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SHORT COMMUNICATION

Induction of manganese peroxidase and laccase by *Lentinula edodes* under liquid culture conditions and their isozyme detection by enzymatic staining on native-PAGE

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Abstract The white-rot basidomycete *Lentinula edodes* often produces the lignin-degrading enzymes manganese peroxidase (MnP; EC 1.11.1.13) and laccase (Lcc; EC 1.10.3.2) in sawdust-based media. In the present study, MnP from *L. edodes* was induced under liquid culture supplemented with sawdust extracts of *Castanopsis cuspidata*. Lcc activity was induced by the addition of 2 mM $CuSO_4 \cdot 5H_2O$ into the same media 7 days after initial inoculation. Phenoloxidase enzymes were distinguished by polyacrylamide gel electrophoresis (native-PAGE), followed by sequential enzymatic staining with an improved staining solution. The isozyme bands detected under MnP-induced conditions were identified as manganese peroxidase (lemnp2) and bands detected under Lcc-induced conditions were identified as laccase (lcc1) by Q-TOF mass spectrometry.

Keywords Breeding · Copper ion · Extracellular enzyme · Phenoloxidases · Shiitake mushroom

Lentinula edodes (Berk.) Pegler, the shiitake mushroom, a white-rot basidiomycete, is one of the most valuable, cultured, edible mushrooms in the world (Chang and Miles 1989). It was traditionally cultivated on Fagaceae logs but is now grown on sawdust-based media. The ability of white-rot basidiomycetes to degrade wood components, especially lignin, affects culture time to harvesting and yields (Smith et al. 1988; Kinugawa and Tanesaka 1990; Tanesaka et al. 1993; Ohga and Kitamoto 1997). *L. edodes* secretes lignin-degrading enzymes, laccase (Lcc) and manganese peroxidase (MnP), on sawdust-based media (Leatham 1985; Tokimoto et al. 1987; Buswell et al. 1995; Makker et al. 2001), but not usually in liquid media. Sakamoto et al. (2009) found the main isozyme produced by *L. edodes* on sawdust media was manganese peroxidase, lemnp2. This finding suggests these enzymes play an important role in degrading sawdust during culture, and the expression and properties of the enzymes will influence mycelia growth and fruit body development (Wood et al. 1988).

There have been several reports on purification and characterization of the lignin-degrading enzymes secreted by *L. edodes* using sophisticated biochemical procedures (Forrester et al. 1990; Nagai et al. 2002, 2003; Sakamoto et al. 2008, 2009). However, these methods are impracticable for routine isozyme analysis during breeding trials. Methods for isozyme detection by electrophoresis using enzyme catalytic properties called "protein activity staining" or "enzymatic staining" are well established in histochemical studies and genetics (Pasteur et al. 1988). The present study reports improved procedures of enzymatic staining following polyacrylamide gel electrophoresis (native-PAGE) to distinguish Lcc and MnP isozymes induced in liquid cultures of *L. edodes*.

A variety of *L. edodes* (Hokken 600: Hokken, Tochigi, Japan) was used as a dikaryotic stock. To induce phenoloxidases, mycelia were cultured on MYPG liquid (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

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sawdust extract (MYPG-S). One gram of sawdust from *Castanopsis cuspidata* (Thunb. ex Murray) Schottky in 30 ml distilled water was autoclaved for 15 min and filtered with filter paper (No. 1; ADVANTEC). The filtrate was used as sawdust extract. MYPG-S liquid media was prepared from MYPG liquid with a half-volume of sawdust extract added in place of distilled water. Mycelia were subcultured at 25°C on MYPG 1% agar plates. After 14 days, three mycelial disks, which were 3 mm in diameter, were taken from the plates and inoculated into 30 ml MYPG-S liquid in a 100-ml flask and statically cultured at 25°C. MnP activity was induced during culture on MYPG-S. Lcc activity was induced by adding 2 mM $CuSO_4 \cdot 5H_2O$ to the same media 7 days after initial inoculation.

To assay activities of extracellular enzymes, 100 µl culture liquid was sampled every few days during culture and centrifuged at 13,000 rpm for 10 min; the supernatant was used as a crude enzyme solution. The crude enzyme solution was assayed for Lcc, Per, and MnP in identical 5-ml test tubes containing the following reaction mixtures: Lcc assay mixture consisted of 0.1 mM *o*-dianisidine in 0.1 M sodium tartrate buffer (pH 5.0); additional H_2O_2 (final concentration 0.1 mM) was added to Lcc assay mixture to assess Per activity; and additional $MnSO_4 \cdot 5H_2O$ (final concentration 0.1 mM) was 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; the mixtures were incubated at 37°C for 10 min and the reaction stopped by addition of 50 µl 40 mM NaN_3. Sodium azide was added to control tubes containing the Lcc assay mixture before incubation to inactivate enzymes. Catalytic products of the reaction were spectrophotometrically assayed with *o*-dianisidine as a substrate at absorbance 460 nm; enzyme products were estimated by subtraction of assay measurements (Szklarz et al. 1989): i.e., Lcc activity = Lcc assay minus measurements of control; Per activity = Per assay minus Lcc assay; and MnP activity = MnP assay minus Per assay, respectively. One unit (U) of enzyme activity was defined as amount of enzyme that catalyzes 1 µmol *o*-dianisidine for 1 min (Paszczynski et al. 1988).

Neither MnP nor Lcc was induced under cultures on MYPG liquid without sawdust extracts (data not shown). Under MnP-induced conditions (cultured on MYPG + S) MnP activity increased suddenly after 20 days, reached maximum activity (60 U/ml) on day 35, and then decreased (Fig. 1a). Yoshikawa et al. (2004) reported that addition of wood tips or sawdust of fagaceous trees, *C. cuspidata* or *Fagus crenata* Blume, in MYPG liquid, induced MnP activity. The results from the present study show that sawdust extracts in hot water also induce MnP. Lcc activity was detected 14 days after inoculation (7 days after the addition of 2 mM $CuSO_4 \cdot 5H_2O$) and continued beyond day

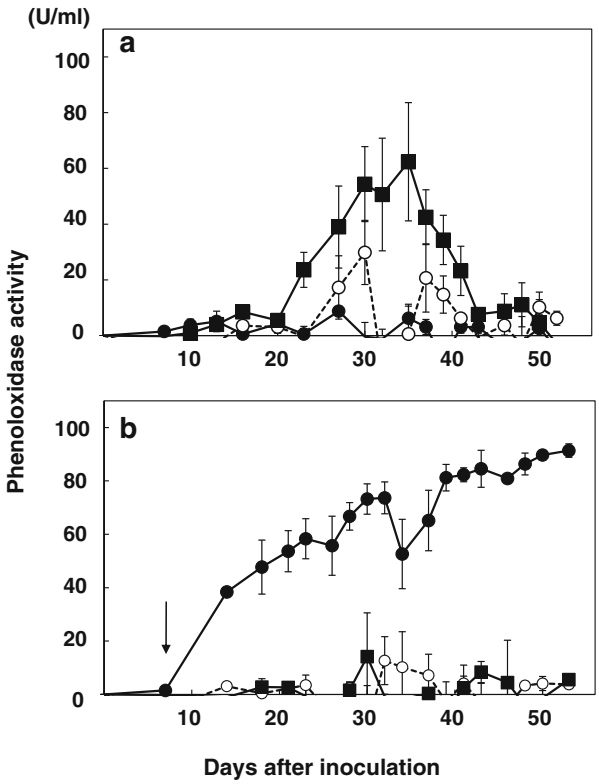

Fig. 1 Phenoloxidase activity, laccase (Lcc) (*filled circles*), peroxidase (Per) (*open circles*), and manganese peroxidase (MnP) (*filled squares*), in liquid culture of *Lentinula edodes* under MnP-induced (**a**) or Lcc-induced conditions (**b**). *Arrow* in **b** indicates the day of $CuSO_4$ addition. Each value represents mean with standard error (*vertical bar*) of three replicate cultures

50, while MnP activity was suppressed (Fig. 1b). Laccase may often be induced by the addition of aromatic compounds (Scheel et al. 2000; Saparrat et al. 2002) or metallic ions including copper (Galhaup and Haltrich 2001; Soden and Dobson 2001; Galhaup et al. 2002; Shutova et al. 2008). In the white-rot basidiomycete *Trametes pubescens* (Schum.: Fr.) Pilát., transcription of the laccase gene is induced within 10 h after the addition of 2 mM $CuSO_4$ (Galhaup et al. 2002). Under the two culture conditions we employed, either Lcc or MnP activity was detected, but not both. These results suggest that induction of MnP and Lcc are controlled by an interlock system, i.e., Lcc induction suppresses the MnP production, or more simply, the addition of $CuSO_4 \cdot 5H_2O$ suppresses the MnP production.

Each phenoloxidase isozyme was detected by native PAGE. Whole cultures were filtered through a nylon stocking and the filtrate collected and centrifuged at 13,000 rpm for 10 min. The supernatant was filtered (filter paper, No. 2) and concentrated to approximately 1/15 of the original volume by ultrafiltration using Centriprep YM-10 (Millipore). Aliquots containing 15 µl filtrate and 1.5 µl loading dye (50% glycerol and 0.5% bromophenol blue,

BPB) were applied to 17.5% polyacrylamide gel. Native-PAGE was run at 15 mA for 15 min followed by 25 mA for 3–4 h. After electrophoresis, the gel was sequentially incubated at 37°C for 30 min in fundamental enzymatic staining solutions to detect each enzyme in the following order: Lcc staining solution (LccS), 1.8 mM *o*-dianisidine, and 0.1 M acetate buffer (pH 4.0); additional H_2O_2 (final concentration, 0.1 mM) to produce Per staining solution (PerS); and additional $MnSO_4{\cdot}5H_2O$ added to the PerS (final concentration 0.1 mM) to produce MnP staining solution (MnPS). Nomenclature of the isozyme follows May et al. (1980). Lcc-e and MnP-e refer to extracellular enzymes of Lcc and MnP, respectively, and the following numerals in parentheses represented the relative mobility of each isozyme to the mobility of bromophenol blue used as a dye marker.

We expected Lcc, Per, and MnP bands to be distinguished on the same gel by subtraction of newly appeared bands from sequential staining in the order of LccS, PerS, and MnPS. In practice, however, unexpected bands (two intense bands) often appeared on gels subjected to LccS staining solution, even on the samples prepared from MnP-induced conditions without Lcc activity (Fig. 2a, lane 1). No additional bands appeared in subsequent staining procedures with PerS or MnPS (Fig. 2b, lane 1). At first, we considered these bands developed from MnP isozymes were caused by Mn^{2+} contamination in the sample solution, gel, or staining solution. However, acetone precipitation of sample solution and pre-run gels at 15 mA for 20 min also gave unexpected results (data not shown).

In subsequent staining procedures, ethylenediaminetetraacetic acid (EDTA) was added at concentrations of 100, 110, 120, 130, 140, and 150 mM into the staining solutions LccS and PerS, named LccS + EDTA and PerS + EDTA, respectively, to eliminate Mn^{2+} contamination, before the gel was stained with MnPS. The gels were rinsed with distilled water between each stain procedure. When gels were stained with LccS + EDTA, the intensity of the bands weakened with increasing concentration of EDTA and disappeared at 130–150 mM (Fig. 2a, lanes 5–7). These bands reappeared following sequential staining with MnPS. This result indicated that these two bands were MnP isozymes, named MnP-e (52) and MnP-e (57), respectively (Fig. 2b). In sample solutions prepared from Lcc-induced conditions, two bands with broad tailing smears were detected by staining with LccS. Intensity of these bands also weakened as EDTA concentration in the staining solution (LccS + EDTA) increased, but did not completely disappear even at 130–150 mM EDTA (Fig. 2c). As this result is independent of Mn^{2+} and developed from Lcc isozymes, named Lcc-e (61) and Lcc-e (67), we deduce that treatment with approximately 130 mM EDTA in the staining solution would enable the distinction of Lcc and MnP isozymes from each other on a single gel.

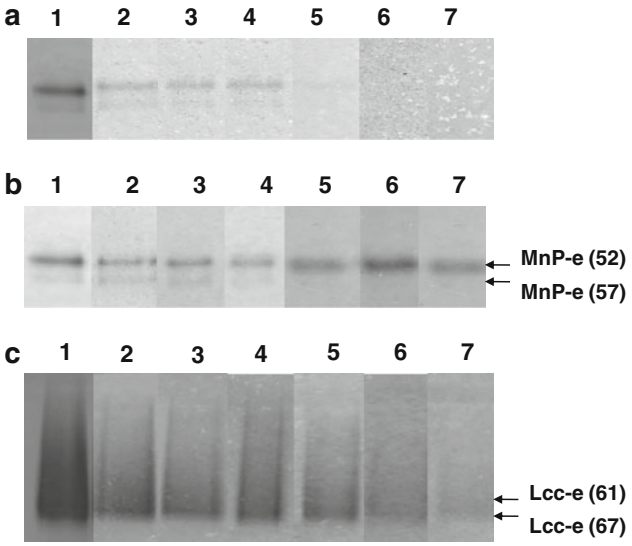

Fig. 2 Effects of the addition of ethylenediaminetetraacetic acid (EDTA) in staining solution on isozyme detection for one gel. **a** Lcc staining with LccS (*lane 1*) or with LccS + EDTA (*lanes 2–7*) conducted on aliquots of the same sample solution prepared from MnP-induced conditions (at 27 days). *Lanes 2–7* contain increasing concentrations of EDTA. EDTA concentration: *lane 1*, 0 mM (LccS); *lane 2*, 100 mM; *lane 3*, 110 mM; *lane 4*, 120 mM; *lane 5*, 130 mM; *lane 6*, 140 mM; *lane 7*, 150 mM. **b** MnP staining with MnPS in sequential staining after staining with LccS and PerS (*lane 1*) or with LccS + EDTA and PerS + EDTA (*lanes 2–7*) on the same gel shown in **a. c** Lcc staining with LccS (*lane 1*) or with LccS + EDTA (*lanes 2–7*) conducted on aliquots of the same sample solution prepared from Lcc-induced conditions (36 days). EDTA concentration of each lane was the same as above

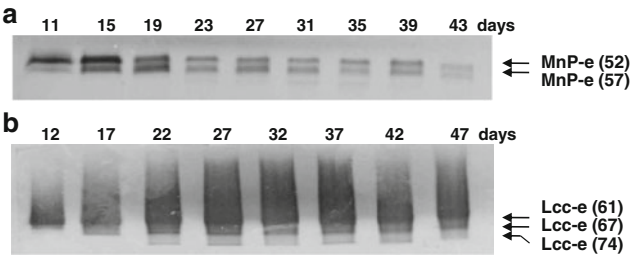

Fig. 3 Phenoloxidase isozymes detected by the improved staining method (see text) during cultures expressed under MnP-induced (**a**) and Lcc-induced (**b**) conditions. Days after inoculation are represented on each *lane*

By using the improved staining method described above, two MnP isozymes, MnP-e (52) and MnP-e (57), were detected during culture under MnP-induced conditions (Fig. 3a). Lcc isozymes, Lcc-e (61) and Lcc-e (67), were also detected as major isozymes during culture under Lcc-induced conditions with an additional isozyme Lcc-e (74), which was weakly detected between the 22th day and 47th day of culture (Fig. 3b).

Isozyme distinction was confirmed by identifying peptides of each isozyme detected on gels. After native-PAGE conducted on the same sample solution on adjacent lanes, each lane of a gel was divided and subjected to enzymatic staining and Coomasie brilliant blue (CBB) staining (Fig. 4). From the CBB staining gel, bands of interest including those showing the same mobility as that of the enzymatic staining were excised using a sterile surgical blade and placed in 1.5-ml tubes. Each polyacrylamide piece was repeatedly rinsed with 50%, 30%, and 50% v/v acetonitrile containing 25 mM NH_4HCO_3 under sonication for 20 min with a micromixer (Taitec, Tokyo, Japan) and finally 100% acetonitrile without NH_4HCO_3 for 5 min. The pieces were air-dried for 5 min and recovered in 100 µl 50 mM NH_4HCO_3 with 10 µg/µl trypsin (sequencing grade, Roche Diagnostics) on ice for 30 min. The solution was drained and the pieces were incubated at 37°C for 16 h. The tryptic fragments were extracted in 50 µl extract buffer consisting 50% acetonitrile and 5% formic acid under sonication for 20 min. The extract buffer was placed into a new tube and replaced with 25 µl fresh extract buffer; this extraction was repeated three or more times. The collected buffer containing the tryptic fragments was concentrated to approximately to 5 µl by drying under vacuum. Analysis of tryptic peptides by tandem mass spectrometry was performed on a nanoelectrospray ionization quadrupole time-of-flight hybrid mass spectrometer (Q-TOF Premier; Waters Micromass) coupled with a nano-HPLC (Cap-LC; Waters Micromass). The peptides were separated on a BEH130C18 column (1.7 µm, 100 µm × 100 mm; Nano Ease, Waters) according to the manufacturer's instruction manual. The peptide sequences obtained were either matched automatically to proteins in a nonredundant database (National Center for Biotechnology Information, NCBI, http://www.ncbi.nlm.nih.gov) using the Mascot MS/MS ions search algorithm (http://www.matrixscience.com) or manual BLAST searches were performed against the current databases DNA Data Bank Japan (DDBJ, http://www.ddbj.nig.ac.jp).

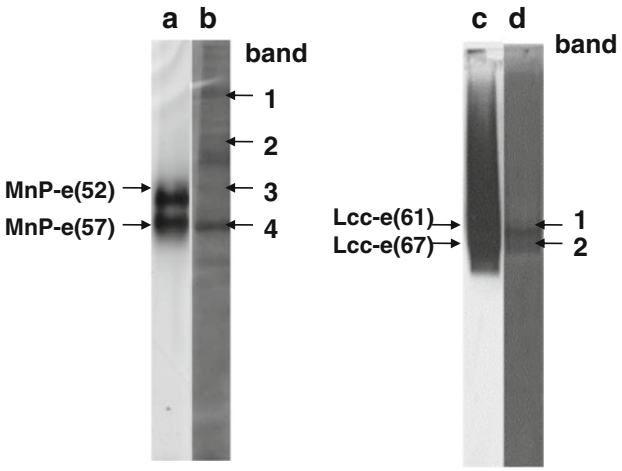

Fig. 4 Protein bands detected by enzymatic staining (**a**, **c**) and corresponding Coomassie brilliant blue (CBB) staining (**b**, **d**) for quadrupole time-of-flight (Q-TOF) mass spectrometry analysis. **a**, **b** Bands detected on MnP-induced conditions (at 22 days). **c**, **d** Bands detected on Lcc-induced conditions (at 30 days)

Table 1 shows protein identifications by Q-TOF mass spectrometry analysis. Two MnP isozymes, MnP-e (52) and MnP-e (57), were both identified as manganese peroxidase, lemnp2, which is known to be a major MnP isozyme secreted into sawdust medium by *L. edodes* (Sakamoto et al. 2009). Other enzymes, *β*-glucosidase and exo-*β*-1,3-glucanase, were also detected under MnP-induced conditions. Makker et al. (2001) reported that *β*-glucosidase is secreted into sawdust culture media of *L. edodes* as a multicomponent protein complex with MnP and Lcc. Two Lcc isozymes, Lcc-e (61) and Lcc-e (67), detected under Lcc-induced conditions were both identified as laccase produced by *L. edodes* (lcc1: Sakamoto et al. 2008). We conclude that MnP and Lcc isozyme detection by the improved enzymatic staining solutions LccS + EDTA, PerS + EDTA, and MnPS correctly distinguishes each phenoloxidase.

Table 1 Extracellular enzymes produced by *Lentinula edodes* under manganese peroxidase (MnP)- and laccase (Lcc)-induced liquid culture conditions

Culture conditions	Band[a]	Protein score[b]	Protein identification		Fungi	References
MnP-induced	1	48	Avenacinase: *β*-glucosidase	EC 3.2.1.21	*Botryotinia fuckeliana*	Quidde et al. (unpublished)[c]
	2	188	exo-*β*-1,3-glucanase	EC 3.2.1.58	*L. edodes*	Sakamoto et al. (2005)
	3	48	lemnp2, manganese peroxidase	EC 1.11.1.13	*L. edodes*	Sakamoto et al. (2009)
	4	171	lemnp2, manganese peroxidase	EC 1.11.1.13	*L. edodes*	Sakamoto et al. (2009)
Lcc-induced	1	527	lcc1, laccase	EC 1.10.3.2	*L. edodes*	Sakamoto et al. (2008)
	2	321	lcc1, laccase	EC 1.10.3.2	*L. edodes*	Sakamoto et al. (2008)

[a] Band numbers are those listed on the Coomassie brilliant blue (CBB) staining gel, Fig. 3

[b] Sum of individual ion scores, where score > 41 indicates identity or extensive homology ($P < 0.05$)

[c] DDBJ accession number, AJ130890

The separation and identification of each isozyme and enzyme activity will provide information for the evaluation and screening of breeding varieties of *L. edodes*. Induction of MnP and Lcc under liquid cultures and the improved method for enzymatic staining presented here are convenient and effective method of screening for isozymes of value in mushroom breeding.

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References

Buswell JA, Cai YJ, Chang ST (1995) Effect of nutrient and manganese on manganese peroxidase and laccase production by *Lentinula* (*Lentinus*) *edodes*. Microbiol Lett 128:81–88

Chang ST, Miles PG (1989) *Lentinus*: a mushrooming mushroom. In: Edible mushrooms and their cultivation. CRC Press, Boca Raton, pp 189–223

Forrester IT, Grabski AC, Mishra C, Kelley BD, Strickland WN, Leatham GF, Burgess RR (1990) Characteristics and N-terminal amino acid sequence of a manganese peroxidase purified from *Lentinula edodes* cultures grown on commercial wood substrate. Appl Microbiol Biotechnol 33:359–365

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

Kinugawa K, Tanesaka E (1990) Changes in the rate of carbon dioxide release from cultures of three basidiomycetes during cultivation. Trans Mycol Soc Jpn 31:489–500

Leatham GF (1985) Extracellular enzymes produced by the cultivated mushroom *Lentinus edodes* during degradation of a lignocellulosic medium. Appl Environ Microbiol 50:859–867

Makker RS, Tsuneda A, Tokuyasu K, Mori Y (2001) *Lentinula edodes* produces a multicomponent protein complex containing manganese (II)-dependent peroxidase, laccase and β-glucosidase. FEMS Microbiol Lett 200:175–179

May B, Stoneking M, Wright JE (1980) Joint segregation of biochemical loci in Salmonidae. II. Linkage association from a hybridized *Salvelinus* genome (*S. namaycush* × *S. fontinalis*). Genetics 95:707–726

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

Nagai M, Kawata M, Watanabe H, Ogawa M, Saito K, Takesawa T, Kanda K, Sato T (2003) Important role of fungal intracellular laccase for melanin synthesis: purification and characterization of an intracellular laccase from the edible mushroom *Lentinula edodes* fruit bodies. Microbiology 149:2455–2462

Ohga S, Kitamoto Y (1997) Future of mushroom production and biotechnology. Food Rev Int 13:461–469

Pasteur N, Pasteur G, Bonhomme F, Catalan J, Britton-Davian J (1988) Practical isozyme genetics. Ellis Horwood, Chichester

Paszczynski A, Crawford RL, Huynh V-B (1988) Manganese peroxidase of *Phanerochaete chrysosporium*: purification. Methods Enzymol 161:264–270

Sakamoto Y, Minato K, Nagai M, Mizuno M, Sato T (2005) Characterization of the *Lentinula edodes* exg2 gene encoding lentinan-degrading exo-beta-1, 3-glucanase. Curr Genet 48:195–203

Sakamoto Y, Nakade K, Yano A, Nakagawa Y, Hirano T, Irie T, Watanabe H, Nagai M, Sato T (2008) Heterologous expression of lcc1 from *Lentinula edodes* in tobacco BY-2 cells results in the production an active, secreted form of fungal laccase. Appl Microbiol Biotechnol 79:971–980

Sakamoto Y, Nakade K, Nagai M, Uchimiya H, Sato T (2009) Cloning of *Lentinula edodes* lemnp2, a manganese peroxidase that is secreted abundantly in sawdust medium. Mycoscience 50:116–122

Saparrat MC, Guillen F, Arambarri AM, Martínez AT, Martínez MJ (2002) Induction, isolation, and characterization of two laccases from the white rot basidiomycete *Coriolopsis rigida*. Appl Environ Microbiol 68:1534–1540

Scheel T, Höfer M, Ludwig S, 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

Shutova VV, Revin VV, Myakushina YU (2008) The effect of ions on the production of laccase by the fungus *Lentinus* (*Panus*) *tigrinus*. Appl Biochem Microbiol 44:683–687

Smith JF, Fermor TR, Zadrazil F (1988) Pretreatment of lignocellulosics for edible fungi. In: Zadrazil F, Reiniger P (eds) Treatment of lignocellulosics with white rot fungi. Elsevier, London, pp 3–13

Soden DM, Dobson AD (2001) Differential regulation of laccase gene expression in *Pleurotus sajor-caju*. Microbiology 147:1755–1763

Szklarz GD, Antibus RK, Sinsabaugh RL, Linkins AE (1989) Production of phenol oxidase and peroxidases by wood-rotting fungi. Mycologia 81:234–240

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

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

Wood DA, Matcham SE, Fermor TR (1988) Production and function of enzymes during lignocelluloses degradation. In: Zadrazil F, Reiniger P (eds) Treatment of lignocellulosics with white rot fungi. Elsevier, London, pp 43–49

Yoshikawa T, Tanesaka E, Yoshida M (2004) Determination of ligninolytic culture conditions in expression of *Lentinula edodes* manganese peroxidase. Breed Res 6(suppl 1):15 (in Japanese)