PULP BLEACHING WITH MANGANESE PEROXIDASE AND XYLANASE: A SYNERGISTIC EFFECT

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ABSTRACT: *Although bleaching of kraft pulps with MnP or xylanase have been studied extensively, their synergistic effects have yet to be demonstrated. We report here for the first time that a combination of MnP and xylanase has pulp bleaching effects far superior to those of the individual enzymes used sequentially. Bleaching of hardwood kraft pulp with MnP and xylanase for 24 hours followed by a single alkaline/hydrogen peroxide treatment increased pulp brightness from 35.3 to 78.1% (ISO) and decreased the kappa number from 13.7 to 4.3. We demonstrated that this bleaching effect was a synergistic action between the enzymes rather than an addition of the brightening abilities of each individual enzyme. An optimal temperature for the pulp bleaching with MnP and xylanase is 40^{o}C. The concentration of hydrogen peroxide in the pulp slurry has very important impacts on pulp bleaching effect. A continuous addition of hydrogen peroxide at an appropriate flow rate could enhance the pulp bleaching effect dramatically.*

KEYWORDS*: Manganese peroxidase, Xylanase, Synergism, Bleaching, Hardwood kraft pulp, Delignification.*

Many studies are currently done to increase the efficient utilization of enzymes in pulp delignification and bleaching as the current trend towards chlorine-free bleaching escalates. Among all the wood degrading enzymes the first reported to be useful for pulp bleaching was xylanase (*1*). A pretreatment of wood pulp with xylanase was found to increase the bleachability of the pulp in subsequent chemical bleaching stages (*2*). Xylanases were demonstrated to save up to 25% of bleaching chemicals required to reach a desired brightness (*3*). Although xylanases are currently used in several pulp mills worldwide, their effect on delignification is limited and indirect. Therefore, utilization of ligninolytic enzymes such as manganese peroxidase (MnP) and laccase might be a more efficient way to delignify and bleach pulp. Although both laccase and

MnP have been demonstrated to degrade lignin and bleach pulp to various extents, neither one is as yet used in commercial bleaching operations, due to their limited efficacy and high costs (*4, 5, 6, 7, 8*). To increase their pulp bleaching effect, a combination of two or more enzymes has been used for pulp bleaching. In pulp bleaching experiments with a combination of a xylanase and a laccase/mediator system, a beneficial effect of these two enzymes has recently been observed (*9*). Olsen claims that pulp can be bleached with xylanase and MnP/lignin peroxidase in two or more bleaching stages (*10*). Niku-Paavola *et al*. (1994) also demonstrated the possibility of using MnP and xylanase together for pulp bleaching (*11*). In our studies, we have further developed a pulp bleaching sequence that utilizes both xylanase and MnP. A very strong synergistic effect of the two enzymes on pulp bleaching has been demonstrated.

Materials and Methods

Source of pulp and chemicals

Unbleached hardwood kraft pulp (HWKP) with initial brightness of 35.3% ISO and kappa number of 13.7 was obtained from a Georgian pulp mill. All chemicals were of analytical grade and obtained from commercial sources.

Enzymes

Manganese peroxidase was produced from *Trametes versicolor* strain 52J kindly provided by Michael G. Paice (Pulp and Paper Research Institute of Canada). Cultivation of the fungus and purification of the enzyme was performed as described by Paice *et al*. (7). The culture medium was modified to contain 10 g/l glucose, 0.1 mM $CuSO_4$ and no pulp.

Partially purified xylanase form *Trichoderma reesei* was kindly provided by Dr. Jeff Tolan (Iogen Corporation, Ottawa, Canada).

Enzyme assays

MnP activity was measured by monitoring the increase in Mn(III)-malonate formation at 270 nm ($_{max}$= 11590 $M^{-1}cm^{-1}$) at 30^{o}C (*12*). Assay mixtures contained malonate buffer (50 mM, pH 4.5), $MnSO_4$ (1 mM), H_2O_2 (0.1 mM), and 100 µl of the MnP samples. One unit of MnP was defined as 1 µmol of product formed per min at 30^{o}C and at pH 4.5. Xylanase activity was determined by the dinitrosalicylic acid (DNS) method (*13*). Xylanase samples (200 µl) were added to 800 µl of malonate buffer (50 mM, pH 4.5) containing a final concentration of 1% birchwood xylan, and incubated at 40^{o}C for 10 min. Reducing sugars released were then estimated as xylose equivalents. One unit of xylanase was defined as the amount of enzyme required to release 1 µg of xylose per min under the above assay conditions.

Enzymatic pulp bleaching

Pulp bleaching experiments were performed in the presence of the purified MnP (50 U/g pulp) or xylanase (4.5 U/g pulp) or of both enzymes together. Each enzymatic treatment was

followed by a wash with deionized water. The pulp was then suspended at 2% consistency in an EDTA solution (pH 5.0, 1.2% EDTA on pulp) at 50°C for 30 min, which was followed by another wash with deionized water. Next, the pulp was bleached in an alkaline/hydrogen peroxide stage previously described by Paice et al. (*14*). The bleached pulp was disintegrated for 30 seconds in a Waring blender and handsheets were formed according to TAPPI test method T218 om-83. Brightness values were determined using TAPPI method T525 om-86 and kappa numbers using the micro kappa number method (*15*). Brightness of the handsheets was measured using a Technibrite TB-1 (Technidyne corporation, New Albany, IN) instrument. Brightness and kappa numbers of control experiments were obtained from the pulps that did not undergo any enzymatic treatment but were bleached with the alkaline/H_2O_2 step described above.

Two percent pulp consistency experiments

A) Pulp bleaching with Xylanase. In these experiments 4.5 U of xylanase was added to a 125 ml flask containing HWKP (1.0 g, OD) in 50 mM malonate buffer (pH 4.5, final volume 50 ml). The flask was shaken in a rotary shaker (150 rpm, 18 mm) at 30, 40, 50 or 60°C for 4 or 24 hours.

B) Pulp bleaching with MnP or with MnP+Xylanase.

i) Experiments with the addition of H_2O_2 (1 mM) at the beginning. In bleaching experiments with MnP, a 125 ml flask contained HWKP (1.0 g, OD), MnP (50 U), $MnSO_4$ (1 mM) and 10% Tween 80 (100 µl). The final volume was brought to 50 ml with malonate buffer (50 mM, pH 4.5), and the bleaching was started by the addition of H_2O_2 (0.034 mmole). In another group of experiments, xylanase (4.5 U) together with MnP was added to the mixture described above. All flasks were kept in a rotary shaker (150 rpm, 18 mm) at 30, 40, 50 or 60°C for 4 or 24 hrs. The remaining MnP and xylanase activities in the pulp bleaching at 40°C were measured after 1, 2, 3, 5, 10 and 24 hrs of incubation. Enzyme activities in control experiments that did not contain pulp were also monitored.

ii) Experiments in which H_2O_2 was added continuously throughout the incubation. Bleaching experiments with MnP or MnP + xylanase (pulp conditions as described above) were repeated. However, instead of addition of 1 mM H_2O_2 at the beginning of the experiments, H_2O_2 was added to the flasks throughout the 24-hour incubation, to a final amount of 0.0408, 0.0612, or 0.204 mmole by means of a multichannel peristaltic pump at flow rates of 0.6, 1.2, or 3 ml/hr, respectively.

Eight percent consistency experiments

A) Pulp bleaching with xylanase. In these experiments, xylanase (4.5 U) was added to a ziploc bag containing HWKP (1.0 g, OD, 8% consistency) and malonate buffer (50 mM, pH 4.5, final volume 12.5 ml). The bag was immersed in water in a waterbath at 40°C for 24 hours.

B) Pulp bleaching with MnP or MnP+xylanase. In the bleaching experiments with MnP, pulp (1.0 g, OD), MnP (50 U), $MnSO_4$ (1 mM), 10% Tween 80 (100 µl), and malonate buffer (50 mM, pH 4.5, final volume 12.5 ml) were thoroughly mixed in a plastic ziploc bag. The incubation started upon addition of H_2O_2 (0.034 mmole). In another group of experiment,

xylanase (4.5 U) together with MnP was added to the mixture described above. All bags were submerged in water in a waterbath at 40°C for 24 hrs.

Sequential bleaching of pulp with MnP and xylanase

Pulps were bleached with either MnP (50 U) or xylanase (4.5 U) for 24 hrs at 40°C, washed with water and then treated with the other enzyme for another 24 hrs (with the addition of 0.0612 mmole of H_2O_2 at a rate of 1.2 ml/hr in both enzymatic bleaching steps). The pulps were then washed, chelated and bleached with alkaline/hydrogen peroxide as described previously.

All data from the pulp bleaching experiments are the means of 2 independent experiments.

Results

Two percent consistency studies

a) Impacts of temperature on pulp bleaching with the addition of H_2O_2 at the beginning of experiments. Among the four different temperatures at which the pulp bleaching experiments were performed, xylanase worked best at 40°C, increasing the brightness from 59.3 to 65.6% ISO and decreasing the kappa number from 8 to 6.2 in 24 hours, (no H_2O_2 added) (Fig. 1 and 2). Xylanase also bleached pulp better at 30°C than at 50°C and 60°C. In MnP treated pulps, when H_2O_2 (0.034 mmole) was added at the beginning, higher brightness was obtained at higher temperatures (**Figures 1a, 1b**). When MnP and xylanase were applied together (0.034 mmole H_2O_2 was added at the beginning), the most effective bleaching was observed at 40°C with an increase in brightness from 59.3 to 70.5% ISO (Fig 1a). Kappa number also decreased from 8 to 5.2 (Fig. 1b).

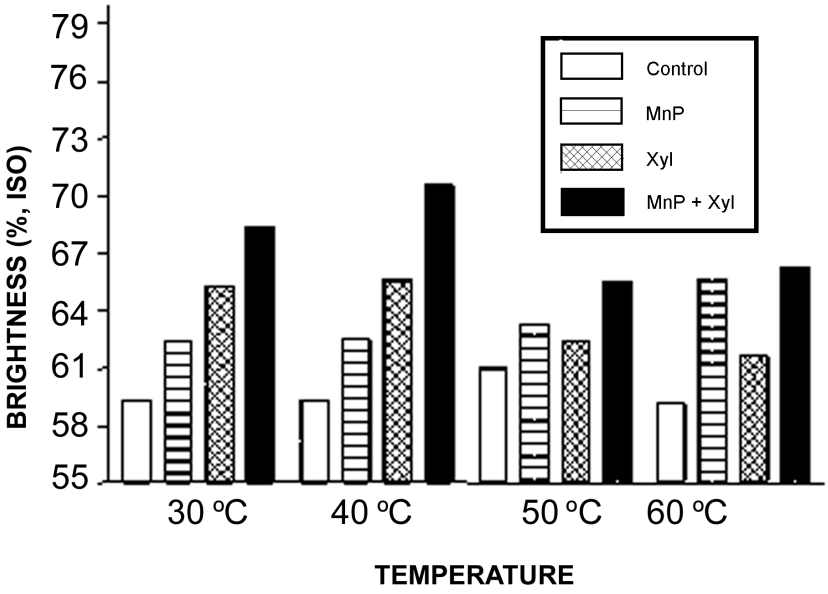


Figure 1a

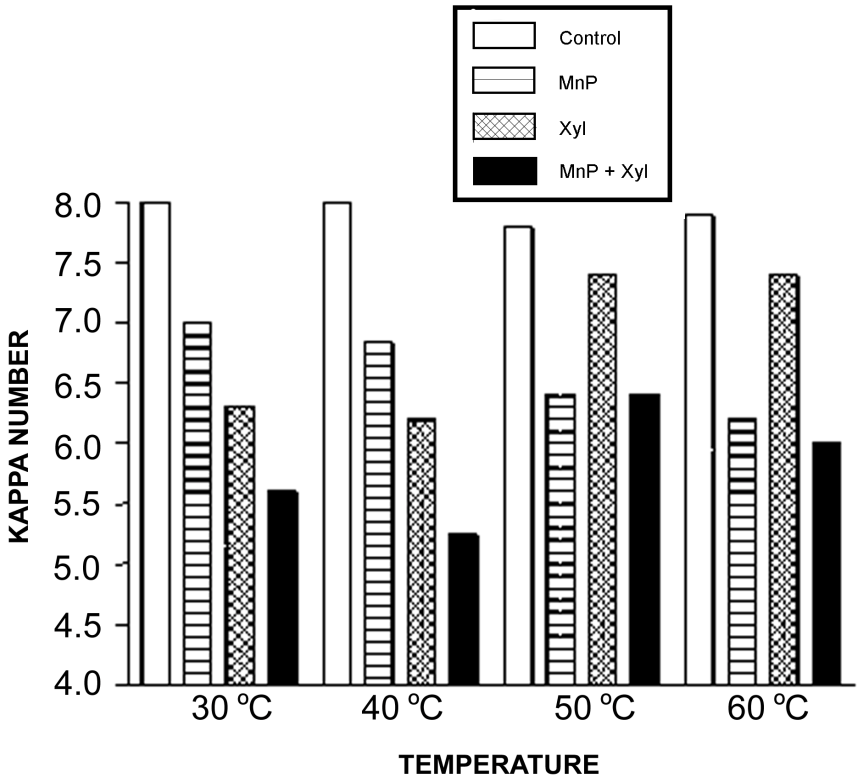


Figure 1b

Figure 1. Pulp bleaching with MnP and xylanase at various temperatures. H_2O_2 (0.034 mmole) was added at the beginning of the experiments.
(Legends: Control; MnP; Xyl; MnP + Xyl)

b) Impacts of temperature on pulp bleaching with continuous addition of H_2O_2 at a rate of 1.2 ml/min. Bleaching experiments with MnP showed that with the slow addition of H_2O_2 (1.2 ml/min, 0.0612 mmole total added), 30°C was the optimal temperature for MnP bleaching effect (**Figures 2a, 2b**). When MnP and xylanase were used together, the pulp bleaching effect was much better than their individual effects. The highest brightness and lowest kappa numbers were achieved at 40°C: the brightness increased from 59.2 to 78.1, and the kappa number decreased from 7.9 to 4.3 (Fig. 2a, 2b).

c) Impacts of H_2O_2 concentrations and the addition rate of H_2O_2 on pulp bleaching. Since 40°C was found to be the optimal temperature for the combined effect of MnP and xylanase, we studied impacts of concentration and addition rate of H_2O_2 on pulp bleaching at this temperature (**Table 1**). When H_2O_2 was added to pulp at a flow rate of 0.6 ml/hr throughout 24 hrs (0.0408 mmole total H_2O_2 added), bleaching with MnP increased the brightness from 59.3 to 67.9, and decreased kappa number from 8.0 to 6.1. Higher increase in brightness and higher decrease in kappa numbers were observed when both MnP and xylanase were added together (Table 1). In comparison with the results shown in Fig. 2a and 2b (the flow rate of 1.2 ml/min, 0.0612 mmole total H_2O_2 added), increase in the concentration of H_2O_2 enhances the pulp bleaching effect. However, further increase in the concentration of H_2O_2 (3 ml/hr flow rate, 0.204 mmole total H_2O_2 added), sharply decreased the gain in brightness.

Since a short bleaching time is more favorable in a commercial application, the bleaching time was reduced from 24 hours to 4 hours. In 4 hour experiments, when H_2O_2 (0.034 mmole) was added at the beginning, the bleaching effects were only slightly lower than those obtained in the 24 hour experiments (Table 1 and Fig. 1a, 1b). Higher brightness and lower kappa number values were obtained with MnP when H_2O_2 was added slowly (Table 1). Especially, with combinations of MnP and xylanase, results were more impressive, brightness increased to 72.7, while kappa number decreased to 5.

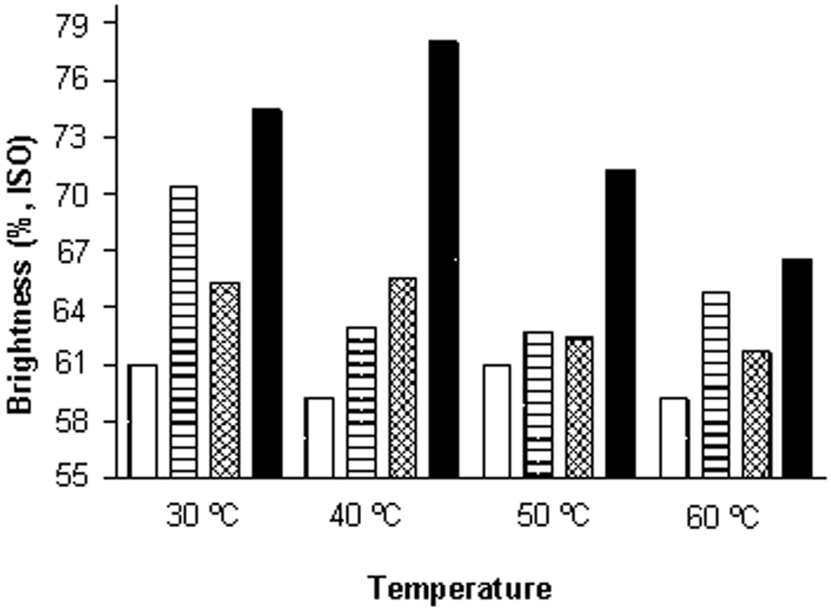


Figure 2a

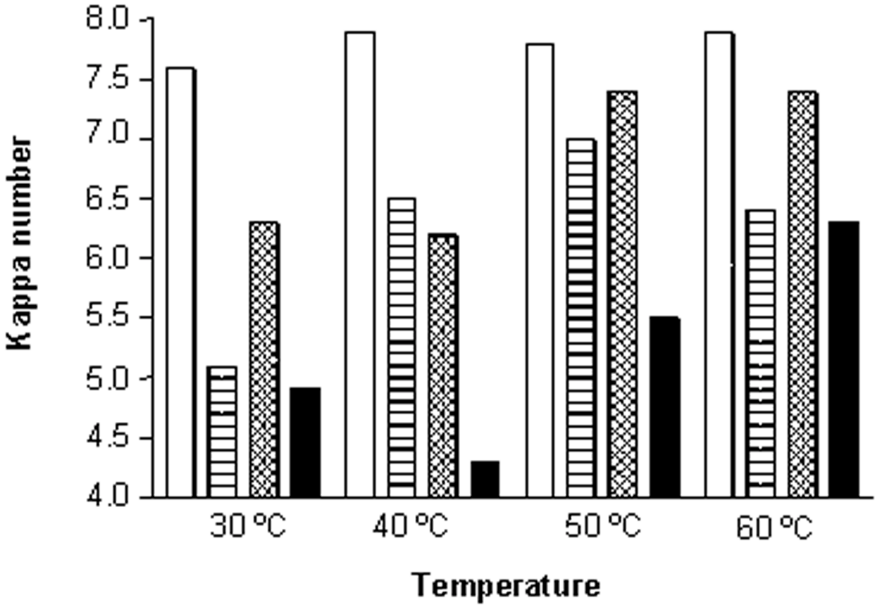


Figure 2b

Figure 2. Pulp bleaching with MnP and xylanase at various temperatures. A total of 0.0612 mmole H_2O_2 was added at a flow rate of 1.2 ml/min, throughout the incubation. (Legends: ▭ Control; ▤ MnP; ▩ Xyl; ■ MnP + Xyl)

Table 1. Effect of concentrations of H_2O_2 on pulp bleaching (2% consistency) with MnP and xylanase.

Control pulps (no enzymatic treatment)		Brightness (%, ISO)	Kappa number
Before alkaline/H_2O_2		35.3	13.7
After alkaline/H_2O_2		59.3	8.0
Pulps treated with enzymes*			
24 hrs incubation, 40°C			
H_2O_2 added at a rate of 0.6 ml/hr (0.0408 mmole total H_2O_2 added)	MnP	67.9	6.1
	MnP+Xylanase	74.3	4.9
H_2O_2 added at a rate of 3 ml/hr (0.204 mmole total H_2O_2 added)	MnP	52.9	6.8
	MnP+Xylanase	58.5	6.1
	Xylanase only	65.6	6.2
4 hrs incubation, 40°C			
H_2O_2 (0.034 mmole) added at the beginning of the experiment	MnP	64.4	6.5
	MnP+Xylanase	68.5	5.9
H_2O_2 added at a rate of 1.2 ml/hr (0.0612 mmole total H_2O_2 added)	MnP	67.7	6.1
	MnP+Xylanase	72.7	5
	Xylanase only	68.6	5.7

* Data were obtained after an alkaline hydrogen peroxide stage in addition to the enzyme treatment described.

Eight percent consistency studies

Pulp bleaching experiments at 8% consistency were all performed at 40°C (0.034 mmole H_2O_2 added at the beginning of incubation). The results of these studies were almost identical to those obtained at 2% consistency (H_2O_2 (0.034 mmole) added at the beginning) (**Figure 3**). Bleaching with MnP and xylanase together was also much more effective than that with individual enzymes.

Sequential treatment of pulp with MnP and xylanase

To understand if pulp bleaching effect by MnP and xylanase is due to the synergistic effect of the two enzymes, pulp was treated with MnP and xylanase sequentially. The brightness and the kappa number of the pulp treated with MnP and then xylanase were 65.9 % ISO and 6.4, respectively. Pulps treated in the reverse order, i.e. first with xylanase and then with MnP did not give significantly different results: 64.4% ISO brightness and 6.6 kappa number, respectively.

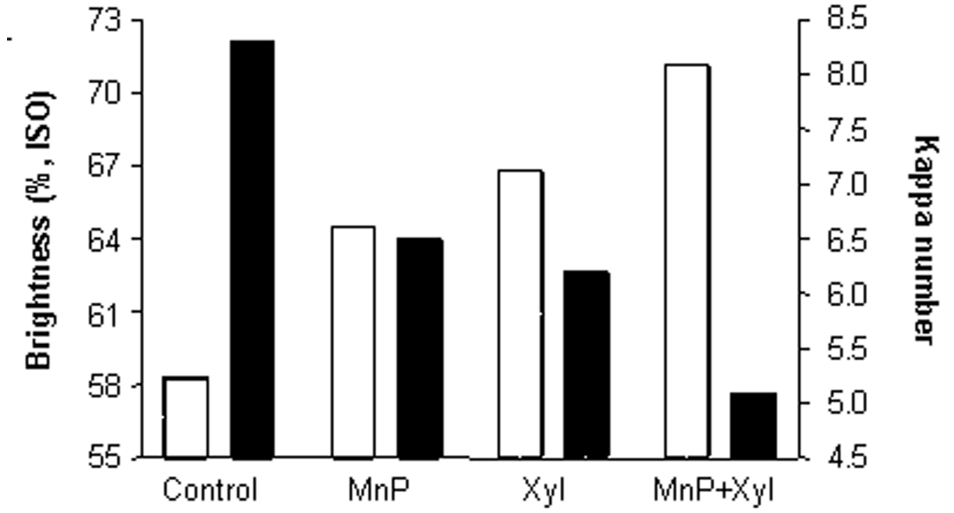


Figure 3. High consistency (8%) enzymatic pulp bleaching experiments at 40°C (24 hrs). (Legends: Brightness (%, ISO); Kappa number).
(Legends: ☐ Control; ▤ MnP; ▩ Xyl; ■ MnP + Xyl)

Stability of MnP and xylanase

During the incubations at 40°C, MnP and xylanase activities were monitored (Fig. 4). In the absence of pulp, MnP activity was stable throughout the 24 hour incubation both with and without xylanase (**Figure 4a**). In the presence of pulp, MnP activity gradually decreased by 60% in 24 hours both in the presence and in the absence of xylanase. In the absence of pulp, xylanase was very stable without MnP, whereas its activity decreased by 65% in 24 hours in the presence of MnP (**Figure 4b**). Similarly, in the presence of pulp xylanase was very stable without MnP while its activity decreased by 30% in the first 3 hours, and remained the same thereafter when MnP was included.

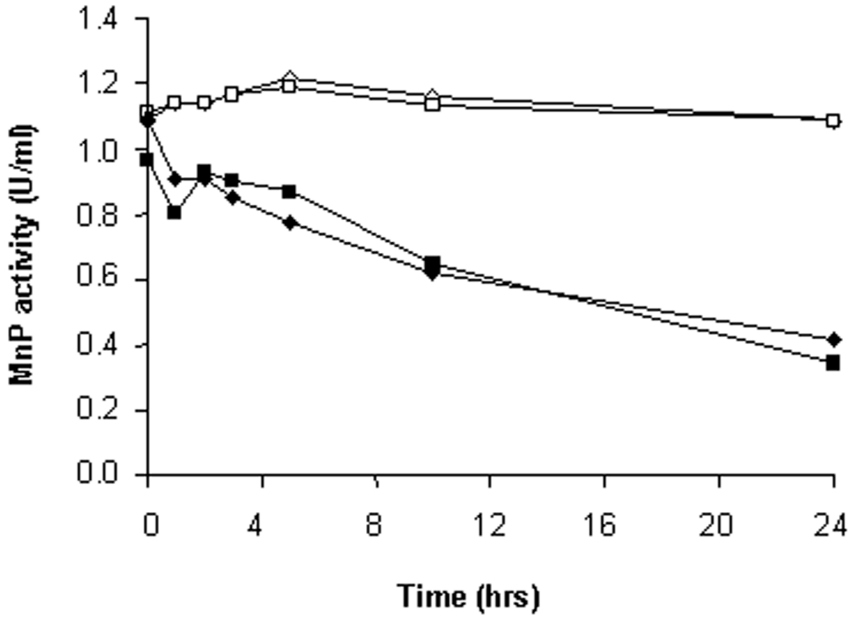


Figure 4a

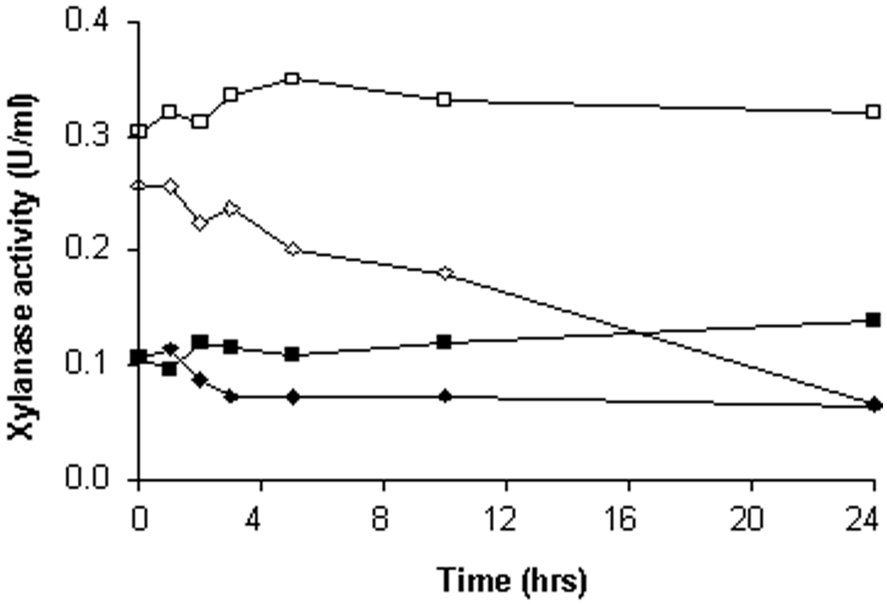


Figure 4b

Figure 4. Stability of enzymes: A. MnP in the absence and the presence of pulp (40°C). (Legends: (□) MnP only (no pulp); (◊) MnP+xylanase (no pulp); (■) MnP only (with pulp); (♦) MnP+xylanase (with pulp)). B. xylanase in the absence and presence of pulp (40°C). (Legends: (□) xylanase only (no pulp); (◊) xylanase+MnP (no pulp); (■) xylanase only (with pulp); (♦) xylanase +MnP (with pulp).

Discussion

It has been demonstrated that the slow addition of the H_2O_2 to the pulp slurry would greatly enhance pulp bleaching effect by MnP (7, 8). We also observed the same effect. As described above, the best effect was achieved when H_2O_2 was added at a rate of 1.2 ml/min (0.0612 mmole H_2O_2 added). The brightness was 8% ISO higher than that obtained in the experiments where 0.034 mmole H_2O_2 was added at the beginning. All of these occur because MnP is rapidly inactivated by the high concentration of H_2O_2 (unpublished results).

We have demonstrated that pulp bleaching effects with a combination of MnP and xylanase are always better than those with individual enzymes. This is in accordance with the previous investigations in the bleaching of oxygen-bleached softwood kraft pulp with MnP and xylanase (*11*). To find out whether the strong bleaching ability of the combined MnP and xylanase was due to their synergistic action or due to the simple addition of pulp bleaching effects of individual enzymes, we studied a sequential pulp bleaching with both enzymes. Pulp bleaching with MnP and xylanase simultaneously gave significantly higher pulp brightness than that obtained after the treatment of pulp with MnP and xylanase sequentially. This indicates that MnP and xylanase exhibit strong synergistic effect on pulp bleaching. The synergistic effect probably results from that xylanase can increase MnP's access to lignin better in a simultaneous treatment than in a sequential treatment. The optimal temperature for the synergistic action of MnP and xylanase was 40°C. At this temperature, the combination of MnP and xylanase

followed by an alkaline/H_2O_2 treatment increased the pulp brightness from 38.7 to 78.1% ISO and decreased the kappa number from 13.1 to 4.3.

While impressive pulp bleaching results were obtained with MnP and xylanase in 2% consistency of pulp at 40°C, the results obtained in bleaching experiments with 8% consistency of pulp was also very effective. This result has indicated the possibility of a commercial use of MnP and xylanase in pulp bleaching since bleaching of a high consistency of pulp is desired in a pulp mill.

At 40°C, the MnP activity did not decrease with time in the absence of pulp, and was not affected by the presence of xylanase. However, in the presence of pulp, MnP activity decreased with time. The drop in MnP activity might be due to adsorption of MnP on the pulp fibers which would give rise to a decrease in the amount of MnP in solution. In the absence of pulp, inactivation of MnP in the presence of excess H_2O_2 or xylanase was not observed, which was due to the stabilizing effect of Mn(II) (unpublished results). In contrast, xylanase activity decreased in the absence of pulp when MnP was present, but did not decrease in the presence of pulp. Since, in the pulp bleaching experiments with xylanase, the incubation started with the addition of xylanase to the pulp first, it can be speculated that the interaction of xylanase with pulp might protect the enzyme. This possible interaction can also be observed in Fig.4b. Although the initial amounts of xylanase in the experiments with or without pulp were the same, the enzyme free in solution with pulp was much lower even at the very beginning of the experiments in comparison to those without pulp.

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