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Differential and synergistic action of *Streptomyces* endoxylanases in prebleaching of kraft pulps

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Microbial endoxylanases reduce chemical demand when they are applied to pulps prior to bleaching sequences. However, their mechanism of action is not fully understood. and the criteria for determining which enzymes are most effective are not well known. We examined the effects of two types of well-characterized endoxylanases from Streptomyces *sp. TUB B-12-2 on the bleachability of kraft softwood and hardwood pulps. xyl 1a is a low molecular mass, high PI xylanase that forms mainly xylotriose but no xylose from oat spelts xylan. xyl 3 is a high molecular mass, low pI xylanase that forms mainly xylose and xylobiose as end products. xyl 3 released more reducing sugars than did xyl 1a, but both enzymes released about the same amount of total sugars. High performance liquid chromatography analysis of product sugars from pulp showed that at pH 7.0. xyl 1a formed oligosaccharides with a high degree of polymerization, but at pH 9.0 the action patterns of the two enzymes were similar. xly 3 released 12% more chromophores from hardwood and 30% more from softwood pulp than did.@ 1a. Both enzymes enhanced brightness while reducing chemical demand, but xyl 3 was more effective. Synergism was noted in the prebleaching of softwood but not hardwood pulp. Both enzymes were active at pH 9, and the presence of pulp increased thermal stability.*

Keywords: Pulp prebleaching; mechanism of action: chromophore release

Introduction

Viikari *et al.* [1] first reported that endoxylanases decrease chemicals needed for bleaching kraft pulp. Many researchers[2,3] have confirmed and extended this observation, and the technology is being commercialized. The mechanism by which xylanases facilitate bleaching is not fully understood. One hypothesis is that they depolymerize hemicellulose precipitated on the surface of the fiber, thereby opening up the pulp structure to access by bleaching chemicals.[4] However, it is also possible that xylanases release chromophores associated with carbohydrates, as suggested by Patel *et al.* [5] Conventionally, removal of lignin by sequential bleaching and extraction of pulp is determined by measuring the kappa number.[6] However, chromophores are not necessarily derived from or even associated with residual lignin. Recent studies by Zibrio[7,8] have implicated carbohydrate degradation rather than lignin as a source of color in kraft pulp and its effluents. The structure and possible association of such chromophores with xylan are not known.

Xylanases seem to play a main role in the prebleaching of kraft pulp,[9] but some synergism with other enzymes such as mannanases has been reported. [10] Although there have been many studies of prebleaching by xylanases, [11] most have been performed with crude preparations. and relatively few have used purified xylanase isoenzymes.[12] Highly xy - lanolytic organisms produce several xylanases with different physiochemical characteristics.[13] Our previous studies showed that purified xylanases from a single organism have different abilities to release chromophores and reduce kappa numbers and we have been interested in knowing how these differences might correlate with action patterns, efficacy in bleaching, or other characteristics.

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Strepromyces sp. TUB B-12-2 is a thermo- and alkaline-tolerant organism that produces five biochemically distinct xylanases[14] which can be classified into two groups based on their physiochemical properties and substrate specificities. Type 1 enzymes (xyl 1a and xyl 1b) are low molecular mass/high pI xylanases that form mostly xylotriose but no xylose from the hydrolysis of oat spelt xylan and xylooligosaccharides. Type 2 enzymes (xyl 2, xyl 3, and xyl 4) are high molecular mass/low pI xylanases that form xylobiose and xylose as main end products. These enzymes have not been cloned, but B-12-2 xyl 3 resembles *Streptomyces lividans* xyl *A* and B-12-2 xyl 1a is similar to *S. lividans xyl* B or xyl C in their physiochemical and catalytic properties.[15]

The objective of the present study was to determine whether enzymes from each of these groups has different effects on the prebleaching of hardwood and softwood kraft pulps. We found that although each purified enzyme was effective alone, one was slightly more effective than the other, and together they attained even higher pulp brightness.

Materials and methods

Substrates and enzymes

Oat spelt **xylan and carboxymethylcellulose were obtained from Sigma (St. Louis, MO). Hardwood kraft pulp (aspen, kappa no. 9) and softwood kraft pulp (mixture of spruce,** red pine. and jack pine, kappa no. 26) were kindly provided by Potlatch. Corp. (Cloquet, MN). Kappa numbers of the washed pulps were determined by Tappi method T-236.[6] Xylanases from *Streptomyces* sp. TUB-B-12-2 were purified from supernatant solutions of cultures grown in 170 oat spelt xylan as previously reported.[14] Xylanase and cellulase activity were measured as previously described.[16]

Enzyme prebleaching

Xylanase prebleaching was carried out at 10% pulp consistency (wv $^{-1}$) in 50 mM potassium phosphate buffer at pH 7.0 and 60°C, unless otherwise noted. The same buffer was used in studies of pH effects (6.0-9.0). Enzyme dosage was 3 IU g^{-1} oven-dried pulp unless otherwise indicated. One unit of enzyme was defined as the amount of enzyme required to produce 1 mmol of xylose equivalents (as determined by the arsenomolybdate method") in 1 min at standard conditions (60°C, pH 7.0). For bleaching trials. samples of pulp (20 g oven-dried) and appropriate volumes of buffer were equilibrated at 60°C for 30 min. The enzymes were then diluted in preheated buffer and immediately added to pulp in sealed plastic bags. The samples were occasionally mixed by kneading the bags several times during a 3-h incubation. Following incubation, pulps were dewatered to about 20% consistency on a Buchner funnel using a Schleicher and Schuell (Keene, NH) no. 595 filter. Pulps were subsequently washed with water and suction-dried.

Chlorine bleaching

The control (buffer only) and xylanase-treated samples were bleached with a conventional bleaching sequence (C70/D30)EDED as described in the following sections.

Hardwood. Pulp samples (20 g on an oven-dried basis) were bleached in polyethylene bags for 30 min at 45°C and 3.5% consistency. The chlorine charge applied was a 0.18 multlple based on a kappa number of 9, or 1.62% active chlorine on oven-dried pulp. A 30% chlorine dioxide substitution was used (C70/D30). The chlorine and chlorine dioxide were added to water and applied simultaneously to the pulp in dilute form. The chlorine solution was distributed manually throughout the pulp prior to placing the samples in a water bath. At the end of chlorination, bleaching chemicals were removed from the pulp samples on a Buchner funnel. The samples were washed thoroughly, dewatered. and extracted with 1 % sodium hydroxide (w w $^{-1}$ oven-dried pulp) for 90 min at 65°C and 10% consistency (E1 stage). The pulp was washed thoroughly after extraction. and 5 g pulp was removed to determine brightness and viscosity. The remaining pulp was bleached with 0.5% chlorine dioxide (calculated as active chlorine on an oven-dry pulp basis) for 3 h at 65°C and 10% consistency (D1 stage). The second extraction (E2 stage) was carried out for 1 h at 60°C using 1% sodium hydroxide and 12% pulp consistency. The pulp was fully bleached with 0.5% chlorine dioxide for 2 h at 65°C and 12% consistency (D2 stage).

Softwood. The same procedure was followed as described for hardwood with the exception of the chemical charge applied. The higher kappa number for the softwood pulp (26) required more chlorine. Based on a 0.2 multiple. 5.2% active chlorine was applied. Again. a 30% chlorine dioxide substitution was used. Extraction was carried out with 3% sodium hydroxide: 1% chlorine dioxide was applied in the D1 stage. E2D2 stages were per-formed as described for hardwood but a 0.6% chlorine dioxide was used in the D2 stage. Brightness was determined by measuring the reflectance at 457 nm on handsheets using a Technidyne (New Albany. IN) brightness meter (Tappi standard method T452 om 92).

Chemical analyses

Pulp samples were solubilized in 72% sulfuric acid (w w-^{1}) for 1 h at 30°C. The acid was diluted to 4.0% (w **w** $^{-1}$) and the samples were hydrolyzed at 120°C for 1 h in an autoclave.[18] The carbohydrate composition (percentage of dry weight) of the pulp was measured by high performance liquid chromatography (HPLC) after acid hydrolysis using a Carbopac PA1 column equipped with a Dionex (Sunnyvale. CA) pulsed amperometric detector. K1ason lignin was determined according to the method of Effland[19] except that ethanol-benzene extraction was omitted. Hardwood kraft pulp contained 80.1% glucose, 19.0% xylose, 0.3% mannose, and 0.03% arabinose. The lignin content measured as klason lignin was 2.1%. Softwood kraft pulp had 79.3% glucose, 9.9% xylose, 5.9% mannose, 0.8% arabinose, and 0.5% galactose. Klason lignin was 5.9%. Oligosaccharides produced during the xylanase treatment were analyzed using the same HPLC system and eluted with a sodium acetate gradient in 100 mM NaOH.

Carbohydrates released by enzyme treatment were analyzed both as reducing sugars (arsenomolybdate method)[17] and total sugars (phenol–H_2SO_4).[20] Chromophore release was determined spectrophotometrically at two wavelengths (237 and 465 nm) as previously reported.[5]

Results

Two purified enzymes (xyl 1a and xyl 3) from *Strepromyces* sp. TUB B-12-2 were applied to hardwood kraft pulp at various doses *(Figure 1a).* xyl 3 released approximately two to three times more reducing sugars than did xyl 1a. By contrast. xyl 3 released only 1.2– 1.6 as much total sugar. The ratio of total sugars to reducing sugars indicated that xyl 1a produced larger oligosaccharides. Release of absorbing materials at 237 nm increased with enzyme dosage and

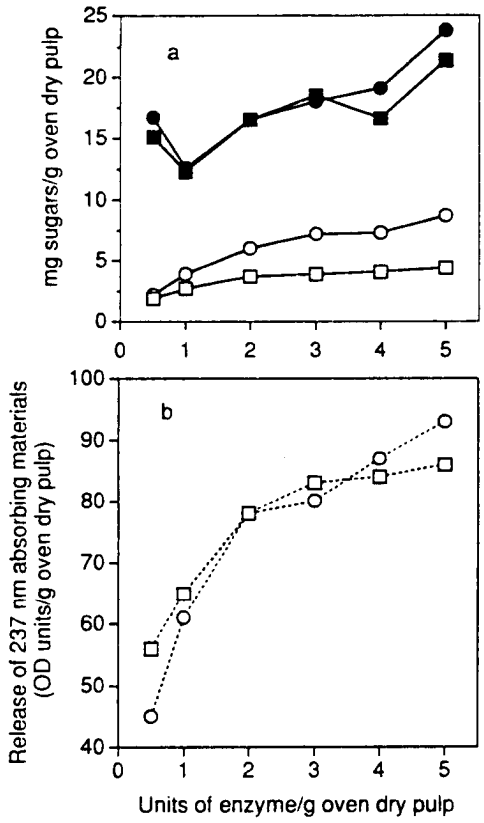

Figure 1 (a) Release of reducing and total sugars from hardwood kraft pulp by xyl 1a and xyl 3 as a function of enzyme dose. □, xyl 1a reducing sugars; ■, xyl 1a total sugars; ○, xyl 3 reducing sugars; ●, xyl 3 total sugars. (b) Release of materials absorbing at 237 nm as a function of enzyme dose. □, xyl 1a; ○, xyl 3

correlated with total sugar release: however. chromophore release as a function of enzyme dose appeared to plateau earlier with xyl 1a *(Figure lb).*

The HPLC analysis of the hydrolysis products from kraft pulp confirmed that xyl 3 released more total sugars and that xyl 1a released oligosaccharides with a greater degree of polymerization (DP). *Table 1* shows the molar percentage composition of the products obtained from hardwood and softwood kraft pulp. The differences were most conspicuous with xylose (X1) and xylobiose (X2). xyl la produced mostly xylotriose (X3) plus xylotetraose (X4) but essentially no xylose from either hardwood or softwood pulp. xyl 3 formed primarily X2 along with xylose and lesser amounts of X3. xyl 3 did not release X4. Neither enzyme produced significant amounts of xylopentaose (X5), but significant amounts of oligosaccharides with DP > 5 were formed. The chromatographic profile of the products with DPs > 5 differed with the pulp and the enzyme used. The amount of DP > 5 products was significantly higher when xyl 3 was applied to softwood kraft pulp. With xyl 1a, the product profiles of the two pulps appeared to be similar. When xyl 3 and xyl 1a were used together, the oligosaccharide pattern was almost identical to that obtained when xyl 3 acted alone. However, the combination of the two enzymes produced a greater amount of sugars as judged by the area of all peaks detected in the chromatogram.

Table 1 Hydrolysis products from reaction of purified *Streptomyces* B-12-2 xylanases with kraft pulp

		Total recovered sugars (mol %)				
Substrate	Enzyme[a]	X1	X2	X3	X4	>X5
Hardwood pulp	xyl 1a	0.2	8.9	24.1	9.9	56.9
	xyl 3	10.8	35.2	16.3	0.2	37.5
	xyl 1a + xyl 3	8.5	31.5	20.5	0.0	39.5
Softwood pulp	xyl 1a	0.2	7.8	22.7	10.9	58.4
	xyl 3	13.0	25.9	10.3	0.0	50.8
	xyl 1a + xyl 3	11.5	24.6	12.5	0.0	51.4

[a]Total activity for each enzyme treatment was 3.0 IU g^{-1} oven-dry pulp

The total sugars released per gram of oven-dried pulp and the chromophore release (as measured at 237 nm) were greater for hardwood pulp than for softwood pulp (*Tables 2 and 3*). However, considering the different xylan contents of the pulps (see MATERIALS AND METHODS), xyl 3 hydrolyzed 55% more of the residual xylan from softwood than from hardwood pulp (17.4% versus 11.2% of total residual xylan, respectively). By contrast, xyl 1a hydrolyzed only about 20% more xylan from softwood than from hardwood pulp (11.4 and 9.5%, respectively). The combination of the two enzymes increased the sugar release only in the case of hardwood pulp, whereas the chromophore release was greater for both pulps. The yield loss, as calculated from the total sugar release, ranged from a low value of 1.1% to a high value of 2.4%. Yield losses were lower and viscosities were higher with xyl 1a (*Tables 2 and 3*).

Table 2 Effect of purified endoxylanases from *Streptomyces* B-12-2 on hardwood kraft pulp

				Brightness (%)			
Enzyme[a]	Chromophore (A_{237} g^{-1} pulp)	Reducing sugar (mg g^{-1} pulp)	Total sugar (mg g^{-1} pulp)	C/DE_1	D_1	E_2D_2	Viscosity (mPa · s)
Control	14.7	0.0	2.9	46.4	62.8	79	27.4
xyl 1a	66.4	2.6	17.9	51.9	68.6	86	33.0
xyl 3	87.4	6.6	19.5	52.4	71.3	86	30.8
xyl 1a + 3	97.3	6.8	24.2	54.6	73.0	85	31.3

[a]Total activity for each enzyme treatment was 3.0 IU g^{-1} oven-dry pulp

Table 3 Effect of purified endoxylanases from *Streptomyces* B-12-2 on softwood kraft pulp

Enzyme[a]	Chromophore (A_{237} g^{-1} pulp)	Reducing sugar (mg g^{-1} pulp)	Total sugars (mg g^{-1} pulp)	Brightness (%) $C_0DE_1D_1E_2D_2$	Viscosity (mPa · s)
Control	9.5	0.0	0.0	68	25.7
xyl 1a	70.0	1.9	11.4	74	31.4
xyl 3	78.4	6.3	17.5	77	30.9
xyl 1a + xyl 3	78.8	5.6	15.9	80	30.8

[a]Total activity for each enzyme treatment was 3.0 IU g^{-1} oven-dry pulp

We analyzed the effects of xylanase treatments on hardwood and softwood kraft pulp bleachability by measuring the brightness of the samples at different stages in a conventional chlorine sequence *(Tables 2 and .3).* As can be noted, xyl *3* performed better than xyl la with both kraft pulps. A1though in the case of hardwood kraft pulp xyl la and xyl *3* achieved the same final brightness with similar yield losses (1.8% versus 1.9%). significant differences in brightness values were observed after E1 and D1 stages. When the combination of the two enzymes was used to treat hardwood kraft pulp. the final brightness did not increase. whereas the yield loss was greater (2.4%). However, the combination of xyl la and xyl 3 showed higher brightness values after the E1 and D1 stages. On the contrary, the combination of xyl la and xyl 3 increased the brightness of softwood kraft pulp more effectively than did either enzyme individually without increasing the yield loss.

Shifting the pH toward the alkaline region had only a slight effect on reducing sugar release by the purified xy - lanases from *Streptomyces* sp. TUB B-12-2. *Figure 2* shows that at pH 9.0. in the presence of the pulp substrate, XVI la and xyl 3 released 62 and 73%, respectively, of the sugars released at their pH optima. In contrast, the two enzymes showed a clear difference in their hydrolysis products released from pulp at these different pH values, xyl 3 produced primarily X2, 8.5% xylose, and no X4 at pH 7.0. At pH 9.0. xylose represented only 0.6% of the total oligosaccharide products, and X2 and .X3 were present at the same molar concentration. xyl la released mostly X3 at pH 7.0. whereas X2 and X4 were present In 1:1 ratio at pH 9.0.

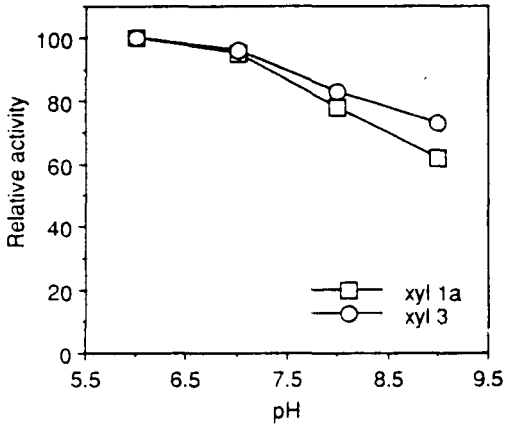

Figure 2 Relative enzymatic activities of xyl 1a and xyl 3 on hardwood kraft pulp

Discussion

Streptomyces sp. TUB B-12-2 exhibits good xylanase production using both pure xylan from oat spelts and inexpensive lignocellulosic material (corn stalks) as a carbon source, [14] Because this organism produces a low level of cellulase, it seems to be a good candidate for industrial application. This strain was selected on the basis of its high xylanase activity and its ability to reduce the kappa number without affecting the viscosity of a commercial softwood kraft pulp. in addition. the crude xylanase retains 40% of its initial activity after 3 h incubation at 60°C, in the absence of substrate at both neutral (7.0) and alkaline (8.5) pH.

The crude xylanase from *Streptomyces* sp. TUB B-12-2 is able to release chromophores from kraft pulp. as has already been reported for other *Streptomyces* species.[5] It also increases the brightness of kraft pulp under alkaline conditions. The purified enzymes from B-12-2 shows the same capabilities. The chromophore release correlates well with the total sugar release: this activity was used throughout our experiments as a simple method to determine the efficacy of the enzyme treatment. We observed also that in most cases greater chromophore release corresponds to higher brightness *(Tables 2 and 3).* This correlation was more evident at the low brightness levels observed in early bleaching stages. Given the difficulty in measuring pulp bleachability, chromophore release might be an easy way to monitor enzyme efficacy.

The results demonstrate that different xylanases are not equivalent with respect to their action against kraft pulp. xyl 3 was able to release more sugars and chromophores than xyl 1 a when the same units of xylanase activity were applied to pulp. Moreover, xyl 3 hydrolyzed a greater amount of xylan from softwood kraft pulp. The hydrolysis products from hardwood and softwood kraft pulp were very similar, but xyl 3 showed an increase in the production of larger oligosaccharides when it was applied to softwood pulp *(Ta-b/e 1).* xyl 3 was also more effective than xyl la in increasing the final brightness of softwood kraft pulp. In contrast, the treatment of hardwood kraft pulp showed that the two enzymes achieved the same final brightness using a single multiple-chlorine charge. Nevertheless, xyl 3 increased the brightness to a greater extent after the E1 and D1 stages. It is common to observe a plateau in the brightness level during the final bleaching stages, and because we fully bleached the hardwood pulp no difference was observed between the two treatments. These results suggest that it is possible to reach a target brightness using a lower chlorine multiple with xyl 3 than with xyl la.

Table 4 Hydrolysis products from reaction of purified *Streptomyces* B-12-2 xylanases with hardwood kraft pulp at neutral and alkaline pH

		Total recovered sugars (mol %)				
Hydrolysis pH	Enzyme[a]	X1	X2	X3	X4	X5
7.0						
	xyl 1a	0.2	8.9	24.1	9.9	2.0
	xyl 3	8.6	32.5	17.4	0.0	0.0
9.0						
	xyl 1a	0.5	14.5	16.4	7.1	2.5
	xyl 3	0.6	17.1	17.8	7.1	1.5

[a]5.0 IU of enzyme g^{-1} oven-dried pulp

The combination of xyl 1a and xyl 3 showed a synergistic effect in increasing the final brightness of softwood kraft pulp, whereas in the case of hardwood kraft pulp the synergism was observed after E1 and D1 but not at the final stage (D2). The highest brightness obtained by using the combination of the two enzymes to treat softwood pulp was not due to a greater hydrolysis of the pulp. In fact, the total sugar release was smaller than when xyl 3 was used alone. Because the type of sugars released by the combination of xyl la and xyl 3 was very similar to the type of sugars produced by xyl 3 alone. it is not possible to conclude that xyl 1a helps in releasing different carbohydrate moieties. On the other hand. when the combination of these enzymes was used to pretreat both hardwood and softwood kraft pulp, we observed higher chromophore release. Furthermore, we previously reported that xyl la is able to solubilize xylan much faster than xyl 3. [14] These results suggest that xyl 1a solubilizes the pulp more rapidly, thereby allowing xyl 3 to release more chromophores. Moreover, the data strongly suggest that using the two *Streptomyces* B-12-2 xylanases leads to a further reduction in chlorine consumption in obtaining a target brightness.

The pH optimum for xyl 1a and xyl 3 was 6.0 and 7.0, respectively. In the absence of substrate. xyl 1 a and xyl 3 retained 10 and 40% of their activities, respectively, at 60°C and pH 9.[14] However, during the prebleaching of kraft pulp both enzymes exhibited a much higher stability *(Figure 2)*. This shows that the pulps had a positive effect on thermal stability at alkaline pH. Kavita *et al.* [21] reported that 3% xylan could prevent thermal inactivation of a xylanase from *Chainia* sp. Because we used 10% pulp consistency (2% xylan concentration). we speculate that xylan in the pulp exercised a similar effect.

The hydrolysis products from kraft pulp obtained by the action of xyl 1a and xyl 3 depended on the pH of the reaction. At pH 7.0, the two enzymes produced significantly different hydrolysis oligosaccharide patterns. but at pH 9.0 their patterns were almost identical. xyl 3 shifted to form higher DP oligosaccharides observed with xyl 1a. To our knowledge, the effect of pH on xylanase action pattern has not been reported previously.

Buchert *et* al. [12] reported only a slight synergism between two xylanases from *Trichoderma reesei* with respect to their action on pine kraft pulp. We detected greater differences using purified xylanases from *Streptomyces sp.* TUB B-12-2. The enzymes from *T. reesei* are mostly active on acetylglucuronoxylan rather than unsubstituted xylan.[22] Because xylan in kraft pulp is not acetylated. the difference in their substrate specificity might not have been enough to obtain a different effect on pulp bleachability. In contrast, xyl la and xyl 3 from *Streptomyces* sp. TUB B-12-2 xylanases show greater activity on unacetylated birch xylan than on acety - lated xylan; moreover, their action patterns on xylooligosaccharides are completely different from the activity patterns observed with the *T. reesei* enzyme. The differences in their catalytic activities could be responsible for their different capabilities in the biobleaching of kraft pulp and for their apparent synergism.

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