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Enzymatic Pretreatment of Kraft Pulps from *Pinus radiata* D Don With Xylanolytic Complex of *Penicillium canescens* (CP1) Fungi

EMILIA CUROTTO,*[1] ALEJANDRA NAZAL,[1] CAROLINA AGUIRRE,[1] VICTORIANO CAMPOS,[2] AND NELSON DURÁN[3]

[1]*Instituto de Quimica, Biochemistry Laboratory, and* [2]*Instituto de Biologia, Microbiology Laboratory, Universidad Católica de Valparaíso, Casilla 4059, Valparaíso, Chile, and* [3]*Instituto de Química, Biological Chemistry Laboratory, C.P. 6154, Campinas, CEP 13083-970, S.P., Brazil*

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ABSTRACT

After screening 14 strains exhibiting high xylanase activities, *Penicillium canescens* (CP1) and *Penicillium janthinellum* Biourge (CP2) strains were selected. The β-xylanases produced had an optimum temperature and pH of 50°C and 4.0, respectively. Using a bleaching sequence of $D_{100}EP$, $D_{80}EP$, and $XD_{80}EP$, the effluent color obtained with $XD_{80}EP$ was lower for CP1 and CP2 than at the D_{100} stage. The color was slightly higher at the $XD_{80}EP$ stage than with the $D_{80}EP$ sequence. In the final pulp obtained with $XD_{80}EP$ pretreatment, the viscosity increase and the Kappa number was similar to that of $D_{100}EP$ in the CP1 and CP2 strains. Brightness in the final pulp was slightly lower than that of control. The selectivity ratio was better for the CP1 and CP2 strains as compared to control. In the $XD_{80}EP$ stage using xylanase extract from CP1 with a pulp consistency of 8 to 15%, the Kappa number was not changed, but the viscosity, brightness, and selectivity ratio were improved proportional to the rise in consistency and delignification. Breaking length, burst and tear index, porosity, and elongation, in the final paper did not change after enzymatic treatment. AOX decreased (26%) in the D_{80} stage effluent as compared with D_{100}, whereas in the XD_{80} stage diminished 42%. The enzymatic treatment with CP1 facilitates the lignin release, decreases the $CL0_2$ load by 20%, and reduces the AOX without any negative effects on the physical properties of the pulp and paper.

*Author to whom all correspondence and reprint requests should be addressed.

INTRODUCTION

Recently β-xylanase enzymes have been the object of numerous studies, mainly for their possible application in enzymatic prebleaching of cellulose pulps. The ability of xylanase to facilitate subsequent chemical bleaching of kraft pulps has been known since 1986 *(1,2)*. The endoxylanases play an important role in the treatment of hardwood Kraft pulps by facilitating the bleaching and the elimination of xylans from the pulp (20–30%). The process was improved with mannanase *(3,4)*. The Kraft pulp treatment (hardwood) with xylanases decreases the amount of chemical products needed for later bleaching sequences *(5,6)*. Similar results were obtained with the OXDPD sequence in hardwood pulp *(7)*. Using the XZP sequence in hardwood pulps (*Eucalyptus grandis*), Kappa values were lower and brightness higher than those obtained with the ZP process. Viscosity of the bleached pulps was generally low in comparison with the reference pulps (*ODEDED*) *(8,9)*. Similar studies with commercial xylanases in the XZED and XDED sequences on softwood pulps (*Pinus radiata*) were done. Enzymatic modification of *Pinus radiata* kraft fiber and handsheets were also studied *(10)*. The effectiveness of the bleaching was good; however, the selectivity is very low *(11)*. The pretreatment of softwood kraft pulps with xylanase had beneficial effects on bleaching with selective oxygen-based bleaching reagents. Optimal biobleaching results were achieved with ozone (XZE), which exhibited enhanced bleaching selectivity and brightness *(12)*. The xylanase treatment of *Eucalyptus* kraft pulp reduced the amount of chlorine needed by 31% and the amount of organic carbon in the effluent by 30% *(13)*. Recently, studies with thermostable xylanases from bacteria showed the effectiveness of these xylanases at a high temperature and pH (over 60°C and 8.0, respectively, refs. *14,15*). Because of the importance of these enzymes, screening of many xylanolytic fungi were carried out *(16–19)* and their production and characterization, particularly in *Penicillium* and *Aspergillus*, were investigated *(20–23)*. The application of these selected xylanases on hardwood and softwood, was researched in order to understand the different behavior of the enzyme extracts on *Eucalyptus* and *Pinus* pulps *(21–25)*. The most recent papers on the xylanase application on pine pulps are related to the enhancement of the process efficiency on ECF and TCF bleaching *(26–33)*. Comparative studies on commercial xylanases on *P. radiata* D Don have been published *(16,18,21,25,34–42)*.

The *Penicillium canescens* was selected from a large screening study on *P. radiata* D Don woods through several years in Chile *(17,19,43)*.

The aim of this work is to study the effect of β-xylanase complex of new fungal strains selected from *P. radiata* woods in an elemental-chlorine-free biobleaching of kraft pulps from the same wood.

MATERIALS AND METHODS

Isolation of Fungi

Isolation of the fungi were carried out as previously described *(16,17,19)*.

Growth Conditions

The isolated fungi were cultivated in 25 mL of Vogel solution *(44)*, in the presence of 1% oat or birchwood xylans and 50 m*M* phosphate buffer pH 6.0, in a 250-mL Erlenmeyer flask. The growth phase was initiated by inoculation of 1 mL of spores at 10^7–10^8 units/mL. The incubation was carried out in an orbital shaker (150 rpm) at 28°C during 4–7 d.

Proteins

The protein measurements were carried out by the Bradford method *(45)*.

Enzymatic Assays

The β-xylanase was determined by measuring the reducing sugars according to the following modified method using birchwood xylan as substrate *(19,46)*. The xylanase activity was measured as xylose μmoles per minute per mL *(47)*.

The β-xylosidase and β-glucosidase activity was measured by published methods *(48)*. Cellulase activity was measured by a published method *(49)*. Endogluconase was measured by the standard method *(50)*. The L-α-arabinofuranosidase and mannanase activities were determined by measuring the *p*-nitrophenolate released by the enzymes from *p*-nitrophenyl-L-α-arabinofuranoside and *p*-nitrophenyl-β-manopyranoside by published methods *(51,52)*. The feruloylesterase activity was measured with the substrate methylferulate and the ferulic acid liberated was evaluated by HPLC *(53)*. Protease activity was measured with either azocoll *(54)* or azocasein as substrate *(55)*.

Enzymatic Pulp Treatment

Xylanase Pretreatment (XDEP Sequence)

Crude kraft pulp from *Pinus radiata* D Don was used after it was neutralized with water; the Kappa number, viscosity, brightness, and properties of refinement, were determined as control. The enzymatic treatment was carried out in plastic bags, containing 30–250 g of dried pulps; with a

consistency of 8–15%, placed in a thermostatized bath (45°C) adjusted to pH 4.0–4.5 with sulfuric acid at 10%, for 60 to 180 min with an enzymatic charge of 1.0–10 U/g dried pulp. After the enzymatic treatment, the unwashed pulp was submitted to delignification for 30 min with 20% less chlorine dioxide than the control pulp, then washed and centrifuged. The pulp was submitted to an alkaline extraction in the presence of H_2O_2 for 60 min. The COD, color, and pH of the filtrate were determined. The Kappa number, viscosity, brightness, and yield in the pulp were also measured. The controls were carried out in the absence of the enzyme with a chlorine dioxide charge with 100% as well as one with 80%.

Chemical and Physical Pulp Properties

All the following parameters were determined by standard methods: Forming handsheet (T 205 OM-88); for physical test of pulp Kappa number of pulp (T 236 OM-85); viscosity of pulp (T 230 OM-89); bursting strength of paper (T 403 OM-85); internal tearing resistance of paper (T 414 OM-88); air resistance of paper (air porosity) (T 460 OM-88); opacity of paper (T 425 OM-86); grammage of paper and paperboard (T 410 OM-88); folding endurance of paper (T 511 OM-88); tensile breaking properties of paper and paperboard (using constant rate of elongation apparatus); (T 494 OM-89); and diffuse brightness of pulp (T 525 OM-86). All ther values in the figures represent at least a duplicated assay. The approximately average standards deviations in the chemical and physical pulp properties are approx 3–5%.

Effluent Analyses

1. The AOX content was measured by standard techniques (DIN 38409, part 14 of the AOX Euroglass).
2. Color: The methodology followed was described by the Hach spectrophotometer with an internal calibration given directly in ppm.
3. COD: The COD was determined by the standard method described by Hach and the results are given directly in mg/L O_2.

Selectivity and Efficiency

The terms are described as:

$$\text{Delignification} = (\text{Kappa before} - \text{Kappa after})/\text{Kappa before} \times 100 \quad \text{Efficiency (\%)} \quad (\text{DEf})$$

$$\text{Selectivity} = \text{DEf}/\text{Viscosity reduction of the treated pulp (\%)}$$

Table 1
Enzymatic Activities Present in the Crude Extracts
Cultivation of the *Penicillium canescens* (CP1)
and *Penicillium janthinellum* Biourge (CP2)

Enzymes	CP1	CP2
Xylanase[a]	370	60
Xylosidase[b]	123	20
Glucosidase[b]	205	23
Acetylesterase[b]	29	n.d.
α-L-Arabinofuranosidase[a]	0.16	n.d.
Feruloylesterase[b]	1	n.d.
Cellulase	0	0
Protease	0	0

[a]U/mL.
[b]U/L.
n.d. not determined.
The assays are in duplicate, from two different liquid cultures.
1% oat xylan, 50 m*M* buffer phosphate (pH 6.0), 28°C and 150 rpm.

RESULTS AND DISCUSSION

Enzyme Production

After screening 14 fungal strains which exhibited high β-xylanase activities induced by birch xylan, *Penicillium canescens* (CP1), *Penicillium janthinellum* Biourge (CP2) and XM3 were chosen *(16,17,19)*. No cellulases or proteases were found in the extracts in the assays conditions. Table 1 shows these results and the activity of α-L-arabinofuranosidase, acetylesterase, mannanase, and feruloylesterase, and other enzymes of xylanolytic complex from CP1. All enzymes showed optimum activity in assay conditions at pH between 4.0 and 8.8 and a temperature of 40 to 70°C. Results are given in Table 2.

Enzymatic Pulp Treatment

Crude extracts produced by the strains CP1, CP2, and XM3 were tested on *Pinus radiata* kraft pulp in a sequence, xylanase extract pretreatment, 80% chlorine dioxide, alkaline extraction, and H_2O_2 ($XD_{80}EP$). Table 3 shows the results in the absence (D_{100}, 100% chlorine dioxide and D_{80}, 80% chlorine dioxide) and presence of the enzyme (XD_{80}). All three extracts increased the delignification (Kappa number) as compared with the D_{80}

Table 2
Partial Characterization of Enzymes from Crude Extract of *Penicillium canescens* (CP1)

CP1 Strain	T (°C)	pH
Xylanase	45–50	4.0–4.5
Xylosidase	40	5.0–5.5
Glucosidase	40	5.0
Acetylesterase	40–45	4.0–4.5
α-L-Arabinofuranosidase	50–70	8.3–8.8
Feruloylesterase	45	5.5

Table 3
Pulp and Effluent Characteristics,[a] with Different Fungus

Fungus	Crude pulp	Control ClO_2 (100%)	Control ClO_2 (80%)	Enzymatic pretreatment CP1	CP2	XM3
Kappa	25.45	3.47	6.67	4.40	4.36	4.60
Viscosity, cP	46.32	27.22	25.24	29.67	30.78	24.50
Bleaching, % ISO	27.20	70.20	60.00	67.90	62.80	68.00
Yield, %		97.28	94.40	95.04	91.75	95.76
Delig. Effic. %		86.60	73.80	82.70	82.80	81.90
Selectivity		2.10	1.62	2.30	2.46	1.73
Color ppm[a]		1450.00	1633.00	1640.00	1933.00	1975.00
COD mg/L O_2[a]		4110.00	3916.00	4170.00	977.00	4350.00

[a]Effluent after EP.
Enzymatic charge 10 U/g pulp, time 60 min, consistency 8% (45°C), $XD_{80}EP$ sequence.

sequence. The criteria for selecting which strain extract to study further were: good industrial pulp yield (95–96%); no big changes in the experimental conditions as compared with the industrial process; and maintenance of the final pulp properties. The extract from the CP2 strain was eliminated from further study because of its low pulp yield even though it displayed better selectivity and delignification efficiency. The results obtained with the CP1 extract, regarding its final pulp characteristics, Kappa index, and viscosity, were better than those from the XM3 strain, therefore, the extract from the CP1 strain, was selected for further study.

Table 4 shows the pulp-consistency effect on the XDEP sequence. We observed that an increase in pulp consistency did not significantly change the Kappa number, viscosity, brightness, and selectivity. The color and COD values obtained with the effluent from the D and XD_{80} stages tended

Table 4
Pulp and Effluent Characteristics[a] with Enzymatic Pretreatment

Consistency %	Control ClO_2 (100%)	Control ClO_2 (80%)	Enzymatic pretreatment 8	10	15
Kappa	3.47	6.67	4.40	4.30	4.17
Viscosity, cP	27.22	25.24	29.67	27.58	27.48
Bleaching, % ISO	70.20	60.00	67.90	69.70	68.80
Yield, %	97.28	94.40	95.04	95.00	94.33
Delig. Effic. %	86.60	73.80	82.70	83.10	83.60
Selectivity	2.10	1.62	2.30	2.05	2.05
Color ppm[a]	1450.00	1633.00	1640.00	1850.34	1917.00
COD mg/L O_2[a]	4110.00	3916.00	4170.00	4850.00	4850.00

[a]Effluent after EP.
Different consistency pulp. Enzymatic charge 10 U/g pulp, time 60 min. (45°C), $XD_{80}EP$ sequence. CP1 fungi.

Table 5
Pulp and Effluent Characteristics[a] with Enzymatic Pretreatment at Different Times

Time, min	Control ClO_2 (100%)	Control ClO_2 (80%)	Enzymatic pretreatment 60	90	120	150	180
Kappa	3.5	6.7	4.3	4.4	4.2	3.9	4.0
Viscosity, cP	27.2	25.2	27.6	28.3	28.5	27.4	26.0
Bleaching, % ISO	70.2	60.0	69.7	70.1	71.5	70.7	70.5
Yield, %	97.3	94.4	95.0	95.5	93.0	96.1	96.6
Delig. Effic. %	86.6	73.8	83.1	82.8	84.0	84.7	84.2
Selectivity	2.1	1.6	2.2	2.1	2.2	2.1	2.0
Color ppm[a]	1450.0	1633.0	1850.0	2010.0	1810.0	1366.7	1933.0
COD mg/L O_2[a]	4110.0	3916.0	4850.0	4960.0	4520.0	4800.0	5000.0

[a]Effluent after EP.
Enzymatic charge 10 U/g pulp, consistency 10% bps, (45°C), $XD_{80}EP$ sequence. CP1 fungi.

to increase with increasing consistency. This is probably caused by the high delignification efficiency in which colored products are eliminated from the pulp (data not shown). This same trend was also observed in the alkaline extraction (Table 4). Considering these results as well as the normal consistency used in the pulp and paper industry, we chose a consistency of 10% for further study.

Table 5 shows data, obtained using different treatment times between 60 and 180 min, with an enzymatic charge of 10 U/g of pulp at 45°C, Kappa values decreased with an increased pretreatment time, and a

Table 6
Pulp and Effluent Characteristics[a] with Enzymatic Pretreatment and Different Enzymatic Charge, Consistency 10% bps, Time 60 min

U/g bps	Control ClO_2 (100%)	Control ClO_2 (80%)	Enzymatic pretreatment 1	3	5	7	10
Kappa	3.47	6.67	4.26	4.30	4.30	4.10	4.30
Viscosity, cP	27.22	25.24	22.61	22.53	20.30	28.05	27.58
Bleaching, % ISO	70.20	60.00	68.40	69.00	68.90	69.40	69.70
Yield, %	97.28	94.40	96.36	97.02	96.14	95.59	95.00
Delig. Effic. %	86.60	73.80	83.10	83.10	83.80	83.10	83.20
Selectivity	2.10	1.62	1.63	1.62	1.48	2.12	2.05
Color ppm[a]	1450.0	1633.0	2216.6	2633.3	2233.3	2566.6	1850.0
COD mg/L O_2[a]	4110.0	3916.0	4966.0	4633.3	4520.0	4833.3	4850.0

[a]Effluent after EP.
(45°C). $XD_{80}EP$ sequence. CP1 fungi.

decrease in viscosity was observed at 120 min. This may be caused by excessive hydrolysis of the xylan components in the pulps, producing a lower polymerization degree in the final fibers. This effect was also observed in the delignification efficiency and the selectivity, which decreased after 180 min of treatment. The final pulp characteristics were similar to those of the $D_{80}EP$ sequence. The COD values increased with the time of pretreatment, but no correlation with color behavior was observed.

Table 6 shows the enzymatic charge effect on the bleaching. An enzymatic charge from 1 to 10 U/g of pulp at 45°C for 60 min was used. The best results were seen with 7 U/g of pulp. A reduction in viscosity and selectivity was observed at a low enzymatic dose. An increase in the color and COD values was seen in the effluent of the D_{100} sequence with a high enzymatic dose. No clear correlation between the color and COD values was observed in the EP stage. A higher enzymatic charge (40–100 U/g of pulp) was also carried out (data not shown), but the final pulp was negatively affected under those conditions.

Since no large differences were seen between an enzymatic charge of 7 or 10 U/g of pulp in the selectivity values, a refining process was studied, using two extreme enzymatic charges (1 and 10 U/g of pulp) at 45°C and 10% consistency during 120 min and compared with those of D_{100} (Figs. 1–6). The most important pulp characteristics are the tear index, burst index, and breaking length. The tear index (Fig. 1) is related to the fiber length and thickness. In general, with increased pulp bleaching, the tear index decreases because of shrinkage in fiber size and thickness caused by delignification and the use of various chemicals. Increasing the

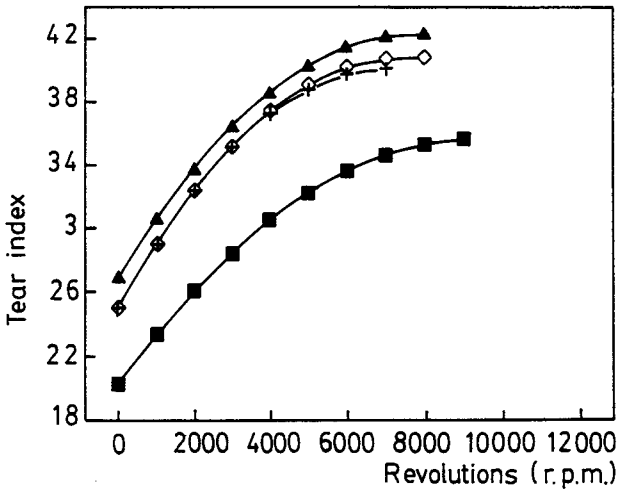

Fig. 1. Relationship between number of revolution in a PFI mill and Tear Index. (■) Crude pulp: bleaching ISO 27.2%, Kappa 25.45, viscosity ISO 46.32 cP. (+) Control 100%: bleaching ISO 70.2%, Kappa 3.47, viscosity ISO 27.22 cP. (◇) Enzymatic pretreatment 1 U/g pulp: bleaching ISO 69.3%, Kappa 4.0, viscosity ISO 26.5 cP. (▼) Enzymatic pretreatment 10 U/g pulp: bleaching ISO 69.5%, Kappa 3.9, viscosity ISO 28 cP.

tear index produced a decrease in the breaking length (Fig. 2) and an increase in the burst index (Fig. 3).

Figure 4 shows data on elongation vs revolution at an enzymatic charge of 10 U/g of pulp. As can be seen, elongation increases in relation to control, demonstrating that the fibers in the pulp have less lignin and high flexibility. However, at 1 U/g of pulp, no significant changes were observed. The same can also be seen in the graph of porosity vs revolution when 10 U/g of pulp was used (Fig. 5).

The Schopper-Riegler index (Fig. 6) is related to the fiber drainage and paper quality. In order to obtain the same Schopper-Riegler Index in the enzyme-treated pulp, it was necessary to use more energy (i.e., more revolutions) than in the untreated one. This means that the treated pulp was harder than the untreated one and depilated less, thereby resulting in a better fiber quality since more fibers remained intact. Although no big enhancement in pulp characteristics was observed in the enzymatic treatment, neither was any diminishment seen, which is good despite the large delignification that was found. Little improvement was seen in pulp with 10 IU/g of pulp as compared to 1 IU/g of pulp, the latter displaying the same characteristics as control. Enzymatic treatment of the pulp significantly reduces absorbable organic halogens (AOX) in the delignification effluent of the D stage. There was a 26.1% and 42% decrease in AOX when D_{80} and XD_{80} sequences were used, respectively, instead of a D_{100} sequence (Table 7).

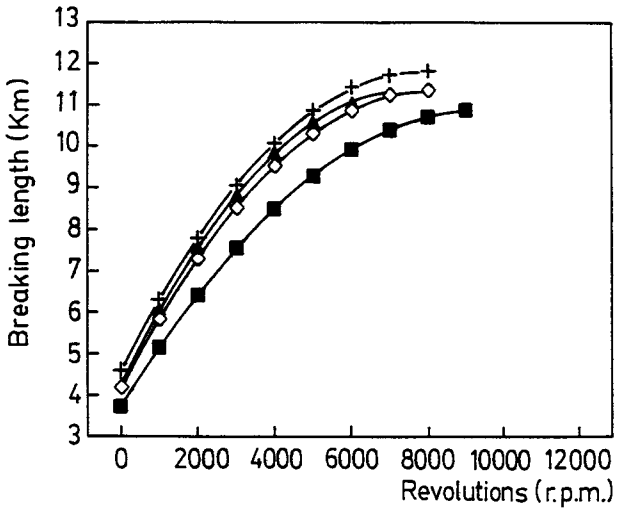

Fig. 2. Relationship between number of revolutions in a PFI mill and breaking length. (■) Crude pulp: bleaching ISO 27.2%, Kappa 25.45, viscosity ISO 46.32 cP. (+) Control 100%: bleaching ISO 70.2%, Kappa 3.47, viscosity ISO 27.22 cP. (◇) Enzymatic pretreatment 1 U/g pulp: bleaching ISO 69.3%, Kappa 4.0, viscosity ISO 26.5 cP. (▼) Enzymatic pretreatment 10 U/g pulp: bleaching ISO 69.5%, Kappa 3.9, viscosity ISO 28 cP.

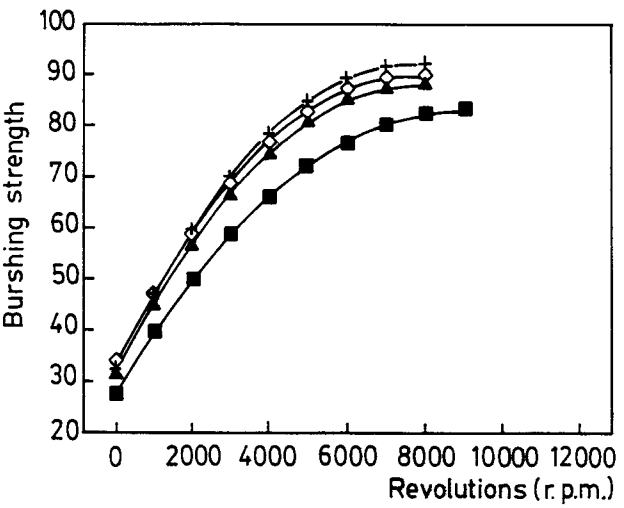

Fig. 3. Relationship between number of revolutions in a PFI mill and Schopper-Rigler Index. (■) Crude pulp: bleaching ISO 27.2%, Kappa 25.45, viscosity ISO 46.32 cP. (+) Control 100%: bleaching ISO 70.2%, Kappa 3.47, viscosity ISO 27.22 cP. (◇) Enzymatic pretreatment 1 IU/g pulp: bleaching ISO 69.3%, Kappa 4.0, viscosity ISO 26.5 cP. (▼) Enzymatic pretreatment 10 IU/g pulp: bleaching ISO 69.5%, Kappa 3.9, viscosity ISO 28 cP.

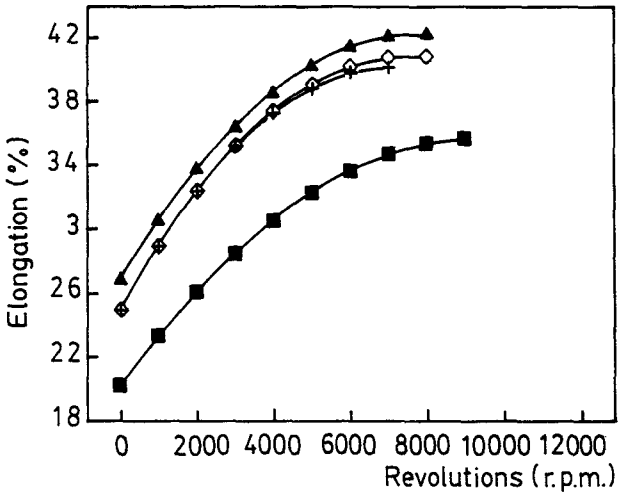

Fig. 4. Relationship between number of revolutions in a PFI mill and brushing strength. (■) Crude pulp: bleaching ISO 27.2%, Kappa 25.45, viscosity ISO 46.32 cP. (+) Control 100%: bleaching ISO 70.2%, Kappa 3.47, viscosity ISO 27.22 cP. (◇) Enzymatic pretreatment 1 IU/g pulp, bleaching ISO 69.3%, Kappa 4.0, viscosity ISO 26.5 cP. (▼) Enzymatic pretreatment 10 IU/g pulp, bleaching ISO 69.5%, Kappa 3.9, viscosity ISO 28 cP.

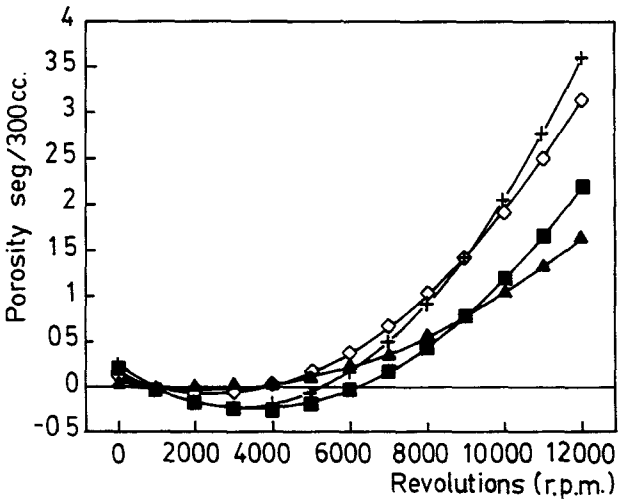

Fig. 5. Relationship between number of revolutions in a PFI mill and porosity. (■) Crude pulp: bleaching ISO 27.2%, Kappa 25.45, viscosity ISO 46.32 cP. (+) Control 100%: bleaching ISO 70.2%, Kappa 3.47, viscosity ISO 27.22 cP. (◇) Enzymatic pretreatment 1 IU/g pulp: bleaching ISO 69.3%, Kappa 4.0, viscosity ISO 26.5 cP. (▼) Enzymatic pretreatment 10 IU/g pulp: bleaching ISO 69.5%, Kappa 3.9, viscosity ISO 28 cP.

Table 7
AOX Determination, Enzymatic Pretreatment, Sequence $XD_{80}EP$, CP1 Fungi

Treatment	mg/L O_2
ClO_2 (100%) control	43.0
ClO_2 (80%) control	31.8
Enzymatic Pretreatment 1 U/g	24.7
Enzymatic Pretreatment 10 U/g	25.3

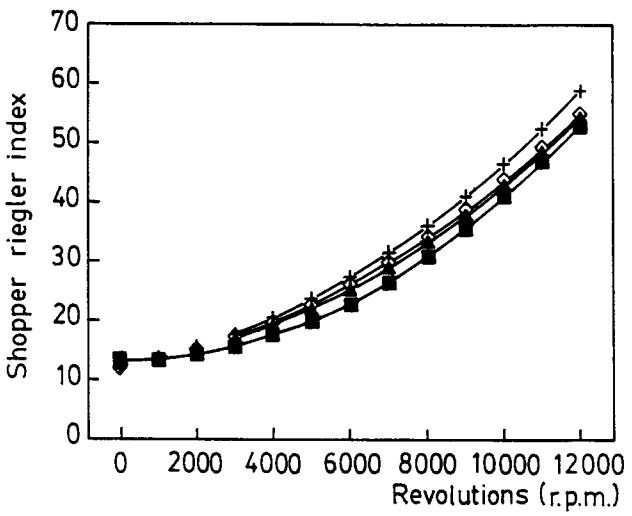

Fig. 6. Relationship between number of revolutions in a PFI mill and elongation. (■) Crude pulp: bleaching ISO 27.2%, Kappa 25.45, viscosity ISO 46.32 cP. (+) Control 100%: bleaching ISO 70.2%, Kappa 3.47, viscosity ISO 27.22 cP. (◇) Enzymatic pretreatment 1 IU/g pulp, bleaching ISO 69.3%, Kappa 4.0, viscosity ISO 26.5 cP. (▼) Enzymatic pretreatment 10 IU/g pulp, bleaching ISO 69.5%, Kappa 3.9, viscosity ISO 28 cP.

In summary, enzymatic treatment with the extract of β-xylanase, xylosidase, feruloylesterase, and L-β-arabinofuranosidase from the CP1 strain on *Pinus radiata* Kraft pulp facilitates lignin liberation and reduces approx 20% of chlorine dioxide used in the first stage of bleaching. This produces no negative effects on the physical characteristics of the pulp and paper, and reduces the amount of AOX released into the environment.

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REFERENCES

1. Noe, P., Chevalier, J., Mora, F., and Comtat, J. (1986), *J. Wood Chem. Technol.* **6,** 167–184.
2. Viikari, L., Ranua, M., Kantelinen, A., Linko, M., and Sundquist, J. (1986), in *Proc. 3rd Intern. Conf. Biotechnology Pup. Paper Ind.*, Stockholm, Sweden. pp. 67–69.
3. Clark, T. A., Steward, D., Bruce, M. E., McDonald, A. J., Singh, A. P., and Senior, D. J. (1991), *APPITA J.* **44,** 389, 390.
4. Allison, R. W., Clark, T. A., and Wrathall, S. H. (1994), *APPITA J.* **47,** 125–129.
5. Senior, D. J. and Hamilton, J. (1993), *TAPPI J.* **76,** 200–206.
6. Daneault, C., Leduc, C., and Valade, J. L. (1994), *TAPPI J.* **77,** 125–130.
7. Eriksson, K.-E. L. (1992), in *Proc. 2nd. Braz. Symp. Chem. Lignin and Other Wood Comp.*, vol. 3, Durán, N. and Esposito, E., eds., UNICAMP Press, Campinas, S., P., Brazil **3,** pp. 274–296.
8. Yang, J. L., Sacon, V. M., Law, S. E., and Eriksson, K.-E. L. (1993), *TAPPI J.* **76,** 91–96.
9. Vicuña, R., Escobar, F., Osses, M., and Jara, A. (1997), *Biotechnol. Lett.* **19,** 575–578.
10. Kibbledwhite, P. and Clark, T. A. (1996), *APPITA J.* **49,** 390–396.
11. Allison, R. W. and Clark, T. A. (1994), *TAPPI J.* **77,** 127–134.
12. Ragauskas, A. J., Poll, K. M., and Cesternino, A. J. (1994), *Enzyme Microb. Technol.* **16,** 492–494.
13. Bajpai, P., Bhardwaj, N. K., Maheshwari, S., and Bajpai, P. K. (1993). *APPITA J.* **46,** 274–276.
14. Davis, S., Gysin, B., Casimir, J., and Zimmermann, W. (1992), *Progress Biotechnol.* **7,** 551–554.
15. Ratto, M., Mathrani, I. M., Ahring, B., and Viikari, L. (1994), *Appl. Microbiol. Biotechnol.* **41,** 130–133.
16. Curotto, E., Aguirre, C., Concha, M., Nazal, A., Campos, V., Esposito, E., Angelo, R., Milagres, A. M. F., and Durán, N. (1993), *Biotechnol. Tech.* **7,** 821, 822.
17. Curotto, E., Aguirre, C., Angelo, R., Esposito, E., and Durán, N. (1993), in *Proc. 3rd. Braz. Symp. Chem. Lignin and Other Wood Comp.*, vol. 4, Veloso, D. P., Donnici, C. L., Carazza, F., and Drumond, M. G., eds., UFMG Press, Belo Horizonte, MG, Brazil, pp. 328–331.
18. Durán, N., Milagres, A. M. F., Esposito, E., and Haun, M. (1995), in *ACS Symposium,* **618,** pp. 253–259.
19. Durán, N., Milagres, A. M. F., Esposito, E., Curotto, E., Teixeira, M. F. S., Carvalho, S. M. S., and Fernades, O. C. C. (1995), *Appl. Biochem. Biotechnol.* **53,** 155–162.
20. Curotto, E., Concha, M., Campos, V., Milagres, A. M. F., and Durán, N. (1994), *Appl. Biochem. Biotechnol.* **48,** 107–116.
21. Durán, N., Curotto, E., Esposito, E., Aguirre, C., and Angelo, R. (1994), in *Advances in Bioprocess Engineering*, Galindo, E. and Ramirez, O. eds., Kluwer Cuernavaca, Mexico, pp. 489–494.
22. Angelo, R., Aguirre, C., Curotto, E., Esposito, E., Fontana, J., Baron, M., Milagres, A. M. F., and Durán, N. (1997), *Appl. Biochem. Biotechnol.* **25,** 19–27.
23. Angelo, R., Ruiz, J., Freer, J., Baeza, J., and Durán, N. (1997), in *Pro. 5th. Braz. Symp. Chem. Lignin and Other Wood Comp.*, vol. 6, Ramos LP, Ed., Curitiba, PR, Brazil, pp. 537–546.
24. Milagres, A. M. F. and Durán, N. (1992), *Progress Biotechnol.* **7,** 539–545.
25. Ruiz, J., Baeza, J., Freer, J., Curotto, E., Esposito, E., and Durán, N. (1993), in *Proc. 3rd. Braz. Symp. Chem. Lignin and Other Wood Comp.*, vol. 4, Veloso, D. P., Donnici, C. L., Carazza, F., and Drumond, M., eds., UFMG Press, Belo Horizonte, MG, Brazil, pp. 324–327.

26. Laine, J., Buchert, J., Viikari, L., and Stenius, P. C. (1996), *Holzforschung*, **50,** 208–214.
27. Teleman, A., Siika-aho, M., Sorsa, H., Buchert, J., Pettula, M., Hausalo, T., and Tenkanen, M. (1996), *Carbohydr. Res.* **293,** 1–13.
28. Buchert, J., Tenkanen, M., Suurnakki, A., and Viikari, L. (1996), in *Biotechnology in the Pulp and Paper Industry. Recent and Advances in Applied and Fundamental Research,* Srebotnik, E. and Messner, K., eds., Facultas-Universitatsverlag. Vienna, Austria, pp. 57–62.
29. Buchert, J., Carlsson, G., and Stoem, G. (1996b), *Holzforschung.* **50,** 69–74.
30. Suurnakki, A., Clark, T. A., Allison, R. W., Viikari, L., and Buchert, J. (1996), *TAPPI J.* **79,** 111–117.
31. Suurnakki, A., Heijnesson, A., Buchert, J., Westermark, U., and Viikari, L. (1996), *J. Pulp Pap. Sci.* **22,** J91–J96.
32. Leite, M., Eremeeva, T., Eisimonte, M., Treimanis, A., and Viesturs, U. (1996), *Vybrane Procesy Chem. Spracovani Dreva.* 141–148.
33. Chen, J., Yang, G., and Li, Z. (1996), *Zhongguo Zaozhi*, **15,** 16–20.
34. Aguirre, C., Ruiz, J., Castillo, C., Nazal, A., Baeza, J., Durán, N., and Curotto, E. (1995), in *Proc. 4th Braz. Symp. Chem. Lignin and other Wood Comp.*, vol. 5, Melo, E. H. M. and Pimentel, M. C. B., eds. pp. 170–183.
35. Vicuña, R., Oyarzun, E., and Osses, M. (1995), *J. Biotechnol.* **40,** 163–168.
36. Vicuña, R., Yeber, M. C., and Osses, M. (1995), *J. Biotechnol.* **42,** 69–73.
37. Suurnakki, A., Heijnesson, A., Buchert, J., Tenkanen, M., Viikari, L., and Westermark, U. (1996), *J. Pulp Pap. Sci.* **22,** J78–J83.
38. Allison, R. W., Clark, T. A., and Suurnakki, A. (1996), *APPITA J.* **49,** 18–22.
39. Clark, T. A., Allison, R. W., and Kibblewhite, R. P. (1996), in *Proc. 50th Appita Ann.* vol. 1, *Gen. Conf.*, Auckland, New Zealand, pp. 47–50.
40. Chang, H. M. and Farrell, R. L. (1996), in *Biotechnology in the Pulp and Paper Industry. Recent and Advances in Applied and Fundamental Research,* Srebotnik, E. and Messner, K., eds., Facultas-Universitatsverlag. Vienna, Austria, pp. 75–80.
41. Clark, T. A. and Allison, R. W. (1996), in *Biotechnology in the Pulp and Paper Industry. Recent and Advances in Applied and Fundamental Research,* Srebotnik, E. and Messner, K., eds., Facultas-Universitatsverlag. Vienna, Austria, pp. 81–86.
42. Gibbs, M. D. and Berquist, P. L. (1996), in *Biotechnology in the Pulp and Paper Industry. Recent and Advances in Applied and Fundamental Research,* Srebotnik, E. and Messner, K., eds., Facultas-Universitatsverlag. Vienna, Austria, pp. 509–512.
43. Curotto, E., Concha, M., Aguirre, C., O'Reilly, S., Ferreira, A., and Durán, N. (1991), in *Proc. Workshop Bio-Organic Chemistry (Brazil-Chile)*, vol. 2, Mansilla, H., Pacheco, P. and Villaseñor, J. eds., Talca, Chile, pp. 88–92.
44. Vogel, H. J. (1956), *Microb Genet Bull.* **13,** 42–43.
45. Bradford, M. M. (1976), *Anal. Biochem.* **72,** 248–254.
46. Bailey, M., Biely, P., and Poutanen, K. (1992), *J. Biotechnol.* **23,** 257–270.
47. Miller, G. L. (1959), *Anal. Chem.* **31,** 426–428.
48. Tan, L. U. L., Mayer, P., and Saddler, J. N. (1987), *Can. J. Microbiol.* **33,** 689–692.
49. Mandels, M., Andreotti, R., and Roche, C. (1976), *Biotechnol. Bioeng. Symp.* **6,** 21–23.
50. Commision of Biotechnology. (1987). *Pure Appl. Chem.* **59,** 245–256.
51. Gilead, S. and Shoham, Y. (1995), *Appl. Environ. Microbiol.* **61,** 170–174.
52. Gomes, D. J. Gomes, J., and Steiner, W. (1994), *J. Biotechnol.* **37,** 11–32.
53. Borneman, W. S., Hartley, R. D., Morrison, W. H., Akin, D. E., and Ljungdahl, L. G. (1990), *Appl. Microbiol. Biotechnol.* **33,** 345–355.
54. O'Reilly, S., Curotto, E., and Gonzalez, G. (1981), *Acta Cient. Venezolana*, **32,** 105–108.
55. Demartino, N. G. (1989), in *Proteolytic Enzymes*, Beyno, R. J. and Bond, J. S., eds., IRL Press, New York, pp. 83–102.