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Correlation of Brightening with Cumulative Enzyme Activity Related to Lignin Biodegradation during Biobleaching of Kraft Pulp by White Rot Fungi in the Solid-State Fermentation System

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Biobleaching of hardwood unbleached kraft pulp (UKP) by *Phanerochaete chrysosporium* and *Trametes versicolor* was studied in the solid-state fermentation system with different culture media. In this fermentation system with low-nitrogen and high-carbon culture medium, pulp brightness increased by 15 and 30 points after 5 days of treatment with *T. versicolor* and *P. chrysosporium*, respectively, and the pulp kappa number decreased with increasing brightness. A comparison of manganese peroxidase (MnP), lignin peroxidase (LiP), and laccase activities assayed by using fungus-treated pulp and the filtrate after homogenizing the fungus-treated pulp in buffer solution indicated that enzymes secreted from fungi were adsorbed onto the UKP and that assays of these enzyme activities should be carried out with the treated pulp. Time course studies of brightness increase and MnP activity during treatment with *P. chrysosporium* suggested that it was difficult to correlate them on the basis of data obtained on a certain day of incubation, because the MnP activity fluctuated dramatically during the treatment time. When brightness increase and cumulative MnP, LiP, and laccase activities were determined, a linear relationship between brightness increase and cumulative MnP activity was found in the solid-state fermentation system with both *P. chrysosporium* and *T. versicolor*. This result suggests that MnP is involved in brightening of UKP by white rot fungi.

Residual lignin in kraft pulp is highly modified by alkaline condensation reactions during pulping and gives the pulp a characteristic dark-brown color. This residual lignin is commercially removed by bleaching with chlorine-based chemicals. It has been reported that chlorinated products derived from lignin during these bleaching procedures are mutagenic (1, 31). They also cause a waste treatment problem because of their toxicity and dark color. Therefore, environmental concerns have led us to seek alternative ways to eliminate, or at least reduce, the use of chlorine-based chemicals in bleaching.

Because lignin biodegradation by *Phanerochaete chrysosporium* and *Trametes versicolor* has been intensively studied (3, 5, 11, 14, 29), much research has been carried out in an attempt to delignify and brighten unbleached kraft pulp (UKP) by these fungi. Kirk and Yang were the first to recognize that *P. chrysosporium* could partially delignify softwood UKP (16). It was also reported that hardwood UKP treated with *T. versicolor* showed an increase in brightness and a corresponding decrease in residual lignin concentration (17, 25). Nishida et al. showed that the white rot fungus IZU-154, which degrades wood lignin more extensively and selectively than do *P. chrysosporium* and *T. versicolor* (23), delignifies hardwood and softwood UKPs and increases pulp brightness significantly. In addition, the use of chlorine-based chemicals and the pollution load of waste liquor in bleaching of UKP were significantly reduced by the biobleaching process, which combined a IZU-154 treatment and chemical bleaching (7, 8, 21).

However, the delignification rate in the biobleaching process is somewhat lower than that in the chemical bleaching process. To overcome this drawback of the fungal treatment, we have attempted to determine the enzymologic basis of fungal delignification. *P. chrysosporium* and *T. versicolor* produce laccase, lignin peroxidase (LiP), and manganese peroxidase (MnP) (4, 10, 12, 18, 33, 34), and it has been suggested that these enzymes are involved in the oxidative breakdown of lignin. Perez and Jeffries showed that increased synthetic lignin mineralization correlates with increased LiP activity, not with increased MnP or laccase activity (28), and LiP has been reported to bleach UKP (2, 6) and to facilitate subsequent chemical bleaching (24). However, there are some reports in which the role of LiP in lignin biodegradation is questioned. Paice et al. reported that LiP activity is not detected during biobleaching of UKP by *T. versicolor* (26), and there are several lignin-degrading fungi that secrete MnP and laccase but not LiP (9, 19, 22, 30). MnP was found to depolymerize synthetic lignin (35) and has been shown to be present at substantial levels during biobleaching with *T. versicolor* (26). Furthermore, Paice et al. showed that MnP is produced by bleaching cultures of *T. versicolor* and that the peak production of the enzyme occurs at the same time as the maximum rate of fungal bleaching (26). Kondo et al. showed that cell-free, membrane-filtered components in the in vitro biobleaching system are capable of delignifying UKP and that a positive correlation between the level of MnP and brightening of the pulp was observed (18). However, the extensive increase in brightness observed in the fungus-treated pulp was not found with MnP alone (26).

In this study, to clarify the role of MnP, LiP, and laccase in biobleaching, we examined the relationship between the brightening of UKP and the activities of these enzymes produced by *P. chrysosporium* and *T. versicolor* in a solid-state fermentation system with different culture media. By a new approach in which the enzyme assay was carried out with fungus-treated pulp instead of with the extracted enzyme from

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TABLE 1. Composition of culture media

Component	Amt of component in:			
	LN-HC	LN-LC	HN-HC	HN-LC
Ammonium tartrate (g)	0.2	0.2	2.0	2.0
Glucose (g)	20.0	2.0	20.0	2.0
KH_2PO_4 (g)	1.0	1.0	1.0	1.0
NaH_2PO_4 (g)	0.2	0.2	0.2	0.2
$MgSO_4 \cdot 7H_2O$ (g)	0.5	0.5	0.5	0.5
Dimethyl succinate (g)	1.46	1.46	1.46	1.46
Thiamine hydrochloride (μg)	100	100	100	100
$CaCl_2$ (μg)	100	100	100	100
$FeSO_4 \cdot 7H_2O$ (μg)	100	100	100	100
$ZnSO_4 \cdot 7H_2O$ (μg)	10	10	10	10
$CuSO_4 \cdot 5H_2O$ (μg)	20	20	20	20
$MnSO_4 \cdot 4H_2O$ (μg)	10	10	10	10
Distilled water (liters)	1.0	1.0	1.0	1.0

the fungus-treated pulp and cumulative enzyme activity was determined, we showed that MnP is the most important enzyme in biobleaching of UKP by *P. chrysosporium* and *T. versicolor*.

MATERIALS AND METHODS

Microorganisms. *P. chrysosporium* ME-446 and *T.* (*Coriolus*) *versicolor* IFO-30340 were used in this study. The fungi were maintained on potato dextrose agar (Difco Laboratories) slants.

Biobleaching of kraft pulp. To delignify and brighten the hardwood UKP with white rot fungi, two treatment systems were used.

Biobleaching in the solid-state fermentation system was performed as follows. PDA plates were inoculated with each white rot fungus and incubated for 5 to 7 days at 30°C. Five disks punched from the grown edge of the mycelium were homogenized for 30 s with 50 ml of PMY medium (3.0% glucose, 1.0% peptone, 1.0% malt extract, 0.4% yeast extract) and then put in a 500-ml Erlenmeyer flask with 150 ml of PMY medium and shaken at 200 rpm to give a mycelium suspension culture. After 3 days, 50 ml of the culture was homogenized once again and then further precultured in a 500-ml Erlenmeyer flask with 250 ml of PMY medium on a rotary shaker (200 rpm) for 5 days at 30°C. The precultured mycelium of white rot fungus was separated from the PMY medium and then aseptically added to a 300-ml Erlenmeyer flask containing 10 g of hardwood UKP (brightness, 29.0%; kappa no., 15.5) and 40 ml of culture medium (Table 1). In this system, the pulp consistency corresponded to about 20%. The UKPs were then incubated statically at 30°C.

Biobleaching in the liquid-state fermentation system was performed as follows. As mentioned above, the precultured mycelium of white rot fungus was separated from the PMY medium and then added to a 100-ml Erlenmeyer flask containing 200 mg of hardwood UKP and 20 ml of culture medium (the pulp consistency corresponded to 1%). The flasks were shaken at 200 rpm and 30°C.

Enzyme assays. In the solid-state fermentation system, the fungus-treated pulp (but not the extracted enzyme from the treated pulp) was added to the reaction mixtures containing substrates, the mixtures were homogenized, and the enzyme activities were determined at 37°C as described below. In the liquid-state fermentation system, the homogenized whole culture containing broth, mycelia, and treated pulp was added to the reaction mixtures. Laccase activity was measured by monitoring the oxidation of 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) at 415 nm (36). The reaction mixture contained 0.5 mM ABTS and 0.1 M sodium acetate buffer (pH 5.0). MnP activity was measured by monitoring the oxidation of ABTS at 415 nm (27). The reaction mixture contained 7.8 mM ABTS, 0.1 mM $MnSO_4$, 50 mM sodium lactate, 0.1 mM H_2O_2, and 20 mM sodium succinate buffer (pH 4.5). LiP activity was measured by monitoring the oxidation of veratryl alcohol at 310 nm (20). The reaction mixture contained 10 mM veratryl alcohol, 0.1 mM H_2O_2, and 20 mM sodium succinate buffer (pH 3.0). One unit of enzyme activity is defined as the amount of enzyme that increases the absorbance by 0.1 unit/min under the above conditions, and enzyme activity was expressed in units per gram of treated pulp.

Pulp properties. After incubation with fungi, pulp samples were washed with water and pulp sheets were prepared with a Buchner funnel (diameter, 11 mm) and air dried. Brightness was determined with a colorimeter (model CR-300; Minolta, Tokyo, Japan). The values determined with the colorimeter were multiplied by a coefficient to adjust them to International Standards Organization (procedure 5269) brightness values. The kappa number is defined as the amount (in milliliters) of a 0.1 N $KMnO_4$ solution consumed by 1 g of moisture-free pulp under standard conditions (Standard T 236 of the Technical Association of the Pulp and Paper Industry, Atlanta, Ga.).

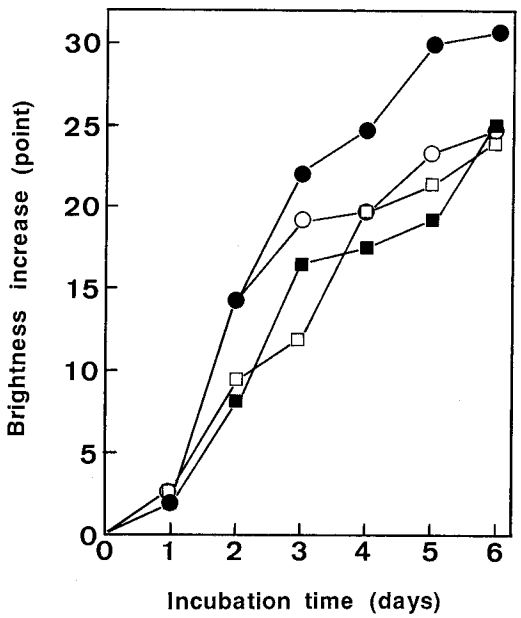

FIG. 1. Time courses of the brightness increase of UKP treated with *P. chrysosporium* in the solid-state fermentation system with four different culture media. Symbols: ●, LN-HC medium; ○, LN-LC medium; ■, HN-HC medium; □, HN-LC medium.

RESULTS

Biobleaching of kraft pulp by *P. chrysosporium* and *T. versicolor*. The hardwood UKP was inoculated with *P. chrysosporium* in the solid-state fermentation system with four different culture media (listed in Table 1) and incubated for 6 days. Figure 1 shows increases in brightness after treatment with *P. chrysosporium*. Marked increases were obtained after 1 day of incubation, and obvious differences in the rate of brightening were observed among four different culture media. The greatest brightness increase was obtained with an LN-HC culture medium (Fig. 1). This was consistent with previous results of lignin biodegradation by white rot fungi under low-nitrogen culture conditions (13, 15). With an LN-HC culture medium, brightness increased by 22 and 30 points after 3 and 5 days of fungal treatment, respectively.

When pulp was treated with *T. versicolor* in the solid-state fermentation system with an LN-HC culture medium, brightness did not increase appreciably during the first 3 days of incubation and then began to increase significantly (Fig. 2). Throughout the scheduled fungal treatment period, the brightness was increased 20 and 30 points for pulp treated with *T. versicolor* and *P. chrysosporium*, respectively. The pulp kappa numbers decreased with increasing brightness, and the kappa numbers reached 7.8 and 5.8 for *T. versicolor* and *P. chrysosporium*, respectively (Fig. 2). Under the culture conditions used here, *P. chrysosporium* has a higher potential for biobleaching of UKP than does *T. versicolor*.

Figure 3 shows the relationship between increase in brightness and decrease in kappa number during treatment with *P. chrysosporium* and *T. versicolor*. The brightness increase obtained by the fungal treatment was accompanied by a decrease in kappa number. This result indicates that the brightness increase was due to the degradation of residual lignin in UKP.

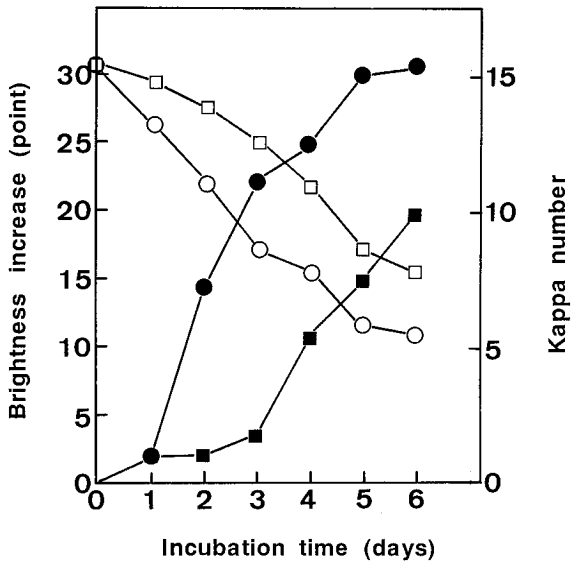

FIG. 2. Time courses of the brightness increase and kappa number decrease of UKP treated with white rot fungi in the solid-state fermentation system with LN-HC culture medium. Symbols: ●, *P. chrysosporium*, brightness increase; ■, *T. versicolor*, brightness increase; ○, *P. chrysosporium*, kappa number; □, *T. versicolor*, kappa number.

Changes in brightness and enzyme activity during biobleaching. In the solid-state fermentation system, the enzymes secreted from fungi may be adsorbed onto the UKP. Therefore, our first effort was focused on establishing the enzyme assay systems. When the filtrate obtained after homogenizing the fungus-treated pulp in buffer solution was used for MnP, LiP, and laccase assays as the enzyme solution, LiP and laccase were not detectable (Table 2). On the other hand, when treated pulp, onto which enzymes might be adsorbed, was added to the buffer solutions containing substrates, MnP activity was increased 1.5-fold and LiP and laccase activities were also detected (Table 2). This clearly indicates that enzymes secreted by fungi were adsorbed onto pulp during biobleaching in the solid-state fermentation system and that assays of these enzymes should be carried out with the treated pulp instead of the extracted enzyme solution.

TABLE 2. Comparison of enzyme activities assayed with fungus-treated pulp and extracted solution during the treatment with *P. chrysosporium* in the solid-state fermentation system with LN-HC culture medium

Enzyme	Enzyme activity (U/g of pulp) in:	
	Fungus-treated pulp	Extracted solution
MnP	334	216
LiP	1.04	0
Laccase	0.62	0

In our enzyme assay system, *P. chrysosporium* was observed to produce laccase activity, which is ABTS-oxidizing activity without addition of hydrogen peroxide. *P. chrysosporium* is not supposed to produce laccase; therefore, it is possible that the laccase activity observed with *P. chrysosporium* was caused by MnP supported by low levels of endogenous Mn and hydrogen peroxide produced by fungus. It was difficult to avoid the effect of endogenous Mn and hydrogen peroxide produced by fungus in our enzyme assay system; therefore, this ABTS-oxidizing activity was regarded as laccase in this study.

Figure 4 shows changes in brightness and MnP activity observed during treatment with *P. chrysosporium*. Brightness continuously increased after the first day of incubation, and MnP activity dramatically fluctuated throughout the treatment. The MnP activity markedly increased during the first 2 days of incubation, decreased rapidly on day 3 of incubation, and increased again thereafter. The time course studies of brightness increases and changes in MnP activity were repeated, with similar results. Therefore, it seems impossible to define the relationship between the brightness increase and the enzyme

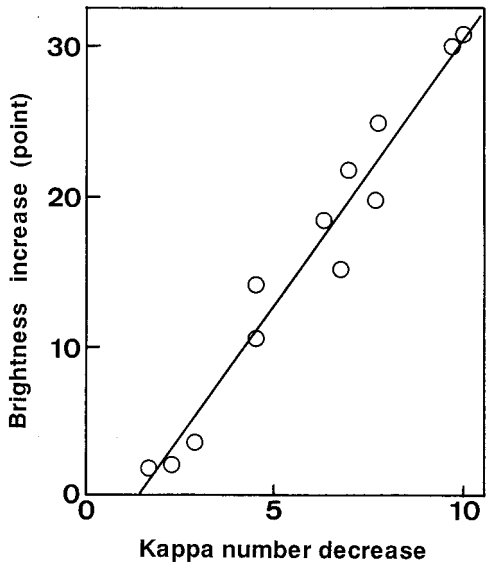

FIG. 3. Relationships between brightness increase and kappa number decrease during treatment with *P. chrysosporium* and *T. versicolor*. The regression equation is $Y = -5 + 3.55X$. The correlation coefficient is 0.973.

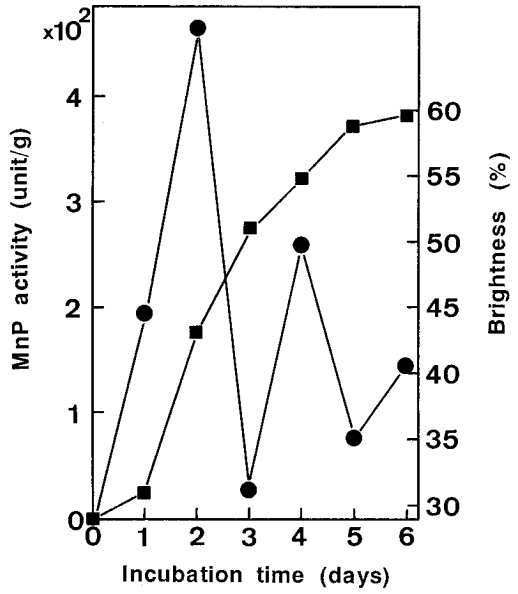

FIG. 4. Changes in the MnP activity and brightness of UKP during treatment with *P. chrysosporium* in the solid-state fermentation system with LN-HC culture medium. Symbols: ●, MnP activity; ■, brightness.

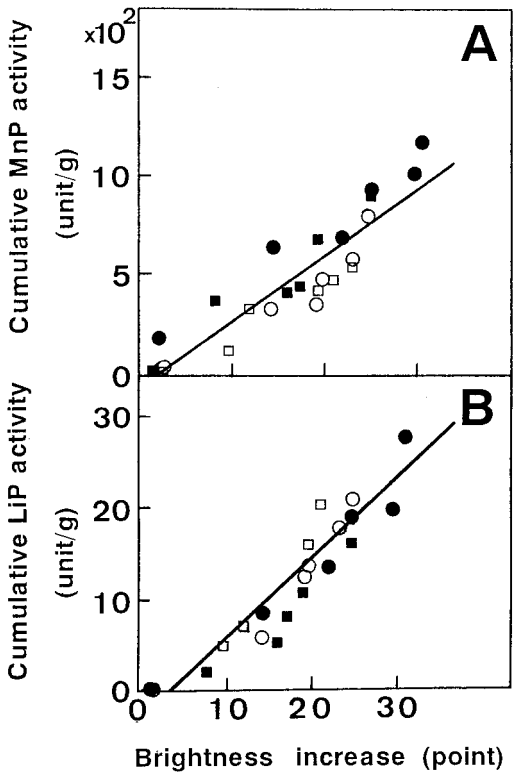

FIG. 5. Relationship between cumulative enzyme activity and brightness increase during treatment with *P. chrysosporium* in the solid-state fermentation system with four different culture media. Symbols: ●, LN-HC medium; ○, LN-LC medium; ■, HN-HC medium; □, HN-LC medium. (A) Cumulative MnP activity. (B) Cumulative LiP activity. For panel A, the regression equation is $Y = -69.7 + 32.6X$ and the correlation coefficient is 0.896. For panel B, the regression equation is $Y = -3.58 + 0.896X$ and the correlation coefficient is 0.907.

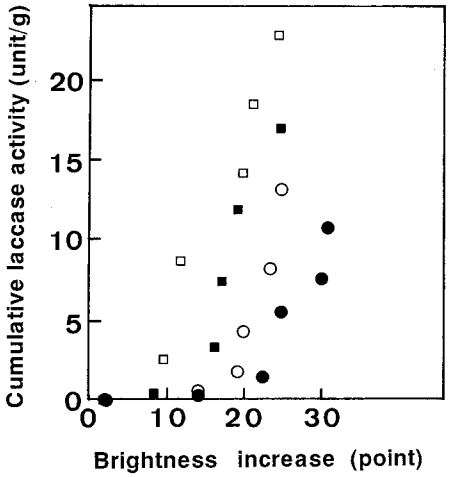

FIG. 6. Relationship between the cumulative laccase activity and brightness increase during treatment with *P. chrysosporium* in the solid-state fermentation system with four different culture media. Symbols: ●, LN-HC medium; ○, LN-LC medium; ■, HN-HC medium; □, HN-LC medium. The correlation coefficient is 0.639.

activity on the basis of data obtained on a certain day during treatment, because the brightness of treated pulp results from the cumulative daily brightening and the observed enzyme activity is a transient activity at a certain point in time. Therefore, the brightness increase and the cumulative enzyme activity were investigated to define the enzyme(s) related to biobleaching of UKP.

Each cumulative MnP, cumulative LiP, and cumulative laccase activity was plotted against brightness increases during treatment with *P. chrysosporium* in the solid-state fermentation system (Fig. 5A and B and 6). There was a linear relationship between cumulative MnP activity and brightness increase under all four different culture conditions (Fig. 5A). The relationship between cumulative LiP activity and brightness increase was also linear (Fig. 5B). Both correlation coefficients were about 0.9, whereas the correlation coefficient for cumulative laccase activity and brightness increase was about 0.6 (Fig. 6). These results suggest that MnP and LiP relate to brightening of the UKP by *P. chrysosporium* but laccase does not.

In the solid-state fermentation system with an LN-HC culture medium, *T. versicolor* produced substantial levels of MnP and laccase but not LiP. Therefore, the relationships between the brightness increase and cumulative MnP activities during treatment with *P. chrysosporium* and *T. versicolor* in the solid-state fermentation system with an LN-HC culture medium were examined. In this fermentation system, a linear relationship was observed between the brightness increase and cumulative activity of MnP produced by the two fungi, indicating that the cumulative MnP activity required to obtain a certain level of brightness increase is the same for treatment with *P. chrysosporium* and treatment with *T. versicolor* (Fig. 7). This result intensively supports the idea that MnP plays the most important role in biobleaching of UKP by white rot fungi.

Further experiments were carried out with the liquid-state fermentation system and an LN-HC culture medium to investigate the relationship between cumulative MnP activity and brightness increase during treatment with *P. chrysosporium* and *T. versicolor*. No significant increase in pulp brightness was obtained, and only about a 5-point increase in brightness was achieved by both fungi after 6 days of incubation (data not shown). However, *P. chrysosporium* and *T. versicolor* in the

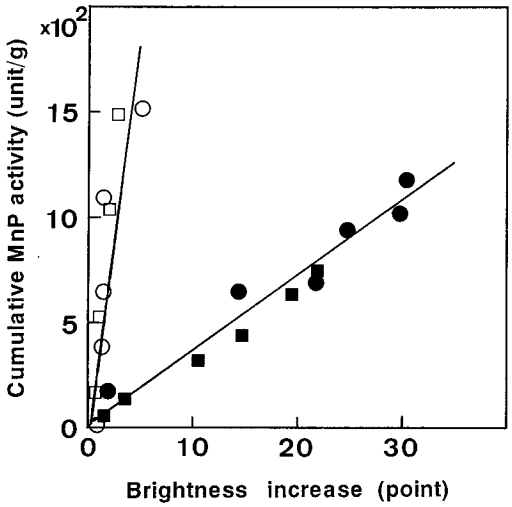

FIG. 7. Comparison of the relationship between the cumulative MnP activity and brightness increase in the solid-state and liquid-state fermentation systems with LN-HC culture medium. Symbols: ●, *P. chrysosporium*, solid-state fermentation system; ■, *T. versicolor*, solid-state fermentation system; ○, *P. chrysosporium*, liquid-state fermentation system; □, *T. versicolor*, liquid-state fermentation system.

liquid-state fermentation system produced the same levels of MnP as were produced in the solid-state fermentation system during 6 days of incubation. The cumulative activities of MnP produced by *P. chrysosporium* and *T. versicolor* in the liquid-state fermentation system were also plotted against the brightness increase, as shown in Fig. 7. A linear relationship between brightness increase and cumulative MnP activities was also observed in this fermentation system. However, the slopes for the two fermentation systems were very different, and the brightness increase was much higher in the solid-state fermentation than in the liquid-state fermentation at the same level of cumulative MnP activity. This suggests that other important factors besides the cumulative MnP activity are involved in biobleaching of UKP by white rot fungi, one of which may be the concentration of MnP and/or hydrogen peroxide in contact with the UKP.

DISCUSSION

Most studies on the biobleaching of UKP by *P. chrysosporium* and *T. versicolor* were carried out in the liquid-state fermentation systems in which the pulp consistencies were about 1.5%. On the other hand, Nishida et al. have reported that the biobleaching of UKP in the solid-state fermentation system (pulp consistency, 20%) by the hyperligninolytic fungus IZU-154 (23) afforded a marked brightness increase. Therefore, in this study, the biobleaching of UKP by *P. chrysosporium* in the solid-state fermentation system was measured with four different culture media (LN-HC, LN-LC, HN-HC, and HN-LC). The greatest brightness increase was obtained in a combination of the solid-state fermentation system with the LN-HC culture medium and *P. chrysosporium*. This result supports the previous reports that many lignin-degrading fungi perform better under solid-state fermentation conditions than under submerged conditions (32) and that the ligninolytic activity of white rot fungi is greatest under nitrogen-limited culture conditions (13, 15).

Since many studies have suggested that MnP, LiP, and laccase are involved in lignin biodegradation (2, 4, 6, 26, 28, 35), the changes in brightness and in these enzyme activities were investigated during a 6-day incubation period. In this study, different culture media were used for biobleaching to define the relationship between these enzyme activities and brightness increase, because the obvious differences in brightness increase under these culture conditions should be manifested as differences in enzyme activities related to brightening of UKP.

For these purposes, the extracellular enzymes have been commonly prepared from the broth of cultures, excluding mycelia and biobleached pulp, in the liquid-state fermentation systems (26). Arbeloa et al. (2), however, reported that LiP was adsorbed onto UKP and that 90% of the LiP activity disappeared immediately after the LiP contacted the hardwood UKP, suggesting that it is difficult to estimate accurately the enzyme activities secreted from fungi by using the enzyme solution prepared from the broth as in the previous studies and that activities of enzymes adsorbed onto pulp should be determined to define the relationship between the brightness increase and the enzyme activity. The results shown in Table 2 clearly indicate that the assays of these enzyme activities in the solid-state fermentation system should be carried out with the treated pulp but not with extracted enzyme solution.

As shown in Fig. 4, the MnP activity dramatically fluctuated while the brightness increased continuously during the treatment, which raises the question of whether it is possible to define the relationship between brightness increase and enzyme activity based on the data on a certain day when the time courses of both the brightness increase and enzyme activity were determined. In our new approach, the relationship between the cumulative enzyme activity and brightness increase was analyzed. The cumulative MnP activity and cumulative LiP activity correlated markedly with the brightness increases in the treatment with *P. chrysosporium* in the solid-state fermentation system with four different culture media (Fig. 5). A positive correlation was also observed between the brightness increase and cumulative MnP activity in the treatments with *T. versicolor* and *P. chrysosporium* in the solid-state fermentation system with the LN-HC culture medium (Fig. 7). It should be noted that a linear relationship between brightness increase and cumulative MnP activity can be obtained in four different culture media with *P. chrysosporium* and in LN-HC medium with *T. versicolor*, indicating that MnP is involved in the brightening of UKP. LiP has been suggested as one of the lignin-degrading enzymes (2, 6, 24, 28), and the results obtained here suggest that this enzyme also contributes to brightening of UKP during treatment with *P. chrysosporium*. On the other hand, *T. versicolor* could brighten UKP, even though this fungus did not produce LiP. It is not possible to clarify the function of LiP in the brightening of UKP from the results of this study, and this should be a subject of future studies.

Kirk et al. used *P. chrysosporium* for biobleaching of softwood UKP in a liquid-state fermentation system and reported that the kappa number of 8-day-treated pulp was decreased by 60% after a subsequent alkali treatment (16). Paice et al. reported that a 15-point increase in brightness was obtained after a 5-day treatment with *T. versicolor* in the liquid-state fermentation system (25). On the other hand, brightness was hardly increased by treatment with *P. chrysosporium* and *T. versicolor* in the liquid-state fermentation system in our study. This may be due to differences in several conditions such as a component of the nutrients, the use of O_2 flashing, and the strains of fungi used. Even though the pulp brightness was not significantly increased in the liquid-state fermentation system, the same level of cumulative MnP was produced by both *P. chrysosporium* and *T. versicolor* in the solid-state and liquid-state fermentation systems (Fig. 7). A possible explanation for the difference in the brightness increase in the two fermentation systems is that the concentrations of MnP and hydrogen peroxide are very different in the liquid-state and solid-state fermentation systems. In the liquid-state fermentation system, the enzyme and hydrogen peroxide were released into the culture fluid, and their concentrations were diluted; consequently, they became ineffective for brightness increases.

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REFERENCES

1. **Ander, P., K.-E. Eriksson, M.-C. Kolar, and K. P. Kringstad.** 1977. Studies on the mutagenic properties of bleaching effluents. Sven. Papperstidn. **80:** 454–459.
2. **Arbeloa, M., J. D. Leseleuc, G. Goma, and J.-C. Pommier.** 1992. An evaluation of the potential of lignin peroxidases to improve pulps. TAPPI J. **75:**215–221.
3. **Crawford, R. L.** 1981. Lignin biodegradation and transformation. John Wiley & Sons, Inc., New York.
4. **Dodson, P. J., C. S. Evans, P. J. Harvey, and J. M. Palmer.** 1987. Production and properties of an extracellular peroxidase from *Coriolus versicolor* which catalyzes cleavage in a lignin model compound. FEMS Microbiol. Lett. **42:**17–22.
5. **Eriksson, K.-E., and U. Lindholm.** 1971. Lignets mikrobiella nedbrytning. Sven. Papperstidn. **74:**701–706.
6. **Farrell, R.** September 1987. U.S. patent 4,690,895.
7. **Fujita, K., R. Kondo, K. Sakai, Y. Kashino, T. Nishida, and Y. Takahara.**

1991. Biobleaching of kraft pulp using white-rot fungus IZU-154. TAPPI J. **74:**123–127.
8. **Fujita, K., R. Kondo, K. Sakai, Y. Kashino, T. Nishida, and Y. Takahara.** 1993. Biobleaching of softwood kraft pulp with white-rot fungus IZU-154. TAPPI J. **76:**81–84.
9. **Galliano, H., G. Gas, J. L. Seris, and A. M. Boudet.** 1991. Lignin degradation by *Rigidoporus lignosus* involves synergistic action of two oxidizing enzymes: Mn peroxidases and laccase. Enzyme Microb. Technol. **13:**478–482.
10. **Gold, M. H., M. Kuwahara, A. A. Chiu, and J. K. Glenn.** 1984. Purification and characterization of extracellular H_2O_2-requiring diaryl propane oxygenase from the white rot basidiomycete, *Phanerochaete chrysosporium*. Arch. Biochem. Biophys. **234:**353–362.
11. **Higuchi, T.** 1985. Lignin biochemistry: biosynthesis and biodegradation. Wood Sci. Technol. **24:**23–63.
12. **Johansson, T., and P. O. Nyman.** 1987. A manganese(II)-dependent extracellular peroxidase from the white-rot fungus *Trametes versicolor*. Acta Chem. Scand. Ser. B **41:**762–765.
13. **Keyser, P., T. K. Kirk, and J. G. Zeikus.** 1978. Ligninolytic enzyme system of *Phanerochaete chrysosporium*: synthesized in the absence of lignin in response to nitrogen stavation. J. Bacteriol. **135:**790–797.
14. **Kirk, T. K.** 1971. Effects of microorganisms on lignin. Annu. Rev. Phytopathol. **9:**185–210.
15. **Kirk, T. K., E. Schultz, W. J. Connors, L. F. Lorenz, and J. G. Zeikus.** 1978. Influence of culture parameters on lignin metabolism by *Phanerochaete chrysosporium*. Arch. Microbiol. **117:**277–285.
16. **Kirk, T. K., and H. H. Yang.** 1979. Partial delignification of unbleached kraft pulp with ligninolytic fungi. Biotechnol. Lett. **1:**347–352.
17. **Kirkpatrick, N., I. Reid, E. Ziomek, C. Ho, and M. G. Paice.** 1989. Relationship between fungal biomass production and the brightening of hardwood kraft pulp by *Coriolus versicolor*. Appl. Environ. Microbiol. **55:**1147–1152.
18. **Kondo, R., K. Kurashiki, and K. Sakai.** 1994. In vitro bleaching of hardwood kraft pulp by extracellular enzymes excreted from white rot fungi in a cultivation system using a membrane filter. Appl. Environ. Microbiol. **60:**921–926.
19. **Leatham, G. F.** 1986. The ligninolytic activities of *Lentinus edodes* and *Phanerochaete chrysosporium*. Appl. Microbiol. Biotechnol. **24:**51–58.
20. **Lee, S. W., and G. Edlin.** 1985. Expression of tetracycline resistance in pBR322 derivatives reduces the reproductive fitness of plasmid-containing *Escherichia coli*. Gene **39:**173–180.
21. **Murata, S., R. Kondo, K. Sakai, Y. Kashino, T. Nishida, and Y. Takahara.** 1992. Chlorine-free bleaching process of kraft pulp with the fungus IZU-154. TAPPI J. **75:**91–94.
22. **Nerud, F., Z. Zouchova, and Z. Misurcovaz.** 1991. Ligninolytic properties of different white-rot fungi. Biotechnol. Lett. **13:**657–660.
23. **Nishida, T., Y. Kashino, A. Mimura, and Y. Takahara.** 1988. Lignin biodegradation by wood-rotting fungi. I. Screening of lignin-degrading fungi. Mokuzai Gakkaishi **34:**530–536.
24. **Olsen, W. L., H. P. Gallagher, A. K. Burris, S. S. Bhattacharjee, J. P. Slocomb, and D. M. Dewitt.** December 1989. Canadian patent application 2,019,411.
25. **Paice, M. G., L. Jurasek, C. Ho, R. Bourbonnais, and F. Archibald.** 1989. Direct biological bleaching of hardwood kraft pulp with the fungus *Coriolus versicolor*. TAPPI J. **72:**217–221.
26. **Paice, M. G., I. D. Reid, R. Bourbonnais, F. S. Archibald, and L. Jurasek.** 1993. Manganese peroxidase, produced by *Trametes versicolor* during pulp bleaching, demethylates and delignifies kraft pulp. Appl. Environ. Microbiol. **59:**260–265.
27. **Paszcyn'ski, A., V.-B. Huynh, and R. Crawford.** 1985. Enzymatic activities of an extracellular, manganese-dependent peroxidase from *Phanerochaete chrysosporium*. FEMS Microbiol. Lett. **29:**37–41.
28. **Perez, J., and T. W. Jeffries.** 1990. Mineralization of C-ring-labeled synthetic lignin correlates with the production of lignin peroxidase, not of manganese peroxidase or laccase. Appl. Environ. Microbiol. **56:**1806–1812.
29. **Perez, J., and T. W. Jeffries.** 1992. Roles of manganese and organic acid chelators in regulating lignin degradation and biosynthesis of peroxidases by *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **58:**2402–2409.
30. **Perie, F. H., and M. H. Gold.** 1991. Manganese regulation of manganese peroxidase expression and lignin degradation by the white-rot fungus *Dichomitus squalens*. Appl. Environ. Microbiol. **57:**2240–2245.
31. **Rappe, C., S. Swanson, B. Glas, K. P. Kringstad, F. D. Sousa, L. Johansson, and Z. Abe.** 1989. On the formation of PCDDs and PCDFs in the bleaching of pulp. Pulp Pap. Can. **90:**T273–T278.
32. **Reid, I. D.** 1989. Solid-state fermentation for biological delignification. Enzyme Microb. Technol. **11:**786–803.
33. **Reinhammar, B.** 1984. Laccase, p. 1–35. *In* R. Lontie (ed.), Copper proteins and copper enzymes, vol. 3. CRC Press, Inc., Boca Raton, Fla.
34. **Tien, M., and T. K. Kirk.** 1983. Lignin-degrading enzyme from the hymenomycete *Phanerochaete chrysosporium* Burd. Science **221:**661–663.
35. **Wariishi, H., K. Valli, and M. H. Gold.** 1991. In vitro depolymerization of lignin by manganese peroxidase of *Phanerochaete chrysosporium*. Biochem. Biophys. Res. Commun. **176:**269–276.
36. **Wolfenden, B. S., and R. L. Willson.** 1982. Radical cations as reference chromogens in kinetics studies of one-electron transfer reactions: pulse radiolysis studies of 2,2′-azinobis(3-ethylbenzthiazoline-6-sulphonate). J. Chem. Soc. Perkin Trans. II **1982:**805–812.