Research Article

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Effectiveness of novel xylanases belonging to different GH families on lignin and hexenuronic acids removal from specialty sisal fibres

Susana Valeria Valenzuela,[a] Cristina Valls,[b] M. Blanca Roncero,[b] Teresa Vidal,[b] Pilar Diaz[a] and F.I. Javier Pastor[a]*

Abstract

BACKGROUND: The effectiveness of xylanases on lignin removal from pulps differs widely depending on the enzyme family, the type of pulp and the bleaching sequence among other factors. Xylanases can also reduce the presence of undesirable hexenuronic acids in the papermaking fibers. The performance of non-commercial xylanases belonging to families GH10, GH30, GH30-CBM35 and GH11, and of the multicomponent xylanase from *Paenibacillus barcinonensis* for lignin and hexenuronic acids removal from sisal (*Agave sisalana*) has been evaluated.

RESULTS: Sisal pulps were bleached by an XP sequence, where X denotes the enzyme treatment and P a hydrogen peroxide extraction stage. Kappa number, brightness, viscosity and hexenuronic acid content of samples were determined. Sugars released from sisal pulps, other non-wood fibres and also eucalyptus fibres, by the treatment with xylanases were also analysed. The best results were obtained with the GH10 xylanase and with crude supernatants of *P. barcinonensis*, which produced a lignin removal of 23% and a reduction of 25% in the hexenuronic acid content of sisal pulps without a significant loss of viscosity.

CONCLUSION: The release of sugars in the effluents from the X stage applied to sisal correlated with the effectiveness of the xylanases tested. The xylan content of wood and non-wood fibres, the type of xylan and its accessibility also had an influence on the xylanase activity on pulps.
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Keywords: biobleaching; hexenuronic acids; sisal; xylanase; effluents

INTRODUCTION

Commercial non-wood pulp production has been estimated to account for about 10% of global pulp production and is expected to increase in the next few years. Sisal (*Agave sisalana*) is a good candidate for such use because of its commercial price, ease of availability and renewability.[1] Sisal fibres, moreover, offer attractive properties, such as their high tear strength, alpha cellulose content, porosity, and folding endurance, for the production of a variety of specialty and high value papers, such as those used in surgical gauze, filters, condensers or even for tea bags.[2]

Xylanases are enzymes that catalyze the hydrolysis of xylan, the second most abundant polysaccharide in nature after cellulose. Most of the xylanases characterized to date belong to glycoside hydrolase families 10 (GH10) and 11 (GH11) (CAZy).[3] These xylanases hydrolyze all types of β (1,4) xylan, branched or not with arabinose or methylglucuronic acid side chains.[4] However, recently a few xylanases that are specific for glucuronoxylans, and do not show activity on arabinoxylans have been characterized.[4,5] They require methylglucuronic acid branches for xylan hydrolysis and have been classified in family GH30.[6,7]

The application of xylanases as bleach boosting agents has gained popularity in the pulp and paper industry, as their use allows a notable reduction in the dose of bleaching chemicals and minimizes the production of pollutants.[8] Recent reports show that xylanase application in pulps can also reduce the content of hexenuronic acids (HexA), formed during the alkaline cooking of wood.[9,10] HexA can increase kappa number and brightness reversion, and also produce consumption of bleaching reagents and can retain metal ions.[11] Xylanase-assisted bleaching of wood pulps has been the focus of several studies designed to identify the most appropriate enzymes and the optimum conditions of application. A comparison of family GH10 and GH11 xylanases has shown that the latter usually perform better in wood pulp bleaching,[12,13] while a recent report has shown that GH30 xylanases, previously classified as belonging to family GH5, can be efficient in eucalyptus pulp bleaching.[14] Despite the growth in interest in the use of agricultural fibres, enzyme application has not achieved parallel levels of industrial use as in the biobleaching of wood pulps. Several reports show the effectiveness of ligninolytic

* *Correspondence to: F.I. Javier Pastor, Department of Microbiology, Faculty of Biology, Universitat de Barcelona, Av. Diagonal 643, E-08028 Barcelona, Spain. E-mail: fpastor@ub.edu*

a *Department of Microbiology, Faculty of Biology, Universitat de Barcelona, Av. Diagonal 643, E-08028 Barcelona, Spain*

b *Department of Textile and Paper Engineering, Universitat Politècnica de Catalunya, Colom 11, E-08222 Terrassa, Spain*

enzymes, such as laccases, on non-wood fibres, including flax and kenaf,[15–17] while the successful application of laccases alone or in combination with xylanases to sisal fibres has recently been reported.[18]

Paenibacillus barcinonensis is a xylanolytic bacteria[19] which produces a complex set of xylanases, some of which have been successfully evaluated in eucalyptus pulp bleaching.[20,21] The aim of this study was to evaluate the effects of five xylanase preparations of *P. barcinonensis*, from different enzyme families, on sisal fibres. All the xylanases were applied separately as a pretreatment to a hydrogen peroxide extraction stage. Their bleach boosting ability was tested by analyzing their effect in reducing the amount of HexA and lignin. Paper and effluent properties were also analyzed. Our study reports for the first time the application of non-commercial xylanases to sisal fibres.

MATERIALS AND METHODS

Raw material

Sisal (*Agave sisalana*) alkaline pulps from soda anthraquinone cooking were supplied by CELESA (Tortosa, Spain). Before the initial characterization, fibres were washed with 1 N H_2SO_4, pH 4 at 2% consistency, for 30 min, filtered and thoroughly washed with de-ionized water. Initial pulp properties were 47.8% ISO brightness, kappa number 8.1, viscosity 778 mL g^{-1} and hexenuronic acid content (HexA) 42.0 μmol g^{-1} oven-dried pulp (odp). Unbleached soda anthraquinone pulps from flax (*Linum usitatissimum*), showing 40.1% ISO brightness, kappa number 10.5 and 12.5 μmol HexA g^{-1} odp; and kenaf (*Hibiscus cannabinus*), showing 35.1% ISO brightness, kappa number 12.9 and 50.1 μmol HexA g^{-1} odp were also supplied by CELESA. Unbleached eucalyptus (*Eucalyptus globulus*) kraft pulp, 38.3% ISO brightness, kappa number 14.3 and 36.5 μmol HexA g^{-1} odp was supplied by ENCE (Pontevedra, Spain).

Xylan content of initial pulps was determined by quantitative acid hydrolysis with 72% sulfuric acid (TAPPI T13m method). The resulting hydrolysates were analyzed for monosaccharides (glucose from cellulose, and xylose from hemicelluloses) by high performance liquid chromatography (HPLC) in an Agilent 1100 HPLC instrument furnished with an Aminex HPX-87H ion-exchange column under the following operating conditions: mobile phase, 0.006 mol L^{-1} sulfuric acid; flow rate, 0.6 mL min^{-1}; column temperature, 60 °C.[22]

Xylanase treatments

The xylanases (EC 3.2.1.8) assayed were Xyn10A from family GH10[23], Xyn30D and Xyn30Dcat from family GH30, [5] and Xyn11E from family GH11.[24] Xyn10A and Xyn11E are single-domain enzymes and thus comprised of a sole catalytic module. Xyn30D is a modular enzyme comprised of a GH30 catalytic module linked to a CBM35 carbohydrate binding module. Xyn30Dcat is a truncated derivative of Xyn30D devoid of the CBM35. All these xylanases were recombinant enzymes from *P. barcinonensis* that were previously cloned in *Escherichia coli* and characterized.[5,23,24] Clarified cell extracts from the recombinant clones expressing each of these enzymes were used for pulp treatments. Pba crude xylanase consisted in the supernatant of cultures of *P. barcinonensis* grown for 3 days at 37 °C on LB broth supplemented with 0.5% rice straw. Xyn10A showed maximum activity at 60 °C and pH 6.5. Xyn30D, Xyn30cat and Xyn11E showed maximum activity at 50 °C and pH 6.5, while Pba crude xylanase showed maximum activity at 50 °C and pH 7.0.

Xylanase treatments (X stage) were performed on 12 g of dried pulp using 10 xylanase units per gram of oven-dried pulp in 50 mmol L^{-1} phosphate buffer, pH 6.0 at 10% consistency. The treatments were carried out in polyethylene bags at 50 °C for 2 h. The enzyme dosage was adjusted for each enzyme in order to apply the same xylanase units g^{-1} odp determined in the treatment conditions (50 °C, pH 6.0). All the xylanases retained more than 80% of their initial activity after 2 h incubation in these conditions. The control reaction contained all the components with the exception of the enzyme. Once treated, the pulp samples were filtered and their residual liquor collected. The samples were thoroughly washed with de-ionized water.[25] Xylanase activity was determined by measuring the amount of reducing sugar released from birchwood xylan by the Nelson–Somogyi method.[26] One unit of enzymatic activity was defined as the amount of enzyme that releases 1 μmol of reducing sugar equivalent per min under the assay conditions described.

Bleaching treatment

The X stage was followed by a hydrogen peroxide bleaching stage (P stage). The P stage was carried out using 2% H_2O_2, 1.5% NaOH, 0.5% DTPA (diethylenetriaminepentaacetic acid) and 0.2% $MgSO_4$ (percentages referring to odp), at 5% pulp consistency, at 90 °C for 2 h, in an Ahiba Spectradye dyeing apparatus (Datacolor) equipped with closed vessels. Treated pulps were then filtered and thoroughly washed with de-ionized water.

Analysis of effluents

Reducing sugar released was mesured by the Nelson–Somogyi method using a standard curve for xylose. Thin layer chromatography (TLC) was performed as described previously.[23]

Analysis of pulp properties

Kappa number, pulp brightness and viscosity were determined according to ISO 302, ISO 2470–1 and ISO 5351/1, respectively. The hexenuronic acid (HexA) content was determined after the P stage following the Gellerstedt and Li method,[27] as modified for UV detection by Chai *et al.*[28] All parameters were measured at least three times in order to calculate the standard deviation.

RESULTS AND DISCUSSION

Xylanase activity on sisal fibres

In a first approach to evaluate the activity of the xylanases belonging to different GH families on sisal fibres, the effluents obtained from the enzyme treatments (X stage) were analyzed to quantify their reducing sugar content and to characterize their oligosaccharide composition. Xyn10A and Pba crude xylanase released high amounts of reducing sugars from sisal fibres, Xyn30Dcat and Xyn11E released lower amounts, while treatment with Xyn30D released only a very small amount of these sugars (Table 1). Analysis by TLC showed that the sugars released by Xyn10A were mostly short chain oligosaccharides with a degree of polymerization ≤ 5, while Xyn30D, Xyn30Dcat and Xyn11E released predominantly longer oligosaccharides (Fig. 1). These results are in accordance with the reported mode of action of these enzymes from different GH families on xylans.[5,23,24] The profile obtained with Pba crude xylanase was very similar to that obtained with Xyn10A, which is in line with the fact that it is the predominant xylanase in the supernatants of *P. barcinonensis* cultures, as shown by SDS-PAGE and zymographic analysis.[20]

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Table 1. Reducing sugars released by xylanase treatment of pulps

	µg of xylose equivalent per mL of effluent			
	Sisal	Eucalyptus	Flax	Kenaf
Control	23.1 ± 17.6	21.6 ± 17.0	27.0 ± 17.7	39.2 ± 17.9
Xyn10A	589.4 ± 28.5	1045.8 ± 40.2	88.1 ± 30.8	697.2 ± 16.7
Xyn30D	54.9 ± 16.7	62.4 ± 17.9	62.4 ± 27.3	142.1 ± 34.4
Xyn30Dcat	138.7 ± 32.0	162.8 ± 19.1	122.1 ± 25.0	182.7 ± 16.7
Xyn11E	132.1 ± 43.7	117.2 ± 20.3	45.8 ± 20.3	111.4 ± 23.8
Pba crude xylanase	323.8 ± 28.5	830.0 ± 40.2	154.5 ± 30.8	464.9 ± 40.2

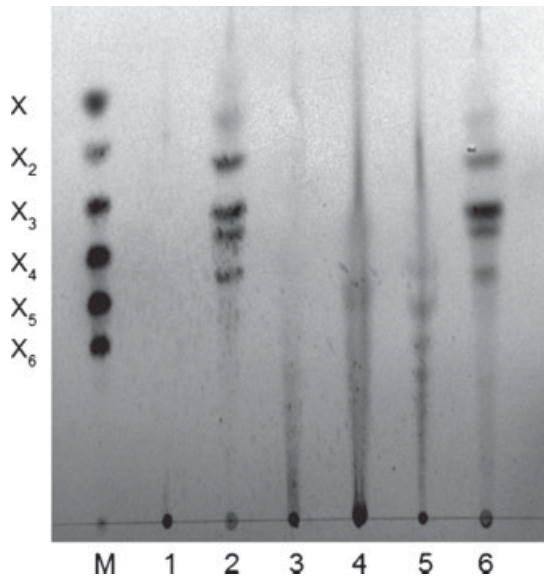

Figure 1. Thin layer chromatogram of effluents from X stage. Analysis of the effluents of sisal pulp treatments with Xyn10A (2), Xyn30D (3), Xyn30Dcat (4), Xyn11E (5), or Pba crude xylanase (6). Effluent from control pulp (1). A mass standard with xylose, xylobiose, xylotriose, xylotetraose, xylopentaose and xylohexaose was included (M).

Analysis of sisal pulp properties

Pulp samples taken after the X and P stages respectively were analyzed in order to evaluate and compare the ability of the xylanases to increase lignin and chromophore removal. Table 2 shows kappa numbers and brightness values of the pulp samples after these two stages. Xylanase treatment led to a decrease in kappa number of most of the samples. These decreases were in accordance with the amount of sugars released by each enzyme. The maximum effects were obtained with Xyn10A and with Pba crude xylanase, which produced kappa number decreases of 22 and 21%, respectively, after the X stage and 23 and 18%, respectively, after the P stage, compared with control values. Xyn30Dcat also presented a marked decrease in kappa number (11%) after the P stage (Table 2). However, treatment with Xyn30D did not reduce kappa number of pulps, while treatment with Xyn11E led to only a minor decrease in kappa number. A recent study of the enzymatic treatment of sisal pulps has shown that commercial xylanases in similar conditions but at a lower dosage, 3 U g^{-1} odp, led to a 14% fall in kappa number, while reductions of 22% were achieved only when the X stage was combined with a laccase-mediator system.[29] These results stress the importance of assessing the enzyme dosages required for an efficient application and are evidence of differences in the lignin removal efficiency of xylanases from different GH families.[21]

Brightness analyses of the pulp samples after the X stage showed that treatment with Xyn10A, Pba crude xylanase and Xyn30Dcat increased brightness by 8.3, 2.8 and 1.5% ISO, respectively, compared with control values (Table 2). After the P stage, all samples, including controls, showed a notable increase in brightness of around 25% ISO, indicative of the bleaching effect of alkaline peroxide. Xylanase treatment led, in most instances, to an additional increase in brightness between 0.9 and 2.4% ISO. Surprisingly, yet in accordance with the absence of any significant contribution to lignin removal, treatment with Xyn11E did not increase pulp brightness, showing that, contrary to what has been suggested, not all family GH11 xylanases are suitable candidates for bleaching.[12,13] Alternatively, its lack of efficiency might be due to putative inhibitors present in pulp that affect the activity of individual xylanases.

Our results show that xylanase effects were less evident after the P stage than they were after the X stage, probably because of an excess of bleaching agent during the second stage. Nevertheless, the bleaching capacity of the xylanases could be detected immediately after the X stage by evaluating changes in pulp properties.[30] Xyn10A was found to be the best candidate among those assayed for improving the bleachability of sisal fibres. However, Pba crude xylanase performed similarly to Xyn10A, the predominant enzyme in the multicomponent xylanase. Thus, it would be considerably cheaper to use Pba crude xylanase, the supernatant of *P. barcinonensis* cultures, than to employ recombinant Xyn10A, due to the costs that its production can imply. A comparison of Xyn30D and Xyn30Dcat shows that better results were obtained by the latter, which is a truncated derivative of Xyn30D. As Xyn30D is a modular xylanase containing the carbohydrate-binding module 35 (CBM-35), it is of greater size (62 kDa) than Xyn30Dcat, which comprises no more than the catalytic module of the enzyme (47 kDa). Even when the catalytic domain is responsible for xylan hydrolysis (and hence its bleaching capability), the extra size provided by CBM-35 could interfere with xylan accessibility. Previous results from the performance evaluation of a modular 120 kDa xylanase from *P. barcinonensis* on eucalyptus fibres showed that the enzyme did not produce any effects on bleaching.[31] These results suggest that the accessory modules may decrease the bleaching efficiency of an enzyme, because of the difficulties a larger molecule encounters in accessing the lignin trapped in pulp fibres. The lower bleaching capacity of xylanase Xyn30D could also be caused by its glucuronoxylan binding ability[5] as this might decrease the free diffusion of the enzyme between fibres.

Even when all treatments with recombinant xylanases were cellulase free, the Pba crude xylanase contained a small amount of cellulase (11.6 U mL^{-1} of xylanase activity vs. 0.13 U mL^{-1} of CMCase activity). Moreover, enzyme treatments can cause damage to the structural integrity of the cellulose. For this reason in order to assess the effect of each treatment on cellulose, the viscosity of the pulps was determined after the P stage (Table 2). In all cases a small decrease in viscosity was observed. However, the enzyme treatments that gave the best results in bleaching (Xyn10A and Pba crude xylanase), were those that presented the smallest losses in viscosity. This is consistent with the fact that xylan removal during the X stage may increase the average degree of polymerization (DP) of fibre carbohydrates, because of its lower DP compared with that of cellulose.[32,33]

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Table 2. Sisal pulp properties after each bleaching stage

	X		P		
	KN	Br (%ISO)	KN	Br (%ISO)	Viscosity (mL g^{-1})
Control	7.7 ± 0.02	47.6 ± 1.9	5.6 ± 0.09	72.6 ± 0.3	772 ± 25
Xyn10A	6.0 ± 0.02	55.9 ± 0.4	4.3 ± 0.08	73.8 ± 0.1	724 ± 18
Xyn30D	8.1 ± 0.11	47.4 ± 0.3	5.5 ± 0.18	73.8 ± 0.1	662 ± 12
Xyn30Dcat	7.2 ± 0.12	49.1 ± 0.5	5.0 ± 0.02	75.0 ± 0.1	620 ± 52
Xyn11E	7.1 ± 0.01	48.2 ± 1.3	5.5 ± 0.18	72.0 ± 0.3	658 ± 18
Pba crude xylanase	6.1 ± 0.02	50.4 ± 0.2	4.6 ± 0.21	73.5 ± 0.1	753 ± 10

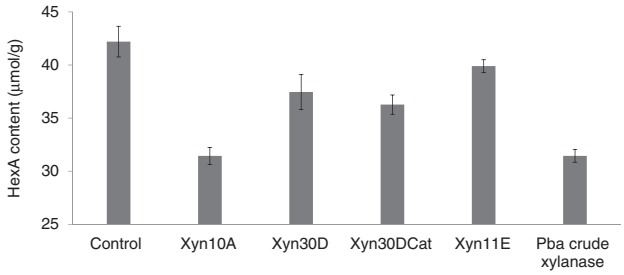

Figure 2. Hexenuronic acids content of pulps. Pulps were bleached by an XP sequence and their hexenuronic acids content determined.

Hexenuronic acid content of sisal fibres

The HexA content of pulps was measured after the P stage. All xylanase treatments reduced HexA content (Fig. 2). Once more the best results were obtained with Xyn10A and Pba crude xylanase, which reduced the total HexA of the pulp samples by around 25%. Xyn30D and Xyn30Dcat treatments led to a HexA reduction of between 11 and 14%, which contrasted with previous findings that report a smaller effect of the xylanases belonging to family GH30 in HexA removal from eucalyptus fibres.[31] The greater effectiveness reported here may correlate either with the higher enzyme dose used (3×), with the difference in the fibres assayed (sisal vs. eucalyptus) or with the different GH30 xylanases applied. A more detailed analysis with more xylanases and a wider range of raw materials is needed to clarify the effect of xylanase application on HexA content of pulps.

Xylanase application on other non-wood and wood fibres

As the amount of reducing-sugar release in the X stage correlated with xylanase bleaching efficiency and their HexA removal capacity, a further three raw materials were assayed to determine the potential effectiveness of the xylanases on these fibres. Eucalyptus, flax and kenaf fibres were treated, as above, with the enzymes under study and the effluents from all treatments were analyzed to measure the amount of reducing-sugar release (Table 1). Maximum activity on eucalyptus fibres was observed with Xyn10A and Pba crude xylanase, which released a considerably greater amount of reducing sugars than they did from sisal. The previously reported bleaching efficiency of Xyn10A on eucalyptus fibres supports the correlation found here between pulp xylan activity and the bleaching properties of this xylanase. Xyn30, Xyn30cat and Xyn11E presented similar results on eucalyptus as they did on sisal fibres, suggesting a similar behaviour in bleaching. Xylanase treatment of flax fibres released only small amounts of sugar in each case, which can probably be attributed to the low xylan content of these fibres, in accordance with its high cellulose content.[34] Kenaf fibres, in contrast, recorded slightly better results than those obtained with sisal, indicating that an enzyme-aided bleaching with Xyn10A or Pba crude xylanase could produce promising results. We believe this result is remarkable since to the best of our knowledge no xylanases have previously been tested in the bleaching of kenaf fibres.

The xylan content of the non-wood and wood fibres tested was determined. They presented different percentages of xylan: eucalyptus pulp had the highest xylan content (18.9%), followed by sisal (16.3%), kenaf (15.1%), and flax (4.4%). Figure 3 shows the relationship between the xylan content of pulps and the release of sugars from them by the xylanases. Figure 3(b) and 3(f) show the marked effect of Xyn10A and Pba crude xylanase in releasing sugars from fibres. For these two enzymes a correlation between pulp sugar release and the xylan content of fibres was found. In the case of Xyn11E (Fig. 3(e)), although the amount of sugars released by this xylanase was significantly lower, the correlation between sugar release and xylan content was maintained. By contrast, this correlation was not observed with xylanases belonging to family GH30 (Fig. 3(c) and 3(d)). These xylanases are specific for glucuronoxylans as a consequence of their requirement of methylglucuronic acid branches to hydrolyze xylan.[6,7] The xylans of the fibres evaluated are glucuronoxylans showing different degrees of substitution.[35] These methylglucuronic acid branches are converted to hexenuronic acid during bleaching in alkaline conditions.[36] We determined the hexenuronic acids content of the pulps, finding considerable differences among them. Kenaf presented the highest content (50.1 µmol HexA g^{-1} odp), followed by sisal (42.0 µmol g^{-1}), eucalyptus (36.5 µmol g^{-1}) and flax (12.5 µmol g^{-1}). We found an increased effectiveness of GH30 enzymes on kenaf fibres (Fig. 3(c) and 3(d)), which can be attributed to the high HexA content in this pulp. However, the smaller effect of GH30 xylanases on sisal and eucalyptus (with only a slightly lower amount of HexAs) suggests that xylan accessibility is better in kenaf pulps. A comparison of Xyn30D and Xyn30Dcat indicates that the lower size of Xyn30Dcat permits this xylanase better accessibility to the xylan polymer of the fibres. Thus, our results indicate that the effectiveness of a xylanase is dependent on the xylan content of fibres, xylan type and xylan accessibility.

CONCLUSIONS

Xyn10A from family GH10 was the most efficient xylanase for lignin removal from sisal fibres and the reduction of their HexA content. Similar results were obtained with Pba crude xylanase, an enzyme cocktail which contains Xyn10A as its predominant component. Xylanases belonging to family GH30 were more efficient when

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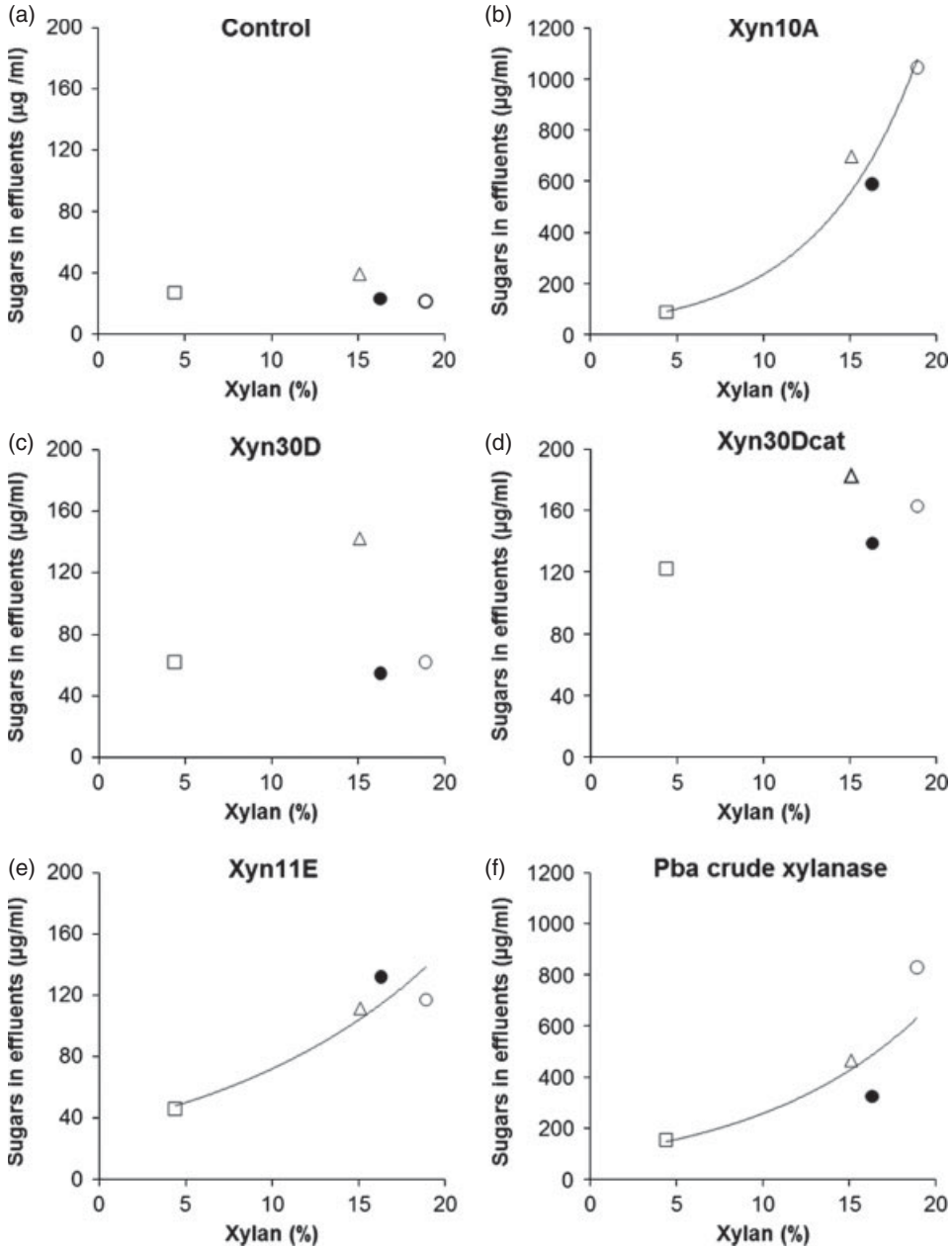

Figure 3. Relationship between the xylan content of flax (open square), kenaf (open triangle), sisal (solid circle) and eucalyptus (open circle), and the reducing sugars equivalent released from fibres by treatment with the xylanases.

applied as a single catalytic domain. By contrast, Xyn11E did not have any significant effects on pulp properties. The release of sugars in the X stage effluents correlates with the effectiveness of the enzymes tested. Moreover, the effectiveness of a xylanase depends on the xylan content of fibres and on the type and accessibility of these xylans.

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