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Biorefinery on-demand: Modulating pretreatments to recover lignin, hemicellulose, and extractives as co-products during ethanol production

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ABSTRACT

In this work, we designed three scenarios to obtain ethanol as the main product from an elephant grass biorefinery and maximize the recovery of extractives, hemicellulose, and lignin. Common pretreatment methodologies, such as diluted acid and alkaline treatments, ball milling and pressurized liquid extractions (PLE) were combined to provide a detailed quantification of the products obtained in each step, enabling an assertive choice for further decision-making depending on the demand for specific products. Scenario 1 was based on an alkaline treatment at mild conditions (85 °C for 20 min, followed by ball milling) and stood out as the most efficient setup for ethanol production (up to 100 kg of ethanol/ton biomass). Scenario 2 was elaborated by adding a diluted acid step prior to the alkaline treatment, enabling the production of up to 77 kg of ethanol/ton biomass, together with the best fractionation results (up to 88 % of biomass use against the 28 % reported in an analogous process, but disregarding the co-products). Finally, the third scenario illustrates how the use of PLE combined with the alkaline treatment could be a clean alternative to recover biomass extractives (37–46 %) as well as produce up to 74 kg of ethanol/ton biomass. Therefore, this study could contribute to the development of greener and more sustainable biorefineries by properly forwarding their normally discarded residues and biomass components to high added-value applications.

1. Introduction

Lignocellulosic biomass is a promising substrate to replace non-renewable raw-materials in the production of several chemical products, mainly due to its rich chemical structure composed by cellulose, hemicellulose, lignin, extractives, and inorganic components (Ragauskas, 2006). Due to the importance of this field, several works are proposing and comparing different physicochemical treatments to fractionate a variety of lignocellulosic biomasses (Camargos et al., 2019; Dávila et al., 2019; Xu et al., 2020). Pretreatment effects had been reported on biomass chemical composition, morphology, and availability to enzymatic hydrolysis (Liu et al., 2020; Rezende et al., 2011; Tsai et al., 2018).

The majority of published works on biomass pretreatments are focused on a single target product derived from either carbohydrates or lignin (Ferreira et al., 2020; Gürbüz et al., 2013; Wang et al., 2018). By neglecting the co-products obtained in parallel, this single-product approach makes the process economically unfeasible and disadvantageous when compared to petroleum derivatives (Alonso et al., 2017). The advance towards greener, cost-effective, and sustainable processes to obtain bio-based products depends on integrated methodologies to optimize the extraction of biomass compounds, minimize wastes and save energy, through an approach termed biorefinery (Attard et al., 2020; Rosales-Calderon and Arantes, 2019; Ubando et al., 2020).

Biorefineries are multi-output systems that convert biological feedstocks into a spectrum of bio-based products (*e.g.* fuels, chemicals, and electricity), using integrated processes (Cherubini, 2010; Jorissen et al., 2020). The term biorefinery is commonly used to designate several types of industrial plants, including those that process sugar, starch, oil, fat, wood, aquatic biomass, agricultural residues and food waste to produce pulp, sugar, bioethanol, biodiesel, oil, textiles, chemicals, *etc.* (Jorissen et al., 2020). Amongst feedstocks that can be processed in biorefineries, non-wood lignocellulosics, such as forage crops and agricultural residues, are particularly beneficial due to their low price, availability, and the possibility to enhance land use, *e.g.* nutrient-poor soils (Badgujar and Bhanage, 2018; Lima et al., 2014). However, a recent study showed that only 5 out of 224 biorefineries in Europe use non-wood lignocellulose as feedstocks (Jorissen et al., 2020), confirming that the implementation of

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real biorefineries remains a challenge and that second-generation biorefineries based on lignocellulose processing have plenty of potential for further developments.

Likewise, the so-called biorefineries currently in operation do not encompass the production of a variety of co-products in most cases but are limited to two or three of them. For biorefineries based on agricultural wastes and forage crops, most of the applications are targeting the co-production of ethanol and electricity (Jorissen et al., 2020; Rabelo et al., 2011); glucose and hemicellulose derivates, such as xylose and xylooligosaccharides (Bhatia et al., 2020; Padilla-Rascón et al., 2020); or organic acids, such as lactic and succinic acids, together with electricity (Alves et al., 2017; Jorissen et al., 2020; Klein et al., 2017). Numerous works were also dedicated to techno-economic analysis in biorefineries, often focusing on the integration of first and second-generation processes to produce bioethanol and recover bioenergy (Dias et al., 2013; Larnaudie et al., 2019; Vasconcelos et al., 2020).

Contributions exploring a more complete fractionation of biomass also covered a restricted number of non-wood biomasses, such as bamboo (Xu et al., 2020), corn stover (Yu et al., 2020), elephant grass (Nascimento and Rezende, 2018), and wheat straw (Xu et al., 2019). An interesting example is the biorefinery proposed by Yu et al., in which corn stover was fractionated by a treatment with *p*-toluenesulfonic acid and produced glucose from cellulose, levulinic acid from hemicellulose, and lignin nanoparticles (Yu et al., 2020). Similarly, Xu et al. applied a hydrothermal treatment onto wheat straw to obtain a liquid fraction composed of xylooligosaccharides (54 % yield) and a solid fraction composed of cellulose and lignin. By enzymatic hydrolysis, cellulose was converted into glucose (89 % yield) and the lignin remaining in the solid fraction was used to produce lithium-sulfur cathodes through carbonization (Xu et al., 2019).

Coupling the recovery of co-products in a biorefinery with the production of second-generation ethanol is an interesting strategy since the treatments acting on the improvement of biomass enzymatic digestibility can be also used to obtain such co-products (Ragauskas, 2006). The interest in 2G ethanol production is mainly assigned to the reduced emission of greenhouse gases in comparison to gasoline (62 % lower) (Wang et al., 2012), and the well-established market for its 1G version (obtained from the fermentation of either sucrose or starch) in the transportation sector of several countries, such as Brazil and USA. The current production of bioethanol in Brazil is estimated at around 32 billion litters (2018/2019) (CONAB, Conselho Nacional de Abastecimento, 2018) and the production of 2G ethanol from structural carbohydrates can augment these values. However, the major bottleneck for large-scale production of 2G ethanol is the cost of the entire process (Rosales-Calderon and Arantes, 2019), a problem that could be overcome by co-products processing and their valorization (Jorissen et al., 2020).

Elephant grass (*Pennisetum purpureum*) is a promising biomass to be used in a biorefinery due to its high productivity (*ca.* 45 ton/ha/year), chemical composition, and high adaptability to diverse soils and climates (Menegol et al., 2017). This forage crop has been recently studied for the production of 2G ethanol (Dien et al., 2020; Rezende et al., 2018; Scholl et al., 2015), cellulose nanoparticles (Nascimento and Rezende, 2018), lignin nanoparticles (Trevisan and Rezende, 2020), and extractives (Scopel et al., 2020). Aiming to improve the enzymatic digestibility of elephant grass to ethanol production, acid-alkali treatments were previously optimized by our research group for this biomass, using a fractional factorial experimental design (Rezende et al., 2018). Based on the earlier results, the acid-alkali methodology was adopted as the main process in the biorefinery routes presented in the present work. Another advantage of these treatments is that they are already industrially used, which facilitates future scaled-up processing (Lorenci Woiciechowski et al., 2020). Compared to Kraft and organosolv pretreatments, the methodologies using diluted acid-alkali are considered less aggressive to the biomass compounds, which reduces the formation of inhibitory compounds, and can be performed under lower temperatures and in shorter times (Lorenci Woiciechowski et al., 2020).

Prior optimization of the acid-alkali pretreatment on elephant grass also indicated that the use of a ball milling step contributed to improvement of sugar release, which is noticeable even under a 10 % reduction of biomass crystallinity (Rezende et al., 2018). Generally, enzymatic hydrolysis is facilitated in amorphous cellulose, so ball milling can be combined with other chemical treatments, aiming at different mechanisms, to improve sugar release (Gu et al., 2018; Rezende et al., 2018).

Another important but often neglected issue in the implementation of biorefineries is the use of extractives, since this is a biomass fraction composed of high value-added organic molecules (Attard et al., 2020). Recently we investigated the use of green extractions, through supercritical fluids (SFE) and pressurized liquids (PLE), to recover high value-added molecules from elephant grass (Scopel et al., 2020). PLE presented higher extraction yields in comparison to SFE and lower demands for energy and time than conventional extraction methods, such as Soxhlet. PLE, as a stand-alone step, did not hinder the enzymatic hydrolysis, which enables the use of sequential treatments for this approach and empowers the biorefinery to extract sterols, phenols, and alcohols prior to the biomass fractionation and enzymatic conversion into fermentable sugars.

According to previously published studies on elephant grass, we carried out a systematic analysis assessing the co-products that can be obtained during the processing of this biomass to produce ethanol in three different scenarios, including combinations of PLE, acid-alkali pretreatments, and ball milling. A detailed quantification of the main product (ethanol) and of co-products (cellulose, hemicellulose, glucose, xylose, arabinose, and extractives) after different processing steps was performed for leaves and stems separately. This approach enables the implementation of an on-demand biorefinery for the complete biomass use, where the most advantageous routes can be selected at a given moment, bearing in mind both price and demand for specific products as well as energy inputs and time required.

2. Material and methods

2.1. Materials

Elephant grass (*Pennisetum purpureum*) plants at 12-month-old were donated by the Institute of Animal Science (Instituto de Zootecnia, Nova Odessa, SP, Brazil). Samples were separated into leaves and stems, and then dried in a convection oven (Tecnal, TE-394/3) at 60 °C for 6 h. Finally, leaves and stems were separately milled in a knife mill (Arthur H. Thomas Co – *Standard model* 3) until passing through a 2 mm sieve. NaOH P.A. and ethanol (99.5 % purity) were purchased from Synth®; and H_2SO_4 (98 % purity) was acquired from LSChemicals. All reactants were used as received.

2.2. Chemical treatments and extractions

A diluted acid treatment was applied directly to *in natura* biomass using H_2SO_4 2 % v/v at 121 °C for 40 min in an autoclave (AV-75 Phoenix), at a 1:10 solid:liquid ratio (Rezende et al., 2018) (samples identified as "Ac"). Alternatively, PLE was applied to *in natura* biomass using a mix of ethanol and water (1:1 v/v) in an accelerated solvent extractor (Dionex ASE 350, Thermo Scientific, Waltham, MA) (Scopel et al., 2020). Extractions were performed in 3 cycles of 15 min at 100 °C, using 5 min of preheating and 120 s to purge the solvent (samples identified as PLE). The extracts were collected, and their detailed compositions were previously reported (Scopel et al., 2020).

Diluted alkaline treatments were applied directly to *in natura* biomass, or to samples previously treated by either acid or PLE steps. Alkaline chemical treatments were performed according to previously optimized conditions (Rezende et al., 2018), using an aqueous NaOH solution 4.5 % w/v at 85 °C in a 1:10 solid:liquid ratio. Two different

reaction times were tested (20 and 100 min) and the alkaline steps are identified as Alk20 or Alk100.

Pretreated solids were separated from the liquid fraction after each step by filtration. Then, samples were rinsed using 10 mL of tap water per g of solid, except for PLE samples, which had left the extraction unit already separated. Pretreatment liquor and this first rinsing water were collected and stored for characterization, while the filtered solid was further rinsed until reaching neutral pH, and then oven-dried at 60 °C for 6 h.

2.3. Physical treatments: ball milling

Ball milling was carried out in a planetary ball mill (MTI Corporation, SFM-1 Desk-Top), using alumina jars and balls of zirconium oxide. The following optimized ball milling conditions were used to achieve improved biomass amorphization: 10 g of pretreated biomass was added into an alumina jar together with 12 balls of zirconium oxide of 20 mm and milled for 2 h at 250 rpm. These conditions were determined by a screening of the experimental conditions using an L_9 orthogonal design of experiments (DOE) (Supplementary Material, Table S1).

2.4. Enzymatic hydrolysis

All samples underwent enzymatic hydrolysis, using the cocktail Cellic® CTec2 (Novozymes), at 25 mg/g substrate (approximately 20 FPU/g substrate) and 2.5 % solid to liquid ratio in citrate buffer (50 mmol.L^{-1}, pH = 5). The system was kept at 50 °C for 72 h at 150 rpm, then the enzymes were denatured by heating at 95 °C for 5 min. The liquid fraction was collected to determine the amount of glucose, xylose, and arabinose released during hydrolysis.

2.5. Fermentation

Fermentation was carried out with *Saccharomyces cerevisiae* (Santa Adélia) in tubes of 15 mL, using the samples with the higher enzymatic hydrolysis yields. The assay was performed at 30 °C and 150 rpm in an orbital shaker, and aliquots of the fermented liquor were collected after 6, 12, 24, and 48 h of fermentation.

2.6. Characterizations

2.6.1. Chemical composition

Chemical compositions of solid and liquid fractions were determined according to the protocol of the National Renewable Energy Laboratory (NREL) (Sluiter and Hames, 2008). Briefly, solid fractions were hydrolyzed using H_2SO_4 72 wt.% at 30 °C, followed by a hydrolysis with H_2SO_4 4% wt.% at 121 °C in an autoclave, while liquid fractions were submitted to the second hydrolysis step only in autoclave. After cooling to room temperature, samples were filtered in a porous-bottom crucible. The filtrate was used to quantify cellulose and hemicellulose sugars by high-performance liquid chromatography (HPLC) (Agilent 1200) and acid-soluble lignin by UV–vis spectroscopy (Agilent, Cary 5000); the residue was utilized to quantify acid-insoluble lignin by calcination in an oven at 800 °C. Concomitantly, extractives from *in natura* samples were quantified by Soxhlet extraction, as previously described (Scopel et al., 2020); whilst ashes were quantified by calcination in an oven at 800 °C.

2.6.2. Sugar quantification

Glucose, xylose, and arabinose from chemical composition or fermentation were measured by HPLC (Agilent 1200) coupled with a refractive index detector. A BIORAD HPX87H column was utilized at 45 °C, using H_2SO_4 5 mmol.L^{-1} aqueous solution as mobile phase.

2.6.3. Crystallinity index

The crystallinity index was analyzed according to height peak method by X-ray diffraction (XRD) (Park et al., 2010) using a Shimadzu XRD7000 (2θ from 5 to 35°, speed of 2°/min and step of 0.02° operating at 40 kV and 30 mA). More details are discussed in Supplementary Material (Figure S1).

3. Results and discussion

3.1. Presenting the three scenarios

Fig. 1 summarizes the three scenarios proposed for an elephant grass biorefinery in this study. Scenario 1 was based on an alkaline step and mainly designed to enhance ethanol production, but also to evaluate the co-products that could be recovered from the alkaline liquid fraction, especially lignin and hemicellulose at smaller quantities. Scenario 2 had two steps and was focused on a more effective extraction of hemicellulose in the acid treatment as well as on obtaining a purer lignin from the alkaline liquid fraction in the second step. Due to a higher extraction of non-cellulosic compounds achieved by the sequential acid-alkali treatment, the remaining solid obtained in this route was significantly rich in cellulose. Scenario 3 was also based on an alkaline pretreatment (similar to Scenario 1), but included a previous step with pressurized liquid extraction (PLE), which stands out as a greener technology to recover the biomass extractives, as previously reported (Scopel et al., 2020). PLE did not hinder the enzymatic hydrolysis of elephant grass and the combination with alkaline treatment enabled the recovery of cellulose to be fermented, lignin, and hemicellulose (just as in Scenario 1), with an extra amount of profitable extractives obtained by a clean methodology.

All the alkaline treatments performed herein were based on a previous optimization designed for enhancing the enzymatic hydrolysis in elephant grass (Rezende et al., 2018). In each scenario, we compared alkaline reactions of 20 and 100 min since the interactions of time with other reaction factors, such as the temperature of the alkaline treatment and NaOH concentration, were considered relevant in previous studies. It is also important to highlight that the temperature used in the alkaline steps (85 °C) is lower than those typically reported for this pretreatment in literature, which is usually above 100 °C (Lorenci Woiciechowski et al., 2020). Low reaction temperatures are important to avoid the formation of degrading products during the pretreatments (such as HMF and furfural in acid media), which were analyzed in this work, but not found in detectable amounts.

Finally, treatments were performed separately for leaves and stems in all scenarios enabling that two parts of biomass could be compared. Whether desired, these parts could be processed together in future applications. Both chemical composition and morphological properties of all samples will be discussed in the next sections to determine the treatment effects on enzymatic digestibility, biomass morphology, and recovery of generated co-products (extractives, hemicellulose, and lignin).

3.2. Chemical composition and morphology of samples

Sample composition in terms of cellulose, hemicellulose, lignin, and ash contents, as well as the remaining mass for all the solids after treatments, are shown in Table 1. Prior to chemical treatments, leaves and stems had similar contents of hemicellulose and lignin, ranging from 21 to 25 %. On the other hand, the cellulose content was 28 % higher in stems, whilst the contents of extractives and ashes were higher in leaves (42 % and 78 %, respectively). Despite the different environmental factors, such as climatic changes and planting/harvest methods, these values are in accordance with other studies that quantified cellulose (32–42 %), hemicellulose (20–25 %), lignin (14–28 %), extractives (8–16 %) and ashes (2–12 %) in elephant grass (Menegol et al., 2014a; Montipó et al., 2018; Nascimento and Rezende, 2018; Santos et al., 2018).

Generally, after chemical treatments, both leaves and stems usually denote similar chemical composition when submitted to equivalent treatments. In this study, all the tested conditions led to an increase in

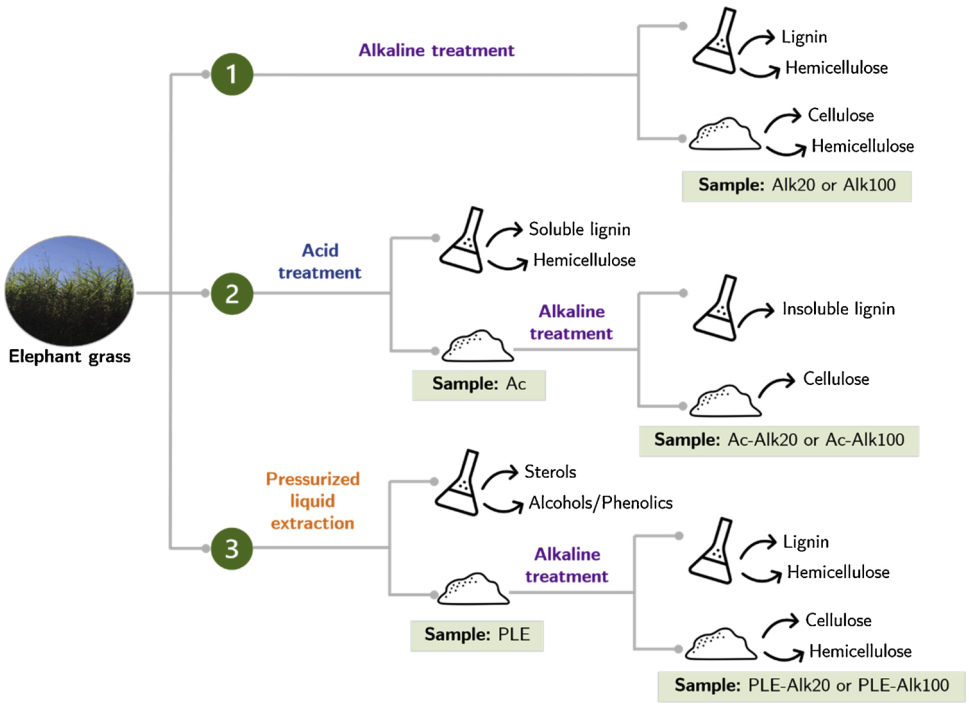

Fig. 1. Summary of the three scenarios proposed for an elephant grass biorefinery using dilute acid, alkaline treatments and pressurized liquid extractions to fractionate the biomass compounds. Sample codes are indicated in each case.

Table 1
Chemical composition and the remaining mass of solids after treatments. Analyses were performed in duplicate and the values are represented as averages ± standard deviation. Total for *in natura* leaves and stems includes extractives.

Sample	Compound (% w/w)					Remaining mass (%)
	Cellulose	Hemicellulose	Lignin	Ashes	Total	
In natura leaves*	30.1 ± 2.2	24.3 ± 2.1	22.5 ± 0.1	5.7 ± 0.1	102.7 ± 3.1	100
Scenario 1						
Alk20	54.7 ± 1.5	24.3 ± 1.5	12.9 ± 1.5	6.0 ± 0.1	102.6 ± 2.1	55.7 ± 0.1
Alk100	63.0 ± 0.7	21.5 ± 0.6	12.5 ± 1.5	6.3 ± 0.2	100.6 ± 1.8	46.3 ± 0.1
Scenario 2						
Acid	54.2 ± 1.5	6.9 ± 0.2	29.0 ± 0.8	7.0 ± 0.2	97.1 ± 1.7	49.1 ± 2.9
Ac-Alk20	73.4 ± 0.4	6.1 ± 0.8	14.0 ± 0.4	3.0 ± 0.2	96.5 ± 1.0	30.1 ± 2.9
Ac-Alk100	82.9 ± 2.3	4.0 ± 1.1	12.3 ± 0.1	1.4 ± 0.1	100.6 ± 2.6	28.1 ± 0.7
Scenario 3						
PLE-Alk20	55.6 ± 0.3	25.6 ± 1.0	13.7 ± 0.3	3.7 ± 0.1	98.6 ± 1.1	45.6 ± 0.3
PLE-Alk100	57.0 ± 2.4	19.5 ± 0.2	16.1 ± 0.2	4.4 ± 0.2	96.9 ± 2.4	39.2 ± 4.1
In natura stems **	38.6 ± 0.5	21.8 ± 0.7	25.5 ± 1.9	3.2 ± 0.1	103.2 ± 2.1	100
Scenario 1						
Alk20	58.0 ± 0.2	23.2 ± 0.2	17.5 ± 0.2	2.8 ± 0.1	101.6 ± 2.1	65.8 ± 4.1
Alk100	62.7 ± 0.1	20.0 ± 0.1	18.2 ± 0.2	2.2 ± 0.1	103.2 ± 0.2	58.7 ± 1.6
Scenario 2						
Acid	58.2 ± 0.2	7.8 ± 0.2	32.8 ± 4.1	1.3 ± 0.2	100.1 ± 4.1	61.0 ± 0.6
Ac-Alk20	71.9 ± 2.1	5.7 ± 0.3	22.3 ± 1.0	1.3 ± 0.2	101.2 ± 2.4	46.0 ± 2.2
Ac-Alk100	79.0 ± 2.1	3.6 ± 0.1	19.1 ± 0.1	1.0 ± 0.1	102.7 ± 2.1	41.6 ± 0.4
Scenario 3						
PLE-Alk20	60.3 ± 1.1	25.6 ± 0.3	13.5 ± 3.0	0.4 ± 0.1	99.8 ± 3.2	59.5 ± 0.6
PLE-Alk100	63.9 ± 1.3	18.9 ± 0.2	12.8 ± 4.1	1.2 ± 0.1	96.8 ± 4.3	54.1 ± 4.2

* Extractives from leaves: 20.2 ± 0.2 %.
** Extractives from stems: 14.2 ± 0.6 %.

the cellulose content of solids between 1.5 and 2.8-fold, reaching maximum cellulose contents (82.9 % in leaves and 79.0 % in stems) after the sequential treatments with acid-alkali solutions (sample Ac-Alk100 in Scenario 2, Table 1).

The remaining mass of solids obtained after the chemical treatments was systematically lower in leaves than in stems (Table 1), which indicates a higher mass transference to the liquid fraction from leaves. This effect is more expressive in Scenario 2, in which the single acid step extracted 50 % of the mass of leaves to the liquid fraction, while 39 % of stems were extracted in the same treatment. After the sequential alkaline treatment, the remaining mass was *ca.* 30 and 28 % in leaves and about 40 % in stems. The different profiles in leaves and stems are probably related to the higher content of cellulose from *in natura* stems. As the chemical treatments mainly act on solubilizing hemicellulose and lignin, cellulose was mostly preserved in these treatments, standing out as a significant result to use solid cellulose in ethanol production.

To facilitate the discussion, Fig. 2 exhibits the percentages of specific components remaining in the solid fraction after various treatments, which were calculated using both compositions and residual masses presented in Table 1. As observed in the diverse samples, treatments were effective to remove hemicellulose, lignin, and ashes from the substrates with a minimum loss of cellulose. In stems, cellulose removal was lower than 15 % in all pretreatments (Fig. 2a), while the maximum cellulose loss was around 25 % for leaves (Fig. 2b). These values are similar to cellulose losses noticed when acid-alkaline treatments were applied in sugarcane bagasse (Rezende et al., 2011; Rocha et al., 2011) and Napier grass (Tsai et al., 2018). A reduced loss of cellulose in pretreatments is ideal to produce 2G ethanol since the solid fraction will be further hydrolyzed and fermented. However, removing lignin and hemicellulose without losing a fraction of cellulose is still a challenge due to the intrinsic network configuration of plant cell wall (Rezende et al., 2011).

Scenario 2 (with 2 steps) was the one leading to higher cellulose removal in both substrates, a result that underlines the need for additional evaluation of this pretreatment. Although the samples from Scenario 2 yielded cellulose-rich solids (Table 1), these samples also showed the highest cellulose extraction for the liquid fraction (Fig. 2), which can compromise the final ethanol yields. In contrast, samples with lower cellulose content (Alk20 and Alk100 in Scenario 1) had almost no loss of cellulose mass.

It is important to highlight that the cellulose contents obtained after 20 or 100 min of treatment are similar regarding the remaining mass (Fig. 2), thus allowing the selection of significantly short reaction times (Yang et al., 2016). The previous optimization of the alkali treatment applied to elephant grass demonstrated that enzymatic digestibility could be significantly enhanced when using mild temperatures (85 °C), but the more appropriate conditions for sugar release (205 mg/g substrate) were achieved when the treatment was used for 100 min (Rezende et al., 2018). At the present work, we showed that 85 °C was also sufficient to obtain solids with elevated content of cellulose (72–73 %) even when the reaction lasted only 20 min. In another work, where elephant grass underwent a more severe acid-alkali pretreatment (121 °C for 1 h in the alkali step), a higher cellulose content was achieved (83.5 %) (Nascimento and Rezende, 2018), though it is important to take into account the considerably higher demand for time and energy. Furthermore, other treatments applied to elephant grass, such as those that use only H_2SO_4 (20 % w/w for 30 min at 121 °C), resulted in a cellulose content of 40 % (Santos et al., 2018), while steam explosion (190 °C for 8 min) yielded *ca.* 58 % of cellulose content (Scholl et al., 2015). Both values are similar but inferior to those obtained here for alkaline treatments alone (between 54 and 63 % in Scenario 1, Table 1) or acid step alone (54–58 % in Scenario 2, Table 1).

The final lignin content in solid samples was 12–16 % for leaves and 12–22 % for stems (Table 1), which was similar to other treatments using acid-alkali extractions in elephant grass or other biomasses (da Silva et al., 2018; Nascimento and Rezende, 2018; Rezende et al., 2011; Santos et al., 2018). Lignin removal took place mainly in the alkaline pretreatment, since hydroxyl groups are known to act on the breakage of both lignin native network and the covalent bonds between cellulose and lignin (Jones et al., 2017). Since the presence of lignin in the substrate is one of the main hurdles to the enzymatic action in cellulosic substrates (Vermaas et al., 2015), its removal in pretreatments is crucial.

Fig. 2 shows that even short pretreatment times, such as the ones applied in sample Alk20 (20 min), are able to remove more than 70 % of the initial amount of lignin from leaves and 50 % from stems under alkaline conditions (NaOH 4.5 % w/v, 85 °C). In the most efficient procedure (Ac-Alk100, in Scenario 2), up to 85 % of lignin was removed from leaves, with removal rates around 35 % during the acid step and around 50 % in the alkali one. Even though acid-alkaline treatments in two-steps are the most efficient method, exclusive alkaline steps could also result in excellent lignin removal, especially from stems, where the remaining fractions of lignin were comparable among all samples that have undergone the alkaline step. This result should be considered in cases where reducing the number of pretreatment steps is important.

Hemicellulose removal was also achieved from both leaves and stems. Final remaining fractions varied considerably (5–70 %) amongst different pretreatments. The acid step in Scenario 2 was crucial to extract hemicellulose (Fig. 2), reducing up to 85 % of this polysaccharide in the solid biomass by its solubilization in oligomers and monomers (mainly xylose and arabinose for elephant grass) (Rezende et al., 2018). These extraction yields are similar to those obtained by alkaline extraction of hemicellulose from switchgrass (*ca.* 80 %) (Farhat et al., 2017) and by steam explosion of slash pine sawdust (*ca.* 90 %) (Stoffel et al., 2017). The acid step applied in Scenario 2 was, therefore, decisive to hemicellulose extraction and its further recovery, playing also an important role on facilitating the obtainment of a liquor richer in lignin during the following alkaline step performed in Scenario 2. Nevertheless, the hemicellulose that remains in the solid fraction (mostly in Scenarios 1 and 3) can be hydrolyzed to produce fermentable sugars by using appropriate enzymatic cocktails, which will contribute to increasing the total fermentable sugars recovered from the biomass.

In all scenarios, hemicellulose was extracted together with a lignin fraction to the reaction liquors, so further separation steps were required to use them individually. However, the purification of lignin from the alkaline liquid fraction is facilitated when a first acid step is performed, as proposed in Scenario 2 (Trevisan and Rezende, 2020). Moreover, the recovery of purer hemicellulose may be possible from the acid liquid fraction due to a relatively lower amount of extracted lignin (*ca.* 40 % and 20 % of lignin from leaves and stems, respectively). In the absence of an acid step, the hemicellulose derivatives must be separated from the

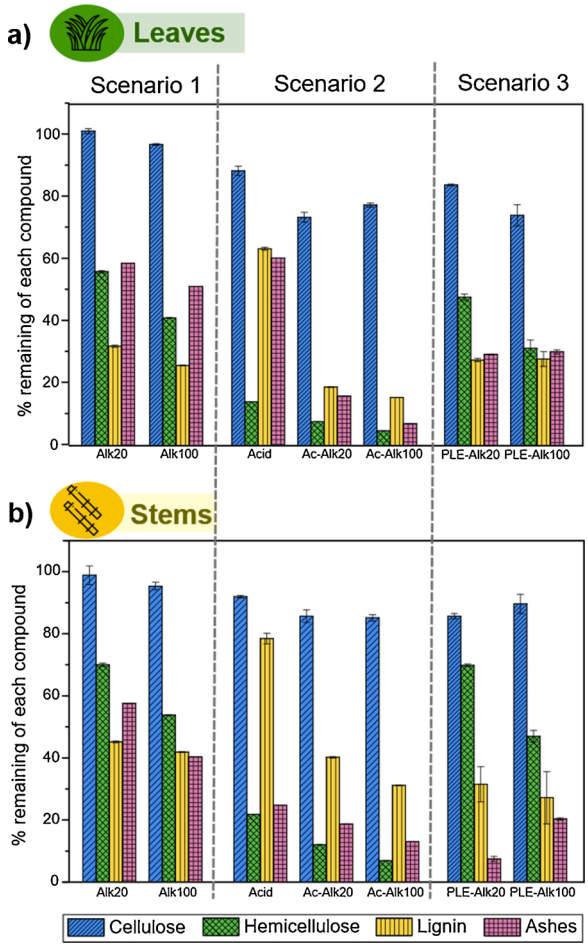

Fig. 2. Percentage of remaining compounds (cellulose, hemicellulose, lignin, and ashes) in the solid samples after treatments: a) leaves; b) stems.

insoluble-lignin fraction, which hinders the co-products use.

Another noteworthy aspect is the similarity of Scenario 1 and 3 regarding the chemical composition of solid samples, thus corroborating the outcomes attained in a previous study, where PLE neither alter the structure nor the composition of elephant grass (Scopel et al., 2020). This extraction approach can, therefore, be performed as the initial step in biorefineries intending to recover phenols and sterols (Herrero and Ibañez, 2018), also allowing the lignin recovery from the alkaline liquid fraction and the use of the solid content to produce fermentable sugars and ethanol, as proposed in Scenario 3. Green extraction methods, such as PLE, are promising technologies for biorefineries, particularly when biomasses with high yields of extractives are used as feedstock. PLE has been mainly utilized in stand-alone applications (Herrero and Ibañez, 2018), and only a few studies considered its association with other biomass pretreatments (Neves et al., 2019; Scopel et al., 2020), but the results obtained here should serve as a baseline for future studies in this area.

Furthermore, ashes, the minor component in Table 1 and Fig. 2, are composed of several types of inorganic materials with different solubilities under acidic or alkaline conditions, which resulted in different final fractions depending on the pretreatment. A more detailed discussion on this topic, including the determination of metal oxides by x-ray fluorescence, is presented in Supplementary Material (Figure S2).

Changes in chemical composition are followed by modifications in biomass morphology, as shown in Figures S3 and S4. The extraction of hemicellulose and lignin by acid and alkali pretreatments was associated with the removal of the parenchymal tissue covering cellulose fibers, grouped in bundles in the *in natura* sample (Figure S3a and Figure S4a) (Rezende et al., 2011). In contrast, cellulose fibers were more exposed in pretreated samples, which will contribute to enhance the enzymatic action. Remarkable alterations can be observed in samples submitted to sequential acid-alkali treatments (Figure S3c-d and Figure S4c-d) in which the higher extraction of hemicellulose and lignin led to a more unstructured substrate. On the other hand, this effect is less pronounced in samples submitted to either alkaline treatments alone (Figure S3e-f and Figure S4e-f) or PLE followed by alkaline treatments (Figure S3g-h and Figure S4g-h).

Similar profiles in terms of biomass composition and morphology were observed when analogous acid-alkaline treatments were applied to sugarcane bagasse (*Saccharum* ssp.) (Ferreira et al., 2020; Rezende et al., 2011), corn biomass (*Zea mays*) (Camargos et al., 2019), *Panicum maximum* (Lima et al., 2014), and *Brachiaria brizantha* (Lima et al., 2014). This indicates that the biorefinery approach proposed here should also be applicable to other biomasses, especially those derived from other grasses. Although small adjustments in experimental conditions may be necessary, the overall proposal of pretreating sequences and quantifying the obtained products tends to be similar.

3.3. Enzymatic digestibility

This work demonstrated that treatments playing a role on the recovery of elephant grass co-products can be also used to improve samples enzymatic digestibility. As shown in Fig. 3, all treatments applied enhanced sugar release, except PLE alone, which did not alter the

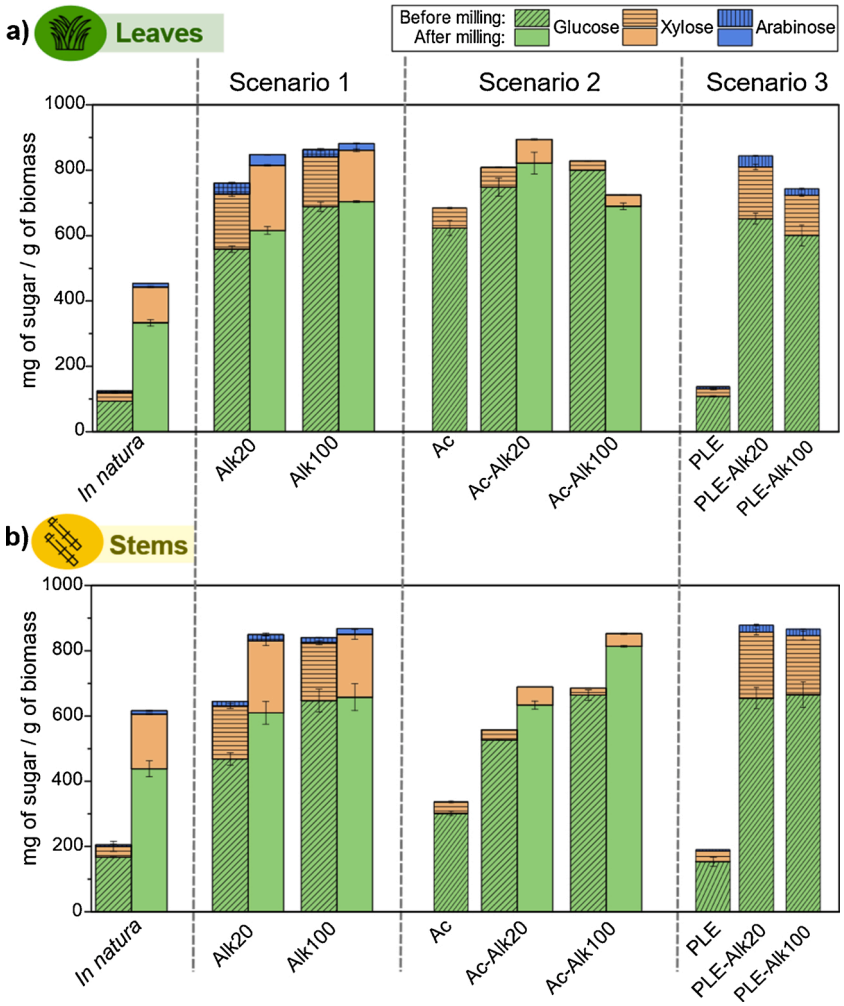

Fig. 3. Quantities of sugars (glucose, xylose, and arabinose) released by enzymatic hydrolysis from leaves (a) and stems (b).

enzymatic digestibility when compared to samples from both *in natura* and sequential acid-alkali treatments (Scopel et al., 2020). The finest improvements in sugar release were observed from 124.4 mg/g of substrate to 894.8 mg/g for leaves (sample Ac-Alk20, after milling) and from 205.6 mg/g to 867.5 mg/g for stems (Alk100, after milling). However, Fig. 3 shows that an elevated release of sugar can be also obtained in other experimental conditions, such as Alk-20 (after milling) and Alk-100 in leaves or Ac-Alk 100 (after milling) and PLE-Alk 20 in steams.

In general, leaves were more susceptible to treatments and their sugar release increased up to 6 times in comparison to *in natura* leaves. Prior to the pretreatments, stems released more sugar than leaves and denoted up to a 3-fold increase. The amount of sugars released in this work was more elevated than previously reported values for elephant grass treated under similar conditions, which were 558.56 mg of sugars/g of biomass, when using NaOH 3 % for 15 min at 120 °C (Menegol et al., 2014a). Comparing with other treatments, such as steam explosion (10 min at 190 °C), these improvements were also higher (Scholl et al., 2015).

In accordance with the similar cellulose amounts obtained from samples treated with alkaline solutions for 20 and 100 min (Table 1), equivalent amounts of sugars were also reported for samples treated for 20 and 100 min in Fig. 3. Condensed treatment time is beneficial to reduce energy inputs and improve the amount of biomass that can be processed in a determined period. Also, the total amount of sugar released from leaves was similar in Scenarios 1, 2, and 3. Therefore, the acid step can be avoided if only ethanol production is desired. In Scenario 2, the acid step alone had a more expressive effect on sugar release from leaves than from stems, which was also reported by Santos et al. (Santos et al., 2018). The use of an acid step defines how the hemicellulose can be processed in this biorefinery, since it will determine whether hemicellulose will be mainly extracted to the acid liquid fraction or if it will remain in the solid fraction before being finally hydrolyzed and fermented. Samples from Scenario 2 released less xylose and no arabinose (Fig. 3), since the removal of these fractions occurred during the acid hydrolysis step.

On the other hand, hemicellulose remained in the solid fraction in Scenario 1 and 3 (Fig. 3) and should be mainly converted to pentoses and fermented. However, the fermentation of pentoses requires more specific conditions than those used for glucose, such as the isomerization of xylose to xylulose prior to fermentation by *Saccharomyces cerevisiae* (Yuan et al., 2011) or the use of specific microorganisms (Agbogbo and Coward-Kelly, 2008). Information on both fractionation and enzymatic action on substrates richer in hemicellulose are important to evaluate more economically feasible alternatives for this polysaccharide processing. Excluding the acid treatment reduces steps in the production of ethanol but limits the use of hemicellulose to the production of fermentable sugars. Chemical treatments improved the cellulose conversion to almost 100 % in most samples (Fig. 4). This result associated with the low loss of cellulose during the pretreatment step (Fig. 2) highlights the efficiency of these treatments to produce fermentable sugars, especially glucose, from the biomass carbohydrates. These

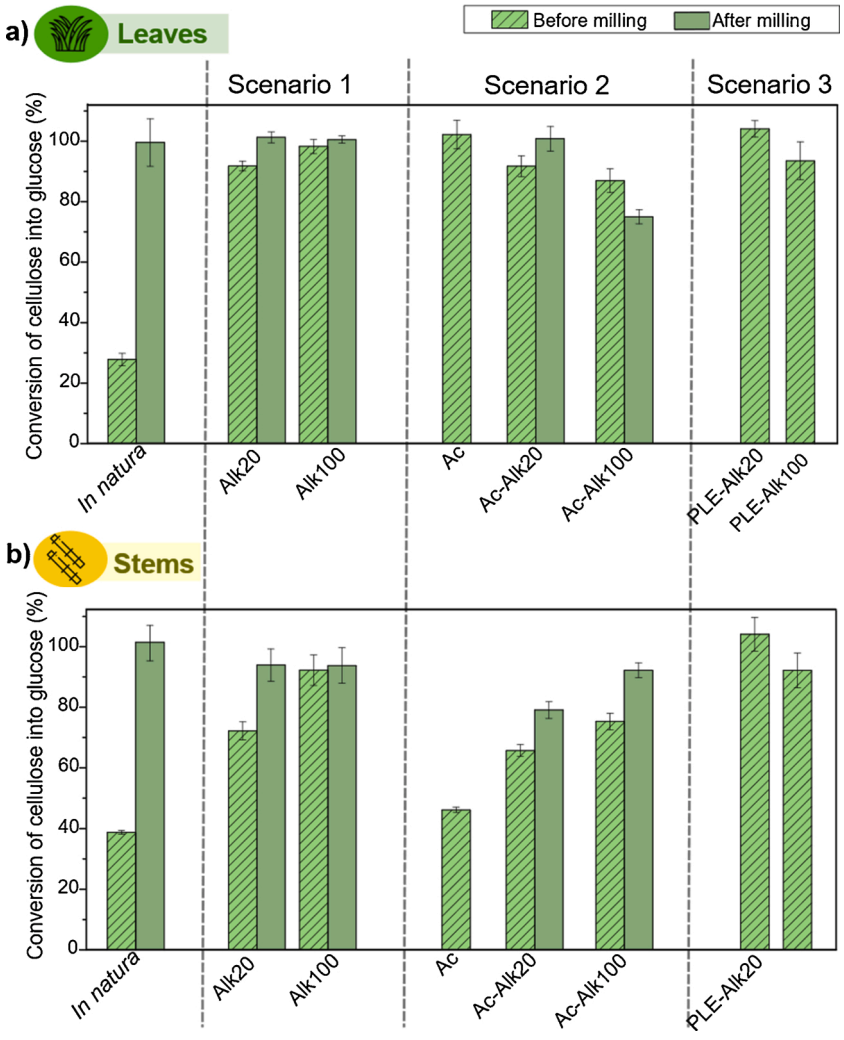

Fig. 4. Conversion of cellulose into glucose for leaves (a) and stems (b).

values were higher than other treatments applied to elephant grass using acid (89 % of conversion) (Santos et al., 2018) or steam explosion (55 %) (Kataria et al., 2017).

Ball milling was performed in this work to evaluate the effect of a reduced crystallinity index in the hydrolysis ability of elephant grass samples. Indeed, milling improved the sugar release up to 30 % in comparison to their pretreated equivalent but non-milled samples (Fig. 3). Surprisingly, when applied directly to *in natura* leaves and stems, ball milling showed a 4 and 3-fold increase in sugar release, respectively, improving the cellulose conversion into glucose from 30 to 100 %. These results demonstrate that a physical treatment based on ball milling is a promising method to enhance the enzymatic digestibility in processes exclusively focused on ethanol production. In the context of our work, however, ball milling by itself would not enable biomass fractionation in extractives, lignin, and hemicellulose; hence, the application of an auxiliary pretreatment was adequate. Another aspect that should be considered is that ball milling is not a process straightforwardly used in industrial applications and its inclusion in a biorefinery should be economically evaluated, balancing its remarkable efficiency with energy demand.

Fig. 5 displays an upsurge of crystallinity index (CrI) observed in the chemical treatments from 54 to 74 % for leaves and from 65 to 76 % for stems due to the extraction of hemicellulose and lignin, which are amorphous compounds (Nascimento et al., 2014; Sheltami et al., 2012). CrI values closer to 70 % were also acquired for sugarcane bagasse (Bernardinelli et al., 2015) and elephant grass (Nascimento and Rezende, 2018), both treated with sequential acid-alkali solutions. Ball milling reduced the sample crystallinity in all samples, but this reduction was more elevated in stems than in leaves, when comparing the identical treatment conditions. This could be related to the different thicknesses of substrates, since the particles from leaves are thinner and hamper milling efficiency in comparison to stems substrates.

Fig. 5 also indicates that a decrease in sample crystallinity was followed by higher sugar release in most samples. Despite the clear improvements in biomass hydrolysis due to ball milling, a linear negative correlation between crystallinity index and sugar release was not observed in these samples (Figure S5), which may be explained by the influence of several factors on hydrolysis yields, such as chemical composition and particle size, which was also reduced by milling (Figure S6). FESEM images of milled samples brought to light that the main consequences of ball milling to sample morphology were: a drastic reduction of particle size; a more compact surface; and the absence of visible fibers after milling. Menegol et al. observed the relation between particle size and sugar released from elephant grass, in which smaller particles released higher amounts of sugars than the larger ones (Menegol et al., 2014b). Indeed, the enzymatic response can be affected by a reduction in either crystallinity index or particle size; however, no clear relationship between these effects that occurred simultaneously was observed in this work (Park et al., 2010).

Regarding sugar concentration, the values increased from 3.1 and 5.1 g/L for *in natura* leaves and stems to *ca.* 20 g/L after treatments (Table S2). These values are similar to those reported for sugarcane bagasse treated with alkaline hydrogen peroxide (22 g/L) using similar solid levels (Rabelo et al., 2011) or elephant grass submitted to ball milling and hydrolyzed at high solid levels (*ca.* 12 g/L) (Menegol et al., 2016).

3.4. Overview of the scenarios

A detailed description of each scenario proposed here for the three biorefineries of elephant grass is shown in Fig. 6, including composition, enzymatic hydrolysis, and fermentation results. Table 2 summarizes the percentages of co-products recovered and the total use of biomass following the scenarios proposed, allowing a comparison to the cases where the same pretreatment method was applied but focused only in ethanol production, disregarding the co-products. The quantity of sugars that can be obtained from 1 ton of biomass (Figure S7) and a detailed quantification of the liquid fractions (Table S4), used to elaborate the scenarios, are reported in Supplementary Material, together with a kinetic assay to optimize ethanol yields during fermentation (Figure S8 and Table S3).

Scenario 1 favored 2G ethanol production, wherein a higher amount of ethanol was obtained from stems than from leaves (78 kg *versus* 70 kg, respectively). In addition, ball milling improved the ethanol production from these substrates in up to 30 % and 10 % for stems and leaves, respectively. Only the conversion of glucose to ethanol was considered in the scenarios proposed here because of *Saccharomyces cerevisiae* use in fermentation. In this case, xylose (94–145 kg per 1 ton of biomass) and arabinose weights (12–19 kg per 1 ton of biomass) presented on the pretreatment liquors remained available for other uses. Fermentation step was not optimized in this work, but the quantity and concentration of ethanol can be enhanced, for instance, by using simultaneous saccharification and fermentation (SSF) (Montipó et al., 2019), higher solid levels in hydrolysis and fermentation (Menegol et al., 2016), surfactants (Sun et al., 2020), or a cocktail able to convert both pentoses and hexoses (Antunes et al., 2019).

Scenario 1 also provided 99 kg and 68 kg of acid-insoluble lignin from 1 ton of leaves and stems, respectively, which can be isolated from the alkaline liquid fraction (Fig. 6a). However, the purity of this lignin may be impaired by the presence of hemicellulose derivates (xylose and arabinose) presented in the same liquor. As shown in Table 2, approximately 60 % of the biomass could be used in this scenario, including the

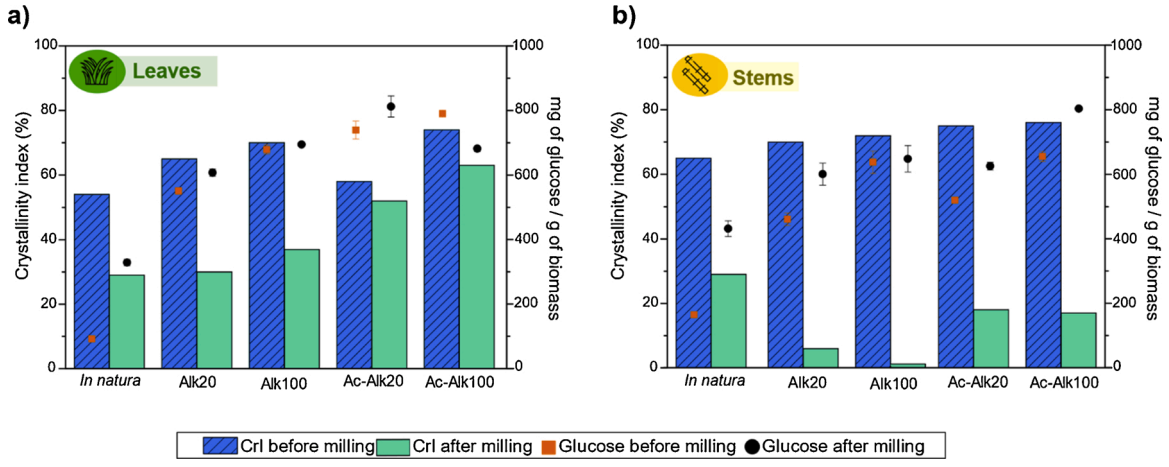

Fig. 5. Crystallinity index (CrI) and glucose released from samples before and after ball milling: a) leaves; b) stems.

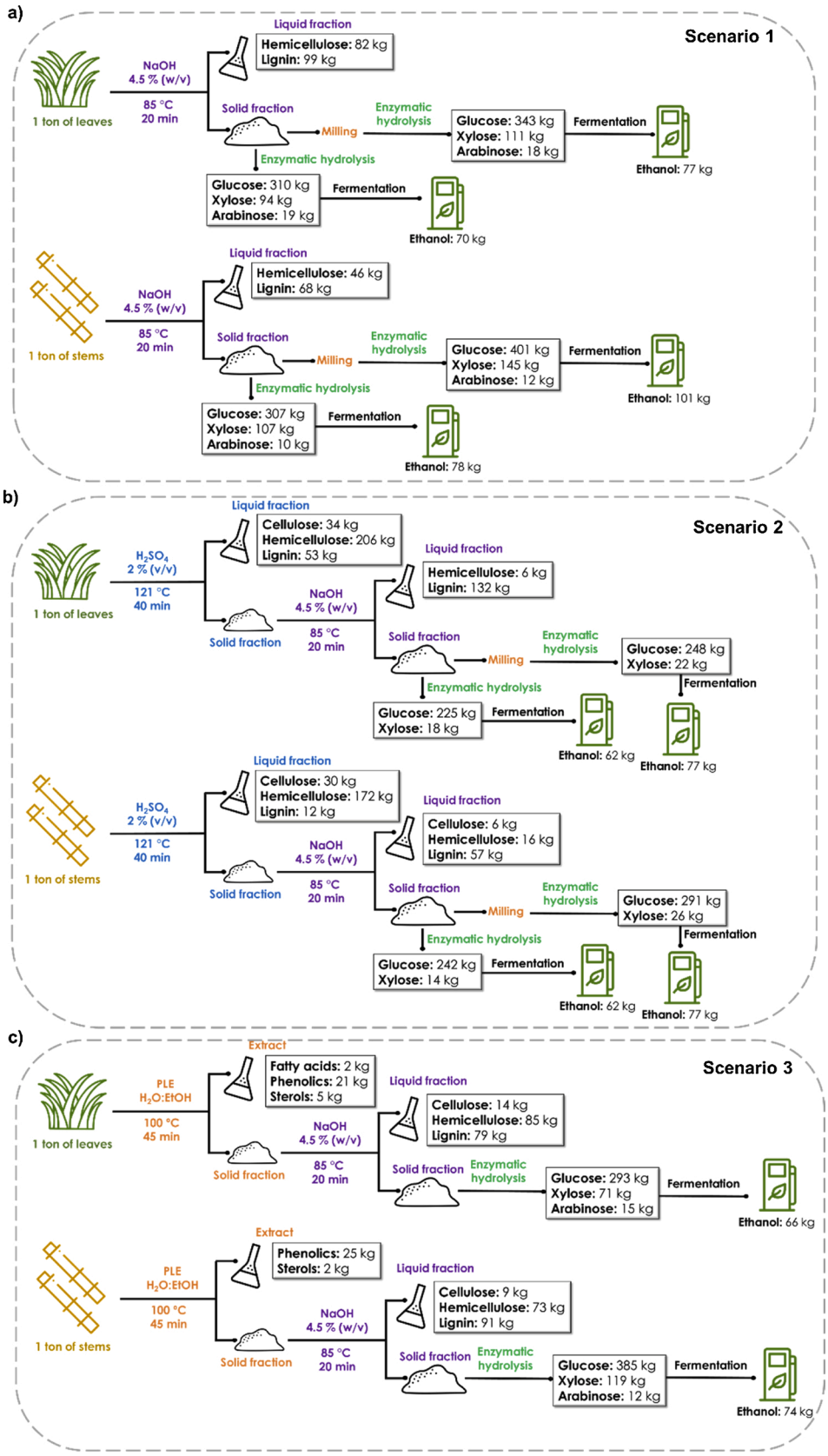

Fig. 6. Quantities of ethanol and co-products that can be produced by elephant grass processing in: a) Scenario 1; b) Scenario 2; c) Scenario 3.

Table 2
Percentage of co-products and total use of the biomass considering in each scenario the recovery of co-products or not.

Scenario	Use of the biomass (%)								
	Lignin	Hemicellulose		Cellulose		Extractives	Only Hyd	Total	
		Liq	Hyd	Liq	Hyd*			Without extractives	With extractives
	Leaves								
	44.0	33.7	40.7	—	100	100*	41.4	60.3	80.5
	Stems								
1 (Alkaline)	31.2	21.1	63.4	—	93.4	100*	49.9	61.3	75.5
	Leaves								
	82.2	87.2	8.0	11.3	74.1	100*	24.2	67.4	87.6
	Stems								
	27.0	86.2	10.5	9.33	67.8	100*	28.5	57.8	72.0
	Leaves								
3 (PLE + Alkaline)	35.1	35.0	31.1	4.6	87.6	37.3	33.9	**	59.3
	Stems								
	35.6	33.5	52.9	2.3	89.8	54.4	46.2	**	71.2

Liq: compound recovered from liquor.
Hyd: compound hydrolyzed by enzymes.
— Compound not identified.
* Maximum of extractable compounds (Scopel et al., 2020).
** Condition not proposed.
¥ Considering ball milling conditions.

recovery of lignin, hemicellulose in the liquors as well as the conversion of hemicellulose and cellulose to pentoses and hexoses, respectively. To assure a more effective use of biomass, an extraction step could also be included in Scenarios 1 and 2. Though initially proposed only for Scenario 3, an extraction (by PLE or any other extraction method) would also improve the other scenarios, since extractives compose 20 % and 14 % of the weight of leaves and stems in elephant grass, respectively (Scopel et al., 2020). Considering the maximum of extractives that can be obtained from this biomass (using Soxhlet extraction in two steps with cyclohexane followed by a mixture of ethanol and water), the total use of the biomass increased to 80 % for leaves and 75 % for stems. Table 2 discloses the remarkable increase achieved when using those extractives in comparison to the application of this pretreatment method focused only on ethanol production, which would use only 40–50 % of biomass. Details about the percentage of the biomass use without the co-products use are presented in Supplementary Material (Figure S5).

In Scenario 2, the sequential treatments enabled the recovery of 212 kg and 188 kg of hemicellulose derivates (xylose and arabinose) from the pretreatment liquors of leaves and stems, respectively (Fig. 6b). These values are 2.5 times higher than the amount of hemicellulose that could be recovered from leaves and 4 times the amount from stems in Scenario 1. On the other hand, the ethanol yield (77 kg) was 10 % lower for leaves and 25 % inferior for stems than in Scenario 1. Lignin presented in pretreatment liquors of leaves was also significantly higher in Scenario 2 (185 kg against 99 kg in Scenario 1). For stems, acid-soluble lignin was obtained in similar amounts in both scenarios (*ca.* 68 kg). These results reinforce the idea that an integrated evaluation of possible applications of hemicellulose and lignin co-products should be considered when ethanol production viability is evaluated. Overall, enhanced recovery of co-products was possible in Scenario 2, which resulted in the use of *ca.* 74 and 56 % of leaves and stems, respectively. These values could possibly reach 88 % and 72 % for leaves and stems if extractives are considered (Table 2), leading to the conclusion that Scenario 2 stood out as the best option for an effective fractionation and further use of biomass co-products. In a single-product approach focused only on ethanol production, 25–30 % of the biomass total weight would be used.

Finally, Scenario 3 presents a pretreatment configuration that included an extraction step using a green methodology. PLE, using water and ethanol as solvents, was used to extract mainly alcohol and phenolic compounds from elephant grass (Scopel et al., 2020). Based on previous work, up to 28 kg of alcohols, phenols and fatty acids can be recovered from 1 ton of leaves (Scopel et al., 2020). These products could be applied in food, pharmaceutical and cosmetical industries, *i.e.* in products with high added value. The amount of ethanol produced in Scenario 3 (66 kg from leaves and 74 kg from stems) is only 5 % lower than the quantity obtained in Scenario 1 (70 and 78 kg, without milling), and similar recoveries of hemicellulose (85 kg from leaves and 73 kg from stems) and lignin (79 kg from leaves and 91 kg from stems) (Fig. 6c) were also noticed.

An important difference in Scenario 3 in comparison to Scenario 1 is the presence of cellulose derivatives (glucose) in the pretreatment liquors (9–14 kg per 1 ton biomass), which could be responsible for the small decrease in ethanol production. Although the same alkaline treatment is applied in both cases, a previous extraction step is probably facilitating biomass alkaline hydrolysis in Scenario 3. Additionally, cellulose conversion yields were improved in PLE-Alk20 samples in both leaves and stems (in comparison to Alk20, Fig. 3), thus indicating that PLE extraction may have a positive effect on enzymatic hydrolysis. In Scenario 3, a total of 59 % of leaves and 71 % of stems were used, which is an important increase in biomass usage in comparison to the 34 and 46 % utilized from leaves and steams, respectively, when co-products were disregarded. Therefore, PLE stand out as a more advantageous method than traditional ones due to its environmentally-friendly character, reduced operation time, and low cost with solvents; however, Soxhlet extraction enables higher extraction yields (Mustafa and Turner, 2011; Scopel et al., 2020).

Table S6 (Supplementary Material) presents an estimation of water use in each scenario, considering the steps of treatment, liquid fraction recovery, and solid washing. Based on our laboratory procedures, 60 to 90 m^3 of water would be used to pretreat 1 ton of dried elephant grass, depending on the route chosen in Fig. 6. However, it is important to consider that the pretreatments used here were not optimized to minimize water waste, but to enhance hydrolysis yields. Besides, the water use in a bench scale may differ from a pilot or a large-scale processing, and methods for water reuse may completely change the estimated values of water use. Analyses on water waste and techno-economic aspects are important and should be investigated to consolidate the best scenarios as well as elucidate the most cost-effective routes within the proposals in biorefineries. Bearing in mind the estimations of energy, water-use, the cost of reagents and of manufacture (COM), an evaluation of feasible investments on processing and their costs would be possible. Then, the expenses could be reduced by a more integral use of the substrate in high value-added applications (Jorissen et al., 2020).

Considering a broaden approach for the scenarios proposed, the acid-alkali treatments used here are not limited to the production of fermentable sugars, but have also been studied to produce cellulose-

based materials, such as nanocelluloses (Nascimento and Rezende, 2018), and lightweight materials (Ferreira et al., 2020). Solid foams, for instance, were produced from sugarcane bagasse using similar conditions to those proposed in Scenario 1, without the acid step. Additionally, the obtained glucose can be converted into other chemicals, such as 5-hydroxymethylfurfural (HMF), which is a chemical platform to produce levulinic and formic acids, dihydroxy methyl tetrahydrofuran, and 2,5-dimethylfuran after subsequent catalyzed reactions (Gallo et al., 2017). Lignin extracted from elephant grass by acid-alkaline treatments can be used to produce lignin nanoparticles (Trevisan and Rezende, 2020) or other lignin-derived materials, such as carbon foams (Alonso et al., 2017), carbon fibers (Kadla et al., 2002), or fillers to nanocomposites (Tian et al., 2017). Lignin is also an important chemical platform to synthetize chemicals, such as vanillin (Wang et al., 2018). Moreover, hemicellulose derivatives extracted in this biorefinery can be converted into ethanol, xylitol, butanediol, furfural, or polyhydroxy butyrate (PHB) by specific micro-organisms (Chandel et al., 2018). Finally, extractives can be used in food, pharmaceutical, and cosmetic industries, mainly to replace the products obtained from oil (Scopel et al., 2020).

It is worth mentioning that our approach accomplishes 5 of the 12 Principles of Green Chemistry, named: (1) Prevention, since the more integral use of biomass prevents the waste formation when compared to processes focused only on one target product; (5) Safer solvents and auxiliaries, especially considering the use of PLE with ethanol and water to replace traditional toxic solvents used to isolate extractives; (6) Design for energy efficiency, since ball milling conditions were enhanced by DOE tools in this work, and the conditions of alkaline pretreatment were also previously optimized (Rezende et al., 2018) to reduce time and temperature of processing; (7) Use of renewable feedstocks; and (11) Real-time analysis for pollution prevention, considering that the reaction liquors were quantified to point out the absence of hazardous substances, such as hydroxymethyl furfural (HMF).

4. Conclusion

We carried out a systematic analysis of co-products from elephant grass leaves and stems in three different scenarios of a biorefinery having ethanol as its main product. In our proposal, all the scenarios proved to be efficient to improve biomass use, by recovering hemicellulose, lignin, and extractives, while maintaining elevated yields of ethanol production (60–100 kg/ ton biomass). The best fractionation results were obtained in Scenario 2, fomenting an increase of biomass use from 24 to 88 % in leaves and from 28 to 72 % in stems. Scenario 3 also encompassed an interesting approach, where a PLE extraction step was incorporated prior to the pretreatments, representing an important contribution to promote the use of green extraction methodologies in biorefineries. This study advances towards more sustainable and integrated processes to fractionate non-wood plant biomasses, aiming at a more integral and profitable use of these renewable resources.

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CRediT authorship contribution statement

Eupídio Scopel: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization. **Camila A. Rezende:** Conceptualization, Resources, Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi: https://doi.org/10.1016/j.indcrop.2021.113336.

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