observation of the system turbidity. The supernatant containing cellulose nanocrystals was then removed and transferred to another vial, being thus separated from the precipitate. The process of rinsing and centrifugation of the precipitate, followed by removal of nanocrystal dispersion was repeated until the supernatant became transparent (*ca.* 4 times). After complete removal of the nanocrystals, cellulose nanofibrils were removed from the centrifuge tube as a solid precipitate. The mass yields of cellulose nanocrystals in dispersion and nanofibrils were quantified in an infrared balance (METTLER TOLEDO MD-20, Columbus-OH, USA).

2.4. Determination of chemical composition of solid samples and liquors obtained in the process steps

Extractive removal and the quantification of cellulose, hemicellulose and lignin were performed in solid samples, as previously described (Rezende et al., 2011). The hydrolysate had its content of soluble lignin determined by absorbance measurements (280 nm), using a UV/VIS diode array spectrophotometer (model 8453, Agilent Technologies, Santa Clara-CA, USA). The hydrolysed sugars were quantitatively determined by HPLC in a chromatographer Agilent series 1200, equipped with a refractive index detector and an Aminex column (HPX-87H, 300 × 7.8 mm, Bio-Rad, Hercules-CA, USA). Analyses were carried out in duplicate at 45 °C, using a 5 mM H_2SO_4 solution as mobile phase at a 0.6 mL/min flow rate. Prior to injection, samples were filtered using Sep-Pak C18 filters (Waters, Milfords-MA, USA).

In the case of the liquors resulting from the different steps of the process, the same protocol was followed for sugar determination, but from the point where the acid is diluted to 4% (w/w) and the mixture is autoclaved. Liquors obtained from pretreatments were collected after filtering and rinsing the solid substrates (20 mL of water to 1 g of solid substrate) and liquors from hydrolysis were collected in the first rinsing/centrifugation step. The final acid concentration of 4% (w/w) was reached by adding a 60% H_2SO_4 (w/w) solution.

2.5. Transmission electron microscopy (TEM)

TEM analysis was carried out in a microscope LIBRA 120 (Carl Zeiss, Oberkochen, Germany). Dispersions of nanocrystals and nanofibrils were sonicated, deposited on 400 mesh copper grids (3 μL) previously coated with a supporting carbon and parlodium film, then stained with 2% (v/v) uranyl acetate and dried in desiccator for 2 h at room temperature prior to analysis. Nanocrystal dimensions were determined, by measuring 50 cellulose nanocrystals, using the software Zeiss Axiovision.

2.6. X-ray diffractometry (XRD)

XRD analyses were carried out using a Shimadzu diffractometer model XRD 7000 (Kyoto, Japan), with Cu target (Kα) operating at 40 kV and 30 mA. Measurements were performed at room temperature at a 2θ angular range from 5 to 60° scanned at 2°θ/min.

2.7. Zeta potential

Zeta potential of cellulose nanocrystals dispersed in deionized water at a concentration of 0.01% w/v was determined using a Zetasizer Nano-2S-ZEN 3600 equipment (Malvern Instruments, Worcestershire, UK). Prior to analysis, the dispersion was sonicated for 2 min and the measurements were performed in triplicate.

3. Results and discussion

3.1. Characterization of elephant grass substrates during pretreatment steps

3.1.1. Chemical composition of the solid substrates

Table 1 shows the mass balance of the solids obtained from elephant grass leaves after every pretreatment step, in terms of cellulose, hemicellulose, lignin and ashes (% w/w) in a dry weight basis. Cellulose content was obtained by the glucose amount quantified by HPLC, while hemicellulose was obtained by summing xylose and arabinose, always correcting by a hydrolysis factor. The lignin amount includes acid soluble and insoluble lignin and the ashes correspond to the inorganic fraction after carbonization.

Concentrations of cellulose, hemicellulose and lignin before pretreatments were 41.8, 24.7 and 28.0% w/w, respectively (Table 1). The mass closure approached 100% for all the samples, which is very good, considering the typical mass closure results obtained in biomass samples. As shown in Table 1, all the pretreatment methodologies were efficient to enrich the substrates in cellulose. Cellulose content increased to 72.5% w/w, after the treatment with concentrated peroxide (CP), to 67.5% after diluted peroxide (DP) and achieved the best results (83.5%) after the acid-alkali (AA) pretreatment.

In terms of lignin removal, which is also important to an efficient nanocrystal preparation, the three pretreatments demonstrate similar effects, decreasing the final lignin content to *ca.* 9–10% w/w. Most of the lignin in the acid-alkali pretreatment is removed in its alkali step. The first acid step in AA is actually not appropriate for lignin removal, and its percentage seems to increase to 32.4% in Table 1. But it is important to notice that this is not a real increase in lignin weight, just an apparent increase in percentage caused by the removal of other cell wall components. The acid step is very efficient into removing hemicellulose from the samples (reduction from 24.7% to 7.4% w/w), and lead to a liquor rich in hemicellulose sugars.

The high efficiency of the acid-alkali method in the removal of hemicellulose and lignin is an interesting result since this is not the traditional pretreatment used for nanocrystal production. The most common methods used for this purpose are the ones based on alkaline peroxides (Teixeira et al., 2011; Teodoro et al., 2011). Table 1 also shows that the first step of all the pretreatments is in general more effective in removing the undesirable cell wall constituents, which is consistent with the fact that it acts on the components more prone to degradation.

3.1.2. Crystallinity index (CI) and morphological analysis

Crystallinity of the solid substrates to be hydrolysed is an important parameter to cellulose nanocrystal production, together with the cellulose content. Crystallinity index (CI) values presented in Table 2 were calculated from x-ray diffraction patterns (data shown in Supplementary Material, Fig. S1), according to the method proposed by Segal and more recently revisited by Park, Baker, Himmel, Parilla and Johnson (2010).

Elephant grass leaves presented increased crystallinity index (CI) after any of the pretreatment steps, which is related to the removal of amorphous biomass constituents such as hemicellulose and lignin (Nascimento et al., 2014; Sheltami et al., 2012). AA pretreatment is the most efficient method for this purpose, increasing CI from 50% (sample *in natura)* to 70% after the pretreatment. The two alkaline peroxide pretreatments also presented significant increase in CI values that reached *ca.* 65–66%. These values agree with the compositional results (Table 1), where the substrates obtained after AA were those with higher cellulose contents and lower amounts of amorphous components, followed by CP and then DP.

The removal of hemicellulose and lignin during pretreatments were followed by morphological changes in the solid elephant grass substrates, which are described in detail in the Supplementary Material (Fig. S2). In general, after all the three pretreatment methodologies, the plant cell wall appears to be rougher and to have increased surface area. So, the higher the removal of components, the greater is the defibrillation, the surface area and the exposure of crystalline fibers of the substrate to hydrolysis.

3.2. Characterization of cellulose nanocrystals

Fig. 1 shows TEM images of cellulose nanocrystals obtained from elephant grass pretreated with CP, after hydrolysis for 40 min ($NCCP_{40}$) and 60 min ($NCCP_{60}$), and also nanocrystals from DP ($NCDP_{60}$) and AA ($NCAA_{60}$), both hydrolysed for 60 min. A shorter hydrolysis time (20 min) was also tested, but cellulose nanocrystals could not be isolated under these conditions. TEM micrographs show elongated needle-shaped nanoparticles for all the samples, with dimensions varying depending on the preparation conditions, as shown in Table 3.

After comparing the aspect ratios in $NCCP_{40}$ and $NCCP_{60}$, the

Table 1
Quantification of cellulose, hemicellulose, lignin and ashes in elephant grass leaves before (*in natura*) and after the various pretreatment steps. Values are expressed as an average ± standard deviation, in samples without extractives and in a dry weight basis.

Sample	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Ashes (%)	Total (%)
In natura[a]	41.8 ± 0.2	24.7 ± 1.0	28.0 ± 1.5	2.0 ± 0.46	96.5 ± 3.2
Concentrated alkaline peroxide (CP)					
NaOH 5%	66.9 ± 0.4	16.7 ± 0.3	12.8 ± 0.3	0.20 ± 0.05	96.6 ± 1.0
NaOH 4% + H_2O_2 7%[b]	72.5 ± 1.5	12.9 ± 0.2	9.2 ± 0.2	0.10 ± 0.01	94.7 ± 2
Diluted alkaline peroxide (DP)					
NaOH 2%	60.8 ± 0.3	19.7 ± 1.5	12.4 ± 0.8	0.68 ± 0.01	93.6 ± 2.6
NaOH 2% + H_2O_2 2.6%	67.5 ± 0.7	18.3 ± 2.0	9.9 ± 0.1	0.40 ± 0.05	96.1 ± 2.9
Acid-alkali (AA)					
H_2SO_4 1%	58.6 ± 0.7	7.4 ± 0.3	32.4 ± 0.4	2.6 ± 0.40	101 ± 1.8
NaOH 2%	83.5 ± 0.1	2.8 ± 1.1	10.5 ± 0.1	0.02 ± 0.01	96.8 ± 1.4

[a] A total of 12.3 ± 0.2% (w/w) of extractives were removed from the sample *in natura*. Pretreated samples do not contain extractives, because they were removed in the process.
[b] Step applied two times.

Table 2
Crystallinity indexes of elephant grass leaves *in natura* and after each step of the pretreatments with concentrated alkaline peroxide (CP), diluted alkaline peroxide (DP) and acid-alkali (AA).

Sample	CI (%)
In natura	50
Concentrated alkaline peroxide (CP)	
NaOH 5%	63
NaOH 4% + H_2O_2 7%	66
Diluted alkaline peroxide (DP)	
NaOH 2%	64
NaOH 2% + H_2O_2 2.6%	65
Acid-alkali (AA)	
H_2SO_4 1%	60
NaOH 2%	70

60 min hydrolysis time with 60% H_2SO_4 at 45 °C was chosen to be used in all samples. The high standard deviations in the aspect ratios indicate the polydispersity of the samples. Despite this, the tendency in the mean values is quite clear. Higher aspect ratios in nanoparticles are associated to more effective reinforcement and improved barrier properties in the nanocomposites where they are incorporated (Johar et al., 2012).

Hydrolysis yields, calculated as the weight of cellulose nanocrystals obtained from pretreated biomass starting in hydrolysis, and the total yield (considering the weight of biomass *in natura*) are also presented in Table 3. The best hydrolysis yields were obtained in $NCCP_{60}$ and $NCAA_{60}$ (52 and 53%, respectively), which can be associate to increased hydrolysis time in these samples, but also to the composition of the substrates to be hydrolysed, since samples pretreated by CP and AA are richer in cellulose than the sample resulting from DP. In terms of the final yield in nanocrystal production, which is the one that takes into account the weight loss in the pretreatments, these are also higher in samples hydrolysed for 60 min, particularly in $NCCP_{60}$ (15.5%). Considering these results, a balance between aspect ratio, total yield and pretreatment time and cost can be established. $NCCP_{60}$ are produced in higher yields but are nanocrystals with lower aspect ratios, as compared to $NCDP_{60}$ and $NCAA_{60}$. These last pretreatments are more economic in time and cost and results in similar total yields but in nanocrystals with higher aspect ratio in $NCDP_{60}$.

The aspect ratio values of the nanocrystals extracted here from elephant grass leaves are within the literature range and have thus a good reinforcing potential. The aspect ratios presented are higher than those obtained from linter cotton (19) (Morais et al., 2013) and rice husk (15–20) (Johar et al., 2012), and lower than the aspect ratio of nanocrystals isolated from golden grass (L/D = 67) (Siqueira, Abdillahi et al., 2010).

The crystallinity index (CI) was also calculated for cellulose nanocrystals based on their XRD patterns (Table 3). All the nanocrystals presented high CI values around 72–77%, showing that the methodologies used here were also efficient in this purpose. These values are consistent with those reported in literature for cellulose nanocrystals from other grasses such as sugarcane bagasse (CI = 70–87%) (Teixeira et al., 2011) and panicum (CI = 62–75%) (Martins et al., 2015).

Finally, the negative surface charge of the cellulose nanocrystals (zeta potential in Table 3) is attributed to the substitution of cellulose surface hydroxyls by sulphate groups during hydrolysis with sulfuric acid (Teixeira et al., 2011). $NCAA_{60}$ presented a higher zeta potential in modulus than $NCDP_{60}$ and $NCCP_{60}$, which is associated to higher colloidal stability in the first sample.

3.3. Characterization of cellulose nanofibrils obtained as the solid residue from hydrolysis

Acid hydrolysis promotes cell wall defibrillation by breaking the hydrogen bonds between crystalline fibers and releasing cellulose nanofibrils, as can be observed in TEM images (Fig. 2). This material was named $NFCP_{60}$, $NFDP_{60}$ and $NFAA_{60}$ for the nanofibrils produced after hydrolysis for 60 min of samples from concentrated and diluted alkaline peroxide and acid-alkali, respectively. After a few centrifugation cycles to separate dispersed nanocrystals and precipitated nanofibrils, the latter were recovered at total yields of 3.8 ± 1.1%, 9.7 ± 0.5% e 6.3 ± 0.8% (w/w), for $NFCP_{60}$, $NFDP_{60}$ and $NFAA_{60}$ respectively. The yields considering only the hydrolysis steps were 12.7 ± 3.8%, 27.9 ± 1.4% e 26.1 ± 2.8% (w/w), respectively. This method provides thus an interesting approach for combined production of nanocrystals and nanofibrils, reducing the energy costs of producing cellulose nanofibrils, which are usually obtained by mechanical processes. Though the total yield of nanofibrils obtained here is low, this amount can be certainly varied depending on the hydrolysis conditions, since they result from incomplete reactions.

These nanofibrils presented high crystallinity, with CI equal to 76, 78 and 80% for $NFCP_{60}$, $NFDP_{60}$ and $NFAA_{60}$, respectively. These values are much greater than the CI obtained in cellulose nanofibers isolated from eucalyptus pulp by sonication (33%) (Tonoli et al., 2012), and similar to the CI of flex fibers isolated by high pressure homogenization (72–77%) (Qua, Hornsby, Sharma, & Lyons, 2011). High values of crystallinity are desirable in both nanocrystals and nanofibrils, because they are associated to high stiffness and to the efficiency of reinforcement fillers in polymer matrices (Cranston et al., 2011; Maia et al., 2017).

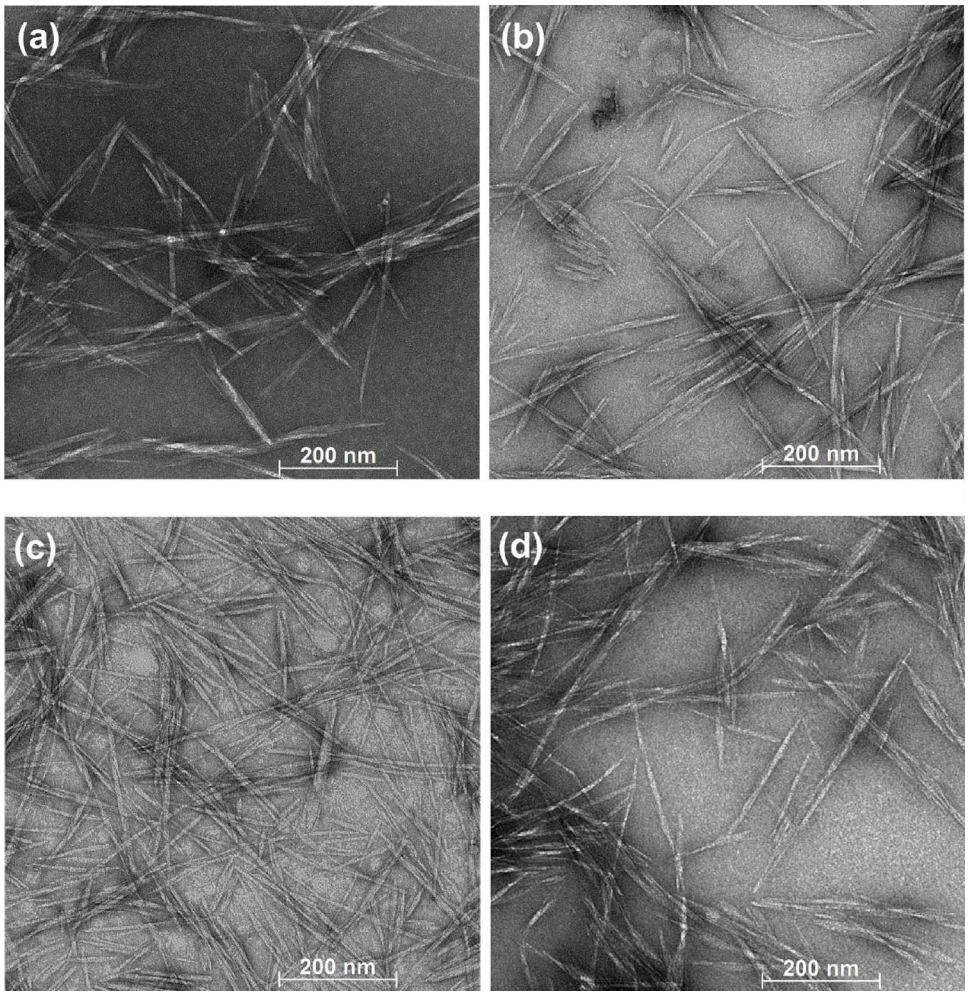

Fig. 1. Transmission electron microscopy (TEM) images of cellulose nanocrystals obtained from elephant grass samples: (a) pretreated with concentrated alkaline peroxide and hydrolysed for 40 min ($NCCP_{40}$); (b) pretreatment under the same conditions, but hydrolysed for 60 min ($NCCP_{60}$); (c) pretreated with diluted alkaline peroxide and hydrolysed for 60 min ($NCDP_{60}$) and (d) pretreated with acid-alkali and hydrolysed for 60 min ($NCAA_{60}$).

Table 3
Properties of cellulose nanocrystals obtained from elephant grass samples after pretreatment with concentrated alkaline peroxide ($NCCP_{40}$) and ($NCCP_{60}$), diluted alkaline peroxide ($NCDP_{60}$) and acid-alkali ($NCAA_{60}$). The indexes 40 or 60 indicate hydrolysis time in minutes.

Properties	$NCCP_{40}$	$NCCP_{60}$	$NCDP_{60}$	$NCAA_{60}$
Length (nm)	169 ± 54	150 ± 44	278 ± 111	167 ± 45
Diameter (nm)	8.0 ± 2.5 nm	5.0 ± 1.0	6.5 ± 2.2	5.2 ± 1.5
Aspect ratio	23 ± 10	30 ± 12	44 ± 18	34 ± 13
Hydrolysis yield (%)[a]	32 ± 3	52 ± 2	34 ± 2	53 ± 2
Crystallinity Index (%)	72	77	72	76
Zeta Potential (mV)	−47 ± 3	−39 ± 4	−47 ± 6	−50 ± 4
Total yield (%)[b]	9.6 ± 0.9	15.5 ± 0.4	11.9 ± 0.5	12.9 ± 0.4

[a] Yield with respect to delignified biomass starting hydrolysis.
[b] Yield with respect to biomass *in natura*.

3.4. Characterization of liquors obtained in pretreatments and hydrolysis

Cellulose, hemicellulose and lignin present in the solid samples are partially hydrolysed during pretreatments and hydrolysis for nanocrystal preparation. Their soluble fractions are thus present in the liquor in the form of sugar monomers and oligomers, sugar derivatives and also lignin fragments. The weight percentages of these components in terms of the initial dry weight of sample *in natura* are shown in Table 4.

Quantification of liquors showed that the hydrolysate from the first pretreatment step is mainly composed by xylose and lignin for all the pretreatments. Xylose can be converted to ethanol, but it is also an important building block to obtain chemicals, such as C3 and C6 carboxylic acids or alcohols as glycerol and sorbitol (Tuck, Pérez, Horváth, Sheldon, & Poliakoff, 2012). Lignin is the only major component in plant cell wall containing aromatic rings, which are useful in the preparation of vanillin and phenolic resins, dispersing agents and emulsifiers (Rinaldi et al., 2016; Tuck et al., 2012; Tejado, Peña, Labidi, Echeverria, & Mondragon, 2007). Other sugars and organic acids, such as arabinose, glucose, cellobiose, acetic and glucuronic acids, are present in small amounts in the liquor of the first step, but their total content do not exceed 6.3% of the initial sample weight in the alkaline peroxide pretreatments and 10.2% in the acid-alkali (Table 4). Of course, they could also be applied within the biorefinery concept to produce chemicals of commercial interest. Furfural and HMF, resulting from the decomposition of pentoses and hexoses, respectively, were not detected in most of the samples. Furfural was detected only after NaOH 5% and H_2SO_4 1% and HMF was not detected in any of the samples, which is a good result. Though HMF is a valuable precursor to chemical synthesis of levulinic acid and dimethylfuran (DMF), among others, it is also a chemical inhibitor in case of yeast fermentation (Rosatella, Simeonov, Frade, & Afonso, 2011).

The liquors from the second step in the acid-alkali pretreatment also contain important contents of lignin (11.3% w/w), and very few of other components, indicating a relatively clean lignin source for various applications. On the other hand, the liquors from the second step in CP and DP are poor in lignin, since these steps have a more bleaching function. In terms of sugar and organic acid recover, the liquors from the second step are also generally poor, and only small amounts of xylose and glucose could be detected, indicating that the first step is

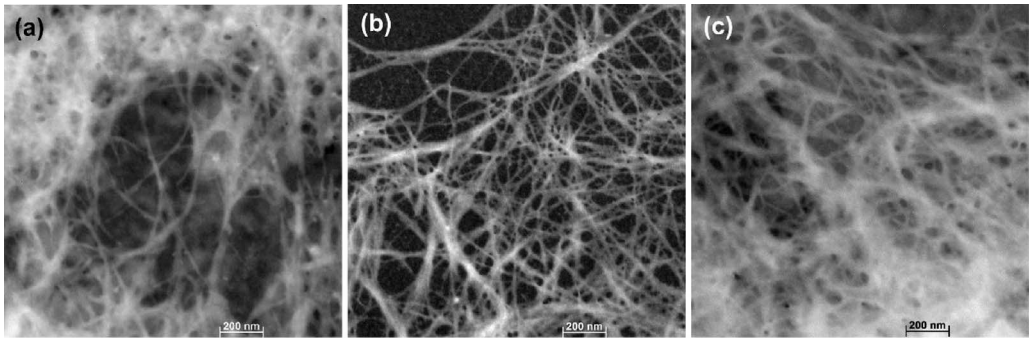

Fig. 2. TEM micrographs of cellulose nanofibrils isolated from the solid residue of hydrolysis with H_2SO_4 (60% w/w) for 60 min: (a) $NFCP_{60}$, (b) $NFDP_{60}$, (c) $NFAA_{60}$.

thus more efficient for sugar recover.

The liquors resulting from hydrolysis for nanocrystal preparation after CP and AA contain mainly glucose in their compositions, in percentages that represent 5.0% and 2.8% (w/w) of the initial dry biomass, respectively (Table 4). The liquors from $NCDP_{60}$ preparation contain also xylose (5.0%) and lignin (3.5%) together with glucose (3.1%). This is consistent with previous results presented herewith, such as solid composition (Table 1) and crystallinity (Table 2), indicating incomplete action of DP. While in the other two procedures, hydrolysis acted in substrates containing cellulosic domains mainly, in the diluted peroxide pretreatment, other components were also hydrolysed, due to the lower efficiency of this pretreatment.

3.5. Overview of the amount of nanocrystals and by-products obtained from elephant grass leaves by three pretreatment methods

Table 5 shows the weight percentages of all the components that were obtained from nanocrystal preparation using each pretreatment method. A total of 73.4, 76.2, 81.8% (w/w) of the initial dry weight of biomass could be recovered by CP, DP and AA, respectively. The remaining fraction of the biomass consists of components that could not be quantified, for instance, oligomers and other hydrolysis products.

It is important to notice that if only the cellulose nanocrystals were to be considered as products in the process, only *ca.* 11–16% (w/w) of the initial dry biomass would be useful in this work. On the other hand, Table 5 shows important weight amounts of co-products in the liquors, such as lignin (16.0–21.1% w/w), nanofibrils (3.8–9.7%), extractives (12.3%), xylose (14.1–15.2%), glucose (4.8–7.3%) and other minor sugars and organic acids derived from cellulose and hemicellulose.

Different pretreatment methodologies can be used to drive the pretreatments to desired products. For instance, as can be observed in Table 5, CP treatment yields more nanocrystals, but less nanofibrils and lignin than the others. On the other hand, DP prioritizes fibrils and AA keeps intermediary yields of nanocrystals and nanofibrils, while maximize the amount of lignin recovered in the various steps. The integral utilization of biomass in the production of fuels, chemicals and high

Table 5
Weight percentages of cellulose nanocrystals, nanofibrils and other components hydrolysed from elephant grass biomass during nanocrystal production, using three different pretreatments and acid hydrolysis for 60 min. Values are expressed as percentages of the dried biomass *in natura*.

	Products (%)	Pretreatments		
		Concentrated alkaline peroxide (CP)	Dilute alkaline peroxide (DP)	Acid-alkali (AA)
	Nanocrystals	**15.5**	**11.9**	**12.9**
	Nanofibrils	**3.8**	**9.7**	**6.3**
	Lignin	**16.0**	**18.5**	**21.1**
	Extractives	**12.3**	**12.3**	**12.3**
	Ashes	**2.0**	**2.0**	**2.0**
Hemicellulose by-products	Xylose	15.1	14.1	15.2
	Arabinose	2.8	3.2	2.2
	Acetic acid	1.6	2.8	3.6
	Glucuronic acid	0.17	0.16	0.47
	Furfural	0.1	***	2.3
	Hemicellulose total	**17.2**	**17.4**	**21.5**
Cellulose by-products	Glucose	7.3	4.8	6.3
	Cellobiose	0.00	0.04	0.02
	Cellulose total	**6.6**	**4.4**	**5.7**
	Total quantified (%)	**73.4**	**76.2**	**81.8**
	Non-quantified components (%)	26.6	23.8	18.2

Table 4
Percentages of monosaccharides (xylose, arabinose, glucose), cellobiose, organic acids (acetic and glucuronic acids), furfural and total lignin present in the liquors after each pretreatment step. Values are expressed in weight percentages of the initial dry weight of biomass *in natura*.

	Components in% (w/w) of the biomass *in natura*							
Samples	Xylose	Arabinose	Glucose	Cellobiose	Acetic acid	Glucuronic acid	Furfural	Lignin
Liquors from concentrated alkaline peroxide pretreatment (CP)								
After NaOH 5%	12.60 ± 0.30	2.24 ± 0.2	2.10 ± 0.70	[a]	1.62 ± 0.04	0.17 ± 0.05	0.10 ± 0.05	14.10 ± 0.43
After NaOH 4% + H_2O_2 7%	[a]	[a]	0.22 ± 0.03	[a]	[a]	[a]	[a]	0.72 ± 0.08
After acid hydrolysis 60 min ($NCCP_{60}$)	2.48 ± 0.04	0.51 ± 0.07	5.01 ± 0.07	[a]	[a]	[a]	[a]	1.13 ± 0.03
Liquors from diluted alkaline peroxide pretreatment (DP)								
After NaOH 2%	7.45 ± 0.20	2.16 ± 0.08	1.19 ± 0.52	0.04 ± 0.01	2.78 ± 0.01	0.16 ± 0.03	[a]	13.40 ± 0.10
NaOH 2% + H_2O_2 2.6%	1.66 ± 0.02	0.80 ± 0.40	0.49 ± 0.05	[a]	[a]	[a]	[a]	1.63 ± 0.01
After acid hydrolysis 60 min ($NCDP_{60}$)	4.96 ± 0.19	0.20 ± 0.09	3.14 ± 0.16	[a]	[a]	[a]	[a]	3.51 ± 0.05
Liquors from acid-alkali pretreatment (AA)								
After H_2SO_4 1%	14.64 ± 0.30	2.22 ± 0.34	3.14 ± 0.01	0.02 ± 0.01	2.10 ± 0.09	0.47 ± 0.02	2.27 ± 0.07	8.85 ± 0.10
After NaOH 2%	0.10 ± 0.01	[a]	0.39 ± 0.05	[a]	1.53 ± 0.07	[a]	[a]	11.3 ± 0.06
After acid hydrolysis 60 min ($NCAA_{60}$)	0.43 ± 0.15	[a]	2.78 ± 0.67	[a]	[a]	[a]	[a]	0.94 ± 0.01

[a] Components at undetectable concentration or not present.

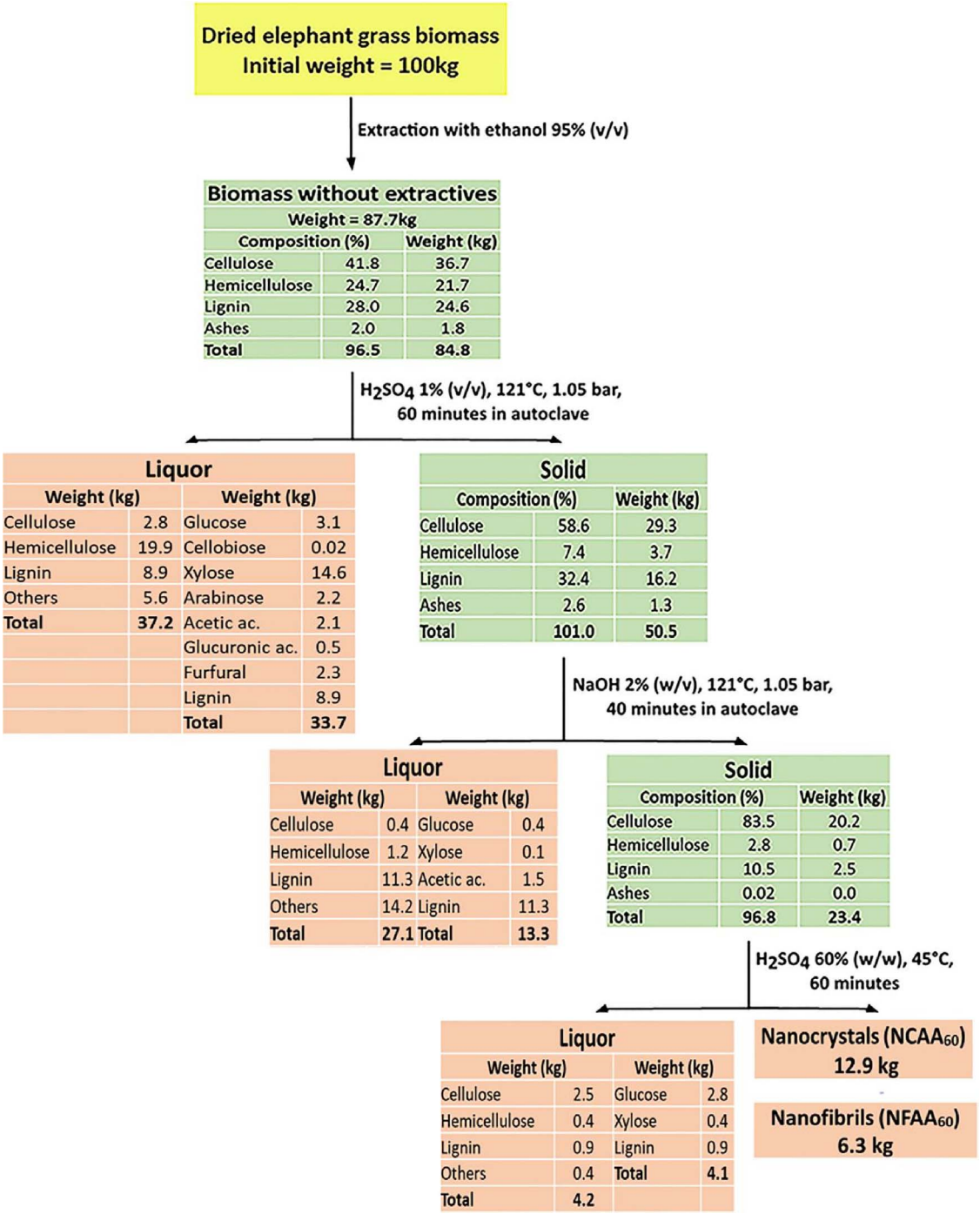

Fig. 3. Overview of the products obtained in elephant grass fractionation to nanocrystal production *via* AA pretreatment. In liquors, individual components and biomass fractions (cellulose, hemicellulose, lignin, others) are expressed in weight, while in the solids, fractions are expressed in percentage (% w/w) or weight (kg).

value-added particles is important to assure the economic viability of these processes and to minimize the environmental impacts of disposing the liquors produced in the process.

Fig. 3 shows an overview of elephant grass fractionation to produce nanocrystals *via* acid alkali pretreatment to illustrate the weight (in kg) that can be obtained from different components in each step in a biorefinery approach. For every fraction (liquor or solid) the composition is expressed in weight of components and in percentage (%w/w) of the initial dried biomass. Liquor composition in terms of cellulose and hemicellulose were obtained from the components detailed on the right, corrected by hydrolysis factors. Similar plots were built to CP and DP pretreatments and are presented in supplementary material (Figs. S3 and S4).

4. Conclusion

The results presented herewith showed that elephant grass is a promising biomass source to produce cellulose nanocrystals with adequate properties, such as high aspect ratio (30–44) and crystallinity indexes (72–77%), to be applied in the preparation of polymer nanocomposites. With three pretreatment methodologies, nanocrystals were obtained with yields between 12–16% (w/w), considering the initial weight of dried elephant grass biomass. Besides, the detailed quantification of the process by-products showed that up to 82% of the starting

biomass could be profitable, in the form of nanofibrils, hydrolysed lignin, extractives, sugars and organic acids, contributing to the process economic viability and sustainability and to the integral use of this biomass.

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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.carbpol.2017.09.099.

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