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The performance of fungal xylan-degrading enzyme preparations in elemental chlorine-free bleaching for *Eucalyptus* pulp

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Cellulase-free xylan-degrading enzyme preparations from *Acrophialophora nainiana*, *Humicola grisea* var. *thermoidea* and two *Trichoderma harzianum* strains were used as bleaching agents for *Eucalyptus* kraft pulp, prior to a chlorine dioxide and alkaline bleaching sequence. In comparison to the control sequence (performed without xylanase pretreatment), the sequence incorporating enzyme treatment was more effective. Removal of residual lignin was indicated by a reduction in kappa number. Overall, enzyme preparations from *T. harzianum* were marginally more effective in reducing pulp viscosity and chlorine chemical consumption and improving the brightness of the kraft pulp. However, the highest reduction in pulp viscosity was mediated by the xylanase preparation from *A. nainiana*. Xylanase pretreatment compares very favorably with that of chemical pulping.
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Introduction

Pulp bleaching technology is changing due to environmental public concerns [17]. Technologies have been developed in order to replace chlorine compounds as bleaching agents. The use of xylanases in pulp bleaching sequences is a technical alternative; many research projects are being developed and there are also some reports on the industrial application of xylanases for this purpose [1]. The potential and use of xylanases as an alternative approach in pulp bleaching was first reported by Finnish researchers [14]. The most significant effects of xylanases on pulp bleaching were reported to be a reduction in the requirement for elemental chlorine, and a decrease in the amount of organochlorine compounds in bleach plant effluents [5,15]. Partial enzymatic hydrolysis of xylan in pulp is thought to render the pulp more permeable by increasing fibre swelling and break lignin–carbohydrate bonds, which facilitates subsequent bleaching [14]. *Acrophialophora nainiana*, *Humicola grisea* var. *thermoidea* and *Trichoderma harzianum* strains 4 and 6 (also named T4 and T6, respectively) were described as good producers of xylan-degrading enzyme preparations [12,15]. In this research, the potential of crude cellulase-free xylanase preparations from these three fungal species was evaluated to determine if any of the preparations could enhance biobleaching in an elemental chlorine-free (ECF) system. This work also represents the first study investigating the potential of a crude cellulase-free xylanase preparation from *A. nainiana* for the specified target application.

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Materials and methods

Growth conditions

The fungi were cultured in 1-l Erlenmeyer flasks containing 1.0% (w/v) fruit stalk, a residual component from the banana plant (*Musa cavendishii*), birchwood or oat spelt xylans in 100 ml of minimal medium with the following composition (w/v): 0.7% KH_2PO_4, 0.2% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.1% $(NH_4)_2SO_4$ supplemented with 0.06% yeast extract. Flasks were inoculated with spore suspensions (1×10^6 ml^{-1}) from routine agar cultures. Liquid cultures were grown for 7 days at 28°C (*T. harzianum* strains 4 and 6) and 40°C (*H. grisea* var. *themoidea* and *A. nainiana*) with shaking at 100 rpm and pH 7.0 media buffered at pH 7.0. Subsequently, the contents of each flask were filtered through Whatman filter paper No. 1. The supernatants, hereafter called crude extract, obtained from the filtration procedure were stored at 4°C until required. The crude extract from *T. harzianum* strain 6 was subjected to ultrafiltration using an Amicon stirred cell apparatus fitted with a 10-kDa cut-off point membrane (PM 10). Xylan-degrading enzyme preparations from both the ultrafiltrate and retentate were tested in bleaching experiments.

Enzyme assays

Cellulase and xylanase activities were determined as described before [8,12].

Kraft pulp pretreatment by xylan-degrading enzyme activities

A kraft industrial pulp obtained from a combination of *Eucalyptus grandis*, *Eucalyptus saligna* and *Eucalyptus urophylla* was used; the pulp was previously delignified with oxygen. It had an initial kappa number of 10.5 and a consistency of 10% (w/v). Xylan-degrading enzyme activity was expressed as the amount of enzyme required to catalyse the transformation of 1 mol of substrate (kraft pulp) per second under specified conditions (katal). A dosage of 100 nkat/g of enzyme activity was incubated with 50 g of pulp under reaction conditions of 50°C and pH 6.5. Brightness and viscosity tests, before and after treatment with xylan-degrading enzyme preparations, were performed according to the recommendations of the Technical Association of the Pulp and Paper Industry (Atlanta, GA), using protocols outlined in Tappi T452 om-92 and Tappi T230 om-94, respectively. The kappa number (Tappi T 236 cm-85) was expressed as the amount (milliliters) of a 0.1 N $KMnO_4$ solution consumed by 1 g of moisture-free pulp. A typical ECF bleaching sequence adopted was: X-D1-E-D2, where X is enzyme, D1 and D2 are first- and second-stage chlorine dioxide steps and E signifies alkaline extraction. A charge of 0.5% (v/v) was used for D1 and D2. In the control sequence, the pulp underwent the same treatment with the omission of enzyme. A corresponding volume of distilled water was used instead of enzyme. After completing the bleaching sequence, the pulp was thoroughly washed three times with tap water, before further analysis. The experiments described were carried out in triplicate. The standard deviation was less than ±20% of the mean.

Results and discussion

Enzyme preparations from three different fungal species, enriched in xylan-degrading enzyme activities, were investigated to evaluate their use in *Eucalyptus* pulp bleaching. The aerobic fungi *T. harzianum* strains 4 and 6, *H. grisea* var. *thermoidea* and *A. nainiana* were reported to produce good levels of extracellular xylan-degrading enzyme activities [9]. Pretreatment of kraft pulp with these enzyme preparations, individually or in combination, at the early stage of pulp bleaching reduced the amount of chlorine chemicals required for subsequent bleaching and enhanced brightness gain. A brightness value of 85.4% and a viscosity of 15.75 cP were determined when the pulp was chemically bleached without the enzyme treatment (control). Enzymatic treatment prior to chlorine dioxide and alkaline extraction procedures yielded an increase in brightness from 86.2% to 88.7%; the best value was obtained with a combination of *T. harzianum* strain 4 and strain 6 enzyme preparations (Table 1). The gain in brightness and reduction of the amount of chlorine compounds after xylanase treatment was reported in the literature [6,15,17]. Pretreatment of kraft pulps using xylanase preparations from *Staphylococcus* sp. SG-13 and *Streptomyces* sp. QG-11-3 enhanced the overall brightness by 11% and 20%, respectively [2]. A brightness increase of 10% has been reported for pulp treatment with xylan-degrading enzyme activity from *Humicola* sp. [13]. However, the brightness of oxygen-bleached hardwood kraft pulp did not increase with increasing amounts of added xylan-degrading preparations from *Aureobasidium pullulans* [16].

Alkaline extraction in combination with xylan-degrading enzyme treatment was reported to improve pulp characteristics [4]. In contrast to work with enzymes from *Coriolus versicolor* [10], our results indicated that the observed brightening of *Eucalyptus* pulp by the enzyme preparations was enhanced by subsequent alkaline extraction. The extraction is suggested to facilitate the dissolution of lignin–carbohydrate fragments in pulp that were previously modified by these enzymes but still remain in pulp because of their large molecular weight [4]. The amount of chlorine residues detected after pulp treatment with xylan-degrading enzyme preparations was higher than the results obtained for pulp treatment with conventional chemical bleaching. The amount of residual chlorine obtained in D1 and D2 stages after pretreatment with individual enzyme or in enzyme cocktails varied from 2.0 to 14.2 g/l and 0.0 to 7.1 g/l, respectively. The best overall effects were obtained with the enzyme combinations containing crude extracts of *T. harzianum* strain 6 and *H. grisea* var. *thermoidea*, *H. grisea* var. *thermoidea* and *A. nainiana*, and *T. harzianum* 6 and *A. nainiana* and both *T. harzianum* strains. A residual chlorine amount of 0.07 g/l was obtained in the chemical bleaching after the D1 stage, while chlorine was not detected in the subsequent D2 stage. The residual chlorine obtained from the bleaching sequences with enzymes is an indication that the charge of chlorine dioxide in the beginning of the stage can be reduced or that the stage parameters can be changed in order to allow an

Table 1 Effect of xylan-degrading enzyme treatment on *Eucalyptus* kraft pulp

Xylan-degrading enzyme source	Brightness (% ISO)	Viscosity (cP)	Kappa number
Control[a]	85.4	15.75	0.707
A. nainiana[b]	86.2	15.85	0.550
H. grisea var. *thermoidea*[b]	87.5	13.03	0.457
T. harzianum strain T4[b]	87.5	15.08	0.430
H. grisea var. *thermoidea*[b] and *T. harzianum* strain T4[b]	87.4	12.76	0.550
H. grisea var. *thermoidea*[b] and *A. nainiana*[b]	88.3	12.76	0.581
T. harzianum strain T6[b] and *H. grisea* var. *thermoidea*[b]	87.2	13.85	0.612
A. nainiana[c]	86.9	17.11	0.519
H. grisea var. *thermoidea*[c]	87.1	16.62	0.457
T. harzianum strain T6[c] and *A. nainiana*[c]	88.0	15.08	0.519
A. nainiana[d]	87.4	16.29	0.519
T. harzianum strain T4[d]	87.7	15.85	0.550
T. harzianum strain T6[d]	88.0	16.29	0.272
T. harzianum strain T6[d]*	87.7	16.11	0.550
H. grisea var. *thermoidea*[d]	87.0	16.29	0.612
T. harzianum strain T6[d] and *H. grisea* var. *thermoidea*[d]	88.5	16.02	0.426
T. harzianum strain T6[d] and *A. nainiana*[d]	88.3	15.85	0.426
T. harzianum strains T4[d] and T6[d]	88.7	15.85	0.581

[a]Pulp bleaching without enzyme pretreatment.
[b]Growth in fruit stalk as carbon source.
[c]Growth in birchwood xylan as carbon source.
[d]Growth in oat spelt xylan as carbon source.
*Ultrafiltrate.

increase in pulp brightness. Therefore, the enzymatic prebleaching procedure of *Eucalyptus* pulp is a suitable alternative approach to lignin removal.

Enzyme treatment improved delignification of *Eucalyptus* spp. kraft pulp, thus reducing the amount of active chlorine required to obtain a given pulp brightness. The best reduction in kappa number was achieved with a xylanase preparation from *T. harzianum* strain 6 (Table 1). The process of release of lignin by xylan removal and further increased swelling of the fibre walls could explain this effect. For most enzyme preparations, the viscosity of kraft pulp produced in the sequence X-D1-E-D2 was higher than that of the control sequence, specially from *A. nainiana* (Table 1). This effect has been attributed to enzyme-mediated hydrolysis of low DP xylans [7,10]. However, a moderate loss of viscosity when a 100 nkat/g charge of enzyme activity was used may be due to some other reason than cellulose-degrading enzyme activity. All xylan-degrading enzyme preparations were not active against filter paper and carboxymethyl cellulose as substrates (results not shown). However, we cannot discard the hypothesis that low levels of synergistic endoglucanase and cellobiohydrolase activities in samples may be responsible for the observed decrease in pulp viscosity. In comparison to our results, the use of xylanase preparations from *Streptomyces lividans* and *A. pullulans* in pretreatment of sulphite and kraft pulps, respectively, resulted in a significant reduction of viscosity [3,11]. A low decrease in pulp viscosity was observed for enzyme pretreatment using crude extract preparation from *A. nainiana* (Table 1). In a previous work [15], we reported a moderate reduction in kraft pulp viscosity using a purified xylanase (Xyn I) from *A. nainiana*.

In conclusion, the pretreatment of *Eucalyptus* kraft pulp with xylan-degrading enzyme preparations from *T. harzianum* strains 4 and 6, *H. grisea* var. *thermoidea* and *A. nainiana* was shown to have beneficial effects on delignification, brightness and viscosity changes. The use of enzyme system combinations can be a technical alternative for ECF bleaching sequence and industrial application of xylanases in pulp bleaching since one can tailor the enzyme combination according to the pulp characteristics.

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