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Cloning, expression in *Escherichia coli*, and enzymatic properties of laccase from *Aeromonas hydrophila* WL-11

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Abstract

A strain WL-11 with high laccase activity was isolated from activated sludge collected from the effluent treatment plant of a textile and dyeing industry. It was identified as *Aeromonas hydrophila* by physiological test and 16S rDNA sequence analysis. A gene encoding of laccase from a newly isolated *Aeromonas hydrophila* WL-11 was cloned and characterized. Nucleotide sequence analysis showed an open reading frame of 1605 bp encoding a polypeptide comprised of 534 amino acids. The primary structure of the enzyme predicted the structural features characteristic of other laccases, including the conserved regions of four histidine-rich copper-binding sites. The predicted amino acid sequence showed a high homology (more than 60%) with bacterial laccases in the genome and protein databases and the highest degree of similarity (61% identity) was observed with the multicopper oxidase of *Klebsiella* sp. 601. When expressed in *Escherichia coli*, the recombinant enzyme was overproduced in the cytoplasm as soluble and active form. The purified enzyme had an optimum pH of 2.6 and 8.0 for ABTS (2,2'-azino-bis(3-ethylbenzthiazolinesulfonic acid) and DMP (2,6-dimethoxyphenol), respectively. The kinetic study on ABTS revealed a higher affinity of this enzyme to this substrate than DMP.

Key words: cloning; expression; enzymatic properties; laccase; *Aeromonas hydrophila*
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Introduction

Laccases (EC 1.10.3.2) catalyze the oxidation of a variety of phenolic compounds and aromatic amines by reducing molecular oxygen to water. They belong to the multicopper oxidase (MCO) family that normally contains four copper atoms, a type I copper (blue copper), a type II copper, and a pair of type III copper center (Solomon et al., 1996). Due to their broad substrate specificity and wide range of reactions that they can catalyze, laccases can be used for several industrial applications, such as biological bleaching in pulp and paper industries, dye decolorization in textile industries, detoxification of recalcitrant environmental pollutants, organic synthesis as biocatalyst and bioremediation (Mayer and Staples, 2002; Rodríguez Couto and Toca Herrera, 2006).

Laccases are widely distributed among plants, fungi (Morozova et al., 2007), insects (Hoegger et al., 2006) and bacteria (Claus, 2003). To date, more than a hundred laccases have been isolated and characterized. Most of them were derived from fungi, including especially white-rot basidiomycetes, whereas laccases from plant are less studied (Mayer and Staples, 2002; Morozova et al., 2007). Fungal laccases with several isoforms are monomeric or oligomeric glycoproteins and are involved in various functions, such as lignin degradation, pigmentation, detoxification, pathogenesis and morphogenesis (Baldrian, 2006; Morozova et al., 2007).

In contrast to fungal laccases, only a few bacterial laccases have been so far studied, although recent rapid progress in whole genome analysis suggests that these enzymes are widespread in bacteria (Alexandre and Zhulin, 2000; Claus, 2003; Sharma et al., 2007). Bacterial laccases have advantageous properties compared to fungal laccases with respect to industrial applications; they are highly active and much more stable at high temperature and high-pH value than fungal laccases (Sharma et al., 2007). Fungal laccases have difficulties in the overproduction of their recombinant forms and thus the lack of sufficient enzyme stocks is the most important obstacles to commercial application of laccases (Rodríguez Couto and Toca Herrera, 2006). However, bacterial laccases can overproduced more easily in heterologous host like *Escherichia coli*, because of their intracellular localizations (Sharma et al., 2007) and development of host-vector system for prokaryotic expression. For the detailed studies on structure and catalytic mechanism of bacterial laccases and their biotechnological applification, it is important to do screening of new sources of bacterial laccases and their gene cloning, followed by successful expression of recombinant enzyme.

In the present study, we have isolated a new strain,

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Aeromonas hydrophila WL-11, that possesses the laccase activity from activated sludge collected from the effluent treatment plant of a textile and dyeing industry. We report the cloning and overexpression of the novel laccase gene in *E. coli*, and characterization of the purified recombinant laccase.

1 Materials and methods

1.1 Materials

Unless described otherwise, all chemicals and substrates including ABTS (2,2'-azino-bis(3-ethylbenzthiazolinesulfonic acid) and DMP (2,6-dimethoxyphenol) were of analytical grade or higher, and purchased from Sigma-Aldrich (South Korea). Enzymes were obtained from Takara Biomedicals (Japan).

1.2 Isolation and identification of laccase-producing bacteria

The laccase-producing bacterial strain was isolated from activated-sludge samples collected from wastewater treatment plants of dyeing industries in Busan, Korea. To isolate laccase-producing bacteria, strains showing dye decolorization were firstly screened, and then a strain displaying laccase activity among them was finally selected by enzyme assay using crude extract obtained after sonication. Screening of the strains for dye decolorization using azo dye Reactive Black-5 was performed by enrichment culture technique using NM9 and DNM9 media as described previously (Xu et al., 2005). After purification by successive single colony isolation on a DNM9 agar plate, strain WL-11 was identified by carbon source utilization patterns using Biolog GN2 microplate (Biolog, USA) and analysis of 16S rDNA sequences. For the 16S rDNA sequence analysis, bacterial genomic DNA was extracted and purified using a Wizard Genomic DNA Prep. Kit (Promega Corp., USA). Two primers annealing to the 5' and 3' end of the 16S rRNA gene were 5'-GAGTTTGATCCTGGCTCAG-3' (positions 9 to 27, *E. coli* 16S rDNA numbering) and 5'-AGAAAGGAGGTGATCCAGCC-3' (positions 1542 to 1525, *E. coli* 16S rDNA numbering), respectively. Polymerase chain reaction (PCR) was performed as follows: pre-denaturation at 95°C for 5 min, 30 cycles at 95°C for 40 sec, 55°C for 40 sec and 72°C for 2 min. The PCR product was subcloned into pGEM-T easy vector (Promega, USA) and its nucleotide sequence was determined by GenoTec Co., Ltd. (South Korea). The partial rDNA sequences were analyzed using a BLAST search algorithm to estimate the degree of similarity to other rDNA sequences obtained from the NCBI/GenBank. Phylogenetic trees were constructed by the ClustalX program (Thomson et al., 1997).

1.3 Cloning of laccase gene

Chromosomal DNA from *A. hydrophila* WL-11 was isolated by the method described previously (Marmur, 1961). The coding sequence for the laccase of *A. hydrophila* WL-11 was available from Genbank (accession no. NC_008570). Amplification of the laccase gene was performed by PCR using Takara LA Taq polymerase with the forward primer LA-1 (5'-TACATATGCAGCGTCGCGATTTTCTC-3') and reverse primer LA-2 (5'-TACTCGAGTTACTCTTCCACC GTGAACG-3'), where the underlines represent the recognition sites of *Nde*I and *Xho*I, respectively, and translation codon ATG and stop codon TAA of open reading frame (ORF) are shown in italics. The PCR was performed as follows: 98°C for 3 min, 95°C for 40 sec, 68°C for 2 min, 30 cycles, and 72°C for 10 min. After the amplified DNA fragment was purified using Geneclean kit (Bio101, USA), it was cloned to pGEM-T easy vector, resulting in pGEMT-LA and the nucleotide sequence of the inserted fragment was confirmed by DNA sequencing. Plasmid pGEMT-LA was digested with *Nde*I and *Xho*I, and then inserted into corresponding sites of pET-15b vector, resulting in the expression plasmid pET15-LA.

1.4 Expression and purification of recombinant laccase

E. coli BL21 (DE3) cells harboring pET15-LA were cultured in 5 mL of LB medium containing 100 μg ampicillin/mL at 37°C overnight. Afterwards, 250 mL of LB supplemented with 200 μg ampicillin/mL was inoculated with 2.5 mL of the prepared culture and incubated at 18°C and 200 r/min. Protein expression was initiated by the addition of 0.2 mmol/L isopropyl β-D-thiogalactoside (IPTG) and 0.2 mmol/L $CuCl_2$ when the culture turbidity at 600 nm reached 0.8. After induction for 8 hr, the cells were collected by centrifugation at 5000 ×*g* at 4°C for 10 min, and then washed with 50 mL of 20 mmol/L Tris-HCl (pH 8.0). The washed cell pellet was re-suspended in 20 mmol/L Tris-HCl at pH 8.0 (10 mL for each gram) and disrupted by ultrasonication. To remove the cell debris, the lysate was centrifuged at 12,000 ×*g* for 20 min. The supernatant as the crude extract was used for purification after filter (0.45 μm).

Purification was performed on the ÄKTA prime and FPLC system (GE health care). The sample was applied to HiTrap Q Sepharose FF column equilibrated with start buffer (20 mmol/L Tris-HCl, pH 8.0), washed with start buffer and eluted by linear gradient with elution buffer (20 mmol/L Tris-HCl, pH 8.0, 1 mol/L NaCl). Fractions containing laccase activity were pooled and dialyzed against 20 mmol/L Tris-HCl (pH 8.0) overnight. Further, the concentrated sample was applied on a HiTrap DEAE Sepharose FF column equilibrated with start buffer (20 mmol/L Tris-HCl, pH 8.0), washed with start buffer and eluted by linear gradient with elution buffer (20 mmol/L Tris-HCl, pH 8.0, 1 mol/L NaCl). The active enzyme fractions with a single peak of protein were pooled and concentrated by ultrafiltration with Centricon-YM30 (Millipore, USA). The enzyme purified as a single band on SDS-PAGE was used for biochemical characterization.

1.5 Enzyme assay and SDS-PAGE

Laccase activities were examined at 37°C using ABTS and DMP as follows. Laccase assay towards substrate

ABTS was determined spectrophotometrically as absorbance increase at 420 nm containing 0.5 mmol/L ABTS, 0.5 mmol/L $CuCl_2$ in 0.1 mol/L sodium acetate buffer, pH 3.6. The assay using substrate DMP was determined by measuring the increase of absorbance at 477 nm containing 0.5 mmol/L DMP and 0.5 mmol/L $CuCl_2$ in 0.1 mol/L Tris-HCl, pH 8.0. The molar extinction coefficients used in activity assay were 36,000 L/(mol·cm) for ABTS, and 14,800 L/(mol·cm) for DMP. One unit is defined as the amount of enzyme that oxidizes 1 mol of substrate per min. Kinetic parameters of the purified enzyme were determined at 37°C using different concentrations of ABTS (0.01–10 mmol/L) or DMP (0.1–10 mmol/L). K_m and K_{cat} values were determined by fitting the data to Lineweaver-Burk plots using SWIFT II Applications Software (Amersham Bioscience). Protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad, USA) according to the manufacturer's instructions, with bovine serum albumin (Sigma-Aldrich, South Korea) as the protein standard for the calibration curve. SDS-PAGE was performed on 10% running gel as described by Laemmli (1970), and the resolved proteins were visualized by Coomassie staining. A mid-range protein standard (Bio-Rad) was used as molecular mass marker. Expression levels were estimated as a percentage of total cellular proteins using Alphaimager™ 1220 Documentation & Analysis system (Alpha Innotech Inc., USA).

1.6 Effect of pH, temperature, and metal ions on enzyme activity

To investigate the effect of pH on the activity of laccase towards ABTS and DMP, different buffers were prepared: 50 mmol/L HAc-NaAc buffer (pH 2.6–5.8), 50 mmol/L Na_2HPO_4-citric acid buffer (pH 5.0–7.4), 50 mmol/L H_2BO_3-$Na_2B_4O_7$ buffer (pH 7.4–9.0). The effect of temperature on laccase stability was determined by incubating aliquot of the purified laccase for 10 min in 20 mmol/L Tris-HCl buffer (pH 8.0) containing 0.5 mmol/L $CuCl_2$ at various temperatures and then by measuring the DMP oxidation. The effects of divalent metal ions on the purified enzyme activity were determined by adding 0.5 mmol/L Cu^{2+}, Ca^{2+}, Ni^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+} to the reaction mixture in the presence of 0.5 mmol/L DMP or 0.5 mmol/L ABTS as substrate. The relative activity was defined as the percentage of activity determined with respect to that measured under the enzyme assay condition described above.

2 Results

2.1 Isolation and characterization of laccase-producing bacteria

A bacterial strain displaying laccase activity, named strain WL-11, was isolated after screening and purification by successive single colony isolation. The strain was a Gram-negative, motile rod and was positive for oxidase and catalase. It showed that the highest similarity to *A. hydrophila* in the carbon-source utilization test. Moreover, a comparison of the 16S rRNA gene sequence of WL-13 with that of other strains using the BLAST(N) program of the National Center for Biotechnology Information (NCBI) also showed the highest homology to *A. hydrophila* with 94% similarity (data not shown). Accordingly, the isolated bacterium WL-11 was named *A. hydrophila* WL-11.

2.2 Cloning and sequence analysis of the laccase gene from *A. hydrophila* WL-11

To clone the laccase gene from *A. hydrophila* WL-11, sequence searches were performed in the GenBank database. A protein noted as "laccase; copper-stimulated phenoloxidase and ferroxidase which may be involved in copper detoxification" was identified in the complete genome database of *A. hydrophila* ATCC 7966 (accession no. NC_008570). Oligonucleotide primers LA-1 and LA-2 were designed based on ORF of this protein from *A. hydrophila* ATCC 7966. A DNA fragment with size of about 1.6 kb was amplified by PCR amplification from genomic DNA of *A. hydrophila* WL-11 and then sequenced. The sequence analysis showed the ORF of *A. hydrophila* WL-11 laccase gene consists of 1605 nucleotides encoding a protein of 534 amino acids with a predicted molecular weight of 58,500 Da (data not shown). The cloned DNA sequence of *A. hydrophila* WL-11 laccase has been deposited in the GenBank database under accession no. FJ797682. The amino acid sequence of *A. hydrophila* WL-11 laccase was found to be rich in alanine (11%), glycine (11%), and leucine (12%) and contained 11% negatively charged and 14% positively charged amino acids. Theoretically calculated isoelectric point value was 6.26. Comparison of the amino acid sequence of *A. hydrophila* WL-11 laccase with those of other bacterial laccases showed the high identity of 92% with putative *A. hydrophila* ATCC 7966 laccase (YP_858059), 61% with *Klebsiella* sp. 601 multicopper oxidase (ABY56791), 61% with *E. coli* Cueo (1KV7_A) (Fig. 1). It also revealed more than 60% identity with other laccases belonging to multicopper oxidase family, whereas no significant similarity was found between *A. hydrophila* WL-11 laccase and the other bacterial oxidoreductases known so far. This result indicated that *A. hydrophila* WL-11 laccase is a member of the multicopper oxidase family. Multiple sequence alignment showed that *A. hydrophila* WL-11 laccase contains four histidine-rich copper-binding domains (domain I from residue 98–104, domain II from residue 138–142, domain III from residue 457–466, and domain IV from residue 511–524) which are characteristic for laccases.

2.3 Overexpression and purification of *A. hydrophila* WL-11 laccase

When *E. coli* BL21(DE3) cells carrying pET15-LA were induced for 8 hr with 0.2 mmol/L IPTG at 18°C, laccase activity was detected in soluble fraction, but not in extracellular and insoluble fractions. Furthermore, a predominant band corresponding to the expected size (58 kDa) of recombinant laccase was also observed in soluble fraction of induced cells (Fig. 1, lane 2). The amount of

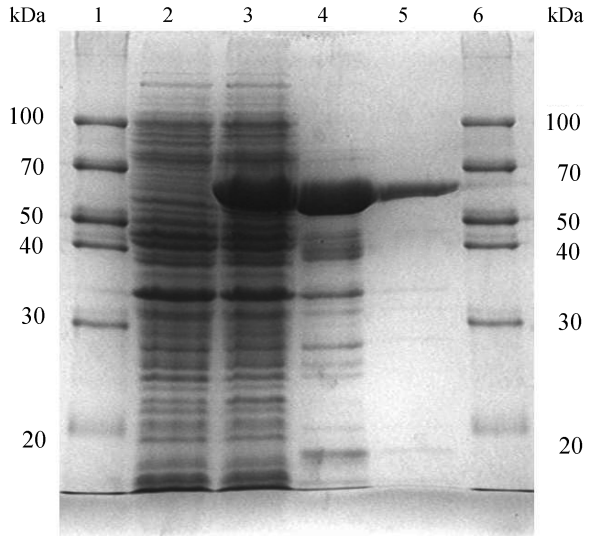

Fig. 1 SDS-PAGE of recombinant laccase produced in *E. coli* BL21 (DE3) cells harboring pET-LA. Lane 1 and 6: molecular weight marker; lane 2: pET 15b vector plasmid (control); lane 3: total fraction; lane 4: sample after HiTrap Q Sephose FF column chromatography; lane 5: sample after HiTrap DEAE Sephose FF column chromatography.

expressed laccase was approximately 12% of the total soluble proteins of intracellular fraction. These results indicate that recombinant laccase was efficiently overexpressed as a soluble and active form in the cytoplasm of *E. coli*. Recombinant laccase was purified from the intracellular fraction of *E. coli* by HiTrap Q Sepharose FF and HiTrap DEAE Sepherose FF column chromatographies. The purified enzyme was identified as a single band with the molecular mass of about 59 kDa on SDS-PAGE (Fig. 1, lane 5), which is in close to 58,500 Da calculated from the amino acid sequence of *A. hydrophila* WL-11 laccase. The process resulted in 3.5-fold purification over the sonicated extract with a 30% recovery of total activity, and the final specific activity of the purified enzyme with ABTS was 0.91 U/mg (data not shown).

2.4 Enzymatic characterization of *A. hydrophila* WL-11 laccase

The enzymatic activity of *A. hydrophila* WL-11 laccase was examined under different conditions. *A. hydrophila* WL-11 laccase displayed activity for ABTS oxidation at pH < 5.0 the and highest activity was detected at pH 2.6. At the same time, the enzyme displayed activity towards DMP at pH 6–9 and the maximal activity was found at pH 8.0 (Fig. 2a). When thermal stability of this enzyme was measured at 70°C for various time, rapid decreases in activity with increase of incubation time were detected. After 10, 20, and 30 min incubation, the observed residual activities were of 40%, 12%, and 6%, respectively (Fig. 2b).

The effects of divalent metal ions on the enzyme activity were at 0.5 mmol/L by using DMP as substrates. As shown in Fig. 3, the results showed that Ca^{2+}, Zn^{2+} did not give any support for enzyme activity, whereas Mg^{2+}, Fe^{2+}, Mn^{2+} and Ni^{2+} gave about 7% activity compared with that of Cu^{2+}.

Kinetic properties of the purified laccase from *A. hydrophila* WL-11 on the ABTS and DMP substrates were estimated at pH 3.6 and pH 8.0, respectively. The dependence of the rate on the substrate concentration showed typical Michaelis-Menten kinetics. The results are summarized in Table 1. The value of K_m and K_{cat} for the enzyme were found to be 0.94 mmol/L and 81.98 sec^{-1} for ABTS and 1.83 mmol/L, 205.99 sec^{-1} for DMP, respectively. The purified enzyme revealed significant differences in its catalytic efficiencies (K_{cat}/K_m) for different substrates; 87.21 and 112.56 mmol/(L·sec) in the presence of ABTS

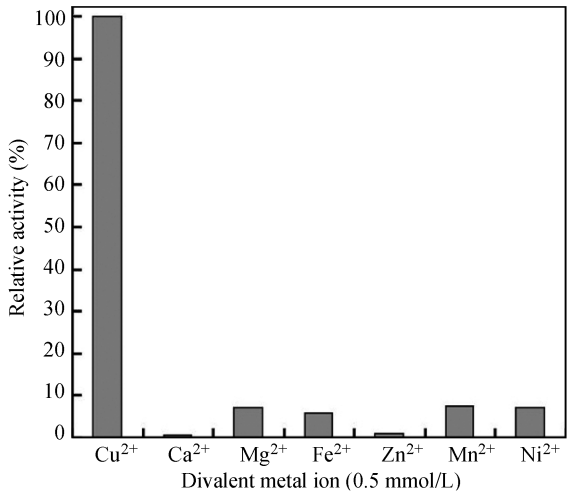

Fig. 3 Effects of divalent metal ions on the laccase activity. Enzyme activity was measured at 37°C in 100 mmol/L Tris-HCl (pH 8.0) containing 0.5 mmol/L metal ions and 0.5 mmol/L DMP.

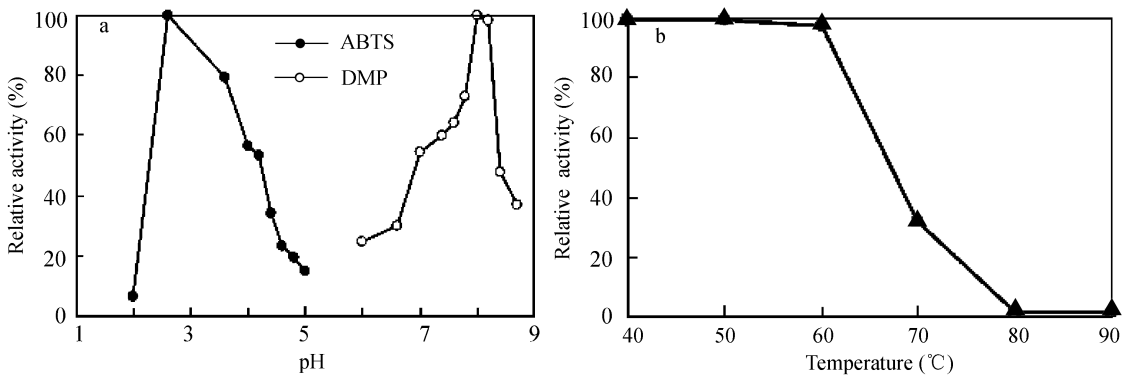

Fig. 2 Effects of pH (a) and temperature (b) on the laccase activity and stability. Enzyme activity was measured using 0.5 mmol/L ABTS or DMP. The remaining activity was measured after 10 min incubation at different temperatures.

Table 1 Kinetic parameters for laccase from *A.* hyrophila WL-11

Substrate	pH	K_m (mmol/L)	K_{cat} (sec^{-1})	K_{cat}/K_m (mmol/(L·sec))
ABTS	3.6	0.94	81.98	87.21
DMP	8.0	1.83	205.99	112.56

and DMP, respectively. These results indicate that *A. hydrophila* WL-11 laccase has a higher affinity toward ABTS than DMP.

3 Discussion

Although nucleotide sequences of the laccase gene from *Aeromonas hydrophila* ATCC 7966 (accession no. NC_008570) are deposited in GenBank database, biochemical characterization of the purified enzyme from *Aeromonas* species has not been reported. In this study, we firstly described the molecular cloning, sequencing, and heterogeneous expression of the gene encoding laccase from a newly isolated *A. hydrophila* WL-11. The enzymatic properties of the purified recombinant enzyme have been also characterized. The laccase gene from *A. hydrophila* WL-11 encoded a protein of 534 amino acids. A comparison of the deduced amino acid sequence of this enzyme with those of other laccases showed a high homology (more than 60%) with bacterial laccases in the genome and protein databases and the highest degree of similarity (61% identity) was observed with the multicopper oxidase of *Klebsiella* sp. 601 (ABY56791). *A. hydrophila* WL-11 laccase contains the conserved regions of four histidine-rich copper-binding sites which are specific for all laccases (Baldrian, 2006; Morozova et al., 2007; Sharma et al., 2007).

In addition, this enzyme has some properties similar to or in the range of most bacterial laccases characterized so far. The molecular mass of *A. hydrophila* WL-11 laccase (about 59 kDa) was similar to the laccases from *P. putida* F6 (59 kDa) (McMahon et al., 2007), *M. mediterranea* (59 kDa) (Jimenez-Juarez et al., 2005), *B. halodurans* (56 kDa) (Ruijssenaars and Hartmans, 2004), and *Klebsiella* sp. 601 (58 kDa) (Li et al., 2008).

The purified enzyme had an optimum pH of 2.6 and 8.0 for ABTS and DMP, respectively. This pH profile was also similar to that reported previously in which the oxidation of ABTS and DMP in most laccases usually occurs at strongly acidic and neutral or alkaline pH (Baldrian, 2006; Morozova et al., 2007; Sharma et al., 2007; Li et al., 2008). However, *A. hydrophila* WL-11 laccase showed a lower thermal stability than other bacterial laccases reported so far (Sharma et al., 2007). The kinetic study on ABTS revealed a higher affinity of *A. hydrophila* WL-11 laccase to this substrate, which was 0.94 vs. 1.83 mmol/L for DMP. This parameter was similar to those of laccases from *B. licheniformis* (Koschorreck et al., 2008) and *P. tephropora* (Ben Younes et al., 2007).

Generally, expression system in *E. coli* offers a means for the rapid and economical production of recombinant proteins compared to fungal expression system which is much more difficult to work with. However, overproduction of heterologous proteins in the cytoplasm of *E. coli* often results in the formation of insoluble and biologically inactive aggregates known as inclusion bodies (Baneyx, 1999). The formation of inclusion bodies is a major obstacle for large-scale production. It has been reported that overproduction of laccases from *B. subtilis* (Martins et al., 2002), *S. lavendulae* (Suzuki et al., 2003) and *Klebsiella* sp. 601 (Li et al., 2008) also resulted in an extensive intracellular aggregation. Therefore, many attempts of avoiding the formation of inclusion bodies have so far focused on increasing the soluble expression of heterologous proteins by manipulating the cellular folding apparatus (Jana and Deb, 2005; Sorensen and Mortensen, 2005). In this study, we have succeeded in high-level production of recombinant laccase as soluble and active form in the cytoplasm in *E. coli*. This will enable structural studies by crystallization followed by X-ray diffraction and commercial application. The recombinant laccase produced and characterized in this study may be a good candidate for the construction of a bioreactor for wastewater treatment containing a variety of phenolic compounds and aromatic amines.

4 Conclusions

The gene encoding laccase was cloned from a newly isolated *A. hydrophila* WL-11 which displays laccase activity, and then successfully expressed in *E. coli* BL21(DE3) cells. The high-level production of recombinant laccase and its characterization suggested that this enzyme can be used as a biocatalyst in biotechnological applications requiring high levels of immobilized laccase.

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