Fungal Pretreatment of *Eucalyptus* Wood Can Strongly Decrease the Amount of Lipophilic Extractives during Chlorine Free Kraft Pulping

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Modern environmentally sound trends in manufacturing of bleached paper pulp involve development of totally chlorine free (TCF) bleaching and zero liquid effluent (ZLE) processes. Lipophilic extractives are among the most problematic wood constituents for both TCF and ZLE processes, since they tend to accumulate in circuits resulting in new manufacturing ("pitch" deposits) and environmental troubles. The extractive-degrading fungi *Bjerkandera adusta, Phlebia radiata, Pleurotus pulmonarius* and *Poria subvermispora* were assayed to remove these compounds from *Eucalyptus globulus* wood using solid-state fermentation conditions. The pretreated chips were subjected to laboratory kraft pulping to assess the effectiveness of the treatment. Evaluation of extractive removal was carried out by gas chromatography−mass spectrometry of extracts from the pretreated wood and the pulps and black liquors after cooking. As a result of the fungal pretreatment, up to 75% decreased levels of problematic compounds (including free and esterified sitosterol) were found in pulps and liquors. Moreover, a significant reduction of potential acute toxicity was found in black liquors from wood pretreated with three of the fungi. The bio-pulps were TCF-bleached, refined and handsheet properties evaluated to select the most advantageous fungi from the point of view of their industrial applicability in environmentally sound pulp manufacturing processes.

Introduction

The bleaching process for the manufacture of paper pulp is undergoing a dynamic development. There are today two strong trends in the process development of bleaching of chemical pulps. One is totally chlorine free (TCF) bleaching. The second is the closed cycle mill concept. Considerable progress has been made in reducing the formation of chlorinated organics in the mills manufacturing bleached chemical pulp. Today, a lot of effort is made in the development of TCF bleaching sequences, and in a few years molecular chlorine traditionally used for pulp bleaching will almost completely be replaced by more environmentally sound bleaching methods, such as hydrogen peroxide, oxygen or ozone (*1*). On the other hand, the closed cycle mill concept is still far away but is nevertheless more realistic than it was few years ago (*2*). This has now reached the point where the attainment of "zero liquid effluent" (ZLE) operation is considered as a serious proposition for many pulp and paper mills. The deterioration in water quality with increased system closure is one of the reasons cited by mills for not reducing water consumption. Among others, one of the major challenges in the closed-cycle TCF mill is the handling of lipophilic wood extractives that tend to accumulate in circuits. These compounds form the so-called *pitch* deposits resulting in low-quality final product and problems in mill operations with important economic losses (*3*). The increasing need for recirculating water in mills and reducing effluents to meet ZLE operation is leading to an increase in pitch concentrations which results in higher deposition. Likewise, some pitch problems became more severe with the introduction of environmentally sound TCF bleaching sequences.

Traditionally, pitch deposits in pulping and subsequent paper manufacture have been reduced by debarking and seasoning logs and wood chips and by adding physicochemical control agents (*4*−*7*). However, the cost is high and often the results are far from satisfactory. As alternatives to the above, biological removal of wood extractives by treatment with enzymes (*8*−*10*) or microorganisms (*11, 12*) have been suggested in recent years for pitch control. The biotechnological preparations commercially available are not effective for pitch control during pulping of *Eucalyptus* wood, which is extensively used as pulp raw material in Spain, Portugal, Brazil and other countries. This is because they are based on enzymes or organisms mainly hydrolyzing triglycerides, which only represent a minor fraction of extractives from *Eucalyptus globulus* and related species (*13*). Moreover, high-quality TCF *E. globulus* pulps are obtained by kraft alkaline cooking, which saponifies triglycerides from pulpwoods (*14*). In a previous screening, a wide set of fungal strains were tested for their ability to remove extractives from *Eucalyptus* wood (*15*), and their action on the most problematic extractives was investigated by gas chromatography−mass spectrometry (GC-MS) (*16*). In the present study, *Eucalyptus* wood chips were treated prior to pulping with four fungal strains selected in the course of the above studies. The amount of wood treated with the fungi was large enough to permit the evaluation of the effectiveness of the biological pretreatment by laboratory kraft cooking followed by TCF bleaching and assessment of refining, mechanical and optical properties of the environmentally sound "bio-pulps" obtained.

Methods

Fungal Treatment of the *Eucalyptus* Wood. Chips of *E. globulus* wood (2−4 × 20−40 mm) were obtained from ENCE pulp mill (Pontevedra, Spain). The following fungal strains were selected for bio-kraft experiments: *Bjerkandera adusta* CBS 230.93, *Phlebia radiata* CBS 184.83, *Pleurotus pulmonarius* CBS 507.85, and *Poria* (synonym: *Ceriporiopsis*) *subvermispora* CBS 347.63. The fungi were grown in 1-L flasks with 200 mL of glucose-ammonium-yeast extract medium (*17*) for 5 d, and the pellets produced were washed and used

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to inoculate the *Eucalyptus* chips. These were incubated under solid-state fermentation (SSF) conditions (water holding capacity) in a rotary fermentor including six 2-L bottles, each of them containing 350 g of chips, sterilized at 120 °C for 20 min (*18*). The bottles were flushed with wet air (165 mL/min), rotated 1 h/day at 1 rpm, and maintained at 28 °C. After 21 d, the treated chips were dried in an aerated oven at 60 °C, ground to sawdust using a knife mill, and extracted with acetone and the lipophilic compounds were analyzed by GC-MS as described below. Klason lignin content was estimated following rule T 222 om-88 (*19*), after hot-water extraction of extractive-free wood.

Laboratory Cooking and Bleaching and Evaluation of Pulp Properties. Kraft cooking was performed in a Lorentzen & Wettre digester at 165 °C (50 min), with 3.5 liquor/wood ratio, and 25% sulfidity. The digester consisted of six 2.5-L autoclaves (loaded with 200 g of wood) which rotated at 2.8 rpm in a poly(ethylene glycol) bath (90 min to reach 165 °C). Five to seven replicates were obtained from each wood sample. Using 15% active alkali the same kappa number was obtained for control and wood treated with *P. pulmonarius* and *B. adusta*, and some increase of the total alkali was necessary after wood treatment with *P. subvermispora* and *P. radiata* (15.5% and 16% respectively). After cooking, the brown pulps were disintegrated, washed and filtered. Yield (%), residual alkali (g/L), kappa number (ISO 302), viscosity (mL/g) (ISO 5351/1), and brightness (%) (ISO 2470) were analyzed (*20*). Brown pulp samples were dried in an aerated oven at 60 °C and extracted for GC-MS analysis of lipophilic compounds.

The TCF bleaching sequence used was OQPoP, where O is an oxygen prebleaching stage, Q represents the chelating stage, and Po and P are two hydrogen peroxide stages, the former under pressurized oxygen. Bleaching experiments were carried out in stainless steel reactors equipped with an internal stirrer, a system injecting oxygen, and automatic controls of pressure and temperature. Kappa number, viscosity and brightness were estimated at the different bleaching stages. In addition, average fiber length (mm) and amount of fines of less than 0.45 mm (%) were estimated in the bleached final pulp (Kajaani FS-200).

To evaluate their papermaking properties, using ISO standard methods (*20*), the TCF-bleached pulps were refined using a PFI mill (ISO 5264/2) by applying different energies, expressed as total beating revolutions. Then, the Schopper-Riegler refining degree (°SR) was estimated (ISO 5702), and handsheets were prepared (grammage 65 g/m^2) (ISO 536). Apparent density (g/cm^3) (ISO 5270), tensile (N·m/g) (ISO 1924), burst (kPa·m^2/g) (ISO 2758) and tear indices (mN·m^2/g) (ISO 1974), scattering coefficient (m^2/kg) (ISO 9416), opacity (%) (ISO 2469), Gurley (s) (ISO 5636/5) and Bendtsen air permeabilities (ml/min) (ISO 5636/3) were estimated in handsheets.

Analysis of Lipophilic Extractives. For chemical analysis of extractives, wood sawdust and dried pulp were Soxhlet-extracted with acetone for 6 h (*19*). In the case of black liquors from cooking, liquid/liquid extractions (× 3) with hexane-acetone (2:1) at pH 12 were performed (*21*). The latter extraction conditions had been optimized for recovery of sterols, as the most problematic compounds during kraft pulping of *Eucalyptus* wood. The extracts were evaporated to dryness and redissolved in chloroform for chromatographic analyses of the lipophilic fraction. The GC-MS analyses were performed in a Varian Star 3400 gas chromatograph with an ion trap detector (ITD, Varian Saturn 2000) using a 15 m × 0.25 mm DB-5HT capillary column (0.1 μm film thickness, J&W Scientific). Helium was used as the carrier gas. Samples were injected with an auto-injector (Varian 8200) directly onto the column using a septum-equipped programmable injector. The temperature of the injector during the injection was 120 °C and 0.1 min after the injection was programmed to 380 °C (10 min) at a rate of 200 °C/min. The oven was programmed from 120 °C (1 min) to 380 °C (5 min) at 10 °C/min. The temperatures of the ITD and transfer line were set at 200 °C and 300 °C, respectively. Compounds were identified by mass spectra comparison with those in the Wiley and Nist libraries, by mass fragmentography and, when possible, using standards. Peaks were quantified by area. A mixture of standard compounds (palmitic acid, sitosterol, cholesteryl oleate and triheptadecanoin) was used to elaborate a calibration curve for quantitation of wood extractives in a concentration range between 0.1 and 1 mg/mL (all correlation coefficients being higher than 0.99).

Ecotoxicological Tests. To determine the potential acute toxicity of black liquors, the Microtox bioassay was used. This is based on inhibition of bioluminescence of the marine bacteria *Photobacterium phosphoreum* after a short exposure to sample (*22*). The evaluation of potential chronic toxicity was carried out using the *Selenastrum* algal growth inhibition test (*23*). Toxicity was expressed as EC50 15′, which is the concentration causing 50% effect on the system used to evaluate toxicity (i.e., 50% decrease of bacterial luminiscence or algal growth) after 15 min exposure.

Results and Discussion

To develop a biotechnological pretreatment overcoming some of the drawbacks in manufacturing TCF kraft pulp from *E. globulus* wood, mill chips were treated under SSF conditions with the extractive-degrading fungi *B. adusta*, *P. radiata*, *P. pulmonarius* and *P. subvermispora* (*15, 16*). After 3-week fungal treatment, the chips were used as raw material for laboratory kraft pulping followed by TCF bleaching and papermaking evaluation. With the only exception of *B. adusta*, which caused 12% wood degradation, the weight loss was moderate (6−7%). In addition to extractive degradation, wood treatment with these fungi also decreased Klason lignin content, up to 24% in the case of *P. radiata*, and 9% by *B. adusta*, 7% by *P. subvermispora*, and only 3% by *P. pulmonarius*.

Samples of pulp and black liquor from laboratory kraft cooking, together with the pretreated wood, were extracted and the lipophilic fraction analyzed by GC-MS. As shown in Figure 1A, esterified (up to 39% of total chromatographied compounds) and free (29%) sterols, steroid hydrocarbons (12%), free fatty acids (12%), and steroid ketones (3%) were the main compounds identified in the wood samples. Changes in the chromatographic profiles of lipophilic compounds were found after kraft pulping, including reduced amount of steroid hydrocarbons and sterol esters in pulp (Figure 1B). The black liquor was characterized by the absence of sterol esters and the presence of a lignan, probably arising from lignin depolymerization (Figure 1C).

The above chromatographic profiles were drastically modified as a consequence of the fungal pretreatment of *Eucalyptus* wood prior to pulping. This is illustrated in Figure 1D−F showing the main lipophilic compounds present in the wood treated with *P. radiata* and in the pulp and black liquor obtained after its kraft cooking. As a result of the fungal pretreatment, the peaks corresponding to sitosterol and sterol esters—the latter including sitosterol, and lower amounts of stigmastanol and citrostadienol, esterified mainly with linoleic and oleic acids—decreased in all samples. Since free and esterified sterols had been found to be the main lipophilic compounds in pitch deposits from different parts of the *Eucalyptus* kraft mills (*16*) and TCF pulp (*24*), these compounds were quantified in the different samples analyzed. Table 1 shows the sterol and sterol ester content in the control wood, pulp and black liquor, and the effect of the fungal

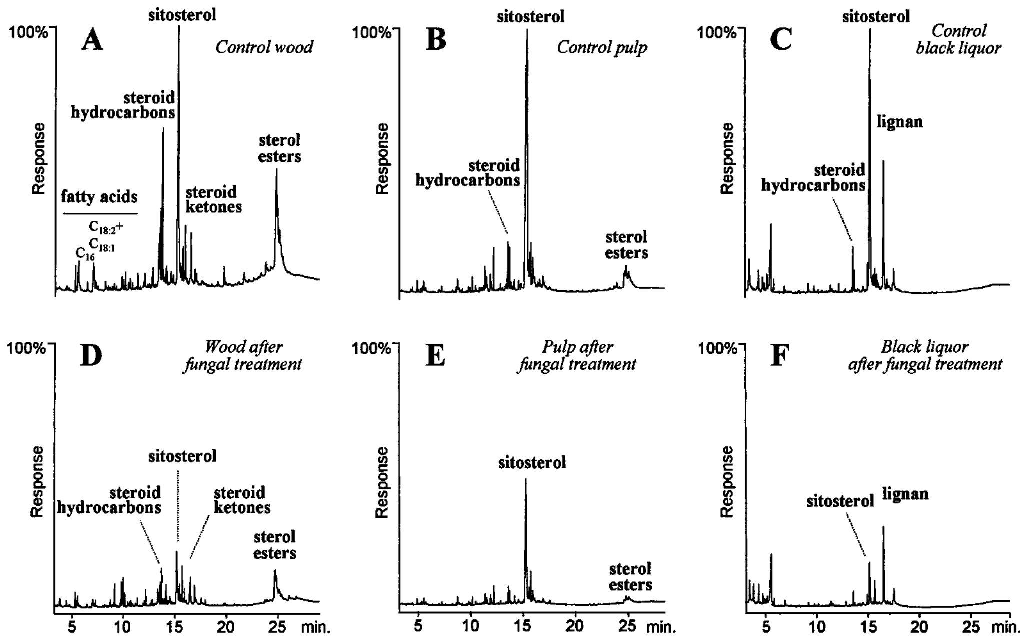

FIGURE 1. Chromatographic analysis of extractives during laboratory kraft cooking of *E. globulus* wood pretreated with *P. radiata* (bottom) and untreated control (top): A comparison of lipophilic compounds in wood (A and D), brown pulp (B and E) and black liquor (C and F) (the same amount of sample was extracted in each case).

TABLE 1. Decreased Lipophilic Compounds by Pretreating *E. globulus* Wood with Fungi Prior to Laboratory Kraft Cooking[a]

		fungal removal (% of control)			
	control[b]	*P. pulmonarius*	*P. radiata*	*B. adusta*	*P. subvermispora*
Wood					
free sterols	218	46	77	56	68
sterol esters	206	32	57	62	51
Pulp					
free sterols	324	48	57	55	44
sterol esters	61	61	61	71	40
Black liquor					
free sterols	8.7	69	76	55	44

[a] Total amount in controls is shown in the first column. [b] mg/kg of wood or pulp and mg/L black liquor.

TABLE 2. Properties of Brown Pulp, Fiber Length and Fines (Final Pulp) and Potential Toxicity (EC50) of Black Liquors after Kraft Cooking of *E. globulus* Wood Pretreated with Fungi and Control Wood

		fungal treatment			
	control	*P. pulmonarius*	*P. radiata*	*B. adusta*	*P. subvermispora*
yield (%)	53.5	53.2	52.7	53.1	51.3
kappa number	15.9	15.9	15.8	15.9	15.3
viscosity (mL/g)	1464	1381	1355	1350	1287
brightness (% ISO)	43.5	44.6	43.5	45.5	44.2
fiber length (mm)	0.63	0.61	0.62	0.60	0.61
fines (%)	17.7	21.1	19.6	21.8	20.6
EC50 (%)	0.026	0.026	0.053	0.067	0.063

pretreatment on the abundance of these compounds in the different samples. Taking into account the wood/liquor ratio in cooking and the final pulp yield (see Table 2) it can be deduced that a part of wood sterol esters are saponified during cooking (they are absent from black liquors) and that some decrease of free sterols is also produced. Although some differences were observed between the four fungi investigated, a significant decrease of sterol esters and/or free sterols as a result of fungal pretreatment was found in all the wood (32−77% decrease), pulp (40−71% decrease), and black liquor (44−76% decrease) samples. Since sitosterol is the main lipophilic compound in the pulps analyzed, wood pretreatment with *P. radiata* and *B. adusta* can be considered as the most advantageous from the point of view of the removal of problematic lipophilic compounds from *Eucalyptus* kraft pulps. The efficiency of these fungi removing sterol esters from hardwood was much higher than reported for commercial *Ophiostoma piliferum* inocula (Cartapip), which in addition are not able to degrade free sterols (*16*, *25*). The capability of liquid cultures of the white-rot fungus *Phanerochaete chrysosporium* to degrade sterol esters from aspen, responsible for pitch troubles (*26*), has been reported (*27*). However, the hydrolysis of esters into their fatty acid and sterol moieties, by this and other fungi, is not enough to

TABLE 3. Beating and Papermaking Properties of Bleached Pulp from Kraft Cooking of *E. globulus* Wood Pretreated with Fungi and Control Wood

	control	fungal treatment			
		P. pulmonarius	*P. radiata*	*B. adusta*	*P. subvermispora*
beating degree (°SR)[a]	33	36	36	37	37
apparent density (g/cm^3)	0.75	0.76	0.77	0.77	0.77
tensile index (N·m/g)	95.7	98.7	98.3	94.0	92.0
burst index (kPa·m^2/g)	7.00	6.89	7.14	7.45	7.05
tear index (mN·m^2/g)	9.02	7.90	7.70	7.40	8.00
Gurley permeability (s)	26	34	44	44	39
Bendsten permeability (mL/min)	800	600	480	400	480
scattering coeff (m^2/kg)	23.9	24.1	24.0	24.1	23.3
opacity (%)	68.2	68.1	68.1	68.0	67.0

[a] All data correspond to same refining energy (a total of 4200 revolutions in PFI mill).

control pitch since it has been shown that free sitosterol is also involved in deposit formation.

Evaluation of cooking, refining and papermaking properties of the pretreated wood was carried out to select the fungal strains with the highest potential to control pitch troubles in manufacturing of TCF kraft pulp from *Eucalyptus* wood. Several properties of the bio-pulps obtained are summarized in Table 2, compared with control pulp from untreated wood. Pulp yield after wood treatment with *B. adusta*, *P. pulmonarius*, and *P. radiata* (around 53%) was similar to that obtained with the untreated chips, whereas a lower yield was obtained after wood pretreatment with *P. subvermispora*. Some reduction of viscosity of unbleached pulp was observed after all fungal treatments (5−12%), but, as mentioned below, the viscosity differences were lower in the final pulp. The same active alkali (around 15%) was used to attain similar kappa numbers (between 15 and 16) in pulps from the wood samples treated with fungi and the untreated control. However, an increase of the unbleached pulp brightness (up to 2% ISO) was produced after wood treatment with *B. adusta*, *P. pulmonarius*, and *P. subvermispora*. Both decrease of kappa number and increase of brightness have been reported during sulfite pulping of *Eucalyptus* wood pretreated with fungi (*28*). The comparison between Microtox and other acute toxicity tests (on invertebrates and fishes) pointed out satisfactory sensitivity for pulp and paper mill effluents (*22*). Using the former method, a strong decrease of potential acute toxicity of the laboratory black liquors resulting in 2−2.6-fold increased of EC50 values (the sample concentration causing 50% reduction of bacterial luminescence after 15 min exposure) was observed after wood pretreatment with *P. radiata*, *P. subvermispora* and *B. adusta* (Table 2). No significant chronic toxicity was found in the black liquors from *Eucalyptus* kraft cooking.

Pulp viscosity and brightness during TCF-bleaching are shown in Figure 2A. Similar brightness degrees (89−91% ISO) were attained at the end of TCF bleaching of pulps from control and pretreated wood, and the differences in viscosity were reduced at the highest bleaching degrees. The best results were obtained with *P. radiata* yielding pulps with very similar final brightness and viscosity than the control. Some decrease of average fiber length and increase of the fine fraction content (up to 3−4% over the control content) were found in bleached pulps due to fungal pretreatment (Table 2). The bleached bio-pulps were refined by applying different beating energies (i.e., total revolutions in the PFI mill) (Figure 2B). Then, tensile (Figure 2C) and other papermaking indices were estimated in the handsheets prepared from pulps at the different refining degrees. For comparative purposes, Table 3 shows the values of resistance, porosity and optical indices at an intermediate beating degree (4200 revolutions) yielding pulps with a refining degree (33−37 °SR) similar to those used for paper manufacture. In general, the pulps from wood pretreated with fungi were easier to refine than the control (i.e., higher refining degrees were obtained for the same beating revolutions), the best results being obtained with *P. subvermispora* (Figure 2B). The fungal treatment of wood also resulted in an increase of apparent density at all the refining degrees assayed. The mechanical properties of the bio-pulps slightly differed from those of control pulp but an improvement of tensile index at the different beating energies assayed was obtained with *P. radiata* and *P. pulmonarius* (Figure 2C). Moreover, some improvement in burst index was obtained with *B. adusta, P. radiata* and *P. subvermispora*, but the tear index was decreased in all cases. The optical properties were conserved after wood pretreatment with most of the fungi, and similar handsheet scattering coefficients and opacities (as well as pulp brightness) were found. The most remarkable difference concerned handsheet porosity, estimated by two air permeability tests. The porosity of bio-pulps dramatically decreased when beating energy increased, especially in the case of those from wood pretreated with *P. subvermispora* (Figure 2D).

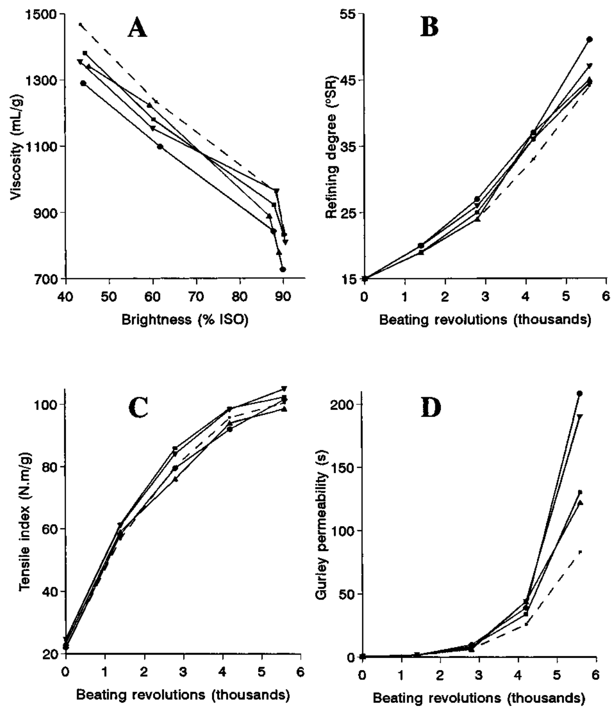

FIGURE 2. TCF bleaching and mechanical properties of pulps after laboratory kraft cooking of *Eucalyptus* wood treated with *P. pulmonarius* (■), *P. radiata* (▼), *B. adusta* (▲) and *P. subvermispora* (●) compared with control wood (dashed line): A. Pulp viscosity vs brightness during TCF bleaching. B−D. Refining degree, tensile index and Gurley porosity vs beating energy.

It is possible to conclude that a fungal pretreatment can strongly decrease the amount of problematic lipophilic compounds (namely sitosterol and sterol esters) during TCF kraft pulping of *Eucalyptus* wood maintaining most pulp properties, some of the most promising results being obtained with *P. radiata*. The decrease of potential acute toxicity of the black liquors is another advantage of the biological pretreatment investigated. Some of the changes observed in pulping and papermaking properties can be considered as positive, such as the reduction of refining energy and the improvement of some mechanical properties. The decrease of total pulp yield (considering wood weight during biological pretreatment and cooking yield) varies from 53% for control wood to 47−50% for wood treated with fungi, and it should be further optimized by modifying the duration of the biological pretreatment or by improving the fungal strains used to treat wood. Concerning other pulp properties, some of the most significant changes (e.g., the increased amount of fines in unrefined pulp, the increased apparent density of handsheets, and the strong decrease of their porosity) seem related to the modification of fiber size distribution during fungal pretreatment. The final selection of the most promising fungal treatment for industrial control of pitch deposition should be a compromise between the optimum in terms of extractive removal, the lowest decrease of pulp yield and the conservation of those pulp properties of interest for the different types of paper to be produced. The ultimate goal is a sustainable production of paper pulp, i.e., a kraft pulp mill in ecological balance with nature, with the use of biotechnological tools enabling a higher degree of system closure in mills and a better performance of environmentally sound TCF bleaching processes.

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