Carbohydrate Polymers 80 (2010) 154–160

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Obtaining low-HexA-content cellulose from eucalypt fibres: Which glycosil hydrolase family is more efficient?

Cristina Valls [a], Teresa Vidal [a], Oscar Gallardo [b], Pilar Diaz [b], Francisco I. Javier Pastor [b], M. Blanca Roncero [a,*]

[a] Textile and Paper Engineering Department, ETSEIAT, Universitat Politècnica de Catalunya, Colom 11, E-08222 Terrassa, Spain
[b] Department of Microbiology, Faculty of Biology, University of Barcelona, Avinguda Diagonal 645, 08028 Barcelona, Spain

ARTICLE INFO

Article history:
Received 1 October 2009
Received in revised form 2 November 2009
Accepted 5 November 2009
Available online 12 November 2009

Keywords:
Biobleaching
Chlorine dioxide
Eucalypt
Hexenuronic acids
Xylanase

ABSTRACT

Four new bacterial xylanases from different glycosyl hydrolase families (11, 10 and 5) were evaluated for hexenuronic acid (HexA) removal capacity and bleach boosting ability of a eucalypt kraft pulp. The family 11 xylanase was the most effective in enhancing HexA removal and also in increasing delignification and brightness. A very innovative point was that a xylanase from family 5 was applied in pulp bleaching for the first time, showing a notable contribution to pulp bleachability. On the contrary, the family 10 xylanases did not modify kappa number or brightness. A remarkable effect was that the tested xylanases reduced pulp HexA content. The effects produced by the different xylanases in pulp properties were closely related with the effects observed in the effluent properties. The effluents from enzymatic treatments showed the dissolved xylans and xylooligosaccharides branched with HexA, as well as lignin. In addition, a simple tool to assess the boost bleaching ability of a xylanase treatment was also used.

1. Introduction

The need to reduce or eliminate the formation of organo-chlorinated compounds of high toxicity, during the bleaching processes has led to the emergence of new products in the market, such as ECF (Elemental Chlorine Free) and TCF (Totally Chlorine Free) pulps (Roncero, Colom, & Vidal, 2003a, 2003b; Shatalov & Pereira, 2005, 2007a). Biotechnology has rapidly gained field in pulping processes; thus, enzyme stages involving xylanases or laccases have so far provided very promising results in pulp bleaching sequences. Xylanase enzymes are applied to enhance the kraft pulp bleachability. Early xylanase treatments were made at mildly acidic pH and low temperatures using low concentration of enzyme. The efforts of most enzyme producers towards improvement resulted in better products which are active within the neutral or alkaline pH range. There is a new generation of enzymes that operate at conditions close to those of most mills, namely, alkaline pH, higher temperatures, and shorter retention times (Fillat, Sacón, Bassa, & Yoshiko, 2008). The mechanism of xylanase action during bleaching has not been clearly established and several theories have been proposed, although the general effect is that the removal of xylans makes it easier the penetration of reactives in subsequent bleaching stages (Roncero, Torres, Colom, & Vidal, 2000; Torres et al., 2000; Valls & Roncero, 2009). Eucalypt pulp, as a hardwood pulp, contains a high amount of xylans, forming part of the hemicelluloses present in the cellulosic fibres. Short-chain xylans precipitate in more or less crystalline forms on the surface of cellulose microfibres during kraft cooking, decreasing the fibre wall accessibility.

A very innovative aspect related with the use of xylanases is that these enzymes can reduce the content of hexenuronic acids (HexA) of the pulps (Valls & Roncero, 2009). HexA are formed during kraft cooking, where the methylglucuronic acid present in xylans from the fibre surface is transformed in the corresponding unsaturated hexenuronic acid (4-deoxy-β-L-threo-hex-4-enopyranosyluronic acid) (Daniel, Neto, Evtuguin, & Silvestre, 2003; Lisboa, Evtuguin, Neto, & Goodfellow, 2005). HexA content in bleaching pulps is important as these acids can adversely affect pulp bleachability by increasing reagent consumption and by facilitating brightness reversion, and also they contribute to increasing the kappa number (Forsström, Wackerberg, Greschik, Jour, & Holtinger, 2007; Vuorinen, Fagerström, Buchert, Tenkanen, & Teleman, 1999). Because of the large molecular mass of xylanases, the more readily accessible xylans on fibre surfaces, which probably contain HexA, should be the first to be hydrolyzed.

Therefore, xylanase treatments applied as a bleaching stage not only can have a bleach boosting effect, but also can reduce the HexA content of pulps, which is a remarkable secondary effect. However, it remains to be known if all xylanases are able to remove HexA and/or to enhance bleachability.

Xylanases were initially classified in two groups: family 10 and family 11 xylanases (Gilkes, Henrissat, Kilburn, Miller, & Warren, 1991; Henrissat & Bairoch, 1996). Soon afterwards, xylanases from family 5 were also described (Collins, Gerday, & Feller, 2005; Keen,

* Corresponding author. Tel.: +34 937398210; fax: +34 937398101.
E-mail address: roncero@etp.upc.edu (M. Blanca Roncero).

0144-8617/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2009.11.006

Boyd, & Henrissat, 1996). Several studies have reported the bleach boosting capacity of xylanases from families 10 and 11, which showed different bleaching abilities and a different way to operate on the xylan polymer (Clarke, Rixon, Ciruela, Gilbert, & Hazlewood, 1997; Esteghlalian et al., 2008; Georis, Giannotta, De Buyl, Granier, & Frere, 2000). However, there have not been reported studies concerning pulp bleaching with xylanases of family 5. Moreover, what is more interesting, it has not been studied which family is more efficient in HexA removal.

The aim of this work was to evaluate the effects produced by new xylanases from different families (11, 10 and 5) and bacterial strains (*Bacillus* sp. BP-7 and *Paenibacillus barcinonensis*) (López, Blanco, & Pastor, 1998; Sánchez et al., 2005) on HexA content and pulp bleachability of eucalypt kraft pulp. The pulp and effluent properties were assessed evaluating in detail the influence of xylanases in HexA removal. In addition, a simple tool to assess the boost bleaching ability of a xylanase treatment was also used.

2. Materials and methods

2.1. Raw material

The raw material used was oxygen delignified eucalypt kraft pulp (*Eucalyptus globulus*) produced by Torraspapel S.A. mill in Zaragoza, Spain. Two kinds of pulp were used. The initial properties of pulp 1 were: 9.2 kappa number; 50.2% ISO brightness; 958 ± 24 mL g^{-1} viscosity, and 44.1 ± 0.42 μmol g^{-1} odp (oven-dried pulp) of hexenuronic acid content. Pulp 2 corresponded to the same pulp washed with 50 mM Tris–HCl buffer (pH 7) at room temperature for 30 min; their initial characteristics were: 8.0 kappa number; 51.1% ISO brightness; 972 ± 20 mL g^{-1} viscosity, and 38.8 ± 0.7 μmol g^{-1} odp of hexenuronic acid content.

2.2. Enzymes

Four new laboratory xylanases isolated and characterized from two bacterial strains, *Bacillus* sp. BP-7 and *P. barcinonensis*, were used. They belonged to different glycosil hydrolase families and were referred as X_A, X_G, X_J and X_K (Table 1). The xylanases samples used were clarified cell extracts from recombinant *Escherichia coli* clones producing the enzymes.

2.3. Bleaching stages

An ECF (XDP) bleaching sequence was carried out, X corresponding to the enzymatic pretreatment stage with xylanase, D to a bleaching stage with chlorine dioxide and P to a bleaching stage with hydrogen peroxide. The effects of xylanases were evaluated in comparison with a control sequence (X_0DP) where the X treatment was carried out at the conditions required by each xylanase but without enzyme addition (X_0).

The application conditions of stage X varied depending on the enzyme used and they are stated in Table 2. The pulps resulting from the X stage were efficiently washed with decalcified water three times and once with distilled water.

D stage was carried out at 10% pulp consistency with 3% odp of chlorine dioxide as active chlorine, at 56 °C for 60 min. The application conditions for the D stage are nowadays being applied in Torraspapel S.A. industry in the Zaragoza factory.

P stage was carried out at 5% consistency with 3% odp of hydrogen peroxide, 1.5% odp of NaOH, 1% odp of DTPA and 0.2% odp of $MgSO_4$, at 90 °C for 120 min (García et al., 2003).

All reagents were for synthesis and from Merck or Sigma.

2.4. Pulp properties

Treated pulp samples were characterized in terms of kappa number, brightness and viscosity according to ISO 302, ISO 3688 and ISO-5351-1, respectively. Kappa number was measured two times and four measures of brightness were obtained in order to calculate a standard deviation, which was found to be 0.1 for both properties. The pulp hexenuronic acid (HexA) content was also determined through UV detection following the method described by Chai et al. (2001).

Delignification, brightness and HexA increases were calculated according to Eqs. (1)–(3), respectively.

$$\text{Delignification } (\%) = \frac{KN_i - KN_f}{KN_i} \times 100 \quad (1)$$

$$\text{Brightness increase } (\%ISO) = Br_X - Br_{Xo} \quad (2)$$

$$\text{HexA removal } (\%) = \frac{HexA_i - HexA_f}{HexA_i} \times 100 \quad (3)$$

where,

Br corresponded to brightness, KN_i and $HexA_i$ to kappa number or HexA content of the initial pulp, and KN_f and $HexA_f$ to kappa number or HexA content of the pulp after each stage (X; D or P).

2.5. Thin-layer chromatography (TLC)

The effluents from the enzymatic stages were analyzed by TLC. 100 μL of each effluent were applied on a silica gel plate constituting the solid phase. 10 μL of an oligomer standard mixture containing X1 (xylose), X2 (xylobiose), X3 (xylotriose), X4 (xylotetraose), G1 (glucose), G2 (cellobiose), G3 (cellotriose), G4 (cellotetraose) and G5 (cellopentaose), 10 mg mL^{-1} each, were applied on the same plate as migration standards. The mobile phase was a mixture of chloroform, glacial acetic acid and H_2O in a 6:7:1 ratio, respectively. The migration was repeated twice and the silica gel plate was then sprayed (spray Fungilab S.A.) with the developing solution, consisting of a 5% solution of H_2SO_4 in ethanol. Finally the plate was introduced in the oven at 100 °C for 5 min, were the spots corresponding to sugar oligomers released from pulps were identified.

2.6. UV/vis absorbance spectra

Absorbance spectra UV/vis were carried out in the effluents from the enzymatic pretreatment with xylanase (X). The absorbance between 190 and 900 nm wavelengths was measured with an UV spectrophotometer (Shimadzu model UV-1603).

Table 1
Characteristics of the new xylanases.

Ref. Xylanase	Glycosil hydrolase family	Molecular weight (kDa)	Bacterial strain	Reference
X_A	10	38.0	*Paenibacillus barcinonensis*	Gallardo, Díaz, and Pastor (2003)
X_G	11	23.5	*Bacillus* sp. BP-7	Gallardo, Díaz, and Pastor (2004)
X_J	5	47.6	*Bacillus* sp. BP-7	Gallardo (2007)
X_K	10	12.1	*Paenibacillus barcinonensis*	Blanco, Díaz, Zueco, Parascandola, and Pastor (1999)

Table 2
Application conditions of the xylanases treatments.

	Treatment	Dose (U g^{-1} odp)	Temp. (°C)	pH	Buffer (50 mM)	Time (h)	Consistence (%)
Pulp 1	X_0	0	40–60	7	Tris–HCl	2	10
	X_G	2	50	7	Tris–HCl	2	10
	X_J	2	60	7	Tris–HCl	2	10
	X_K	2	40	7	Tris–HCl	2	10
Pulp 2	X'_0	0	40–50	7–8	Tris–HCl	2	10
	X'_A	2	40	8	Tris–HCl	2	10
	X'_G	2	50	7	Tris–HCl	2	10

The absorbance spectra of a solution of the different xylanases alone (at the concentration they are applied in the treatments) were assessed, where some absorption peaks (between 280 and 230 nm and near 200 nm) were observed. These absorbance spectra as well as the absorbance spectra from the respective control treatments were subtracted from the corresponding treatment absorbance spectra (Abs. Spec.) (Eq. (4)).

$$\text{Absorbance spectra} = \text{Treatment Abs.Spec.} - \text{Control Abs.Spec.} - \text{Xylanase Abs.Spec.} \quad (4)$$

3. Results and discussion

3.1. Effects of xylanases on pulp properties

Concerning the effects produced on kappa number (Table 3), control treatment (X_0) decreased kappa number (8%) of pulp 1. However, kappa number of pulp 2, washed pulp 1, did not substantially change during the X'_0 control treatment. Therefore, the operational conditions of the enzymatic treatment did not produce a significant delignification. On the other hand, kappa number strongly diminished (55–60%) during the chlorine dioxide stage, and 30% due to the effect of hydrogen peroxide.

Comparison of performance of the xylanases tested showed that X_G xylanase (family 11) was the most efficient in increasing delignification in both pulps (Fig. 1a). On pulp 1, the maximum delignification effect of X_G was observed after D stage (6% delignification increase when compared to control treatment), while on pulp 2 the maximum effect was observed after X stage (6.3%). The X_J xylanase (family 5) increased delignification after X and P stages being the highest effect after P (4% of delignification increase). On the contrary, with xylanases X_K and X_A (family 10) only a slight effect was observed after D and P stages.

Brightness increased during both, D (25% ISO) and P (15% ISO) stages (Table 3). The different xylanase treatments produced slight effects on pulp brightness where the highest brightness increase with respect to the control treatments (1.4% ISO) was produced after the X_JDP sequence (family 5) as can be observed in Fig. 1b.

In enzymatic treatments carried out with pulp 1, and always compared to control, brightness only increased after X stage in treatment with X_J; after D stage no increasing in brightness was observed, and after P stage brightness increased in X_GDP (family 11) and X_JDP (family 5) sequences. Pulp treated with xylanase X_K (family 10) showed always lower brightness than the control treatment. In samples of pulp 2, brightness increased in X'_G and in X'_AD treatments. As well as in kappa number, the effect of the X_G xylanase depended on the kind of pulp; on pulp 1 the highest effect was observed after P stage while on pulp 2 brightness only increased after X stage (Fig. 1b).

The effects produced on kappa number and brightness by the different xylanases were not always correlated, and this phenomenon has been previously observed by other authors (Shatalov & Pereira, 2007b). Theoretically, the xylanase pretreatment facilitates the penetration of reactive in subsequent bleaching stages and therefore it is expected that its effect may not appear at the enzymatic stage but in later bleaching stages. Nevertheless, results showed that in some cases the enzymatic stage caused an increase in the pulp delignification and brightness. This effect was probably produced by the attack of some xylan–lignin complexes or by the release of chromophore compounds derived from xylan, producing direct delignification and bleaching as suggested by different authors (de Jong, Wong, & Saddler, 1997; Patel, Grabski, & Jeffries, 1993; Roncero, Torres, Colom, & Vidal, 2003c; Shatalov & Pereira, 2007b).

Concerning the viscosity values, they did not decrease during the enzymatic treatments, and it demonstrated that the xylanases used were specific for xylans and did not show any cellulase activity (Table 3). This is an important quality for a xylanase because cellulose will not be deteriorated. Chlorine dioxide did not affect the pulp cellulose since viscosity was not significantly affected.

The different effects produced by the several xylanases may be related with their glycosyl hydrolase family. Several authors (Clarke et al., 1997; Esteghlalian et al., 2008; Georis et al., 2000) compared the bleach boosting effect of different xylanases from families 10 and 11, and they reported the highest efficacy of

Table 3
Pulp properties after each bleaching stage.

	X			XD			XDP	
	KN	Br (%ISO)	Viscosity (mL g^{-1})	KN	Br (%ISO)	Viscosity (mL g^{-1})	KN	Br (%ISO)
Pulp 1	9.2	50.2	958 ± 24	–	–	–	–	–
X_0	8.5	47.1	981 ± 30	3.6	74.2	980 ± 22	2.6	89.2
X_G	8.2	47.2	929 ± 63	3.0	74.3	965 ± 43	2.0	90.2
X_J	8.3	48.2	945 ± 11	3.6	74.3	958 ± 66	2.2	90.6
X_K	8.7	47.0	1005 ± 58	3.5	74.0	939 ± 46	3.0	84.5
Pulp 2	8.0	51.1	972 ± 20	–	–	–	–	–
X'_0	7.9	51.8	997 ± 59	3.5	75.3	994 ± 30	2.5	89.4
X'_A	8.0	51.4	1001 ± 5	3.6	76.0	1013 ± 36	2.4	89.4
X'_G	7.4	52.8	945 ± 63	3.2	74.9	965 ± 15	2.2	89.2

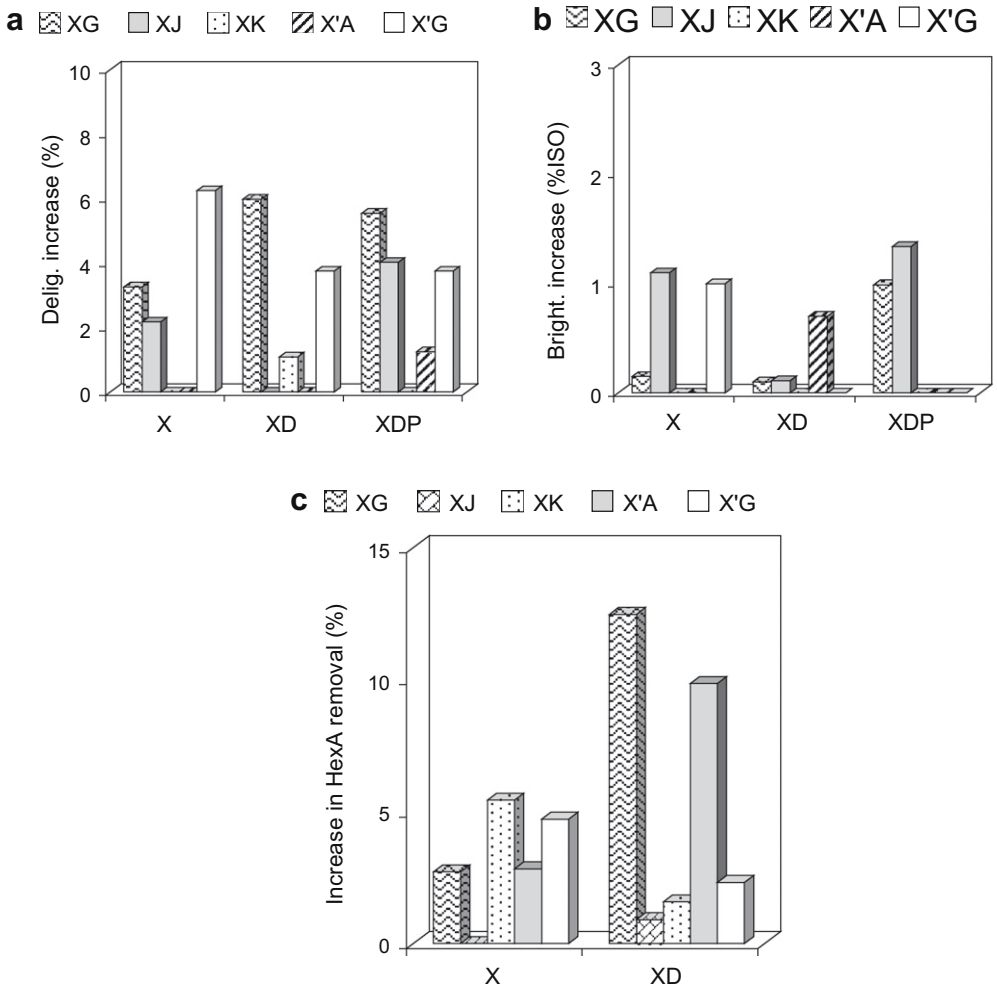

Fig. 1. Increase in delignification (a) in brightness (b) and in HexA removal (c) produced by the different xylanases in comparison with the control treatment.

xylanases from family 11. The results obtained in this study also shown that the family 11 xylanase (X_G) was the most efficient one in enhance pulp bleachability.

The family 10 xylanases (X'_A and X_K) did not produce significant effects in delignification and brightness. These xylanases were both isolated from *P. barcinonensis*. X_A xylanase has an intracellular localization and its natural function may be the hydrolysation of small parts of the xylan polymer that had entered in the cell (Gallardo, Díaz, & Pastor, 2007). Thus, it would not act directly on the xylan, in agreement with the results obtained. As suggested by different authors, the behaviour of a xylanase depends on several factors like the xylan polymer nature, the chain length and the presence of substituents (Li et al., 2000) as well as on the amount of xylans (Wong, Allison, & Spehr, 2001).

An outstanding result is that the family 5 xylanase (X_J) produced a similar effect in increasing delignification and brightness than the family 11 tested (xylanase X_G). This xylanase was very promising since xylanases from family 5 have been more recently discovered and there are no former studies about the application of these xylanases on pulp bleaching.

3.2. Effect of xylanases in the pulp HexA content

The pulp HexA content was measured after X and D stages (Table 4). Control treatment had no a significant effect on HexA content of pulp 2 (X'_0). However, during the control treatment (X_0) of pulp 1, HexA considerably decreased probably due to the dissolution of xylans that contained these compounds. During the D stage of control samples HexA strongly decreased (50%), which is in agreement with reported data that describe chlorine dioxide as an electrophylic oxidant that may attack and destroy the HexA double bond (Costa & Colodette, 2007).

During the enzymatic stage, all xylanases produced a HexA removal except X_J xylanase (family 5), which as described above increased delignification and brightness. On the other hand, the X_K and X'_A treatments (both from family 10), which did not produce any significant effect in kappa number or brightness during X stage, increased the HexA removal (Fig. 1c).

Table 4
HexA (μmol/g odp) content of the pulps after X and D stages.

	HexA (μmol/g odp)	
	X	XD
Pulp 1	44.1 ± 0.4	–
X_0	37.8 ± 0.7	18.1 ± 0.7
X_G	36.6 ± 0.9	12.6 ± 0.7
X_J	39.3 ± 1.1	17.7 ± 1.2
X_K	35.4 ± 1.2	17.4 ± 0.1
Pulp 2	38.8 ± 0.7	–
X'_0	37.0 ± 0.6	16.5 ± 0.4
X'_A	35.9 ± 0.5	12.7 ± 0.6
X'_G	35.2 ± 1.0	15.6 ± 0.2

After D stage, all the xylanase pretreatments increased the HexA removal in comparison with their control treatment (Fig. 1c). The greatest effect was produced with the X_G xylanase (family 11) which also produced delignification and brightness increase. The X'_AD treatment, that did not produce effects on kappa number, slightly increased brightness but boosted considerably the HexA removal (10%). Therefore, it seems that some xylanase enzymes could enhance HexA removal but not pulp bleachability (family 10). But family 11 is a good option to obtain both positive effects.

An important finding from the results obtained is the reduction of HexA content by enzyme treatment with the new xylanases. This can be related with a reduction of the reactive consumption and with the advantage of a pulp with greater brightness stability.

3.3. Thin-layer chromatography (TLC) of effluents

In order to evaluate the action of each xylanase on the xylan polymer, the effluents from X stages were evaluated by TLC (Fig. 2). Effluents from xylanase treatments of pulp 1 (lanes 1–6) or pulp 2 (lanes 7–10) are shown. Control samples and enzyme treated samples are odd in and even numbers, respectively.

Effluents from the control treatment of pulp 1 (X_0) (columns 1, 3 and 5) showed dissolved xylooligosaccharides, resulting from the previous stage of oxygen delignification carried out in the mill. During the control treatment they were removed from pulp (Roncero, Torres, Colom, & Vidal, 2005) and dissolved due to the conditions of the control treatment. In accordance with this, effluents from control treatment of pulp 2 (X'_0) (lanes 7 and 9) did not show any dissolved product in the effluents. These products could be xylooligosaccharides containing hexenuronic acids, in accordance with the HexA decrease produced during X stage. In the treatments with xylanases X_J and X_G (lanes 2 and 4, respectively), although the pattern of dissolved products was similar to control effluents, the spots were more intense suggesting that these xylanases boosted the xylan removal.

On the other hand, in the effluents corresponding to the xylanase X_K (lane 6) it emerged some products that did not appear in any other treatment. Among them, xylose, and two products of higher mobility than xylose and xylotriose, that could be xylan-uronic products or xylooligosaccharides branched with hexenuronic acid or methylglucuronic acid. Xylanase X_K (family 10) had no influence on kappa number or brightness properties but it increased the HexA removal during the enzymatic stage, suggesting that some

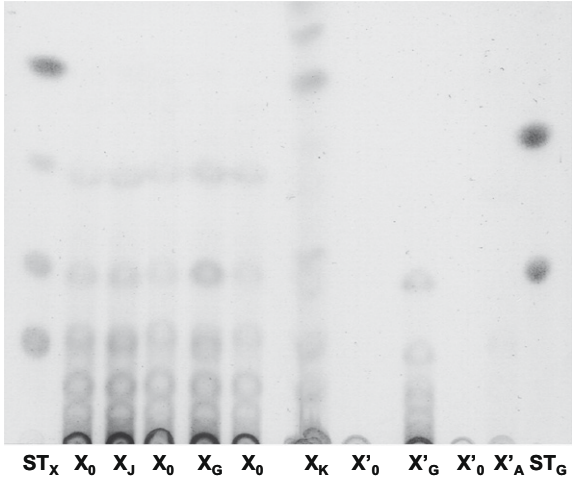

Fig. 2. Thin-layer chromatography (TLC) of the effluents in X stage (ST_X, standard of xylooligosaccharides; ST_G, standard of glucooligosaccharides).

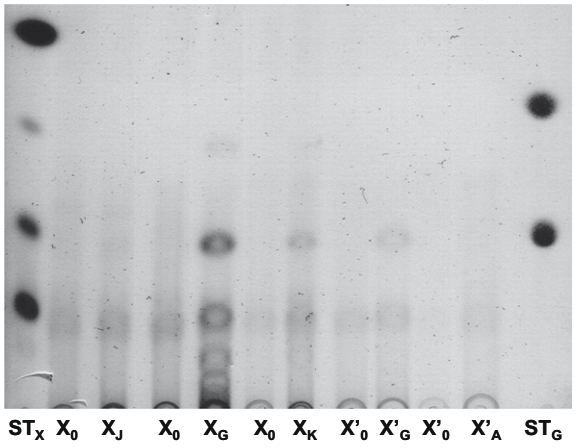

Fig. 3. Thin-layer chromatography (TLC) of the effluents in D stage (ST_X, standard of xylooligosaccharides; ST_G, standard of glucooligosaccharides).

of the products observed by TLC were xylooligosaccharides branched with hexenuronic acids.

Lanes 7–10 in Fig. 2 corresponded to treatments performed on pulp 2. This pulp was washed in the laboratory with the objective to remove the dissolved xylans contained in pulp 1. In the effluents from this control treatment (lanes 7 and 9) there were no soluble xylooligosaccharides, while they were clearly observed in effluents from X'_G xylanase (lane 8). This xylanase produced the hydrolysis of the xylan polymer releasing xylotriose, xylotetraose and other products of intermediate mobility, which, similarly to those mentioned above, can be branched xylooligosaccharides. Shatalov and Pereira (2007b) also reported that xylanase release this kind of products to the effluents. The X'_A treatment did not produce as much effect as X'_G since practically no spots appeared, although a slight spot corresponding to xylotetraose was appreciated. The effects observed in the effluents were closely related with the effects produced in the pulp properties. The X'_G treatment was the one that produced the highest effect in increasing delignification and brightness, and also in the HexA removal. This could be related with its greater action on the xylan polymer. On the other hand, X'_A treatment increased exclusively the HexA removal, but not enhanced the pulp bleachability as much as X'_G treatment.

Consequently, the xylan polymer was partially solved by means of a pretreatment with xylanase, but the removal pattern of xylans depended on the type of xylanase. Moreover, the amount of sugars released by the xylanases was correlated with their bleaching capacity.

According to Clarke et al. (1997) some xylanases attack the xylan of the fibre surface, but they have no capacity to depolymerise the xylan located among the inner walls of the fibre, whose release or depolymerisation is important to reach an efficient bleaching process. Moreover, the xylan polymer length or its substituents may have an influence on the effectiveness of a xylanase (Li et al., 2000).

In conclusion, the removal of xylooligosaccharides observed by TLC was closely related with the HexA removal suggesting that xylanases hydrolyze the xylans of the fibre surface that contain these compounds.

The dissolution of xylooligosaccharides during the D stage was further analyzed (Fig. 3). The effluents from control treatment (lanes 1, 3, 5, 7 and 9) showed faint spots corresponding to xylotriose and xylotetraose, released probably due to the effect of chlorine dioxide. The greatest dissolution of xylans during this stage was observed in the X_GD treatment on pulp 1 (lane 4), where deep spots of xylan hydrolysis products appeared in comparison with other treatments. X_GD effluents showed xylotriose, xylotetraose and higher molecular weight xylooligomers as main products

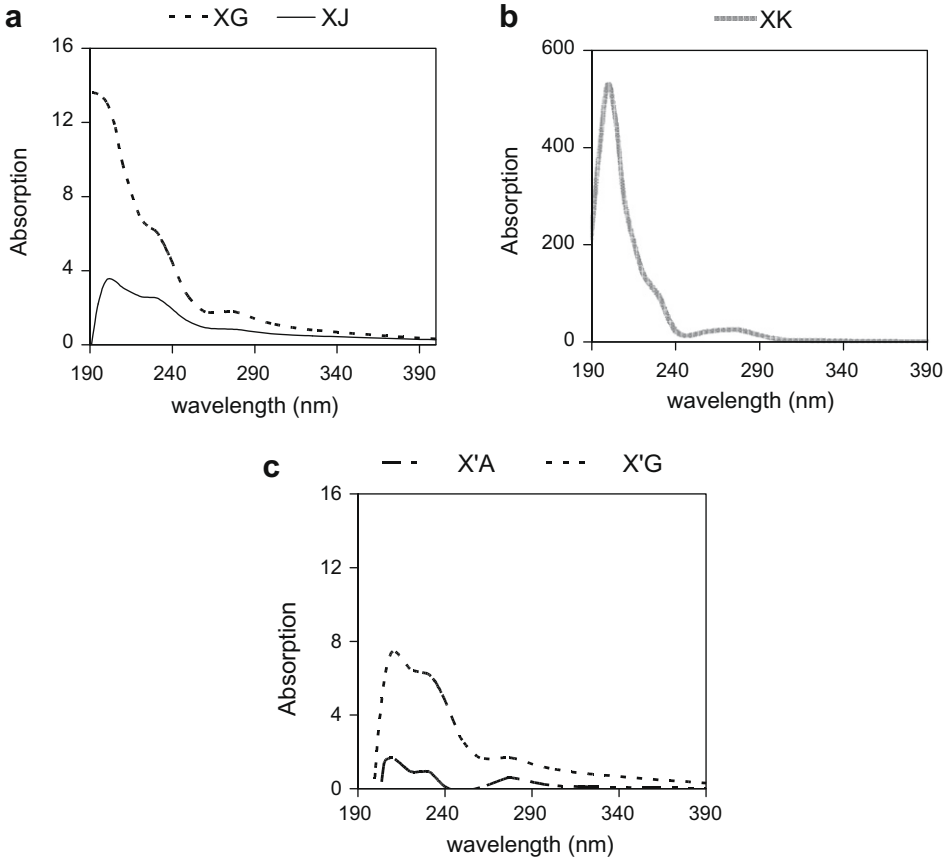

Fig. 4. Absorption spectra of effluents in X stage. Treatments on pulp 1: X_G and X_J (a) and X_K (b). Treatments on pulp 2: X'_A and X'_G (c).

released from pulps, while xylobiose was also detected. Concerning the X_JD, X_KD, X'_GD and X'_AD (lanes 2, 6, 8 and 10, respectively) treatments, the spots were slightly deeper than the control.

It can be concluded that xylanases can act during the enzymatic stage hydrolyzing the xylan polymer and leaving the fibre more accessible for the chlorine dioxide penetration. This effect depends on the xylanase used being it more clearly observed with the X_GD treatment (family 11 xylanase).

A beneficial effect observed was that neither in X stage nor in D stage, cellulose degradation products appeared since no spots corresponding to glucose or cellobiose emerged. This fact corresponded with the viscosity results obtained, where there were no variations due to the treatment with xylanases or to the effect of chlorine dioxide.

3.4. UV/vis absorbance spectra of effluents from X stage

Dissolved lignin in effluents absorbs at 205 and 280 nm, while HexA compounds absorb at 235 nm (Bikova & Treimanis, 2004; Jiang, Van Lierop, & Berry, 2000) due to their double bond. In this way, the UV/vis absorbance spectrum of effluents may be an indicator of the primary action of a xylanase.

The absorbance spectra observed in Fig. 4 corresponded to the products released by the xylanase pretreatments. The same pattern of released products was observed in all the xylanase treatments. An absorbance peak observed around 280 nm was produced by the dissolved lignin, while a second peak, between 230 and 240 nm, corresponded to HexA-xylooligosaccharides. However, the intensity of the absorbance peaks produced by X_G treatment (family 11) was higher than those produced by X_J (family 5) and X'_A (family 10) treatments (Fig. 4a and c).

Due to low specific activity of the X_K enzyme, effluents from X_K treatment had high protein content, which gave high absorption. For this reason, it was not possible to compare its absorbance spectra with those of the other treatments (Fig. 4c). However, the peaks at 280 nm that corresponded to lignin, and that at 220–240 nm that corresponded to the HexA-xylooligosaccharides were also observed. With this xylanase HexA decreased during the enzymatic stage and the products observed by thin-layer chromatography were different to the other xylanases.

In conclusion, the determination of the UV/vis absorbance spectra after the enzymatic treatment with xylanase could be accurately correlated with the boost bleaching ability of a xylanase in order to obtain a simple tool to asses the bleaching reaction.

4. Conclusions

The xylanase from the family 11 (X_G) resulted to be the most efficient one in producing HexA removal and also in increasing delignification and brightness. The effluent properties corroborated the effectiveness of this xylanase. A novelty is that a xylanase from family 5 (X_J) was applied in pulp bleaching for the first time and it produced a similar effect in increasing delignification and brightness than the family 11 tested (X_G). The results obtained with this xylanase from family 5 are of high relevance for further studies to test the application of the enzyme at different conditions.

The two xylanases of family 10 (X_A and X_K) did not produce effects in kappa number and brightness but reduced the pulp HexA content. A remarkable effect was that HexA were removed by all the new xylanases tested except by X_J xylanase (family 5). It seems that some xylanase enzymes could enhance HexA removal but not

pulp bleachability (family 10). But family 11 is a good option to obtain both positive effects.

The effluent properties confirmed that all the xylanase released HexA-xylooligosaccharides and/or also some lignin although not in all cases this was traduced in a bleach boosting effect. Finally, it was shown that the UV/vis absorbance spectra could be a quick tool to evaluate the bleach boosting effect of a xylanase.

Acknowledgements

This work was funded within the framework of Spain's MEC CTQ2007-68003-C02-01 and CTQ2009-12904 projects. Torraspapel S.A. (Zaragoza, Spain) is gratefully acknowledged for supplying the pulp used.

References

Bikova, T., & Treimanis, A. (2004). UV-absorbance of oxidized xylan and monocarboxyl cellulose in alkaline solutions. *Carbohydrate Polymers, 55*, 315–322.

Blanco, A., Díaz, P., Zueco, J., Parascandola, P., & Pastor, F. I. J. (1999). A multidomain xylanase from a *Bacillus* sp. with a region homologous to thermostabilizing domains of thermophilic enzymes. *Microbiology, 145*, 2163–2170.

Chai, X. S., Zhu, J. Y., & Li, J. (2001). A simple and rapid method to determine hexeneuronic acid groups in chemical pulps. *Journal of Pulp and Paper Science, 27*, 165–170.

Clarke, J. H., Rixon, J. E., Ciruela, A., Gilbert, H. J., & Hazlewood, G. P. (1997). Family-10 and family-11 xylanases differ in their capacity to enhance the bleachability of hardwood and softwood paper pulps. *Applied Microbiology and Biotechnology, 48*, 177–183.

Collins, T., Gerday, C., & Feller, G. (2005). Xylanases, xylanase families and extremophilic xylanases. *FEMS Microbiology Reviews, 29*, 3–23.

Costa, M. M., & Colodette, J. L. (2007). The impact of kappa number composition on eucalyptus kraft pulp bleachability. *Brazilian Journal of Chemical Engineering, 24*, 61–71.

Daniel, A. I. D., Neto, C. P., Evtuguin, D. V., & Silvestre, A. J. D. (2003). Hexenuronic acid contents of *Eucalyptus globulus* kraft pulps: Variation with pulping conditions and effect on ECF bleachability. *Tappi Journal, 2*, 3–8.

de Jong, E., Wong, K. K. Y., & Saddler, J. N. (1997). The mechanism of xylanase prebleaching of kraft pulp: An examination using model pulps prepared by depositing lignin and xylan on cellulose fibers. *Holzforschung, 51*, 19–26.

Esteghlalian, A. R., Kazaoka, M. M., Lowery, B. A., Varvak, A., Hancock, B., Woodward, T., et al. (2008). Prebleaching of softwood and hardwood pulps by a high performance xylanase belonging to a novel clade of glycosyl hydrolase family 11. *Enzyme and Microbial Technology, 42*, 395–403.

Fillat, U., Sacón, V. M., Bassa, A., & Yoshiko, C. (2008). *A study in the application of commercial xylanases in the bleaching sequence at the Jacarei Mill (Votorantim Celulose e Papel)* (pp. 207–209). Québec, Canada: IPBC, International Pulp Bleaching Conference.

Forsström, A., Wackerberg, E., Greschik, T., Jour, P., & Holtinger, L. (2007). High brightness stability in standard ECF sequences. *O Papel, 68*, 101–112.

Gallardo, O. (2007). Caracterización de nuevas xilanasas bacterianas. Ingeniería de enzimas con la xilanasa B de ''*Paenibacilus barcinonensis*". Doctoral Thesis. Department of Microbiology, Faculty of Biology, University of Barcelona.

Gallardo, O., Díaz, P., & Pastor, F. I. J. (2003). Characterization of a *Paenibacillus* cell-associated xylanase with high activity on aryl-xylosides: A new subclass of family 10 xylanases. *Applied Microbiology and Biotechnology, 61*, 226–233.

Gallardo, O., Díaz, P., & Pastor, F. I. J. (2004). Cloning and characterisation of xylanase A from the strain *Bacillus* sp. BP-7: Comparison with alkaline pI-low molecular weight xylanases of family 11. *Current Microbiology, 48*, 276–279.

Gallardo, O., Díaz, P., & Pastor, F. I. J. (2007). Cloning and production of xylanase B from *Paenibacillus barcinonensis* in *Bacillus subtillis* hosts. *Biocatalysis and Biotransformation, 25*, 157–162.

García, O., Camarero, S., Colom, J. F., Martínez, A. T., Martínez, M. J., Monje, R., et al. (2003). Optimization of a laccase-mediator stage for TCF bleaching of flax pulp. *Holzforschung, 57*, 513–519.

Georis, J., Giannotta, F., De Buyl, E., Granier, B., & Frere, J. M. (2000). Purification and properties of three endo-[beta]-1, 4-xylanases produced by Streptomyces sp. strain S38 which differ in their ability to enhance the bleaching of kraft pulps. *Enzyme and Microbial Technology, 26*, 178–186.

Gilkes, N. R., Henrissat, B., Kilburn, D. G., Miller, R. C., & Warren, R. A. (1991). Domains in microbial beta-1,4-glycanases: Sequence conservation, function, and enzyme families. *Microbiology and Molecular Biology Reviews, 55*, 303–315.

Henrissat, B., & Bairoch, A. (1996). Updating the sequence-based classification of glycosyl hydrolases. *Biochemical Journal, 316*, 695–696.

Jiang, Z., Van Lierop, B., & Berry, R. (2000). Hexenuronic acid groups in pulping and bleaching chemistry. *Tappi Journal, 83*, 167–175.

Keen, N. T., Boyd, C., & Henrissat, B. (1996). Cloning and characterization of a xylanase gene from corn strains of *Erwinia chrysanthemi*. *Molecular Plant–Microbe Interactions, 9*, 651–657.

Li, K. C., Azadi, P., Collins, R., Tolan, J., Kim, J. S., & Eriksson, K. E. L. (2000). Relationships between activities of xylanases and xylan structures. *Enzyme and Microbial Technology, 27*, 89–94.

Lisboa, S. A., Evtuguin, D. V., Neto, C. P., & Goodfellow, B. J. (2005). Isolation and structural characterization of polysaccharides dissolved in *Eucalyptus globulus* kraft black liquors. *Carbohydrate Polymers, 60*, 77–85.

López, C., Blanco, A., & Pastor, F. I. J. (1998). Xylanase production by a new alkali-tolerant isolate of *Bacillus*. *Biotechnology Letters, 20*, 243–246.

Patel, R. N., Grabski, A. C., & Jeffries, T. W. (1993). Chromophore release from kraft pulp by purified *Streptomyces roseiscleroticus* xylanases. *Applied Microbiology and Biotechnology, 39*, 405–412.

Roncero, M. B., Colom, J. F., & Vidal, T. (2003a). Cellulose protection during ozone treatments of oxygen delignified eucalyptus kraft pulp. *Carbohydrate Polymers, 51*, 243–254.

Roncero, M. B., Colom, J. F., & Vidal, T. (2003b). Why oxalic acid protects cellulose during ozone treatments? *Carbohydrate Polymers, 52*, 411–422.

Roncero, M. B., Torres, A. L., Colom, J. F., & Vidal, T. (2000). Using xylanase before oxygen delignification on TCF bleaching. Influence on fibre surfaces by SEM. *Process Biochemistry, 36*, 45–50.

Roncero, M. B., Torres, A. L., Colom, J. F., & Vidal, T. (2003c). Effect of xylanase on ozone bleaching kinetics and properties of eucalyptus kraft pulp. *Journal of Chemical Technology and Biotechnology, 78*, 1023–1031.

Roncero, M. B., Torres, A. L., Colom, J. F., & Vidal, T. (2005). The effect of xylanase on lignocellulosic components during the bleaching of wood pulps. *Bioresource Technology, 96*, 21–30.

Sánchez, M. M., Fritze, D., Blanco, A., Sproer, C., Tindall, B. J., Schumann, P., et al. (2005). *Paenibacillus barcinonensis* sp. nov., a xylanase-producing bacterium isolated from a rice field in the Ebro River delta. *International Journal of Systematic and Evolutionary Microbiology, 55*, 935–939.

Shatalov, A. A., & Pereira, H. (2005). Kinetics of polysaccharide degradation during ethanol–alkali delignification of giant reed – Part 1. Cellulose and xylan. *Carbohydrate Polymers, 59*, 435–442.

Shatalov, A. A., & Pereira, H. (2007a). Polysaccharide degradation during ozone-based TCF bleaching of non-wood organosolv pulps. *Carbohydrate Polymers, 67*, 275–281.

Shatalov, A. A., & Pereira, H. (2007b). Xylanase pre-treatment of giant reed organosolv pulps: Direct bleaching effect and bleach boosting. *Industrial Crops and Products, 25*, 248–256.

Torres, A. L., Roncero, M. B., Colom, J. F., Pastor, F. I. J., Blanco, A., & Vidal, T. (2000). Effect of a novel enzyme on fibre morphology during ECF bleaching of oxygen delignified eucalyptus kraft pulps. *Bioresource Technology, 74*, 135–140.

Valls, C., & Roncero, M. B. (2009). Using both xylanase and laccase enzymes for pulp bleaching. *Bioresource Technology, 100*, 2032–2039.

Vuorinen, T., Fagerström, P., Buchert, J., Tenkanen, M., & Teleman, A. (1999). Selective hydrolysis of hexenuronic acid groups and its application in ECF and TCF bleaching of kraft pulps. *Journal of Pulp and Paper Science, 25*, 155–162.

Wong, K. K. Y., Allison, R. W., & Spehr, S. (2001). Effects of alkali and oxygen extractions of kraft pulp on xylanase-aided bleaching. *Journal of Pulp and Paper Science, 27*, 229–234.