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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Molecular cloning and characterization of a novel metagenome-derived multicopper oxidase with alkaline laccase activity and highly soluble expression

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Abstract *Lac591*, a gene encoding a novel multicopper oxidase with laccase activity, was identified through activity-based functional screening of a metagenomic library from mangrove soil. Sequence analysis revealed that *lac591* encodes a protein of 500 amino acids with a predicted molecular mass of 57.4 kDa. Lac591 was over-expressed heterologously as soluble active enzyme in *Escherichia coli* and purified, giving rise to 380 mg of purified enzyme from 1 l induced culture, which is the highest expression report for bacterial laccase genes so far. Furthermore, the recombinant enzyme demonstrated activity toward classical laccase substrates syringaldazine (SGZ), guaiacol, and 2, 6-dimethoxyphenol (2, 6-DMP). The purified Lac591 exhibited maximal activity at 55°C and pH7.5 with guaiacol as substrate and was found to be stable in the pH range of 7.0–10.0. The substrate specificity on different substrates was studied with the purified enzyme, and the optimal substrates were in the order of 2, 6-DMP > catechol > α-naphthol > guaiacol > SGZ > 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid). The alkaline activity and highly soluble expression of Lac591 make it a good candidate of laccases in industrial applications for which classical laccases are unsuitable, such as biobleaching of paper pulp and dyestuffs processing.

Keywords Multicopper oxidase · Alkaline laccase · Gene cloning · Enzyme characterization · Metagenome

Introduction

Laccases (benzenediol/oxygen oxidoreductases, EC 1.10.3.2) are the most numerous members of the multicopper oxidases family, which also includes ascorbate oxidase, monoxygenases, and dioxygenases (Morozova et al. 2007). Laccase catalyze reduction of molecular oxygen to water with the oxidation of a wide variety of phenolic and nonphenolic compounds, including industrial dyes, polycyclic aromatic hydrocarbons, pesticides, and alquenes. Furthermore, laccases are also capable of performing polymerization, depolymerization, methylation, and demethylation reactions (Solomon et al. 1996). Due to the broad substrate spectrum of laccases and their wide spectrum of catalyzed reactions, they are considered to be industrially relevant enzymes for a variety of applications, including decolorization of different types of recalcitrant dyes, bioremediation of soils and water, and kraft pulp biobleaching, the synthesis of natural products like pigments and antioxidants through dimerization of phenolic and nonphenolic acids, as well as in other biotechnological applications (Morozova et al. 2007; Mustafa et al. 2005; Riva 2006).

Laccases are widely distributed among fungi and higher plants; thus, classical laccases are considered to be associated exclusively with plants and fungi. The plant laccases have not been characterized or used extensively despite their wide occurrence because their detection and

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purification is often difficult, as the crude plant extracts contain a large number of oxidative enzymes with broad substrate specificities (Ranocha et al. 1999). So far, the majority of laccases characterized have been derived from fungi especially from white-rot basidiomycetes which are efficient lignin degraders, and some of them have been used in biotechnology processes (Xu et al. 2007). Currently, laccases are also found to be widespread among bacteria, based on homology searches in protein databases and bacterial genomes (Claus 2003; Sharma et al. 2007), and actual laccase activity has been found in *Escherichia coli* (Roberts et al. 2001), *Bacillus subtilis* (Martins et al. 2002), *Bacillus halodurans* (Ruijssenaars and Hartmans 2004), *Bacillus licheniformis* (Koschorreck et al. 2008), γ-proteobacterium (Singh et al. 2007), *Thermus thermophilus* (Miyazaki 2005), and several streptomycetes (Arias et al. 2003; Endo et al. 2003). However, little attention has been paid to bacterial laccases with respect to industrial applications to date. Bacterial laccases may have advantageous properties compared to classical fungi laccases. The highly efficient expression of fungal laccases is often much more difficult than that of bacterial enzymes. Furthermore, existence of intron in fungal laccase genes, formation of disulfide bridges, and glycosylation of fungal laccase are also frequently obstructive. In addition, most fungal laccases have an acidic pH optimum, and few have a near neutral pH optimum (Yaver et al. 1999; Kiiskinen et al. 2002). Comparing with fungal laccases, bacterial laccases have highly efficient expression, much higher thermostability, even some of them exhibit alkaline laccase activity (such as Lbh1 from *B. halodurans* C-125) (Ruijssenaars and Hartmans 2004). Despite these advantageous properties, until now, only a few bacterial laccases have been completely purified and characterized (Sharma et al. 2007; Singh et al. 2007; Koschorreck et al. 2008). Therefore, it is urgently in demand to find novel bacterial laccases with potential industrial relevance through the exploration of biological diversity.

As we know, more than 99% of bacteria in the environment cannot be cultured using conventional methods (Amann et al. 1995; Rappé and Giovannoni 2003); therefore, identification of novel biocatalysts using conventional laboratory methods from uncultured microorganisms is limited, and thus, metagenomics has been in the spotlight since the 1990s (Handelsman et al. 1998). The metagenomic approach, direct cloning of DNA from environmental samples and thereby accessing the potential of unculturable organisms, has proven to be a powerful tool for the isolation of novel biocatalyst genes. Current computed estimates of soil diversity are in the range of a million species per 1 g of soil (Curtis and Sloan 2005; Gans et al. 2005), and metagenomic strategy has led to the discovery of quite diverse and novel enzymes for a broad range of applications (Daniel 2005). Metagenome-derived enzymes include polysaccharide degrading/modifying enzymes, lipases, nitrile hydratases, nitrilases, amidases, dehydrogenases, oxidoreductases, and proteases (Steele et al. 2009). Many of them displayed novel enzymatic characteristics and thus have potential industrial applications.

In this paper, a metagenomic library from mangrove soil of Shenzhen City in China was constructed for the screening of clones with laccase activity. A clone with laccase activity and highly soluble expression was identified, and purified enzyme was used for further characterization including the optimum and stability of pH and temperature, effect of metal ions, kinetic parameters, and substrate specificity. To our knowledge, it is the first report on bacterial laccase gene from soil metagenomic library so far. An excellent bacterial laccase gene with potential industrial relevance is likely to be obtained after further study.

Materials and methods

Materials

2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), 2, 6-dimethoxyphenol (DMP), and syringaldazine (SGZ) were purchased from Sigma-Aldrich (St. Louis, MO, USA), respectively. All other chemicals and reagents were of analytical grade and were purchased from commercial sources, unless otherwise stated.

Strains, media, and plasmids

E. coli DH5α was used as the host for recombinant plasmids. The pET-32a (+) (Novagen, Madison, WI, USA) was used as an overexpression vector to produce the target protein. *E. coli* BL21 (DE3; Novagen) was used as the host for expression of *lac591* gene under the control of the T7 promoter. *E. coli* transformants were grown at 37°C in Luria–Bertani (LB) broth, when necessary; the LB medium was supplemented 100 μg/ml ampicillin, unless otherwise stated.

DNA manipulation

Routine DNA manipulations were carried out according to standard techniques. Restriction enzymes and DNA polymerase were purchased from TaKaRa (Dalian, China). Each enzyme was used according to the recommendations of the manufacturer. DNA ligations were performed using T4 DNA ligase (TaKaRa). Plasmids were prepared from *E. coli* by using a QIAGEN miniplasmid purification kit according to the manufacturer's instructions (QIAGEN Inc., Hilden, Germany). DNA fragments were isolated from agarose gels by

using a QIAquick gel extraction kit (QIAGEN Inc.). Electroporation was performed with a Gene-Pulser II electroporation apparatus (Bio-Rad Laboratories, Hercules, CA, USA).

Sequencing and analysis of positive clones

Sequencing reactions were performed using a BigDye sequencing kit according to the instructions of the manufacturer. DNA sequencing of positive clone (pUC118-*lac591*) was analyzed on ABI 377 DNA sequencer. Database homology search was performed with BLAST program provided by NCBI. To establish multiple sequence alignment of different laccase genes, a web-based version of ClustalW was used with default settings (http://www.ebi.ac.uk/clustalw/; Thompson et al. 1994).

DNA extraction from soil samples

The topsoil samples (5–10 cm) from mangrove were used for the experiments. Samples were collected and stored at −80°C until the DNA extraction was performed. Extraction of the total genomic DNA from mangrove soil was performed using the Fast DNA® SPIN kit for soil according to the recommendations of suppliers (MP Biomedicals, Solon, OH, USA).

Construction of genomic libraries and screening for laccase activity gene

The metagenomic library was constructed from environmental DNA isolated from mangrove soil. DNA fragments (2.5–7.5 kb) obtained after partial *Sau*3AI digestion were inserted into the *Bam*HI restriction site of the pUC118 vector (TaKaRa) with T4 DNA ligase. *E. coli* DH5α was transformed with the library by electroporation and plated onto LB agar plates containing 100 μg/ml ampicillin, 40 μg/ml 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal), and 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). A total of approximate 8,000 transformants were generated. Single clones grown on plate were picked and transferred into 100-ml flask containing 10 ml LB medium (supplemented with 100 μg/ml ampicillin and 1 mM IPTG) at 37°C to overnight. Two-milliliter liquid culture was harvested by centrifugation and lysed with 200 μl lysis reagent (50 mM Tris-HCl, pH7.5, 1% Triton X-100, and 1 mg/ml lysozyme) and then added with 1 mM guaiacol. A functional laccase screening was visually performed by brownish red color, which was resulted from the hydrolysis of guaiacol. To avoid the isolation of false-positive clones, plasmid DNA was extracted from the positive clones obtained in the initial screening and retransformed, and the new clones were examined by the same method for laccase activity. Subsequently, the plasmid was subjected to restriction analysis with *Bam*HI to identify the uniqueness of the clone followed by sequencing.

Cloning and overexpression of the laccase gene in *E. coli* and purification of the recombinant protein

The putative laccase activity gene was amplified from the pUC118-*lac591* plasmid by using the primers introduced *Hin*dIII and *Eco*RI restriction sites for cloning to the pET-32a (+). The following primers were used: *lac591*-F (5′-CCG GAA TTC ATG AAA AAA AGC TAT GGC GTG ATG GGC GGC GT-3′) and *lac591*-R (5′-AGC AAG CTT TTC CGG CAT ATT CGG AAT ATT CGG ATC-3′). The underlined sequences represent the recognition sites of restriction enzymes *Hin*dIII and *Eco*RI, respectively. The PCR product was digested with *Eco*RI/*Hin*dIII and then ligated into *Eco*RI/*Hin*dIII-treated expression vector pET-32a (+) and transformed into *E. coli* BL21 (DE3). The *E. coli* cells transformed with this plasmid were plated on LB agar containing 100 μg/ml ampicillin. The transformant was grown in a 100-ml flask containing 10 ml LB medium supplemented with 100 μg/ml ampicillin at 37°C until the optical density at 600 nm reached to 0.6–1.0, and then 0.8 mM IPTG and 0.1 mM $CuSO_4$ of the final concentration were added to induce target protein expression. After incubation at 30°C for more than 8 h with shaking at 200 rpm, cells were harvested by centrifugation (6,000×*g* for 20 min at 4°C) and washed twice with cold 50 mM Tris-HCl buffer (pH7.2), and the cell pellet was stored at −20°C for later purification. All purification steps were performed according to the instruction of His•Bind® Purification Kit (Novagen). In brief, the cells were resuspended in 10 ml ice-cold 1× binding buffer followed by sonication on ice. Lysate was centrifuged at 14,000×*g* for 20 min to remove debris. Desired amount of His•Bind resin was transferred to column, and the following washing solutions were used sequentially for charging and equilibrating column: sterile deionized water, 1× charge buffer, and 1× binding buffer. Column was loaded with prepared extract and then was washed with 1× binding buffer and 1× wash buffer. Bound protein was eluted with 1× elution buffer. The purity of the enzyme in the eluted fractions was estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), using 10% polyacrylamide running gels. Protein concentration was determined by the method of Bradford, and bovine serum albumin (Sigma-Aldrich) was used as standard for calibration. Enzyme samples were stored at −20°C until further use.

Determination of molecular mass

The molecular mass of the denatured protein was determined by SDS-PAGE. Proteins were stained with Coomas-

sie brilliant blue G-250. The molecular mass of the enzyme subunit was estimated using protein marker (TaKaRa) as standards.

Analysis of laccase activity

Laccase Lac591 activity was measured in UV–Vis Spectrophotometer (UV-1700 pharmaSpec, SHIMADZU, Japan), using guaiacol as the oxidation substrate ($\varepsilon_{465\ nm}$= 12,000 $M^{-1}cm^{-1}$). The standard assay temperature was 55°C. The assay mixture contained Mcllvaine's citrate–phosphate buffer (pH7.5), supplemented with 1 mM $CuSO_4$, and the reaction was started by adding guaiacol to a final concentration of 10 mM. One unit is defined as the amount of enzyme that oxidizes 1 μmol of substrate per minute. All assays were performed in triplicate.

Effect of pH on enzymatic activity and stability

The effect of pH on Lac591 activity with guaiacol as substrate was evaluated at the optimal temperature (55°C) over a pH range of 4.0–10.0, using Britton–Robinson buffer. Further study on the pH stability of the Lac591 was carried out by pre-incubating the enzyme solutions at 4°C for 1 h in the aforementioned buffer systems in the absence of substrate. The pH values of various reaction solutions were adjusted to pH7.5, and then they were subjected to laccase activity assay.

Effect of temperature on enzymatic activity and thermostability

The temperature optimum was measured by performing the laccase activity assay at various temperatures under pH7.5 using Mcllvaine's citrate–phosphate buffer. Guaiacol was used as substrate for determining activity. The thermostability of Lac591 was investigated by pre-incubation of the enzyme solutions for 15 min in the absence of substrate in Mcllvaine's citrate–phosphate buffer (pH7.5), at temperatures 30°C, 35°C, 40°C, 45°C, 50°C, 55°C, and 60°C, respectively. Residual activities were determined under laccase activity assay conditions.

Effect of metal ions on enzymatic activity

After pre-incubating the enzyme solutions containing each individual metal ions at pH7.5 and 50 mM Tris-HCl buffer at 4°C for 15 min, substrate guaiacol (10 mM) and 1 mM $CuSO_4$ were then added, and the enzyme activity was measured as described above under standard conditions. A control without metal ion was also performed. The amount of enzymatic activity measured was calculated as a percentage of the activity comparing to that of the control. The ions tested were 1 and 100 mM of $MnCl_2$, $MgCl_2$, $CaCl_2$, $ZnSO_4$, $CoCl_2$, Ni_2SO_4, NaCl, KCl, and $AlCl_3$.

Kinetic parameters and substrate specificity

To investigate the substrate specificity of the enzyme, different substrates were chosen according to the positions of substituents on the phenolic ring and the type and/or length of the substituents. Alternative substrates for measurement of laccase activity were ABTS ($\varepsilon_{420\ nm}$= 36,000 $M^{-1}cm^{-1}$), guaiacol, 2, 6-DMP ($\varepsilon_{468\ nm}$= 14,800 $M^{-1}cm^{-1}$), SGZ ($\varepsilon_{530\ nm}$=64,000 $M^{-1}cm^{-1}$), 1, 2-dihydroxybenzene (catechol, $\varepsilon_{450\ nm}$=2,211 $M^{-1}cm^{-1}$), and 2-hydroxynaphthalene (α-naphthol, $\varepsilon_{330\ nm}$=2,200 $M^{-1}cm^{-1}$). The temperature used in the activity assays was 55°C. The kinetic parameters (K_m and k_{cat}) for the recombinant enzyme were determined by assaying the enzymatic activity in a Mcllvaine's citrate–phosphate buffer at 55°C with different substrates. All kinetic studies were performed at least three times, and kinetic data were fitted to hyperbola by using the Michaelis–Menton equation. Kinetic analyses by curve fitting were performed with the Origin software (OriginLab Corporation, Northampton, MA, USA). Finally, the optimal substrate order of Lac591 was determined by comparing catalytic efficiencies (k_{cat}/K_m) of Lac591 to these substrates.

Nucleotide sequence accession number

The nucleotide sequence data reported here have been deposited in the nucleotide sequence databases (GenBank) under accession number (GQ468313).

Results

Construction of a metagenomic library and screening for clones encoding laccase activity

To obtain genes associated with laccase activity, a metagenomic library containing 8,000 clones was generated using DNA extracted from mangrove soil, where contains rich ligninolytic enzymes. The quality and size of inserts were tested by restriction analysis of 20 randomly picked clones from the library. Almost all of analyzed clones carried inserts of approximately 2.5–7.5 kb, and the average insert size was about 4.5 kb. The metagenomic library represented about 36 MB of soil microbial community DNA. The result of restriction analysis revealed a high diversity of the cloned DNA fragments in the metagenomic library. Out of approximately 8,000 colonies, one positive clone was identified by its red brown color. Sequence data from this clone showed the presence of one open reading

frame of 1.5 kb encoding a full-length multicopper oxidase gene, *lac591*. The gene *lac591* translates into a protein of 500 amino acids with a predicted molecular mass (M_r) of 57.4 kDa.

Sequence analysis of Lac591

A BLAST search in the databases of NCBI revealed that the Lac591 was a member of the multicopper oxidase family protein. Multiple sequence alignment, performed using ClustalW, showed that Lac591 from metagenomic library of mangrove soil contains four histidine-rich copper-binding domains which are characteristic for laccases (Fig. 1). Four conserved copper-binding domains from Lac591 and several representatives of fungal and bacterial laccases were analyzed, and result showed that Lac591 has a very high similarity to these laccases in four conserved copper-binding domains. However, Lac591 only showed moderate protein sequence identities to multicopper oxidase/laccase from the following bacteria: *B. halodurans* C-125 (52%), *B. cereus* Rock 3–44 (45%), *B. cereus* AH621 (42%), *Bacillus mycoides* DSM 2048 (42%), and *Bacillus thuringiensis* serovar kurstaki (42%), and no protein sequence identity of fungal laccases was found, which suggested that the overall similarity of Lac591 to bacterial laccases is not very high, but higher than that to fungal laccases.

Overexpression and purification of recombinant Lac591

To investigate the biochemical property of Lac591, the laccase gene was subcloned in frame with a six-histidine tag sequence into a T7 RNA polymerase driven *E. coli* expression vector of pET-32a (+) and expressed in *E. coli* BL21 (DE3) and induced with 0.8 mM IPTG at 30°C. The cells were harvested and disrupted by sonication in ice-water bath. The cell lysates was found fully clear, and no inclusion bodies were formed, which suggested that the recombinant Lac591 was highly soluble. The recombinant Lac591 was purified by Ni-NTA chromatography, and purified protein and supernatant from cell lysates were applied to SDS-PAGE (Fig. 2) together to determine the molecular mass and expression level of recombinant protein. Taking into consideration that the resulting recombinant protein should consist of Lac591 (500 amino acids, its calculated molecular mass is about 57.4 kDa) and an N-terminal fusion of 156 amino acids (about 18 kDa) corresponding to thioredoxin tag (Trx·Tag), polyhistidine tag (His·Tag), S·tag epitope (S·Tag), and a unique thrombin cleavage site (thrombin), its total molecular mass should be about 75.4 kDa. A clear protein band of approximate 75 kDa was observed in Fig. 2, which was in accordant with the predicted molecular mass. In addition, Fig. 2 also showed that the expression level of the recombinant Lac591 was very high, displaying a strong band of target protein in

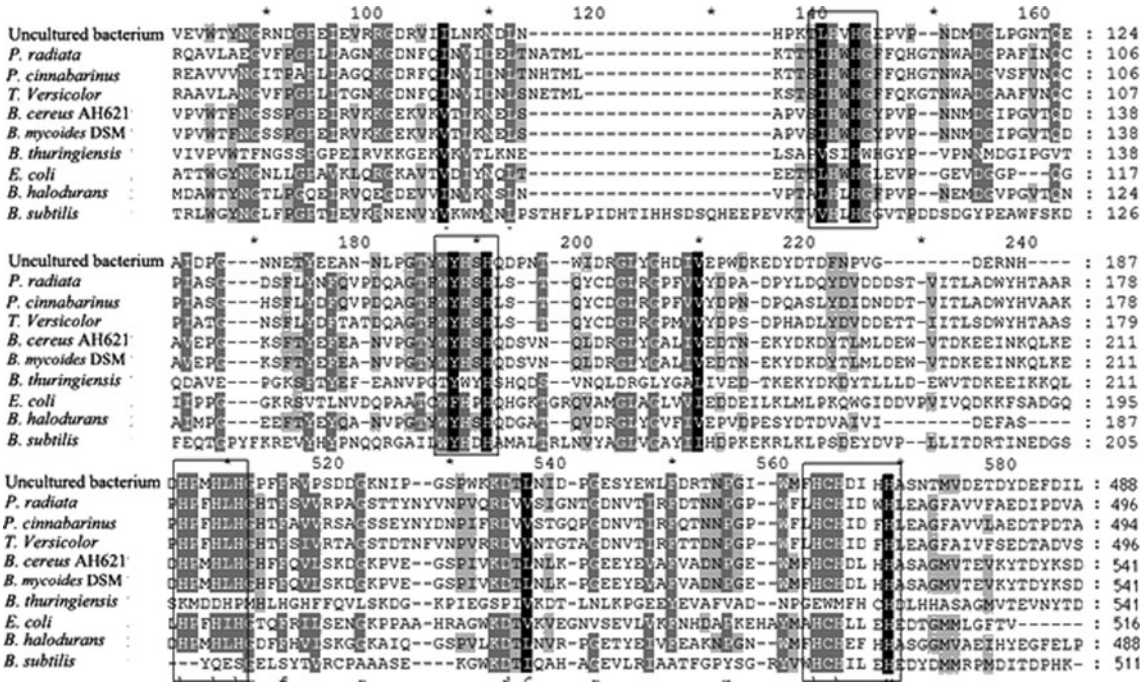

Fig. 1 Alignment of the four copper-binding domains of Lac591, several bacterial laccases, and fungal laccases. The four conserved copper-binding domains are shown (*boxed*). The protein sequences were retrieved from GenBank under following accession numbers: uncultured bacterium of this study (ACV83921), *Phlebia radiata* (CAA36379.2), *Pycnoporus cinnabarinus* (AAC39469.1), *Trametes versicolor* (CAA59161.1), *B. cereus* AH621 (ZP_04294553.1), *B. mycoides* DSM 2048 (ZP_04168427.1), *B. thuringiensis* serovar kurstaki (ZP_04114398.1), *E. coli* (NP_414665.1), *B. halodurans* C-125 (NP_242948.1), and *B. subtilis* (AAB62305.1)

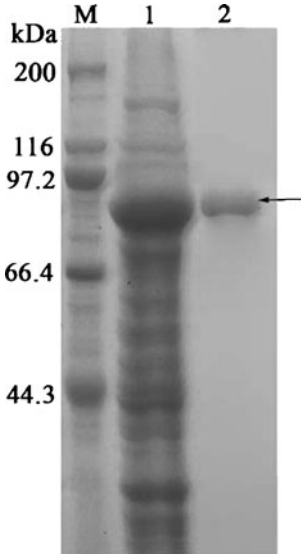

Fig. 2 SDS-PAGE analysis of recombinant Lac591 from supernatant of *E. coli* BL21 (DE3) cell lysates and purified Lac591 by affinity chromatography. *Line M* standard protein molecular mass marker (TaKaRa, sizes in kilodaltons are indicated on the *right*), *lane 1* unpurified Lac591 recombinant protein, *lane 2* purified Lac591 recombinant protein

the SDS-PAGE. The highest expression level of Lac591 (OD_{600}=2.4) was about 380 mg/l and its content in total soluble protein reached up to 52.7% according to Quantity One software (Bio-Rad Laboratories Inc., Hercules, CA, USA) for protein band visualization. The enzymatic activity of purified Lac591 was determined and yielded a highest specific activity of 12.73 U/mg for 2, 6-DMP. Moreover, Lac591 also displayed specific activity toward several classical laccase substrates, including α-naphthol (5.43 U/mg), catechol (7.35 U/mg), guaiacol (1.02 U/mg), SGZ (0.51 U/mg), and ABTS (0.15 U/mg). These results indicated that Lac591 is a bacterial laccase with potent activity and highly soluble expression and considered to have potential for industrial applications. The purified recombinant protein was used for studies on biochemical characterizations of Lac591.

Effect of pH and temperature on catalytic activity and stability

To determine the optimal pH for recombinant Lac591, we measured the enzyme activity at various pH values (pH4.0–10.0), using guaiacol as substrate. The pH-activity profile of the enzyme was bell-shaped. The pH value for optimal activity of Lac591 was determined to be 7.5, with about 60% of maximum activity being retained in pH10.0 (Fig. 3a). Moreover, the enzyme was found to be stable in the pH range of 7.0–10.0, and more than 80% of the activity was remained (Fig. 3a), confirming that Lac591 has broader alkaline pH activity range. The optimal temperature for the enzyme was 55°C (Fig. 3b). The enzyme was stable under 50°C; however, its activity was drastically reduced over 50°C, and the remained activity was less than 30% after pre-incubation of the enzyme for 15 min at 55°C (Fig. 3b).

Effect of metal ions on enzyme activity

The effect of different metal ions on the Lac591 activity was investigated by using 10 mM guaiacol as substrate (Table 1). The results revealed that some metal ions imparted distinct effect on the enzymatic activity. When added at low concentrations (1 mM), Ca^{2+} and Fe^{2+} increased the enzymatic activity evidently, while Mn^{2+}, Mg^{2+}, Co^{2+}, Ni^{+}, Na^{+}, and K^{+} slightly decreased the enzymatic activity and Zn^{2+} and Al^{3+} decreased the enzymatic activity remarkably. When added at high concentration (100 mM), Ca^{2+} enhanced the enzyme activity markedly, and the enzyme activity reached up to 2-fold of original that. Moreover, 100 mM of Mg^{2+}, Na^{+}, and K^{+} slightly increased the enzymatic activity. However, the enzymatic activity was completely inhibited in the presence of 100 mM of Co^{2+}, Zn^{2+}, Fe^{2+}, Ni^{+}, and Al^{3+}.

Kinetic parameters and substrate specificity

In general, the catalytic efficiencies (k_{cat}/K_m) are considered as a measurement of the enzymatic specificity. To investigate the kinetic parameters for the enzymatic activity of Lac591, the initial reaction rates at various substrate concentrations were determined, and kinetic parameters of different substrates determined for Lac591 were summarized in Table 2. The kinetic parameters of Lac 591 for various substrate fall within the ranges reported for other bacterial laccases (Koschorreck et al. 2008; Miyazaki 2005; Roberts et al. 2001; Singh et al. 2007). K_m value of Lac591 for guaiacol (1,037 μM) was similar with that for α-naphthol (1,419 μM), while k_{cat}/K_m value of Lac591 for α-naphthol (0.025 $s^{-1}\,\mu M^{-1}$) was about two times higher than that for guaiacol (0.010 $s^{-1}\,\mu M^{-1}$). 2, 6-DMP was the best substrate considering its highest k_{cat}/K_m value (0.28 $s^{-1}\,\mu M^{-1}$), and the other optimal substrates were in the order of catechol > α-naphthol > guaiacol > SGZ > ABTS according to their k_{cat}/K_m value.

Discussion

Laccases are well-known biocatalysts and are considered to be some of the most promising enzymes for future industrial applications (Xu 2005; Xu et al. 2007). Fungal laccases have found wide applications ranging from the pharmaceutical sector to the pulp and paper industry to reduce the kappa number and enhance the bleaching of kraft pulp when they are used in the presence of chemical mediators, such as ABTS (Bourbonnais and Paice 1996). Although laccases are widespread in bacteria as well, only a few of them have been characterized till now. There are rich ligninolytic enzymes in mangrove soil, such as laccase.

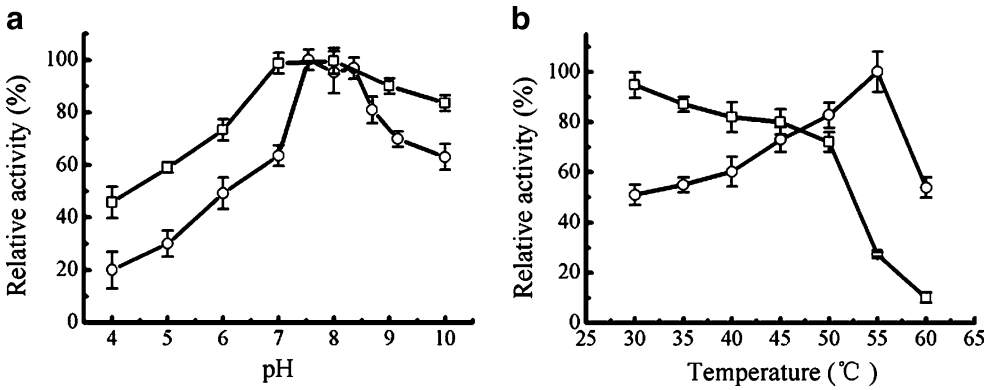

Fig. 3 Effect of pH (**a**) and temperature (**b**) on activity (*circle*) and stability (*square*) of Lac591 using guaiacol as substrate. Data points are the average of triplicate measurements; *error bars* represent ±1 SD

Therefore, to obtain novel bacterial laccase, a metagenomic library of mangrove soil was constructed, and a new bacterial laccase gene was cloned, expressed, and characterized.

Generally, most fungal laccases have an acidic pH optimum (Baldrian 2006), and most bacterial laccases are active at pH7.5–8.5. Recently, an alkaline fungal laccase from *Acremonium murorum* was found (Gouka et al. 2001). This enzyme has a pH optimum of 9.0 with SGZ as the substrate. However, this enzyme is more related to bilirubin oxidase than to other fungal laccases and has less overall similarity to fungal laccases. In addition, the alkaline bacterial laccase (Lbh1) from *B. halodurans* C-125 was identified by genome mining in 2004 (Ruijssenaars and Hartmans 2004), and the pH optimum of the enzyme is about 7.5–8.0 with SGZ as the substrate. However, the pH optimum of the enzyme for other substrates was not reported. Another alkaline bacterial laccase from γ-proteobacterium JB was also identified recently (Singh et al. 2007), but this enzyme displayed its alkaline pH optimum (pH7.2) only with *p*-phenylenediamine as substrate. In this study, Lac591 showed an alkaline pH optimum for all tested substrates such as guaiacol (pH 7.5), ABTS (pH7.4), 2, 6-DMP (pH8.0), SGZ (pH8.0), α-naphthol (pH8.2), and catechol (pH9.0), confirming that Lac591 is an alkaline laccase. This property underlines its potential in industrial applications such as biobleaching of paper pulp and dyestuffs processing, for which most fungal laccases are unsuitable (Singh et al. 2009).

Another attractive property of Lac591 is its highly soluble expression at 30°C in *E. coli*. The main purpose of recombinant protein expression is often to obtain a high degree of accumulation of soluble product in the bacterial cell. However, this strategy is not always accepted by the metabolic system of the host and in some situations a cellular stress response is encountered. Another response encountered in recombinant systems is the accumulation of target proteins into insoluble aggregates known as inclusion bodies. These aggregated proteins are in general misfolded and thus biologically inactive (Villaverde and Carrio 2003). Recently, a bacterial laccase gene *cotA* from *B. subtilis* was overexpressed in *E. coli* (Martins et al. 2002); however, most of the recombinant CotA protein was found in the insoluble fraction obtained after centrifugation of the broken cell suspension, presumably in the form of inclusion bodies, and attempts to recover the soluble protein from this fraction were unsuccessful. Consequently, the content of soluble protein is only about 10% of all the recombinant CotA protein. The overexpression in *E. coli* of another bacterial laccase gene *cotA* from *B. licheniformis* was also investigated not long ago (Koschorreck et al. 2008), and the expression of *cotA* was induced at different temperatures (16°C, 18°C, 20°C, 25°C, and 30°C). The highest volu-

Table 1 Effect of metal ions on the activity of recombinant Lac591

Metal ions	Metal salts	Relative activity (%)	
		1 mM	100 mM
None	None	100	100
Ca^{2+}	Calcium chloride	150	200
Mn^{2+}	Manganese chloride	80	70
Mg^{2+}	Magnesium chloride	85	125
Co^{2+}	Cobalt chloride	92	0
Zn^{2+}	Zinc chloride	55	0
Fe^{2+}	Ferrous gluconate	140	0
Ni^{+}	Nickel sulfate	77	0
Na^{+}	Sodium chloride	80	120
K^{+}	Potassium chloride	98	110
Al^{3+}	Aluminum chloride	68	0

Table 2 Kinetic properties of recombinant Lac591

Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})
2, 6-DMP	340	96.25	0.28
Guaiacol	1,037	10.81	0.010
Catechol	70	4.36	0.062
α-Naphthol	1,419	35.32	0.025
SGZ	8	0.031	0.0039
ABTS	90	0.042	0.00047

metric activity was obtained after an overnight expression at 18°C, which suggested that inclusion bodies were formed over 18°C. The property of low temperature induction of *cotA* from *B. licheniformis* was unsuited for scale production. Therefore, in prokaryotic expression system, the solubility of recombinant protein at normal temperature (such as 30°C) is an emerging matter of concern, especially when the obtained products are to be used for functional analyses (de Marco 2008). The solubility of recombinant protein could be dependent on its amino acid sequence, growth rates, culture temperature, and doses of gene expression inductor (Sorensen and Mortensen 2005a, b). Thus, to find a suitable gene with high solubility in prokaryotic system, it is a feasible way to screen a great deal of genes using metagenomic method from uncultured microorganisms, which containing rich gene resources. In this study, a bacterial laccase gene (*lac591*) was cloned from metagenomic library of mangrove soil, and this gene displayed a very high solubility when it was expressed in *E. coli*. Lac591 was induced at 30°C, and no any inclusion bodies were found. Furthermore, the expression level of *lac591* in *E. coli* was very high, and its highest expression level was approximately 380 mg/l. To our knowledge, this is the highest expression report for bacterial laccase genes so far, comparing favorably with those high expression reports of other laccase genes including a laccase gene from *Streptomyces coelicolor* with the expression level of 350 mg/l (Dubé et al. 2008) and a *cotA* gene from *B. licheniformis* with the expression level of 300 mg/l (Koschorreck et al. 2009).

In summary, a novel multicopper oxidase gene with laccase activity was successfully cloned via metagenomic approach combined with activity-based functional screening from mangrove soil; the recombinant Lac591 was purified and characterized. Results showed that its properties of alkaline activity and highly soluble expression in *E. coli* were distinct from other bacterial laccases reported before. It indicated that metagenomic method provide an alternative way to find laccase-like proteins with diverse and novel enzymatic characteristics for a wide range of applications. Lac591 exhibits novel enzymatic characteristics including high alkaline pH optimum and highly soluble expression in *E. coli*; these unusual properties make Lac591 an interesting enzyme in scale production and some industrial applications for which classical laccases are unsuited, such as lignocellulose treatment, biobleaching of paper pulp, and dyestuffs processing.

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