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Full Length Research Paper

Purification, characterization and application of laccase from *Trametes versicolor* for colour and phenolic removal of olive mill wastewater in the presence of 1-hydroxybenzotriazole

**Rosana C. Minussi[1,2], Márcio A. Miranda[3], José A. Silva[4], Carmen V. Ferreira[3], Hiroshi Aoyama[3], Sérgio Marangoni[4], Domenico Rotilio[5], Gláucia M. Pastore[1] and Nelson Durán[2,6]*

[1]Faculdade de Engenharia de Alimentos Universidade Estadual de Campinas, Campinas - SP, Brazil
[2] Instituto de Química Universidade Estadual de Campinas, Campinas - SP, Brazil
[3]Instituto de BiologiaUniversidade Estadual de Campinas, Campinas - SP, Brazil
[5]Consorzio Mario Negri Sud, "Gennaro Paone" Environmental Health Center Santa Maria Imbaro, Italy.
[6]Núcleo de Ciências Ambientais, Universidade de Mogi das Cruzes, Mogi das Cruzes - SP, Brazil.

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Laccase forms (L1 and L2) from *Trametes versicolor* CCT 4521 showed a molecular mass of 66 kDa and optimum temperature around 40°C. The optimum pH (4.0 and 5.0) and K_m (28.6 and 5 µM) values using syringaldazine as substrate were found for L1 and L2, respectively. The enzymes were able to oxidize several compounds and were strongly inhibited by sodium azide, L-cysteine and dithiothreitol. The 75% of the N-terminal sequences were identical in both forms and similarities around 40 - 60% of laccases from wood-degrading fungi were observed. The use of 1-hydroxybenzotriazole as a mediator increased the compounds oxidized by laccases in olive mill wastewater.

Key words: Laccase, *Trametes versicolor*, 1-hydroxybenzotriazole, mediator, olive mill wastewater.

INTRODUCTION

Laccase (benzenediol: oxygen oxidoreductases; EC 1.10.3.2) is an enzyme that oxidizes a large variety of organic substrates. The enzyme is widely distributed in fungi; however its biological function is still not totally clarified (Duran et al., 2002; Gianfreda and Rao, 2004). Fungal laccases have been implicated in sporulation, rhizomorph formation, pathogenesis, formation of fruity bodies and lignin degradation (Mayer and Staples, 2002). There has been great interest in using fungal laccases for biotechnological processes due to their chemical and catalytic features (Heinzkill et al., 1998; Minussi et al., 2002). In recent years, interest in laccases has been fuelled by their potential uses in detoxification of environmental pollutants, prevention of wine decoloration, paper processing, enzymatic conversion of chemical intermediates and production of useful chemicals from lignin (Robles et al., 2000; Minussi et al., 2003).

Comparative studies of fungal laccases have shown that these enzymes are similar in their catalytic activity with phenolic compounds, regardless of their origin, but differ markedly in their inducibility, number of enzyme forms, molecular mass and optimum pH (Fortina et al., 1996; Minussi et al., 2006). Laccase alone has a limited effect on bioremediation due to its specificity for phenolic subunits in lignin. Bourbonnais and Paice (1990) found that the range of substrate catalysed by laccase can be extended to non phenolic subunits of lignin by inclusion of a mediator, such as 2, 2'-azinobis (3-ethylbenzthiazoline-6-sulfonate) (ABTS). The laccase-ABTS couple has been shown to effectively demethylate and delignify kraft pulp (Bourbonnais and Paice, 1992). Furthermore, 1-hydroxybenzotriazole (HBT) has been found to mediate pulp delignification in the presence of laccase (Call, 1994),

*Corresponding author. E-mail: duran@iqm.unicamp.br. Tel: (0055) 19-3521-3149. Fax: (0055) 19-3521-3023.

phenothiazines have been found to bleach and prevent redeposition of azo dyes in textile manufacturing (Schneider and Pedersen, 1995) and 3-hydroxyanthranilic acid was observed to oxidize non phenolic lignin model compounds (Eggert et al., 1996ab). The use of a laccase–mediator system is one of the promising possibilities for an environmentally benign pulp-bleaching process (Li et al., 1998) and decolorization of textile dyes (Soares et al., 2001; Durán and Durán, 2000). New mediators such as hydroxamic acids derivatives and N-hydroxycetanilide and others has been previously discussed in the literature (Minussi et al., 2007)

In the extraction process of the olive oil production, the following two by-products are obtained along with the oil (which accounts for 20% of the total): a solid residue (30% of the total) and a black wastewater (50% of the total) called olive mill wastewater (OMW). Most of the solid residue is used as fuel, but the OMW is an environmental problem for Mediterranean countries (Pérez et al., 1998). An important step in the degradation of OMW is the breakdown of colored polymeric phenolics (decolorization) to monomers, which can subsequently be mineralised.

Previous studies (Minussi et al., 2001; Cordi et al., 2006; Minussi et al., 2006) have shown that *Trametes versicolor* CCT 4521 was the best laccase producer in a liquid medium containing 2, 5-xylidine as inducer in the presence of copper sulphate and produced different isoenzymes. *T. versicolor* was also able to produce laccase isoenzymes in submerged culture medium depending on the lignocellulose material employed with ratio of activity Lac II/Lac I from 0.9 (barley straw) to 4.4 (grapes stalks) (Moldes et al., 2004). For *Trametes* sp. I-62 (Arana-Cuenca et al., 2004) with veratryl alcohol induction, a main laccase band which had an apparent molar mass which was similar to that of *T. versicolor* induce by 2,5-xylidine (Lac I 67 kDa and Lac II 70 kDa) (Bourbonnais et al., 1995) was found.

The aim of this work was to develop a procedure for the purification of extracellular laccases from *T. versicolor* CCT 4521, to determine the individual physicochemical and catalytic properties of these enzymes and to study the effects of laccase-mediator system on the treatment of olive mill wastewater, since no study in this direction has been published.

MATERIALS AND METHODS

Fungal strain, culture conditions and laccase assay

T. versicolor CCT 4521 is a white-rot fungus isolated in Brazil by Dr. Elisa Esposito (NCA-Universidade de Mogi das Cruzes, S.P., Brazil). This fungal strain was maintained on malt extract agar plates at 28ºC. Laccase was obtained by growing the fungus in a liquid medium containing (g/L): peptone, 10; malt extract, 5; $CuSO_4 .5H_2O$, 0.005 and glucose, 20; at pH 5.4. Laccase induction consists of the addition of 1.0 mM 2, 5-xylidine at 96 h of growth. The 1 L Erlenmeyer flasks containing 200 ml of culture medium were inoculated with 8 fungal disks (7 mm diameter) and incubated for 20 days at 28ºC and 240 rpm on a rotary shaker.

The enzymatic activity was assayed by measuring oxidation of syringaldazine at 525 nm for 5 min (Leonowicz and Grzywnowicz, 1981). The assay mixture contained 0.1 ml of 1.0 mM syringaldazine, 0.3 ml of 50 mM citrate-phosphate buffer (pH 5.0) and 0.6 ml of culture filtrate. One unit of laccase activity was defined as 1 µmol of syringaldazine oxidized per min per litre of the culture filtrate. The effect of pH on laccase activity was measured over the range of 3.0 to 6.0 in 50 mM citrate-phosphate buffer and 7.0 to 8.0 in 50 mM HEPES buffer. The effects of temperature on enzyme activity and stability (after the incubation time of 20 min) and the optimum pH of each laccase isoform with 50 mM citrate-phosphate buffer in a range of 25 to 80^{o}C were measured. All spectrophotometric measurements were carried out on an Agilent 8453E UV-visible Spectrophotometer System coupled with temperature control. All values represent the mean of duplicate measurement with sample mean deviation of less than 5%.

Laccase activity with different substrates was determined by monitoring the absorbance with a UV-Vis spectrophotometer following the addition of the following substrates: ABTS, catechol, guaiacol, hydroquinone (Ogel et al. 2006), 2, 6-dimethoxyphenol (Tetsch et al., 2006) and o-dinisidine (Irzua et al., 1995).

Purification of extracellular laccase

The fungal culture filtrate (380 mesh sieve) was frozen, thawed, filtrated (Millipore 0.45 µm) and lyophilized. The concentrated crude extract was applied to an ion exchange DEAE Sephadex A-50 column (4 by 20 cm; Pharmacia Biotech, Uppsala, Sweden) equilibrated with 0.05 M citrate phosphate buffer, pH 5.0. Fractions containing laccase activity were eluted with the equilibrating buffer in two distinct peaks (L1 and L2). The fractions corresponded to L2 peak were lyophilized and loaded onto a gel filtration Sephacryl S-200-HR column (2.5 by 95 cm; Sigma) equilibrated with the same buffer containing 0.15 M NaCl in a FPLC System (Pharmacia). Laccase containing fractions were pooled, concentrated, dialyzed and stored at -20^{o}C until further use.

Determination of protein concentration and carbohydrate content

Protein concentrations were determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard. The protein concentration of the fractions collected during chromatography was estimated by the absorbance at 280 nm. The neutral carbohydrate content of laccase was determined according to the method of Dubois et al. (1956) using mannose as standard.

Gel electrophoresis and molecular mass

The molecular mass under native conditions was estimated by gel filtration chromatography (Superdex 200, Pharmacia). Markers with molecular masses ranging from 14 to 94 kDa were used as standards. Apparent molecular mass under denaturing conditions and subunit composition of the laccase were estimated by 15% SDS-PAGE according to the method of Laemmli (1970). Purified laccase isoenzymes were subjected to SDS-PAGE under both reducing (0.1 M DTT) and non-reducing conditions. The gel was stained with Coomassie Blue R-250. Native 12.5% polyacrylamide gel was used for carbohydrate content determination using periodic acid-Schiff's reagent as described by Korn and Wright (1973). Albumin and asialofetuin were used as glycosylated controls (positive staining) and trypsin was used as non-glycosylated control.

Amino acid composition and N-terminal amino acid sequence

The purity of the laccase isoenzymes was also assessed by reversed-phase HPLC analysis conducted on a µ-Bondapack C18 column ((Waters System) (0.39 x 30 cm, 5µm) (Marangoni et al., 1995). The column was preequilibrated with 0.1% trifluoroacetic acid (TFA, v/v) in water (solvent A) and the elution program was as follows: isocratic elution in 1005 A for 80 min followed by a three-steps gradient of acetonitrile in 0.1% TFA (0 - 45% in 10 min, 45 - 74% in 70 min, and 75 - 100% in 75 min). The elution profile was monitored at 280 nm.

Amino acid analysis was performed with a Pico-Tag amino acid analyzer (Waters System). The purified sample was hydrolyzed with 6N HCl containing 1% phenol (v/v) at 106°C for 24 h. Hydrolyzates reacted with 20 µL of fresh derivatization solution (v/v, 7:11:1:1; ethanol: triethylamine: water: phenylisothiocyanate) for 1 h at room temperature. The phenylthiocarbamyl (PTC) amino acids were identified by HPLC.

A sample of 500 pmoles of the enzyme was used to determine its N-terminal sequence in an automatic Edman sequenciator (Applied Biosystems model 477A sequencer). Phenylthiohydantoin (PTH) amino acids were identified in a model 120-A PTH-amino acid analyzer, according to the retention times of a 20 PTH-amino acid standard. The sequence was submitted to automatic alignment, which was performed using the NCBI-Blast search system.

Substrate specificity, inhibition and kinetic parameters

Spectrophotometric measurements of substrate oxidation by *T. versicolor* laccases were carried out at 40°C in a 1 mL reaction volume containing the test substrates in 50 mM citrate-phosphate buffer (optimal pH of each laccase). The concentrations of both enzymes were adjusted to give the same oxidation rate of ABTS. The effect of potential inhibitors of the laccase activity was monitored with 0.1 mM syringaldazine as substrate in 0.05 mM citrate phosphate buffer (pH optimal of each laccase). Michaelis constant (K_m) was calculated using syringaldazine as substrate (concentrations between 4 and 100 µM) from a Lineweaver-Burk plot.

Olive mill wastewater treatment

The fungal culture filtrate (Millipore 0.45 µm) was lyophilized, resuspended in 50 mM citrate-phosphate buffer (pH 5.0) and precipitated with 90% ammonium sulfate. The enzyme was eluted in a gel chromatography column (Sephacryl S-200, Sigma) and lyophilized. The laccase activity in a liquid stock solution was around 100,000.0 U/L

The olive mill wastewater (Abruzzo, Italy) was treated with laccase (2,000.0 U/L) in the presence and absence of the mediator 1-hydroxybenzotriazole (HBT) at 0.5 mM (0.07 g/L) concentration (mass ratio of mediator to phenolic compounds is over 30). Total phenols (2.1 g/L) (Clesceri et al., 1989) and colour (Livernoche et al., 1983) (initial colour units: 8.2) were monitored from 0 to 24 h of treatment. During the treatment the effluent was kept in the dark with slow agitation.

RESULTS AND DISCUSSION

Laccases were purified to homogeneity according to the procedure summarized in Table 1. Two chromatographic steps were required to purify the laccases. DEAE Sephadex A-50 chromatography removed a large amount of the brown-coloured pigments and yielded two distinct fractions with high laccase activity (L1 and L2). L1 fraction was absent of pigments or another enzymes. The L2 fraction with the presence of brown pigments was further purified to homogeneity with a gel filtration chromatography. The overall yield of the purification was around 38% with a purification fold of 35 for L2 and 8 for L1.

The molecular mass of purified laccases was found to be approximately 66 kDa, as determined by calibrated gel filtration and SDS-PAGE (Figure 1a). Native PAGE of crude extract (Figure 1b) and purified laccases (Figure 1c) showed the presence of pure isoenzyme L1 and L2.

This value is consistent with most fungal laccases. Laccases showed activity after SDS-PAGE. This was reportted for another laccase and may be caused by the SDS protection (Gonçalves and Steiner, 1996). The enzymes were found to be glycoprotein (data not shown) containing a carbohydrate content of approximately 7%.

A typical blue laccase normally contains three types of copper sites. The purified laccases had a UV-Visible spectrum with peaks of maximal absorption around 597 (L1) and 613 nm (L2), corresponding to the type-1 copper site, which is responsible for the deep blue colour of the enzymes.

The effect of pH value on the activity was examined in the range 3.0 - 8.0, using syringaldazine as substrate. L1 showed to be active in acidic pH range, with an optimum at pH 4.0, whereas a relative activity of 32.3 and 10.3% at pH 3.0 and 6.0 respectively, were found. The optimum pH for L2 was 5.0, with residual activity of 68.7 and 29.0% at pH 4.0 and 6.0, respectively. Both forms showed to be inactive at pH 8.0. Both isoenzymes exhibited an optimum temperature at 40°C and relative activities at 60 and 70°C were 65.0 and 37.0%, respectively. Stability experiments showed that L1 was stable at 60°C retaining 100% activity after 20 min incubation while the amount of residual activity at 70°C amounted to 47%. On the other hand, the L2 isoenzyme was less stable retaining only 28.1% initial activity upon 20 min incubation at 60°C. Incubation of L1 at 80°C and L2 at 70°C for 20 min caused a complete loss of activity.

The substrate specificity of purified laccases was studied with several compounds. Like other laccases (Koroljova et al., 1999; Jung et al., 2002), L1 and L2 were able to oxidize a variety of phenolic compounds, including simple diphenols (hydroquinone, catechol), methoxy-substituted monophenols (guaiacol, 2, 6-dimetoxyphenol) and other substrates such as ABTS and O-dianisidine, including syringaldazine which is considered a specific substrate for laccase. As expected for a laccase-like enzyme, no activity towards tyrosine was observed. The Lineweaver-Burk plot yielded a K_m value of 28.6 µM for L1 and 5 µM for L2 using syringaldazine as substrate. Compared with the laccase isoenzymes isolated from *T. versicolor* (ATCC 20869) induced by 2, 5-xylidine (Bourbonnais et al., 1995), the reactivity of syringaldazine is

Table 1. Purification of extracellular laccases by *Trametes versicolor* CCT 4521.

Purification step	Volume (ml)	Total protein (mg)	Total activity (U)	Sp. Act. (U/mg)	Yield (%)	Purification (fold)
Culture filtrate	200.0	762.9	2,227.7	2.9	100.0	
DEAE-Sephadex A-50 (L1)	58.0	5.4	127.3	23.6		8.1
DEAE-Sephadex A-50 (L2)	58.5	221.0	898.9	4.1	46.1[a]	1.4
Sephacryl S-200 (L2)	61.0	7.2	727.3	101.0	38.4[b]	34.8

[a]This value is the yield percentage for DEAE-Sephadex A-50 purification step (L1 and L2). The laccase activity was carried out by syringaldazine.
[b]This value is the yield percentage for DEAE-Sephadex A-50 (L1) and Sephacryl S-200 (L2) purification steps.

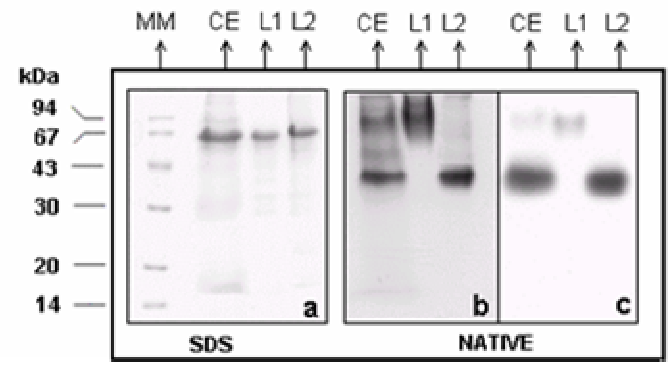

Figure 1. Polyacrylamide gel electrophoresis of extracellular laccases by *T. versicolor* CCT 4521. SDS-PAGE (a) stained with Coomassie Blue R-250 for protein. MM, molecular mass markers; CE, crude extract. Native PAGE of crude extract and purified laccases stained with Coomassie Blue R-250 for proteins (b) and with 0.1 mM ABTS in citrate-phosphate buffer, pH 5.0, for laccase activity (c).

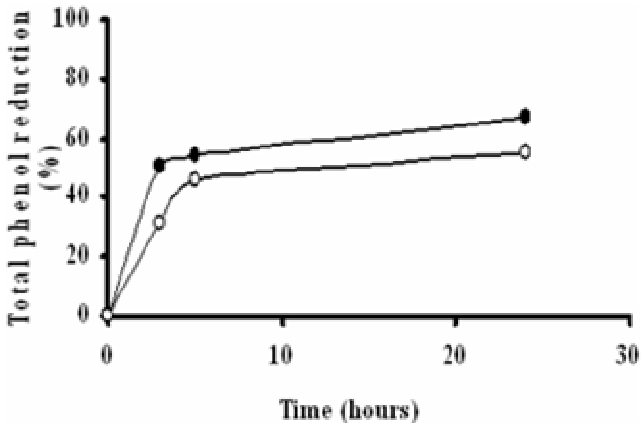

Figure 2. Total phenol of OMW treated with laccase in the presence (●) and absence (○) of 0.5 mM HBT.

completely different from that of the present strain (laccase activity in our case was 36.6% (Table 2) and 77% in Bourbonnais et al. (1995) compared with ABTS (100%) reactivity for L2 isoenzyme).

The inactivation of laccases by various concentrations of potential inhibitors is shown in Table 3. The purified laccases were strongly inhibited by sodium azide, L-cysteine and dithiothreitol, whereas EDTA affected laccase activities to a lesser extent.

Table 4 presents the amino acid composition of laccases which are in agreement with other laccases (Froehner and Erisson, 1974; Koroljova et al., 1999) but not completely with Bourbonnais et al. (1995). In order to acquire information on the primary structure of the proteins, their N-terminal sequence was determined. N-terminal sequences of L1 and L2 isoenzymes showed a 75% similarity (Table 5).

Olive-mill wastewaters, arising from olive oil extraction plants, are characterized by both high organic loads and significant ecotoxicity towards several biological systems (Lolos et al., 1994). Two opposite approaches that are conventionally used with this effluent mainly include sewage disposal and spreading on soils. In this respect, a drastic reduction in phenol concentration is a prerequisite for both techniques. The treatment of OMW with semi-purified laccase by *T. versicolor* CCT 4521 indicated a significant total phenol reduction in the mediator (HBT) presence and absence, although the kinetic of phenolic reduction was faster and higher in the presence of HBT (Figure 2A). In 3 h of treatment, 50 % and 30% of phenollic reductions were observed in the presence and absentce of HBT, respectively. Phenolic reductions around 67 and 55% were observed after 24 h in the presence and absence of HBT, respectively. The presence of HBT also enhanced colour reduction of OMW and the results are shown in Figure 3. Colour removals of 27 and 40% were achieved in the presence of HBT, whereas only 7 and 15% were observed in the absence of this mediator after 5 and 24 h of treatment, respectively.

The efficiency of laccase in polyphenol reduction of OMW is known (D'Annibale et al., 1998, 1999, 2000; Dias et al., 2004), but there is no report about the laccase-mediator system applied in this kind of effluent. In previous reports, laccases by *T. versicolor* CCT 4521 was able to decolorize some reactive dyes in free and immobilized forms in the presence of HBT (Peralta-Zamora et al., 2001). Although, the laccase-HBT system did not inc-

Table 2. Substrate specificity of laccases by *Trametes versicolor* CCT 4521.

Substrate	Conc. (mM)	ϵ_{max} ($M^{-1}cm^{-1}$)	Wavelength (nm)	Laccase activity (%)[a]	
				L1	L2
ABTS	1.0	36,000	420	100.0	100.0
Hydroquinone	1.0	17,542	248	85.1	88.8
2,6-Dimetoxyphenol	1.0	35,645	470	44.1	40.3
Guaiacol	1.0	6,400	436	31.2	21.3
Syringaldazine	0.1	65,000	525	23.1	36.6
Catechol	1.0	2,211	392	22.3	17.3
o-Dianisidine	0.1	29,400	460	9.9	12.6
Tyrosine	1.0	-	280	ND[b]	ND

[a]All values represent the mean of duplicate measurements with a sample mean deviation of less than 5%.
[b]ND, not detected.

Table 3. Effect of inhibitors on the oxidation of syringaldazine by purified *T. ver*sicolor CCT 4521 laccases.

Compound	Concentration (mM)	Inhibition (%)[a]	
		L1	L2
Sodium azide	0.0001	16.0	14.3
	0.0005	62.7	20.0
	0.0010	100.0	30.3
	0.0100	100.0	75.6
	0.1000	100.0	100.0
EDTA	50.0	8.4	25.3
L-Cysteine	0.05	73.0	88.1
	0.10	86.9	91.7
	0.50	100.0	100.0
Dithiothreitol	0.05	49.8	87.7
	0.10	66.4	95.0
	0.50	100.0	100.0

[a]All values represent the mean of duplicate measurements with a sample deviation of less than 5%.

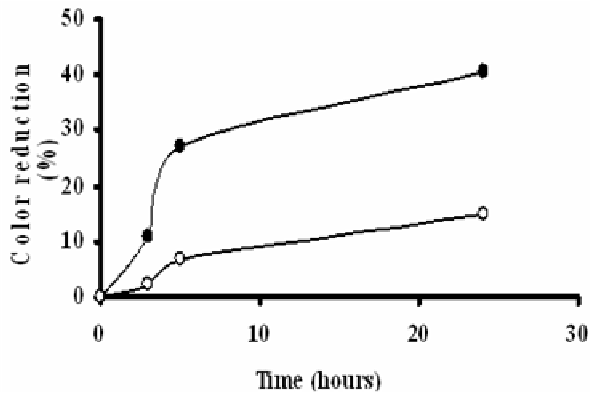

Figure 3. Colour reduction of OMW treated with laccase in the presence (●) and absence (○) of 0.5 mM HBT.

rease phenol and colour reduction in paper and pulp effluent (E_1 effluent) (Minussi et al., 2001). In that case, the laccase-acetohydroxamic acid appears as the best system (Minussi et al., 2007).

Thus, laccases by *T. versicolor* CCT 4521 cultivated in the presence of 2, 5-xylidine were purified by an easy and fast process. The purified laccases physicochemical and catalytic properties are similar to those of analogous enzymes of other basidiomycetes and they have high potential for industrial application, mainly in the immobilized form. These preliminary results open the possibility to apply laccase-mediator system in phenolic and colour reduction of olive mill wastewater.

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Table 4. Amino acid composition of purified *T. versicolor* CCT4521 laccases.

Amino acid	Number of amino acid residues/molecule of laccase	
	L1	L2
Asp	63	68
Glu	38	33
Ser	43	49
Gly	45	55
His	17	16
Arg	18	12
Thr	44	39
Ala	56	56
Pro	42	48
Tyr	17	6
Val	40	46
Met	6	3
Cys	3	0
Ile	31	31
Leu	51	28
Phe	49	94
Lys	18	9

Table 5. Comparison of N-terminal amino acid sequences of *T. versicolor* CCT4521 and other fungal laccases.

Microorganism	Reference	N-terminal amino acid sequence
T. versicolor CCT4521 (L1)		**A I G P V A L L A** T **A P N A A** V **P D G F L R A A V V** S V
(L2)		**A I G P** K **A L L A** D **A P N A A** D **P D G** I **L R A A V** F V N
T. villosa (L1)	**yaver**	G **I G P V A** D **L** T I T N A **A** V S **P D G F** S **R** Q **A V V** V N
(L2 and L3)		**A I G P V A** D **L** V V **A** N A P V S **P D G F L R** D **A** I **V** V N
T. versicolor (I)	Bour.	**A I G P V A** S **L** V V **A** N A P V S **P D G F L R** D **A** I **V** V N
(II)		G **I G P V A** D **L** T I T D A **A** V S **P D G F** S **R** Q **A V V** V N
Coriolus hirsutus	kojima	A **I G P** T **A** D **L** T I S N A E V S **P D G F** A **R** Q **A V V** V N
Pycnoporus cinnabarinus	eggert	**A I G P V A** D **L** T L T N A **A** V S **P D G F** S
Ceriporiopsis subvermispora	fukushima	**A I G P V** T D I E I T D A F V S **P D G** P
Pleurotus ostreatus	palmieri	**A I G P** A G N M Y I V N E D V S **P D G F**
Phlebia radiata	saloheimo	S **I G P V** T D F H I V N A **A** V S **P D G F**

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