

An evaluation of the potential of lignin peroxidases to improve pulps

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ABSTRACT *Lignin degradation by lignin peroxidases was studied for its potential to improve the optical and mechanical properties of thermomechanical pulp and to improve the bleachability of kraft pulps. Optimum conditions for enzyme use were determined. For TMP, the enzyme treatment decreased pulp brightness but had no effect on mechanical properties or lignin content. By contrast, enzyme treatment prior to chemical bleaching increased brightness and decreased lignin content in kraft pulps.*

KEYWORDS

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Pulp and paper manufacturers can use biotechnology to improve processes and products. Aside from fungi, certain enzymes could be used to degrade cellulose, hemicellulose, and lignin in wood (1-4). Hemicellulases could be used, for instance, to improve the bleaching of chemical pulps (5, 6). As another example, a process we recently proposed uses cellulases and hemicellulases to improve the drainage of recycled papers (2, 7, 8). Though lignin removal is usually achieved by mechanical, thermal, and chemical means, it could also be performed using enzymes.

Our aim in this work was to evaluate lignin peroxidases for their potential (a) for improving the mechanical and optical properties of high-yield pulps and (b) for enhancing the bleachability of kraft pulps.

Background

Lignin peroxidases seem to be especially useful enzymes for lignin degradation. These enzymes were first isolated from the white-rot fungus *Phanerochaete chrysosporium* in 1983 (9, 10). At that time, the enzymatic degradation of milled wood lignin was reported, and various uses of these enzymes were considered for pulping, bleaching, and effluent treatment.

Several studies have dealt with the

direct growth of fungi on chips or pulp to reduce lignin content. The main findings are a reduction of the energy required for refining (11) and an improvement of the mechanical properties of the pulp (12, 13). A patent has also been filed on the use of lignin peroxidases to improve the optical properties and strength of mechanical pulps (14).

Another use of lignin-degrading enzymes is in the bleaching of chemical pulps. Kappa number reduction and increased brightness of kraft pulps have been obtained in organic solvent with peroxidases (15), and patents have been claimed on the use of lignin peroxidases on kraft pulps to increase brightness (16, 17).

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Finally, several research teams are at work on the treatment of effluents with *P. chrysosporium* or other fungi. The treatment produces a biodegradation of chlorinated xenobiotics from bleaching effluents (18, 19) or a polymerization of lignosulfonates (20).

At La Cellulose du Pin, which has been involved in using biocatalysts in the pulp and paper industry for several years, we have worked on lignin biodegradation. Although the fungi that act on wood or pulp seem promising, it is doubtful that they will be put to practical industrial use in the near future. Therefore, we decided to test enzymes, for which it is more feasible to evaluate direct action on pulp.

Overview of experiments

Our experiments fell into three groups. In these, our efforts were to:

1. Optimize the enzymatic reaction
2. Determine the effects of lignin peroxidases on various pulps
3. Determine the behavior of enzymes on pulp.

In the first series of experiments, we examined the conditions that affect lignin peroxidase activity. The enzymatic reaction of lignin peroxidases is initiated by the addition of hydrogen peroxide. The reaction is enhanced by the presence of veratryl alcohol, which also helps the *in vitro* oxidation of various phenolic and nonphenolic substrates by the enzymes. An excess of hydrogen peroxide, however, denatures lignin peroxidases. Furthermore, the maximum activity of veratryl alcohol is achieved when the pH of the reaction mixture is set to pH 3 (21), but enzyme stability is best at pH 6 (22). We therefore determined the optimum values for the rate of hydrogen peroxide addition, the pH of the reaction mixture, and the concentration of veratryl alcohol.

In the second series of experiments, we studied the effects of lignin peroxidases on (a) the mechanical and optical properties of high-yield pulps and (b) the bleachability of kraft pulps. The composition of lignin differs between softwood and hardwood, and the lignin content of these pulps is variable. Additionally, the chemical structure of the lignin in softwood and hardwood pulp is modified by the cooking process in kraft

pulping, as compared with the structure of unmodified lignin in thermomechanical pulping. Because of these differences in lignin, we studied the action of lignin peroxidases using thermomechanical pulp and hardwood and softwood pulps. The effect of lignin content on the action of lignin peroxidases was studied further using softwood pulps with different lignin contents.

The third series of experiments pertained to the dependency of enzyme behavior on pulp type. We conducted a study of enzyme activity as a function of time using different pulps to see if the differences in lignin could lead to a difference in enzyme behavior. We also studied the method of the decrease in enzyme activity.

Materials

Enzymes

The enzyme preparation used in our experiments was produced by Cultor Ltd. (Helsinki, Finland) from *Phanerochaete chrysosporium* BKM-1767 after Q-Sepharose (Pharmacia) separation (where Q stands for "quaternary amine"). Approximately 95% of the proteins in the preparation were peroxidases: 15% were manganese-dependent peroxidases, and 80% were lignin peroxidases.

Lignin peroxidase activity was defined as the oxidation of veratryl alcohol to veratraldehyde according to the procedure described by Tien and Kirk (23).

Reagents measurements

The concentration of hydrogen peroxide (Prolabo, France) was determined according to Bailey and Boltz (24). Veratryl alcohol (undistilled) and veratraldehyde (Aldrich) were analyzed by reverse-phase HPLC performed on a Milton Roy C4000 chromatograph using a Nucleosil C₁₈ column from SFCC (Société Française de Chromatographie Colonne). The elution was run in an isocratic solvent system of H₂O and CH₃OH (50:50 mixture) at a flow rate of 1 mL/min and was monitored at 280 nm (Isco (USA) Spectrophotometer 1840). The analysis was recorded and processed by computer using the Baseline 810 software package (Dynamics Solutions, Division of Millipore).

Pulps

Thermomechanical pulp (TMP) was produced from pine (*Pinus pinaster*) by La Cellulose du Pin. Softwood kraft pulp was either provided by Papeteries de Gascogne or produced by La Cellulose du Pin (Facture Mill). Hardwood kraft pulp was produced in Papeteries de Condat. Softwood kraft pulps with variable lignin contents were produced in the laboratory by oxygen delignification of an industrial pulp.

Methods

Evaluation of pulp properties

The laboratory handsheets were prepared on Noble and Wood equipment according to International Standards Organization procedure ISO-5269. Properties were determined by the following procedures:

Burst index: ISO-2758

Tear index: ISO-1974

Breaking length: ISO-1924/2

Brightness: ISO-2470.

Other properties measured on the pulp were:

Klason lignin: NFT-12014 (French)

Kappa number: ISO-302.

The water retention value was defined as the weight of water retained per unit weight of oven-dried pulp after centrifuging at 900 g for 15 min.

Bleaching

Kraft pulps were bleached with a CEHD sequence under the following conditions:

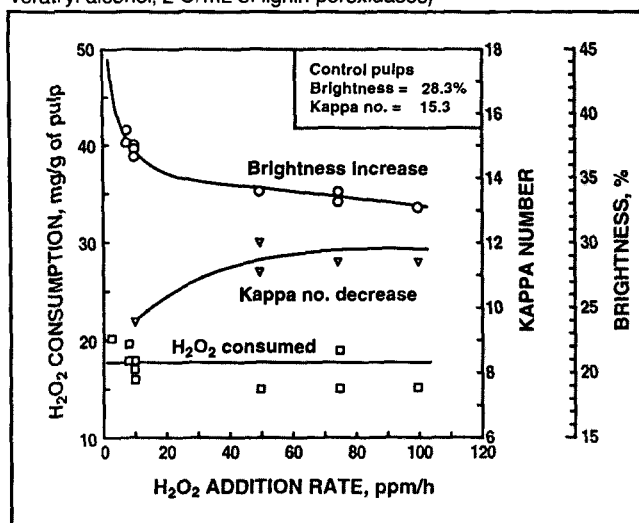
For the C (chlorine) stage, pulp was suspended at 3% consistency in a stoppered flask for 20 min at room temperature, with the percentage of active chlorine set to $(0.2 \times \text{kappa no.})$.

The E (extraction) stage was performed at 10% consistency with 2.5% NaOH at 58°C for 50 min.

The H (hypochlorite) stage was performed at 5% consistency with 2.7% NaOCl (as active chlorine) for 170 min at 50°C. When necessary, sodium hydroxide was added to keep the pH higher than 10.

For the D (chlorine dioxide) stage, 2.4% ClO₂ (as active chlorine) was applied to pulp at 6% consistency for 170 min at 90°C. The reaction was performed in a stoppered flask.

1. Effects of hydrogen peroxide addition rate on hydrogen peroxide consumption, brightness, and kappa number. (pH = 3, 2 mmol/L of veratryl alcohol, 2 U/mL of lignin peroxidases)



TMP was bleached with hydrogen peroxide at a concentration of 3% H₂O₂. Sodium hydroxide (2.3%) and sodium silicate (4%) were also added to the reaction. The reaction was performed at 12% consistency at 70°C for 180 min.

Enzymatic pulp treatment

The pulp was suspended at 1% consistency in distilled water, and the pH was adjusted to the desired value (from pH 3 to 5.5) with lactic acid. The suspension was warmed to 30°C in a thermostatic bath. The enzyme preparation was added such that the concentration in the reaction medium was 2 U/mL (activity units per milliliter), and veratryl alcohol was added at the desired concentration (from 0 to 15 mmol/L).

The enzymatic reaction was started by the addition of hydrogen peroxide solution. This solution was added continuously at a flow rate of 2 mL/h with a peristaltic pump. The rate of addition of hydrogen peroxide was adjusted from 2 ppm/h to 100 ppm/h. This rate is calculated as the amount added to the reaction medium by volume and time.

Samples of the medium were taken regularly during the reaction and were filtered on sintered glass filters. Veratryl alcohol, veratraldehyde, and hydrogen peroxide contents were determined on filtrates.

The reaction was stopped when hydrogen peroxide was detected in the filtrates (1 h to 75 h, depending on the conditions).

The pulp was washed with water, and its properties were determined. Handsheets were made and were used in the measurement of mechanical and optical properties.

Controls pulps were treated similarly but without either enzyme treatment or hydrogen peroxide.

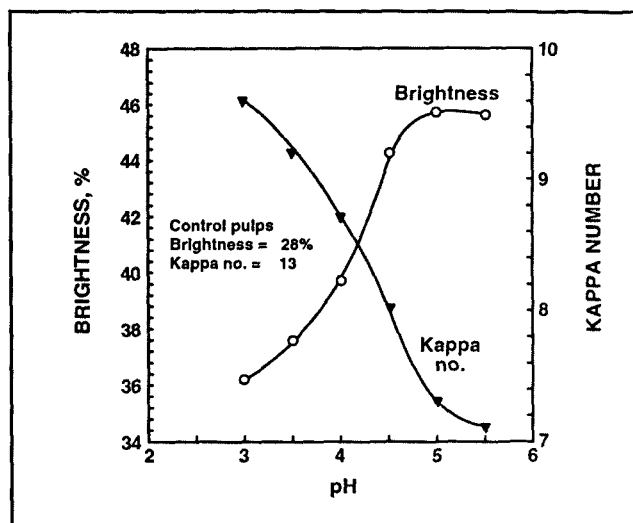
Enzyme activity over time

The enzyme activity over time was studied by adding LIPS to TMP and softwood and hardwood kraft pulps in the absence of veratryl alcohol and hydrogen peroxide at pH 4.5. Enzyme activity in the filtrate was measured at various times from 0 min to either 500 min or until no enzyme activity was detected.

Furthermore, to determine if the decrease in enzyme activity was caused by enzyme inhibition, inactivation, or adsorption, we measured brightness and kappa number in kraft pulp after four different treatment procedures. Lignin peroxidases were mixed with a pulp suspension, and hydrogen peroxide was added continuously (50 ppm/h) at pH 3. The activity was measured in the supernatant during the course of the reaction. When no residual activity was detected, hydrogen peroxide addition was stopped. [Residual activity was measured by the same method (23) applied to the filtrate.]

The pulp was separated into two parts. On one part (designated as Part B in the upcoming Fig. 6), hydrogen peroxide addition continued at the same rate (50 ppm/h). The other part

2. Effect of pH on brightness and kappa number. (2 mmol/L of veratryl alcohol, H₂O₂ added at 10 ppm/h)



was filtered, and the pulp cake and filtrate were recovered. The pulp cake was suspended in new buffer (Part C), while new pulp was mixed with the separated filtrate (Part D). No enzyme was added to Parts C and D, but hydrogen peroxide was added continuously at the same rate (50 ppm/h). A control pulp (A) was processed under the same conditions as Part B but with water instead of lignin peroxidases.

Optimization of the enzymatic reaction

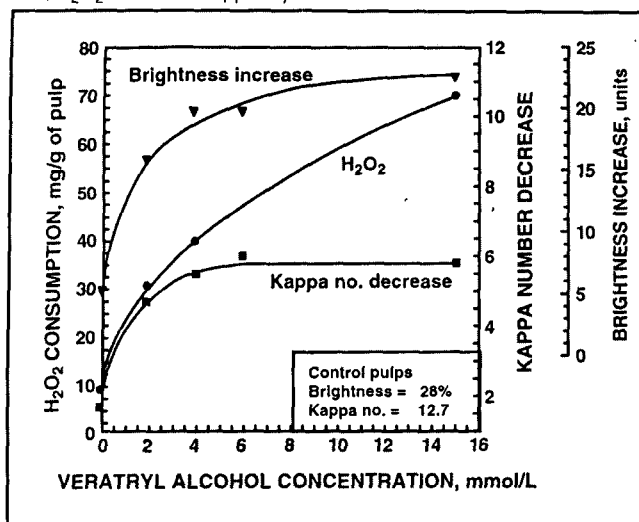
All the following experiments were performed on hardwood kraft pulp, because preliminary tests showed that lignin peroxidase treatment increased brightness and decreased kappa number on this kind of pulp.

In Figs. 1-3, initial values for brightness and kappa number are not given for the pulps. Instead, the data presented are increases or decreases in relation to the averages of final values reached by the control pulps. Differences in the final values for the control pulps were very slight.

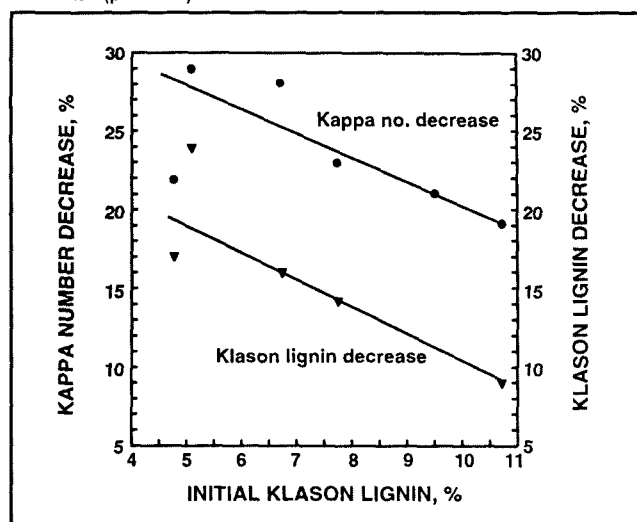
Effect of hydrogen peroxide addition rate

Figure 1 shows the effects of enzyme treatment on the changes in brightness and kappa number for addition rates of hydrogen peroxide ranging from 2 ppm/h to 100 ppm/h. In all cases, an increase in brightness and a decrease in kappa number occur, but the figure does not show absolute values. Instead the curves reflect the

3. Effects of veratryl alcohol concentration on hydrogen peroxide consumed, brightness increase, and kappa number decrease (pH = 4.5, H₂O₂ added at 10 ppm/h)



4. Influence of the lignin content (measured as Klason lignin) on the enzyme action as it affects kappa number decrease and Klason lignin decrease (pH = 4.5)



rates of change. Maximum effects are observed for low addition rates, and the effects become less pronounced as the rate of hydrogen peroxide addition increases.

At addition rates below 10 ppm/h, the brightness effect falls rapidly with small increases in the addition rate. Above 10 ppm/h, changes in the addition rate did not greatly affect the brightness. Similarly, the kappa number must have increased more rapidly at low rates of addition (data not shown), since an inverse correlation can be established for brightness and kappa number.

The hydrogen peroxide consumed for the reaction stayed constant at about 15 mg/g of pulp. Under these conditions, enzymes were stable during the oxidation of veratryl alcohol (data not shown). Increasing hydrogen peroxide concentrations above the constant amount produced no further effect on brightness or kappa number.

(Various control pulps were treated under the same conditions but without enzymes. For control pulps without enzymes, the average brightness and kappa number were 28.3% and 15.3. Control pulps with enzyme in the absence of hydrogen peroxide had a brightness of 28% and a kappa number of 15.)

Effect of pH

As the pH was increased from 3.0 to 5.5, brightness and kappa number improved as a result of greater enzyme efficiency (Fig. 2). Similarly, the hydrogen peroxide consumption

increased (data not shown). The maximum action was observed when the pH was between 4.5 and 5.5, at which brightness increased over the control pulp by about 18 brightness units and the kappa number decreased by about 6 in comparison to the control.

(A control pulp was treated under the same conditions without enzyme for each pH value. For the control without enzymes, the average brightness was 28%, and the average kappa number was 13. A control pulp with enzymes, without H₂O₂, also had a brightness of 28% and a kappa number of 13.)

Effect of veratryl alcohol concentration

The results presented in Fig. 3 indicate that veratryl alcohol enhanced the efficiency of lignin peroxidases on pulp. An optimum concentration for veratryl alcohol appeared around 4 mmol/L. At higher concentrations, no more effect was observed on the pulp although hydrogen peroxide consumption increased. For all concentrations, approximately 70% of veratryl alcohol was oxidized to veratraldehyde.

(For each veratryl alcohol concentration, a control pulp was treated under the same conditions without enzyme. For the control pulps, the average brightness and kappa number were 28% and 12.7.)

Effects of lignin peroxidases on various pulps

Softwood TMP

As shown in Table I, enzymatic treatment led to a brightness decrease in the absence (6 brightness points) and presence (16 brightness points) of 10mM veratryl alcohol. The enzyme-treated pulp did not reach the brightness level of the control even after chemical bleaching.

The Klason lignin content remained constant, indicating that the enzyme treatment did not depolymerize lignin. Slight increases in the mechanical properties were observed.

Kraft pulps

Effects on optical properties. The results of enzymatic treatment on brightness and kappa number of softwood and hardwood pulps, before and after chemical bleaching, are shown in Table II.

After enzyme treatment, the kappa numbers of pulps decreased. The brightness of hardwood pulp increased by 8 points, and the brightness of softwood pulp decreased by 5 points.

While a correlation between kappa number decrease and brightness increase was seen with hardwood pulp, no such correlation was observed with softwood. However, a decrease in the brightness of the softwood pulp was observed in all cases.

Since treated kraft pulps yielded higher values for brightness than

I. Effect of enzyme treatment on mechanical and optical properties of TMP

	Control (no enzyme treatment)	Enzyme treatment: veratryl alcohol, mmol/L	
		0	10
Brightness, %			
After lignin peroxidase	53.5	47.1	37.4
After bleaching	68	65	61
Water retention value, %	126	128	132
Klason lignin, %	30.0	n.d.	30.4
Physical properties			
Breaking length, m	1490	1565	1738
Burst index, kPa·m ² /g	0.77	0.82	0.94
Tear index, 100 mN·m ² /g	594	579	656

pH 3. H₂O₂ added at 10 ppm/h. n.d. = not determined.

II. Effects of enzyme treatment on kappa number and brightness in softwood and hardwood kraft pulps

	After treatment		After bleaching
	Kappa number	Bright- ness, %	Bright- ness, %
Softwood			
Control	29.7	29.1	88.8
Treated	26.9	24.1	90.7
Hardwood			
Control	14.6	31.0	88.6
Treated	11.8	39.0	89.4

pH 3.
H₂O₂ added at 10 ppm/h.
2 mmol/L veratryl alcohol.

control kraft pulps after chemical bleaching, the meaningful parameter in the enzyme treatment is the kappa number decrease.

Influence of the lignin content of softwood kraft pulp. Because the effects on brightness are opposite on softwood and hardwood pulps (Table II), we were interested in whether the difference could be due to the lignin content of pulp, which is much higher in softwood kraft. Therefore, softwood pulp of high lignin content (10.7%) was oxygen delignified to obtain pulps of different lignin contents. All these pulps were treated with lignin peroxidases under the same experimental conditions. (The reactions were performed at pH 4.5. In each case, a control pulp was treated under the same conditions in the absence of enzyme.)

The effects on kappa number and lignin content (measured as Klason lignin) are presented in Fig. 4. Both parameters decrease after enzyme treatment. The higher the initial lignin content, the lower the effect of the lignin peroxidases. For example, a 30% reduction of the kappa number and 20% lignin reduction were obtained using 5% initial lignin content. With an initial lignin content of 10.5%, the reductions in kappa number and lignin were only 20% and 10%, respectively.

The Klason lignin content reductions show that a depolymerization of the lignin occurred under the action of lignin peroxidases. The kappa number is also an indirect evaluation

of the lignin content but is affected by the oxidation of the lignin. Since the kappa number decrease is proportionally higher than the lignin content decrease, it is reasonable that the lignin peroxidases action leads to an oxidation of the lignin and partly to depolymerization. The oxidized lignin remains on the fibers and is detected by the Klason method.

Behavior of enzymes in acting on pulp

The decrease in enzyme activity as a function of time is shown in Fig. 5. For all three pulps, 20–30% of the enzyme activity disappeared immediately after the enzymes contacted the pulp. For TMP, a slight decrease of the activity occurred during the first hour and remained constant at about 55% of the initial activity thereafter. For hardwood and softwood, no residual activity was detectable after 20 min and 120 min, respectively.

The results of experiments designed to determine the method of the decrease in enzyme activity are shown in Fig. 6. Nearly identical values for brightness and kappa number were reached by the pulp under normal treatment, Part B, and by the separated pulp, Part C. On the other hand, the separated filtrate, Part D, yielded the same results as the control, Part A, indicating that there were no enzymes in the separated filtrate. These results demonstrate that the enzymes adsorbed onto the pulp and reacted when hydrogen peroxide was

added to the reaction medium.

Discussion

The reaction of lignin peroxidases is a complex process. They require a cosubstrate (hydrogen peroxide), and their action is considerably enhanced by veratryl alcohol. Most of the reactions previously reported have been performed on lignin model compounds or synthetic lignin. As this study shows, lignin peroxidases are also active on pulps.

The reaction parameters were optimized on hardwood kraft pulp so that the reaction would reach a significant effect. The optimum pH for the initial rate of veratryl alcohol oxidation has been reported to be around 3 (21). However, the enzyme stability and the products formed depend on the pH, with the optimum pH for enzyme stability being about 6 (22). According to our results, an optimum pH for the action of the enzymes on pulp appears at about pH 5. Therefore, the optimum pH for the action on pulp results from a compromise between the optimum pH for enzyme reactivity and the optimum pH for enzyme stability.

It is known that the heme (in LIPs, protein containing iron protoporphyrin IX) is denatured by excess hydrogen peroxide (25). Our preliminary experiments showed that enzyme treatment did not affect the pulp when a large amount of hydrogen peroxide was added only at the beginning of pulp treatment. Therefore,

this cosubstrate was added continuously at a flow rate low enough that its concentration in the reaction medium did not denature the enzyme.

Still, the effect on the pulp decreased as the hydrogen peroxide addition rate increased. In fact, when the addition rate increased, the rate of veratryl alcohol oxidation to aldehyde was enhanced to the detriment of the reaction rate on the pulp (data not shown).

The explanation for these results depends on the reaction mechanism. As the hydrogen peroxide addition rate increases, part of the peroxide denatures the enzyme. This denaturation can be irreversible, or it can lead to the formation of an inactive enzyme complex (named Compound III-H₂O₂). This compound can revert to the active form (Compound II) in the presence of veratryl alcohol, which is oxidized to veratraldehyde (26). The irreversible denaturation partly explains the decrease of the effect on pulp.

On the other hand, if the limiting rate is the pulp oxidation, the enzyme competition between pulp and veratryl alcohol will lead to veratraldehyde forming more rapidly, when the hydrogen peroxide addition rate increases, than the rate at which the pulp oxidizes.

Furthermore, beyond a certain veratryl alcohol concentration (around 4 mmol/L), no additional effect occurs on the pulp. Therefore, it seems that a limitation exists on the substrate accessibility.

Our results show that lignin peroxidases produce a delignification of kraft pulps. This effect is a function of the pulp lignin content; when lignin content increases, the enzyme effect decreases. This phenomenon can be explained by a limit in substrate accessibility.

The kappa number decrease caused by enzyme action is much greater than the lignin decrease. In pulp and paper processes, a kappa number decrease correlates with a decrease in lignin content. In our results, this correlation was not found. Apparently, the lignin peroxidases action leads partly to lignin depolymerization and partly to lignin oxidation only. This contention is supported by the effects observed on TMP, for which an oxidation occurs without any lignin depolymerization (constant Klason lignin).

Enzymes adsorb at different rates

with different pulps, and a better enzyme effect on pulp correlates with better adsorption. This finding is particularly important when treating TMP, which only adsorbed about 50% of the applied enzymes. This result can be compared to the results recently presented by Ruel and Joseleau (27), who showed that hemicelluloses prevent the penetration of lignin peroxidases into wood and pulp fibers.

For the application of enzymes in the pulping industry, the modification of mechanical and optical properties of various pulps has been studied. The lignin peroxidases reacted with all the pulps, but the effect was different in each case.

The brightness of TMP decreased from enzyme treatment, indicating that an oxidation of the lignin occurred. Even after chemical bleaching, the brightness was not higher than that of the control pulp. This effect has also been reported for *in vivo* experiments (12, 13). Furthermore, we found no effect on brightness reversion (results not shown). The effect on lignin was not substantial enough to produce a significant effect on mechanical properties.

Our results on TMP are opposite to those reported previously (14). In that patent by Farrell, an improvement in mechanical properties and better brightness stability were claimed as a result of treatment of the pulp with lignin peroxidases. In those experiments, hydrogen peroxide was added at the beginning of the reaction.

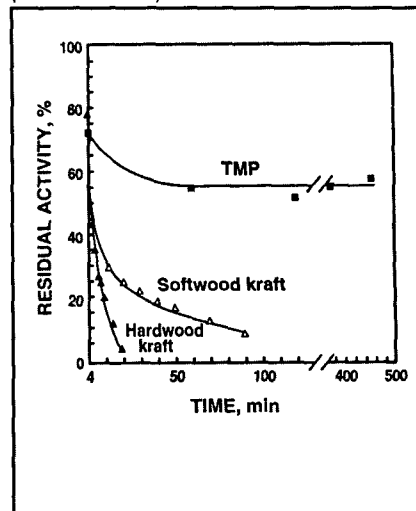
In the case of kraft pulps, the lignin oxidation and delignification by lignin peroxidases improved the final brightness of the pulp after chemical bleaching (by 1-2 brightness units). Therefore, the enzyme action can be used as a prebleaching stage—that is, a lignin peroxidase treatment step preceding a traditional bleaching sequence.

Conclusions

Here, we have presented a treatment of pulps by lignin peroxidases. To achieve measurable effects on pulp, one of the key points is to feed the hydrogen peroxide slowly. The results suggest interesting possibilities in the bleaching of kraft pulps.

By using lignin peroxidases, the final brightness of the pulps after chemical bleaching can be increased.

5. The activity of lignin peroxidases in the reaction medium as a function of time. (pH = 4.5, neither veratryl alcohol nor hydrogen peroxide added)



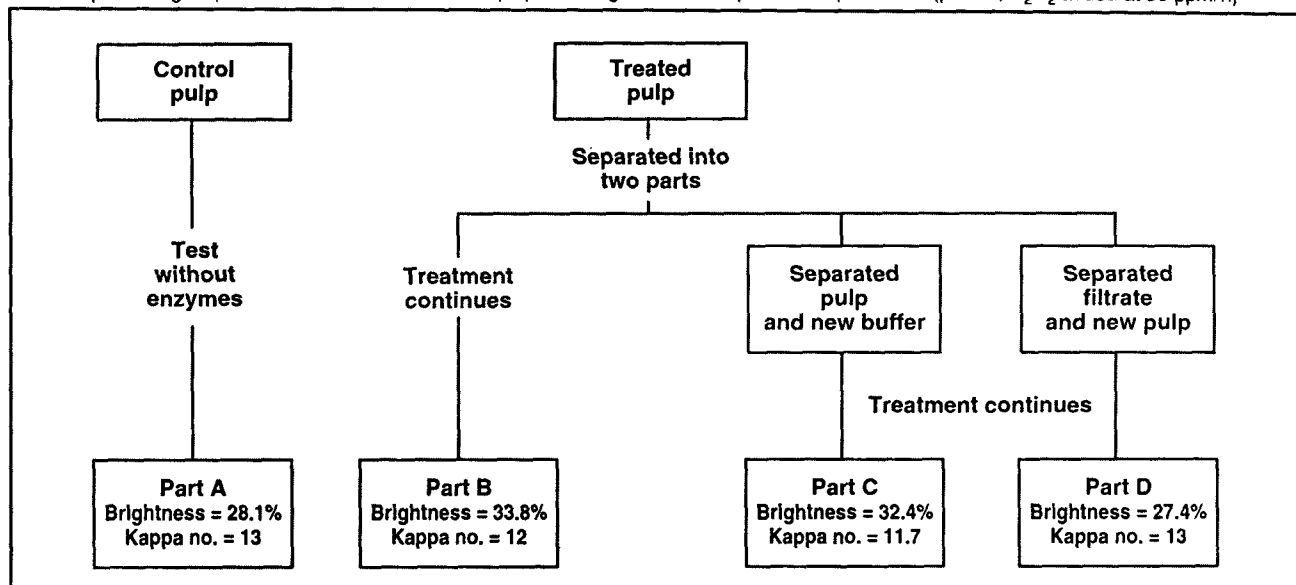
Another way to take advantage of the enzyme treatment is to decrease the chemical reagents used during bleaching to reduce the content of chlorinated compounds in effluents.

Simultaneous or sequential use of lignin peroxidases with hemicellulases is still promising. However, this process requires the addition of two reagents (hydrogen peroxide and veratryl alcohol). Furthermore, the cost of enzymes is still high, and the enzymes cannot be recovered easily after treatment, because they adsorb onto the pulp. Currently, all these problems constitute drawbacks to the utilization of lignin peroxidases and delay their application in industry. □

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