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Cloning and expression of thermo-alkali-stable laccase of *Bacillus licheniformis* in *Pichia pastoris* and its characterization

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HIGHLIGHTS

- The laccase gene from *Bacillus licheniformis* was expressed in *Pichia pastoris*.
- The recombinant enzyme was successfully secreted from yeast cells in an active form.
- The purified laccase was thermo-alkali-stable and resistant towards organic solvents.
- More than 93% of the tested dyes were decolorized after 6 h treatment by the laccase.

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ABSTRACT

A thermo-alkali-stable laccase gene from *Bacillus licheniformis* was cloned and expressed in *Pichia pastoris*. The recombinant laccase was secreted into the culture medium with a maximum activity of 227.9 U/L. The purified laccase is a monomeric glycoprotein, and its molecular weight was estimated to be 65 kDa on SDS–PAGE after deglycosylation. Optimal enzyme activity was observed at pH 6.2 and 70 °C with syringaldazine as substrate. The recombinant laccase was highly stable in the pH range 7–9 after 10 days at 30 °C. The enzyme displayed remarkable thermostability at 50–70 °C, with a half-life of inactivation at 70 °C of 6.9 h. It also exhibited high tolerance to NaCl and organic solvents like the native spore laccase. The purified laccase could rapidly decolorize reactive blue 19, reactive black 5 and indigo carmine in the presence of acetosyringone. More than 93% of the tested dyes were decolorized in 4 h at pH 9.0.

1. Introduction

Laccases (benzenediol:oxygen oxidoreductases, EC 1.10.3.2) belong to the multicopper oxidases (MCOs) family that normally contain four copper atoms. These enzymes are of particular interest with regard to various industrial applications based on their ability to utilize a broad range of substrates (Dwivedi et al., 2011). Industrial processes are often carried out at harsh environment, such as high temperature, high salt concentration or extremely acidic or alkaline pH. Therefore, resistant laccases are required to maintain high activity under these adverse physicochemical conditions (Santhanam et al., 2011). Fungal laccases usually show high redox potential and production yield than bacterial laccases (Dwivedi et al., 2011; Uzan et al., 2010; Fan et al., 2011). However, the majority of fungal laccases suffer from the drawbacks of low thermostability, narrow pH range as well as susceptible to salts, which limited their practical application (Fang et al., 2012; Santhanam et al., 2011).

In recent years, rapid progress has been made in finding and application of prokaryotic laccase. Bacterial laccases have great potential as biocatalysts due to their intrinsic properties of high thermal and alkaline pH stability (Singh et al., 2011). The major obstacle for their commercial application is the lack of sufficient enzyme stocks, as the production yield from native sources is usually very low (Dubé et al., 2008b). Recombinant production of enzymes in easily cultivable and handling hosts is often one of the best choices to achieve higher productivity. The most common host for heterologous protein expression is *Escherichia coli*, which is characterized for its fast growth and easy genetic manipulation (Banerjee et al., 2009). To date, several bacterial laccases have been successfully expressed in *E. coli* (Santhanam et al., 2011). However, all these recombinant enzymes were located intracellularly, which was difficult to purify and thus increased the production cost (Santhanam et al., 2011). Moreover, the recombinant laccase often formed insoluble aggregates, leading to significantly low yield (Martins et al., 2002; Suzuki et al., 2003; Fang et al., 2011). To solve these problems, extracellular protein expression may be an ideal alternative.

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Pichia pastoris is one of the most effective expression systems for the secretion of recombinant proteins (Damasceno et al., 2012). It offers several advantageous features including ease of genetic manipulation and rapid growth rate like bacteria. Meanwhile, foreign proteins can be secreted into the culture medium, which avoids the intracellular accumulation of target protein and simplifies the purification steps (Damasceno et al., 2012). We have recently reported the characterization and decolorization ability of a novel spore-associated laccase from *Bacillus licheniformis* (Lu et al., 2012). The spore laccase was featured by its excellent tolerance towards high temperature, alkaline pH and chloride, which make it a promising candidate for dye decolorization in textile industry. However, it is difficult to obtain this enzyme in a robust and inexpensive way due to its spore-bound nature (Hullo et al., 2001; Martins et al., 2002). In this investigation, we have cloned the laccase gene of *B. licheniformis* to investigate its effective expression in *P. pastoris*. The recombinant enzyme was purified, characterized and tested for its ability in dye decolorization.

2. Methods

2.1. Materials

2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS), syringaldazine (SGZ), 2,6-dimethoxyphenol (2,6-DMP), reactive blue 19, reactive black 5, indigo carmine and acetosyringone were all Sigma-Aldrich products (St. Louis, MO, USA). Bacteria DNA Kit, Gel Extraction Kit and Plasmid Mini Kit I were purchased from Omega Bio-Tek (Norcross, GA, USA). *Ex Taq* DNA polymerase, T4 DNA ligase, primers, pMD18-T plasmid and restriction enzymes were obtained from TaKaRa (Dalian, China). PageRuler prestained protein ladder was purchased from Fermentas (Ontario, Canada). Zeocin and expression vector pPICZαA were purchased from Invitrogen (Carlsbad, CA, USA). Other chemicals were of analytical regent grade.

2.2. Microbial strains and media

B. licheniformis LS04 was isolated from forest soil (Lu et al., 2012), and was deposited in China General Microbiological Culture Collection Center (CGMCC No. 4263). It was grown overnight in Luria-Bertani (LB) medium at 37 °C and 200 rpm. *E. coli* Top 10 competent cells (Tiangen, Beijing, China) were used for subcloning procedures and were grown in Low Salt LB medium. *P. pastoris* SMD1168H was products of Invitrogen (Carlsbad, CA, USA). Yeast extract-peptone-dextrose (YPD), buffered glycerol-complex (BMGY) and buffered minimal methanol (BMM) media were prepared according to the manual of the EasySelect *Pichia* Expression Kit (Invitrogen).

2.3. Cloning of laccase gene and homology modeling

Genomic DNA of *B. licheniformis* LS04 was prepared using the Bacteria DNA Kit. Amplication of the laccase gene was performed by PCR with forward primer 5′-CCGGAATTCAAACTTGAAAAATT CGTTG-3′and reverse primer 5′-CGGGGTACCTTATTGATGACGAA-CATCTG-3′. Recognition sites of *EcoR* I and *Kpn* I are indicated by underline. The PCR amplication program was initiated at 94 °C for 4 min, followed by 30 cycles of 94 °C for 45 s, 53 °C for 45 s and 72 °C for 2 min, and a final extension at 72 °C for 10 min. The amplified DNA fragment was purified using Gel Extraction Kit, and was cloned into pMD18-T vector. The recombinant pMD18-T vector containing the laccase gene was digested with *Eco*RI and *Kpn*I and then ligated between corresponding sites of the digested pPICZαA vector. The ligation mixture was transformed into *E. coli* Top10, and transformants were selected on Low Salt LB medium supplemented with 25 μg/mL Zeocin. A map of the recombinant plasmid pPICZαA/lac as confirmed by sequence analysis is shown in Supplementary data Fig. S1.

Homology modeling of the *B. licheniformis* LS04 laccase was performed using the SWISS-MODEL server (http://swissmodel.expasy.org/) based on the template of *Bacillus subtilis* CotA protein (PDB ID: 1W8E).

2.4. Expression and purification of recombinant laccase

The pPICZαA/lac vector was linearized with *Sac*I, and transformed into competent cells of *P. pastoris* by electroporation (Eppendorf Eporator, Humburg, Germany) at 1.5 kV with a 0.2-cm cuvette. Positive clones were selected on YPD plates supplemented with 100 μg/mL Zeocin, and then transferred to BMM plates containing 0.5 mM ABTS and 0.1 mM $CuSO_4$. The BMM plates were incubated at 28 °C and 100 μL of methanol was added to the lid each day. Laccase-producing transformants were identified by the presence of a dark green halo around the *Pichia* colonies.

Transformants with high laccase activity was inoculated in 25 mL BMGY medium, and incubated at 30 °C, 200 rpm. The cells were harvested by centrifugation at 3000 g, 4 °C for 5 min when the OD_{600} of the culture reached 2.0–6.0. The cell pellets were resuspended in BMM medium containing 0.1 mM $CuSO_4$ to an OD_{600} of about 1.0. The flasks were cultivated at 30 °C, 200 rpm, with 0.5% (v/v, final concentration) of methanol being added daily. Aliquots of culture supernatant were sampled every day to measure the enzyme activity. The supernatant was collected after 5 days by centrifugation at 3000*g*, 4 °C for 5 min. Purification of the recombinant laccase was performed using ultrafiltration, anion-exchange chromatography, and gel filtration according to Lu et al. (2007). Proteins were eluted with 20 mM sodium phosphate buffer (pH 7.5).

2.5. Enzyme assay

Laccase activity was measured at 30 °C using ABTS, syringaldazine (SGZ) and 2,6-dimethoxyphenol (2,6-DMP) as substrates (Lu et al., 2012). One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 μmol of substrate per minute. Protein concentration was determined using the Bradford Protein Assay Kit (Tiangen, Beijing, China) with bovine serum albumin as the standard. All assays were carried out in triplicate.

2.6. Characterization of the recombinant laccase

The molecular weight of the purified protein was determined by SDS–PAGE and gel filtration. SDS–PAGE was carried out using 12% polyacrylamide and stained with Coomassie Brilliant Blue R-250. Gel filtration was performed by Sephadex G-75 column calibrated with protein standard mixture containing lysozyme (14 kDa), chymotrypsinogen A (25 kDa), ovalbumin (43 kDa) and albumin (66 kDa). Deglycosylation of the purified laccase was performed using PNGase F (New England Biolabs, Ipswich, MA) according to the supplier's instructions. Zymography analysis for laccase activity was performed by separating unheated protein sample on 12% SDS polyacrylamide gel. The gel was washed with 0.1 M citrate-phosphate buffer (pH 4.0) for 5 min after electrophoresis. Protein bands with laccase activity were then visualized by immersing the gel in the same buffer containing 1 mM ABTS.

The effects of pH on laccase activity towards ABTS, SGZ and 2,6-DMP were determined in 0.1 M citrate-phosphate buffer (pH 3.0–7.0) and 0.1 M Tris–HCl buffer (pH 7.0–9.0). The enzyme stability against pH was assayed by measuring the residual activity after incubation at 30 °C in pH 3.0, 7.0 and 9.0. The temperature