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Laccase from *Trametes versicolor*

Stability at Temperature and Alkaline Conditions and Its Effect on Biobleaching of Hardwood Kraft Pulp

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Abstract

The enzyme laccase was produced by the white-rot fungus *Trametes versicolor* in repeated batches cultures with immobilized mycelium. Two different culture conditions were used. Enzymes produced were evaluated regarding their stability at high temperatures (55°C and 65°C) and at alkaline conditions (pH 7.0 and pH 8.0) having in view the application of these enzymes in biobleaching of hardwood Kraft pulp.

Biobleaching experiments were divided in two parts, enzymatic prebleaching followed by chemical bleaching. In the enzymatic prebleaching the enzyme laccase was used at two conditions of pH and temperature, whereas the reaction time was fixed at 1 h in all pretreatments. In the chemical bleaching the DEDED and DEpDED sequences were used.

The enzyme action was evaluated by Kappa number, viscosity, and brightness at the end of bleaching sequences. There were obtained values of Kappa numbers lower than control assays, viscosities compatible with industrial pulps, and brightness higher than controls, when pulps were pretreated for 1 h with laccase at pH 8.0 and 55°C.

Index Entries: *Trametes versicolor*; laccase; biobleaching; kappa number reduction; brightness increase.

Introduction

The growing pressure from authorities, consumers and environmental groups to reduce the use of chlorine for pulp bleaching and the concomitant production of great amounts of organochlorinated compounds in bleach plant effluents have led the pulp and paper industry to investigate alternative

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ways of reducing the use of chlorine and its derivatives in bleaching sequences. The new technologies include extended delignification, use of anthraquinone, oxygen delignification, elemental chlorine-free (ECF), total chlorine-free (TCF) bleaching sequences, and also, enzymatic technology *(1–9)*.

Enzymes can act on hemicellulose like xylanases or on lignin such as lignin peroxidases (LiPs), manganese peroxidases (MnP), and laccases *(10–13)*. White-rot fungus *Trametes versicolor* is able to produce the three enzymes involved in the lignin biodegradation and its use in biomechanical pulping and biobleaching of cellulosic pulps has been studied by several authors *(14–16)*.

At present, some factors restrict the use of enzymes as a step of industrial bleaching. These factors include the presence of cellulolytic activities in crude enzyme preparations, pH and thermal stability requirements necessary for the enzyme utilization, and the amount of enzyme required for industrial use.

In this study, the effects of laccase pretreatment associated to chemical bleaching sequences (DEDED/DEpDED) for bleaching of eucalyptus kraft pulp were investigated. Laccase production using the immobilized microorganism under different fermentation conditions and stabilities of the crude enzyme at high temperature and alkaline conditions were also evaluated.

Materials and Methods

Strain Maintenance and Activation

The fungus *Trametes versicolor* (ATCC 20869) was used for enzyme production. The strain was maintained on 2% malt agar slants. Slants of the same agar were used for production of spores, which were used as inoculum in all fermentations. A dense sporulation was observed after 7 d of incubation at 30°C. The concentration of spores was determined using a standard curve that correlated absorbance at 650 nm with spores concentration in terms of number of spores/mL.

Enzyme Production

Experiments were performed using immobilized mycelium. For the immobilization step, shake-flask fermentations using a medium containing 5 g/L glucose, 10 g/L peptone, trace metals, and 20 m*M* ammonium tartrate buffer at pH 5.0 were carried out in the presence of nylon-web cubes. The 500-mL flasks containing 200 mL of the culture medium were inoculated with 1.0×10^7 spores, incubated at 30°C, and shaken at 200 rpm. This enzyme produced in a buffered medium was called laccase A. Fermentation was also carried out using sterilized tap water instead of buffer and trace metals. The crude enzyme produced in this way was named laccase B. Laccase production was performed also in a semi-continuous mode and

in this case, at defined time periods the extracellular medium was replaced by fresh medium.

Enzyme Assays

Enzyme concentration was determined by measuring the level of activity of the cultivation medium. Laccase activity was determined as described by Szklarz et al. *(17)* with syringaldazine as substrate. The reaction was monitored by change in absorbance at 525 nm ($\varepsilon = 65000/M/cm$) for 1 min. One unit (U) of enzyme activity is the amount of enzyme that oxidizes 1 micromole of the substrate per minute. Endoglucanase activity was determined according to Mandels et al. *(18)* using carboxymethyl cellulose (CMC) as substrate. One unit of enzyme activity produces one micromole of reducing sugars/min.

Enzyme Stability

For assays on stability, laccase A and laccase B were stored at different values of pH and temperature. The values of pH used were the optimum value for enzyme activity, pH 5.0, the final pH of production for laccase A, pH 5.7, and laccase B, pH 6.5, and two other values of advantage for industrial bleaching. Samples were maintained at temperatures of 55°C, 60°C, and 65°C. Crude preparations of laccase A with 684 U/L and laccase B with 874 U/L were used. Enzymes were separated into various samples and the pH were adjusted to the desired values. Each one of these samples was divided in 3-mL aliquots and stored at different temperatures. After incubation for the desired time period, the activity was measured according to Szklarz et al. *(17)*.

Pulp Characteristics

The pulp used in the experiments was an unbleached hardwood kraft pulp, which contained a mixture of *Eucalyptus grandis* and *Eucalyptus urophylla*, and was obtained from Brazilian mills. The initial Kappa number and viscosity values were 15.5 and 1245 dm^3/kg, respectively.

Enzymatic Prebleaching

The conditions used are listed in Table 1. Both prebleaching and bleaching sequences were performed in polyethylene bags in a water bath at the required time periods and temperatures. Enzymatic treatment assays were followed by a conventional alkaline extraction stage at 65°C for 1 h. After prebleaching and after each bleaching stage, the pulp was filtered and washed with distilled water.

Bleaching

The chemical bleaching sequences were performed as indicated in Table 2. All concentrations are expressed in terms of air-dried pulp. Besides the DEDED and DEpDED bleaching controls, other controls were used.

Table 1
Enzymatic Prebleaching Conditions[a]

	Enzymatic stage			
Conditions	Laccase A[b]		Laccase B[c]	
Treatment	A_1	A_2	B_1	B_2
Temperature (°C)	30	55	30	55
Consistency (%)	10	10	10	10
Time (h)	1	1	1	1
pH	5	8	6.5	7
Laccase (U/g pulp)	2	2	2	2

[a]Enzymatic prebleaching was carried out in absence of ABTS: 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonate), according to Monteiro and de Carvalho *(16)*.
[b]Laccase A was produced in a buffered medium.
[c]Laccase B was produced in a nonbuffered medium.
Alkaline extraction stage (E)
2.0% NaOH/65°C/1 h/10% consistency

Table 2
Elemental Chlorine Free (ECF) Bleaching Conditions

	Conditions					
Sequences	ClO_2 (%)	NaAOH (%)	H_2O_2 (%)	Consistency (%)	Temperature (°C)	Time (h)
D[a]	0.65			6	70	3
E[b]		2.0		10	65	1
D	0.40			10	70	2
E		2.0		10	65	1
D	0.40			10	70	2
D	0.65			6	70	3
Ep[c]		2.0	0.50	10	65	1
D	0.40			10	70	2
E		2.0		10	65	1
D	0.40			10	70	2

[a]Chlorine dioxide stage (D).
[b]NaOH extraction stage (E).
[c]NaOH/H_2O_2 extraction stage (Ep).

(Enzymatic pretreatment, EDEDED, and EDEpDED controls were submitted to a conventional alkaline extraction stage [E] before chemical bleaching sequences, whereas, in *EDEDED and *EDEpDED controls were used at same conditions of enzymatic prebleaching [Table 1], but without enzyme [*E], followed by chemical bleaching.)

Evaluation of Pulp Treatments

All determinations of Kappa numbers, viscosities, and brightness were made according to standard TAPPI methods. Brightness, Kappa numbers,

and pulp viscosities values were determined at the end of bleaching sequences. All values reported are the average of four replicate experiments.

Results and Discussion

Enzyme Production

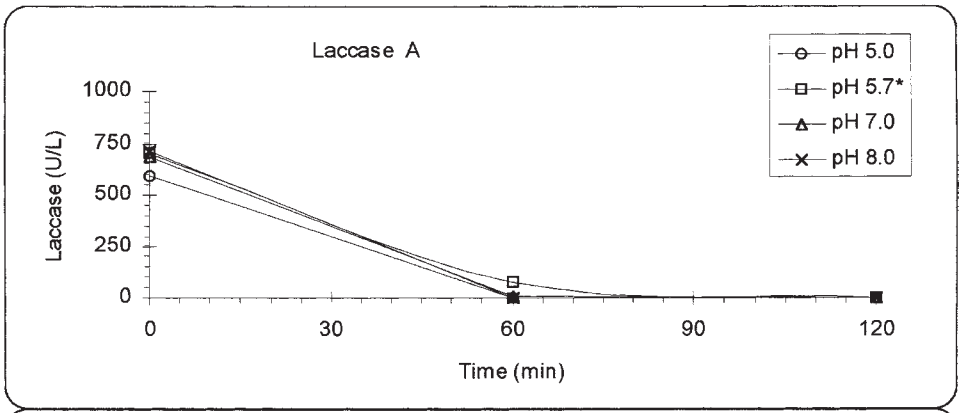
In previous work, the best conditions for laccase production by *Trametes versicolor* in repeated batches cultures with immobilized mycelium was determined *(16)*. In that case, a medium containing 5 g/L glucose, 10 g/L peptone, and buffered to pH 5.0 with 20 m*M* diammonium tartrate was used. Now, the production of laccase was investigated using the same conditions as aforementioned but in a nonbuffered medium, in which the buffer and trace metals solution were replaced by sterilized tap water. The fungus was able to produce the enzyme in both media, but the activity levels and final pH values of the cultures were quite different. The enzyme produced on the buffered medium (laccase A) achieved the maximum activity level (1000 U/L) in about 15 d, whereas the enzyme production carried out on the nonbuffered medium (laccase B) resulted in activity level of 850 U/L in the same fermentation time. Endoglucanase activity was detected only in the initial phase of both cultures.

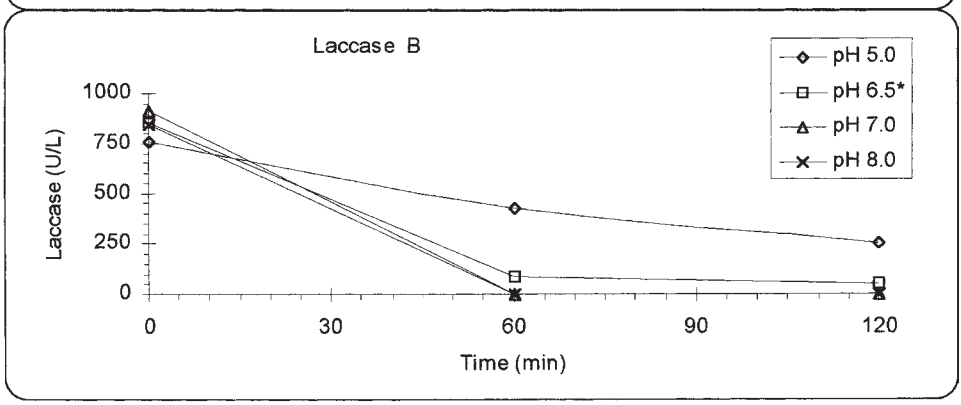
pH and Thermal Stability

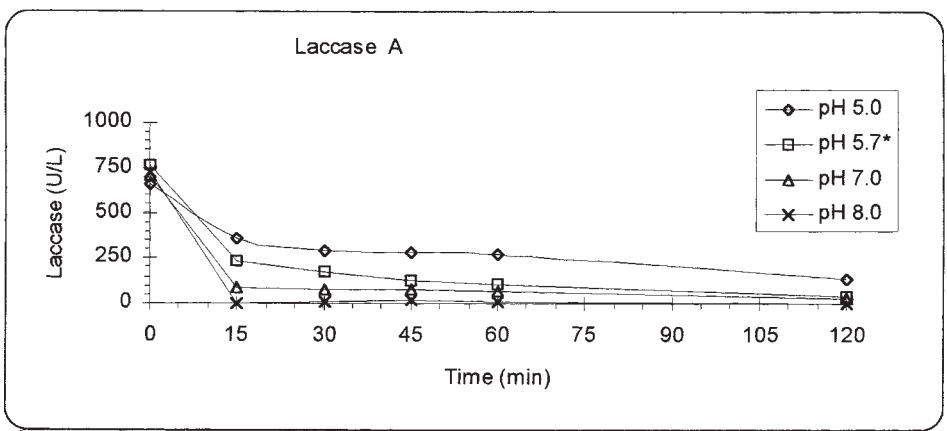
The effects of different pH and temperature values on measured activity of laccase A and laccase B are shown in Figs. 1, 2, and 3. It can be seen that the laccase A was inactivated at 65°C after incubation time of 1 h independent of the pH value tested (Fig. 1). The samples stored at pH 5.7 retained about 11% of the initial activity after this time period. Laccase B showed a similar profile except for samples maintained at acid conditions, pH 5.0, for which the remaining activity after incubation times of 1 and 2 h was 50% and 30% of the initial activity, respectively.

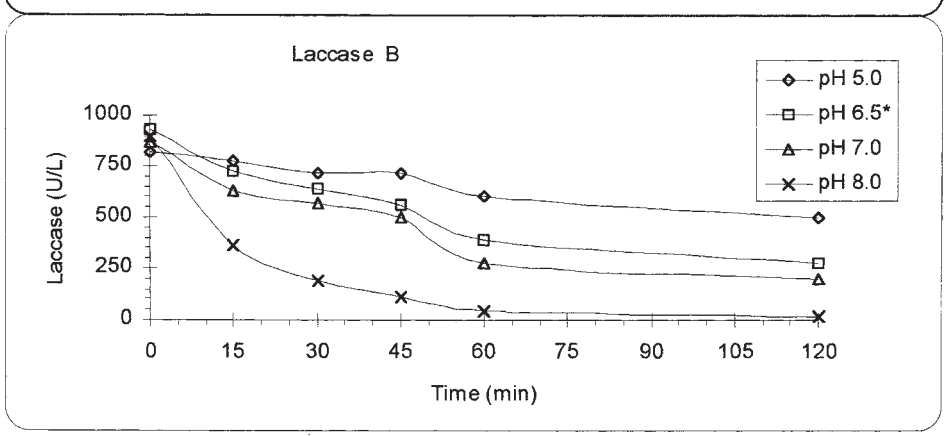
As shown in Fig. 2, laccase A incubated at pH 7.0 and 8.0 was inactivated after 15 min at 60°C, whereas, samples kept at pH 5.0 and 5.7 retained around 53% and 35% of the initial activity, respectively. After 1 h, only 39% of the initial activity remained at pH 5.0. Laccase B was more resistant at this temperature. At pH 5.0, around 70% of the initial activity was maintained. At pH 8.0, nearly 40% of the initial activity was retained after 15 min but the inactivation was complete in 1 h, whereas at pH 7.0, only 35% of activity was present after this incubation time. This rapid drop in activity indicated the use of the enzyme at alkaline pH range and 60°C in biobleaching assays was impracticable.

Figure 3 shows the stability of laccases A and B at 55°C. Good results were achieved when laccase A was maintained at pH 7.0 and 8.0. In this case, a drop in activity less than 10% was obtained after 6 h incubation. With laccase B at pH 7.0, a drop in activity of 15% was observed in the first hour of incubation. This inactivation reached 50% at pH 8.0 after the same time


Laccase A
pH 5.0
pH 5.7*
pH 7.0
pH 8.0
Laccase (U/L)
1000
750
500
250
0
0
30
60
90
120
Time (min)


Laccase B
pH 5.0
pH 6.5*
pH 7.0
pH 8.0
Laccase (U/L)
1000
750
500
250
0
0
30
60
90
120
Time (min)


Laccase A
pH 5.0
pH 5.7*
pH 7.0
pH 8.0
Laccase (U/L)
1000
750
500
250
0
0
15
30
45
60
75
90
105
120
Time (min)


Laccase B
pH 5.0
pH 6.5*
pH 7.0
pH 8.0
Laccase (U/L)
1000
750
500
250
0
0
15
30
45
60
75
90
105
120
Time (min)

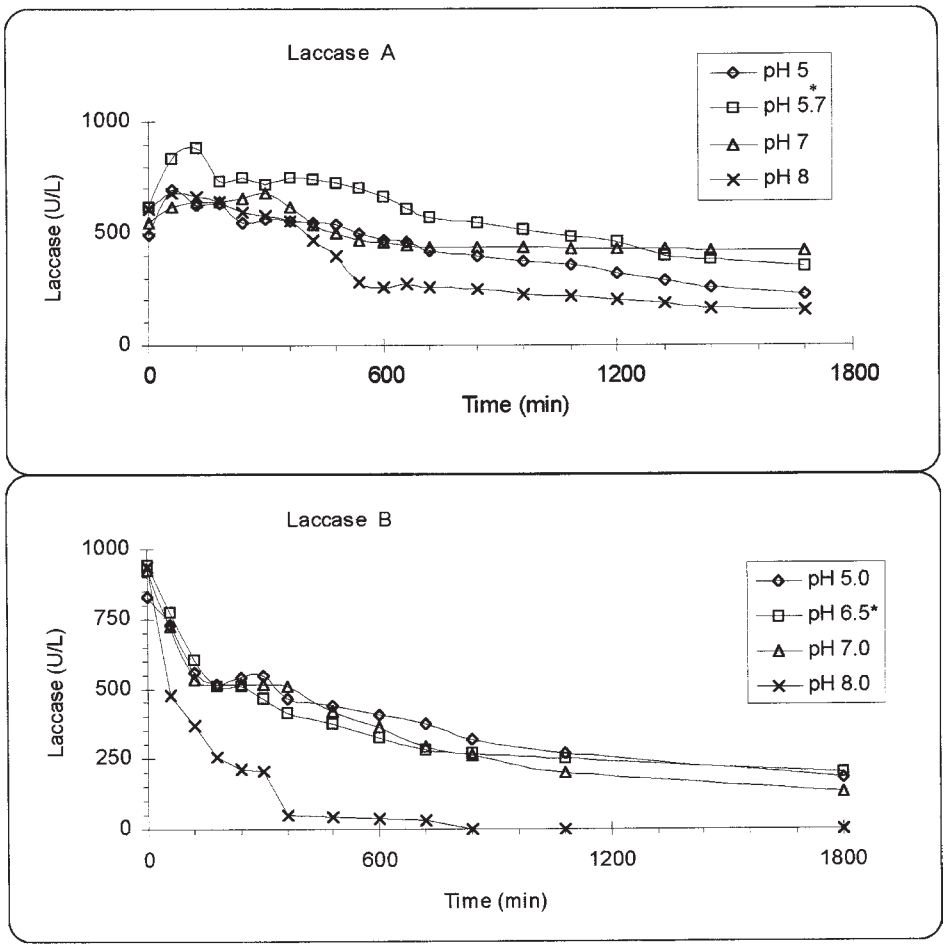


Fig. 3. pH stability of laccase A and laccase B at 55°C.

period. Under these conditions, laccase A was more stable than laccase B. However, both enzymes can be employed in biobleaching experiments at 55°C and an alkaline pH range because they were stable for the time required for enzymatic treatments.

Biobleaching

Four enzymatic treatments were investigated, two at 30°C and two others at 55°C, according to the conditions indicated in Table 1. The conditions of chemical bleaching sequences applied to pulp are listed in Table 2 and the controls were made according to those listed in the Material and Methods section.

Fig. 1. *(previous page; top)* pH stability of laccase A and laccase B at 65°C.
Fig. 2. *(previous page; bottom)* pH stability of laccase A and laccase B at 60°C.

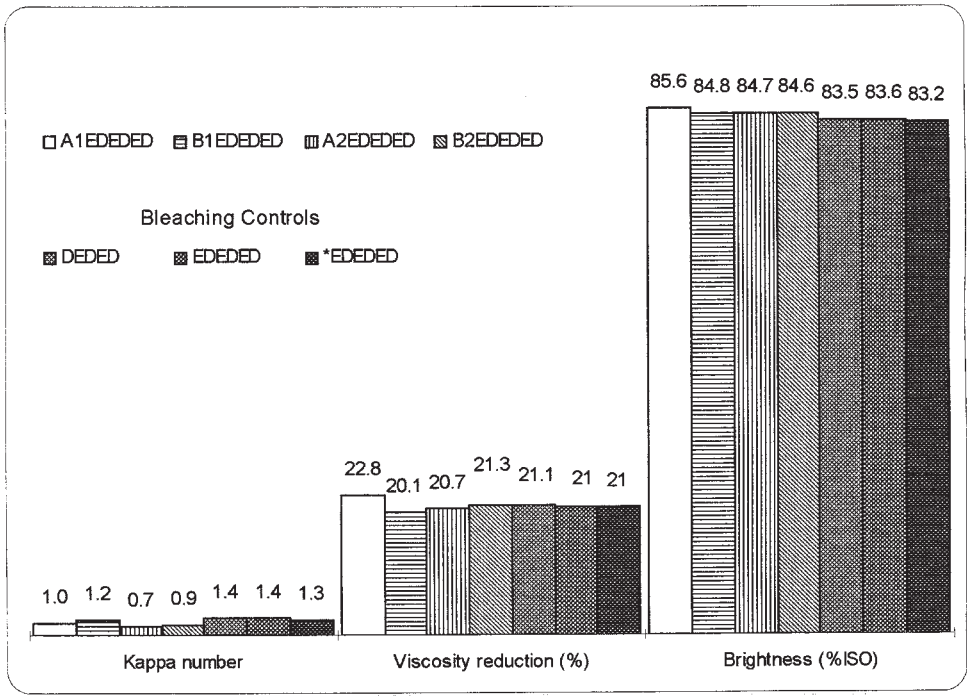


Fig. 4. Characterization of kraft pulp pretreated by laccase and bleached by DEDED sequence.

Figure 4 shows final Kappa numbers, viscosity reduction and brightness of pulps enzymatically pretreated and bleached by the DEDED chemical bleaching sequence. All pulps pretreated by laccases showed Kappa numbers smaller than controls. High delignification (95.5%) was obtained when pulp was bleached by A_2EDEDED sequence (laccase A/55°C/pH 8.0). A good result was also observed in pulps bleached by sequence B_2EDEDED (laccase B/55°C/pH 7.0), in which Kappa number of 0.9 was smaller than those obtained for controls. The sequences A_2EDEDED and B_2EDEDED applied on pulp yielded brightness of 84.7% ISO and 84.6% ISO, respectively. These brightness values were greater than those observed for the controls, whose brightness were 83.5% ISO; 83.6% ISO, and 83.2% ISO for the DEDED, EDEDED, and *EDEDED controls, respectively.

When pulps were bleached by the B_1EDEDED sequence (laccase B/30°C/pH 6.5), they showed Kappa number of 1.2 corresponding to a delignification of 92.3%. Although the delignification was greater than controls, it was smaller than other enzymatic sequences. However, it showed the smallest viscosity reduction and brightness higher than those observed for the A_2EDEDED and B_2EDEDED enzymatic sequences and for the controls.

The best result was obtained when pulps were treated using the A_1EDEDED sequence (laccase A/30°C/pH 5.0). This sequence produced

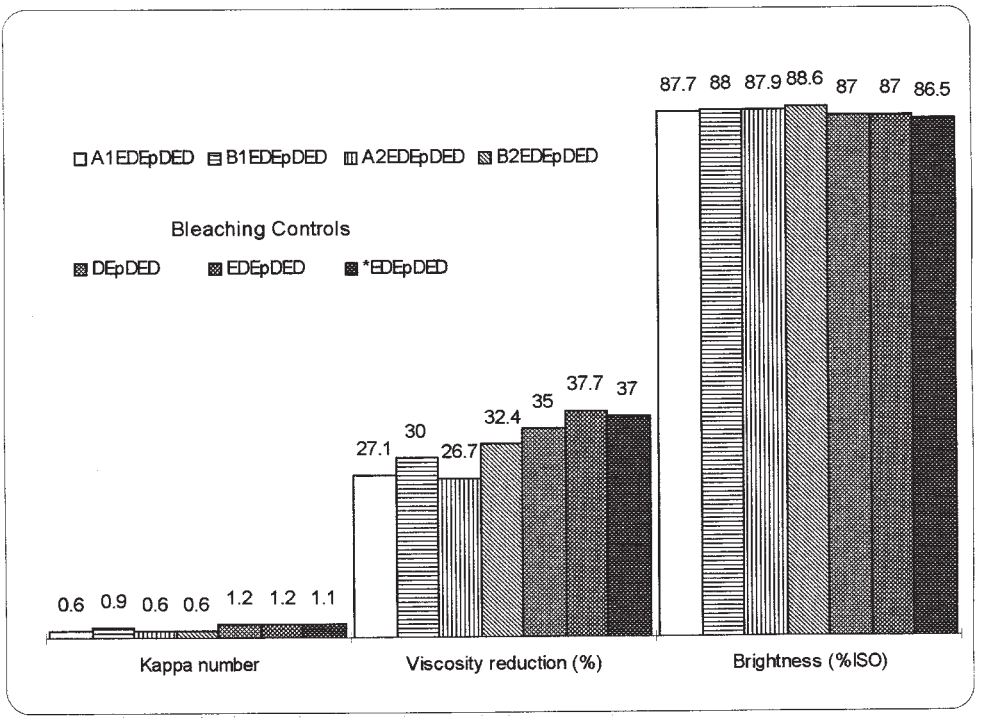


Fig. 5. Characterization of kraft pulp pretreated by laccase and bleached by DEpDED sequence.

samples with a delignification of 93.5% and a viscosity reduction of 22.8%, values greater than controls. Moreover, it also produces the best brightness (85.6% ISO). These results indicate that enzymatic bleaching can be used in two strategic manners: to obtain an increase of the brightness values or to reduce the Kappa number.

Figure 5 shows the results obtained when pulps were enzymatically pretreated and bleached by DEpDED sequence. All pulps pretreated with enzymes resulted in Kappa numbers smaller than the controls. The same occurred in viscosity reduction. In this case, A_2EDEpDED sequence (laccase A/55°C/pH 8.0) was more efficient and resulted in a brightness of 87.9% ISO with a viscosity reduction of only 26.7%.

When a DEpDED chemical bleaching sequence was used, the best results were observed in pulps pretreated with laccase B. When this enzyme was applied on pulp in a B_1EDEpDED sequence (laccase B/30°C/pH 6.5), the samples yielded a Kappa number of 0.9, viscosity reduction of about 30% and brightness of 88% ISO. Although B_1EDEpDED samples have shown Kappa numbers greater than those observed in A_1EDEpDED (laccase A/30°C/pH 5.0) and A_2EDEpDED (laccase A/55°C/pH 8.0) samples, they exhibited brightness greater than the other two sequences. The highest brightness value was obtained by use of B_2EDEpDED sequence (laccase B/55°C/pH 7.0).

All results showed that laccase A (produced in buffered medium) and laccase B (produced in nonbuffered medium) act on pulps increasing the bleachability of these pulps during the chemical bleaching.

These enzymes can act in different manners depending on the conditions in which they are applied. It was also observed that brightness does not depend directly on the Kappa number. Pulps showing smaller Kappa numbers did not produce the best brightness values, probably owing to different lignin degradation modes. The enzymatic pretreatments are made during a short reaction time, in which the lignin present on pulp is not completely degraded. The laccase caused some type of degradation of lignin, making it more susceptible to chemical bleaching. Lignin degradation is restricted owing to the heterogeneity of this substrate and the difficult access of the enzyme to lignin molecule. Thus, only modifications occur on lignin structure. The success of biobleaching depends on the enzyme actuation, on the conditions in which it is applied and also on the chemical bleaching sequence used.

Conclusions

Assays of pH and thermal stability showed that under the conditions tested, laccase A was more stable at 55°C with a drop in activity less than 10% of the initial activity after 6 h of incubation at pH 7.0 or 8.0. On the other hand, laccase B was more stable at 55°C with an activity reduction around 15% of the initial activity after 1 h of incubation at pH 7.0.

The effect of laccase treatment associated with the chemical bleaching sequences (DEDED and DEpDED) for biobleaching eucalyptus kraft pulp was also investigated. The best results for Kappa number reduction were obtained in samples biobleached by A_2EDEDED (laccase A/55°C/pH 8.0), A_1EDEpDED (laccase A/30°C/pH 5.0), B_1EDEpDED (laccase B/30°C/pH 6.5) and B_2EDEpDED (laccase B/55°C/pH 7.0) sequences, whose Kappa number values were smaller than controls.

In the case of brightness enhancement, good results were observed in pulps biobleached by A_1EDEDED sequence (laccase A/30°C/pH 5.0) and by B_2EDEDED sequence (laccase B/55°C/pH 7.0), whose brightness values were 85.6% ISO and 88.6% ISO, respectively.

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