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Expression of CotA laccase in *Pichia pastoris* and its electrocatalytic sensing application for hydrogen peroxide

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Abstract The CotA laccase from *Bacillus subtilis* WD23 was successfully overexpressed in *Pichia pastoris*, and the production level reached 891.2 U/L. The recombinant CotA laccase was purified to homogeneity. The optimal enzymatic activity was found at pH 4.6, 6.6, and 6.8 for 2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonate) (ABTS), 4-hydroxy-3, 5-dimethoxybenzaldehyde azine (SGZ), and 2, 6-dimethoxyphenol (2, 6-DMP) oxidation, respectively. The maximal enzyme activity was observed at 80 °C with SGZ as a substrate. The kinetic constant K_m values for ABTS, SGZ, and 2, 6-DMP were 162 ± 20, 24 ± 2, and 166 ± 18 μM, respectively, with corresponding k_{cat} values of 15 ± 1.0, 7.6 ± 1.5, and 0.87 ± 0.1 s^{-1}. Remarkably, the laccase activity increased to 561.9 % of its initial activity at pH 9.0 after 7 days of incubation and the half-life of laccase inactivation was approximately 3 h at 80 °C, which indicated that the recombinant CotA was a highly thermo-alkali-stable laccase. Bioelectrocatalytic reduction of H_2O_2 by the CotA laccase was detected when the recombinant CotA was adsorbed on pyrogenation graphite electrodes. Based on the bioelectrocatalytic reduction, a mediator-free amperometric biosensor for hydrogen peroxide was designed. The linear range of the H_2O_2 biosensor was from 0.05 to 4.75 mM, with a detection limit of 3.1 μM. The amperometric biosensor for H_2O_2 by CotA-modified electrode is a novel application for CotA laccase.

Keywords CotA · Laccase · Secretory expression · Bioelectrocatalytic · H_2O_2 biosensor

Introduction

Laccases belong to the family of multi-copper oxidases that catalyze the oxidation of a variety of phenolic and non-phenolic aromatic compounds. In these oxidation reactions, the transfer of four electrons from laccase to oxygen results in one oxygen molecule being reduced to water (Giardina et al. 2010; Solomon et al. 1996). The wide substrate range and the use of available oxygen as an electron acceptor make laccases highly useful biocatalysts for various industrial and biotechnological applications (Dwivedi et al. 2011). Laccases are widely distributed in many species including plants, fungi, bacteria, and insects (Baldrian 2005; Dittmer et al. 2004; Sharma et al. 2007). Bacterial laccases have attracted considerable attention over the last decade (Fang et al. 2011; Koschorreck et al. 2008; Lu et al. 2012). Compared with fungal laccases, bacterial laccases are able to overcome the disadvantages of instability. Moreover, the intrinsic property of high thermal stability and high alkaline tolerance render bacterial laccases great potential for industrial applications (Singh et al. 2011).

The CotA protein from *Bacillus subtilis* is a thermoactive and intrinsically thermostable bacterial laccase (Martins et al. 2002). This CotA protein is a structural component of the *B. subtilis* endospore coat and interacts tightly with other proteins to form the coat layer of the endospore (Driks 1999). Therefore, it is difficult to isolate and purify CotA protein from the endospore protein complex. In recent years, the

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CotA laccase has been expressed in *Escherichia coli*, but the recombinant laccases often form inactive inclusion bodies (Brander et al. 2014; Durao et al. 2008; Loncar et al. 2013). In addition, the recombinant CotA is mixed with intracellular proteins in *E. coli* that add more hardness to the purification. The low production yield of CotA makes the study and application of the enzyme difficult, so it is necessary to find a better expression system. Using secretory expression of CotA in the yeast system would be a good way to enhance the productivity of CotA isolation and purification. *Pichia pastoris* can serve as a potential host for heterologous protein production because of its efficient secretion of extracellular proteins, high expression levels, and ease of genetic manipulation (Damasceno et al. 2012). The *P. pastoris* expression system has been widely used for heterologous production of fungal laccases, which indicates that this system is suitable for laccase expression (Garg et al. 2012; Jolivalt et al. 2005; Lu et al. 2013; Otterbein et al. 2000).

In recent years, most research into the application of CotA laccases has focused on decolorization of dyes (Loncar et al. 2013; Pereira et al. 2009), biofuel cells (Beneyton et al. 2011; Durand et al. 2012), and organic synthesis (Koschorreck et al. 2008). However, there is little information available in literature about electron transfer between CotA and the electrode and its biosensor applications. CotA laccases have great potential as ideal sensing materials due to their intrinsic properties of high thermal and alkaline stability. The development of H_2O_2 sensors was necessary because H_2O_2 was not only the product of many enzymatic reactions but also an essential mediator in food, pharmaceutical, and environmental analysis (Safavi and Farjami 2010; Li et al. 2011). Recently, it was found that H_2O_2 could be reduced efficiently on laccase-modified electrode, which implies the potential for construction of H_2O_2 biosensor by laccase-modified electrode (Zhao et al. 2013).

In this study, we have constructed a *P. pastoris* strain for the secretory expression of CotA laccase from *B. subtilis*. The recombinant CotA laccase was purified and characterized with respect to its biochemical and catalytic properties. The direct electron transfer reactions and bioelectrocatalytic reduction of H_2O_2 catalyzed by recombinant CotA laccases were studied when the CotA proteins were adsorbed on pyrogenation graphite electrode. Furthermore, a mediator-free amperometric biosensor was designed for H_2O_2 detection. This research would provide a new insight into the application of CotA in H_2O_2 biosensor.

Materials and methods

Materials

2, 2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonate) (ABTS), 2, 6-dimethoxyphenol (2, 6-DMP), 4-hydroxy-3, 5-dimethoxybenzaldehyde azine (syringaldazine, SGZ), and nafion (perfluorinated ion exchange resin) were supplied by Sigma-Aldrich (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). Zeocin and expression vector pPICZαB were purchased from Invitrogen (Carlsbad, CA, USA). Bradford Protein Assay Kit and protein molecular weight marker were supplied by Tiangen (Beijing, China) and Transgen (Beijing, China), respectively. All other chemicals were of analytical regent grade.

Microorganism and media

B. subtilis WD23 was isolated from forest soil (Wang et al. 2013). The strain was grown in LB medium at 37 °C (180 r/min). *E. coli* DH5α-competent cells (Tiangen, Beijing, China) were used for the construction and routine propagation of vectors. *P. pastoris* SMD1168H was obtained from Invitrogen (Carlsbad, CA, USA). Yeast extract-peptone-dextrose, buffered glycerol-complex, and buffered minimal methanol (BMM) media containing 0.1 mM $CuSO_4$ and 0.8 % (g: vol.) DL-alanine were used to culture *P. pastoris* according to the manual of the Easy Select *Pichia* Expression Kit.

Cloning CotA gene of *B. subtilis* WD23

The genomic DNA of *B. subtilis* WD23 was extracted using the Bacteria DNA Kit, and the *cotA* gene was amplified by polymerase chain reaction (PCR) using the forward primer cotAs (GAACTGCAGGCACACTTGAAAAATTTG) containing a *Pst*I restriction site and the reverse primer cotAa (TCCCCGCGGTTATTTATGGGGATCAG) containing a *Sac*II site. A PCR amplification program was initiated at 94 °C for 5 min, followed by 35 cycles of 94 °C for 45 s, 59 °C for 45 s and 72 °C for 1.5 min, and a final extension at 72 °C for 7 min. The PCR products were purified using a Gel Extraction Kit and were inserted into the pMD18-T vector.

The recombinant pMD18-T-cotA was digested with *Pst*I and *Sac*II and then ligated between corresponding sites of the digested pPICZαB vector. The ligation mixture was transformed into *E. coli* DH5α, and transformants were selected on low-salt LB medium supplemented with 25 μg/mL Zeocin. The expression plasmid of pPICZαB-*cotA* was extracted and sequenced on both strands.

Enzyme assay

The laccase activity measurements were performed using a spectrophotometer. The assay temperature was 30 °C. The oxidation of ABTS (1 mM) was measured at 420 nm

(ε = 36,000 M^{-1} cm^{-1}) in 0.1 M citrate-phosphate buffer (pH 4.6). The oxidation of SGZ (0.1 mM) was detected at 525 nm (ε = 65,000 M^{-1} cm^{-1}) in 0.1 M citrate-phosphate buffer (pH 6.6). The oxidation of 2, 6-DMP (2 mM) was determined at 470 nm (ε = 49,600 M^{-1} cm^{-1}) in 0.1 M citrate-phosphate buffer (pH 6.8). One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 μmol of substrate per min at 30 °C.

Overproduction and purification of recombinant CotA

The recombinant expression vector pPICZαB-cotA was linearized with restriction enzyme *Sac*I and then transformed into *P. pastoris* through electroporation. The DNA from the *P. pastoris* transformed with pPICZαB-*cotA* was isolated and purified. The genomic DNA was used directly as a PCR template, and the *cotA* gene was amplified with the forward primer cotAs and the reverse primer cotAa. The transformation and expression of CotA laccase were performed according to Lu et al. (2013). Positive clones available on YPDS agar plates containing 100 mg/mL Zeocin were further screened on buffered minimal medium (BMM) plates containing 0.5 mM ABTS and 0.1 mM $CuSO_4$. The cultured supernatant of laccase-producing transformants was collected after 12 days by centrifugation at 6600×*g* (4 °C for 10 min). The cell-free culture medium was concentrated in a dialysis bag (molecular weight 8000–14,000 cutoff) with polyethylene glycol 2000. Then, the concentrated laccase was loaded onto a DEAE Sepharose FF column (GE, Uppsala, Sweden) equilibrated with citrate-phosphate buffer (20 mM pH 7.5). The column was washed with the same buffer, and the absorbed proteins were eluted by a linear NaCl gradient (300 mL, 0–1 M) at 1 mL/min. The factions with laccase activity were pooled and concentrated by ultrafiltration (10 kDa cutoff; Amicon, Bedford, MA, USA). The resulting sample was then applied to a Sephadex G-75 column (GE, Uppsala, Sweden) equilibrated with the same buffer at 0.2 mL/min, and the fractions with laccase activity were also pooled and concentrated as described above. The purification products were detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and native-polyacrylamide gel electrophoresis (native-PAGE). The purity and molecular mass of the purified protein were determined by SDS-PAGE using a 12 % resolving gel. Protein bands were stained with Coomassie Brilliant Blue R-250. The molecular mass of the purified protein was determined by its relative mobility. Zymography analysis for laccase activity was performed by native-PAGE on 12 % polyacrylamide gel. The gel was stained in 0.1 M citrate-phosphate buffer (pH 4.0) containing 1 mM ABTS at 40 °C.

Catalytic properties of the CotA laccase

The effects of pH on CotA laccase activity towards ABTS (1 mM), SGZ (0.1 mM), and 2, 6-DMP (2 mM) were measured in 0.1 M citrate-phosphate buffer (pH 3.4–8.0) at 30 °C. The temperature optimum for laccase activity was determined using SGZ (0.1 mM) as the substrate in 0.1 M citrate-phosphate buffer (pH 6.6) at temperatures ranging from 0 to 100 °C. To determine the pH stability, the remaining activity of the laccase was measured after it was incubated for 1, 2, 3, 4, and 7 days at 0.1 M citrate-phosphate buffer (pH 3, 7, and 9) at 24 °C. To estimate the thermostability, the remaining activity of the enzyme solution was measured after 1 to 10 h of incubation at temperatures ranging from 60 to 90 °C in 0.1 M citrate-phosphate buffer (pH 6.6). The remaining activities were determined using SGZ as the substrate in 0.1 M citrate-phosphate buffer (pH 6.6) at 30 °C. Kinetic parameters for purified laccase were determined at 37 °C using different concentrations of ABTS (10–1000 μM), SGZ (5–100 μM), and 2, 6-DMP (50–2000 μM). The solutions used to determine kinetic values were air-saturated. The protein concentration was determined using the Bradford Protein Assay Kit. The Cu content was measured by inductively coupled plasma atomic emission spectrometry. All assays were conducted in triplicate.

Preparation of CotA-modified electrode

The pyrogenation graphite (GP) electrodes were thoroughly polished and washed before use: the electrodes (3 mm in diameter) were polished sequentially with 1.0, 0.3, and 0.05 mm alumina slurry, and then washed ultrasonically in water and ethanol for 1 min to remove all physically adsorbed particles. To form the CotA-modified electrode by simple adsorption, the GP electrodes were covered with 10 μL (1 μg/μL) purified CotA laccase and maintained at room temperature until dry. To form the CotA-modified electrode by nafion immobilization, the 10 μL (1 μg/μL) purified CotA laccase was mixed with 10 μL 0.5 % nafion. The 20 μL mixture was dropped on the pretreated GP electrode and dried under ambient conditions for 3 h. The two CotA-modified electrodes are referred to as CotA/GP electrode and CotA/Nafion/GP electrode.

Electrochemical measurements

The cyclic voltammetric and amperometric measurements were performed using the electrochemical workstation Chenhua CHI660D (Shanghai, China). A three-electrode set-up was used for all electrochemical experiments. The GP/CotA electrode was used as the working electrode. A platinum wire and an Ag/AgCl (saturated KCl) electrode were used as the counter electrode and the reference electrode, respectively. Nitrogen-saturated citrate-phosphate buffer (20 mM, pH 7.6)

was used as the electrolyte for electrochemical measurements. All pH 7.6 citrate-phosphate buffers were deoxygenated by bubbling highly pure nitrogen through for at least 20 min before use. The electrocatalytic reduction of hydrogen peroxide on the CotA/GP electrode was detected through cyclic voltammetry measurement. The following parameters were applied: initial voltage of −0.8 V, high voltage of 1 V, low voltage of −0.8 V, scan rate 0.1 V/s, sample interval 0.001 V, quiet time 2 s, and sensitivity 10^{-4} A/V. The amperometric response of the CotA/GP electrode to H_2O_2 was measured by amperometric measurements, and the parameters of amperometric measurements were as follows: initial voltage of −0.7 V, sample interval 0.1 s and sensitivity 10^{-4} A/V. All measurements were carried out at room temperature. The long-term stability of CotA/GP electrode and CotA/Nafion/GP electrode was tested by measuring its current response to 0.5 mM H_2O_2 for 1 week. The two modified electrodes were stored under ambient conditions, and the current responses were tested every day.

Nucleotide sequence accession number

The newly determined open reading frame of *B. subtilis* WD23 CotA laccase gene is available from GenBank under the accession number KJ722789.

Collection and strain number

The *B. subtilis* WD23 strain in this study is available in the China Center of Industries Culture Collection (CICC) that belongs to WDCM under the deposited number CICC 23830.

Results

Cloning of cotA from *B. subtilis* WD23

The open reading frame of *B. subtilis* WD23 *cotA* gene was cloned, and the cloned sequence was deposited in the GenBank database under accession number KJ722789. Sequence analysis showed that the DNA sequence of the *cotA* gene was 1542 bp encoding a 513 amino acid protein. The CotA laccase from *B. subtilis* WD23 had an amino acid identity of 98 % with laccase from *Bacillus vallismortis* (AGR50961.1), 68 % with CotA from *Bacillus pumilus* (AFK33221.1) and 66 % with CotA from *Bacillus licheniformis* (WP 020,450,420.1). The ligands that bind T1 copper (H419, C492, H497, M502), T2 copper (H105, H422), and T3 copper (H107, H153, H155, H424, H491, H493) in *B. subtilis* WD23 CotA were all highly conserved in CotA laccases. These ligands and some adjacent amino acids constituted the four histidine-rich copper-binding regions that were conserved for bacterial laccase. According to the classification of laccase based on the $E^{\circ\prime}$ value of the T1 copper, CotA laccase belongs to the low redox potential enzymes with methionine as an axial ligand of the T1 copper (Shleev et al. 2005b).

Secretory expression of CotA laccase in *P. pastoris*

The *cotA* gene was inserted into the vector pPICZαB within the framework of the α-factor secretion signal gene, under the control of the *AOX1* promoter. The vector was transformed into the electro-competent *P. pastoris* SMD1168H cells. The integration of the *cotA* gene into genome of *P. pastoris* was checked by PCR amplification and DNA sequencing. The transformants were tested for laccase expression by being cultured on the BMM plates under methanol-inducible conditions. The BMM plate was supplemented with 0.5 mM ABTS and 0.1 mM $CuSO_4$. A dark green halo zone was observed to form around the *P. pastoris* that contained the recombinant plasmid, whereas no green halo was observed around the *P. pastoris* SMD1168H colony. The clone, which showed the darkest green zone on the plates, was chosen to produce the recombinant laccase using liquid cultures. To investigate the laccase activity of recombinant CotA in the liquid cultures, the oxidation of laccase substrates ABTS, SGZ, and 2, 6-DMP was tested. These three substrates were successfully oxidized by crude recombinant CotA laccase; however, in liquid cultures of wild-type *P. pastoris*, no significant catalysis was observed. These phenomena indicated that an α-factor signal peptide could effectively direct the secretion of the recombinant CotA into the liquid cultures with laccase activity.

The relationship between the production of laccase and the growth of the yeast was studied. Meanwhile, the pH of the cultures was determined. Because of the addition of 0.8 % alanine in BMM, the pH decrease of the cultures during the 12-day fermentation was merely one unit. Seven days after the culture began, cell concentration and laccase activity increased gradually. From day 7 to day 12, the biomass reached the maximum, and laccase activity increased dramatically. The maximum laccase activity of 891.2 U/L (ABTS) was observed 12 days after the initiation of induction (Fig. 1).

Purification and characterization of the CotA laccase

Culture supernatant fractions containing laccase were harvested and concentrated. The recombinant laccase was purified using anion-exchange chromatography and gel filtration. SDS-PAGE and native-PAGE were performed to confirm protein purification and enzymatic activity. SDS-PAGE analysis of the enzyme showed a single band with a molecular weight of 65 kDa (Fig. 2a). The green band on native-PAGE was oxidized ABTS, which indicated the laccase activity of the purified protein (Fig. 2b).

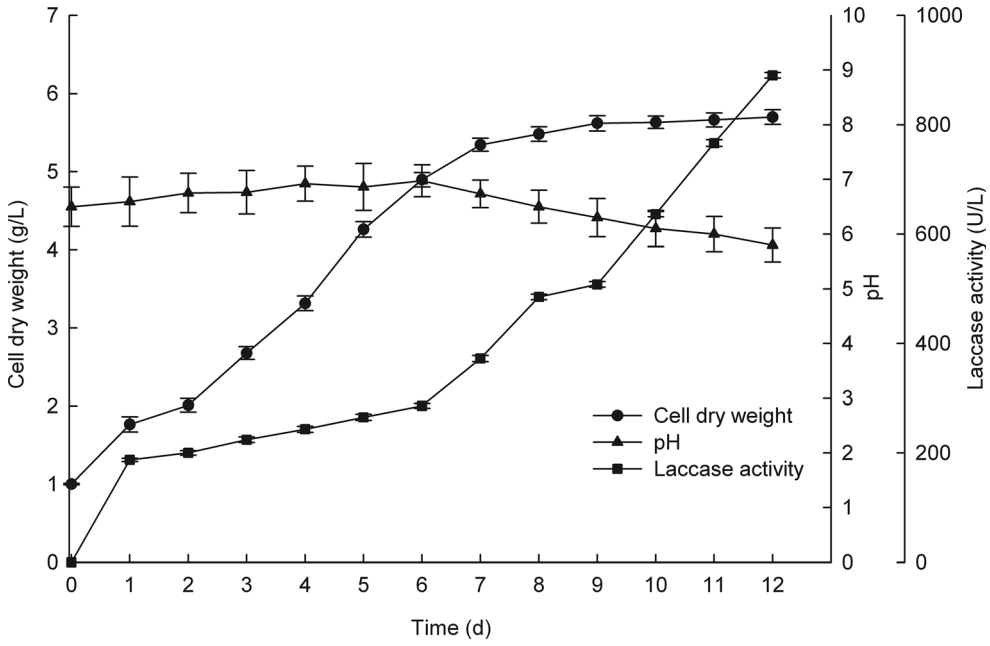

Fig. 1 Time course of *P. pastoris* transformant growth, recombinant laccase production, and pH change in BMM medium at 30 °C. The laccase activity measurements were performed using ABTS as substrate in 0.1 M citrate-phosphate buffer (pH 4.6)

The optimum pH values of CotA laccase for oxidizing ABTS, SGZ, and 2, 6-DMP were 4.6, 6.6, and 6.8, respectively (Fig. 3a). These three substrates could easily be oxidized under acidic conditions. The pH stability studies showed that the recombinant CotA laccase was unstable at pH 3.0 and lost 71.3 % of its activity after 24 h. However, the enzyme was highly stable under neutral and alkaline conditions. The enzyme activity increased to 348.7 and 561.9 % of its initial activity at pH 7.0 and pH 9.0 after 7 days of incubation under room temperature (Fig. 3b). These results indicated that the CotA laccase possessed alkaliphilic property.

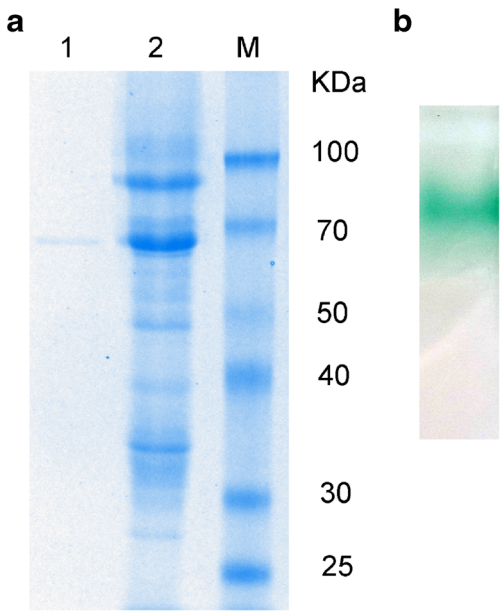

Fig. 2 SDS-PAGE (**a**) and native-PAGE (**b**) analysis of CotA laccase. **a** The gel was stained with Coomassie Brilliant Blue. *Lane 1*: purified protein; *Lane 2*: concentrated culture supernatant; *Lane M*: protein marker. **b** Zymogram detection with 1 mM ABTS at 40 °C in 0.1 M citrate-phosphate buffer (pH 4.0)

Another property of CotA laccase was its ability to tolerate high temperatures. The highest activity of recombinant CotA laccases from *B. subtilis* WD23 was observed at 80 °C under the conditions of SGZ oxidation. A prominent peak of the relative activity was observed as the temperature was raised from 60 to 80 °C (Fig. 4a). The thermostability of the CotA laccase was tested at different temperatures (60–90 °C) for 0 to 10 h (Fig. 4b). The thermal denaturation profile indicated that the laccase was significantly thermostable at 60 and 70 °C. The CotA laccase retained more than 72 and 57 % of its initial activity after the incubation for 10 h. The half-life of laccase inactivation was approximately 3 h at 80 °C, and it retained 22 % of its initial activity even after 2 h at 90 °C.

The initial reaction rate at various concentrations of ABTS, SGZ, and 2, 6-DMP was determined at optimum pH for the three substrates. The dependence of the rate on the substrate concentration followed Michaelis-Menten kinetics. From Lineweaver-Burk plots, the values of K_m, V_{max}, and k_{cat} were calculated and compared to the constants of CotA from *B. subtilis*. As shown in Table 1, the values of K_m and k_{cat} obtained were similar to the values of recombinant CotA expressed by *E. coli* (Martins et al. 2002). The CotA presented a higher affinity for the SGZ than for the ABTS and 2, 6-DMP. Compared with the holoCotA, the CotA exhibited enormous differences regarding the calculated k_{cat} values. The catalytic constant of holoCotA for the three substrates was much larger than the catalytic constant of the CotA. The inductively coupled plasma atomic emission spectrometry was performed to measure the content of copper atoms in the purified CotA

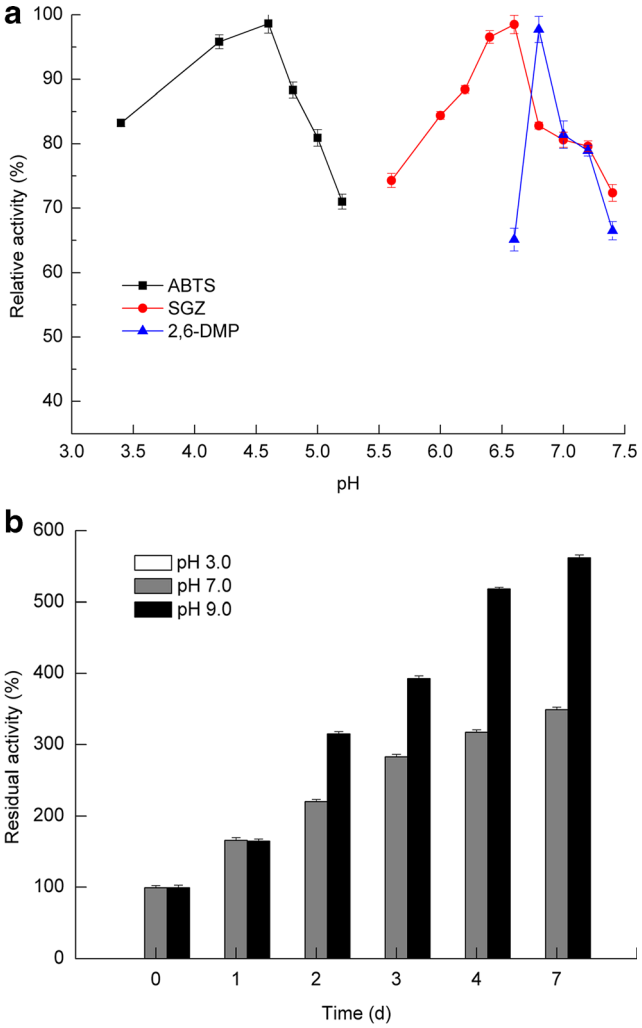

Fig. 3 Effect of pH on activity (**a**) and stability (**b**) of the recombinant CotA laccase at 30 °C. **a** The effects of pH on CotA laccase activity towards ABTS (1 mM), SGZ (0.1 mM), and 2, 6-DMP (2 mM) were measured in 0.1 M citrate-phosphate buffer at 30 °C. **b** Residual activity was measured after incubation in 0.1 M citrate-phosphate buffer pH 3, 7, and 9 at 24 °C

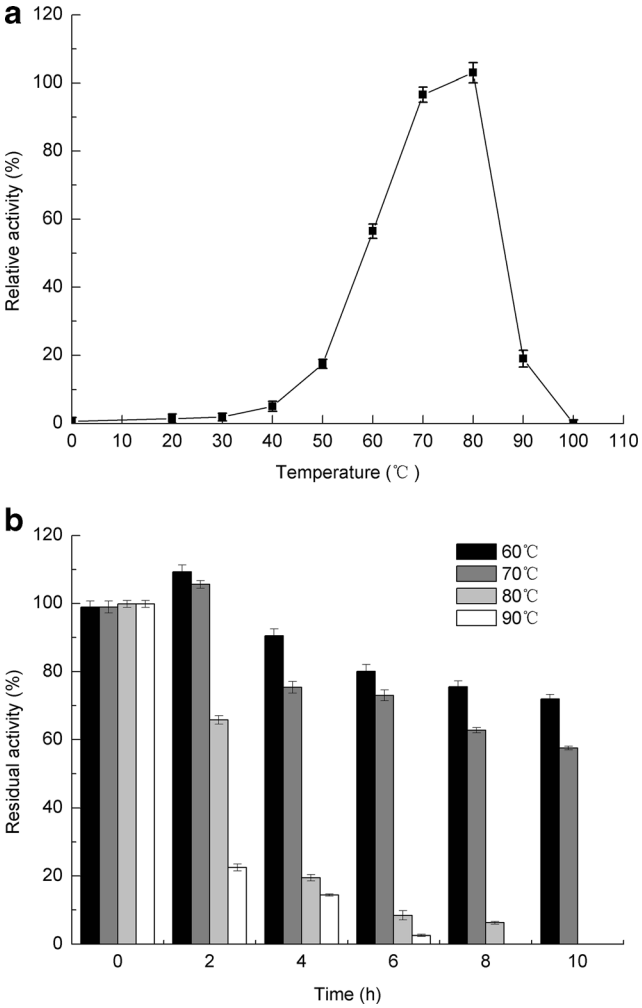

Fig. 4 Effect of temperature on the activity (**a**) and stability (**b**) of recombinant CotA laccase. **a** The temperature optimum for laccase activity was determined using SGZ (0.1 mM) as the substrate in 0.1 M citrate-phosphate buffer (pH 6.6). **b** Residual activity was measured after 1 to 10 h incubation at temperatures ranging from 60 to 90 °C in 0.1 M citrate-phosphate buffer (pH 6.6)

protein. The quantitative analysis resulted in 2.8 copper atoms per protein molecule, which indicated that the CotA was not a holoCotA form with four Cu atoms per protein. The low ratio of copper/protein (2.8:1) justified the relatively low efficiency of the purified CotA enzyme.

Electrocatalytic activity and amperometric biosensor

The direct electrochemistry of CotA on the pyrogenation graphite electrode was studied to understand the electrotransfer process and develop amperometric biosensors for H_2O_2. The electrocatalytic activity of CotA towards reduction of H_2O_2 was examined. The cyclic voltammogram of the CotA/GP electrode showed the reduction peak at −0.7 V, whereas no reduction peak was detected with the same concentration of H_2O_2 using the bare GP electrode (Fig. 5). The changes in redox currents of CotA displayed an electrocatalytic behavior of immobilized CotA to the reduction of H_2O_2. The results demonstrated that CotA was able to catalyze the reduction of H_2O_2. The electrocatalytic process can be proposed as follows:

$$CotA_{OX} + 2e^- \rightarrow CotA_{red} \quad (1)$$

$$H_2O_2 + CotA_{red} + 2H^+ \rightarrow 2H_2O + CotA_{OX} \quad (2)$$

The electrons were transferred from the electrode to the $CotA_{ox}$ by the direct electron transfer, and then the $CotA_{ox}$ was reduced to $CotA_{red}$. Consequently, the electrons were transferred from $CotA_{red}$ to the H_2O_2. $CotA_{red}$ converted to its oxidized form, and H_2O_2 was reduced to H_2O.

Upon the addition of H_2O_2, the reduction peak current increased dramatically. The amperometric response of the

Table 1 Kinetic parameters of recombinant CotA laccase from *B. subtilis*

Laccase	ABTS		SGZ		2, 6-DMP		References
	K_m (μM)	k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})	
CotA	162 ± 20	15 ± 1	24 ± 2	7.6 ± 1.5	166 ± 18	0.87 ± 0.1	This study
CotA	106 ± 11	16.8 ± 0.8	26 ± 2	3.7 ± 0.1			Martins et al. (2002)
HoloCotA[a]	124 ± 17	322 ± 20	18 ± 3	80 ± 4	216 ± 35	29 ± 4	Durao et al. (2008)

[a] HoloCotA means the fully copper loaded CotA with four Cu atoms per protein

CotA/GP electrode to H_2O_2 is shown in Fig. 6. The reduction current increased steeply to reach a steady-state value with the addition of aliquots of H_2O_2. The electrode achieved a steady-state current in less than 25 s, which means that the electrocatalytic response was fast. The inset in Fig. 6 shows the linear calibration curve of the CotA/GP electrode under the optimized experimental conditions. The linear range of the H_2O_2 sensor ranged from 0.05 to 4.75 mM, with a correlation coefficient of 0.9932. The detection limit was estimated to be 3.1 μM at a signal to noise ratio of 3. The long-term stability of the CotA/GP electrode and the CotA/Nafion/GP electrode was studied. The current responses of the two electrodes to 0.5 mM H_2O_2 were tested every day. The CotA/GP electrode retained approximately 72 % of the initial response current. As for the CotA/Nafion/GP electrode, 93 % of its initial response current was retained after 1 week (Fig. 7).

Discussion

The CotA protein is an important bacterial laccase and is widely distributed in the *Bacillus* genus, for example, *B. subtilis*, *B. pumilus*, *Bacillus clausii*, *B. licheniformis*, and *Bacillus amyloliquefaciens* (Brander et al. 2014; Guan et al. 2014; Koschorreck et al. 2008; Loncar et al. 2013; Martins et al. 2002). The activity and stability at high pH and temperature make the CotA laccases alternatives for some special fields where the fungal laccases are inactive. However, the low production yield and difficulties in protein purification are barriers to the widespread application of CotA laccase. In this study, we constructed the recombinant *P. pastoris* strains to enhance the production yields of the laccase. The *cot*A gene was cloned into *P. pastoris* using an *AOX1* promoter and a native *Saccharomyces cerevisiae* α-factor secretion signal. The promoter allowed the methanol-inducible, high-level expression of CotA in *P. pastoris*, and the secretion signal directed the secretion of active recombinant CotA laccase. The laccase activity were measured as 891.2 U/L in liquid cultures of pPICZαB-*cotA*-transformed *P. pastoris* strain, showing that this *P. pastoris* was an efficient CotA laccase producer compared with the *Yarrowia lipolytica*, which possessed an activity of 230 U/L (Jolivalt et al. 2005).

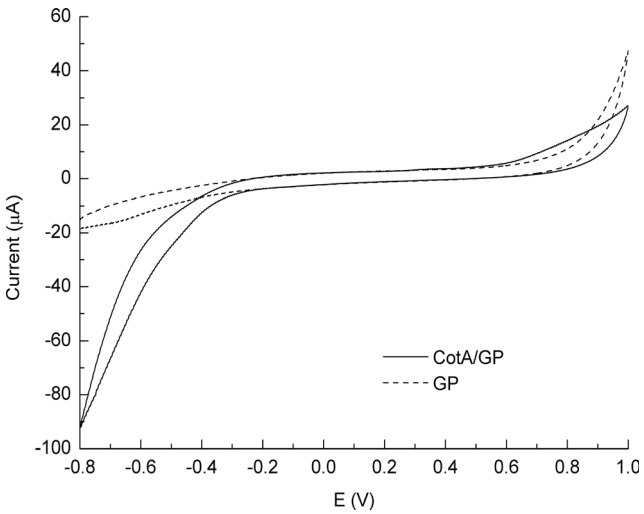

Fig. 5 Cyclic voltammograms obtained on the bare GP and CotA/GP electrodes. The experiments were conducted in 20 mM nitrogen-saturated citrate-phosphate buffer (pH 7.6) with 25 mM H_2O_2

The previous studies of laccase expression in *P. pastoris* demonstrated the importance of pH in laccase production (O'Callaghan et al. 2002). The pH of cultures was observed to decrease from 7 to 3.5 during the 10 days of fermentation (Lu et al. 2013). However, the CotA laccase was unstable under acidic condition. In our study, the alanine was added to the medium to stabilize the pH of the culture medium because it is possible that the ammonia released during the metabolism process of the alanine was able to neutralize the acidic end products of the methanol metabolism process (O'Callaghan et al. 2002). As we added 0.8 % alanine to the medium, the pH decrease observed in the cultures during the 12 days of fermentation was merely one unit, and the increase in laccase activity continued until the end of the fermentation process. The laccase activity of the liquid cultures reached 891.2 U/L. In cultures without alanine, the pH decrease of the cultures during the 10 days of fermentation was three units, and recombinant CotA laccase production leveled off on the 7th day, with a maximum activity of 227.9 U/L (Lu et al. 2013).

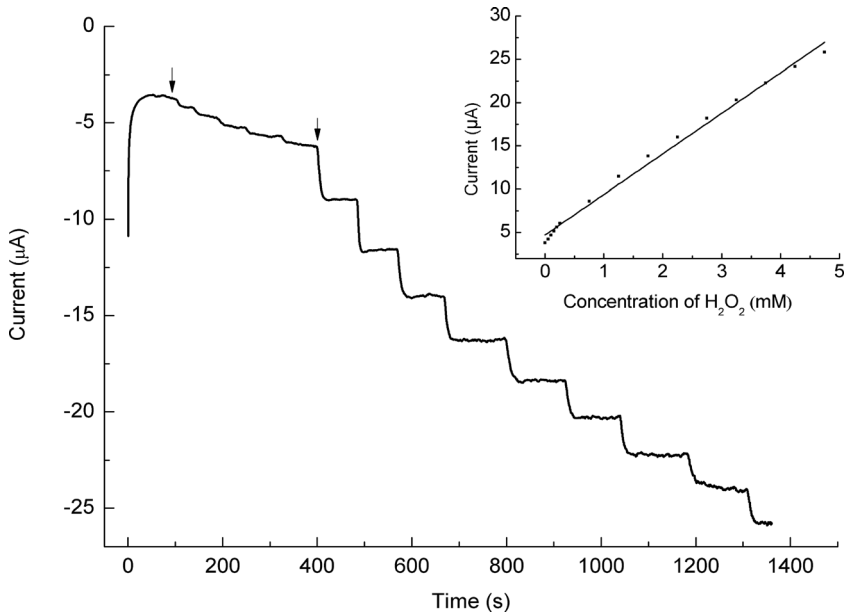

Fig. 6 Amperometric response of the CotA/GP electrode with successive additions of 50 μM and 0.5 mM H_2O_2. *Inset*: The linear calibration curve for H_2O_2 determination. The measurements were made at an applied potential of −0.7 V vs. Ag/AgCl electrode in 20 mM nitrogen-saturated citrate-phosphate buffer (pH 7.6) at room temperature

The alkaline pH stability and the high thermostability are striking properties of CotA laccases from the *Bacillus* genus. The recombinant CotA laccase from *B. subtilis* WD23 had 561.9 % of its initial activity at pH 9.0 after 7 days. The activity of the CotA laccase increased during storage at pH 9. A similar phenomenon has been reported in some of the literature. For example, the recombinant CotA laccase from *B. subtilis* LS02 showed high stability under alkaline condition and the activity increased to 637 % of its initial activity after a 10-day incubation at pH 9 (Wang et al. 2015). The CotA laccase from *B. subtilis* X1 had approximately 163 % of the initial activity when incubated at 37 °C for 10 days (Guan et al. 2014). The CotA laccase from *B. licheniformis* was also stable at pH 9.0, and the activity increased to 237.7 % of its activity after 10 days at 30 °C (Lu et al. 2013). However, to date, there is no clear explanation for this phenomenon. Therefore, further studies are needed to explain the reason why the laccase activity increases during storage under the alkaline condition. Compared with the laccase from fungi such as *Myrothecium verrucaria* NF-05 and *Lentinula edodes*, the recombinant CotA laccase was more stable over a wider pH range, especially under alkaline conditions (Nagai et al. 2002; Zhao et al. 2012). The pH optimum of *B. subtilis* CotA for ABTS, SGZ, and 2, 6-DMP oxidation was similar to the pH optimum of the *B. licheniformis* and *B. pumilus* Cot A laccases (Guan et al. 2014; Koschorreck et al. 2008; Lu et al. 2013; Reiss et al. 2011).

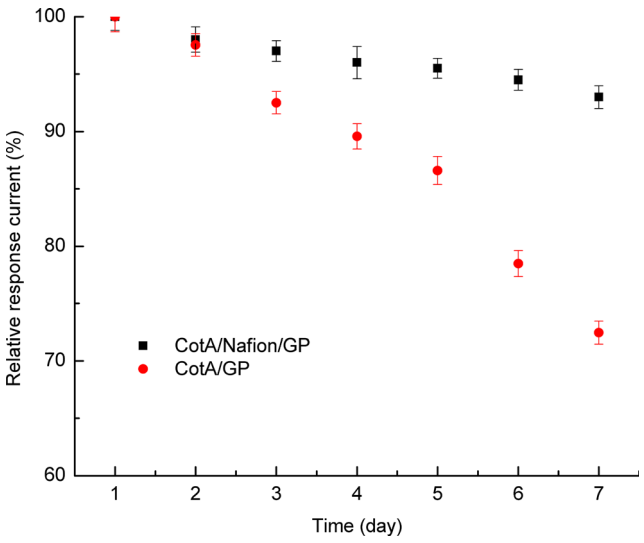

Fig. 7 Long-term stability of CotA/GP electrode and CotA/Nafion/GP electrode. The response current was tested in 20 mM nitrogen-saturated citrate-phosphate buffer (pH 7.6) with addition of 0.5 mM H_2O_2 at an applied potential of −0.7 V vs. Ag/AgCl electrode

Another remarkable property of the CotA enzyme from *B. subtilis* was its high intrinsic thermostablity. The relatively high thermostability of the CotA laccase from *B. subtilis* has been reported. The half-life of inactivation was 112 min at 80 °C for the purified CotA laccase from *B. subtilis* MB24 (Martins et al. 2002). For CotA laccase from *B. subtilis* X1, the half-life exceeded 6 h at 70 °C and was 2.5 h at 80 °C (Guan et al. 2014). In this study, the recombinant CotA laccase from *B. subtilis* WD23 behaved in a significantly stable manner at 80 and 90 °C. The half-life of laccase inactivation was approximately 3 h at 80 °C and retained 22 % of its initial activity even after 2 h at 90 °C. The recombinant CotA laccase from *B. subtilis* exhibited higher thermostability than the laccase from *B. amyloliquefaciens* and *B. licheniformis* (Koschorreck et al. 2008; Loncar et al. 2013; Lu et al. 2013). The high degree of domain packing, the high proline content, and the highly packed atomic core around the copper centers could be three factors that promote the thermostability

of CotA laccase from *B. subtilis*. A larger number of hydrophobic interactions between domains 1 and 2 as well as the external connections could contribute to the higher degree of domain packing in CotA laccase (Enguita et al. 2003).

Direct electron transfer (DET) reactions between proteins and electrodes have been shown for many enzymes from different sources (Shleev et al. 2005a; Shleev et al. 2005b). Laccases have frequently been applied to the reduction of O_2 based on DET from the electrodes to the enzymes without any electron transfer mediators (Beneyton et al. 2011; Kuwahara et al. 2013). The use of CotA for reducing H_2O_2 on electrodes has, however, not previously been explored to our knowledge. The CotA-modified electrode displayed an electrocatalytic reduction behavior towards H_2O_2. The catalytic efficiency of the GP/CotA electrode was 4.5 times the catalytic efficiency of the bare GP electrode, and the initial reduction potential of H_2O_2 positive increased 0.4 V compared with the bare GP electrode. The electrocatalytic reduction behavior towards H_2O_2 has also been researched using a fungal laccase-modified electrode (Zhao et al. 2013). The catalytic efficiency of CotA was significantly higher than the catalytic efficiency of the laccase from *M. verrucaria NF-05* (Zhao et al. 2013).

In this study, the cyclic voltammogram of the CotA/GP electrode showed a reduction peak at an applied potential of −0.7 V vs. Ag/AgCl electrode, whereas no reduction peak was detected with the same concentration of H_2O_2 using the bare GP electrode. Because of the addition of the CotA protein, the reduction peak of H_2O_2 was found, and the reduction peak current increased with the concentration of H_2O_2. This result indicated that the CotA laccase was able to transfer electrons from the pyrogenation graphite electrode to H_2O_2 concomitantly with the H_2O_2 reduction when the electrolyte was deoxygenated by bubbling highly pure nitrogen. This study proves that the CotA has the ability to reduce hydrogen peroxide. The X-ray structure determination of CotA laccase in a complex with hydrogen peroxide allowed the proposal of the mechanism for the O_2 reductive reaction (Bento et al. 2005, 2010). The proposed mechanism involves binding the dioxygen into the trinuclear center such that the dioxygen is located approximately symmetrically between the two type 3 copper ions, with one oxygen atom close to the type 2 copper ion. Further stages involve the formation of a peroxide intermediate, and following the splitting of this intermediate, the migration of the hydroxide moieties towards the solvent exit channel. In this mechanism, H_2O_2 was an intermediate when CotA catalyzed the reduction of O_2 to H_2O, so the CotA laccase can catalyze the reduction of H_2O_2 to H_2O. Besides dioxygen, H_2O_2 is another electron acceptor leading to the formation of water at the T2/T3 trinuclear center of the enzyme. Our research results agree with this mechanism that the CotA laccase has the ability to catalyze the reduction of H_2O_2 to H_2O.

The CotA-modified electrodes presented good long-term stability. The CotA/GP electrode retained approximately 72 % of the initial response current and the CotA/Nafion/GP electrode retained 93 % of its initial response current after 1 week. The CotA/Nafion-modified elctrode showed better long-term stability than the one which simply adsorbed CotA since the nafion film formed can prevent the CotA protein losing from the electrode surface. The CotA-modified electrode was utilized to fabricate a biosensor for the sensing of H_2O_2. The biosensor displayed a good and quick electrocatalytic response to the reduction of H_2O_2. The performance of the H_2O_2 biosensor illustrated the potentiality and the advantage of CotA as a transducer for H_2O_2 detection and preparation of amperometric biosensors. To date, a number of H_2O_2 biosensors have been reported (ElKaoutit et al. 2008; Li et al. 2011; Xu et al. 2007). Among these reports, horseradish peroxidase was generally used to fabricate the biosensors for the detection of H_2O_2. Compared with these biosensors, the laccase CotA from *B. subtilis* endospore coat is a thermophilic enzyme and is an ideal sensing material because of its ability to tolerate high temperatures and alkaline conditions. Therefore, the CotA laccase would be a good candidate for preparing H_2O_2 biosensors. The sensitive and rapid determination of H_2O_2 by the CotA-modified electrode is of great importance because H_2O_2 is associated with the diagnostic response in monitoring the blood glucose (Thomé-Duret et al. 1996). The main product of the oxidation of glucose by glucose oxidase is H_2O_2. Thus, there is the potential to achieve the determination of glucose when the electrocatalytic reduction of H_2O_2 by CotA is coupled with the oxidation of glucose by glucose oxidase.

In conclusion, the *cotA* gene of *B. subtilis* WD23 was cloned and successfully expressed in the culture supernatant of *P. pastoris.* The biochemical properties of the purified CotA laccase showed that the enzyme exhibited high stability towards an alkaline pH and high temperatures. The purified CotA laccase had been immobilized on the GP electrode, and the direct reduction of H_2O_2 by the catalysis of the immobilized CotA was achieved. The biosensor exhibited a fast amperometric response, a wide linear range, and good long-term stability. This work not only provided a highly efficient method to improve the yield of bacterial laccases but also broadened the practical application of CotA protein in the biosensor field.

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Ethical statement This article does not contain any studies with human participants or animals performed by any of the authors. Informed consent was obtained from all individual participants included in the study.

Conflict of interest The authors declare that they have no competing interests.

References

Baldrian P (2005) Fungal laccases-occurrence and properties. FEMS Microbiol Rev 30:215–242. doi:10.1111/j.1574-4976.2005.00010.x

Beneyton T, El Harrak A, Griffiths AD, Hellwig P, Taly V (2011) Immobilization of CotA, an extremophilic laccase from *Bacillus subtilis*, on glassy carbon electrodes for biofuel cell applications. Electrochem Commun 13:24–27. doi:10.1016/j.elecom.2010.11.003

Bento I, Martins LO, Lopes GG, Carrondo MA, Lindley PF (2005) Dioxygen reduction by multi-copper oxidases; a structural perspective. Dalton Trans 21:3507–3513. doi:10.1039/B504806K

Bento I, Silva CS, Chen Z, Martins LO, Lindley PF, Soares CM (2010) Mechanisms underlying dioxygen reduction in laccases. Structural and modeling studies focusing on proton transfer. BMC Struct Biol 10:28. dio:10.1186/1472-6807-10-28

Brander S, Mikkelsen JD, Kepp KP (2014) Characterization of an alkali- and halide-resistant laccase expressed in *E coli*: CotA from *Bacillus clausii*. PLoS ONE 9:e99402. doi:10.1371/journal.pone.0099402

Damasceno LM, Huang CJ, Batt CA (2012) Protein secretion in *Pichia pastoris* and advances in protein production. Appl Microbiol Biotechnol 93:31–39. doi:10.1007/s00253-011-3654-z

Dittmer NT, Suderman RJ, Jiang H, Zhu YC, Gorman MJ, Kramer KJ, Kanost MR (2004) Characterization of cDNAs encoding putative laccase-like multicopper oxidases and developmental expression in the tobacco hornworm, *Manduca sexta*, and the malaria mosquito, *Anopheles gambiae*. Insect Biochem Mol Biol 34:29–41. doi:10.1016/j.ibmb.2003.08.003

Driks A (1999) *Bacillus subtilis* spore coat. Microbiol Mol Biol Rev 63:1–20 doi: 1092-2172/99/$04.00+0

Durand F, Kjaergaard CH, Suraniti E, Gounel S, Hadt RG, Solomon EI, Mano N (2012) Bilirubin oxidase from *Bacillus pumilus*: a promising enzyme for the elaboration of efficient cathodes in biofuel cells. Biosens Bioelectron 35:140–146. doi:10.1016/j.bios.2012.02.033

Durao P, Chen Z, Fernandes AT, Hildebrandt P, Murgida DH, Todorovic S, Pereira MM, Melo EP, Martins LO (2008) Copper incorporation into recombinant CotA laccase from *Bacillus subtilis*: characterization of fully copper loaded enzymes. J Biol Inorg Chem 13:183–193. doi:10.1007/s00775-007-0312-0

Dwivedi UN, Singh P, Pandey VP, Kumar A (2011) Structure-function relationship among bacterial, fungal and plant laccases. J Mol Catal B Enzym 68:117–128. doi:10.1016/j.molcatb.2010.11.002

ElKaoutit M, Naranjo-Rodriguez I, Domínguez M, Hernández-Artiga MP, Bellido-Milla D, Hidalgo-Hidalgo de Cisneros JL (2008) A third-generation hydrogen peroxide biosensor based on horseradish peroxidase (HRP) enzyme immobilized in a nafion-sonogel-carbon composite. Electrochim Acta 53:7131–7137. doi:10.1016/j.electacta.2008.04.086

Enguita FJ, Martins LO, Henriques AO, Carrondo MA (2003) Crystal structure of a bacterial endospore coat component. A laccase with enhanced thermostability properties. J Biol Chem 278:19416–19425. doi:10.1074/jbc.M301251200

Fang Z, Li T, Wang Q, Zhang X, Peng H, Fang W, Hong Y, Ge H, Xiao Y (2011) A bacterial laccase from marine microbial metagenome exhibiting chloride tolerance and dye decolorization ability. Appl Microbiol Biotechnol 89:1103–1110. doi:10.1007/s00253-010-2934-3

Garg N, Bieler N, Kenzom T, Chhabra M, Ansorge-Schumacher M, Mishra S (2012) Cloning, sequence analysis, expression of *Cyathus bulleri* laccase in *Pichia pastoris* and characterization of recombinant laccase. BMC Biotechnol 12:75–87. doi:10.1186/1472-6750-12-75

Giardina P, Faraco V, Pezzella C, Piscitelli A, Vanhulle S, Sannia G (2010) Laccases: a never-ending story. Cell Mol Life Sci 67:369–385. doi:10.1007/s00018-009-0169-1

Guan ZB, Song CM, Zhang N, Zhou W, Xu CW, Zhou LX, Zhao H, Cai Y-J, Liao X-R (2014) Overexpression, characterization, and dye-decolorizing ability of a thermostable, pH-stable, and organic solvent-tolerant laccase from *Bacillus pumilus* W3. J Mol Catal B Enzym 101:1–6. doi:10.1016/j.molcatb.2013.11.009

Jolivalt C, Madzak C, Brault A, Caminade E, Malosse C, Mougin C (2005) Expression of laccase IIIb from the white-rot fungus *Trametes versicolor* in the yeast *Yarrowia lipolytica* for environmental applications. Appl Microbiol Biotechnol 66:450–456. doi:10.1007/s00253-004-1717-0

Koschorreck K, Richter SM, Ene AB, Roduner E, Schmid RD, Urlacher VB (2008) Cloning and characterization of a new laccase from *Bacillus licheniformis* catalyzing dimerization of phenolic acids. Appl Microbiol Biotechnol 79:217–224. doi:10.1007/s00253-008-1417-2

Kuwahara T, Asano T, Kondo M, Shimomura M (2013) Bioelectrocatalytic O_2 reduction with a laccase-bearing poly(3-methylthiophene) film based on direct electron transfer from the polymer to laccase. Bioelectrochemistry 91:28–31. doi:10.1016/j.bioelechem.2012.12.002

Li M, Xu S, Tang M, Liu L, Gao F, Wang Y (2011) Direct electrochemistry of horseradish peroxidase on graphene-modified electrode for electrocatalytic reduction towards H_2O_2. Electrochim Acta 56:1144–1149. doi:10.1016/j.electacta.2010.10.034

Loncar N, Bozic N, Lopez-Santin J, Vujcic Z (2013) *Bacillus amyloliquefaciens* laccase-from soil bacteria to recombinant enzyme for wastewater decolorization. Bioresour Technol 147:177–183. doi:10.1016/j.biortech.2013.08.056

Lu L, Zhao M, Wang TN, Zhao LY, Du MH, Li TL, Li DB (2012) Characterization and dye decolorization ability of an alkaline resistant and organic solvents tolerant laccase from *Bacillus licheniformis* LS04. Bioresour Technol 115:35–40. doi:10.1016/j.biortech.2011.07.111

Lu L, Wang TN, Xu TF, Wang JY, Wang CL, Zhao M (2013) Cloning and expression of thermo-alkali-stable laccase of *Bacillus licheniformis* in *Pichia pastoris* and its characterization. Bioresour Technol 134:81–86. doi:10.1016/j.biortech.2013.02.015

Martins LO, Soares CM, Pereira MM, Teixeira M, Costa T, Jones GH, Henriques AO (2002) Molecular and biochemical characterization of a highly stable bacterial laccase that occurs as a structural component of the *Bacillus subtilis* endospore coat. J Biolumin Chemilumin 277:18849–18859. doi:10.1074/jbc.M200827200

Nagai M, Sato T, Watanabe H, Saito K, Kawata M, Enei H (2002) Purification and characterization of an extracellular laccase from the edible mushroom *Lentinula edodes*, and decolorization of chemically different dyes. Appl Microbiol Biotechnol 60:327–335. doi:10.1007/s00253-002-1109-2

O'Callaghan J, O'Brien MM, McClean K, Dobson ADW (2002) Optimisation of the expression of a *Trametes versicolor* laccase gene in *Pichia pastoris*. J Ind Microbiol Biotechnol 29:55–59. doi:10.1038/sj.jim.7000268

Otterbein L, Record E, Longhi S, Asther M, Moukha S (2000) Molecular cloning of the cDNA encoding laccase from *Pycnoporus cinnabarinus* I-937 and expression in *Pichia pastoris*. Eur J Biochem 267:1619–1625. doi:10.1046/j.1432-1327.2000.01166.x

Pereira L, Coelho AV, Viegas CA, Santos MM, Robalo MP, Martins LO (2009) Enzymatic biotransformation of the azo dye Sudan Orange G with bacterial CotA-laccase. J Biotechnol 139:68–77. doi:10.1016/j.jbiotec.2008.09.001

Reiss R, Ihssen J, Thöny-Meyer L (2011) *Bacillus pumilus* laccase: a heat stable enzyme with a wide substrate spectrum. BMC Biotechnol 11:9. doi:10.1186/1472-6750-11-9

Safavi A, Farjami F (2010) Hydrogen peroxide biosensor based on a myoglobin/hydrophilic room temperature ionic liquid film. Anal Biochem 402:20–25. doi:10.1016/j.ab.2010.03.013

Sharma P, Goel R, Capalash N (2007) Bacterial laccases. World J Microbiol Biotechnol 23:823–832. doi:10.1007/s11274-006-9305-3

Shleev S, Jarosz-Wilkolazka A, Khalunina A, Morozova O, Yaropolov A, Ruzgas T, Gorton L (2005a) Direct electron transfer reactions of laccases from different origins on carbon electrodes. Bioelectrochemistry 67:115–124. doi:10.1016/j.bioelechem.2005.02.004

Shleev S, Tkac J, Christenson A, Ruzgas T, Yaropolov AI, Whittaker JW, Gorton L (2005b) Direct electron transfer between copper-containing proteins and electrodes. Biosens Bioelectron 20:2517–2554. doi:10.1016/j.bios.2004.10.003

Singh G, Bhalla A, Kaur P, Capalash N, Sharma P (2011) Laccase from prokaryotes: a new source for an old enzyme. Rev Environ Sci Biotechnol 10:309–326. doi:10.1007/s11157-011-9257-4

Solomon EI, Sundaram UM, Machonkin TE (1996) Multicopper oxidases and oxygenases. Chem Rev 96:2563–2605. doi:10.1021/cr950046o

Thomé-Duret V, Reach G, Gangnerau MN, Lemonnier F, Klein JC, Zhang Y, Hu Y, Wilson GS (1996) Use of a subcutaneous glucose sensor to detect decreases in glucose concentration prior to observation in blood. Anal Chem 68:3822–3826. doi:10.1021/ac960069i

Wang CL, Zhao M, Li DB, Cui DZ, Lu L, Wei XD (2013) Isolation and characterization of a novel *Bacillus subtilis* WD23 exhibiting laccase activity from forest soil. Afr J Biotechnol 9:5496–5502. doi:10.4028/www.scientific.net/AMR.113-116.725

Wang TN, Lu L, Wang JY, Xv TF, Li J, Zhao M (2015) Enhanced expression of an industry applicable CotA laccase from *Bacillus subtilis* in *Pichia pastoris* by non-repressing carbon sources together with pH adjustment: recombinant enzyme characterization and dye decolorization. Process Biochem 50:97–103. doi:10.1016/j.procbio.2014.10.009

Xu S, Peng B, Han X (2007) A third-generation H_2O_2 biosensor based on horseradish peroxidase-labeled Au nanoparticles self-assembled to hollow porous polymeric nanopheres. Biosens Bioelectron 22:1807–1810. doi:10.1016/j.bios.2006.07.008

Zhao D, Zhang X, Cui D, Zhao M (2012) Characterisation of a novel white laccase from the deuteromycete fungus *Myrothecium verrucaria* NF-05 and its decolourisation of dyes. PLoS ONE 7, e38817. doi:10.1371/journal.pone.0038817

Zhao D, Cui DZ, Zhang X, Zhao M (2013) Oxidation of aromatic compounds and bioelectrocatalysis of peroxide by a novel white laccase from *Myrothecium verrucaria* NF-05. Catal Commun 31:48–51. doi:10.1016/j.catcom.2012.11.010