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ORIGINAL ARTICLE

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Studies on hypersaline-tolerant white-rot fungi IV: effects of Mn^{2+} and NH_4^+ on manganese peroxidase production and Poly R-478 decolorization by the marine isolate *Phlebia* sp. MG-60 under saline conditions

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Abstract Manganese peroxidase (MnP) production by *Phlebia* sp. MG-60 is strongly regulated by Mn^{2+} and NH_4^+ at various sea salt incubation conditions. Extra-added Mn^{2+} and NH_4^+ obviously inhibited MnP production, but sea salts relieved the inhibition partially or completely. Three media were prepared: Kirk medium with addition of 0%–5% sea salts (KS medium), a high level of Mn^{2+} (300 mg/l) in KS medium (HM-KS medium), and a high level of NH_4^+ (430 mg/l) in KS medium (HN-KS medium). Without addition of sea salts, the dye Poly R-478 was significantly decolorized by low MnP activity (about 200 U/l) and a low level of laccase activity (about 100 U/l) in KS and HM-KS media. In the cultures in which laccase activity was almost completely inhibited by 3% and 5% sea salts, MnP activity higher than 400 U/l was necessary for Poly R-478 decolorization in all of the three media. We first report the linear correlation of MnP activity and decolorization of Poly R-478 under saline conditions and the effect of laccase on this relation.

Key words White-rot fungus · MnP production · Poly R-478 · Decolorization · Sea salts

Introduction

White-rot fungi are regarded as the microorganisms responsible for biodegradation of lignin, the second most abundant natural polymer on earth. The ligninolytic enzymes lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase are all or partly secreted by white rot fungi and regulated by culture conditions.[1–5] Several important culture parameters (e.g., carbon source, nutrient nitrogen concentration, ion strength, oxygen, and pH) have been widely investigated to understand the factors that affect ligninolytic enzyme production and lignin degradation.[6–8]

In previous reports we observed that a white rot marine isolate, *Phlebia* sp. MG-60, showed high delignification and dye decolorization ability,[9,10] and its MnP production was strongly enhanced by sea salts.[11] Until now, our understanding of the MnP secreted by saline-tolerant white rot fungi under marine incubation conditions has been limited. To increase our knowledge of the factors that influence MnP production by the salt-tolerant white rot marine isolate *Phlebia* sp. MG-60, we investigated the effects of Mn^{2+} and NH_4^+ on its MnP production and Poly R-478 decolorization under various sea salt incubation conditions. The dependence of Poly R-478 decolorization on ligninolytic enzymes at various incubation conditions was also studied, and the results are summarized in this paper.

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Materials and methods

Microorganism

The white-rot marine isolate *Phlebia* sp. MG-60[9] was maintained in potato-dextrose agar (PDA) (Difco Laboratories) slants at 4°C before being used.

Medium preparations

A liquid medium that contained glucose 10.00 g/l, ammonium tartrate 0.221 g/l, sodium acetate 1.64 g/l, Tween 80 1.00 g/l, Kirk's salt solution, and trace elements[12] was used as the basal medium in the present research. All factors that affect MnP production and decolorization of Poly R-478 were investigated at various salt concentrations: 0%, 3%, or 5% (w/v) sea salts (Sigma). The medium containing

different concentrations of sea salts is called KS medium. To determine the effect of Mn^{2+} on MnP production and decolorization, the Mn^{2+} level in KS medium (60 mg/l) was adjusted to 300 mg/l (HM-KS) by adding appropriate amounts of $MnSO_4 \cdot H_2O$, and the pH was kept at 4.5. To determine the effect of NH_4^+ on MnP production and decolorization, the low concentration of nitrogen in the KS medium[11] was replaced by a high concentration of nitrogen (HN-KS); the NH_4^+ concentration was 430 mg/l.

Culture conditions and crude enzyme preparations

The strain *Phlebia* sp. MG-60 was preincubated on PDA in petri dishes (diameter 9 cm) at 30°C for 7 days. The preincubated strain was then inoculated into a 100-ml Erlenmeyer flask containing 10 ml of KS, HM-KS, or HN-KS medium. After stationary incubation at 30°C, the culture was separated by filtration with glass fiber paper and 0.45 μm membrane filters (Toyo Roshi Kaisha, Japan), and the supernatant was used as crude enzymes.

Decolorization of Poly R-478

The ability of *Phlebia* sp. MG-60 to degrade Poly R-478 was estimated in KS, HM-KS, and HN-KS media, respectively. KS, HM-KS, and HN-KS media (10 ml) containing 0.02% Poly R-478 was sterilized, inoculated, and then incubated at 30°C without shaking. After incubation, the culture was filtered with glass fiber paper and 0.45-μm membrane filters. The supernatant was used to determine absorbance at 513 nm and 362 nm for decolorization ability[13] and enzyme activity. Noninoculated medium that contained 0.02% Poly R-478 was used as the control. The colors of the mycelia incubated under the various incubation conditions were compared with that of the control. No obvious changes were observed. Therefore, Poly R-478 was not adsorbed by the mycelium. The decolorization ability was expressed as follows.

$$\text{Decolorization}(\%) = \left[(A_0 - A)/A_0\right] \times 100\%$$

$$A_0 = \left[\lambda_{513}/\lambda_{362}\right]_{\text{control}}$$

$$A = \left[\lambda_{513}/\lambda_{362}\right]_{\text{sample}}$$

Enzyme assays

The MnP activity was determined by monitoring the oxidation of 2,6-dimethoxyphenol at 470 nm.[14] Crude 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), and the reaction was initiated with hydrogen peroxide (0.2 mM) in a final volume of 1 ml. Laccase activity was measured by monitoring the oxidation of 2,6-dimethoxyphenol at 470 nm in 0.1 M phosphate buffer (pH 6.0).[14] One unit of enzyme activity is defined as 1 μmol quinone dimer of 2,6-DMP produced in 1 min ($\varepsilon = 49600\,\text{m}^{-1}\,\text{cm}^{-1}$).

Results

Effect of Mn^{2+} on MnP production

The MnP production in KS medium at various sea salt cultures was described in our previous report[11] and is shown here in Fig. 1 to compare the MnP activities at various Mn^{2+} and NH_4^+ conditions. Figure 2 shows the effect of Mn^{2+} on MnP production. The peak activity of MnP produced at 0% sea salts in HM-KS medium occurred around the same time (day 10 of incubation) as that in the KS medium, and the activity was only one-third of that in the KS medium. That is, the high Mn^{2+} concentration (300 mg/l) inhibited MnP production at the 0% sea salt incubation condition, but it did not obviously affect MnP secretion in HM-KS media at 3% sea salts. More interestingly, the MnP activity in HM-KS was enhanced about fourfold by 5% sea salts compared with that in KS medium at the same salt condition.

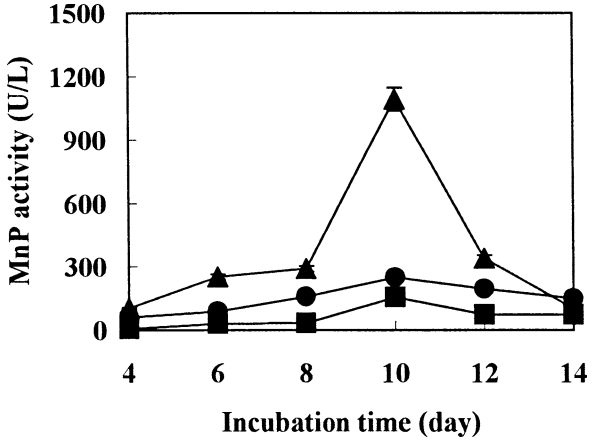

Fig. 1. Production of manganese peroxidases (MnPs) by *Phlebia* sp. MG-60 at 0% (*circles*), 3% (*triangles*), and 5% (*squares*) sea salt concentrations under stationary incubation conditions ($n = 4$)

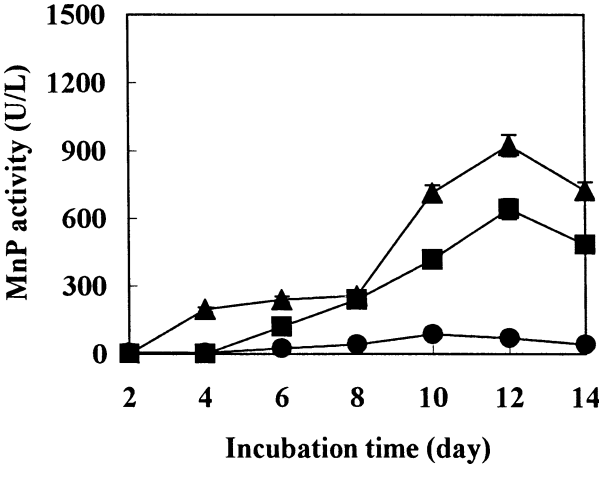

Fig. 2. MnP activity of *Phlebia* sp. MG-60 incubated in Kirk medium with added Mn^{2+} (300 mg/l) (HM-KS) medium with addition of 0% (*circles*), 3% (*triangles*), and 5% (*squares*) sea salts ($n = 3$)

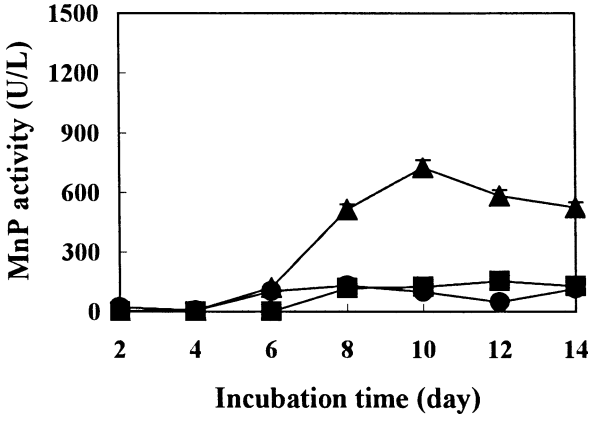

Fig. 3. MnP production by *Phlebia* sp. MG-60 in Kirk medium with added NH_4^+ (430 mg/l) (HN-KS) medium at 0% (*circles*), 3% (*triangles*), and 5% (*squares*) sea salt conditions ($n = 3$)

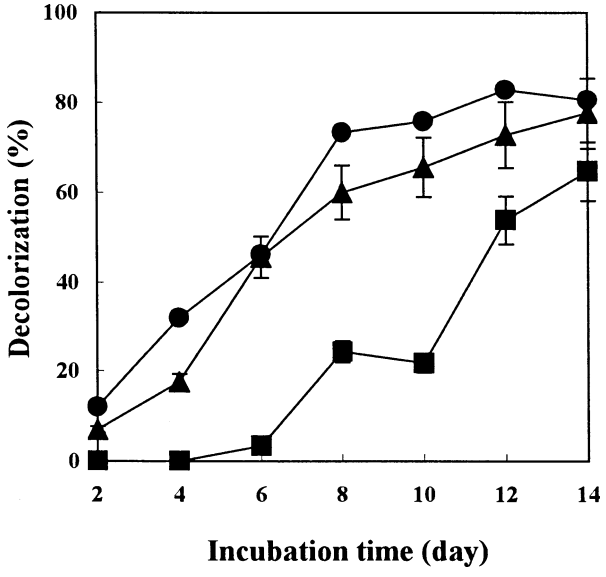

Fig. 4. Decolorization of Poly R-478 by *Phlebia* sp. MG-60 in KS medium at 0% (*circles*), 3% (*triangles*), and 5% (*squares*) sea salt conditions ($n = 3$)

Effect of NH_4^+ on MnP production

MnP production in HN-KS medium with or without addition of sea salts is illustrated in Fig. 3. With or without the addition of sea salts to the culture, MnP production was inhibited by the higher NH_4^+ concentration in HN-KS medium than in KS medium. Among the three sea salt conditions, MnP production was least inhibited at 5%.

Effect of sea salts on Poly R-478 decolorization

Decolorization of Poly R-478 by *Phlebia* sp. MG-60 in KS medium is shown in Fig. 4. The results demonstrated that decolorization of Poly R-478 was obviously inhibited by high concentrations of sea salts at the beginning of the incubation, although it was increased to an almost comparable degree at 2 weeks after inoculation.

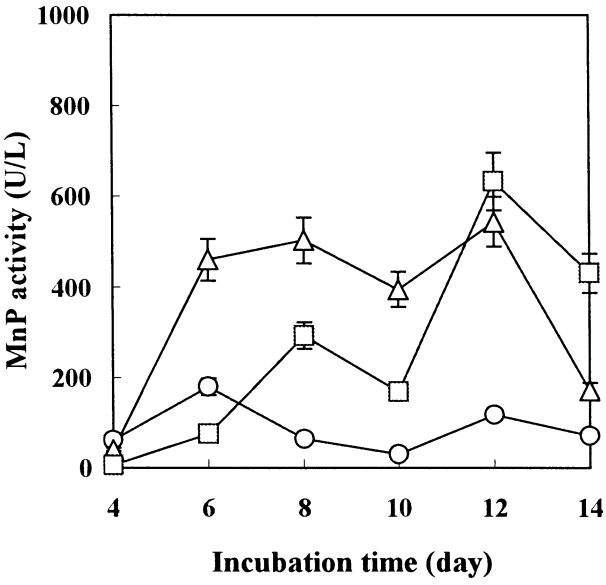

Fig. 5. MnP production by *Phlebia* sp. MG-60 during decolorization of Poly R-478 in KS medium at 0% (*circles*), 3% (*triangles*), and 5% (*squares*) sea salt conditions ($n = 3$)

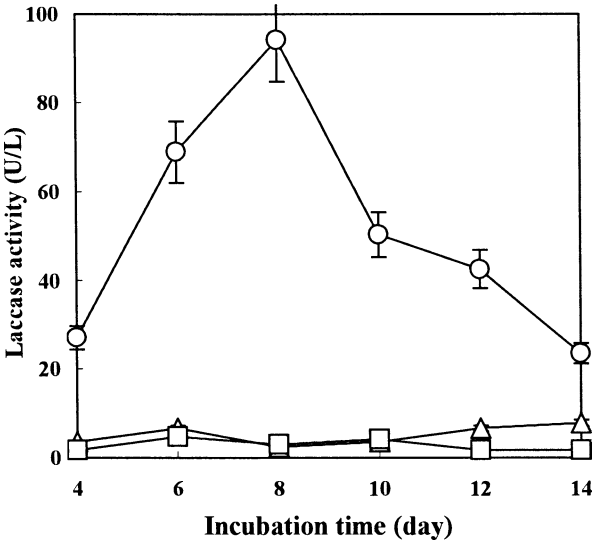

Fig. 6. Laccase production by *Phlebia* sp. MG-60 during decolorization of Poly R-478 in KS medium at 0% (*circles*), 3% (*triangles*), and 5% (*squares*) sea salt conditions ($n = 3$)

The lignin-modifying enzyme activities were determined during decolorization in KS medium (Figs. 5, 6). Sea salts at both 3% and 5% concentrations enhanced MnP production when the fungus was used to decolorize Poly R-478 in KS medium. At 3% or 5% sea salt concentrations, laccase activity was strongly inhibited during decolorization of Poly R-478 (Fig. 6).

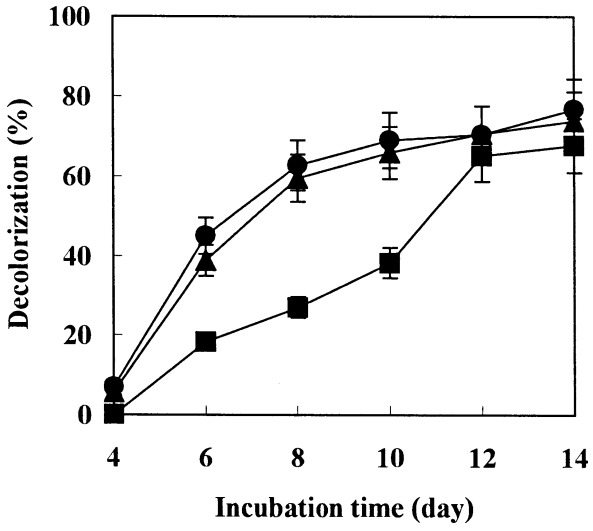

Fig. 7. Decolorization of Poly R-478 by *Phlebia* sp. MG-60 in HM-KS medium at 0% (*circles*), 3% (*triangles*), and 5% (*squares*) sea salt conditions ($n = 3$)

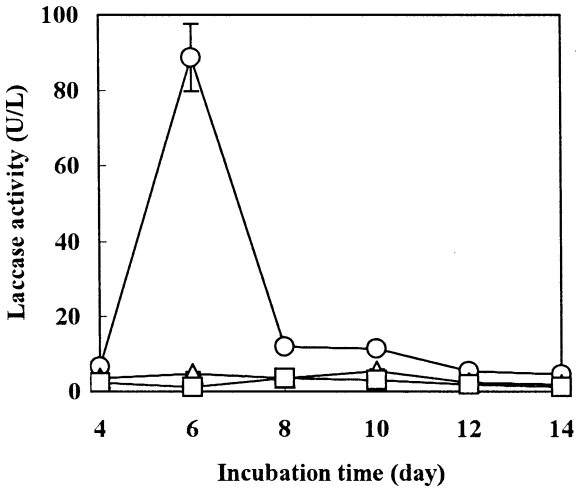

Fig. 9. Laccase production by *Phlebia* sp. MG-60 during decolorization of Poly R-478 in HM-KS medium at 0% (*circles*), 3% (*triangles*), and 5% (*squares*) sea salt conditions ($n = 3$)

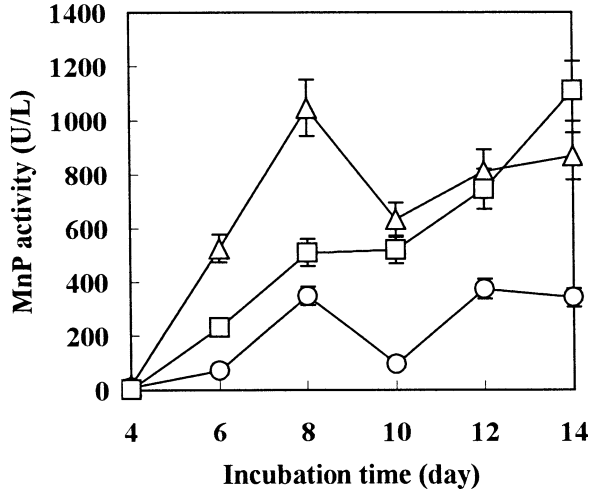

Fig. 8. MnP production by *Phlebia* sp. MG-60 during decolorization of Poly R-478 in HM-KS medium at 0% (*circles*), 3% (*triangles*), and 5% (*squares*) sea salt conditions ($n = 3$)

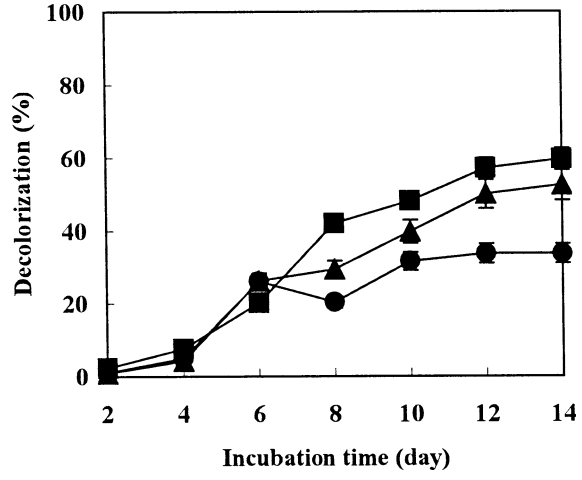

Fig. 10. Decolorization of Poly R-478 by *Phlebia* sp. MG-60 in HN-KS medium at 0% (*circles*), 3% (*triangles*), and 5% (*squares*) sea salt conditions ($n = 3$)

Effect of Mn^{2+} on Poly R-478 decolorization

The effect of Mn^{2+} on Poly R-478 decolorization can be observed by comparing Fig. 7 with Fig. 4. When 3% sea salts was or was not added to the culture, higher Mn^{2+} inhibited decolorization of Poly R-478 slightly more in HM-KS medium than that in KS medium. In contrast, Mn^{2+} enhanced the decolorization process at the beginning of incubation under the 5% sea salt condition.

The MnP and laccase activities were examined to determine if there is a relation between ligninolytic enzymes and decolorization of Poly R-478. The results are shown in Figs. 8 and 9. Similar to that in KS medium, sea salts enhanced MnP production in HM-KS medium during the decolorization process. At 3% and 5% sea salt concentrations, Mn^{2+} contents in either KS or HM-KS medium did not have any effect on laccase activity. However, the cultivation day of the peak laccase activity was different for the KS and HM-KS media under the 0% sea salt incubation condition, although their maximum activity was not obviously different.

Effect of NH_4^+ on Poly R-478 decolorization

The effect of NH_4^+ concentration on Poly R-478 decolorization can be investigated by comparing Fig. 10 with Fig. 4. In HN-KS medium decolorization was inhibited strongly at 0% sea salts, inhibited at 3% sea salts, but accelerated at 5% sea salts.

The time courses of MnP activity of MG-60 during decolorization in HN-KS medium are shown in Fig. 11. Compar-

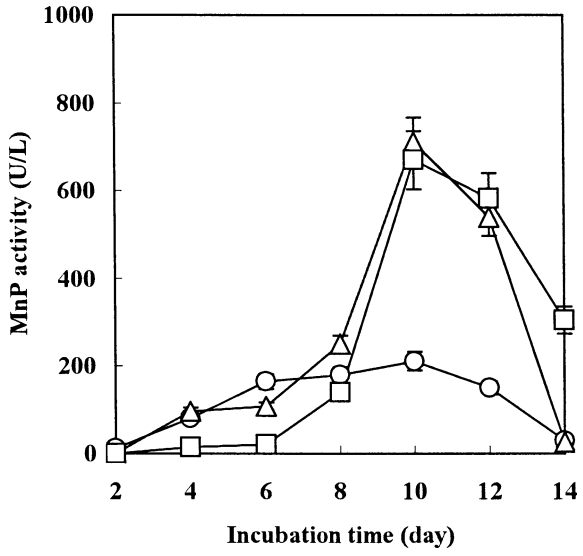

Fig. 11. MnP activity of *Phlebia* sp. MG-60 during decolorization of Poly R-478 in HN-KS medium at 0% (*circles*), 3% (*triangles*), and 5% (*squares*) sea salt conditions ($n = 3$)

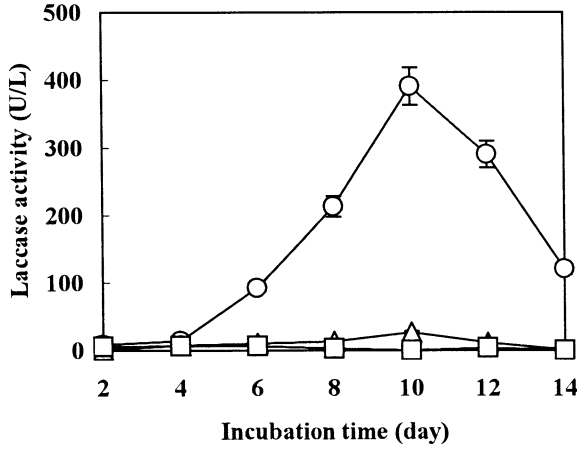

Fig. 12. Laccase production by *Phlebia* sp. MG-60 during decolorization of Poly R-478 in HN-KS medium at 0% (*circles*), 3% (*triangles*), and 5% (*squares*) sea salt conditions ($n = 3$)

ing Fig. 11 with Fig. 5 shows that the maximum MnP activity did not obviously change in KS or HN-KS media at 0% sea salts. In contrast, maximum MnP activity occurred earlier (day 10) in HN-KS medium than in KS medium (day 12) (Fig. 5) with 5% sea salt concentration. Laccase production was enhanced about fivefold in HN-KS medium compared to that in KS medium under the 0% sea salt condition (Fig. 12).

Relations between cumulate MnP activity and decolorization

The relations between the cumulate MnP activity and decolorization of Poly R-478 in KS, HM-KS, and HN-KS media are shown in Table 1. Regardless of the medium, laccase activity was totally inhibited by 5% sea salts (Figs. 6, 9, 12), and the decolorization of Poly R-478 had good linear correlation with the cumulate MnP activity at this sea salt condition. Laccase activity was not totally inhibited at 3% sea salt conditions (Figs. 6, 9, 12), and the linear correlation between MnP activity and decolorization was not as good as that at the 5% sea salt condition but better than that at 0% sea salt concentration. The fungus secreted laccase in the three media at 0% sea salts (Figs. 6, 9, 12), and the laccase seemed to affect greatly the linear relation between the cumulate MnP activity and decolorization of Poly R-478.

Table 1. The r^2 of linear regression of the equation $Y = aX + b$[a]

Medium	r^2		
	0% Sea salts	3% Sea salts	5% Sea salts
KS	0.84	0.91	0.97
HM-KS	0.65	0.79	0.90
HN-KS	0.86	0.86	0.91

KS, Kirk medium with 0%–5% sea salts; HM-KS, KS medium with high-level Mn^{2+} (300 mg/l); HN-KS, KS medium with high-level NH_4^+ (430 mg/l)

[a] X and Y are the cumulate MnP activity (in units) and Poly R-478 decolorization (%), respectively ($n = 3$)

Discussion

It has been demonstrated that MnP production could be enhanced by adding Mn^{2+} to the medium,[5,15] although it appeared to be inhibited by an Mn^{2+} concentration higher than 40 ppm.[3] In the present study, the added Mn^{2+} (240 ppm) in HM-KS medium obviously inhibited MnP production by *Phlebia* sp. MG-60 without the addition of sea salts, but the inhibition was almost completely relieved with 3% sea salts. Moreover, MnP production was improved by Mn^{2+} at a 5% sea salt concentration in the HM-KS medium. The results indicated that a high Mn^{2+} concentration inhibits MnP production by *Phlebia* sp. MG-60, but sea salts can relieve this inhibition.

Although many reports demonstrated that most white rot fungi secrete more MnP at a low nitrogen concentration than that at a high nitrogen concentration, MnP production by some white rot fungi was higher in nitrogen-rich culture than in nitrogen-limited culture.[5,7,16] Our results indicated that NH_4^+ had a different influence on MnP production by *Phlebia* sp. MG-60 under different sea salt concentrations. As the sea salts relieved the inhibition caused by Mn^{2+} on MnP production by *Phlebia* sp. MG-60, they also reduced the inhibitory effect of NH_4^+ on MnP production. The added Mn^{2+} and NH_4^+ inhibited MnP production by *Phlebia* sp. MG-60 in KS medium, but the inhibition was partially or completely relieved by the presence of sea salts, especially 5% sea salts. This interesting phenomenon will be further investigated in our laboratory.

Decolorization of polymeric dyes by microorganisms is regarded as a simple, rapid method to investigate their ligninolytic system.[17] To investigate the effects of Mn^{2+} and

NH_4^+ on lignin degradation by *Phlebia* sp. MG-60, Poly R-478 was added to the incubation cultures and the decolorization ability of the fungus was evaluated under various incubation conditions.

The effects of Mn^{2+} on decolorization of Poly R-478 were different at 0%, 3%, and 5% sea salt concentrations. Without addition of sea salts to the system, a high Mn^{2+} concentration in HM-KS inhibited the decolorization ability of the fungus. After adding 5% sea salts, however, the decolorization ability improved, although it was still lower than that at 0% and 3% sea salt concentrations. Based on these results, it can be concluded that sea salts and Mn^{2+} have certain coordinated effects on the fungal decolorization of Poly R-478. The effect of NH_4^+ on decolorization of Poly R-478 at different sea salt concentrations gave results similar to those seen with Mn^{2+}; that is, sea salts at 5% released the inhibition of NH_4^+ on decolorization.

It has been reported that a minimum MnP concentration of about 200 U/l was needed to start Poly R-478 decolorization, and higher amounts of MnP did not improve the decolorization levels.[17] In our study, however, MnP activity higher than 400 U/l was necessary for Poly R-478 decolorization in all three media when laccase activity was almost completely inhibited by 3% and 5% sea salts. When laccase (about 100 U/l) was produced in KS and HM-KS media at 0% sea salts, an MnP concentration lower than 200 U/l also efficiently decolorized Poly R-478. The relation between the cumulate MnP activity and decolorization of Poly R-478 at various sea salt concentrations in three media are showed in Table 1. The decolorization of Poly R-478 had the best linear correlation (r^2) with cumulate MnP activity under 5% sea salt conditions in the three media. The linear correlation between the cumulate MnP activity and Poly R-478 decolorization was not as good under the 0% sea salt incubation condition as that at 5% sea salts because it was strongly affected by laccase. We therefore conclude that MnP plays the most important role in Poly R-478 decolorization, but a certain amount of laccase activity is necessary.

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