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Production and Characterization of *Phanerochaete chrysosporium* Lignin Peroxidases for Pulp Bleaching

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ABSTRACT

The production of lignin peroxidase from *Phanerochaete chrysosporium* was studied using immobilized mycelia in nylon-web cubes in semicontinuous fermentation using glucose pulses or ammonium tartrate pulses. Consistent enzyme production was achieved when glucose pulses were used, leading to an average activity of 253 U/L. The crude enzyme was added to eucalyptus kraft pulp before conventional and ECF bleaching sequences. Optimization of the enzymatic pretreatment led to the following operational conditions: enzyme load of 2 U/g of pulp, hydrogen peroxide addition rate of 10 ppm/h, and reaction time of 60 min. Pulp final characteristics were dependent on the chemical treatment sequence that followed enzymatic pretreatment. The chief advantage of enzymatic pretreatment was pulp viscosity preservation, which was observed in most of the experiments carried out with seven different chemical treatment sequences.

Index Entries: *Phanerochaete chrysosporium*; lignin peroxidase; enzymatic prebleaching; eucalyptus kraft pulp; selectivity.

INTRODUCTION

World production of market pulp was estimated at 30 million metric tons in 1995, consisting of 55% softwood pulp and 45% hardwood pulp. The main exporters of bleached sulfate pulp are Canada, United States, Sweden, Brazil, Finland, and Chile *(1)*.

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Conventional industrial bleaching with chlorine produces effluents with chlorinated organic compounds that adversely effect the environment. As a consequence, pulp and paper industries are under growing pressure from authorities, consumers, and environmental groups to reduce their pollution load *(2)*.

The environmental concerns also contribute to the development of new technologies of delignification, bleaching, and effluent treatment. In the case of bleaching, new technologies include use of ClO_2 instead of chlorine; bleaching without chlorine; use of hydrogen peroxide, oxygen, ozone, peracids; and, finally, bleaching with the fungal enzymes, lignin peroxidases (LiP), manganese-peroxidases (MnP), laccases and xylanases, known as biobleaching *(3–6)*.

Several studies have also dealt with the direct cultivation of the fungus *Phanerochaete chrysosporium* on chips or pulps. In some cases, it was observed reduction of the energy required for refining and improvement of the mechanical properties of the pulp, Kappa number reduction, and brightness increase. Biopulping, however, presents the major drawback of extended time of treatment *(7)*.

The major components of the ligninolytic system of the white rot fungus *P. chrysosporium* are the two extracellular heme peroxidases, LiP and MnP *(8,9)*. The production of these enzymes for industrial purposes, however, has been hindered by their low stability under usual fermentation conditions. Considering that enzyme inactivation can be caused by the simultaneous presence of proteases in the culture medium, some reported strategies for enzyme production involve the use of glucose pulses, which would selectively repress protease production *(10–14)*.

Among the possible applications of LiP and MnP in the pulp and paper industry, the utilization in biobleaching is the most promising because the enzymes may be very efficient, and can be used under industrial conditions. LiP prebleaching may enhance bleachability of the pulp, preserving its strength properties *(15)*. Xylanase has also been studied for prebleaching, and its use is reported to facilitate the subsequent chemical bleaching of kraft pulp, although the mode of the enzyme action is not fully understood. According to Kantelinen et al. *(6)*, hemicellulases remove xylan from the fiber surface and renders it more permeable. This facilitates the further stages of pulp treatment with bleaching chemicals. LiP and xylanase pretreatment can be applied to any traditional or modern bleaching sequence without significant investments in existing plants. The objectives of the enzymatic treatment are to decrease the consumption of chemicals, to reduce the pollution load, and to increase final pulp brightness.

The main objective of this study was to investigate the adequacy of LiP pretreatment for bleaching eucalyptus kraft pulp. Enzyme production and stability under fermentation conditions were also studied. Semicontinuous fermentations were carried out, using glucose pulses in the absence of nitrogen source, and ammonium tartrate pulses in absence of glucose.

MATERIALS AND METHODS

Strain Maintenance and Activation

The fungus *P. chrysosporium* ATCC 24725 was used for enzyme production. The strain was maintained on 2% malt-agar slants, under refrigeration. Slopes of the same agar were used for production of spores, which were used as inoculum in all fermentations. A dense sporulation was observed within 1 wk of incubation at room temperature. To obtain the spore suspension to be used as inoculum, 5.0 mL of sterile dH_2O were carefully added to a slope. The resultant spore suspension was filtered through sterile glass wool to remove hyphal fragments. The concentration of spores was determined using a standard curve that correlated absorbance at 650 nm with spores concentration in spores/mL.

Fermentations

Experiments were performed using immobilized mycelium. For the immobilization step, shake-flask fermentations, using a chemically defined, carbon-limited medium, were carried out in the presence of nylon-web cubes *(16)*. Flasks containing 75 mL of the culture medium were inoculated with 1.65×10^7 spores, incubated at 37°C, and shaken at 150 rpm. The spores were found to germinate preferentially inside the particles *(17)*. After the growth phase, which was coincident to glucose depletion, veratryl alcohol (2.5 m*M*) was added to the culture, and both immobilized mycelium and culture medium were transferred to a packed-bed reactor. The bioreactor consisted of a 120-mL jacketed glass column (L/D ratio, 5:1) containing a fixed bed of nylon-web cubes. Reactor operation was performed at 37°C in a semicontinuous mode. At defined time intervals, one-third of the liquid volume was replaced by fresh medium containing 1 g/L glucose or 0.22 g/L ammonium tartrate. Veratryl alcohol (2.5 m*M*) and Tween-80 (1.3 g/L) were also added to the fresh medium. Glucose or ammonium tartrate were used, in order to perform pulses during the enzyme production stage. Oxygen gas was directly fed to the reactor bottom at a constant specific rate of 0.22 mL O_2/mL/min.

Enzyme Assays

All enzyme concentration measurements were carried out in the supernatant.

LiP activity was measured by oxidation of veratryl alcohol to veratraldehyde, in presence of hydrogen peroxide, according to Tien and Kirk *(18)*. Oxidation of veratryl alcohol was monitored by absorbance at 310 nm ($\epsilon_{310} = 9300\ m^{-1}cm^{-1}$). One unit (U) of enzyme activity was defined as the amount of enzyme that oxidizes one μmol of veratryl alcohol to veratraldehyde in 1 min.

MnP activity was determined as described by Kuwahara et al. *(8)*. Reaction was monitored at 610 nm for 60 s (ϵ_{610} = 4460 $m^{-1}cm^{-1}$). One U of enzyme activity was defined as the amount of enzyme that oxidized 1 μmol of phenol red per min in presence of Mn(II) and hydrogen peroxide.

Endoglucanase activity was measured as described by Mandels et al. *(19)*, using carboxymethyl cellulose (CMC) as substrate. One U of enzyme activity produced 1 μmol of reducing sugars per min.

Protease activity was measured according to Charney and Tomarelli *(20)*, using azocasein as substrate. Samples (0.5 mL) were incubated for 40 min at 37°C, with 0.5 mL of 0.5% (w/v) azocasein solution in 50 m*M* acetate buffer. The reaction was stopped by the addition of 0.5 mL of 10% (w/v) trichloroacetic acid solution. The residual substrate was removed by centrifugation at 7000 × g for 5 min. To 1 mL of supernatant, 1 mL of 5 *N* KOH was added, and the mixture was spectrophotometrically assayed at 428 nm. The determinations were carried out against both substrate and enzyme blanks. One U of enzyme activity catalyzed the release of azo dye, causing a change in absorbance at 428 nm of 0.001/min.

Analytical

Glucose was measured as reducing sugars by the method of Nelson *(21)*. Hydrogen peroxide was measured, using a specific Peroxide test (Merckoquant 10011).

Pulp Characteristics

The industrial pulp used in the experiments was an unbleached hardwood kraft pulp, which was obtained using a mixture of several eucalyptus varieties. The initial characteristics of the pulp were: Kappa number 15; viscosity 31.7 cp., and brightness 32% ISO.

Enzymatic Pretreatment

The following conditions were investigated: enzyme loadings from 2 to 10 U/g pulp basis, reaction time from 60 to 180 min, and the rate of hydrogen peroxide addition from 10 and 20 ppm/h. Pulp at 10% consistency was acidified to pH 4.5 with lactic acid. Enzyme and hydrogen peroxide at the desired load, and 4 m*M* veratryl alcohol, were added to the pulp, and the mixture was incubated at 30°C for the required time. As a control, pulp was treated under identical conditions without enzyme. After the enzymatic treatment carried out under the studied conditions, the pulp was submitted to a short bleaching sequence (CE), and analyzed for Kappa number and viscosity. According to the results, optimized conditions for enzymatic prebleaching were defined. These conditions were used before the conventional and ECF bleaching sequences. Both enzymatic and chemical treatments were performed in polyethylene bags, in a water bath, at the desired temperatures and time intervals.

Table 1
Conditions of Conventional and ECF Bleaching Chemical Sequences

Conventional bleaching sequences
Sequence 1
A: C3E sequence
Conditions: Chlorination: 3% chlorine, 30°C, 30 min, 3% consistency
Extraction: 2% NaOH, 60°C, 60 min, 10% consistency
B: C2E sequence
Conditions: Same as sequence 1A, except with 2% chlorine
Sequence 2
CEHH sequence
Conditions: Chlorination: 3% chlorine, 30°C, 30 min, 3% consistency
Extraction: 2% NaOH, 60°C, 60 min, 10% consistency
First and second hypochlorination: 1.7% HClO, 50°C, 120 min, 10% consistency
Sequence 3
A: CEHD sequence
Conditions: Chlorination: 2,5% chlorine, 50°C, 30 min, 4% consistency
Extraction: 2.2% NaOH, 65°C, 65 min, 12% consistency
Hypochlorination: 1.0% HClO, 50°C, 120 min, 12% consistency
Dioxidation: 1.0% ClO_2, 75°C, 180 min, 12% consistency
B: CEpHD sequence
Conditions: Same as sequence 3A, except with 0.5% H_2O_2 in extraction stage
ECF Bleaching sequences
Sequence 4
DED sequence
Conditions: First dioxidation: 1.0% ClO_2, 70°C, 180 min, 6% consistency
Extraction: 2% NaOH, 60°C, 60 min, 10% consistency
Second dioxidation: 0.4% ClO_2, 70°C, 120 min, 10% consistency
Sequence 5
DEDED sequence
Conditions: First dioxidation: 1.0% ClO_2, 70°C, 180 min, 6% consistency
First and second extraction: 2% NaOH, 60°C, 60 min, 10% consistency
Second and third dioxidation: 0.4% ClO_2, 70°C, 120 min, 10% consistency

Bleaching

The conventional and ECF bleaching chemical sequences were performed as indicated in Table 1. All concentrations were expressed in terms of air-dried brownstock pulp. At the end of each stage, the pulp was filtered and washed with dH_2O.

Evaluation of Pulp Treatments

All determinations of brightness, Kappa number, and viscosities were made according to standard Technical Association of the Pulp and Paper

Industry methods. Selectivity was defined as the ratio between the degree of delignification and loss of pulp viscosity. Kappa numbers and pulp viscosities values were determined after the extraction stage and at the end of the bleaching sequences, respectively.

RESULTS AND DISCUSSION

Fermentations

Fermentation experiments were carried out to achieve consistent lignin peroxidase production. In the fermenter, feed scheme pulses of ammonium tartrate, which can be used by the microorganism as carbon (C) and nitrogen (N) source, were compared to glucose pulses. The advantages of using the salt, instead of glucose, include handling simplicity and lower chances of contamination in such a long operation.

According to the data presented in Figs. 1 and 2, which show lignin peroxidase activity and pH variation against time, using glucose or salt pulses every 12 h, overall better results were observed for the glucose scheme. These results confirm previous findings for 24-h frequency glucose pulses *(10)*, although more regular enzyme production was observed for the 12-h time interval. Enzyme production was observed for both the glucose and ammonium tartrate-operated reactors, which were run for 660 h. In the salt feed reactor, however, lignin peroxidase activity dropped at 560 h. This was concomitant to a pH increase that suggested a decrease in the viability of the immobilized mycelia.

Considering the profile of protease production (Fig. 2), the glucose feed mode was more effective in keeping a lower protease accumulation. This would explain the higher LiP activity when glucose was used.

According to the foregoing, the crude enzyme preparation used in the present work was obtained using the glucose-operated reactor. This preparation showed LiP activity within the range of 70–350 U/L. The ratio between LiP and MnP activities in the crude preparation showed an average value of 0.10. No endoglucanase activity was observed.

Bleaching

The factors affecting the efficiency of pulp treatment by LiP are pH, temperature, presence, or absence of veratryl alcohol, hydrogen peroxide concentration, reaction time, and enzyme concentration.

The presence of veratryl alcohol is an important factor for enzymatic treatment performance, because it stabilizes lignin peroxidase under excess of hydrogen peroxide, and acts as mediator in the electron transfer process between enzyme and substrate *(22)*. In this work, a concentration of 4 m*M* was applied, because, according to the literature, this reagent is used within the range of 2.5–4 m*M* *(23,24)*.

Eucalyptus kraft pulp was treated with LiP according to the conditions indicated in Table 1. In this first set of experiments, the reaction time

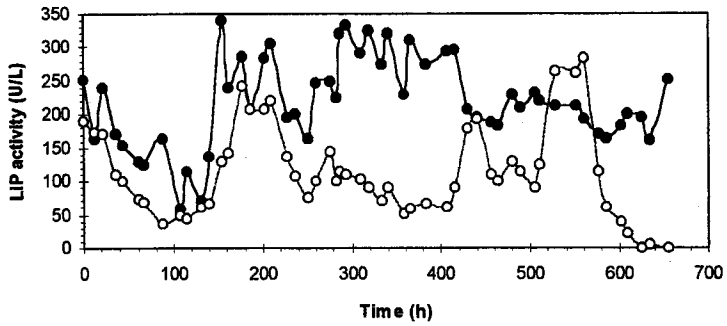

Fig. 1. Lignin Peroxidase production in a packed-bed biorreactor submitted to glucose and ammonium tartrate pulses, symbol: ● glucose pulses, and ○ ammonium tartrate pulses.

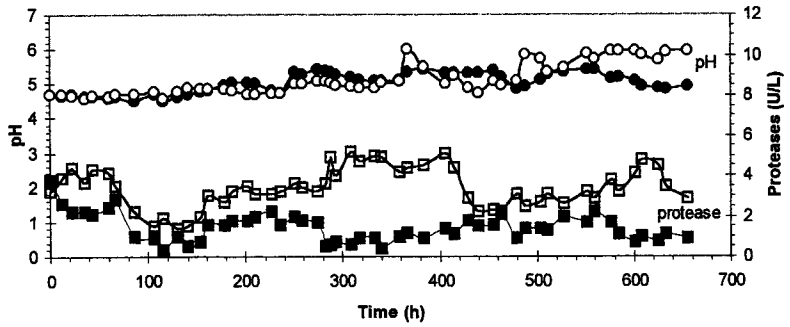

Fig. 2. Protease production and pH variation in a packed-bed biorreactor submitted to glucose and ammonium tartrate pulses, symbol: ● glucose pulses, ■ glucose pulses, ○ ammonium tartrate pulses, and □ amonium tartrate pulses.

was fixed at 180 min, which was compatible to both enzymatic reaction and industrial bleaching processes. After enzymatic prebleaching, the pulps were submitted to bleaching sequences 1A and 1B, which are listed in Table 1.

Delignification and percentage of viscosity reduction are shown in Fig. 3. The percentage of delignification remained comparable for pulps treated with enzyme loads of 2 and 5 U/g. Moreover, treatment with 10 U/g did not significantly improve delignification. Beyond 5 U/g, no appreciable viscosity reduction was observed.

Pulps treated with 2 and 5 U/g, and with 10 and 20 ppm/h of H_2O_2, showed delignification levels around 70%, when submitted to a CE bleaching sequence with 3% of chlorine. When the same sequence, with a lower level of chlorine (2%), was used, only 50% of delignification was achieved, and high viscosities were observed. In both cases, the increase of the rate of H_2O_2 addition did not improve pulp delignification. However, viscosity reduction was affected by the increase of H_2O_2 supply in most cases, inde-

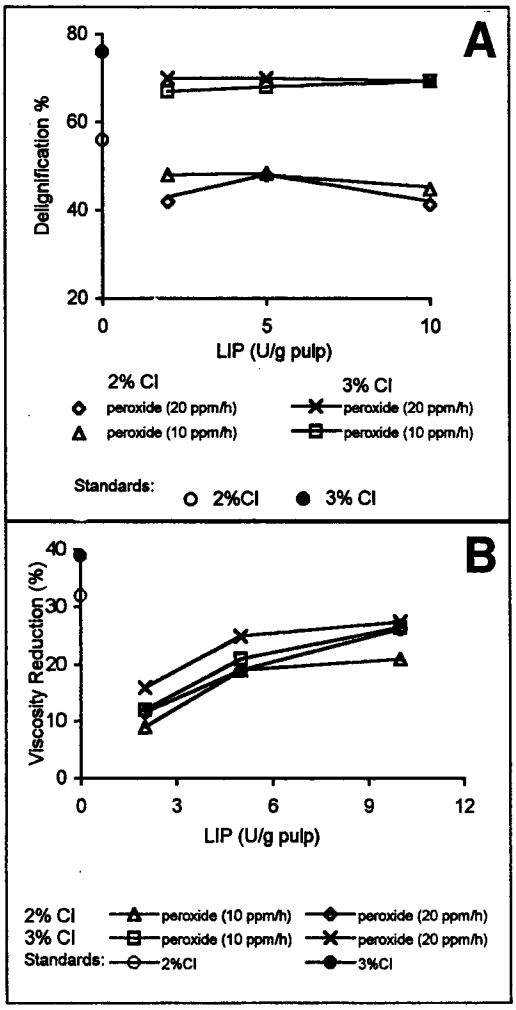

Fig. 3. Effect of enzyme and peroxide addition rate on the degree of delignification **(A)** and viscosity reduction **(B)**.

pendent of chlorine concentration, as illustrated by the results shown in Table 2. Increase of enzyme load from 2 to 5 U/g did not improve pulp delignification, as expressed by Kappa number values of 5.1 and 5.0, respectively. Final pulp viscosity was affected by both H_2O_2 supply rate and enzyme load, as illustrated in Fig. 4. As shown in Table 2, the percentages of viscosity reduction for all pulps treated with LIP were lower than those of control tests in which no enzyme or no peroxide was used.

These results (Fig. 3 and Table 2) could be explained by modifications of lignin molecules caused by the enzymatic action. The modified lignin would be more resistant to alkaline extraction. The viscosity, however, was favored by the enzymatic treatment. This could be because of the protection of the cellulose fibers by the modified lignin.

Table 2
Effect of Enzyme Load and H_2O_2 Addition Rate on Enzymatic Treatment and on Pulp Properties

LiP (U/g pulp)	H_2O_2 (ppm/h)	Chlorine %	Delignif. %	Viscosity reduction %	Selectivity
2	10	2	48	9	5.3
2	10	3	67	12	5.6
2	20	2	42	11.6	3.6
2	20	3	70	16	4.3
5	10	2	48.4	19	2.5
5	10	3	68	21	2.7
5	20	2	48	19	2.5
5	20	3	70	25	2.8
Standard					
	–	2	56	32	1.8
CE	–	3	76	39	2.0

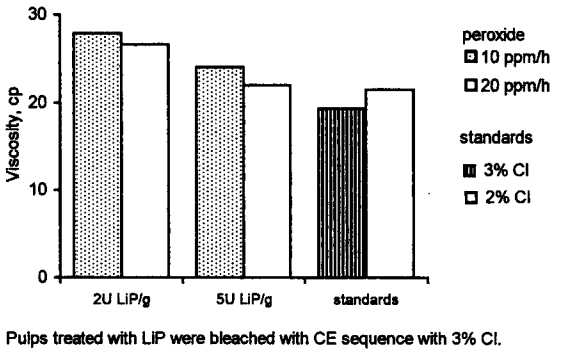

Fig. 4. Viscosities of pulps treated with LiP prebleaching.

Table 3 compares the selectivity values for pulps treated with 2 and 5 U/g to standard pulps treated with CE sequence. The results of the experiments, which were conducted for different periods of time, indicated that optimal enzyme dosage was 2 U/g. Moreover, a short reaction time led to high selectivity values. The delignification range was within 65–72%, and therefore comparable to the control experiment. LiP treatment, however, caused a marked improvement in viscosity, and it also seems to improve bleacheability in the subsequent bleaching stages (results not shown).

Experiments with pulps submitted to enzymatic prebleaching followed by ECF (elemental chlorine-free) bleaching sequences were also conducted. When a DED sequence was used (Table 4), higher selectivity was obtained for the shorter reaction time (60 min) and the lower enzyme

Table 3
Effect of Reaction Time on Kappa Number, Viscosity, and Selectivity of Pulps Treated with Lignin Peroxidase and C_3E Bleaching Sequence

LiP (U/g pulp)	Reaction time (min)	Kappa number	Delignification %	Viscosity cp	Viscosity reduction %	Selectivity
2	60	4.7	68.7	29.0	8.5	8.0
2	120	4.9	67.4	29.0	8.5	7.9
2	180	5.2	65.5	28.4	10.4	6.3
5	60	4.5	69.9	28.5	10.1	6.9
5	120	4.3	71.8	28.3	10.7	6.7
5	180	4.3	71.8	27.6	12.9	5.6
C_3E^a	180	4.4	70.6	25.5	19.6	3.6
C_2E^a	180	6.1	59.4	26.8	15.4	3.8

[a] Standard: treatment without enzyme and with H_2O_2 (10 ppm/h) and VA (4 m*M*).

Table 4
Effect of Reaction Time and Enzyme Load on Properties of Pulp Submitted to Lignin Peroxidase Prebleaching and ECF Bleaching

LiP (U/g pulp)	Reaction time (min)	Delignification (%)	Viscosity reduction (%)	Selectivity
2	60	55.0	12.6	4.4
2	180	55.3	25.8	2.1
5	60	48.0	20.2	2.4
5	180	52.6	18.3	2.9
DED[a]	60	53.5	26.5	2.1
DED[a]	180	53.3	28.4	1.9

[a] Standard with VA (4 m*M*) and peroxide (10 ppm/h), without LiP.

concentration (2 U/g) tested. In these experiments, an improvement of selectivity was observed for higher reaction times (180 min) when the enzyme load was 5 U/g.

Selectivity was also affected by the bleaching sequence used. When pulps were submitted to prebleaching (2 U/g) and subsequent bleaching with CE sequence with 3 and 2% of chlorine, those pulps showed selectivities of 5.6 and 5.3, respectively (Table 5). However, when the pulps were submitted to a DED sequence, the selectivity decreased to 4.4. Pulp characteristics resulting from several treatment sequences are also shown in Table 5.

Concerning selectivity, the best results (5.4, 4.4, and 4.0) were obtained when sequences L_2CEHD, L_2*DED, and L_2*DEDED were used, respectively. The low selectivity value observed when the sequence L_2CE_pHD

Table 5
Selectivity and Brightness of Pulp Treated with Lignin Peroxidase and Several Bleaching Sequences

Sequences	Kappa number	Viscosity cp	Selectivity	Brightness %ISO
CEHD	3.8	18.0	1.7	81.3
L_2C_3E	5.1	27.9	5.6	nd
L_2C_2E	7.8	28.8	5.3	nd
L_2CEHH	5.2	11.2	1.1	nd
L_2CEHD	5.6	28.0	5.4	75.3
L_2CEpHD	4.7	17.7	1.6	81.2
L_2*DED	6.9	27.7	4.4	nd
L_2*DEDED	6.7	27.0	4.0	nd

All conditions of bleaching sequences are listed in Table 1.
L_2: 2U LiP/g pulp/180 min.
L_2*: 2U LiP/g pulp/60 min.

was employed may be attributed to the utilization of hydrogen peroxide, which reduced pulp viscosity. However, the addition of H_2O_2 in the extraction stage of the sequence L_2CE_pHD increased pulp brightness from 75.3% to 81.2% ISO (Table 5).

The L_2CEHH sequence showed very low selectivity values. In this case, severe viscosity reduction occurred in the final bleaching stages. Sodium hypochloride promotes depolymerization of the cellulose chain, reducing pulp viscosity.

When ECF sequences were used (Chlorine dioxide sequences), namely, L_2*DED, and L_2*DEDED, selectivity results were similar to those obtained with a conventional chlorine sequence (L_2CEHD). This is an important result, because it allows the use of dioxide as a substitute of elemental chlorine. Table 5 shows that viscosities were preserved in both cases, and, as a consequence, the strength properties of pulp were also maintained. Kappa numbers were higher for pulps treated with dioxide (L_2*DED and L_2*DEDED) than for pulps treated with a conventional bleaching sequence using chlorine (L_2CEHD). The use of LiP prebleaching, associated with severe bleaching chemical sequences (L_2*DED and L_2*DEDED), may produce pulps with low Kappa numbers, high viscosities, and brightness compatible to or higher than, that of conventional pulps.

CONCLUSION

Consistent lignin peroxidase production was observed using a packed-bed reactor containing *P. chrysosporium* immobilized in sponge cubes. The reactor was operated in a semicontinuous mode, with glucose

pulses every 12 h over 660 h. Under these conditions average values of LiP activity of 253 U/L were observed. The crude enzyme preparation was shown to be effective for the biobleaching of eucalyptus kraft pulp.

Pretreatment of the eucalyptus kraft pulp with crude lignin peroxidase from *P. chrysosporium* under optimized conditions, i.e., enzyme load of 2 U/g of pulp, hydrogen peroxide addition rate of 10 ppm/h, during 60 min at 30°C, yielded a great improvement of the pulp selectivity. The delignification degree was not higher in comparison to the conventional treatment, but LiP treatment was beneficial because the pulp viscosity was preserved, even under the drastic conditions of the chemical treatments.

Considering the use of LiP on conventional and ECF sequences, the improvement on the selectivity depended on the conditions used in both the enzymatic pretreatment and the chemical bleaching sequences.

REFERENCES

1. Macedo, A. R. P., Valenca, A. C. V., and Lima, A. S. (1996) *O Papel* **11,** 45–58.
2. Mehta, V. and Gupta, J. K. (1992) *Tappi J.* **75,** 151–152.
3. McDonough, T. J. (1995) *Tappi J.* **78,** 55–62.
4. Liebergoth, N. (1996) *Pulp and Paper Canada,* **97,** 73–75.
5. Reid, I. D. and Paice, M. G. (1994) *FEMS Microbiol. Rev.* **13,** 369–376.
6. Kantelinen, A., Hortling, B., Ranua, M., and Viikari, L. (1993) *Holzforschung* **47,** 29–35.
7. Eriksson, K.-E. (1985) *Tappi J.* **68,** 46–55.
8. Kuwahara, M., Glenn, J. K., Morgan, M. A., and Gold, M. H. (1984) *FEBS Lett.* **169,** 247–250.
9. Paszczynski, A., Huynh, V. B., and Crawford, R. L. (1985) *FEMS Microbiol. Lett.* **29,** 37–41.
10. Linko, S. (1988) *Enzyme Microbiol. Technol.* **10,** 410–417.
11. Linko, S. (1988) *J. Biotechnol.* **6,** 229–243.
12. Dosoretz, C. G., Chen, H. C., and Grethlein, H. E. (1990) *Appl. Environ. Microbiol.* **56,** 395–400.
13. Dosoretz, C. G., Dass, S. B., Reddy, C. A., and Grethlein, H. E. (1990) *Appl. Environ. Microbiol.* **56,** 3429–3434.
14. Feijoo, G., Dosoretz, C. and Lema, J. M. (1995) *J. Biotechnol.* **42,** 247–253.
15. Eriksson, K.-E. (1990) *Wood Sci. Technol.* **24,** 79–101.
16. Linko, S. and Zhong, L.-C. (1987) *Biotechnol. Techn.* **1,** 249–254.
17. Bon, E. P. S. and Webb, C. (1989) *Enzyme Microbiol. Technol.* **11,** 495–499.
18. Tien, M. and Kirk, T. K. (1984) *Proc. Natl. Acad. Sci.* **81,** 2280–2284.
19. Mandels, M., Andreotti, R., and Roche, C. (1976) *Biotech. Bioeng. Symp.* **6,** 17–34.
20. Charney, J. and Tomarelli, R. M. (1947) *J. Biol. Chem.* **171,** 501–505.
21. Nelson, N. (1944) *J. Biol. Chem.* **153,** 375–380.
22. Goodwin, D. C., Aust, S. D., and Grover, T. A. (1995) *Biochemistry* **34,** 5060–5065.
23. Faison, B. D., Kirk, T. K., and Farrel, R. L. (1986) *Appl. Environ. Microbiol.* **52,** 251–254.
24. Arbeloa, M., Leseleuc, J., Goma, G. and Pommier, J. C. (1992) *Tappi J.* **7,** 215–221.