Exploring the effects of treatments with carbohydrases to obtain a high-cellulose-content pulp from a non-wood alkaline pulp

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Abstract

In this work, treatments with a xylanase (X) and carbohydrases mixture (Cx) were applied on a TCF bleached sisal pulp in order to obtain high-cellulose content fibers applicable on a wide range of uses. A limit of ≈12% w/w final content in hemicelluloses was found regardless of the enzymatic treatment assessed. An extraction with 4% and 9% w/v NaOH was performed for further hemicelluloses removal. We found that NaOH

dose could be strongly reduced if combined with Cx or Cx+X treatments. Also, if necessary, a stronger reduction could be obtained with 9% w/v NaOH, which was found to be boosted in a 14% if performed after a treatment with Cx. An end-product with a low content in xylans (≈2.9% w/w) and in HexA (5.8 µmol/odp) was obtained. Pulp Fock solubility was also increased (≈30%) by enzymatic treatments. HPLC analysis of effluents provided useful information of enzymatic catalytic mechanisms.

Keywords

Carbohydrase, Enzymatic treatment, Xylanase, endoglucanase, high-cellulose content, non-wood pulp.

Introduction

There is a growing interest in society to move towards a bio-based economy where a bigger part of our daily products can be provided by agriculture (Ibarra, Kopcke, Larsson, Jaaskelainen, & Ek, 2010). In this direction, making agriculture not just a food provider, but a producer of other raw materials, by taking advantage of non-food residues generated upon food cropping or cultivating other fiber-providing species, can be a good way to move towards this new economic model. Fibers quality improvement from paper grade to high cellulose content has attracted plenty of interest in recent years (Li, Zhang, Duan, Liu, & Ni, 2015). Traditionally, pulps with low hemicelluloses content have been obtained through acid sulphite or pre-hydrolysis Kraft process (Li et al., 2015). On these processes, hemicelluloses that are present on pulp suffer a greater attack than during alkaline processes such as Kraft or NaOH-AQ, reducing their presence on final product. However, pulps obtained through these processes have some drawbacks related to quality of final product or the pollution they

generate. Also, these pulping processes imply higher costs than alkaline ones in terms of chemical consumption, production rate, inventories and storage space (Barlow & Hillman, 2006). For these reasons, several methods have been studied in order to carry out the selective elimination of hemicelluloses from alkaline pulps (Bajpai & Bajpai, 2001; Jackson, Heitmann Jr., & Joyce, 1998; Kopcke, Ibarra, & Ek, 2008). These methods include nitren, cuen and alkaline extraction. Besides them, enzymatic hydrolysis of different components of lignocellulose has attracted special attention because of its potential as a "green" process. It is well known that biomass availability to enzymes is hindered by diverse factors (Zhu, O'Dwyer, Chang, Granda, & Holtzapple, 2008). Because of this, several methods have been studied for enhancing enzymatic biomass conversion, including physical, chemical, biological or thermophysical pretreatments (Maache-Rezzoug, Pierre, Nouviaire, Maugard, & Rezzoug, 2011; Pierre, Maache-Rezzoug, Sannier, Rezzoug, & Maugard, 2011). Among enzymes, xylanases have been traditionally used in pulp and paper industry for pulp bleaching (Fillat, Roncero, & Vidal, 2011; Valls & Roncero, 2009). In this work, however, they are applied on bleached pulps with the purpose of removing hemicelluloses. Other enzymes, such as endoglucanases (cellulases), have been mainly used by authors for fibers biorefining (Garcia-Ubasart, Torres, Vila, Javier Pastor, & Vidal, 2013), biomass saccharification (Pierre, Maache-Rezzoug, et al., 2011; Pihlajaniemi, Sipponen, Sipponen, Pastinen, & Laakso, 2014; Zhang, Tang, & Viikari, 2012) or increasing cellulose reactivity (Kopcke et al., 2008; Miao et al., 2014; Pierre, Sannier, et al., 2011).

Among the formerly stated fiber-providing species, sisal constitutes a raw material with a great potential for several applications. These fibers have traditionally been used to manufacture natural ropes, cordage and sacking. Regarding their potential

as a raw material for pulp and paper industry, sisal fibers present some positive features including a high tear resistance, alpha cellulose content, porosity, bulk, absorbency and folding endurance, making it excellent for a variety of specialty papers (Aracri & Vidal, 2012). In addition, as sisal fibers have better physical properties than softwood kraft fibers, they become a good raw material for reinforcing fiber in paper with high recycled content, or for reducing basis weight while maintaining product quality (Maddern & French, 1995). This study focuses on the possibility of using a bleached non-wood pulp, from sisal (*Agave sisalana*), to obtain a high cellulose-content pulp by means of enzymatic treatments, combined with alkaline extractions. A bleached sisal pulp was used and different enzymatic treatments with new carbohydrases were applied in order to modify this pulp. Although similar works have been published in literature (Henriksson, Christiernin, & Agnemo, 2005; Ibarra, Kopcke, & Ek, 2009; Wang et al., 2014), this study introduced new enzymes, a xylanase and a carbohydrases mixture (containing endoglucanase and xylanase activities, not still commercially available) together with a NaOH extraction. Enzymes also permitted a reduction in the use of NaOH. Furthermore, possibilities of enzymatic treatments were studied by assessing different ways of applications including a newly focused evaluation of xylanolytic treatments. Also, a comprehensive study of enzymatic effects on fibers was performed for better understanding the effects of these new catalysts.

Materials and methods

Pulp

A totally chlorine free (TCF) bleached pulp from sisal (*Agave sisalana*) was used as a raw material. Pulp was provided by Celesa (Spain), and was obtained by an alkaline NaOH-AQ process. Pulp initial parameters were: Content in hemicelluloses (Xylans, %

w/w) = 16.1 ± 0.3; Kappa Number (KN) = 4.7 ± 0.2; ISO Brightness (%) = 82.1 ± 0.3; Viscosity (mL/g) = 616 ± 41; HexA content (µmol/g odp) = 45.1 ± 1.5; Fock solubility 13.2 ± 0.2%.

Enzymes

A xylanase (X) and a carbohydrases mixture (Cx) were used for treatments, both provided by Fungal Bioproducts (Spain) and obtained from *Cerrena sp* fungus. Carbohydrases mixture (Cx) had both Carboxymethylcellulase (CMCase) and xylanase activities. Activities as U/g dried enzyme powder were: 11000 U/g for the xylanase (X), 1700 U/g and 680 U/g for the cellulase and xylanase activity on the mixture (Cx), respectively. Enzymatic activity was determined at application conditions (50 ºC and pH 7 for X; and 55 ºC and pH 5 for Cx). An activity unit (U) is defined as the amount of enzyme capable of converting 1 µmol of substrate per minute. Enzymatic activity was determined using Spiro method to quantify released reducing sugars (Spiro, 1966) after a microscale enzymatic reaction carried out for 15 minutes. Substrate was carboxymethyl cellulose (CMC) or birchwood xylan for measurement of cellulolytic and xylanolitic activity, respectively. Prior to treatments, sugar presence on enzymatic preparations was analyzed using the same method as for effluents (described below). None of the studied oligosaccharides was found on these preparations.

Enzymatic treatments

Enzymatic treatments were held using X enzyme (Figure 1A) and combined treatments using Cx and X enzymes (Figure 1B). Letter "K" in a sample name indicates a control (*e.g.* KX indicate control for X treatments, and KCx for Cx). Control samples were prepared using the same conditions as in enzymatic treatments, but with no enzyme addition. After treatments, an aliquot of effluents was saved for analysis. Enzymatic

reactions were then stopped washing pulps with decalcified water three times and one time with deionized water.

1. Treatments with xylanase (X) (Figure 1A) were applied in plastic bags on a thermostatic bath with a 10 U/g oven-dried pulp (odp) dose at 50 ºC, 10 % consistency, pH 7 (adjusted with 50 mM Tris-HCl buffer) and manual agitation every 10 min according to two different procedures:
   1.1 Direct (X/KX): Reaction was carried out up to 5h, and samples were collected after each hour for characterization (X_1, X_2, X_3, X_4 and X_5).
   1.2 Stepwise addition (Xs/KXs): 2U/g odp xylanase were added 1h periods which were immediately followed by washing with deionized water (Xs_1, Xs_2, Xs_3, Xs_4 and Xs_5). At the end of treatment (5h) a final dose of 10 U/g odp was applied, equivalent to that used on "direct" treatment.
2. Treatments with carbohydrases mixture (Cx) and xylanase (X) (Figure 1B):
   2.1. Treatments with Cx: Performed at 55 ºC and pH 5 adjusted with 50mM sodium acetate buffer. Applied dose was 10 CMCase U/g odp. Two different conditions were assessed:
      2.1.1. Cx treatment: Carried out for 2 h in plastic bags at 10 % consistency, with manual agitation every 10 min.
      2.1.2. Cx24h treatment: Carried out in an Ahiba Easydye oscillating reactor for 24 h at 5 % consistency with agitation set at 20rpm.
   2.2. Combined treatments: After Cx treatment (2 h), X was applied for 2 and 5 h with a 10 U/g odp dose, 50ºC, 10% consistency and pH 7 (Tris-HCl), as for X2, X5 (part 1.1).

Alkaline extraction (E stage)

An alkaline extraction with NaOH was performed after enzymatic treatments for further hemicelluloses removal. Two different conditions were assessed: 4 % (w/v) NaOH for 2 h, 25 ºC and 5 % consistency; and 9 % (w/v) NaOH for 1 h, 25 ºC and 5 % consistency. Pulps were extensively washed with deionized water after alkaline extraction.

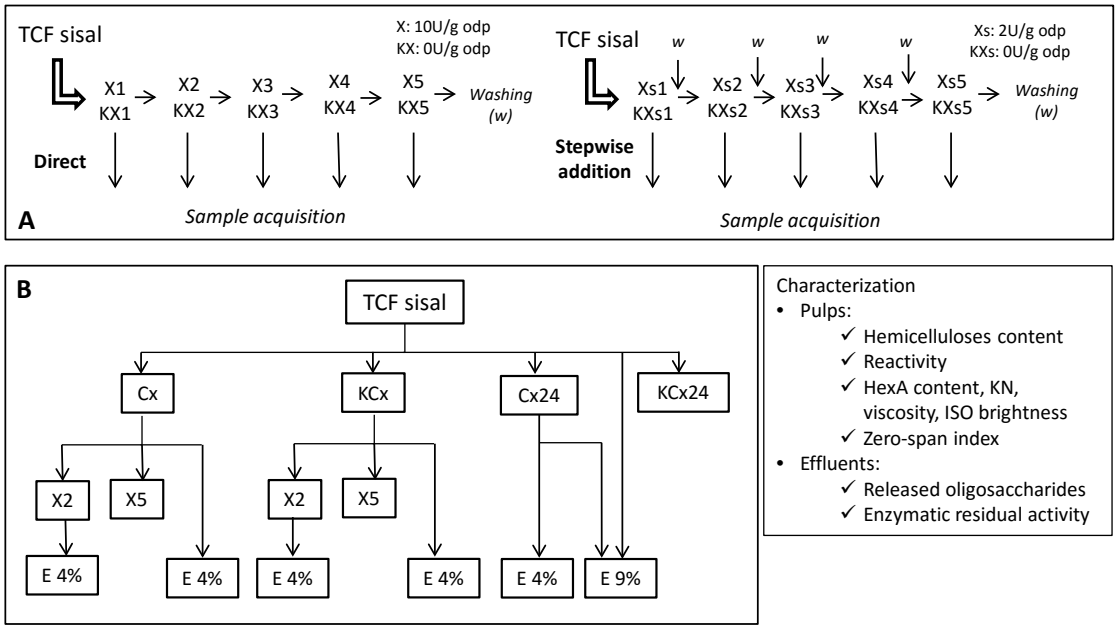


Figure 1: Work scheme of the present study

Pulp properties

Kappa number (KN), ISO brightness, viscosity, wet zero span index and hexenuronic acid (HexA) content of initial and treated pulps were determined according to ISO 302:2004, ISO 3688:1999, ISO 5351:2010, ISO 15361:2000 and TAPPI T282 om-13, 2013, respectively.

KN_{lignin}/KN_{HexA}: An estimate of the actual lignin content of samples was obtained by determining KN due to lignin (KN_{lignin}). This involved determining KN after HexA removal with mercury acetate and efficient washing with distilled water. KN_{HexA} was estimated by the difference of total KN and $KN_{lignin.}$ This procedure was already used to clarify enzymes action over pulp (Valls et al., 2010).

Chain scission number: Pulp degradation can also be assessed *via* the number of scissions in the cellulose strain. Chain scission number (CSN) is defined mathematically as (Jean Bouchard, Méthot, & Jordan, 2006):

$$CSN = \left[\frac{1}{DP} - \frac{1}{DPo}\right] DPo$$

Where: DP_O is the degree of polymerization of the initial pulp, and DP that after treatment. The degree of polymerization is calculated from the intrinsic viscosity [η], using the equation of (SCAN-CM 15:88): $DP^{0.085} = 1.1 \cdot [\eta]$

Carbohydrate composition: Carbohydrate composition of initial and treated pulps was determined using high performance liquid chromatography (HPLC) following a modified version of TAPPI T 249 cm-09, 2009 method (Aracri & Vidal, 2011). Chromatographic analysis was performed using a 1100 Agilent HPLC instrument furnished with a BIO RAD Aminex HPX-87H ion-exchange column. Data was collected by the refractive index detector (RID). Operating conditions were as follows: 0.6 mL/min, mobile phase H_2SO_4 6 mM and temperature 60 ºC. Concentrations were calculated by interpolation into calibration curves run from standards of glucose, xylose, ramnose and arabinose. Because the column fails to resolve xylose, mannose and galactose, their combined content is expressed as xylose.

Moist heat ageing: Pulps were aged in an ageing vessel at 80 ºC and 65 % of relative humidity (RH) during 72 h according to ISO 5630-3:1996. After ageing essays, brightness loss index (BLI) was calculated using the following formula:

$$Brightness\ loss\ index\ (\%) = \frac{B_0 - B_{72}}{B_0} * 100$$

Where: B_0 is the initial brightness value for pulp and B_{72} is the brightness value after 72 h of moist heat exposure.

Cellulose Fock Solubility (reactivity): Reactivity values of samples were determined according to slightly modified version of Fock's method (Kopcke et al., 2008). The Fock method is a micro-scale process simulating the industrial viscose process for the manufacture of regenerated cellulose. Prior to analysis, samples were dried overnight in a controlled atmosphere (25 ºC and 50% RH). Reactivity measurements were expressed as the regenerated cellulose yield (Ibarra et al., 2010):

$$X = 100 * 10(a)\ \frac{M(V_1C_1 - V_2C_2 * \frac{100}{40\ (b)})/6)}{4Y}$$

Where: X is the reacted cellulose (%), Y is the weight of sample (g), M is the molecular mass of glucopyranosyl residue, $C_6H_{10}O_5$ (162 g/mol), V_1 is the volume of added $K_2Cr_2O_7$, V_2 is the volume of titrated $Na_2S_2O_3$ (L), C_1 is the concentration of $K_2Cr_2O_7$ (mol/L), C_2 is the concentration of $Na_2S_2O_3$ (mol/L), *a* is the first dilution to 100 g and outtake of 10 mL (10.4 g) = 100/10.4 = 9.62, and *b* is the second dilution of the sample to 100 mL and outtake of 40 mL = 100/40.

SEM microscopy: Small pieces of paper of each sample were used for SEM analysis with a JEOL JSM-6400 microscope operating at 10 kV. Samples were first coated with a very thin layer (14 nm thick) of gold–palladium in a sputter coater SCD005 in order to obtain a conductive surface.

Effluent properties

Residual enzymatic activity: It was determined using an adapted version of Somogyi-Nelson method to determine reducing sugar concentrations on a solution (Spiro, 1966).

Released carbohydrates: They were identified and quantified on effluents using an 1100 Agilent HPLC instrument furnished with a BIO RAD Aminex HPX-42A ion-exchange column. Samples were filtered using a 0.45 µm pore size Whatman membrane and their pH was neutralized (to pH 7) using HCl or NaOH. Operating conditions were: 0.35 ml/min flow, column temperature 65 ºC and the mobile phase was MQ water. Identification and quantification of compounds was done by interpolation into calibration curves run from standards.

Protein content: Protein content in effluents was measured using Bradford's micromethod (Bradford, 1976).

Results and discussion

1. Xylanase treatments

This work was focused on the removal of the highest possible amount of xylans (hemicelluloses) from a TCF bleached sisal pulp with high hemicelluloses content (16.1 ± 0.2% w/w). For this purpose a higher dose of xylanase (10 U/g odp), than those commonly applied on bleaching processes (3 U/g odp) (Valls & Roncero, 2009) was applied. In order to find the best option, we decided to administrate the same dose according to two different procedures (Figure 1A), while only after 5 treatment hours xylanase dose was equivalent. The "*direct* (X)" treatment allowed the evaluation of xylanase effects on this higher dosage. "Stepwise addition" (Xs) of xylanase was carried out with the intention of studying the performance of the enzyme in a fresh environment for reaction each hour. As described elsewhere, this application procedure could led to better results in enzymatic hydrolysis of biomass (Pihlajaniemi et al., 2014).

Carbohydrate composition was determined for samples, with special interest on xylans content. As shown on Figure 2a, X enzyme produced deeper effects removing hemicelluloses on X (direct) treatments than did on Xs (stepwise addition). Pulps with xylans content close to 12% w/w were obtained already at 3 h of interaction with enzyme, suggesting that the maximal limit of accessible hemicelluloses was hydrolyzed. Xs treatment, by its side, did not provide any evidence of reaching this availability limit as a final value of xylans of 13.9% w/w was reached (Xs5). Enzymatic hydrolysis of biomass is limited by factors traditionally divided into two groups: biomass structural features and enzyme mechanisms (Zhu et al., 2008). Regarding physical structural features, it has been proposed that accessibility problems could be caused by: xylanase size (being too big to enter into fiber structure), fiber pores being too small or too few and/or the existence of an insufficient surface area, with a lower ratio than optimal (Ibarra et al., 2010). Chemical structural features of xylans such as composition, ramification or acetylation (Zhu et al., 2008); or their linkage to cellulose or lignin (Dammström, Salmén, & Gatenholm, 2009) could also affect their digestibility. Finally, enzyme features such as their diffusion or product inhibition could also hinder these reactions. Carbohydrate composition of samples provided evidence that direct addition of 10 U/g odp of X enzyme was more efficient for xylans removal than the stepwise admixture.

Besides the removal of hemicelluloses, X and Xs treatments influenced other pulp characteristics. A 2.5 % ISO brightness increase was obtained with X treatment and a 1 % ISO after Xs, representing an improvement in pulp quality (Figure 2b). KN (Figure 2d) decreased a 31 % with X treatment and a 24 % after Xs treatment (Samples X5 and Xs5, respectively). KN can be influenced not only by lignin, but by any other component capable of being oxidized by potassium permanganate (Gellerstedt & Li,

1996). Because of this, KN values were also expressed as produced by lignin or HexA, while KN_{lignin}+KN_{HexA}= Total KN (Figure 2d). Figure 2c showed a reduction of about 25 % in HexA content on X5 pulp compared to starting pulp, supporting data from KN_{HexA}. This reduction was similar to reductions reported by other authors (≈27 %) (Aracri & Vidal, 2011), observed on a non-bleached sisal pulp also with xylanase treatments. HexA removal importance is due to their negative effects on some pulp properties, such as brightness reversion (Cadena, Vidal, & Torres, 2010). Finally, a linear correlation seemed to exist between xylans content of pulps and HexA content (Figure 2a and c), which was logical attending the fact that HexA are contained in hemicelluloses (Valls et al., 2010). Regarding viscosity, no statistically significant difference was observed between starting, treated pulps and controls (Table 1). However, a small increasing tendency was observed, which could have been due to that removal of hemicelluloses, short polysaccharides, increased average degree of polymerization and thereafter, viscosity (Fillat et al., 2011; Roncero, Colom, Vidal, & Roncero, M B; Colom, J M; Vidal, 2003).

	Viscosity (mL/g)					
Time (h)	Starting	1	2	3	4	5
X	616 ± 41	644 ± 7	657 ± 20	644 ± 24	657 ± 33	692 ± 22
Xs	616 ± 41	610 ± 27	628 ± 29	646 ± 27	642 ± 22	651 ± 2
KX	616 ± 41	-	617 ± 20	-	-	641 ± 8
KXs	616 ± 41	642 ± 26	640 ± 15	652 ± 16	646 ± 41	650 ± 17

Table 1: Viscosity values of starting, xylanase treated and control pulps. Mean value ± confidence interval.

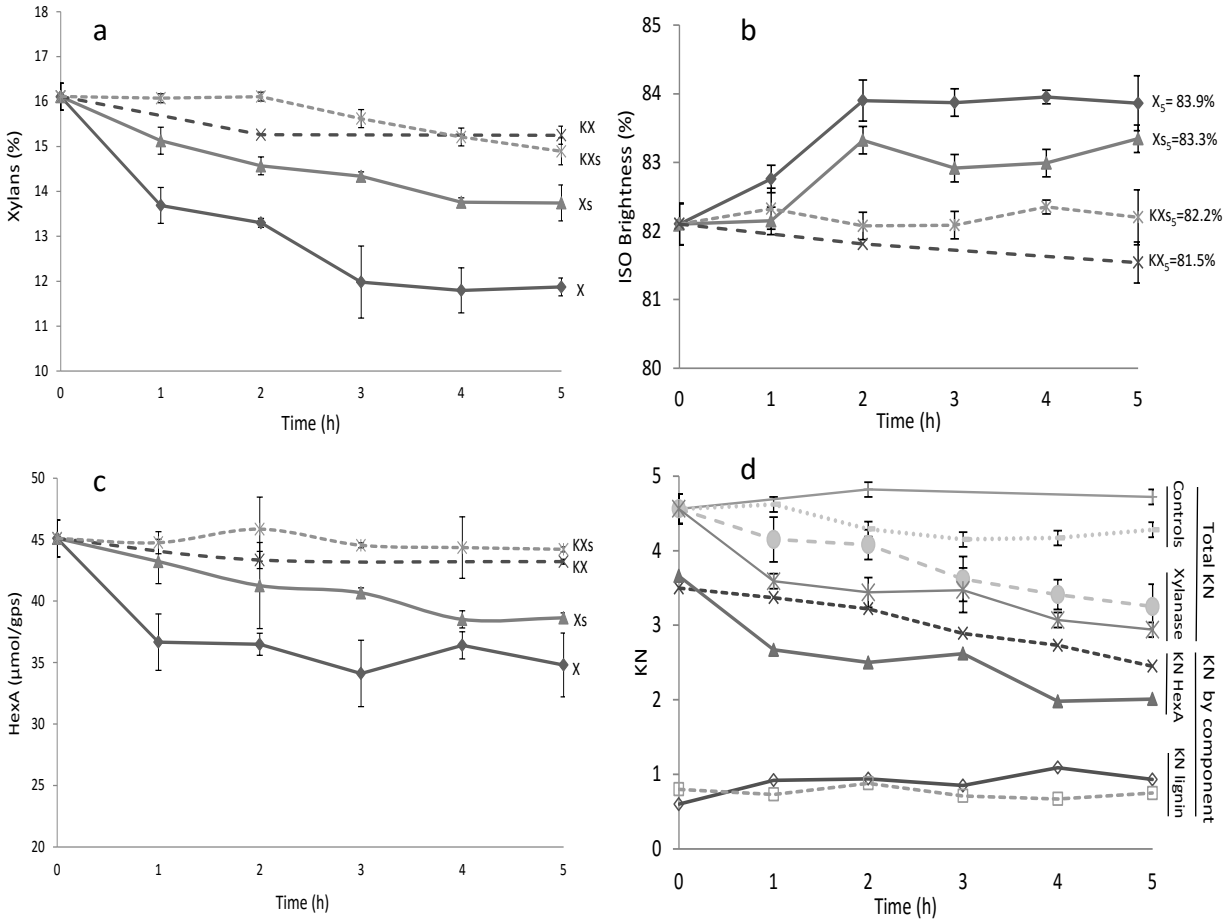


Figure 2: a- Xylan content (%); b ISO Brightness (%); c – HexA content (µmol/g odp) and d- KN values along xylanolytic treatment hours. Specific values of KN due to HexA and to lignin are also indicated. Mean values are represented, error bars indicate confidence intervals.

Ageing of pulps, measured as ISO brightness loss (%) is a process associated with many compounds present on pulp, such as lignin and HexA (Cadena et al., 2010). A reduction in pulp brightness loss implies an increase in pulp quality and durability. Treatments with X seemed to reduce brightness loss (Table 2), accounting for higher reductions after longer treatments, suggesting that these treatments were useful increasing fibers quality.

Time (h)	1	2	3	4	5
X	37.9 ± 0.3	17.5 ± 0.1	14.2 ± 0.1	11.8 ± 0.1	11.8 ± 0.2
KX	-	31.6 ± 0.4	-	-	32.5 ± 0.6

Table 2: Brightness loss index (BLI, %) after 72h of moist heat ageing

After treatments with xylanase, enzymatic activity was measured in effluents and it was expressed as a % of the initial dose that remained active. Re-using effluents for treating pulps instead of using fresh enzyme would reduce the economic cost related to their use, reducing one of the obstacles to their usage. Results indicated that a high proportion of initial activity was conserved even after 5h of treatment, as in all cases more than 60 % of initial activity was still present on effluents (data not shown). Finally, at the end of xylanolytic treaments (X and Xs) we found the same protein amount as initially administrated for treatments, confirming that X enzyme did no remain attached to fibers after reaction.

For a better understanding of enzymatic effects, oligosaccharides released from pulp were measured on reaction effluents following a similar protocol as reported by other authors (Garcia-Ubasart et al., 2013; Zhang et al., 2012). Sugar concentrations on effluents increased along treatment time (Figure 3a and b). Regarding enzyme action mechanism, Figure 3a and 3b provided evidence that this enzyme might be cleaving xylans chains preferably by 2 -3 subunits. This evidence showed that X enzyme produced xylo-oligosaccharides of lower molecular weight than some novel xylanases applied by Valls et al. (Valls et al., 2010). Xylans were also cut into xylose (X1 on chart), but in a lower amount than the other two sugars. For both treatments, the release pattern, by concentrations was Xilobiose> Xilotriose> Xilose. Data on charts (Figure 3) supported the evidence that direct treatment (X) was more efficient to our purposes than the stepwise admixture (Xs), as higher final concentrations of xylooligomers were released from pulp on the former. This differs from other works where stepwise addition of enzymes provided better results than direct one (Pihlajaniemi et al., 2014), showing that each enzyme behavior is unique.

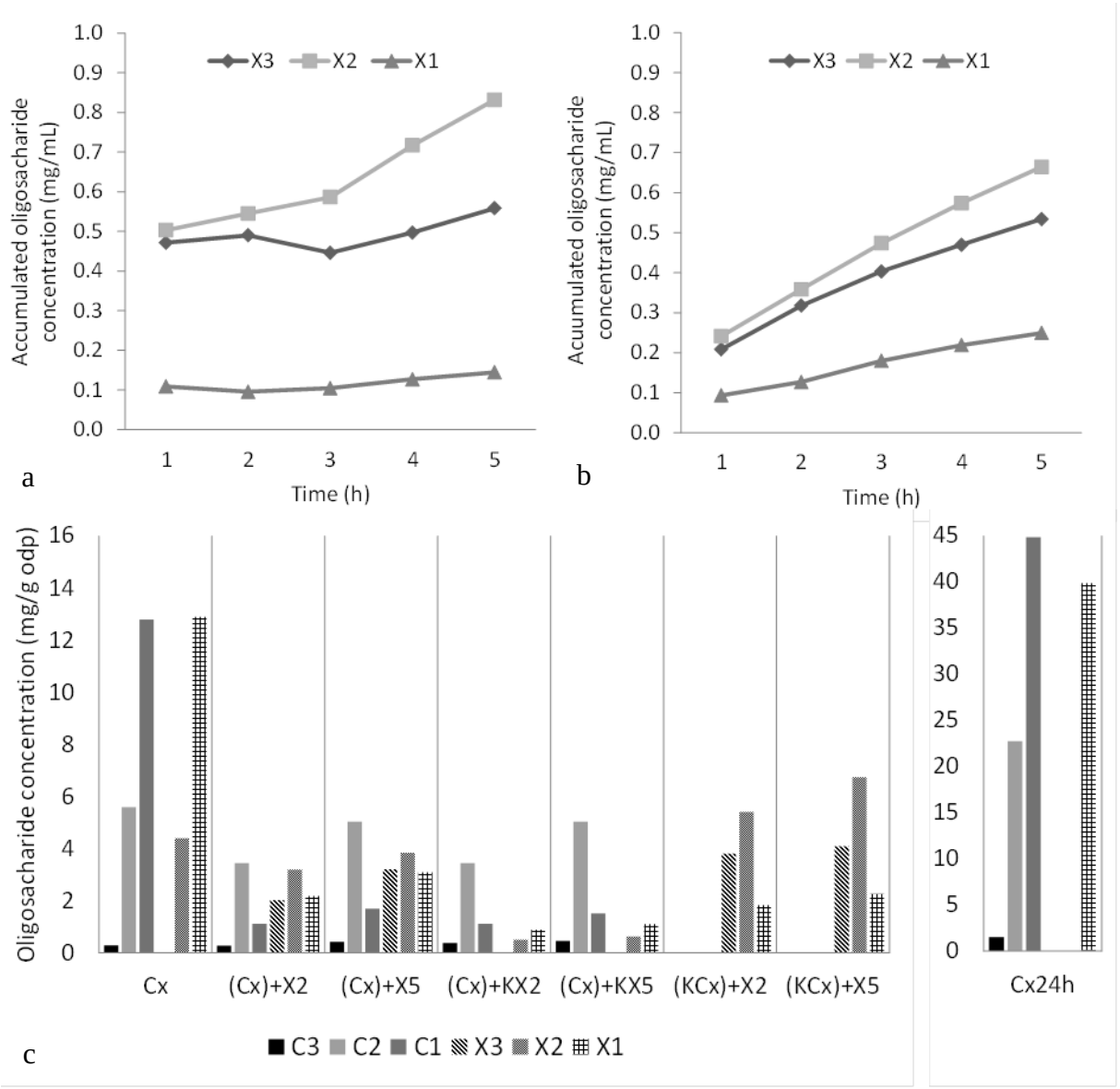


Figure 3 a and b: Accumulated concentration (*i.e.* adding the amount released each hour) of reducing sugars on effluents of treatments with X (a: X treatment, b: Xs treatment). Figure c: Concentration of sugars on effluents (expressed in relation to pulp mass) of combined treatments. Previous treatment, when available, is indicated between parenthesis and only data of effluents of the second treatment is shown. X3, X2 and X1 stands for xilotriose, xilobiose and xylose, respectively; C3, C2 and C1 stand for cellotriose, cellobiose and glucose, respectively

2. Combined enzymatic treatments

A carbohydrase mixture (Cx) was applied on pulp alone and followed by X enzyme with the intention of further reducing fibers xylans content. However, on Figure 4a, it can be seen that a 25% reduction was achieved combining Cx and X5 treatments,

reaching a final content of xylans of 12 % w/w, the same obtained with X5 treatment alone. Again, a limit to enzymatically degradable xylans seemed to be found. Also, we found that X enzyme applied after Cx produced a lower effect than when applied alone. Observing data at Figure 4a, we noticed that xylanase contained into Cx seemed to be more efficient than X enzyme. Cx was applied based on CMCase activity, and when 10 CMCase U/g odp were applied for 2 h, the applied equivalent xylanase dose (of Cx) was 4 U/g opd. Attributing all hemicelluloses removal to this xylanase activity, we assumed that this 4 U/g caused 11% elimination in 2 h, compared to the 15 % elimination provoked by 10 U/g of X produced within 2 h. Calculating the effect *per U* it can be seen that Cx xylanase activity seemed to be twice as effective X, applied alone or after KCx. Because of this, a very prolonged treatment (24 h) was performed with Cx in order to study the potential of this enzyme. Enzymatic residual activity confirmed that Cx was still active at the end of this treatment. A 12.4 % w/w final content in hemicelluloses was reached after Cx24h treatment, while Cx treatment leaded to a final value of 13.5 % w/w, removing 23 % and 16 % of xylans from initial pulp, respectively (Figure 4a). Regarding other characteristics, it can be observed that Cx produced similar effects than X enzyme: Removing HexA, decreasing KN and increasing ISO Brightness (Figure 4b, c and d).

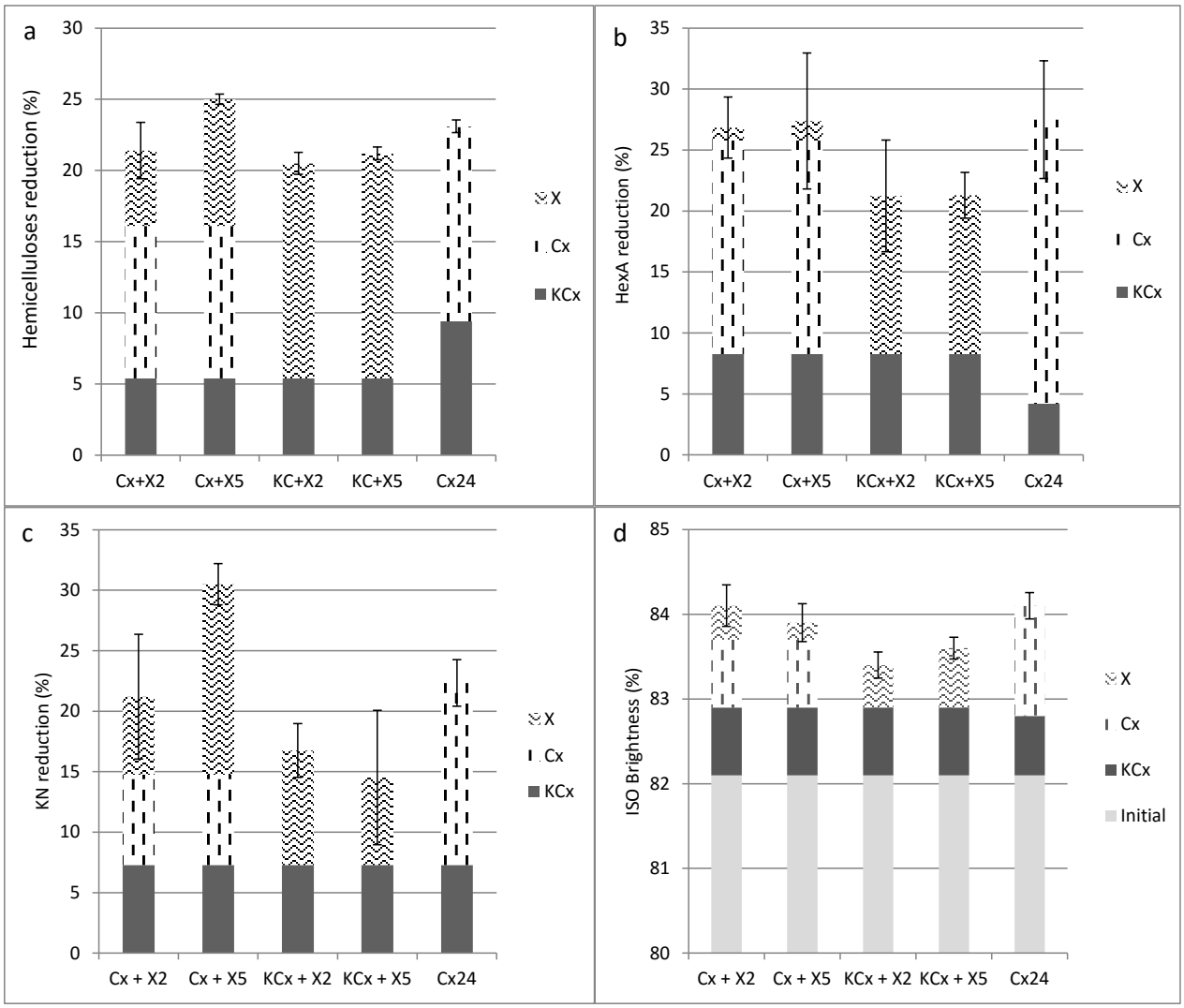
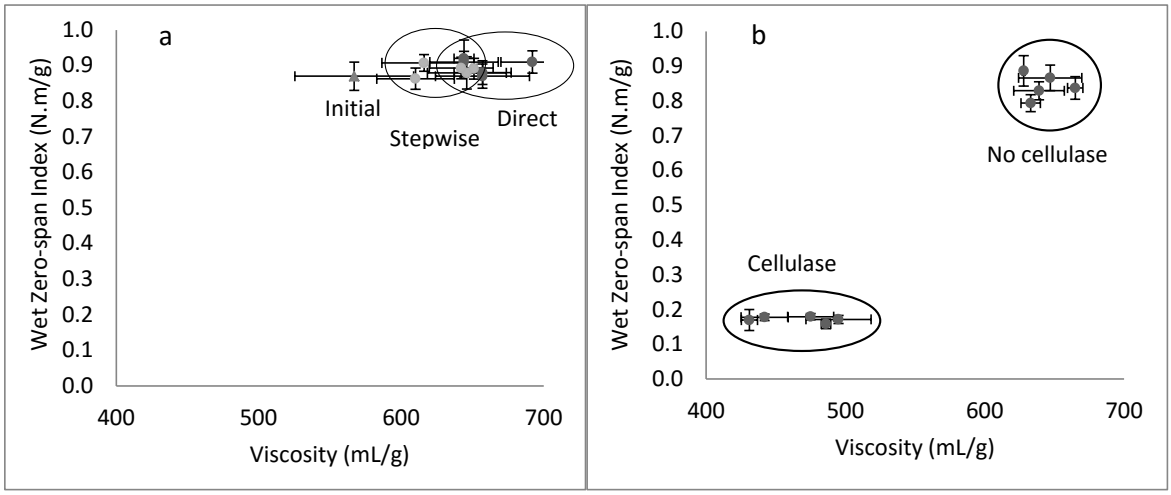


Figure 4: Reduction in hemicelluloses content (a), HexA content (b) and KN (c) compared to initial pulp, and increase in brightness (d) produced by combined treatments, indicated as a contribution of each enzymatic step. Error bars indicate confidence intervals.

Cx pre-treatment also produced a decrease on fibers viscosity (Table 3), as other authors have reported for these type of enzymes (Wang et al., 2014). Cleavage of cellulose chains by Cx leaded to a reduction on degree of polymerization (DP) of cellulose, reducing the viscosity of pulp. By its side, again, X enzyme action produced a slight increase on this parameter (Table 3). Wet zero-span fiber resistance represents a measure of fibers intrinsic strength, *i.e.*, in independence of fibers network (Hägglund, Gradin, & Tarakameh, 2004). It indirectly indicates the physical integrity of fibers, and

346 can provide more information of treatments influence on pulp. We observed that X
347 treatments did not affect fibers mechanical resistance (Figure 5a), while Cx application
348 produced a reduction in zero-span index (Figure 5b). This reduction was probably
349 provoked by cellulolytic action, as previously reported (Garcia-Ubasart et al., 2013;
350 Suchy et al., 2009), and went accompanied with a reduction in cellulose viscosity.
351 Figure 5 suggested that fibers intrinsic strength and their viscosity were two related
352 parameters.



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354 **Figure 5: Wet zero-span index values expressed in front of viscosity for (a) xylanase treatments (X, Xs) and (b)**
355 **Carbohydrase mixture (Cx) + xylanase (X) treatments. Error bars indicate confidence intervals.**

	Cx	KCx	Cx24	KCx24	Cx + X2
Fock reactivity (%)	56.1 ± 2.4	26.7 ± 1.4	61.3 ± 1	30 ± 1.3	53.9 ± 0.4
Viscosity (mL/g)	431 ± 6	628 ± 1	365 ± 8	607 ± 27	486 ± 3

Table 3: Fock reactivity (as % of reacted cellulose) and viscosity values of samples

356

357 Cx treatments produced both glucose and xylose oligosaccharides (Figure 3c)
358 Released oligosaccharides from Cx treatments were now expressed in relation to pulp,
359 not as concentration in effluents because of the existing differences in consistency
360 among treatments. Cx xylanase activity released xylose in the first place and xylobiose

in a lower amout after 2 h of reaction, while only xylose was found after 24 h. This finding suggests the existence of a beta-xylosidase activity on Cx enzyme. Higher amounts of xylo-oligosaccharides were released after 24 h compared to 2 h (expressed as xylose-equivalents), results that were consistent with the higher xylan elimination. Regarding glucose oligosaccharides, proportions between released oligosaccharides were maintained during both treatments, with larger releases after 24h compared to 2h. Of total released, cellotriose represented ≈2%, cellobiose a 31% and glucose a 67% after Cx and Cx24 treatments (Figure 3c). Cx cellulolytic activities removal pattern was then glucose> cellobiose> cellotriose. This pattern was different from others reported for other cellulolytic enzymes (Garcia-Ubasart et al., 2013), where cellobiose> glucose> cellohexose> cellotriose was found. Data from Cx treatment effluents suggested that this enzymatic preparation contained a β-glucosidase activity converting larger oligosaccharides to glucose. Cellotriose and cellobiose could act as a cellulase inhibitors (Philippidis, Smith, & Wyman, 1993), so the existence of this activity would improve the efficiency of the enzyme. Finally, X enzyme applied after Cx had a similar behavior than when applied alone.

3. Achieving a better pulp quality

As commented in previous sections, our enzymes seemed to found a limit of degradable xylans in sisal fibers. For a further reduction, some authors proposed a treatment with NaOH (Hakala, Liitiä, & Suurnäkki, 2012; Ibarra et al., 2010). In the present work, two different NaOH concentrations were assessed. It is proposed that the two types of xylans previously mentioned (Dammström et al., 2009) were being preferably attacked by enzyme and the others by NaOH.

Data on Figure 6 showed that xylans and HexA elimination caused by 4% w/v NaOH (E4%) was similar in all cases (≈30% reductions) achieving total reductions of ≈50% after both enzymatic and chemical stages. Previous works using a enzymatic treatment combined with 4% w/v NaOH reported a similar total reduction in xylan content, around 50% (Hakala et al., 2012). For greater reductions, a stronger extraction, with NaOH 9 % w/v was performed after Cx24 treatment and also on initial pulp, achieving total reductions of 82 % and 45% of xylan content, respectively (Figure 6a). Combining Cx24 and E 9%, final xylans content in pulp of 2.9% w/w was obtained, a satisfactory value for our purposes. Furthermore, we found that Cx + X2 and Cx24 treatments, combined with E 4% produced higher xylans removal than E 9%, allowing a reduction of 55% in the amount of NaOH needed to reach a given purity degree. Also, E 9% removed a 59% of hemicelluloses after Cx24 treatment, achieving a greater reduction than when performed on initial fibers. This evidence shows that combining enzymatic treatments with NaOH not only provided a higher total reduction (82%), but also seemed to produce a synergetic effect, as Cx treatment boosted E 9% effect by increasing it effect in 14%. This synergy would also permit a reduction in the need of NaOH for reaching a specific purity, highlighting the potential of enzymes as green catalysts. By its side, Figure 6b showed that HexA content was reduced in similar proportions to xylans content, as expected. E stage also influenced other pulp properties. It reduced KN up to 3 units, it increased ISO brightness, with gains from 1.5 % to 3 % ISO and it did not produce remarkable effects on viscosity. Finally, E stage did not affect mechanical resistance of fibers either, as very similar zero-span values as those obtained after enzymatic stages were obtained (data not shown).

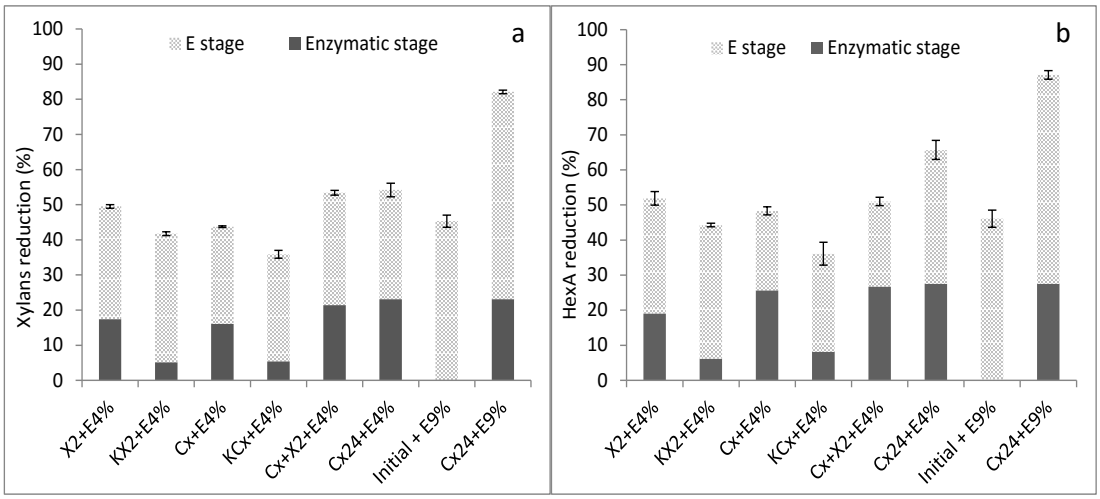

Figure 6: Xylans and HexA content reduction from initial pulp after different enzymatic steps and alkaline extractions with NaOH. Error bars indicate confidence intervals.

Observing SEM images (Figure 7) no major change seemed to be produced by X enzyme on the surface of fibers but the apparition of small fibrils on surface (Figure 7a and b). Cx treatment seemed to lead to the apparition of breaking points on fibers after 2h (Figure 7c and d). These spots probably represent enzyme placement site where hydrolysis started (Igarashi et al., 2011). After 24 h, Cx effects were more noticeable than after 2h (Figure 7e) as fibers were cut a result of treatments, compared to KCx24 sample (Figure 7f), being this effect also observable macroscopically. These weaker fragments produced by cellulase might be the responsible for the loss in zero-span resistance (Figure 5b). Finally, NaOH did not seem to produce any noticeable change on fibers surface (Figure 7g and h).

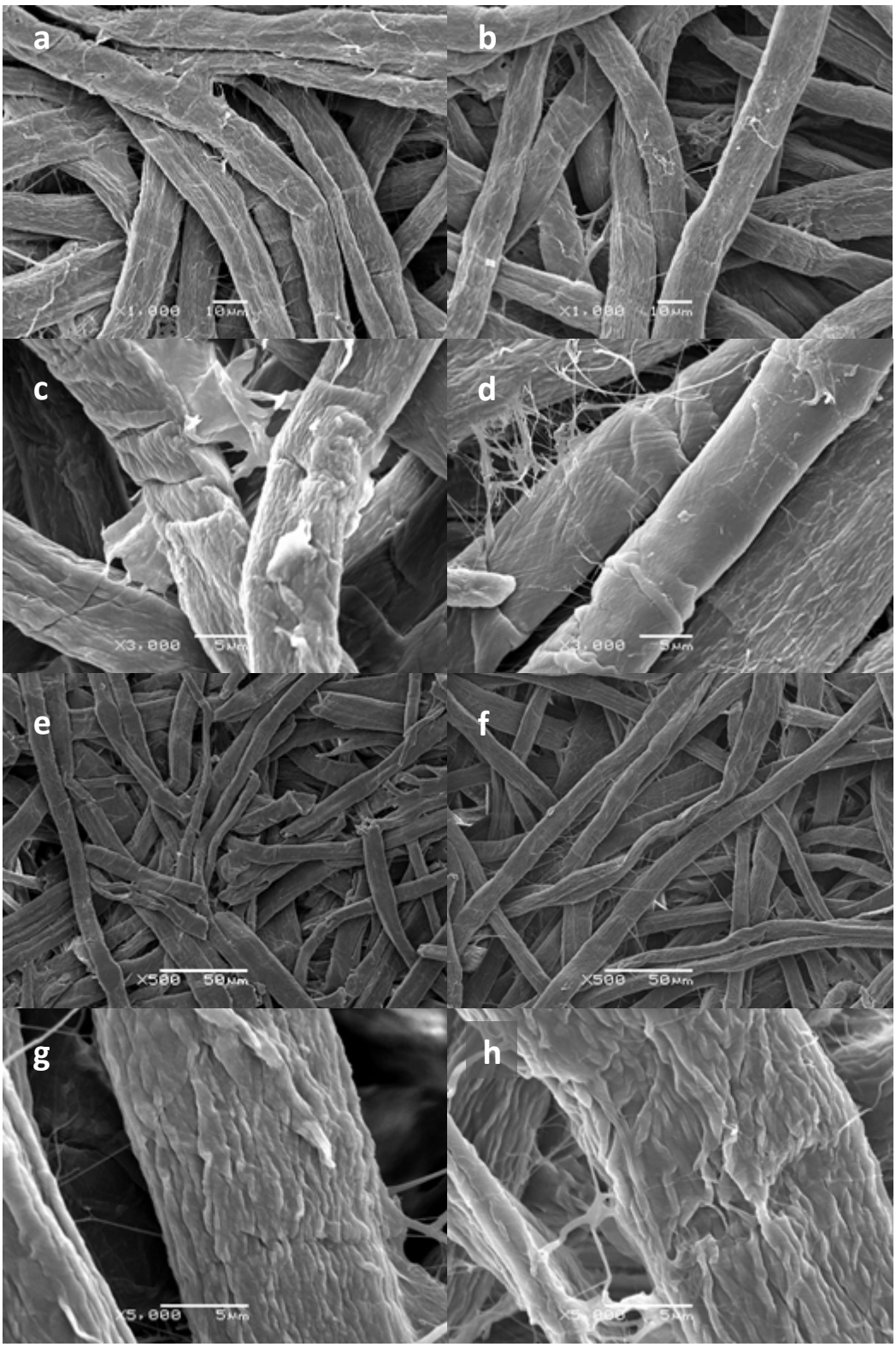

Figure 7: SEM images of treated fibers. Pictures represent following samples: a) X2, b) KX2, c) Cx, d) KCx, e) Cx24, f) KCx24, g) X2 and h) X2+E

The objective of this work was to achieve a strong reduction of xylans content on pulps with the aid of enzymatic treatments. High-cellulose content pulps have a variety of potential uses requiring a high-purity cellulose source. For example, they could be used for nanocellulose production (Fortunati et al., 2013) or other cellulose derivatives, such as viscose rayon (Ibarra et al., 2009). For this last application, besides the low content of hemicelluloses, it is also important to have high reactivity values (Ibarra et al., 2010). Pulp reactivity provides an idea of the capability of pulps to be transformed into its derivative during viscose process (Ibarra et al., 2009; Quintana et al., 2015). Fock solubility is a convenient method to provide insight of the performance a pulp will have on a further process. Treatments with cellulases and particularly endoglucanases could increase pulps Fock solubility (Hakala et al., 2012; Henriksson et al., 2005; Kvarnlöf, Germgärd, Jönson, & Carl-Axel, 2007; Miao et al., 2014). Two mechanisms were proposed to explain this increase: Degradation of amorphous zones of cellulose could lead to a reduction of structural diversity on fibrils surface, increasing its swelling and therefore, its contact with reagents; while degradation of amorphous regions causes a cleavage on fibrils reducing their size and increasing its swellability (Henriksson et al., 2005). In the present study, Cx treatment provoked an increase of about 30% of Fock solubility, reaching a final value of 61% (Table 3). A similar increase of around 30 % has been reported applying an endoglucanase on sisal pulp (Ibarra et al., 2010).

Concerning viscosity, it has been reported that for pulps intended for viscose manufacture desirable values are around 300 mL/g, as too high viscosities could affect cellulose processability during viscose process (Batalha & Colodette, 2011; Henriksson et al., 2005). As can be observed in Table 3, ≈ 250 ml/g reduction produced by Cx24,

446 bigger than the ≈ 180 mL/g accounted with Cx treatment, leaded to a more convenient
447 final value for this application.

448 Chain scission number (CSN) is defined as the average chain cuts produced by a
449 certain process (J Bouchard, Morelli, & Berry, 2000). This parameter is calculated using
450 DP values, which experienced slight increases after some treatments (X or E stage) and
451 because of this, some small negative values were obtained (Figure 8a). Also, it is shown
452 that X enzyme and E stage did not modify pulp CSN or pulp reactivity. Cx treatment,
453 on the other hand, produced an increase in both CSN and Fock solubility. This evidence
454 fitted very well with previous explanations, as the increase in scission points provoked
455 by cellulolytic activity could have increased cellulose swellability and thereafter, its
456 reactivity towards viscose process. In relative terms, compared to control samples,
457 reactivity increases of about 100-110 % were obtained. These values were in the good
458 direction to meet specialty cellulose requirements, compared with the 65-70 %
459 reactivity presented by commercial dissolving pulps (Kopcke et al., 2008). Figure 8b
460 shows that both X and E stages removed hemicelluloses, but produced no effect in pulp
461 reactivity. However, Cx enzyme showed effectivity removing hemicelluloses, boosting
462 further alkaline extraction and increasing reactivity, making it a very promising single
463 catalyst for the studied process. After treatments, combining Cx enzyme and NaOH 9%,
464 a final product with low xylan and HexA content, low KN, high ISO brightness and
465 increased reactivity was achieved (Table 4).

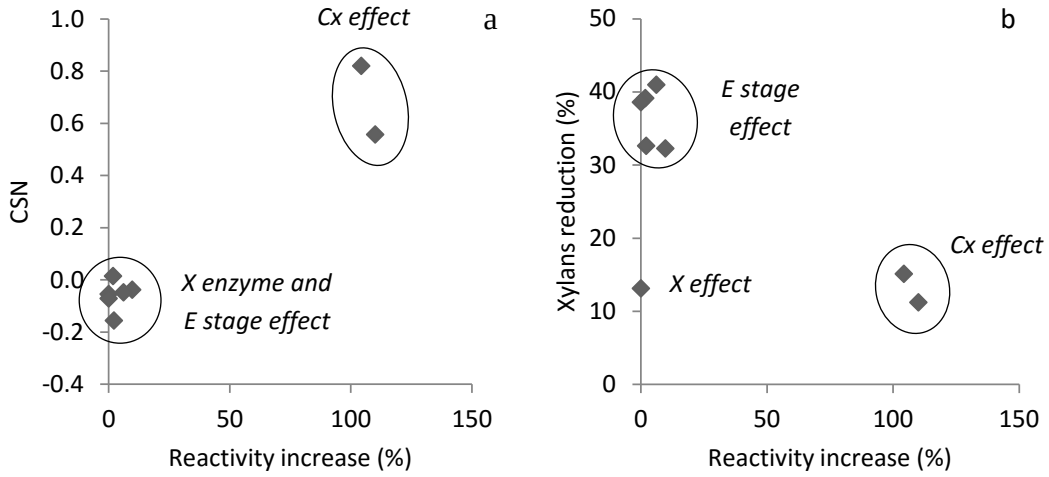

Figure 8: CSN (a) and xylans content reduction (as %) (b), both represented in front of reactivity increase. All variations were calculated in comparison to the previous stage, not initial pulp.

	X2 + E 4%	Cx + E 4%	Cx + X2 + E 4%	Cx24 + E 4%	Cx24 + E 9%
Xylans (%)	8.1 ± 0.1	9.1 ± 0.1	7.5 ± 0.1	7.4 ± 0.3	2.9 ± 0.1
HexA (µmol/g odp)	21.7 ± 0.9	23.3 ± 0.5	22.1 ± 0.5	15.5 ± 1.2	5.8 ± 1.1
KN	2.5 ± 0.1	2.5± 0.3	2.2 ± 0.2	2.1 ± 0.1	0.7 ± 0.1
Brightness (% ISO)	85.4 ± 0.4	86.6 ± 0.2	87.1 ± 0.1	86.1 ± 0.1	86.2 ± 0.2
Viscosity (mL/g)	649 ± 1	498 ± 13	507 ± 18	450 ± 7	503 ± 18

Table 4: Final properties of pulps after enzymatic treatments and E stage.

Conclusions

Treatments with new carbohydrases were effective modifying sisal fibers to produce high cellulose content pulps. Xylanase treatments were found to work better with the complete dosage compared to the stepwise addition. Carbohydrase mixture appeared as a new very promising catalyst, capable itself of removing hemicelluloses and, increase pulp reactivity. We found that one single enzymatic treatment combined with an alkaline extraction led to a high-quality end product with a wide range of potential uses. Enzymatic treatments also boosted NaOH effects, reducing the necessary

amount to reach a given cellulose purity level, and making them suitable treatments for green processes.

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