Enzymatic extraction of xylans from alkaline-sulfite pretreated sugarcane bagasse and their incorporation onto eucalyptus kraf pulps

Aline Antunes[1], Adriane Milagres[1], and Andre Ferraz[2]

[1]University of Sao Paulo
[2]University of São Paulo School of Engineering of Lorena - Campus II

May 5, 2020

Abstract

Hemicellulose-rich substrates produced in the lignocellulose biorefinery context can yield macromolecular hemicellulose structures with assorted application in the chemical industry. In the current work, alkaline-active xylanase aided to extract a varied group of xylan fractions from alkaline-sulfite pretreated sugarcane bagasse. Extraction yields (12-44%) depended on the reaction time and the xylanase load used in the extraction procedure. Prepared fractions contained low levels of lignin contamination (4-9%) and 4-O-methyl glucuronic acid arabinoxylan structures. Molar masses of fractions ranged from 2.3 kDa to 34 kDa. Prepared xylans were incorporated onto eucalyptus pulp fibers up to 4.7 g xylan/100 g pulp. The efficiency of xylan incorporation was dependent on the xylan structures, where low molar mass and low substitution degree favored high incorporation levels. Enzymatic xylan extraction proved a distinguishing procedure to add value in the lignocellulosic biorefinery chain of sugarcane bagasse once extracted xylans are suitable for incorporation onto cellulosic fibers.

1. Introduction

Lignocellulosic biomass has gained relevance as a source of renewable chemicals in the context of modern biorefineries. Major components from lignocellulosic biomass, cellulose, hemicellulose and lignin, can be converted into a variety of new products, serving as alternative for the current petroleum-based industry (Amoah, Kahar, Ogino, & Kondo, 2019). In the particular case of hemicellulose, several of the current biorefinery schedules include hemicellulose conversion to monosaccharides for subsequent production of bioethanol or other biobased products (Kuhad, Gupta, Khasa, Singh, & Zhang, 2011; Mendes, Vasconcelos, Dias, Ferraz, Milagres, Santos, Jesus, Watanabe, Junqueira, & Bonomi, 2018). One alternative approach has focused on the recovery and use of hemicellulose on its macromolecular structure, since this polysaccharide delivers a competitive material to the chemical industries (Deutschmann, & Dekker, 2012; Mandegari, Farzad, van Rensburg, & Görgens, 2017). Drug delivery matrices, hydrogels, barrier films and advanced fibers can be produced with aid of macromolecular structures of hemicellulose (Liu et al., 2016; Naidu, Hlangothi, & John, 2018). In the last case, the incorporation of hemicellulose on cellulosic pulps conveys positive effects on pulp beating and pulp strength properties as well as increases in pulp yield (Ban & Van Heiningen, 2011; Han et al., 2012; Muguet, Pedrazzi, & Colodette, 2011; Tamminen et al., 2018).

Several factors influence the levels of hemicellulose incorporation onto cellulosic materials, including the characteristics of the cellulosic material and structural characteristics of hemicellulose, such as its type and degree of substitution, its molar mass/molar mass distribution, and its level of contamination with residual lignin. For example, scarcely substituted xylan fractions favored adsorption on bacterial cellulose (Kabel, van den Borne, Vincken, Voragen, & Schols, 2007). Similarly, Chimphango, Görgens, & van Zyl, (2016) studied xylan adsorption on cotton lint using xylans prepared by alkaline extraction from four different lignocellulosic

sources. One of the major factors governing xylan adsorption levels was associated with low contents of 4-O-methyl glucuronic acid substituents, since xylan treatments with α-glucuronidase significantly increased their adsorption levels. Comparing the different sources of xylans, the authors also demonstrated that grass xylans (from sugarcane bagasse and bamboo) provided higher adsorption levels as compared with eucalyptus xylans. Regarding xylan molar mass, some authors reported that smaller xylan molecules tend to adsorb less to the cellulose surface than the larger ones (Kabel et al., 2007). These authors reached this conclusion based on a set of oat spelts xylan fractions with different molar masses prepared by controlled enzymatic hydrolysis and bacterial cellulose as a support. In contrast, adsorption studies of xyloglucan and xyloglucan oligosaccharides on crystalline cellulose revealed a greater affinity of low molar mass oligosaccharides on cellulose (Vincken, Keizer, Beldman, & Voragen, 1995). Additional studies of hemicellulose adsorption on cellulosic materials have been reported using commercial xylans (Han et al., 2012), xylans recovered from pulping liquor (Danielsson & Lindstrom, 2009; Magaton, Silva, Colodette, Piló-Veloso, & Milagres, 2013), alkali-extracted xylans from different sources (Köhnke, Brelid, & Westman, (2009); Kohnke, Pujolras, Roubroeks, & Gatenholm), and enzymatically prepared xylan hydrogels (Chimphango, 2019).

From reported work, it is clear that specific hemicelluloses produced in each biorefinery schedule require detailed evaluation regarding production procedures and adsorption studies, since the adsorption levels on cellulosic materials are closely dependent to the source of lignocellulosic material, the extraction procedure, the main type of hemicellulose backbone and its degree of substitution, and the molar mass/molar mass distribution.

Up to date, few studies have explored how the characteristics of diverse hemicellulose structures prepared from sugarcane bagasse, a major lignocellulosic biomass available for biorefineries (Mendes et al., 2018), affects its incorporation on cellulosic pulps (Chimphango et al., 2016). In the few studied cases, alkali-extracted hemicelluloses containing significant amounts of residual lignin have been evaluated, biasing interpretations regarding the effects related to the structure of hemicellulose on the incorporation efficiency. In a recent report, we demonstrated that xylans with low levels of lignin contamination could be recovered from alkaline-sulfite pretreated sugarcane bagasse by enzymatic extraction (Sporck et al., 2017). However, the extracted xylan showed low average molar mass and large molar mass dispersity owing to the long reaction time with xylanases. Within this context, the current work evaluates enzymatic extraction of xylan at varied reaction periods, associated with xylan fractionation with ethanol precipitation. This approach permitted to prepare four different xylans with varied molar mass distributions and structural characteristics, enabling studies of xylan incorporation onto cellulosic pulps fibers. Once prepared xylans presented varied structural characteristics, the features providing high xylan incorporation onto pulp fibers were established.

2. Materials and methods

2.1. Chemicals and lignocellulosic materials

Beechwood xylan and monosaccharides were purchased from Sigma-Aldrich (Saint Louis, USA). Xylo-oligosaccharides (XOs) were purchased from Megazyme (Wicklow, Ireland). Dextrans standards were purchased from Pharmacosmos (Holbaek, Denmark). All other chemicals were at least of analytical grade and supplied by Sigma-Aldrich unless otherwise specified.

Sugarcane bagasse was collected in a sugar and ethanol mill after sugarcane processing for sucrose extraction. The bagasse containing 10% humidity (600 g, oven dry basis) was impregnated with alkaline sulfite liquor (10% (w/w) of Na_2SO_3 and 5% (w/w) of NaOH) at a bagasse/liquor ratio of 1:10 (w/v) and pretreated as described by Mendes, Siqueira, Carvalho, Ferraz, & Milagres (2011). Pretreated bagasse was used as the substrate for enzymatic extraction of xylans.

Unbleached kraft pulp and Kraft O_2 pulp were provided by a local pulping mill that uses hybrid eucalyptus (*E. urophila* x*E. grandis*) as raw material. The pulp fibers were used as model fiber materials for xylan incorporation experiments.

2.2 Enzymatic extraction of xylan from pretreated sugarcane bagasse

Enzymatic extraction of xylan was performed in Erlenmeyer flasks of 50 mL under reciprocal agitation (120 rpm) in a water bath maintained at . The substrate of enzymatic reaction was alkaline-sulfite pretreated sugarcane bagasse suspended at 5% (w/v) in phosphate buffer at pH a working volume of 25 mL. The commercial xylanase (Luminase PB-200, BASF) was loaded to the reaction media at a dosage ranging from 5 to 100 IU/g \soutof pretreated bagasse. Samples of 1 mL were withdrawn at reaction times ranging from 3 to 24 h and boiled in water bath for 5 min to stop the enzymatic reaction. After cooling, the samples were centrifuged and the supernatants were analyzed for carbohydrates content. All reactions were performed in triplicate and the means and standard deviations are reported in the text. The yield of xylan extraction was determined based on mass balances of xylan detected in the extraction solution and the xylan contained in the pretreated sugarcane bagasse (Sporck et al., 2017).

To detect xylan concentrations in the reaction media, extracted xylans were submitted to an acid post-hydrolysis with diluted sulfuric acid as described by Sluiter et al., (2008). Xylose and arabinose were quantified by HPLC as described in 2.5. Monomer contents were converted to anhydrous-monomers using the hydration factor of 0.88.

To detect xylo-oligosaccharides (XOS) formed during xylan extraction, aliquots from the reaction media were analyzed by thin layer chromatography (TLC). Samples of 2 µL of the reaction media were developed on silica gel 60 F254 (Merck, Darmstadt, Germany) plates according to Puchart & Biely (2008). Xylose and XOs (xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylopentose (X5) and xylohexose (X6)) were analyzed as internal standards.

To detect endo-xylanase activity of commercial Luminase (PB-200, BASF), a procedure previously described (Bailey, Biely, & Poutanen, 1992) was adapted to evaluate alkaline-active xylanases. An aliquot of 0.1 mL of the commercial xylanase was incubated in 0.9 mL 1% (w/v) beechwood xylan \soutat homogenously suspended in sodium phosphate buffer (pH 8). The xylanase test was performed at for 5 min. The reducing carbohydrates formed in the reaction medium were measured by monitoring absorbance at 540 nm by comparison with standard curves using xylose as reference. One international unit (IU) of enzyme activity was expressed as micromoles of xylose released from beechwood xylan per minute at pH 8 and 50 °C.

2.3 Scale-up preparation of xylans and fractionation by precipitation in ethanol

Selected reaction conditions were used for scaled-up preparation of xylans. The reactions were performed with 50 g of pre-treated sugarcane bagasse in 2 L Erlenmeyer flasks with a working volume of . The reactions were performed with 5 IU xylanase/g substrate for 6 or 24 h. The xylans extracted for 6 hours of enzymatic reaction were recovered by graded ethanol precipitation. The pH of the reaction medium was adjusted to 5.5 with 1 mol/L HCl and 15% (v/v) ethanol final concentration was added. After standing for 24 h at 5 °C, the precipitate was separated by centrifugation at for 15 minutes (Peng et al., 2009). The supernatant was collected and mixed with ethanol to reach a final concentration of 30% (v/v) and the recovery procedure was repeated. The precipitated xylans were collected, freeze dried and designated as xylan I and II, respectively. A third addition of ethanol to reach a final concentration of 60% (v/v) was made, but no precipitate was observed. Therefore, all the liquid was concentrated under reduced pressure to eliminate ethanol and then freeze-dried, giving the xylan III fraction.

A forth xylan fraction was prepared in a similar reaction lasting for 24 hours. The reaction medium was fully recovered and then freeze-dried to yield the xylan IV fraction.

2.4 Evaluation of molar mass distribution of xylans

Molar mass distribution was estimated by size exclusion chromatography using a Biogel P30 column (× I.D; Biorad, Hercules, CA, USA) that has been equilibrated with phosphate buffer, pH 8.0 and eluted at 0.3 mL/min with the same buffer at . A total of 500 µL of each sample was applied to the column, and eluted fractions were assayed using a phenol–sulfuric acid reagent to determine total sugars (Dubois, Gilles, Hamilton, Rebers, Smith, 1956). The column was calibrated using a mixture of xylose (180 g/mol), dextrans of 3,500 g/mol, 6,000 g/mol, 10,000 g/mol, 20,000 g/mol and blue dextran (2,000,000 g/mol).

2.5 Determination of chemical composition of xylans, pretreated sugarcane bagasse and kraft pulps

The chemical compositions of pretreated bagasse, extracted xylan and kraft pulps before and after treatment with xylans were determined as previously described (Ferraz, Baeza, Rodriguez, & Freer, 2000). The xylans were further evaluated for the glucuronic acid contents using the carbazole-sulfuric acid method (Li, Kisara, Danielsson, Lindstrom, & Gellerstedt, 2007). In the case of xylans, the aromatic compounds content was estimated by UV spectroscopic evaluation of the acid hydrolysates. For this, the hydrolysates were adjusted to pH 12 and the absorbance at 280 nm was recorded in UV/Vis spectrophotometer. Total aromatics was estimated using the extinction coefficient of 23.7 $L.g^{-1}.cm^{-1}$ (Gouveia, Nascimento, Souto-Maior, & Rocha 2009).

2.6 Studies of xylan incorporation onto kraft pulp fibers

The eucalyptus Kraft pulps used in this study corresponded to unbleached Kraft and Oxygen delignified Kraft (Kraft O_2) pulps. The chemical composition of both pulps determined according to Ferraz et al. (2000) was estimated as 70% glucan, 15.1% xylan, 4.9% lignin for Kraft and 78.6% glucan, 13.7% xylan, 2.8% lignin for Kraft O_2.

The never dried pulps were transferred into glass tubes (on oven dry basis) and mixed with xylan dissolved in 4 mL NaOH (pH 10) (Ban & Van Heiningen, 2011). After 2 h of static incubation at 70 °C, the supernatant was poured out and replaced by 10 mL of distilled water. The mixtures remained at room temperature for 1 h and the excess of liquid was removed and the pulps were freeze-dried. The same procedure was performed with the pulps without the addition of xylan as a control reaction. In some experiments, treated pulps were further washed two times (1 h each washing) with NaOH 0.1 mM. The amount of xylan incorporated onto the pulps was quantified by chemical analysis described in 2.5, before and after treatment with selected xylans. The xylan amount incorporated in the pulps was calculated as the increased amounts of xylans detected in the treated samples.

3. Results and discussion

3.1 Preparing enzymatically-extracted xylans with varied molar mass distribution and structural characteristics

Alkaline-sulfite pretreated sugarcane bagasse was the source of enzymatically-extracted xylans for the current study because it is a xylan-rich, low-recalcitrance substrate (Laurito-Friend, Mendes, Reinoso, Ferraz & Milagres, 2015) prepared during sugarcane bagasse bioprocessing in the biorefinery context (Mendes et al., 2018). Alkaline-active xylanases proved useful for extraction of xylans with low lignin contamination from this substrate, despite low molar mass xylans result from these enzymatic extraction procedures (Santos, Reinoso, Tavilla, Ferraz, & Milagres, 2019; Sporck et al., 2017). In the current work, xylans with varied structural characteristics were prepared from alkaline-sulfite pretreated sugarcane bagasse with aid of an alkaline-active xylanase and precipitations using increasingly ethanol concentrations. The aim was to found proper features of xylan structures regulating their incorporation onto pulp fibers.

An assortment of enzyme loads and reaction periods initially assessed the total yields of extracted xylans (Figure 1a). The xylan extraction yields reached 32% and 44% for enzyme loads of 5 and 100 IU xylanase/g substrate at the longest reaction time, respectively. Increasingly enzyme loads resulted in limited increases in final xylan extraction yields, suggesting that a significant proportion of xylan contained in the substrate is inaccessible even to the highest dose of xylanase (100 IU/g) (Figure 1b). In fact, a previous study has reported enrichment of xylans in the external fiber surfaces of alkaline-sulfite pretreated sugarcane bagasse (Mendes et al., 2015). It is probable that this xylan fraction is promptly extracted even at low xylanase loads, whereas the xylan fraction closely associated with inner parts of the cell wall is inaccessible to the enzymes.

The total xylan extraction yields determined in the current work reflect any size of xylan backbone extracted from the substrate, since the yields are calculated with basis on the xylose released from extracted xylans after a mild analytical acid hydrolysis (see M&M for details). To gain some information on the nature

of xylan/xylo-oligosaccharides produced during reaction, thin layer chromatography (TLC) evaluated the samples from the reaction using the lowest xylanase load (data not shown). Even with the lowest enzyme load and short reaction periods (3 and 6 h), TLC revealed the occurrence of short oligosaccharides (xylotriose, xylotetraose and xylopentaose) in the reaction hydrolysate. Longer reaction periods (24h) provided the additional accumulation of xylobiose, demonstrating that with the extension of the reaction time the enzyme extracted more xylan (Figure 1a), but also acts by hydrolyzing the solubilized oligomers.

Based on the initial screening of variables affecting xylan extraction yields and structural xylan characteristics, low enzyme loadings (5 IU xylanase/g substrate) were used for scaled up xylan extractions. Higher loads of xylanases did not enhance the extraction yields significantly (only 1.4-fold increase in extraction yield by 20 times increased in enzyme load). Reaction time was fixed at 6h to avoid further hydrolysis of the extracted xylan. Xylan extracted after 6 h reaction with 5 IU xylanase/g substrate was further fractionated by precipitation with increasingly concentrations of ethanol, as described in other studies (Bian, Peng, Peng, Xu, & Sun, 2010, Peng et al., 2009; Swennen, Courtin, Lindemans, & Delcour, 2006). A reference xylan was also prepared with the longest reaction time (24 h) without precipitation with ethanol.

The relative amounts of the xylan fractions obtained by graded ethanol precipitation are shown in Table 1. Fractions precipitated by 15% and 30% ethanol were designated as xylan I and xylan II, respectively, representing only 2.6% and 2.8% of the originally extracted xylan. Most of the remaining xylan (94.6%) did not precipitate even when ethanol concentration in the extract was increased to 60%. Therefore, a xylan III fraction was prepared by freeze-drying the solution remaining after ethanol concentration was raised to 60%. The fourth xylan fraction (denoted as xylan IV) was obtained after 24 h extraction and freeze-drying of the prepared extract without any fractionation.

All xylan fractions were characterized for their carbohydrate composition and residual aromatic contents (Table 1). As expected for sugarcane xylans (Gomes, Chimphango, & Gorgens, 2015; Sporck et al., 2017; Sun, Sun, Sun, & Su, 2004; Xu, Sun, Liu, & Sun, 2006), xylose, arabinose and 4-O-methyl glucuronic acid constituted the major part of the carbohydrates. Residual aromatic components, mainly derived from lignin, occurred at 4-9% with xylan I fraction containing the highest aromatics content and concomitant lower relative xylan content. The molar ratios for Xyl/Ara and Xyl/GlucAc indicated predominant substitution of xylan chains with arabinose in all fractions. Xylan III was the least substituted in the group illustrating some different populations of xylans, regarding arabinose and 4-O-methyl glucuronic acid substitution.

Molar mass distribution of the xylans indicated that xylan III presented the narrowest distribution and an average apparent molar mass of 2.9 kDa (Figure 2). In contrast, xylan I and II (precipitated by 15% and 30% (v/v) ethanol, respectively) presented wider molar mass distribution and high average apparent molar masses of 34 kDa and 28 kDa, respectively. The xylan IV, generated by the longest reaction time (24 h), contained mostly a small fraction with average apparent molar mass of 2.3 kDa and another fraction with average molar mass of 30.8 kDa. Molar mass distribution data suggest that xylan chains with higher molar mass are predominantly precipitated at relatively lower ethanol concentrations, while xylans with lower molar mass are recovered at higher ethanol concentrations or did not precipitate, corroborating previous work reported by Bian, Peng, Peng, Xu, & Sun, (2010).

3.2 Incorporation of xylans onto eucalyptus pulp fibers

Incorporation of prepared xylans onto fibrous materials was assessed using commercial kraft pulps from eucalyptus wood. Xylans I to IV with varied molar masses and structural characteristics were used to discriminate xylan features enabling incorporation onto pulp fibers.

The experimental procedure used to incorporate xylans onto pulp fibers included an initial equilibrium period contacting fibers and dissolved xylan at pH 10 (see M&M for details). After separation of the pulp suspension, a second equilibrium period contacting the previously treated pulp and water at neutral pH was followed by another separation step, which is designed to release non-adsorbed xylans from the pulp fibers (Han et al., 2012). This premise was confirmed by microscopic evaluation of unbleached kraft fibers treated with the low molar mass xylan IV, since this xylan sample indeed incorporated into the fiber pores or

adsorbed on exposed fiber surfaces, providing a homogeneous treated material without any xylan precipitate (Figure 3). However, part of the high molar mass xylan I precipitated during the second equilibrium period and the xylan agglomerates remained entrapped by the fibers even after washing out with water. In this case, only two additional washings at pH 10 removed the entrapped xylan agglomerates (Figure 3). Similar results were already reported for xylans extracted from elephant grass in adsorption experiments on eucalyptus pulp fibers (Tamminen et al., 2018). Therefore, three types of xylan incorporation on the pulp fibers can be considered for further discussion in this article: a) xylan incorporated in the pores of the fibers; b) xylan adsorbed on the exposed surfaces of the fibers; and c) precipitated xylan entrapped by the fiber net.

Considering that the major monomeric sugar constituting prepared xylans was xylose (Table 1), the increases in xylan contents of the treated fibers was used to quantify xylan incorporation onto the studied pulps (Figure 4). At a mixing ratio of 10 xylan/100 g pulp, the high molar mass xylans I and II were poorly retained in both types of evaluated pulp fibers. In contrast, low molar mass xylans III and IV were incorporated at higher amounts, reaching the highest levels in kraft O_2 pulps. Kraft O_2 pulps appeared more susceptible for retaining the studied xylans probably because they have lower lignin contents (2.8 % and 4.9% in Kraft O_2 and unbleached Kraft pulps, respectively) and a consequent higher hydrophilicity (Miletzky, et al., 2015).

Poor incorporation of xylan I and xylan II on pulp fibers can be attributed to their higher molar masses (Figure 2), which difficult inclusion in fiber pores (Vincken et al, 1995), and the presence of more branched structures (Table 1), which restrain adsorption on flat cellulose chains (Kohnke et al., 2008; Kabel et al., 2007). In contrast, xylans III and IV presented the highest incorporation levels in both studied pulps, probably associated with their lower molar masses and lower substitution degrees. The xylan IV presented an interesting combination of structures containing fractions of low and high molar masses (Figure 2) in a single sample, and also the highest incorporation levels (Figure 4). These data suggest that the larger xylan fraction could adsorb on the exposed fiber surfaces, whereas the smaller xylan chains could penetrate into the pores of the fibers, keeping more firmly adsorbed to them (Muguet et al., 2011; Kabel et al. 2007; Vincken et al. 1995).

The efficient adsorption of less substitute xylans presenting low molar mass may be advantageous for papermaking processes. Barbosa, Colodette, Muguet, Gomes, & Oliveira, (2016) determined that this type of xylans adsorbs better to the fibers, and are more resistant to the bleaching step and to the mechanical forces involved in refining for papermaking. In addition, Danielsson & Lindström (2009) evaluated the adsorption of xylans from pulping black liquors and their influence on the quality of produced papers. Liquors containing xylans of low molar mass and low degree of substitution produced pulps with superior strength. These works corroborate with others demonstrating that the level of interaction between xylan and cellulose depends on the xylan structure (Kabel et al., 2007; Kohnke et al., 2008; Muguet et al., 2011).

The xylan IV, which presented the highest levels of incorporation onto pulp fibers, was further assayed at progressive initial loadings in incorporation experiments. Microscopic evaluation of the treated pulps showed that even the low molar mass xylan IV can precipitate during the washing procedure with water at neutral pH when mixed with fibers at loads from 250 g xylan/100 g pulp (Figure 5). In this case, xylan IV agglomerates appeared less frequently then observed for xylan I (Figure 3), but, again, only alkaline washing removed these entrapped xylan agglomerates.

Quantification of xylan IV incorporated onto the fibers demonstrated that an increase of the initial xylan to fiber ratio in the adsorption experiments contributed to increase incorporation levels (Figure 6). However, initial xylan loads from 250 g/100 pulp caused precipitation of part of the added xylan in form of entrapped agglomerates (Figure 5). These data taken together suggest that increasing xylan IV loadings provided increased levels of xylan incorporation in the pores and adsorbed on the exposed surfaces of the fibers, reaching xylan incorporation levels up to 4.7 g xylan/100 g pulp without any formation of xylan agglomerates. Initial xylan IV loads higher than 100 g/100 g pulp would only add entrapped xylan in the fiber net (Figure 6).

4. Conclusion

A variety of enzymatically-extracted xylans were prepared from alkaline-sulfite pretreated sugarcane bagasse with extraction yields from 12% to 44% depending on the reaction time and the xylanase load used in the extraction procedure. Short reaction time (6h) was useful to prepare high molar mass fractions that precipitated at ethanol concentrations up to 30%, but with low yield. A major fraction (94.6%) with low molar mass (2.9 kDa) did not precipitate even at 60% ethanol. Longer reaction time (24h) yielded a mix of xylan structures with varied molar masses. When these xylans were incorporated onto eucalyptus kraft pulps, the high molar mass fractions tended to precipitate and remain entrapped in the fiber net, whereas the low molar mass fractions have been adsorbed on fiber surfaces or included into the fiber pores. The highest xylan incorporation levels required low molar mass xylan fractions. The results allowed concluding that enzymatic xylan extraction from pretreated sugarcane is a distinguishing procedure to add value in the lignocellulose biorefinery chain once extracted xylans are suitable for incorporation onto kraft pulp fibers up to 4.7 g xylan/100 g pulp.

Acknowledgments

This work was supported by FAPESP (2014/06923-6) and CNPq (308570/2017-0; 303416/2018-1). The Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) - Finance Code 001 also partially financed this study. The authors would like to thank J. M. Silva for technical assistance.

References

Amoah, J., Kahar, P., Ogino, C., & Kondo, A. (2019). Bioenergy and biorefinery: feedstock, biotechnological conversion, and products.*Biotechnology Journal* , *14* (6), 1800494.

Bailey, M.J., Biely, P., & Poutanen, K. (1992). Interlaboratory testing of methods for assay of xylanase activity. *Journal of Biotechnology* , *23* , 257-270.

Ban, W.P., & Van Heiningen, A. (2011). Adsorption of hemicellulose extracts from hardwood onto cellulosic fibers. 1. Effects of adsorption and optimization factors. *Cellulose Chemistry and Technology* ,*45* , 57-65.

Barbosa, B.M., Colodette, J.L., Muguet, M.C.S., Gomes, V.J, & Oliveira, R.C. (2016). Effects of xylan in eucalyptus pulp production.*Cerne* , *22* (2), 207-214.

Bian, J., Peng, F., Peng, P., Xu, F., & Sun, R.C. (2010). Isolation and fractionation of hemicelluloses by graded ethanol precipitation from*Caragana korshinskii* . *Carbohydrate Research* , *345* , 802-809.

Chimphango, A.F.A. (2019). Sorption behaviour of enzymatically and chemically formed beechwood (*Fagus sylvatica*) xylan hydrogels onto cellulosic materials under different sorption conditions.*Journal of Polymers and the Environment* , *27* (3), 561-570.

Chimphango, A.F.A., Görgens, J.F., & van Zyl, W.H. (2016). In situ enzyme aided adsorption of soluble xylan biopolymers onto cellulosic material. *Carbohydrate Polymers* , *143,* 172-178.

Danielsson, S., & Lindström, M.E. (2009). The effect of black liquor exchange on the tensile properties of *Eucalyptus urograndis* kraft pulp. *O Papel* , *70* , 35–50.

Deutschmann, R., & Dekker, R.F.H. (2012). From plant biomass to bio-based chemicals: Latest developments in xylan research.*Biotechnology Advances* , *30* ,1627–1640.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., & Smith, F. (1956). Calorimetric method for determination of sugars and related substances. *Analytical Chemistry* , *28* , 350-356.

Ferraz, A., Baeza, J., Rodriguez, J., & Freer, J. (2000). Estimating the chemical composition of biodegraded pine and eucalyptus wood by DRIFT spectroscopy and multivariate analysis. *Bioresource Technology* , *74* , 201-212.

Gomes, K.R., Chimphango, A.F.A., & Gorgens, J.F. (2015). Modifying solubility of polymeric xylan extracted from *Eucalyptus grandis*and sugarcane bagasse by suitable side chain removing enzymes.*Carbohydrate polymers, 131* , 177-185.

Gouveia, R., Nascimento, R.T.D., Souto-Maior, A.M., & Rocha, G.J.D.M. (2009). Validação de metodologia para a caracterização química de bagaço de cana-de-açúcar. *Química Nova* , *32* (6), 1500-1503.

Han, W.J., Zhao, C.S., Elder, T., Chen, K.F., Yang, R.D., Kim, D., Pu, Y.Q., Hsieh, J., & Ragauskas, A.J. (2012). Study on the modification of bleached eucalyptus kraft pulp using birch xylan. *Carbohydrate Polymers* , *88* , 719-725.

Kabel, M.S., van den Borne, H., Vincken, J.P., Voragen, A.G.J., & Schols, H.A. (2007). Structural differences of xylans affect their interaction with cellulose. *Carbohydrate Polymers* , *69* (1), 94–105.

Köhnke, T., Brelid, H., & Westman, G. (2009). Adsorption of cationized barley husk xylan on kraft pulp fibres: Influence of degree of cationization on adsorption characteristics. *Cellulose* ,*16* (6), 1109–1121.

Kohnke, T., Pujolras, C, Roubroeks, J.P., & Gatenholm, P. (2008) The effect of barley husk arabinoxylan adsorption on the properties of cellulose fibres. *Cellulose, 15* , 537–546.

Kuhad, R.C., Gupta, R., Khasa, Y.P., Singh, A., & Zhang, Y.H.P. (2011). Bioethanol production from pentose sugars: Current status and future prospects. *Renewable & Sustainable energy* , 15, 4950-4962.

Laurito-Friend, D.F., Mendes, F.M., Reinoso, F.M., Ferraz, A., & Milagres, A.M.F. (2015). Sugarcane hybrids with original low lignin contents and high field productivity are useful to reach high glucose yields from bagasse. *Biomass and Bioenergy* , 75, 65-74.

Li, J., Kisara, K., Danielsson, S., Lindstrom, M.E., & Gellerstedt, G. (2007). An improved methodology for the quantification of uronic acid units in xylans and other polysaccharides. *Carbohydrate Research* ,*342* (11), 1442–1449.

Liu, X.X., Lin, Q.X., Yan, Y.H., Peng, F., Sun, R.C., & Ren, J.L. (2019). Hemicellulose from plant biomass in medical and pharmaceutical application: A critical review. *Current Medicinal Chemistry* ,*26* , 2430-2455.

Liu, J., Carrasco, G.C., Cheng, F., Xu, W., Willfor, S.M., Syverud, K., & Xu, C. (2016). Hemicellulose-reinforced nanocellulose hydrogels for wound healing application. *Cellulose, 23* , 3129-3143.

Magaton, A.S., Silva, T.C.F., Colodette, J.L, Piló-Veloso, D., & Milagres, F.R. (2013). Behavior of xylans from Eucalyptus species. Part 1. The influence of structural features of eucalyptus xylans on their retention in kraft pulp.*Holzforschung* , *67* (2), 115-122.

Mandegari, M.A., Farzad, S., van Rensburg, E., & Görgens, J. F. (2017). Multi-criteria analysis of a biorefinery for co-production of lactic acid and ethanol from sugarcane lignocellulose. *Biofuels, Bioproducts and Biorefining, 11* (6), 971–990.

Mendes, F.M., Heikkilä, E., Fonseca, M.B., Milagres, A.M.F., Ferraz, A., & Fardim, P. (2015). Topochemical characterization of sugar cane pretreated with alkaline sulfite.*Industrial Crops and Products, 69* , 60-67.

Mendes, F.M., Siqueira, G., Carvalho, W., Ferraz, A., & Milagres, A.M.F. (2011). Enzymatic hydrolysis of chemithermomechanically pretreated sugarcane bagasse and samples with reduced initial lignin content. *Biotechnology progress* ,*27* (2), 395-401.

Mendes, F.M., Vasconcelos, M.H., Dias, M.O.S., Ferraz, A., Milagres, A.M.F., Santos, J.C., Jesus, C.D.F., Watanabe, M.D.B., Junqueira, T.L., & Bonomi, A. (2018). Alkaline sulfite pretreatment for integrated first and second generation ethanol production: A techno-economic assessment of sugarcane hybrids. *Biomass and Bioenergy* , *119* , 341-321.

Miletzky, A., Punz, M., Zankel, A., Schlader, S., Czibula, C., Ganser, C., Teichert, C., Spirk, S., Zohrer, S., Bauer, W., & Schennach, R. (2015). Modifying cellulose fibers by adsorption/precipitation of xylan. *Cellulose* , *22* ,189-201.

Muguet, M., Pedrazzi, C., & Colodette, J.L. (2011). Xylan deposition onto pulp eucalypt pulp fibers during oxygen delignification.*Holzforschung* , *65* , 605-612.

Naidu, D.S., Hlangothi, S.P., & John, M.J. (2018). Bio-based products from xylan: A review. *Carbohydrate Polymers* , *179* , 28-41.

Peng, F., Ren, J.L., Xu, F., Bian, J., Peng, P., & Sun, R.C. (2009). Comparative study of hemicelluloses obtained by graded ethanol precipitation from sugarcane bagasse. *Journal of Agricultural and Food Chemistry* , *57* (14), 6305-6317.

Puchart, V., & Biely, P. (2008). Simultaneous production of endo-β-1,4-xylanase and branched xylooligosaccharides by *Thermomyces lanuginosus* , *Journal of Biotechnology* ,*137* (1), 34-43.

Santos, M.P., Reinoso, F.A.M., Tavilla, V., Ferraz, A., & Milagres, A.M.F. (2019). On-site produced and commercially available alkali-active xylanases compared for xylan extraction from sugarcane bagasse. *Biocatalysis and Agricultural Biotechnology* , *18* , 101081.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., & Templeton, D. (2008). Determination of sugars, byproducts, and degradation products in liquid fraction process samples. Laboratory Analytical Procedure (LAP). National Renewable Energy Laboratory. Technical Report, 14p.

Sporck, D., Reinoso, F.A.M., Rencoret, J., Gutiérrez, A., Rio, J.C.D., Ferraz, A., & Milagres, A.M.F. (2017). Xylan extraction from pretreated sugarcane bagasse using alkaline and enzymatic approaches. *Biotechnology for Biofuels* , *10* , 296.

Sun, J.X., Sun, X.F., Sun, R.C., & Su, Y.Q. (2004). Fractional extraction and structural characterization of sugarcane bagasse hemicelluloses. *Carbohydrate polymers* , *56* , 195-204.

Swennen, K., Courtin, C.M., Lindemans, G.C.J.E., & Delcour, J.A. (2006). Large-scale production and characterisation of wheat bran arabinoxylooligosaccharides. *Journal of the Science Food Agriculture* , *86* , 1722-1731.

Tamminen, T., Mikkelson, A., Siika-aho, M., Kruus, K. Pere, J., Gomes, F.J.B., & Colodette, J.L. (2018). Deposition of xylan isolated from *Pennisetum purpureum* on fibres of *Eucalyptus globulus* and characterisation of the composition of the surface xylans by immunolabelling and enzymatic peeling. *Holzforschung* , *72* (11), 915-922.

Vincken, J-P., Keizer, A., Beldman, G., & Voragen, A.G.J. (1995). Fractionation of xyloglucan fragments and their interaction with cellulose. *Plant Physiology* , *108* , 1579-1585.

Xu, F., Sun, J.X., Liu, C.F., & Sun, R.C. (2006). Comparative study of alkali-and acidic organic solvent-soluble hemicellulosic fraction polysaccharides from sugarcane bagasse. *Carbohydrate research* ,*341* , 253-261.

Table 1. Composition of enzymatically-extracted xylans prepared from alkaline-sulfite pretreated sugarcane bagasse as a function of varied extraction periods and graded precipitation with different ethanol concentrations.

Xylan sample	Relative amount (%, w/w)[a]	Chemical Composition (%, w/w)	Chemical Composition (%, w/w)	Chem
		Xylose	Arabinose	Glucu
Xylan I	2.6	57.5 ± 0.4	9.2 ± 0.1	6.0 ±
Xylan II	2.8	69 ± 2	12.8 ± 0.5	5.1 ±
Xylan III	94.6	70 ± 2	8.7 ± 0.3	4.3 ±
Xylan IV	whole	66.7 ± 0.4	9.5 ± 0.1	4.8 ±

(a) Xyl I to III corresponded to fractions of the total extracted xylan after 6 h extraction with xylanase. Xyl I and Xyl II precipitated at 15% and 30% ethanol, respectively. Xyl III did not precipitated even at 60% (v/v) ethanol concentration, being recovered by freeze-drying. Xyl IV corresponds to the whole material obtained after 24 h extraction and recovered by freeze-drying. Extraction experiments performed with 5% (m/v) pretreated sugarcane bagasse at 50 °C and pH 8.0.

Figure legends

Figure 1. Time course of xylan extraction from alkaline-sulfite pretreated sugarcane bagasse at varied xylanase loads (a) and effect of the xylanase load on the extraction yield after 24 h reaction (b). Extraction experiments performed with 5% (m/v) of pretreated sugarcane bagasse at 50 °C and pH 8.0.

Figure 2. Molar mass distribution of enzymatically-extracted xylans from alkaline-sulfite pretreated sugarcane bagasse. Xyl I to III corresponded to fractions of the xylan extracted after 6 h reaction with xylanase. Xyl I and Xyl II precipitated at 15% and 30% ethanol, respectively. Xyl III did not precipitated even at 60% (v/v) ethanol concentration, being recovered by freeze-drying. Xyl IV corresponds to the whole material obtained after 24 h extraction and recovered by freeze-drying. Extraction experiments performed with 5% (m/v) pretreated sugarcane bagasse at 50 °C and pH 8.0. For the sack of clarity, the relative absorbance values recorded for each xylan sample are displaced each other by 1.0 absorbance units.

Figure 3 . Microscopic evaluation of the unbleached kraft pulp fibers before and after treatment with a xylan solution at 10 xylan/100 g pulp. Bars indicate 50 μm for a magnification of 200 x. Arrows indicate the xylan agglomerates entrapped by the fibers. A wider view of the fiber net observed at 40x magnification presented similar results (data not shown), suggesting that the above presented microscopies fit well to the overall observation of the samples.

Figure 4. Incorporation of enzymatically-extracted xylans on unbleached Kraft and Kraft O_2 eucalyptus pulp fibers. Initial xylan loads corresponded to 10 g xylan/100 g pulp in all cases. Xyl I to III were obtained after 6 h extraction with xylanase and were recovered by precipitation with increasingly ethanol concentrations of 15% (Xyl I) and 30% (Xyl II). Xyl III did not precipitated even at 60% (v/v) ethanol concentration, being recovered by freeze-drying. Xyl IV was obtained after 24 h reaction and recovered by freeze-drying.

Figure 5. Microscopic evaluation of the unbleached kraft pulp fibers before and after treatment with xylan IV at progressive loadings in the incorporation experiments. Arrows indicate the xylan agglomerates entrapped by the fibers. Bars indicate 50 μm (magnification of 200 x). A wider view of the fiber net observed at 40x magnification presented similar results (data not shown), suggesting that the above presented microscopies fit well to the overall observation of the samples.

Figure 6. Incorporation of xylan IV on unbleached kraft pulp fibers as related to the initial xylan loading in the incorporation experiments.

Hosted file

`Figures.pdf` available at https://authorea.com/users/291191/articles/418329-enzymatic-extraction-of-xylans-from-alkaline-sulfite-pretreated-sugarcane-bagasse-and-their-incorporation-onto-eucalyptus-kraf-pulps