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Studies on hypersaline-tolerant white-rot fungi III: biobleaching of unbleached kraft pulp by hypersaline-tolerant manganese peroxidase from a marine white rot isolate, *Phlebia* sp. MG-60

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Abstract A marine white rot isolate, *Phlebia* sp. MG-60, secreted lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase under different sea salt incubation conditions. Its MnP production was strongly enhanced by adding 3% sea salts, and the MnP showed high tolerance to sea salts and NaCl. The crude enzyme secreted at 3% sea salt concentration by *Phlebia* sp. MG-60, in which the main component was MnP (cMnP), was then used to bleach unbleached hardwood kraft pulp (UKP) in vitro. The pulp was brightened 11 points by 4 U of cMnP, and the kappa number was decreased 6 points when only 0.5 mM H_2O_2 was added continuously. When 0.5 mM H_2O_2 (1.22 mg H_2O_2 /g pulp) was added at the initial bleaching, the pulp brightness increased 6 points with a dosage of 4 U of cMnP. When crude MnPs were employed to bleach UKP with organic-free model white-water instead of the Milli Q water usually used, the pulp was brightened 10 and 13 points by 4 and 20 U of cMnP, respectively, and 5 and 6 points by 4 and 20 U of MnP, respectively, of *Phanerochaete chrysosporium*.

Key words White-rot fungus · Manganese peroxidase · Biobleaching · Hypersaline tolerance · Model white water

Introduction

Since Kirk and Young first reported that *Phanerochaete chrysosporium* could partially delignify softwood unbleached pulp,[1] white rot fungi have been widely studied regarding their ability to bleach unbleached kraft pulp (UKP).[2–6] The extracellular ligninolytic enzymes of white rot fungi – lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase – have shown significant advancements as replacements for chemical bleaching agents over the past few decades.

Although Paice et al. reported that UKP could be delignified by the crude or purified MnP from white rot fungi in 1993,[7] the role of the excreted extracellular enzymes during long-term incubation of biobleaching preparations containing white rot fungi was not clear at that time. Kondo et al. used a cultivation system to bleach UKP and found that MnP played an important role in the bleaching process;[8] as a result MnP was widely studied as a bleaching agent for UKP.[9–11]

Mangrove and sea grasses provide a natural habitat for marine fungi, which play an important role as the primary decomposers in marine ecosystems. Researchers have paid attention to their underestimated but important role in the degradation of organic substrates in marine and hypersaline ecosystems.[12–15]

In previous studies we isolated a marine fungus, *Phlebia* sp. MG-60, based on its high decolorization and delignification.[16] The strain and its enzymes were used to degrade sugar cane bagasse.[17] The lignin-modifying enzyme system of the fungus was investigated, and the MnP production was strongly enhanced by added 3% sea salts to the incubation medium (X. Li, R. Kondo, and K. Sakai, unpublished data, 2001). In this article we report that MnP secreted by *Phlebia* sp. MG-60 incubated at 3% sea salt concentration was used to bleach UKP in vitro.

Owing to environmental and economic concerns white-water recycling systems have been adopted by pulp and paper mills, even though their use has caused many problems, such as the buildup of inorganic ions, a decrease in pH, and increased temperature during the papermaking process.[18] The high tolerance of the MnP secreted by *Phlebia* sp. MG-60 to sea salts at 3% sea salt incubation condition caused us to apply it to bleach UKP with the recycled white-water. We therefore monitored organic-free model white-water and then used it as a medium in which UKP was bleached with the crude MnP secreted by *Phlebia* sp. MG-60 under 3% sea salt condition. The MnP from

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P. chrysosporium was also used to bleach the pulp for comparison.

Materials and methods

Materials

Unbleached hardwood kraft pulp was obtained from a pulp and paper mill in Japan. It was air-dried once.

Phlebia sp. MG-60[16] and *P. chrysosporium* ME-446[19] were maintained on potato dextrose agar (Difco Laboratories) slants at 4°C before use.

Methods

Preparation of crude enzyme. A liquid culture medium was monitored for enzyme production; it contained glucose 10 g/l, ammonium tartrate 0.221 g/l, sodium acetate 20 mM, Tween 80 1.0 g/l, Kirk's trace elements,[20] and sea salts 3% (w/v) (Sigma). The pH was adjusted to 4.5. *Phlebia* sp. MG-60 was incubated on potato dextrose agar in petri plates (diameter 9 cm) at 30°C for 7 days. One quarter of each plate was homogenized in the liquid medium using a Waring blender and then inoculated into a total volume of 200 ml of the liquid medium in 500-ml Erlenmeyer flasks. After 10 days of incubation at 30°C and 150 rpm, the supernatant was filtered with glass fiber and 0.45-μm membrane filters (Toyo Roshi Kaisha, Tokyo, Japan) and was used as crude MnP.

Enzyme assays. MnP activity was determined by monitoring the oxidation of 2,6-dimethoxyphenol at 470 nm and was expressed in international units.[21] Enzyme was added to a solution containing 1.0 mM 2,6-dimethoxyphenol and 1.0 mM $MnSO_4$ in 50 mM malonate buffer (pH 4.5). The reaction was initiated with hydrogen peroxide (0.2 mM) in a final volume of 1 ml.

Pulp treatments. Air-dried UKP was suspended at a consistency of 1% in 50 mM malonate buffer (pH 4.5), in which was contained 0.1 mM $MnSO_4$ and 0.05% Tween 80. The pulp slurry was maintained at 37°C, and proper MnP solution was added. The enzymatic reaction was initiated by the addition of hydrogen peroxide. As a control, UKP was treated in the meantime without the MnP addition.

Effect of H_2O_2 on biobleaching in vitro. Hydrogen peroxide at various concentrations was added continuously to the biobleaching system at a flow rate of 3 ml/h with a peristaltic pump, or it was added once at the start of the biobleaching (0.5 mM). The pulp properties were then evaluated.

Biobleaching with organic-free model white-water. The organic-free model white-water was prepared with the composition shown in Table 1.[21] Instead of Milli-Q water, we used the model white-water to prepare 50 mM malonate buffer and to test the biobleaching process. The pulp was biobleached as described above.

Table 1. Composition of organic-free model white water

Agent	Content (mg/l)
$MgSO_4$	125.0
CaO	182.0
NaCl	75.4
$Al_2(SO_4)_3$	146.6
$FeSO_4$	217.1
SO_4^{2-}	350.0

Pulp properties. The treated pulp was filtered, washed with distilled water, homogenized for 30 s in a Waring blender, and then made into handsheets with a Buchner funnel. After being air-dried on a blotter, each handsheet was tested for brightness and its kappa number. Brightness was determined by measuring the reflectance and then multiplying the reflectance by a coefficient to International Standards Organisation (ISO) brightness. The kappa number was determined following the standard method (ISO302–1981).

Results

Effect of crude enzyme on bleaching in vitro

The main component of the crude enzyme preparation was MnP (Li et al., unpublished observations, 2001), so we used the prepared crude enzyme from the 3% sea salt incubation condition (represented as cMnP) to bleach UKP and calculated the dosage of the crude enzyme by assessing MnP activity. The pulp brightness increased more than 10 points compared with the control when 0.5 mM H_2O_2 was continuously added at the rate of 3 ml/h for 24 h to the pulp suspension containing 4 U of cMnP; the brightness increase was not obviously changed by adding more than 4 U of cMnP (Fig. 1). The kappa number of the treated pulp decreased as the brightness increased.

With the addition of 4 U of cMnP, the pulp brightness increased along with the incubation time (Fig. 2). During the first 3 h the pulp brightness sharply increased 7 points, but the increase slowed during the incubation period that followed. We suggest that the biobleaching process with the crude enzyme can be accomplished within a few hours.

Effect of H_2O_2 on biobleaching in vitro

It was reported previously that MnP required a source of H_2O_2 but that MnP activity was inhibited by a high concentration of H_2O_2. To test the effect of the H_2O_2 concentration on the enzyme treatment, various concentrations of H_2O_2 were continuously pumped into the bleaching system containing 4 U of cMnP. The pulp was brightened to the maximum, an 11-point increase, when 0.5 mM H_2O_2 was added (Fig. 3).

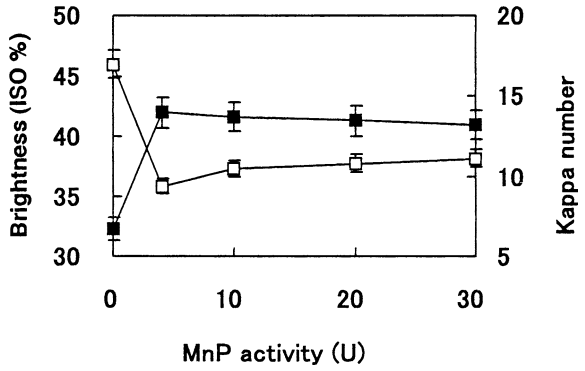

Fig. 1. Effect of crude manganese peroxide (cMnP) from *Phlebia* sp. MG-60 on bleaching of unbleached kraft pulp (UKP) at 37°C for 24 h. *Filled squares*, brightness; *open squares*, kappa number. $n = 3$. *ISO*, International Standards Organization

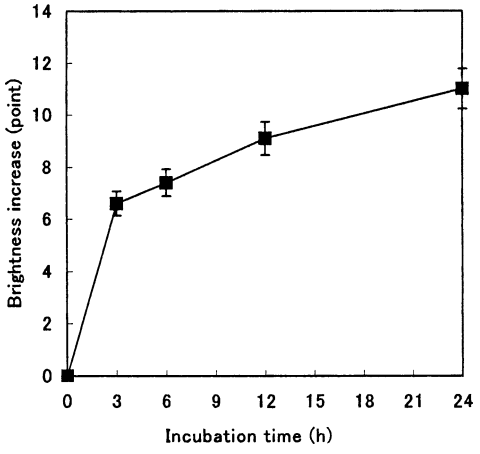

Fig. 2. Time course of pulp brightness increase with treatment with 4 U of cMnP from *Phlebia* sp. MG-60. $n = 3$

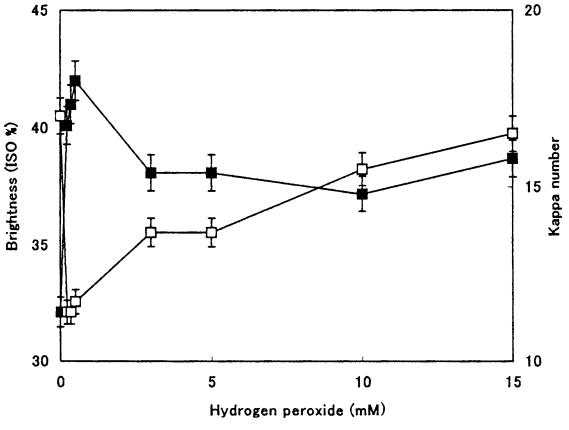

Fig. 3. Effect of continuously added hydrogen peroxide concentration on bleaching of UKP with 4 U of cMnP from *Phlebia* sp. MG-60 at 37°C for 24 h. *Filled squares*, brightness; *open squares*, kappa number. $n = 3$

To investigate the effect of MnP on pulp brightness when 0.5 mM H_2O_2 (1.22 mg H_2O_2/g pulp) was added just once at the beginning of biobleaching, 4, 10, and 20 U of cMnP were employed to bleach the UKP. The results in Fig. 4 show that

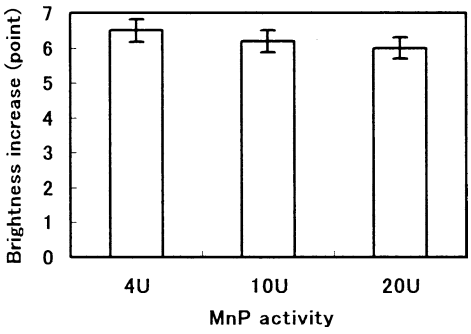

Fig. 4. cMnP from *Phlebia* sp. MG-60 caused increased brightness when hydrogen peroxide was added during the initial period of biobleaching. Treatment time 3 h. $n = 3$

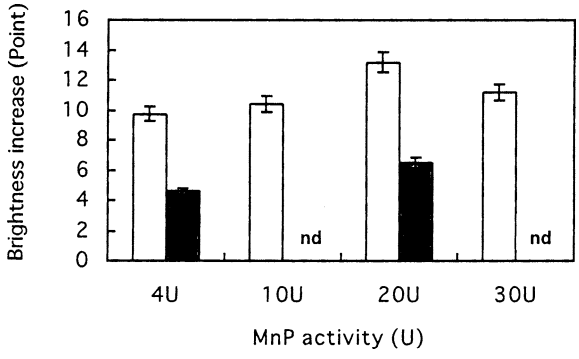

Fig. 5. Brightness increases of UKP bleached with the organic-free model white water by cMnP (*open bars*) from *Phlebia* sp. MG-60 and MnP (*filled bars*) from *P. chrysosporium* at 37°C for 24 h. $n = 3$. *nd*, not determined

4 U of cMnP could increase the pulp brightness about 7 points during the first 3 h. An increase in MnP dosage did not improve the pulp brightness. At the same dosage of 4 U of MnP, the same brightness increase of the bleached pulp was achieved with either continuous or one-time addition of H_2O_2 during the first 3 h of incubation.

Biobleaching with the organic-free model white-water

The MnP secreted by *Phlebia* sp. MG-60 that was incubated at different sea salt concentrations had a certain tolerance to sea salts (Li et al., unpublished observations, 2001), which suggests that the MnPs can maintain their activities in a proper salt environment. White-water contains a variety of soluble salts and organic materials. To investigate the possibility of white-water recycling during the biobleaching process, an organic-free model white-water was prepared (Table 1) and used in the bleaching system. The results of biobleaching with the organic-free model white-water are shown in Fig. 5. The pulp brightness was increased about 13 points, compared with the control, when 20 U of cMnP was used. When the MnP from *P. chrysosporium* was used in the bleaching system, there was only 5 and 6 points of increase in pulp brightness with 4 and 20 U MnP additions, respectively.

Discussion

A strong positive correlation between the MnP level and the pulp brightness increase has been verified.[7,21–23] More than 20 U of MnP from *Phanerochaete sordida* YK-624 brightened UKP 10 points,[9] whereas only 4 U of cMnP from *Phlebia* sp. MG-60 increased the pulp brightness about 11 points. Although the brightness of the treated UKP increases with an increasing amount of MnP during bleaching of UKP with MnP, it is also strongly affected by the concentration of Mn^{2+} and hydrogen peroxide. The effect of the Mn^{2+} concentration on the brightness increase was also investigated, and the results are shown in Table 2. The brightness increase of treated UKP with 4 U cMnP was higher than that with 20 U of cMnP from *Phlebia* sp. MG-60 under various Mn^{2+} concentrations. The results pointed out that brightness increase of treated UKP was inhibited by high amounts of MnP from *Phlebia* sp. MG-60, and it seems that part of the Mn^{3+} malonate produced by the MnP reaction might be disproportionate to Mn^{4+} (MnO_2) and Mn^{2+}, and MnO_2 may affect the brightness of the treated pulp, moreover the radical of residual lignin produced by the MnP reaction may be combined under high amount of MnP conditions.[21]

Manganese peroxidase requires H_2O_2 as a co-substrate and catalyst for oxidizing Mn^{2+} to Mn^{3+}, but the sensitivity of the enzyme to the H_2O_2 concentration is still a problem. Hydrogen peroxide can be generated from glucose and glucose oxidase during the fungal bleaching process, but it must be determined how and how much H_2O_2 should be added to the in vitro biobleaching system. With continuous addition of 10 mM H_2O_2, 20 U of MnP secreted by *Phanerochaete sordida* YK-624 was reported to brighten UKP 10 points and to decrease the kappa number about 6 points.[21] Surprisingly, the pulp was brightened by MnP-3 from *Phlebia* sp. MG-60 more than 10 points with only 0.5 mM of H_2O_2 added throughout the biobleaching process in the present study. This illustrates that only 0.5 mM H_2O_2 was required when MnP-3 from *Phlebia* sp. MG-60 was used to bleach UKP.

The bleaching process would be more convenient and simpler if H_2O_2 could be added at one time to the biobleaching system in vitro. Some experiments have dealt with this aspect of biobleaching, but researchers reported that MnP was inactivated by concentrations above 0.1 mM; and even when the H_2O_2 concentration was kept below 0.1 mM, the MnP became relatively inactive.[24] Surprisingly, when 0.5 mM H_2O_2 was added to the enzyme bleaching system at the start of the biobleaching in our study, the brightness increased about 6 points during the first 3 h. This increase is comparable to the results obtained with continual addition of H_2O_2. We therefore inferred that the MnP secreted by *Phlebia* sp. MG-60 had good stability in the presence of H_2O_2. Despite the 6-point increase in pulp brightness during the first 3 h, the rate of increase became slower and slower during the period that followed. Moreover, the brightness with the one-time addition of H_2O_2 did not reach the level obtained with the continuous addition of the same amount of H_2O_2. These findings imply that the MnP activity may be partly inhibited by the one-time addition of H_2O_2. Another possibility is that MnP reacted with the high concentration of H_2O_2 before it penetrated the pulp fiber.

Table 2. Effect of Mn^{2+} concentration on brightness increase of UKP treated by MnP from *Phlebia* sp. MG-60

$MnSO_4$ (mM)	Brightness increase (points)	
	4 U	20 U
0.1	11.0	9.0
0.2	9.2	9.0
0.5	9.0	8.7

Biobleaching in vivo requires more time than that in vitro. The enzyme treatment of pulp would be less costly and more advantageous if it could be finished in a short time. Treatment of pulp with MnP usually takes as long as 24 h, so the biobleaching process has not been commercialized in the pulp and paper industry. Regardless of whether 0.5 mM H_2O_2 was added once or continuously, the pulp was brightened about 6 points by cMnP produced by *Phlebia* sp. MG-60 during the first 3 h. When 0.5 mM H_2O_2 was added continuously for 3 h, only about 0.15 mg H_2O_2/g pulp was added in the MnP reaction system, whereas 1.22 mg H_2O_2/g pulp was added in the MnP reaction when H_2O_2 was added only once. The results indicated that cMnP had high tolerance to H_2O_2, which will be further studied in our laboratory. The 3-h treatment and its corresponding achievement would probably be found acceptable by the pulp and paper industry.

Environmental and economic factors are forcing pulp and paper mills to adopt closed-water systems. Although the white-water is cleaned before it is recycled and used in the pulp and paper process, most of the dissolved chemicals remain in the white-water system, which causes problems during the papermaking process, such as reduced drainage rates, corrosion, and increased deposits.[25] To decrease the negative effects, biological treatments are being developed and have shown great potential in recent years. When the fungal culture filtrate produced by *Trametes versicolor* was used to treat both model white-water and mill white-water, both showed a significant decrease in total dissolved and colloidal substances.[26,27] Until now there has been no report on biobleaching with white-water as a medium. In our research, the organic-free model white-water, used instead of the Milli-Q water normally used in laboratory-scale biobleaching, was used to bleach UKP by cMnP from *Phlebia* sp. MG-60, and remarkable results were obtained (Fig. 5). When 0.5 mM H_2O_2 was added continually, 4 U of cMnP could brighten UKP about 10 points and 20 U of cMnP could brighten UKP about 13 points; in contrast, the MnP of *P. chrysosporium* could brighten UKP only about 5 and 6 points under the same conditions. These results indicated that the cMnP had better bleaching ability when Milli-Q water was replaced by the organic-free model white-water; when cMnP was used to bleach UKP, the MnP reaction was enhanced by salts in the organic-free model

white-water. Our further studies demonstrated that the MnP activity of cMnP can be enhanced by salts (Li et al. unpublished observations, 2001), and that cMnP has some special characteristics and can be used under salt conditions. The present investigation indicates that cMnP from *Phlebia* sp. MG-60 has great potential for use in white-water recycling during the biobleaching process.

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