

Research Paper

Cloning, characterization and expression of a novel laccase gene *Pclac2* from *Phytophthora capsici*

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Abstract

Laccases are blue copper oxidases (E.C. 1.10.3.2) that catalyze the one-electron oxidation of phenolics, aromatic amines, and other electron-rich substrates with the concomitant reduction of O₂ to H₂O. A novel laccase gene *pclac2* and its corresponding full-length cDNA were cloned and characterized from *Phytophthora capsici* for the first time. The 1683 bp full-length cDNA of *pclac2* encoded a mature laccase protein containing 560 amino acids preceded by a signal peptide of 23 amino acids. The deduced protein sequence of PCLAC2 showed high similarity with other known fungal laccases and contained four copper-binding conserved domains of typical laccase protein. In order to achieve a high level secretion and full activity expression of PCLAC2, expression vector pPIC9K with the *Pichia pastoris* expression system was used. The recombinant PCLAC2 protein was purified and showed on SDS-PAGE as a single band with an apparent molecular weight ca. 68 kDa. The high activity of purified PCLAC2, 84 U/mL, at the seventh day induced with methanol, was observed with 2,2'-azino-di-(3-ethylbenzothiazolin-6-sulfonic acid) (ABTS) as substrate. The optimum pH and temperature for ABTS were 4.0 and 30 °C, respectively. The reported data add a new piece to the knowledge about *P. capsici* laccase multigene family and shed light on potential function about biotechnological and industrial applications of the individual laccase isoforms in oomycetes.

Key words: *Phytophthora capsici*, laccase, expression, purification, activity.

Introduction

Like fungi, oomycetes have a global distribution and prosper in diverse environments. The oomycetes belong to the kingdom Stramenopile and contain a lot of pathogenic species (Baldauf *et al.*, 2000). The genus *Phytophthora* contains a large number of phytopathogens, such as *P. capsici*, *P. infestans*, *P. sojae*, and so on. And most *Phytophthora* species are the cause of blight, crown rot, as well as stem, leaf, and fruit lesions on many plants. Moreover, the *P. capsici* has been identified as a devastating pathogen on solanaceous and cucurbitaceous hosts including pepper, cucumber, eggplant, squash, pumpkin, tomato, melon, and zucchini (Lamour and Hausbeck 2004).

Laccase (EC 1.10.3.2) is a group of enzymes called blue copper oxidases capable of oxidizing phenols and aromatic amines by reducing molecular oxygen to water. They are widely distributed in nature and have been found in

plants, fungi, bacteria and insects and believed to be involved in lignin degradation and lignin biosynthesis (Gianfreda *et al.*, 1999). Recent studies about biological function of fungal laccases have suggested this enzyme maybe play an important role in fungal morphogenesis, fungal virulence and pigmentation (Litvintseva and Henson 2002; Baldrian 2006). Laccases are also very valuable enzyme in the global carbon cycle and industrial applications, such as biodegradation of lignin without polluting the environment, degrading a wide range of different recalcitrant compounds, and biological bleaching in paper industry (Gianfreda *et al.*, 1999).

Several laccase genes have been cloned from different fungal sources and heterologously expressed with the specific purpose of using laccase more efficiently in biotechnology (Galhaup *et al.*, 2002; Soden *et al.*, 2002; Xiao *et al.*, 2006). Among many host systems, the *Pi. pastoris* has been considered as an excellent host system widely

used in the basic research and industrial application. The *Pi. pastoris* expression system offers ease of genetic manipulation, strong inducible alcohol oxidase1 (AOX1) gene promoter, the ability to perform complex eukaryotic post-translational modifications, capacity to grow at very high cell densities, and only secreting small amount of intrinsic protein. In fact, heterologous expression has been often used as a strategy to get around the problem of obtaining laccase isoforms not easily producible in natural hosts (Bleve *et al.*, 2008; Pezzella 2009).

Furthermore, a variety of heterologous proteins have been successfully produced to address some targets (Cereghino and Cregg 2000; Piscitelli *et al.*, 2001). For instance, laccase from *Cryptococcus neoformans* have been conducted expression to ascribe a pathogenic function (Williamson *et al.*, 1998). However, to date, there have been no studies in which the laccase gene of *P. capsici* has been expressed in *Pi. pastoris* system. In this study, we characterized a novel laccase gene from *P. capsici* cDNA and performed expression and purification of PCLAC2 in *Pi. pastoris*. Enzyme activity analysis of PCLAC2 showed high activity with ABTS as substrate, which allows further biochemical studies for laccases.

Materials and Methods

Strains, vectors and culture conditions

A virulent *P. capsici* strain Phyc12 was used and maintained at 25 °C on 10% V8 juice agar medium as previously described (Tyler *et al.*, 1995; Feng *et al.*, 2010). The pPIC9K vector and GS115 *Pi. pastoris* strain were used for heterologous expression of *Pclac2* purchased from Invitrogen. Media components including yeast extract, bacto-peptone, yeast nitrogen base and casamino acids were from Difco (Detroit, MI). MD, MM, YPD, BMGY, and BMMY were all prepared following the Invitrogen expression manuals. Restriction enzymes and T4 ligase were purchased from Sigma.

Total RNA isolation

Phyc12 mycelium grown in 10% V8 liquid medium for three days were collected and were ground in liquid nitrogen. Total RNA was extracted using a fungal RNA kit (OMEGA) following the manufacturer's recommendations. The RNA was quantified by measuring absorbance at

260/280 nm with a spectrophotometer and the quality was examined by electrophoresis on a 1.2% formaldehyde agarose gel. 10 µg of total RNA was treated 4 units of Rnase-free DNaseI (Takara) at 37 °C for 30 min, and then was used for reverse transcription by Omniscript RT kit (Qiagen).

Cloning of laccase gene *Pclac2*

Cloning of *Pclac2* gene was achieved using the primers, lac-1 and lac-2 (Table 1), derived from the genome sequence of *P. capsici* (<http://genome.jgi-psf.org/PhycaF7/PhycaF7.download.html>). The primers were designed using Primer Express 3.0 software and the PCR parameters were as follows: 94 °C for 4 min, then 30 cycles of 94 °C for 1 min, 57 °C for 45 s, and 72 °C for 60 s, and then 72 °C for 10 min for a final extension.

Construction of pPIC9K-his6-*Pclac2*

In order to express *Pclac2*, a construct was made by insertion of the DNA encoding the mature peptide of *Pclac2* into the XhoI/NotI sites of the pPIC9K vector. To obtain the mature protein, the *Pclac2* cDNA was used as template with primers Lac-Utag (including XhoI in bold and 6*his tag underlined) and Lac-Dntag (including NotI in bold) (Table 1). PCR parameters were as follows: 94 °C for 4 min, 35 cycles of 94 °C for 1 min, 60 °C for 45 s, 72 °C 1 min, and a final extension at 72 °C for 10 min. The PCR products were digested with XhoI and NotI, and then were cloned in XhoI-NotI-digested pPIC9K to generate expression vector pPIC9K/*Pclac2*.

The recombinant transformants were identified firstly by restriction digestion. And then plasmids with correct insertion and right open reading frame of his6-*Pclac2* in pPIC9K were also confirmed by DNA sequencing.

Transformation and selection of *Pi. pastoris* clones secreting *Pclac2*

Prior to transformation of the *Pi. pastoris* GS115 host strain, the pPIC9K/*Pclac2* was linearized with StuI to direct integration of the expression vector into the AOX1 locus, resulting in a methanol-utilisation positive (Mut⁺) phenotype. The *Pi. pastoris* host strain was then transformed by electro-poration essentially according to the Invitrogen guide-lines. Transformants (His⁺/Mut⁺) were selected for

Table 1 - Primers used in this study.

Primer	Sequence(5'-3')
lac-1	CTCCACTGGCACGGACTCAAGC
lac-2	CCGGCCTCCAAATGCCAATCGATGTGG
Lac-Utag	CGTACTCGAGC ACCACCACCACCACCAC CGACCTTGCACTTATGACTGCGCGT
Lac-Dntag	CGTAGCGGCCGCTACAACGCGTGGATGGTCGAGTTGA
5'-AOX1 3'-AOX1	GACTGGTTCCAATTGACAAGC GCAAATGGCATTCTGACATCC

multiple integrated copies of the vector as described previously (Fairlie *et al.*, 1999) by transferring, 36 h after transformation, His⁺ colonies grown on YPD (1% yeast extract, 2% bactopectone, 2% dextrose) agar plates containing G418 at concentrations of 1-4 mg/ml. Resultant colonies were screened by PCR using 5'-AOX1/3'-AOX1, Lac-UPtag/Lac-DNtag as listed in Table 1. The obtained transformants were grown in 100 mL buffered methanol complex medium (BMMY) in 250 mL flasks and methanol added to a final concentration of 1% (v/v) for 14 days induction at 30 °C. Expression cultures were subjected to 12% SDS-PAGE analysis. The empty vector pPIC9K was used as a control.

Purification of *Pclac2*

For purification, the culture was collected from 1-14 days by centrifuge (Eppendorf) at 8000 g for 10 min. Then the supernatant was treated with (NH₄)₂SO₄ at 70% saturation, the precipitate was collected by centrifugation at 10,000 g for 15 min, dissolved in native binding buffer (containing 50 mM sodium phosphate, 500 mM NaCl, 10 mM imidazole, pH 8.0), and dialyzed overnight against the same buffer. The solution obtained from dialysis was loaded on a Ni-NTA column (Invitrogen, USA) which had been pre-equilibrated with the same buffer. Wash solution was first performed with the wash buffer (containing 50 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole, pH 8.0) followed by elution. Elution was done with elution buffer (containing 50 mM sodium phosphate, 500 mM NaCl, 250 mM imidazole, pH 8.0). The OD₂₈₀ of each fraction was taken using a UV/vis spectrophotometer. The fractions displaying the highest absorbance were analyzed on an SDS-PAGE gel. The product was designated PCLAC2.

Laccase Enzyme Assay

Laccase activity was determined with ABTS as the substrate (Bourbonnais *et al.*, 1998). The nonphenolic dye ABTS is oxidized by laccase to the more stable and preferred state of the cation radical. The concentration of the cation radical responsible for the intense blue-green color can be correlated to enzyme activity and is read at 420 nm (Majcherczyk *et al.*, 1998). Oxidation of ABTS was monitored by determining the increase in A₄₂₀ (ϵ_{420} , $3.6 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$).

The reaction mixture contained 0.5 mM substrate (ABTS), 2.8 mL of 0.1 M sodium acetate buffer of pH 4.0, 100 μL of PCLAC2 and was incubated at 30 °C for 5 min. Absorbance was read at 420 nm in a spectrophotometer against a suitable blank. One unit was defined as the amount of the laccase that oxidized 1 μmol of ABTS substrate per min. Purified PCLAC2 concentration was determined by the dye-binding method using BSA as standard.

Different culture conditions of adding various carbon sources (glucose, cellulose and starch) were tested respec-

tively. The obtained transformants of *Pi. pastoris* GS115 were grown in 100 mL BMMY and 1% (w/v) different carbon sources under the condition described above. Then laccases activity of culture was determined as described above.

The optimum pH was determined using ABTS as substrate in 0.1 M sodium acetate buffer (pH 2.5-7.5). The optimum temperature for the enzyme was estimated by measuring the enzyme activity at various temperatures ranging from 20 °C to 90 °C in 0.1 M sodium acetate buffer (pH 4.0).

Results

Cloning and analysis of laccase gene *Pclac2*

One complete sequence *Pclac2* gene was identified and submitted to GenBank (accession number: JQ683129). The open reading frame of *Pclac2* contains 1683 bp and encodes a polypeptide of 560 amino acid residues with a predicted molecular mass of 62.04 kDa. It contains a signal peptide of 23-amino acid residues, and has six N-glycosylation sites, but does not exhibit an intron. Figure 1 shows the structure of *Pclac2*, which was analyzed using the NCBI online database to confirm that *Pclac2* was homologous to laccases from fungi and other oomycete pathogens. On the basis of alignment of most of the amino acid sequences, *Pclac2* contains four copper-binding conserved domains of typical laccase: CuI (HWHGLNQ), CuII (HGHEEVQ), CuIII (HPFHLHAHSP), and CuIV (MHCHIDWH) as described by Fan *et al.* (2011) and Piontek *et al.* (2002). The deduced amino acid sequence of *Pclac2* protein was aligned with BLAST in the NCBI online database, which showed *Pclac2* protein was closest to *P. infestans* T30-4 (XP_002896810) with an identity of 88%.

Transformation and Selection

By high efficiency transformation of the StuI-linearized pPIC9K-his6-*Pclac2* into the *Pi. pastoris* strains, about 60 His⁺ transformants of GS115 were isolated, among them 45 of GS115 had the ability to grow well on YPD medium containing G418 (2.0 mg/ml or higher). The subsequent Mut⁺ phenotype identification showed that the His⁺G418-transformants obtained from GS115 were all Mut⁺, indicating that the StuI-linearized pPIC9K-his6-*Pclac2* was integrated into the AOX1 sites of *Pi. pastoris* genome. The genomic DNA PCR amplification with the AOX1 primers and the specific primers Lac-UPtag/Lac-DNtag further confirmed the correct integration of pPIC9K-his6-*Pclac2* into the corresponding chromosomal locus of *Pi. Pastoris* (Data not shown).

Expression and Purification of *Pclac2*

A protein (PCLAC2) with an apparent molecular weight about 68 kDa was detected by SDS-PAGE in the

1 ATGAGTCTCAAGCGCTTCTGCTGTAGCATCGGCTTATTCGGCGTCTTATCAACCTT
 1 M S L K R F S A V A S A L F A A S I N I
 61 AGCGCCCGACCTTGTCACTTATGACTGGCGGTTACATCTTTCCAGCGAGTAGCAT
 21 S A A D L V T Y D W R V T S L S S E Y D
 121 GGCGTCTACATCCACTCTCGGAATCAACAACAAGCCGCGCAAGCTGTGATCGAC
 41 G V Y I H S L G I N N K P A D Q A V I D
 181 GTGGAGCTGGCCAAGAATCGAGGTTCCGCTGACCAACGAGCTCGATGAGCCTACGTGT
 61 V E L G Q E V E V R V T N E L D E P T C
 241 CTCCACTGGCAGGACTCAAGCAGCTCGGTACTCAGGAGATGGACGGCAGCTCCGAAATC
 81 L H W H G L K Q L G T Q E M D G T S E I
 301 ACCGAGTGTACATCGACCCGACGGAACGGCTATCTACCGCTCATGCCGACAAGGCG
 101 T Q C H I D P N G T A I Y R F M P D K A
 361 GGCACTTCTGGTGGCAGTACCACATGGCGAGTACGGTTCGGCTTCGTGGTCTCT
 121 G T F W W H S H H M A Q Y A F G L R G P
 421 CTGGTGTCCACGCCCTCGAAATCAACGACAGGACTGGGAGAAGGACATTGACGGGGAG
 141 L V V H A P A N Q R Q D W E K D I D G E
 481 TACAGATTCAATGGCGGATCTGTATCACCAGCATCCGCTGCCAACCCGATGTGGAT
 161 Y T I Q M A D L Y H R H P L P T R M W D
 541 AACATCTGATCAACAACCGTGGCGTTACAATGCACCGGGCAGCTCACCACAACCTC
 181 N I L I N N R G R Y N C T A A A H H N F
 601 ACCGAGTGCACAGACTTCAATCGTTCGAAATCCATTTCCAGGCTGGAAACAAGTAC
 201 T E C T D L Q S F S K F H F Q A G N K Y
 661 CTGCTCCGTCTCAACATGGCGGCTTGTCCCTATCGTCTTCCAGATTGACGACCAC
 221 L L R L I N M A A L S P I V F S I D D H
 721 GAGTCCGCGTGTGCTGCTGACGGTACCACCTCCAGCGCTCAGAGCTCATCAACTCA
 241 E F R V V A A D G D H L Q P S E L I N S
 781 GTCACATGAACCGCGTACGATATCGTGGTGAAGCCAAAGCTAATTCGAAG
 261 V T L N A G Q R Y D I V V E A K A N S K
 841 CAGAACCAATGGTTCCTTGGATGCGTGCATGGACTTCCGAGCTCCGTGGACA
 281 Q N P I G S F W M R A N G L H G L P W T
 901 CGAGGAACGCGCAATCGCTGGCAAGCTACAGTACGAAGGCTTGGTGTAACTCTCT
 301 R G T A A I A G E G Y T Y E G L G V I S
 961 TACGATGCTGAGAGCAGGACCCGACGTACAGAAGCAACAGAACCTGACGACCATC
 321 Y D A E S E T D P T S Q K Q Q N L T T I
 1021 AACGAGTTCGACTTCCTCCCTGGTGCCGATTGCCCTCCGTAAGCTGCTCGGACCGT
 341 N E F D F L P L V P I A L P E A A S D R
 1081 GCTGTGCTACAGTTCAGATGCAAGTGGTAAGGGCCACTTCTCGATCGATGGTGGAGC
 361 A V L Q F K M Q D G K G H F S I D G G D
 1141 TTTAACCACTTCGTCACCCGAGGATCCCCACTTTTCTCGATCGCCGGTGTATGAAG
 381 F N H F V H P E D P P L F S I A G G M K
 1201 ACTGAGGAGCTCCGCCAACGCCAAGCTCGTAAGATCGAGTACGGCAAGCACATTGAG
 401 T E E L P A N A N A R K I E Y G K H I E
 1261 GTGGTACTGGTCAAGTCAAGGACGACACCCGTTCCACATGCACAGCATTCTCCG
 421 V V L V N V K D E Q H P F H M H T H S P
 1321 TGGGTTGTGGGACCGGTGGCTTCGATCGAACAGATTGAGAAGACGAGCTACCCCT
 441 W V V G S G V A S I E Q I Q K N E L P P
 1381 CTCAAGCTGTAAACCGATGACCCGTGACGTCTACTGTCCTCCCTTGCACCTCAGAC
 461 L K L L N P M T R D V Y T V P P C T S D
 1441 GGCAAGGGCGGCTGTGATGCGGTTACCTGGTACTGCGCTTCACTGCTGACAACCCG
 481 G K G G C L D A G Y L V L R F T A D N P
 1501 GCGTGTGGATTTCCACTGCCACATCGATTGGCATTGGAGGCGGTTGTGATGATC
 501 G V W I F H C H I D W H L E A G L S M I
 1561 CTGGTGGAGGGCGAGGCGAACTGCAACAAAAGGGGTCAGTGCCTTCTCAACACTATT
 521 L V E G E A E L Q Q K G V S A F S N T I
 1621 CTCAGTGTGCGGCGCTAACGCAAGTCTCACCCTCAACTCGACCATCCACGCGTTG
 541 L S V C G A N S K F S P S N S T I H A L
 1681 TAG
 561 *

Figure 1 - Nucleotide and deduced amino acid sequences of *pclac2*. Signal peptides are in gray. Four conserved regions are shown in red box, representing CuI domain, CuII domain, CuIII domain, and CuIV domain respectively as previously described. Six potential N-glycosylation sites are underlined.

culture after methanol induction and there was no band appeared in the negative control with empty pPIC9K (Figure 2).

Due to the 6*Histidine tag of PCLAC2, purification of the secreted PCLAC2 was easily achieved in small scale by using the His-bind Ni-NTA purification method. The purified PCLAC2 were then electrophoresed on SDS-PAGE and a novel protein single band of approximate 68 kDa on 12% polyacrylamide gel (Figure 2).

PCLAC2 Activity Assay

The activity of purified PCLAC2 was measured daily in different carbon sources growth at 30 °C from 1-14 days (Figure 3). The maximum laccase activity (approx. 84 U/mL) was obtained after seven-day cultivation with glucose as additional carbon source and better enzyme activity (approx. 76 U/mL) were obtained in BMMY without complement. Cellulose and starch resulted in relatively low laccase activity. However, no extracellular laccase activity was detected in culture supernatants of the negative control (pPIC9K).

The effect of pH on the enzyme was determined using ABTS as substrate in 0.1 M sodium acetate buffer at pH values ranging from 2.5-7.5 (Figure 4A). The optimum pH was for the enzyme was identified as 4.0. Meanwhile, the optimum temperature of the enzyme for ABTS oxidation was 30 °C (Figure 4B), which was estimated by measuring the enzyme activity at various temperatures ranging from 20 °C to 90 °C in 0.1 M sodium acetate buffer (pH 4.0).

Discussion

In the study, we have reported for the first time identified and partially characterized one laccase gene (*Pclac2*) from a plant-pathogenic oomycete, *P. capsici*. This gene

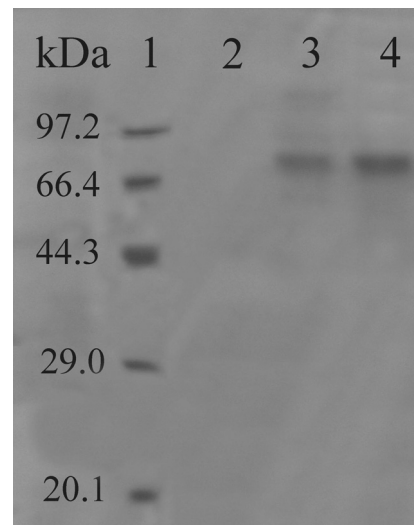


Figure 2 - The recombinant protein expression of PCLAC2 in *Pi. Pastoris* GS115. lane 1: low molecular weight marker; lane 2: pPIC9k (empty vector); lane 3: recombinant protein expression after induction; lane 4: Purified PCLAC2.

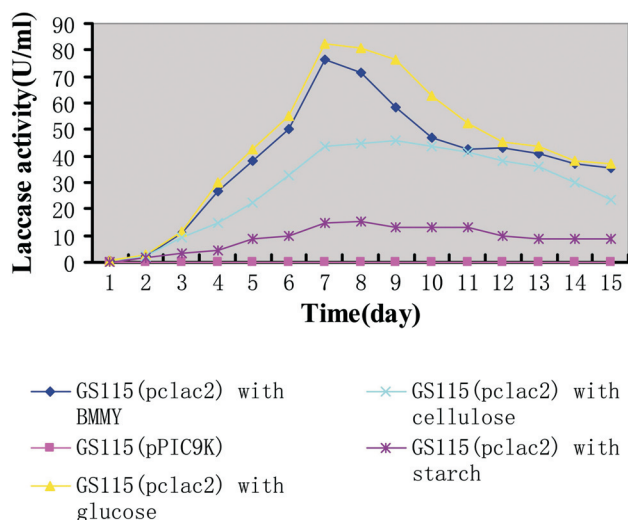


Figure 3 - Effect of various carbon sources on the activity of PCLAC2 in cultures from 1-14 days.

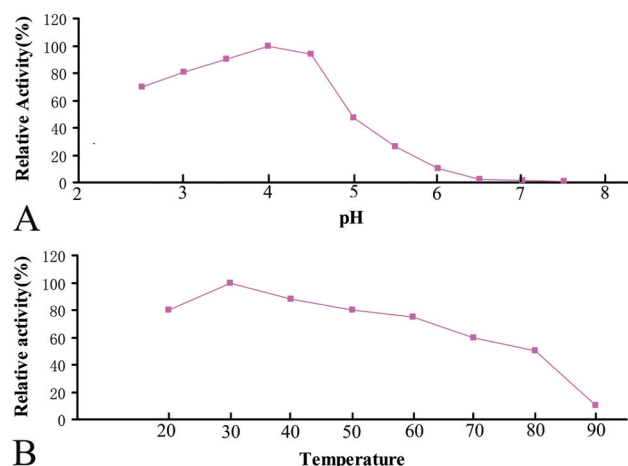


Figure 4 - Effect of pH (A) and temperature (B) on the activity of PCLAC2.

encoding active laccase was confirmed by the successful expression of *Pclac2* in *Pi. pastoris*. Then the protein PCLAC2 was expressed and purified due to the 6*Histidine tag by using the His-bind Ni-NTA purification method. And the analysis of laccase activity showed a highest yield at the seventh day after induced by 1% (v/v) methanol at 30 °C. Also we investigated the optimum pH and temperature of the enzyme for ABTS oxidation.

Based on multiple sequence alignments described previously, four conserved copper oxidases sites (I, II, III, and IV) acted as the copper ligands consisting of 12 amino acids (Larrondo *et al.*, 2003). They contain one type-1 Cu atom (T1), bound as a mononuclear center that gives the proteins their blue color, as well as one type-2 (T2) and two type-3 (T3) Cu atoms, which together form a trinuclear center. The T1 site functions as a primary electron acceptor,

extracting electrons from the substrate. Electrons are then transferred to the T2/T3 center, where reduction of molecular oxygen to water takes place (Ducros *et al.*, 1998; McGuirl and Dooley 1999). According to a recent analysis of the *Coprinus cinereus* laccase crystal structure (Ducros *et al.*, 1998) and site-directed mutagenesis studies of *Myceliophthora thermophila* and *R. solani* laccases (Xu *et al.*, 1998), a pentapeptide segment located downstream of the second conserved histidine in the T1 site (H512 in *Pclac2* Figure 1) has a major effect on the redox potential and specificity of the enzyme. The amino acid composition in this region was unique for *Pclac2* in *P. capsici* (Figure 1), which implied that the enzymes had different substrate specificities and probably different functions in the fungus.

In the present report, we chose the *Pi. pastoris* expression system to express our target gene under the control of the highly methanol-inducible promoter of AOX1, and purification of the secreted protein was easily achieved due to the relatively specific 6*histidine tag. Compared to the other expression systems, the *Pi. pastoris* system not only has the feature of eukaryotic protein synthesis and modification pathway, but also has its own characteristic advantages such as high level expression, proteolytic processing, disulfide bridge formation, and glycosylation (Patrick *et al.*, 2005; Shi *et al.*, 2007). Molecular weights of most fungal laccase proteins fall between 43 kDa and 110 kDa (Xiao *et al.*, 2003). And a majority of laccases from basidiomycete fungi were reported to have molecular weights in the range of 55 kDa to 72 kDa (Petroski *et al.*, 1980). The result of SDS-PAGE results showed that PCLAC2 from *P. capsici* had a molecular weight ca. 68 kDa (Figure 2), while the predicted molecular mass was 62.04 kDa. The difference could be attributed, at least partially, to glycosylation of the protein, as the deduced sequence presents six potential residues for N-glycosylation (Figure 1).

The high activity of purified PCLAC2 was observed with ABTS as substrate as reported for many other laccases like those from *Pycnoporus cinnabarinus* and *Coriolus hirsutus* (Eggert *et al.*, 1996) and for *Trichophyton rubrum* laccase (Jung *et al.*, 2002). The laccase activity was affected by several factors, including culture conditions, pH, temperature, and so on (Hou *et al.*, 2004; Sugareva *et al.*, 2006; Miao *et al.*, 2010;). In our study, the maximum laccase activity was obtained after seven-day cultivation with glucose, while cellulose and starch resulted in low laccase activity (Figure 3). The optimum pH was for PCLAC2 was identified as 4.0 (Figure 4A). Other studies have also reported very low optimal pH (between 3.0 and 5.7) for fungal laccases, except for the laccase from *Rhizoctonia praticola*, which exhibited a neutral optimal pH with various substrates (Park and Park 2008). Meanwhile, the optimum temperature of the PCLAC2 for ABTS oxidation was 30°C (Figure 4B), which was lower than the optimum temperatures previously reported for

other fungal laccases, ranging from 40°C to 50°C (Hou *et al.*, 2004; Park and Park 2008). PCLAC2 is a novel protein with possible biotechnological, industrial, and environmental applications, especially for the enzymatic degradation of aromatic pollutants. Consequently, further studies need to be carried out in order to determine whether the chemical and genetic characteristics of laccase present in *P. capsici* are different from others.

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