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ORIGINAL PAPER

Influence of temperature, pH and metal ions on guaiacol oxidation of purified laccase from *Leptographium qinlingensis*

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Abstract The bark beetle *Dendroctonus armandi* is able to kill living *Pinus armandi* and has caused serious damage to pine forest in Northern China. As the most important symbiotic fungus of *D. armandi*, *Leptographium qinlingensis* plays an important role in the invasion process of the bark beetle. The laccase secreted by it are involved in lignin degradation to provide utilizable nutrition for *D. armandi*, and catalyze some biochemical reactions, causing the damages of tree tissue. In present study, the extracellular laccase of *L. qinlingensis* was purified by using the ammonium sulfate precipitation and DEAE-cellulose (DE-52) column chromatography. Furthermore, the effects of temperature, pH value and metal ions on it were investigated and characterized. The purified enzyme exerted its optimal activity with guaiacol. The catalytic efficiencies K_m and V_{max} determined for substrate guaiacol were 15.4 μM and 372.9 IU mg^{-1}, respectively. The optimum pH and temperature for the purified enzyme was 4.4 and 45 °C, respectively, with the highest enzyme specific activity of 7,000 IU mg^{-1}. Moreover, the metal ions, Co^{2+}, Mn^{2+}, Ca^{2+}, Mg^{2+}, Fe^{2+} and Cd^{2+}, especially Hg^{2+}, showed significantly inhibition effects on its activity. To understand the characteristics of this laccase might provide an opportunity and theoretical basis to promote integrated pest management of *D. armandi*.

Keywords Bark beetle · Symbiotic fungus · Laccase · Purification · Characterization

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Introduction

Bark beetles, especially *Dendroctonus* species, are considered to be serious pests of the coniferous forests and have brought big economic losses (Coulson and Stark 1982). Most bark beetle species engage in symbiotic relationships with fungi (Paine et al. 1997; Klepzig and Six 2004), and rely on fungi to overcome their limited metabolic abilities (Janson et al. 2008; Gibson and Hunter 2010). These symbiotic fungi may be compared to an 'external stomach' of the insect host because of their ability to concentrate large amounts of phloem nutrients (Hulcr and Dunn 2011).

Chinese white pine beetle (*Dendroctonus armandi* Tsai *et* Li, Scolytidae) kills living *Pinus armandi* and has caused serious damage to *P. armandi* forest in the Qinling and Bashan Mountains in Northern China since 1954 (Chen and Tang 2007). As the most important symbiotic fungus associated with *D. armandi*, *Leptographium qinlingensis* plays a key role in the invasion process of beetle to the host pine tree. *D. armandi* can not directly digest and utilize the woody lignin, while, *L. qinlingensis* secrets laccase are involved in lignin degradation and the removal of potentially toxic phenols arising during this degradation to provide utilizable nutrition for *D. armandi*. At the same time, laccase also can catalyze some biochemical reactions, causing the damages of tree tissue, inhibition of the resin secretion as well as destroys of the nutrient and moisture transport channels (Chen and Tang 2007). Above all, laccase secreted by *L. qinlingensis* is a crucial factor for invasion of *D. armandi*.

Laccase (p-diphenol: oxygen oxidoreductase; EC 1.10.3.2) is multi-copper oxidase that catalyzes the oxidation of a wide range of phenolic substrates by coupling them to the reduction of O_2 to water (Téllez-Téllez et al.

2005; Forootanfar et al. 2011; Garg and Tripathi 2011). Moreover, the activity of laccase can be influenced by the temperature, pH value and metal ions (Palmieri et al. 2000; Nagai et al. 2002; Mouso et al. 2003; Dekker et al. 2007; Fonseca et al. 2010). As the important role of this laccase played in the relationship of *D. armandi* and *P. armandi*, the extracellular laccase of *L. qinlingensis* was purified and characterized. In order to save forests, finding new and innovative environmentally friendly approaches in wood-boring insect pest management is more important than ever. This study might provide an opportunity and theoretical basis to promote integrated pest management (IPM) for *D. armandi* control.

Materials and methods

Organism and culture conditions

Leptographium qinlingensis, isolated from harmed *P. armandi* by *D. armandi* in Huoditang forestry region of Qinling Mountains (Tang et al. 2004), the ITS sequence cloned was deposited in Genbank under the accession number KF002408. Three mycelial discs (6 mm in diameter) were inoculated into malt extract agar to produce laccase. Phloem powder of *P. armandi* (6 g L^{-1}) (Wang et al. 2012) and $CuSO_4{\cdot}5H_2O$ (0.6 mM L^{-1}) (Galhaup and Haltrich 2001) were added as inducer of laccase. The flasks were shaken (130 rpm) at 27 °C for 11 days.

Protein concentration and laccase activity assay

The culture liquid was filtered through 8 pieces of sterile gauze and centrifuged at 8,000 rpm for 30 min (4 °C). The culture supernatant was collected and protein concentration was estimated following the method of Bradford (1976) with bovine serum albumin as standard.

Laccase activity was determined by using guaiacol as substrate. 5 ml of the reaction mixture, containing 3.9 ml acetate buffer (10 mM, pH 5.0), 1 mL guaiacol (1.76 mM) and 0.1 mL of the culture supernatant, was incubated at 25 °C for 0.5 h. Subsequently, the absorbance value was detected at 450 nm. In the blank, guaiacol was substituted by acetate buffer (Arora and Sandhu 1985). A unit of enzyme activity was defined as the amount of enzyme oxidizing 1 μmol of guaiacol per min (Nyanhongo et al. 2006).

Laccase purification

Culture supernatant was precipitated by ammonium sulfate from 30 to 80 % saturation. The protein precipitate was collected by centrifugation (8,000 rpm for 20 min, 4 °C), dissolved in sodium acetate buffer (50 mM; pH 5.0) and dialyzed extensively against 10 mM sodium acetate buffer at 4 °C to remove salt, and then dialyzed against 70 % polyethylene glycol 6000 (PEG 6000) for 30 min by using dialysis bags with a 14,000-molecular-weight (MW) cutoff membrane. 10 ml concentrated solution was got, that was concentrated by 10 times. The concentrated enzyme preparation was loaded to a DEAE-cellulose (DEAE-52, Whatman) chromatographic column (1.6 cm × 26 cm) equilibrated with 20 mM Tris–HCl buffer (pH 7.2) overnight. The enzyme fractions were eluted with a linear concentration gradient of 0–0.2 M NaCl at a flow rate of 0.4 ml/min. The eluent was collected every 10 min (Wang and Wang 2008). The absorbance value of each eluent was estimated at 280 nm and their enzyme activities were assayed. Fractions shown laccase activity were pooled, concentrated, dialyzed, and stored at −20 °C, until further use.

Characterization of the purified enzyme

Electrophoretic analyses

The enzyme purity was confirmed by sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE), which was carried out following the protocol of Laemmli (1970) with 5 % w/v stacking gel and 10 % w/v resolving gel and Coomassie brilliant blue R-250 staining. Native-PAGE was proformed under non-denaturing conditions with 6 % w/v gel, and then the gel was stained with guaiacol.

Effects of temperature and pH on enzyme activity

The optimum temperature of the purified laccase was determined in a temperature range from 25 to 75 °C for 0.5 h. In order to investigate the thermal stability of laccase, it was incubated for 2 and 10 h, respectively, and then the residual enzyme activities were assayed. The optimum pH of the purified laccase was tested at citrate–phosphate buffer (pH 2.0–6.8) for 0.5 h. The laccase was then incubated for 1 and 10 h at the same pH range respectively to investigate pH stability by assaying the residual enzyme activities. All the experiments were performed in triplicate.

Effects of acid radical ions and metal ions on the enzyme activity

Effects of acid radical ions of were determined by pre-incubation with the enzyme solution at 25 °C for 0.5 h before the addition of substrate guaiacol. The relative activity was determined under standard assay condition.

Different concentrations (1, 10 and 100 mM) of NaCl, Na_2SO_4, Na_2CO_3 and $NaNO_3$ were used for assay of these effects. As to the effects of metal ions on activity of laccase, $CuSO_4$, $CoCl_2$, $MnCl_2$, $CaCl_2$, $MgSO_4$, NaCl, $FeSO_4$, KCl, $HgCl_2$ and $Cd(NO_3)_2$ were added and their ultimate concentrations were adjust to 1 mM and 10 mM, respectively. After being incubated at 25 °C for 0.5 h, residual activities of laccase were assayed. The experiments were performed in triplicate and the data were assayed by SPSS 19.0. The level of significance was set to $P < 0.05$. Differences between means with $P < 0.05$ were accepted as being statistically significant.

Kinetic studies

The laccase kinetic constants were determined for guaiacol as substrates. Kinetic studies were performed in triplicate and the data obtained fitted to a hyperbola by means of the Michaelis–Menten equation. The K_m and V_{max} values were then determined through non-linear regression analysis using the program Origin 5.0.

Results

Purification of laccase

A summary to every purification procedure of extracellular laccase was shown in Table 1. The total activity and specific activity of laccase in culture filtrate were 709.4 IU L^{-1} and 7.6 IU mg^{-1}, respectively. After being precipitated by ammonium sulfate, the specific activity in concentrated solution was increased 1.7 times, up to 13 IU mg^{-1}. The third procedure made great effect to purify the laccase. Concentrated liquid was through the ion-exchange column chromatography, and three absorption peaks could be seen in 280 nm from the column chromatography figure (Fig. 1a) of DEAE-cellulose (DE-52). Only fractions at the third peak had high laccase activity. 16 ml purified enzyme liquid was got by combining fractions from the 28th to 31st. The purification was 25.6 fold, and the total obtained protein was 0.9 mg. The fractions laccase activity was 5,210 IU L^{-1}, and the specific activity was sharply increased to 195 IU mg^{-1}.

Table 1 Purification of laccase from *L. qinlingensis*

Purification process	Protein (mg)	Total activity (IU)	Specific activity (IU mg^{-1})	Recovery yield (%)	Purification fold
Culture filtrate	93.8	709.4	7.6	100	1
Ammonium sulphate precipitation	22.3	291.3	13	41.1	1.7
DEAE-cellulose column chromatography	0.9	166.7	195	23.5	25.6

Activity values represent means of triplicate measurements (sample mean deviations ≤5 %)

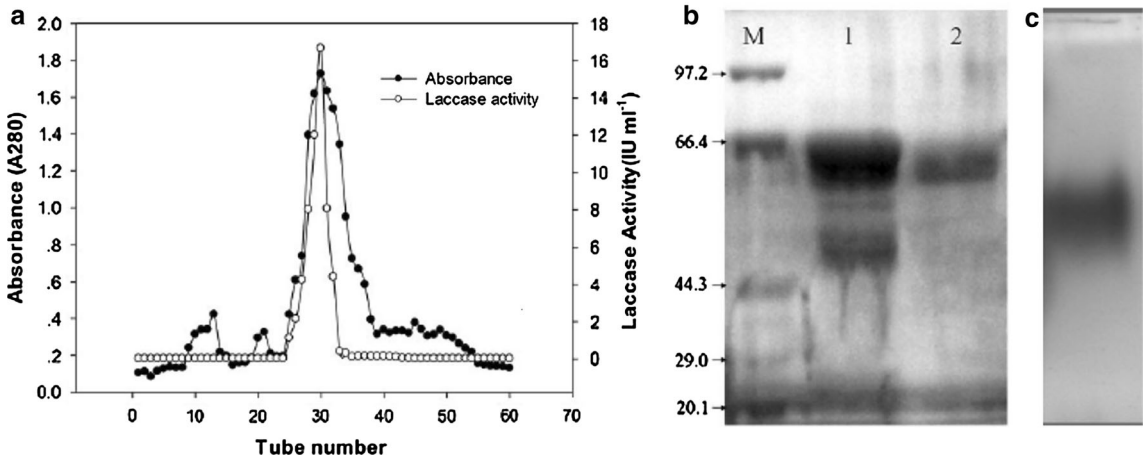

Fig. 1 The purification processes of laccase secreted by *L. qinlingensis*. **a** UV–visible absorption value and laccase activity of each eluent. **b** SDS-PAGE of partial purified laccase. *Lane 1*, culture filtrate; *lane 2*, partial purified laccase; *M* standard protein marker, consisting of phosphorylase B (97.2 kDa), bovin serum albumen (66.4 kDa), ovalbumen (44.3 kDa), carbonic anhydrase (29.0 kDa), soybean trypsin inhibitor (20.1 kDa). **c** Guaiacol stained native gel

Purified laccase showed a single protein band on SDS-PAGE stained with Coomassie blue R-250. The apparent molecular mass of the purified laccase was 61.0 kDa (Fig. 1b). Native-gel under non-denaturing conditions after activity staining with guaiacol revealed that *L. qinlingensis* produced one band displaying laccase activity (Fig. 1c).

Laccase enzymatic properties

The influence of temperature on laccase activity

The optimum temperature of purified laccase was 45 °C and the highest enzyme activity was 5,640 IU L^{-1} (Fig. 2a). This laccase had relative thermostability at 25–35 °C, even it was incubated for 2 h, residual enzyme activities were still more than 90 % of the largest value at both two temperatures. With increasing of the temperature, the residual enzyme activities decreased to different extents. When this laccase was incubated at 45 °C for 2 h, the residual activities decreased 20 %. As to 10 h incubation, obviously, the residual activities were appeared more serious downtrend and significantly lower than 2 h incubation at all of temperature gradient except for 25 °C. When the temperature reached 75 °C, all of the residual relative activities were less than 3 %.

The influence of pH on laccase activity

The activity of laccase reached the highest value at pH 4.4 (6,305 IU L^{-1}) (Fig. 2b). After being kept warm at different pH value for 1 and 10 h, the residual activities were obviously decreased at all of the tested pH conditions, and almost no significantly difference between the two tested time points from pH 2.8–4.4 (Fig. 2b). Even being held for 10 h at pH 4.4, the residual enzyme activity (5,302 IU L^{-1}) still achieved 84.10 % of the maximum.

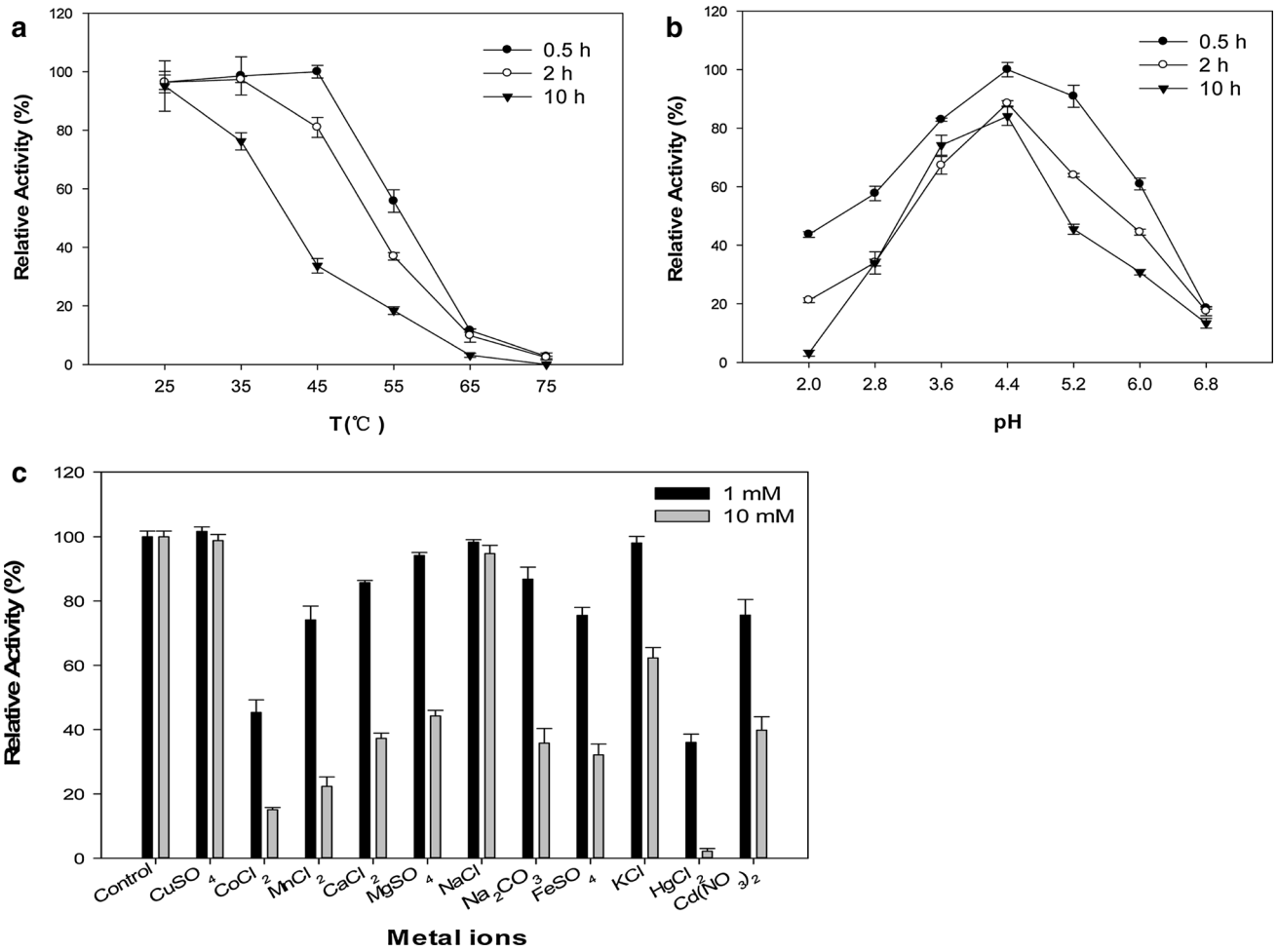

Fig. 2 Effect of temperature,pH and metal ions on enzyme activity (regarding the highest enzyme activity as 100 %). **a** Effect of temperature on enzyme activity. **b** Effect of pH on enzyme activity. **c** Response of partial purified *L. qinlingensis* laccase activity to metal ions at the different concentration. **a**–**b**, the average values and standard errors were presented as data points and *error bars*, respectively; **c** the average values and standard errors were presented as data columns and *error bars*

Table 2 Effect of acid radical ions on the laccase activity

	Relative activity (%)		
	1 mM	10 mM	100 mM
Control	100	100	100
NaCl	97	93	49
Na_2SO_4	102	99	93
Na_2CO_3	86	34	29
$NaNO_3$	97	94	50

However, the residual activity was only 3.13 % after 10 h incubation at pH 2.0.

Kinetic analysis

The apparent K_m value of the enzyme for guaiacol determined from the Lineweaver–Burk plot was estimated to be 15.3 μM, and the corresponding V_{max} value was 372.9 IU mg^{-1}.

Influence of acid radical ions and metal ions on enzyme activity

From Table 2, we could found 1 mM and 10 mM Cl^-, SO_4^{2-}, NO_3^- showed no significant influence ($P > 0.05$) on the laccase activity, while, 100 mM acid radical ions resulted in significant inhibition ($P < 0.05$) of the enzyme activity except for 100 mM SO_4^{2-} ($P > 0.05$).

Different metal ions showed different influence on enzyme activity, and concentrations of these metal ions also appeared dissimilarity (Fig. 2c). Compared to control, the residual laccase activities showed no significant difference ($P > 0.05$) in the presence of 1 mM K^+, 1 and 10 mM Cu^{2+}. While, the others metal ions inhibited laccase activity in different extent, especially for Hg^{2+}, both 1 mM Hg^{2+} (64 % inhibition) and 10 mM Hg^{2+} (98 % inhibition) showing significant deference ($P < 0.001$).

Discussions

This study, the purification fold of laccase secreted by *L. qinlingensis* reached 25.6, confirming the connection of ammonium sulfate precipitation and DEAE-cellulose (DE-52) column chromatography was an effective way for purification of laccase.

The optimal reaction temperature for laccase from *L. qinlingensis* was 45 °C, and the thermal stability was relatively high from 25 to 35 °C, with more than 90 % relative residual activity. Over 35 °C, the higher the temperature was and the longer the heat preservation time was, the lower the activity and stability of the enzyme was. The reason might be the long time heat preservation affected the senior structure of laccase protein and changed its properties. Moreover, laccase secreted by *L. qinlingensis* was suitable to react in acidity conditions, especially at pH 4.4. The closer the reaction pH to this value, the higher the activity was. The stability of laccase activity was higher from pH 2.8–4.4. This might related to the isoelectric point of protein. The pH condition might change the state of disassociation of electriferous groups of the enzyme surface, causing the change in the characteristics of enzyme.

The presence of Cu^{2+} at both concentrations had no significant influence on the purified laccase from *L. qinlingensis*. Similarly, Couto et al. found that both 1 mM and 10 mM Cu^{2+} were no significant effect on laccase stability (Couto et al. 2005). Wang and Wang also reported 5 mM Cu^{2+} had no significant effect on laccase activity derived from *Ganoderma lucidum* Karst Mutant G1502 (Wang and Wang 2008). However, Nagai et al. reported that a purified laccase from the edible mushroom *Lentinula edodes* was activated by 40 % in the presence of 10 mM Cu^{2+} (Nagai et al. 2002). Laccase activity of *Pleurotus ostreatus* was increased in the presence of 0.05–50.0 mM Cu^{2+} (Baldrian and Gabriel 2002). Laccase activity was not significantly inhibited by 1 mM K^+, while significantly restrained by 10 mM K^+ ($P < 0.01$). Same result had been reported by Couto et al. (Couto et al. 2005). Whereas, Nagai et al. reported that laccase was inhibited by 1 mM K^+ (Nagai et al. 2002). On the other hand, Wang and Wang reported that 5 mM K^+ had a positive effect on laccase activity (Wang and Wang 2008). The other metal ions, Co^{2+}, Mn^{2+}, Ca^{2+}, Mg^{2+}, Fe^{2+}, Cd^{2+}, showed significant inhibition effects on laccase activity from *L. qinlingensis* both at low and high concentrations, similar to previous studies (Baldrian and Gabriel 2002; Nagai et al. 2002; Couto et al. 2005). Besides, the addition of Hg^{2+} influenced laccase activity extremely ($P < 0.001$) at both concentrations, with 64 and 98 % inhibition, respectively. Study of Baldrian and Gabriel also indicated the drastic negative effect of low concentration Hg^{2+} on laccase activity of the white-rot fungus *P. ostreatus* (Baldrian and Gabriel 2002). Couto and others found that both 1 mM and 10 mM Hg^{2+} did significantly negative influence on laccase stability (Couto et al. 2005). Metal ions could also influence the degradation reactions by the regulation of other factors affecting biodegradation (Palmieri et al. 2001).

Dendroctonus armandi and its symbiotic fungus *L. qinlingensis* harm the host trees jointly. As precursors, *L. qinlingensis* overcome the host resistance system, destroy the bleeding cells, block up resin ducts, disorder the metabolic system and made wood blue-stain (Tang and Chen 1999). Laccase could stably and effectively act on the lignin composition (Dong et al. 2013) and indirectly

overcome the self-physical defense mechanism of the host (Chen et al. 2004). In order to save forests, finding new and innovative environmentally friendly approaches in wood-boring insect pest management is more important than ever. To understand the characteristics of this laccase might provide an opportunity and theoretical basis to promote IPM of *D. armandi*.

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References

Arora DA, Sandhu DK (1985) Laccase production and wood degradation by a white-rot fungus *Daedalea flavida*. Enzyme Microb Technol 7:405–408

Baldrian P, Gabriel J (2002) Copper and cadmium increase laccase activity in *Pleurotus ostreatus*. FEMS Microbiol Lett 206:69–74

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Chen H, Tang M (2007) Spatial and temporal dynamics of bark beetles in Chinese white pine in Qinling Mountains of Shaanxi Province, China. Environ Entomol 36:1124–1130

Chen H, Tang M, Zhu CJ, Hu JJ (2004) The enzymes in the secretions of *Dendroctonus armandi* (Scolytidae) and their symbiotic fungus of *Leptographium qinlingensis*. Scientia Silvae Sinicae 40:123–126

Coulson RN, Stark RW (1982) Integrated management of bark beetles. In: Mitton JB, Sturgeon KB (eds) Bark beetles in North American conifers. University of Texas Press, Austin, pp 315–350

Couto SR, Sanromán M, Gübitz GM (2005) Influence of redox mediators and metal ions on synthetic acid dye decolourization by crude laccase from *Trametes hirsute*. Chemosphere 58: 417–422

Dekker RF, Barbosa AM, Giese EC, Godoy SD, Covizzi LG (2007) Influence of nutrients on enhancing laccase production by *Botryosphaeria rhodina* MAMB-05. Int Microbiol 10:177–185

Dong XQ, Yang JS, Zhu N, Wang ET, Yuan HL (2013) Sugarcane bagasse degradation and characterization of three white-rot fungi. Bioresource Technol 131:443–451

Fonseca MI, Shimizu E, Zapata PD, Villalba LL (2010) Copper inducing effect on laccase production of white rot fungi native from Misiones (Argentina). Enzyme Microb Tech 46:534–539

Forootanfar H, Faramarzi MA, Shahverdi AR, Yazdi MT (2011) Purification and biochemical characterization of extracellular laccase from the the ascomycete *Paraconiothyrium variabile*. Bioresource Technol 102:1808–1814

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

Garg SK, Tripathi M (2011) Strategies for decolorization and detoxification of pulp and paper mill effluent. Rev Environ Contam Toxicol 212:113–136

Gibson CM, Hunter MS (2010) Extraordinarily widespread and fantastically complex: comparative biology of endosymbiotic bacterial and fungal mutualists of insects. Ecol Lett 13:223–234

Hulcr J, Dunn RR (2011) The sudden emergence of pathogenicity in insect-fungus symbioses threatens naive forest ecosystems. Proc R Soc B: Biol Sci 278:2866–2873

Janson EM, Stireman JO III, Singer MS, Abbot P (2008) Phytophagous insect-microbe mutualisms and adaptive evolutionary diversification. Evolution 62:997–1012

Klepzig KD, Six DL (2004) Bark beetle-fungal symbiosis: context dependency in complex associations. Symbiosis 37:189–205

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Mouso N, Papinutti L, Forchiassin F (2003) Efecto combinado del cobre y pH inicial del medio de cultivo sobre la producción de lacasa y manganeso peroxidasa por *Stereum hirsutum* (Willd) Pers. Rev Iberoam Micol 20:176–178

Nagai T, Ibata K, Park ES, Kubota M, Mikoshiba K, Miyawaki A (2002) A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications. Nat Biotechnol 20:87–90

Nyanhongo GS, Couto SR, Guebitz GM (2006) Coupling of 2, 4, 6-trinitrotoluene (TNT) metabolites onto humic monomers by a new laccase from *Trametes modesta*. Chemosphere 64:359–370

Paine TD, Raffa KF, Harrington TC (1997) Interactions among scolytid bark beetles, their associated fungi, and live host conifers. Ann Rev Entomol 42:179–206

Palmieri G, Giardina P, Bianco C, Fontanella B, Sannia G (2000) Copper induction of laccase isoenzymes in the ligninolytic fungus *Pleurotus ostreatus*. Appl Environ Microbiol 66:920–940

Palmieri G, Bianco C, Cennamo G, Giardina P, Marino G, Monti M, Sannia G (2001) Purification, characterization and functional role of a novel extracelluar protease from *Pleurotus ostreatus*. Appl Environ Microbiol 67:2754–2759

Tang M, Chen H (1999) Effect of symbiotic fungi of *Dendroctonus armandi* on host trees. Scientia Silvae Sinicae 35:63–66

Tang M, Chen H, Zhao JP, Zhu CJ (2004) *Leptographium qinlingensis* sp. nov. associated with *Dendroctonus armandi* in *Pinus armandi*. J Huazhong (Central China) Agric Univ 23:5–6

Téllez-Téllez M, Sánchez C, Loera O, Díaz-Godínez G (2005) Differential patterns of constitutive intracellular laccases of the vegetative phase of *Pleurotus* species. Biotechnol Lett 27:1391–1394

Wang SL, Wang QB (2008) Purification and enzymology characteristics of laccase derived from *Ganoderma lucidum* Karst Mutant G1502. Food Sci 29:287–291

Wang L, Chen H, Hu X, Ma C (2012) Laccase activity of *Leptographium qinlingensis* under different culture conditions. Scientia Silvae Sinicae 48:164–167