Phenol-Oxidizing Enzyme Expression in *Lentinula edodes* by the Addition of Sawdust Extract, Aromatic Compounds, or Copper in Liquid Culture Media

EIJI TANESAKA*, HIRONORI TAKEDA† AND MOTONOBU YOSHIDA

Faculty of Agriculture, Kinki University, 3327-204 Nakamachi, Nara 631-8505, Japan
†Present address: Yukiguni Maitake Co., Ltd., 89 Yokawa, Minami-Uonuma City, Niigata 949-6695, Japan

Received 31 January, 2013/Accepted 13 March, 2013

This study examined how the addition of a sawdust extract from *Castanopsis cuspidata*, several aromatic compounds, and copper affected the expression of a phenol-oxidizing enzyme in the white-rot basidiomycete, *Lentinula edodes*. Compared to liquid media that had not been supplemented with sawdust extract (MYPG), MYPG containing low (MYPG-S100) or high (MYPG-S500) concentrations of sawdust extract had a marked effect on the promotion of mycelial growth. No manganese peroxidase (MnP) production was observed in either MYPG or MYPG-S100 media until 35 days after inoculation. However, MnP production was enhanced by culture in MYPG-S500, with a marked increase observed suddenly at 14 days after inoculation. Northern blot analysis revealed that the transcription of the *lemnp2* gene coding extracellular MnP was initially observed at detectable levels at day 10 after the initial inoculation of MYPG-S500, increasing gradually thereafter until days 22-25. However, laccase (Lcc) production was not observed in any of the media until 35 days after inoculation. Addition of 10 mM aromatic compounds – 1,2-benzenediol, 2-methoxyphenol, hydroquinone, and 4-anisidine – into the MYPG-S500 medium completely inhibited MnP production and did not enhance any Lcc production. While the addition of 1 or 2 mM Cu²⁺ (CuSO₄·5H₂O) to MYPG-S500 medium completely inhibited MnP production, this Cu²⁺ addition caused a marked increase in Lcc production at 17 and 6 days after the addition, respectively.

Key words : Copper / Laccase / Manganese peroxidase / Sawdust extract / Shiitake.

INTRODUCTION

The shiitake mushroom, *Lentinula edodes* (Berk.) Pegler, a white-rot basidiomycete, is one of the most valuable edible mushrooms cultured in the world (Chang and Miles, 1989). This fungus has traditionally been cultivated on Fagaceae logs or, in recent decades, on sawdust-based media. White-rot basidiomycetes are capable of degrading whole wood components including lignin and are frequently among the primary colonizers of decaying wood in nature (Shortle and Cowling, 1978; Tanesaka et al., 1993; Tanesaka, 2012). In addition, these mushrooms have been reported to cause serious economic damage to timber (Dickinson, 1982). Although *L. edodes* secretes the phenol-oxidizing enzymes involved in lignin-degradation, laccase (Lcc, EC 1.10.3.2) and manganese peroxidase (MnP, EC 1.11.1.13), when cultivated on sawdust-based media (Tokimoto et al., 1987; Makker et al., 2001; Sakamoto et al., 2009; Nagai et al., 2009), it does not typically secrete these enzymes in liquid media (Ikegaya et al., 1993; Buswell et al., 1995; Nagai et al., 2002; Cavallazzi et al., 2005; Saeki et al., 2011). Lcc production in white-rot basidiomycetes including *L. edodes* can also be promoted by the addition of various metals, particularly Cu²⁺ (Collins et al., 1997; Palmieri et al., 2000; Galhaup et al., 2001, 2002; Soden et al., 2001, 2003; Faraco et al., 2003), or by aromatic compounds (Fahraeus, 1962; Scheel et al., 2000).
Wood meal spread on the surface of agar media has been used for preparing ligninolytic culture conditions for the white- and brown-rot basidiomycetes and the soft-rot deuteromycetes (Enoki et al., 1988; Tanaka et al., 1999, 2000). Similarly, a marked increase in Lcc production has been reported in *Pleurotus ostreatus* cultured together with cotton stalk extract (Ardon et al., 1996).

The substrates used for mushroom cultivation are typically dependent on the availability of agricultural wastes (Smith et al., 1988; Hadar et al., 1992) or the natural resources prevalent in a particular region. Japanese chinquapin, *Castanopsis cuspidata* (Thunb. ex Murray) Schottky, a member of the Fagaceae, is abundant in the warm, temperate, broad-leaved forests of Korea and Japan, and is commonly used as a component in sawdust-based media for *L. edodes* cultivation. We recently reported that the production of the primary MnP isozyme by *L. edodes* cultured on sawdust-based media, LeMnP2 (Sakamoto et al., 2009), was strongly promoted during liquid cultures supplemented with *C. cuspidata* sawdust extract (Saeki et al., 2011). In addition, the expression of the Lcc isozyme, Lcc1 (Nagai et al., 2002), was strongly promoted by the addition of 2 mM Cu²⁺ seven days after the copper was added to the same media (Saeki et al., 2001). Liquid cultures supplemented with sawdust extract made it easy to collect mycelia separately from the culture liquid compared with cultures supplemented with solid sawdust or wood powder. However, when cultured in liquid medium supplemented with the sawdust extract, *L. edodes* produced either MnP or Lcc, but not both. These observations suggest that the expression of MnP and/or Lcc in *L. edodes* is controlled by a negative feedback interaction, i.e., where MnP production inhibits Lcc production and MnP-inhibition enhances Lcc production (Tanesaka et al., 2012).

This study therefore examined the expression of phenol-oxidizing enzymes in *L. edodes* under liquid culture conditions in response to the addition of varying combinations of the following putative inducers of phenol-oxidizing enzymes: different concentrations of sawdust extract, 10⁵ mg test tubes containing an Lcc assay mixture consisting of 0.1 mM o-dianisidine in 0.1 M sodium tartrate buffer (pH 5.0) and the addition of H₂O₂ (final concentration 0.1 mM) to assess Per activity. MnSO₄·5H₂O (final concentration 0.1 mM) was then added to the Per assay mixture to assess MnP activity. Aliquots (20 µl) of crude enzyme solution were added to test tubes containing 980 µl of each reaction mixture, and after incubating these test tubes at 37°C for 10 min, the reactions were stopped by the addition of 50 µl of 40 mM NaN₃. To inactivate the enzymes in the control tubes, sodium azide was added to tubes that contained the Lcc assay mixture before incubation.

**Materials and Methods**

**Fungal stock and culture conditions**

The *L. edodes* variety, ‘Hokken 600’ (Hokken Co., Ltd., Tochigi, Japan), which is well suited to sawdust cultivation, was used in this study. To enhance the expression of phenol-oxidizing enzymes in liquid media, mycelia were cultured in MYPG liquid medium (2.5 g malt extract; 1.0 g yeast extract; 1.0 g peptone; 5.0 g glucose in 1,000 ml distilled water) supplemented with sawdust extract (MYPG-S). The sawdust extract was prepared by adding 1 g of sawdust (*C. cuspidata*) to 30 ml of distilled water and then autoclaving the mixture at 121°C for 15 min before filtering it through filter paper (No. 1, Advantec, Tokyo) and collecting the extract. Two concentrations of MYPG-S liquid media were then prepared by supplementing MYPG liquid medium with either a 1/10 or 1/2 volume of sawdust extract instead of distilled water, which gave MYPG-S containing extracts from 100 mg (MYPG-S₁₀₀) or 500 mg (MYPG-S₅₀₀) of sawdust in 30 ml media, respectively. Mycelia were then sub-cultured at 25°C on MYPG 1% agar plates for 14 days. Three mycelial disks measuring 3 mm in diameter were then harvested from the plates and used to inoculate 30 ml liquid medium in a 100 ml flask, which was then statically cultured at 25°C. All media treatments were performed in triplicate, and measurements of mycelial dry weight and enzyme activity were performed as described below.

**Dry weight of mycelia**

Mycelia samples were collected by filtration through a double nylon stocking mesh at 3-, 4- or 7-day intervals until sampling ended at day 35 of incubation. After washing the mycelia twice with distilled water, excess water was removed by blotting with a paper towel. The mycelia were then dried overnight at 60°C and then at 105°C for 1 h before being weighed.

**Enzyme assay**

The phenol-oxidizing enzymes were assayed as described previously (Saeki et al., 2011; Tanesaka et al., 2012). Briefly, a 100 µl aliquot of culture medium was collected during culture and centrifuged at 13,000 rpm for 10 min. The obtained supernatant was used as the crude enzyme solution, and was assayed for Lcc, peroxidases (Per, EC 1.11.1.x), and MnP in identical 5 ml test tubes containing an Lcc assay mixture consisting of 0.1 mM o-dianisidine in 0.1 M sodium tartrate buffer (pH 5.0) and the addition of H₂O₂ (final concentration 0.1 mM) to assess Per activity. MnSO₄·5H₂O (final concentration 0.1 mM) was then added to the Per assay mixture to assess MnP activity. Aliquots (20 µl) of crude enzyme solution were added to test tubes containing 980 µl of each reaction mixture, and after incubating these test tubes at 37°C for 10 min, the reactions were stopped by the addition of 50 µl of 40 mM NaN₃. To inactivate the enzymes in the control tubes, sodium azide was added to tubes that contained the Lcc assay mixture before incubation.
photometrically assayed using o-dianisidine as a substrate, and the activity of enzyme products was estimated by subtracting the respective absorbance values at 460 nm (Szklarz et al., 1989): i.e., Lcc activity = Lcc assay minus the control assay; Per activity = Per assay minus the Lcc assay; and MnP activity = MnP assay minus the Per assay. One unit (U) of enzyme activity was defined as the amount of enzyme required to catalyze 1 μmol of o-dianisidine in 1 min (ε₄₆₀ = 29,400 M⁻¹cm⁻¹) (Paszczynski et al., 1988). Lignin peroxidase (ligninase, LiP, EC 1.11.1.14) was assayed in the reaction mixture using veratryl alcohol as a substrate at a final concentration of 2 mM with 0.1 mM H₂O₂ in 0.1 M sodium tartrate buffer. This mixture was incubated at 37°C for 10 min and the absorbance of veratryl aldehyde was measured at 310 nm (Tien and Kirk, 1984).

To examine the effects of aromatic compounds on phenol-oxidizing enzyme production, the following aromatic compounds were used: 1,2-benzenediol (catechol), hydroquinone, 2-methoxyphenol (guaiacol), and 4-anisidine. Specifically, each substance was added to MYPG or MYPG-S₅₀₀ culture media to make 10 mM at day 10 after the initial inoculation, and enzyme activities were assayed daily, or at 2- to 3-day intervals thereafter. In addition, the effect of 1 or 2 mM Cu²⁺ (CuSO₄·5H₂O) on phenol-oxidizing enzyme production was also examined. All substance-addition treatments were conducted in triplicate, except for the 1 and 2 mM copper-addition treatments which were performed in duplicate.

RNA isolation and Northern blot analysis

Total RNA was extracted from mycelia using TRIzol Reagent (Invitrogen, CA) after varying incubation periods on MYPG-S₅₀₀. cDNA was synthesized from total RNA using an RNA PCR Kit Ver.3.0 (Takara Bio, Japan), and amplified using the primer set LeMnP2En5f (5’-TCCGACAGTGAATGACCTCGCTC) and LeMnP2En13r (5’-GTCAGTGGAGATTGGGAGGGGC), which were designed based on the highly conserved lemnp2 region (DDBJ Acc. No. AB306944; Sakamoto et al., 2009). A fragment measuring approximately 700 bp was then excised from the 1% agarose–formaldehyde gel, purified, and sequenced using an ABI Prism 3100-Avant Genetic Analyzer (Applied Biosystems, CA) according to the manufacturer’s instructions. Segments with sequences matching lemnp2 were then labeled using a PCR-based digoxigenin (DIG)-dUTP labeling kit (PCR DIG Probe Synthesis Kit; Roche Diagnostics, Germany) according to the manufacturer’s instructions. The resulting DIG-labeled probe, lemnp2, was then used for Northern hybridization after blotting 10 μg of total RNA onto a Hybond-N* membrane (GE Healthcare, Switzerland) using an established protocol (Sambrook et al., 1989).

Data analysis

Statistical analysis was performed using the STATISTICA statistical package (ver.6, StatSoft, Tulsa, OK). Analysis of variance (ANOVA) and Tukey’s multiple comparison tests were performed to compare the dry weight of mycelia and enzyme activities between treatments.

RESULTS

Promotion of growth and enzyme production by sawdust extract

Compared to the MYPG medium (with no sawdust extract), the media supplemented with sawdust extract (MYPG-S₁₀₀ and MYPG-S₅₀₀) had a marked effect on promoting mycelial growth (FIG. 1). Indeed, significant differences in mycelial growth were observed among the three media 7 days after the initial inoculation ($F = 10.05, P = 0.012$). Mycelial growth in MYPG-S₁₀₀ and MYPG-S₅₀₀ liquid media was four-to-five-fold higher than that observed in MYPG alone ($P < 0.001$ at day 35, Tukey’s test).

Figure 2 shows the activities of the phenol-oxidizing enzyme in different culture media. Lcc production was not observed in any of the media until 35 days after inoculation. Although Per activity was detected after 25 days of culture in MYPG medium and after 14 days of culture in MYPG-S₅₀₀ medium, the activities at day 35 were not significantly different between the two types of media. No obvious MnP-production was observed when mycelia were cultured in either MYPG or MYPG-S₁₀₀ media. Conversely, a marked increase in MnP activity was observed from day 14 on the
coincided approximately with the changes observed in MnP activities in the liquid culture medium.

Effects of aromatic compound and copper addition on enzyme activities

The effect of adding either 10 mM of aromatic compounds or Cu$^{2+}$ (1 and 2 mM) on phenol-oxidizing enzyme activities 10 days after the initial inoculation in MYPG-S$_{500}$ medium are summarized in TABLE 1. In this experiment, MnP activity in the control samples (no additives) exceeded 20 U/ml 20 days after inoculation, which was relatively lower than activities exceeding 50 U/ml that were observed after 14 days in a previous experiment (FIG. 2). This disparity in the timing of MnP production between experiments may have been due to slight differences in the preparation of the sawdust extract or the physiological condition of the mycelia in the culture flasks. Even so, at approximately 60 U/ml, MnP activity at 30 days after inoculation was similar between experiments. Compared to the control samples, the addition of all substances – catechol, hydroquinone, guaiacol, anisidine, 1 and 2 mM Cu$^{2+}$ – completely inhibited MnP production (TABLE 1). Instead of inhibiting MnP, the addition of 1 mM Cu$^{2+}$ enhanced Lcc production after 17 days, with activities exceeding 45 U/ml by day 31. Similarly, the addition of 2 mM Cu$^{2+}$ enhanced Lcc production after 6 days, with an activity exceeding 100 U/ml by day 31. Consequently, the increase in Lcc production observed after the addition of 2 mM Cu$^{2+}$ was quicker and there was a higher activity than that observed after the addition of 1 mM Cu$^{2+}$. Conversely, no noticeable increase in Lcc production was observed in the MYPG liquid medium following the addition of any of the aromatic substances or Cu$^{2+}$ (data not shown).

Manganese peroxidase gene transcription

The sequence of the fragment amplified from cDNA, which was prepared from a mixed pool of total RNA obtained from the mycelia of 10-, 15- and 18-day-old cultures cultured in MYPG-S$_{500}$, was identical to that of lemnp2, which is known to code for extracellular MnP (LeMnP2), but different from lemnp1, which is known to code for intracellular MnP (data not shown). The finding indicated that sawdust extract induced the secretion of the same isozyme, LeMnP2, which is a major MnP isozyme that is secreted into sawdust media (Sakamoto et al., 2009). The results of the Northern blotting analysis with a probe lemnp2 are shown in Figure 3. During culture on MYPG-S$_{500}$, a detectable amount of lemnp2 mRNA was present at days 10-19 during the initial stage of culture, with the transcription increasing from day 22 to day 25 and then decreasing at day 28. These changes in lemnp2 transcription coincided approximately with the changes observed in MnP activities in the liquid culture medium.
TABLE 1. Addition of copper, phenolic and non-phenolic compounds and their effect on phenol oxidizing activities (U/ml) in L. edodes cultured on MYPG+S500.

| Substance     | Enzymes | Days after substance was added* |
|---------------|---------|---------------------------------|
|               |         | 0     | 4      | 6      | 10     | 17     | 20     | 31    |
| Control       | Lcc     | 0.3   | 0.0 ab| 0.1 a  | 0.2 a  | 0.2 a  | -0.3 a | 0.3 a |
|               | Per     | -0.1  | 0.7   | 2 a    | 7.2 b  | 16.4 c | 22.2 c | 27.9  |
|               | MnP     | 0.3   | 2.5   | 5.7    | 20.1 c | 45.8 b | 57.0 b | 65.9 b|
| Catechol      | Lcc     | 0.2   | 0.3 ab| 0.5 a  | 1.5 a  | ne     | ne     | ne    |
|               | Per     | 0.2   | 3.2   | 3.2 a  | 2.0 a  | ne     | ne     | ne    |
|               | MnP     | 2.3   | 5.0   | 4.9    | 4.1 a  | ne     | ne     | ne    |
| Hydroquinone  | Lcc     | 0.3   | -0.3 a| 1.0 a  | 0.0 a  | ne     | ne     | ne    |
|               | Per     | 0.1   | 1.3   | 1.0 a  | 0.8 a  | ne     | ne     | ne    |
|               | MnP     | 0.7   | 1.0   | 1.5    | 0.9 ab | ne     | ne     | ne    |
| Guaiacol      | Lcc     | 0.3   | 0.1 ab| -0.3 a | 0.5 a  | ne     | ne     | ne    |
|               | Per     | 0.4   | 0.7   | 0.5 a  | 0.7 a  | ne     | ne     | ne    |
|               | MnP     | 0.1   | 1.2   | 1.2    | 1.9 ab | ne     | ne     | ne    |
| Anisidine     | Lcc     | 0.4   | 0.3 ab| 0.3 a  | -0.3 a | ne     | ne     | ne    |
|               | Per     | 0.6   | 3.2   | 3.1 a  | 1.4 a  | ne     | ne     | ne    |
|               | MnP     | 1.7   | 8.0   | 7.7    | 3.7 ab | ne     | ne     | ne    |
| 1 mM Cu²⁺     | Lcc     | 0.5   | 0.5 ab| 0.3 a  | 0.2 a  | 12.2 b | 27.0 b | 45.9 b|
|               | Per     | 0.0   | 0.4   | 0.2 a  | -0.1 a | -0.9 ab| -0.1 a | 4.6   |
|               | MnP     | 0.3   | 1.4   | 0.8    | 0.4 ab | 0.6 a  | 0.7 a  | -3.1 a|
| 2 mM Cu²⁺     | Lcc     | 0.6   | 0.9 b | 23.0 b | 63.9 b | 98.0 c | 95.1 c | 109 c |
|               | Per     | 0.1   | 4.4   | 9.1 b  | 3.6 ab | -4.0 a | 2.7 ab | 9.2   |
|               | MnP     | 0.5   | 0.2   | 0.2    | -2.3 a | 4.7 a  | -0.1 a | 3.4 a |

*: Each substance was added 10 days after initial inoculation
†: Each value represents the mean of three replicate flasks or two replicate flasks for Cu²⁺
‡: Values with different lowercase letters are significantly different at the 5% level between treatments.
Values with no lowercase letters are not significantly different between any pair of treatments on a given day.

DISCUSSION

The expression of lignin-degrading enzymes in white-rot basidiomycetes has been attributed to the production of secondary metabolites under various growth and culture conditions (Kirk and Fenn, 1982; Cullen and Kersten 2004). Although L. edodes has been reported to secrete the lignin-degrading enzymes Lcc and/or MnP when cultivated on sawdust-based media (Tokimoto et al., 1987; Makker et al., 2001; Sakamoto et al., 2009; Nagai et al., 2009), the findings of the present study corroborated our previous studies (Saeki et al., 2011) and showed that extracts produced by autoclaving sawdust in hot water can also promote MnP activity. Compared to mycelial growth on MYPG medium, growth on MYPG-S100 and MYPG-S500 media was considerably more vigorous. Although the components in the sawdust extract are unclear, the compound(s) responsible for the observed vigorous growth may be flavonoids (Fahraeus, 1962; Ardon et al., 1996). Interestingly, although mycelial growth was promoted in both MYPG-S100 and MYPG-S500 media, a marked increase in MnP production was only observed in cultures grown in MYPG-S500, and not in MYPG-S100. These observations suggested that MnP is only enhanced by specific, concentration-dependent, functional compounds in the high-concentration sawdust extract (MYPG-S500). In other words, MnP enhancement was not only due to the vigorous growth, which was also observed in the low-concentration sawdust extract (MYPG-S100). Transcription of the lemnp2 gene, which encodes LeMnP2, was initially observed at detectable levels by Northern blot analysis of cultures grown on MYPG-S500 at day 10 after inoculation. Based on these results, the addition of aromatic compounds and copper 10 days after inoculation appeared to coincide, approximately, with the initiation of lemnp2 gene transcription.

In several white-rot fungi, Lcc production is promoted by aromatic substances (Fahraeus, 1962; Scheel et al., 2000; Piscitelli et al., 2011). However, in the present study on L. edodes, although the sawdust extract likely contained a variety of phenolic and nonphenolic aromatic compounds (e.g. flavonoids), none of the 10 mM aromatic compounds tested in this study enhanced Lcc production. In addition, all of the aromatic compounds
tested completely inhibited MnP production. These findings corroborated those of a similar study on *L. edodes* which found that none of the 1 mM aromatic compounds tested (i.e. gallic acid, catechol, hydroxybenzoic acid, or vanillin) enhanced Lcc production; rather, the aromatic compounds either inhibited or repressed Lcc production (Cavallazzi et al., 2005). In contrast, Ikekaya et al. (1993) reported that the addition of 50 μg/ml (0.33 mM) vanillin in a peptone-glucose liquid medium significantly promoted mycelial growth, laccase production and fruit-body formation by *L. edodes*. The differences observed in Lcc expression between studies may be attributable to differences in the concentrations of substances added, nutritional conditions, or stocks and strains used.

The addition of metallic ions such as Cu²⁺ to media has also been used to induce Lcc production in several fungi, including *L. edodes* (Nagai et al., 2002; Cavallazzi et al., 2005; Saeki et al., 2011). Indeed, Cu²⁺ is considered to be a strong Lcc inducer in the white-rot fungi, *Pleurotus ostreatus* (Palmieri et al., 2000; Faraco et al., 2003), *P. sajor-caju* (Soden et al., 2001, 2003), *Trametes pubescens* (Galhaup et al., 2001, 2002), and *T. versicolor* (Collins et al., 1997). For example, compared to a control treatment (no Cu²⁺ added), a 1.5-fold increase in Lcc production was achieved in a liquid culture of *L. edodes* by the addition of 10 mM Cu²⁺ to the culture medium (Nagai et al., 2002). Similarly, more than a 25-fold increase in Lcc production was achieved by the addition of 250 μM or 2 mM Cu²⁺ to a liquid culture of *L. edodes* (Cavallazzi et al., 2005; Saeki et al., 2011). The promoter region of the gene coding the major Lcc isozyme (LAP2) of *T. pubescens* has copper-sensitive metal responsive elements (MRE), and Lcc gene transcription is induced within 10 h after the addition of 2 mM Cu²⁺ (Galhaup et al., 2001, 2002). Copper regulation of Lcc by MRE consensus sequences has also been reported in *Pleurotus* spp (Faraco et al., 2003; Soden et al., 2003).

The main findings of this study were as follows: i) marked mycelial growth was observed in *L. edodes* cultured in low- and high-concentration sawdust extract produced from *C. cuspidata*. However, MnP production was only enhanced by the high-concentration sawdust extract; ii) all of the aromatic compounds tested completely inhibited MnP production and did not enhance Lcc production; iii) the addition of both 1 and 2 mM Cu²⁺ completely inhibited MnP production, but markedly enhanced Lcc production. Although the simultaneous expression of MnP and Lcc was not observed in this study, the hypothesis of whether a negative feedback interaction exists between MnP and Lcc is still unclear, i.e. whether MnP production initially inhibits Lcc production and MnP inhibition promotes Lcc production. However, in this study, MnP inhibition by the addition of aromatic compounds did not enhance Lcc production. These enzyme expression dynamics are highly sensitive to nutrient nitrogen and manganese (Buswell et al., 1995), as well as the fungal response to Cu²⁺ and aromatic compounds as discussed above. Indeed, the findings presented here do not provide sufficient evidence for even the first part of the proposed negative feedback interaction involving these two enzymes. However, the findings do show that MnP inhibition and Lcc production are affected by Cu²⁺ addition and that this inhibition-production relationship might be functionally independent.

Importantly, the experimental conditions employed in this study, in which sawdust extract was shown to enhance mycelial growth and MnP production, were similar to the conditions employed to cultivate edible mushrooms (e.g., autoclaved sawdust-based media). The liquid-culture media described here could therefore be employed to evaluate the lignin-degrading ability of cultivated mushroom varieties. Further analyses are therefore necessary to examine the functional component(s) in the sawdust extract and the correlation between enzyme expression and regulation using this experimental system.

**ACKNOWLEDGMENTS**

This work was supported by the “Academic Frontier” Project for Private Universities with a matching fund subsidy from the Ministry of Education, Culture, Sports, Science and Technology (2004–2008), Japan. This work was also partly supported by a fund from the Farmers Corporation, “JA-Nakanoshi”, Nakano, Nagano, Japan.

**REFERENCES**

Ardon, O., Kerem, Z., and Hadar, Y. (1996) Enhancement of laccase activity in liquid cultures of the ligninolytic fungus *Pleurotus ostreatus* by cotton stalk extract. *J. Biotechnol.*, 51, 201-207.

Buswell, J., Cai, Y., and Chang, S-T. (1995) Effect of nutrient and manganese on manganese peroxidase and laccase production by *Lentinula* (Lentinus) *edodes*. *FEMS Microbiol. Lett.*, 128, 81-88.

Cavallazzi, J. R. P., Kasuya, C. M., and Soares, M. A. (2005) Screening of inducers for laccase production by *Lentinula edodes* in liquid medium. *Brazilian J. Microbiol.*, 36, 383-387.

Chang, S-T., and Miles, P. (1989) *Edible mushrooms and their cultivation*. CRC Press, Boca Raton, Florida, USA.

Collins, P., and Dobson, A. (1997) Regulation of laccase gene transcription in *Trametes versicolor*. *Appl. Environ. Microbiol.*, 63, 3444-3450.

Cullen, D., and Kersten, P. (2004) Enzymology and molecular biology of lignin degradation. In *The Mycota III*;
Biochemistry and molecular biology. 2nd edition (Brambl, R. and Marzuf, G., ed.), pp. 249-273, Springer, Berlin, Germany.

Dickinson, D. J. (1982) The decay of commercial timbers. In Decomposer basidiomycetes: their biology and ecology (Frankland, J., Hedger, J., Swift, M., ed.), pp. 179-190, Cambridge University Press, New York.

Enoki, A., Tanaka, H., and Fuse, G. (1989) Relationship between degradation of wood and production of H2O2-producing or one-electron oxidases by brown-rot fungi. *Wood Sci. Technol.*, 23, 1-12.

Fahraeus, G. (1962) Aromatic compounds as growth substance for laccase producing rot fungi. *Physiol. Plant.*, 15, 572-579.

Faraco, V., Giardina, P., and Sannia, G. (2003) Metal-responsive elements in *Pleurotus ostreatus* laccase gene promoters. *Microbiology*, 149, 2155-2162.

Galhaup, C., and Haltrich, D. (2001) Enhanced formation of laccase activity by the white-rot fungus *Trametes pubescens* in the presence of copper. *Appl. Microbiol. Biotechnol.*, 56, 225-232.

Galhaup, C., Goller, S., Peterbauer, C., Strauss, J., and Haltrich, D. (2002) Characterization of the major laccase isozyme from *Trametes pubescens* and regulation of synthesis by metal ions. *Microbiology*, 148, 2159-2169.

Hadar, Y., Kerem, Z., Gorodecki, B., and Ardon, O. (1992) Utilization of lignocellulosic waste by the edible mushroom *Pleurotus*. *Biodegradation*, 3, 189-205.

Ikegaya, N., Goto, M., and Hayashi, Y. (1993) Effects of phenolic compounds and urocanines on the activities of extracellular enzyme during vegetative growth and fruit-body formation of *Lentinus edodes*. *Trans. Mycol. Soc. Japan*, 34, 195-207. (in Japanese)

Kirk, T., and Fenn, P. (1982) Formation and action of the ligninolytic system in basidiomycetes. In *Decomposer basidiomycetes: their biology and ecology*. (Frankland, J., Hedger, J., and Swift, M., ed.), pp 67-90, Cambridge University Press, New York.

Makker, R., Tsuneda, A., Tokuyasu, K., and Mori, Y. (2001) *Lentinula edodes* produces a multicomponent protein complex containing manganese (II)-dependent peroxidase, laccase and β-glucosidase, *FEMS Microbiol. Lett.*, 200, 175-183.

Nagai, M., Sato, T., Watanabe, H., Saito, K., Kawata, M., and Enei, H. (2002) Purification and characterization of an extracellular laccase from the edible mushroom *Lentinula edodes*, and decolorization of chemically different dyes. *Appl. Microbiol. Biotechnol.*, 60, 327-335.

Nagai, M., Sakamoto, Y., Nakade, K., and Sato, T. (2009) Purification of a novel extracellular laccase from solid-state culture of the edible mushroom *Lentinula edodes*. *Mycoscience*, 50, 308-312.

Palmieri, G., Giardina, P., Bianco, C., Fontanella, B., and Sannia, G. (2000) Copper induction of laccase isozymes in the ligninolytic fungus *Pleurotus ostreatus*. *Appl. Environ. Microbiol.*, 66, 920-924.

Paszczynski, A., Crawford, R., and Huyhn, V-B. (1988) Manganese peroxidase of *Phanerochaete chrysosporium*: Purification. In *Methods in enzymology*. Vol.161, *Biomass, Part B, Lignin, pectin and chitin* (Wood, W., and Kellogg, S., ed.), pp 264-270, San Diego, CA.

Saeki, N., Takeda, H., Tanesaka, E., and Yoshida, M. (2011) Induction of manganese peroxidase and laccase by *Lentinula edodes* under liquid culture conditions and their isoyme detection by enzymatic staining on native-PAGE. *Mycoscience*, 52, 132-136.

Sakamoto, Y., Nakacho, K., Nagai, M., Uchimiya, H., and Sato, T. (2009) Cloning of *Lentinula edodes* lmmnp2, a manganese peroxidase that is secreted abundantly in sawdust medium. *Mycoscience*, 50, 116-122.

Sambrook, J.; Fritsch E. & Maniatis, T. (1989) *Molecular cloning: a laboratory manual*, Book 2, 2nd edition, Cold Spring Harbor Laboratory Press, New York.

Scheel, T., Höfer, M., Ludwig, S., and Hölker, U. (2000) Differential expression of manganese peroxidase and laccase in white-rot fungi in the presence of manganese or aromatic compounds. *Appl. Microbiol. Biotechnol.*, 54, 686-691.

Shortle, W. C., and Cowling, E. B. (1978) Development of discoloration, decay, and micro-organisms following wounding of Sweetgum and Yellow poplar trees. *Phytopathology*, 68, 609-616.

Smith, J., Fermor, T., and Zadrazil, F. (1988) Pretreatment of lignocellulosics for edible fungi. In *Treatment of lignocellulosics with white rot fungi* (Zadrazil, F., and Reiniger, P., ed.), pp. 3-13, Elsevier Applied Science, Essex, UK.

Soden, D., and Dobson, A. (2001) Differential regulation of laccase gene expression in *Pleurotus sajor-caju*. *Microbiology*, 147, 1755-1763.

Soden, D., and Dobson, A. (2003) The use of amplified flanking region-PCR in the isolation of laccase promoter sequences from the edible fungus *Pleurotus sajor-caju*. *J. Appl. Microbiol.*, 95, 553-562.

Szklarz, G. D., Antibus, R. K., Sinsabaugh, R. L., and Linkins, A. E. (1989) Production of phenol oxidase and peroxidases by wood-rotting fungi. *Mycologia*, 81, 234-240.

Tanaka, H., Itakura, S., and Enoki, A. (1999) Hydroxyl radical generation by a extracellular low-molecular weight substance and phenol oxidase activity during wood degradation by the white-rot basidiomycete *Trametes versicolor*. *J. Biotechnol.*, 75, 57-70.

Tanaka, H., Itakura, S., and Enoki, A. (2000) Laccase production and one-electron oxidation activity in wood degradation by the soft-rot deuteromycete *Graphium sp*. *Biocontrol Sci.*, 5, 39-46.

Tanesaka, E. (2012) Colonizing success of saprotrophic and ectomycorrhizal basidiomycetes on islands. *Mycologia*, 104, 345-352.

Tanesaka, E., Masuda, H., and Kinugawa, K. (1993) Wood degrading ability of basidiomycetes that are wood decomposers, litter decomposers, or mycorrhizal symbionts. *Mycologia*, 81, 347-354.

Tanesaka, E., Saeki, N., Kochi, A., and Yoshida, M. (2012) Enzymatic staining for detection of phenol-oxidizing isozymes involved in lignin-degradation by *Lentinula edodes*. In *Gen electrophoresis – Advanced techniques* (Magelaudín, S., ed.), pp. 393-412, InTech, Rijeka, Croatia.

Tien, M., and Kirk, T. (1984) Lignin-degrading enzyme from *Phanerochaete chrysosporium*: Purification, characterization and catalytic properties of a unique hydrogen peroxide-requiring oxidase. *Proc. Natl. Acad. Sci. USA*, 81, 2280-2284.

Tokimoto, K., Fukuda, M., Kishimoto, H., and Koshitani, H. (1987) Activities of enzymes in bedlogs of *Lentinus edodes* during fruitbody development. *Rept. Tottori Mycol. Inst.*, 25, 24-35 (in Japanese).