Appl Biochem Biotechnol (2014) 172:1147–1157
DOI 10.1007/s12010-013-0614-3

Molecular Cloning, Characterization, and Dye-Decolorizing Ability of a Temperature- and pH-Stable Laccase from *Bacillus subtilis* X1

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Received: 16 July 2013 / Accepted: 29 October 2013 /
Published online: 12 November 2013

Abstract Laccases from fungal origin are typically unstable at high temperatures and alkaline conditions. This characteristic limits their practical applications. In this study, a new bacterial strain exhibiting laccase activity was isolated from raw fennel honey samples and identified as *Bacillus subtilis* X1. The CotA-laccase gene was cloned from strain X1 and efficiently expressed in *Escherichia coli* in a biologically active form. The purified recombinant laccase demonstrated an extensive pH range for catalyzing substrates and high stability toward alkaline pH and high temperatures. No loss of laccase activity was observed at pH 9.0 after 10 days of incubation, and approximately 21 % of the initial activity was detected after 10 h at 80 °C. Two anthraquinonic dyes (reactive blue 4 and reactive yellow brown) and two azo dyes (reactive red 11 and reactive brilliant orange) could be partially decolorized by purified laccase in the absence of a mediator. The decolorization process was efficiently promoted when methylsyringate was present, with more than 90 % of color removal occurring in 3 h at pH 7.0 or 9.0. These unusual properties indicated a high potential of the novel CotA-laccase for industrial applications.

Keywords CotA · Laccase · *Bacillus subtilis* · Gene cloning · Recombinant expression · Dye decolorization

Introduction

Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) are multicopper-containing enzymes that can catalyze the oxidation of an extensive range of phenolic and non-phenolic

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aromatic compounds, along with the reduction of molecular oxygen to water [1, 2]. Laccases belong to the multicopper oxidase family, which typically contains four copper atoms: a type I copper (blue copper), a type II copper, and a pair of type III copper centers [3]. Their extensive substrate range makes laccases excellent candidates for various industrial and biotechnological applications, such as biological bleaching in the pulp and paper industry, textile dye decolorization, construction of biosensors for detecting phenolic pollutants, detoxification of recalcitrant environmental pollutants, and bioremediation [4].

Laccases are widely distributed among fungi, higher plants, and bacteria [5]. To date, most laccases studied are of fungal origin, and only fungal laccases are used in industrial processes [2]. However, fungal laccases are usually unstable at high temperatures and alkaline conditions. This characteristic limits their practical applications. Although bacterial laccases have a lower redox potential than fungal laccases and are less frequently investigated, they are more stable to high temperatures and a wider pH range, less dependent on metal ions, and less susceptible to inhibitory agents. Thus, bacterial laccases have significant potential in various industrial applications [6]. Furthermore, bacterial laccases are suitable for overproduction in *Escherichia coli*; and their expression level, stability, and catalytic properties are considerably easier to improve by directed evolution compared with their fungal counterparts [7].

The best-studied bacterial laccase is CotA, i.e., the endospore coat component of *Bacillus subtilis*. CotA participates in the biosynthesis of brown spore pigment, which is also considered to be a melanin-like product [8], and appears to be responsible for the protection afforded by the spore coat against ultraviolet light and hydrogen peroxide [9, 10]. In this study, we isolated a new strain, *B. subtilis* X1, which has high laccase activity. The CotA-laccase gene of this strain was cloned, which revealed less than 95 % similarity either at the nucleotide or amino acid level with all other known *B. subtilis* CotA genes deposited in the GenBank database. The corresponding gene was then expressed in *E. coli*, purified, and characterized with respect to its biochemical and catalytic properties. Furthermore, the ability of the aforementioned gene in decolorizing synthetic dyes was analyzed.

Materials and Methods

Chemicals

2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS), 2,6-dimethoxyphenol (2,6-DMP), 4-hydroxy-3,5-dimethoxybenzaldehyde azine (syringaldazine, SGZ), reactive blue 4, reactive yellow brown, reactive red 11, reactive brilliant orange, and methylsyringate were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). A bacterial DNA extraction kit, gel extraction kit, Ex Taq DNA polymerase, pMD18-T vector, and isopropyl-β-D-thiogalactoside (IPTG) were purchased from TaKaRa (Dalian, China). All other chemicals were standard reagent grade.

Isolating the Laccase-Producing Strain

The laccase-producing bacterial strain was isolated from raw fennel honey samples collected in Fuhuan City, Guangxi Province, P. R. China. A sample of 10 g raw honey was added to 100 mL Luria–Bertani (LB) medium supplemented with 0.25 mM $CuSO_4$ and incubated at 37 °C for 48 h. Then, isolation was carried out via standard serial dilution plate technique using the LB medium with 0.25 mM $CuSO_4$. Plates were incubated at 37 °C for 3 day. Screening of laccase-producing strains was performed by adding two to three drops of laccase

substrate solutions (1 mM ABTS, SGZ, or 2,6-DMP) to the bacterial colonies. Positive colonies were indicated by the appearance of green, pink, or orange color (for ABTS, SGZ, or 2,6-DMP, respectively). Representative colonies were selected and further purified through single-colony isolation.

Identifying the Strain

Morphological observations of the isolated strain were conducted via scanning electron microscopy (FEI Quanta-200, The Netherlands). Physiological and biochemical tests were conducted according to conventional methods. The genomic DNA of the isolated strain was extracted using the bacterial DNA extraction kit. The 16S rRNA gene was amplified through primers p1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and p2 (5′- ACGGTTACCTTGTTACGA CTT-3′) [11]. Amplification was performed by initial denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 2 min, and a final extension at 72 °C for 10 min. Polymerase chain reaction (PCR) fragments were purified using the gel extraction kit. The purified products were inserted into the pMD18-T vector, and then transformed into the competent cell of *E. coli* DH5α. Positive white colonies were selected on the LB/Amp/X-gal/IPTG plates and validated through colony PCR assay. Finally, the 16S rDNA was sequenced. The BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used for the sequence similarity search with the standard program default. Multiple sequence alignment and data analysis were performed using the software package MEGA version 4.0 [12].

Molecular Cloning of *B. subtilis* X1 CotA-Type Laccase Gene

Amplifying the laccase gene was achieved by PCR using Ex Taq polymerase with the forward primer p3 (5′-ATGACACTTGAAAAATTTGTGGATGCTC-3′) and reverse primer p4 (5′-TTATTTATGGGGATCAGTTATATCCATC-3′). PCR was performed as follows: 94 °C for 5 min, 94 °C for 30 s, 55 °C for 30 s, 72 °C for 90 s, 30 cycles, and 72 °C for 10 min. After the amplified DNA fragment was purified using the gel extraction kit, the fragment was subcloned into pMD18-T vector and the nucleotide sequence of the inserted fragment was sequenced.

Three-Dimensional (3D) Modeling of *B. subtilis* X1 Laccase

A suitable structural template for *B. subtilis* X1 laccase and *B. subtilis* MB24 laccase PDB file 2x88A was identified through a BLAST search as implemented in the SWISS-MODEL protein modeling server. The automatic sequence alignment obtained from this process was used for homology modeling with SWISS-MODEL [13]. The resulting theoretical model of a protein monomer was displayed and analyzed using the RasWin Molecular Graphics Program (RasMol, version 2.7.2) software package (http://www.umass.edu/microbio/rasmol/).

Expressing and Purifying the Recombinant Laccase

The DNA fragment encoding laccase was generated by PCR using the primers p5 (5′-TCAG GAGGTACCATGACACTTGAAAAATTTGTG-3′) and p6 (5′-ACTGAGAAGCTTTTATTT ATGGGGATCAGT-3′), which introduced unique *Kpn* I and *Hin*d III restriction sites, respectively. The resulting PCR product was subcloned into a cold-shock expression vector, pColdII (TaKaRa, Japan), which formed a sequence encoding a fusion protein of laccase and an NH_2-terminal His6 tag. This recombinant plasmid, called pColdII-laccase, was sequenced on both

strands. Then, this construct was transformed into *E. coli* BL21 (DE3) to express His6 tag laccase. The bacteria were briefly cultured in LB medium with vigorous shaking (250 rpm) at 37 °C at the density of OD_{600}≈0.4. The induction scheme was subsequently performed according to the instructions of the manufacturer of pCold™ expression vector with several minor modifications. The concentration of IPTG was 0.1 mM and supplemented with 0.25 mM of $CuSO_4$; the temperature of induction was 15 °C; the time of induction was 24 h, and the shaking speed of induction was 160 rpm. The harvested cells were resuspended in 20 mM Tris–HCl, pH 8.0, with 0.5 M NaCl, 5 mM imidazole, and 1 mM β-mercaptoethanol. Then, the cells were sonicated at 10 kHz using 20 s strokes with 30 s intervals, and centrifuged at 15,000×*g* for 15 min. The recombinant laccase proteins were purified from the soluble fraction using a HiTrap™ chelating HP column (Amersham Biosciences, USA) according to the instructions of the manufacturer. Finally, the purified laccase was dialyzed against phosphate-buffered saline and sterilized by filtration.

The expression of recombinant laccase was analyzed via SDS-PAGE and non-denaturing PAGE (omitting the SDS, β-mercaptoethanol, and heat treatment), which were carried out according to the method of Laemmli [14]. Western blot analysis using an anti-His6 tag mouse monoclonal antibody (Shengxing, China) was performed according to Guan et al. [15]. Bacterial proteins from BL21 (DE3) strain transformed with empty pColdII plasmids were prepared and used as control proteins.

Enzyme Assay

Laccase activity was assayed at 37 °C using ABTS, SGZ, and 2,6-DMP as substrates. The oxidation of ABTS (1 mM) was measured at 420 nm in a 0.1 M citrate–phosphate buffer (pH 3.0–6.5). The oxidation of SGZ (0.1 mM) was detected at 525 nm in 0.1 M citrate–phosphate buffer or 0.1 M Tris–HCl buffer. The oxidation of 2,6-DMP (2 mM) was determined at 470 nm in a 0.1 M Tris–HCl buffer. The formation of oxidized product was monitored using an UV–Visible spectrophotometer (Hitachi UV-2800). One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 μmol of substrate per minute. All assays were carried out in triplicate.

Effects of Temperature and pH on Laccase Activity and Stability

The optimum temperature for activity was determined using ABTS as the substrate at different temperatures (20 °C to 90 °C). To determine temperature stability, purified laccase was incubated for 0 h to 10 h at different temperatures (50, 60, 70, and 80 °C); then, the residual activity was measured. The effect of pH on the activity of purified laccase was determined at 37 °C in a 0.1 M citrate–phosphate buffer (pH 2.6 to pH 7.0) or 0.1 M Tris–HCl buffer (pH 7.0 to pH 9.0). The pH stability was assayed by incubating the purified laccase at 37 °C at pH 3.0, 7.0, or 9.0, and then measuring the residual activity. The activity at the optimum temperature or optimum pH was used as the control to determine the respective effect of temperature or pH on the laccase. The test system, which was managed at the same conditions except with a heat-inactivated laccase (30 min in boiling water bath), was used as the control for the temperature or pH stability experiments.

Decolorizing Synthetic Dyes

A dye decolorization experiment was conducted using two anthraquinonic dyes, reactive blue 4 (λ_{max}=595 nm) and reactive yellow brown (λ_{max}=600 nm), and two azo dyes, reactive red

11 (λ_{max}=520 nm) and reactive brilliant orange (λ_{max}=490 nm). Decolorization was tested in the presence or absence of methylsyringate as a mediator. The reaction mixture (6 mL) contained 0.1 M citrate–phosphate buffer (pH 7.0) or 0.1 M Tris–HCl buffer (pH 9.0), dye (5 mM), purified laccase (0.12 U), and the mediator (0.1 mM). Reactions were initiated by adding laccase, and incubated at 50 °C under mild shaking conditions. The control samples were run in parallel without the addition of laccase. The decolorization percentage was determined spectrophotometrically as the relative decrease in absorbance at each maximal absorbance wavelength of the dyes. All reactions were performed in triplicate.

Results and Discussion

Isolating and Identifying the Strain

Six different isolates exhibiting high laccase activity were obtained from the raw fennel honey sample. One isolate, called strain X1, was selected for further study. The colonies of X1 could oxidize ABTS, SGZ, and 2,6-DMP within 5 min. Strain X1 was Gram-positive, spore-forming, and had motile long rods. The 16S rDNA sequence of strain X1 was amplified and sequenced. The BLAST search results of the 1,513-bp long sequence indicated that this strain belonged to the genus *Bacillus*. The phylogenetic tree showed that strain X1 was closely related to other species of *Bacillus*, and the most closely related species was *B. subtilis* (GenBank no. EU661710). The isolated strain X1 was finally identified as *B. subtilis* based on the results of the morphological observation, physiological, and biochemical tests, as well as the 16S rDNA sequence analysis (data not shown).

Cloning the *B. subtilis* X1 CotA-Laccase Gene

The open reading frame of *B. subtilis* X1 CotA-laccase gene consists of 1,542 bp, which encodes a protein of 513 amino acids [2, 6, 10]. The sequence of the laccase gene from *B. subtilis* X1 has been deposited in the GenBank database under accession no. KC751428. Multiple sequence alignment showed that *B. subtilis* X1 laccase has four histidine-rich copper-binding regions which are highly conserved for other laccases [16]. Sequence identity at the amino acid level between *B. subtilis* X1 laccase and those of other *B. subtilis* strains was less than 95 %, and less than 63 and 65 % with those of *B. licheniformis* and *B. pumilus*, respectively. The 3D structure of *B. subtilis* X1 laccase was predicted by comparative modeling using the *B. subtilis* MB24 laccase crystal structure (PDB id: 2x88A) as template. Despite 31-aa differences existing at the amino acid level, the predicted 3D structure of the strain X1 laccase monomer, which also had three common Cu-oxidase domains, looks similar to the strain MB24 counterpart (Fig. 1).

Expressing and Purifying *B. subtilis* X1 Laccase

Cold-shock expression vectors, pCold DNA series, are designed to perform efficient protein expression in *E. coli* using a promoter derived from the *cspA* gene, which is one of the cold-shock genes [17]. These vectors selectively induce target protein synthesis at a low temperature (15 °C), a condition that suppresses the expression of host proteins and decreases protease activity. Laccase proteins have been reported to easily form inclusion bodies when expressed in *E. coli* cells at a relatively high temperature [10, 18]. Therefore, pColdII, a cold-induced expression vector from the pCold DNA series, was selected to express the

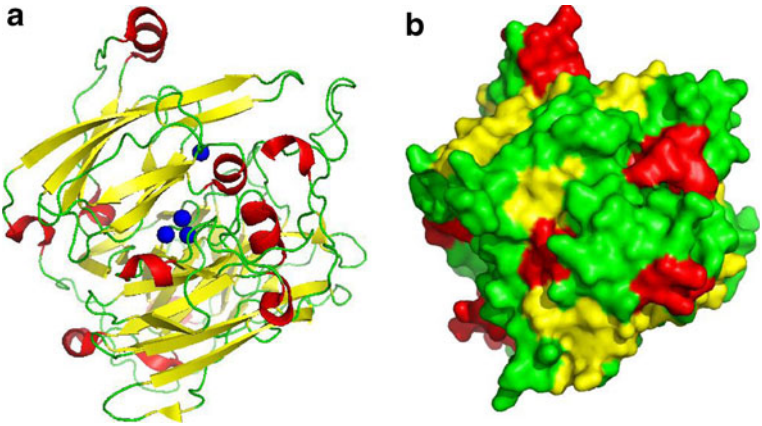

Fig. 1 Homology modeling of the *B. subtilis* X1 laccase based on the *B. subtilis* MB24 laccase crystal structure (PDB id: 2x88A) as template. Cartoon displays (**a**) and surface displays (**b**) of the structure models are shown. *Yellow* denotes β-strands, *red* denotes α-helixes, and four copper ions in the structure are depicted as *blue spheres*

recombinant laccase in this study. The pColdII-laccase construct was transformed into *E. coli* BL21 (DE3), and the laccase was efficiently expressed in a biologically active form at an optimal condition. Highest volumetric activity of 1.26 U/mL as measured with ABTS was reached after an induction expression of 24 h at 15 °C in LB medium. To simplify the purification process, a His6 tag was introduced to the N-terminus of the recombinant laccase because this process enables easy one-step purification through immobilized metal affinity chromatography. When the supernatants of the bacteria extract were filtrated through the nickel column, the protein fraction with blue fluorescence was bound to the sorbent. The soluble recombinant enzyme was finally eluted by 1 M imidazole under natural conditions. According to the SDS-PAGE analysis, a ~65-kDa band corresponding to the predicted size of the fusion and absent in BL21 (DE3) transformed with empty pColdII was detected in BL21 (DE3) transformed with pColdII-laccase after IPTG induction (Fig. 2). After the one-step purification of soluble fusions, the yield of the eluted soluble protein with no less than 90 % purity was approximately 13.5 mg/L bacterial culture, as assessed by the Bradford method. The expressed laccase was recognized through an anti-His6 tag mouse monoclonal antibody (Fig. 2, lane 5).

Effects of Temperature and pH on Laccase Activity and Stability

The purified recombinant laccase of *B. subtilis* X1 demonstrated an extensive pH range for catalyzing substrates (Fig. 3a). It oxidized ABTS in acidic conditions with an optimum pH of 4.4 similar to most fungal laccases [19] and CotA-laccases from other *Bacillus* species [10, 18]. The optimum pH levels for oxidation of SGZ (6.9) and 2,6-DMP (7.3) were higher than those for other bacterial laccases. For example, the optimum pH levels for the oxidation of SGZ and 2,6-DMP were reported to be 6.2 and 6.6, respectively, for *Bacillus licheniformis* LS04 laccase [20], and 6.5 and 7.0, respectively, for *Bacillus pumilus* DSM 27 laccase [21]. Stability studies showed that the enzyme was unstable at pH 3.0, and that only 23 and 15 % of the original activity remained after incubation for 1 and 3 days, respectively (Fig. 3b). However, the enzyme was highly stable at pH 7.0 and pH 9.0 when incubated at 37 °C for 10 days, and retained approximately 126 and 163 % of the initial activity, respectively. The

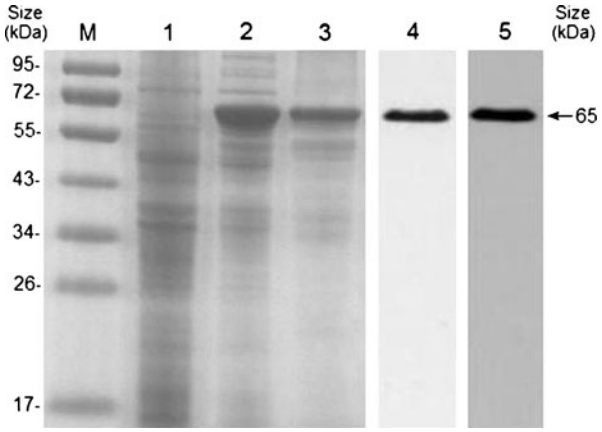

Fig. 2 Analysis of the recombinant laccase expressed in *E. coli* BL21 (DE3). *Lanes 1, 2, and 3* respectively represent the SDS-PAGE analysis of the cell lysates of bacteria transformed with empty pColdII plasmids under IPTG (0.1 mM) induction for 24 h at 15 °C, cell lysates of bacteria transformed with pColdII-laccase under IPTG (0.1 mM) induction for 24 h at 15 °C, and the recombinant laccase purified with Ni^{2+} affinity chromatography. The gel was stained with Coomassie blue R250. *Lane 4* indicates the activity staining band of purified enzyme using 1 mM ABTS as a substrate. *Lane 5* shows the Western blot analysis of the purified laccase recognized by an anti-His6 tag mouse monoclonal antibody. The molecular weight marker is shown in the *M lane*. The *arrowhead* indicates the band of the recombinant laccase protein

laccase from alkaline tolerant γ-proteobacterium JB is also a pH-stable enzyme, which can maintain its activity at pH 9.0 for 60 days at 4 °C [22]. By contrast, laccases from certain fungi such as *Paraconiothyrium variabile* [23], *Aspergillus ochraceus* [24], and *Cladosporium cladosporioides* [25] are restrictedly stable in the acid-to-neutral pH range. It indicated that bacterial laccases can act and are more stable at wider pH range [26, 27]

The X1 laccase exhibited its maximum activity at 60 °C, and could maintain high activity at 50 °C to 70 °C (Fig. 4a). The most remarkable property exhibited by CotA-laccase was its high thermostability [10]. The residual activities of purified laccase incubated for 0 to 10 h at different temperatures (50 to 80 °C) are presented in Fig. 4b. The recombinant laccase behaved in a significantly stable manner at 50C and 60 °C. No loss of laccase activity was observed at 50 and 60 °C after 10 h incubation. The half-life of the laccase exceeded 6 h at 70 °C, and was approximately 2.5 h at 80 °C. The laccase retained approximately 35 and 21 % of its initial activity with SGZ as the substrate at pH 6.9 after 4 and 10 h, respectively, at 80 °C (Fig. 4b). The half-life of inactivation at 80 °C was higher than that of laccase from *B. subtilis* MB24 (~1.7 h) [10]. Moreover, laccase from *B. licheniformis* demonstrated a lower thermostability, and lost approximately 92 % of its activity after 1 h incubation at 80 °C [18]. In addition, a thermostable fungal laccase from *C. cladosporioides* lost 52 % of its activity after 5 min at 80 °C [25]. The results of the pH and temperature stability experiments indicated the extreme robustness of the recombinant *B. subtilis* X1 laccase.

Decolorizing Dye

Two anthraquinonic dyes and two azo dyes were used to evaluate the decolorization ability of the recombinant X1 laccase. The assays were performed with or without methylsyringate as a redox mediator. The results showed that the purified laccase could decolorize all the tested dyes in the absence of methylsyringate at pH 7.0. However, the decolorization of dyes

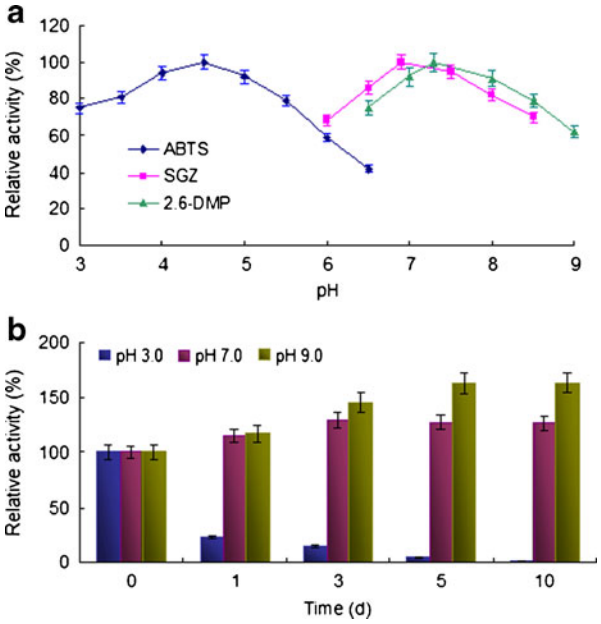

Fig. 3 Effect of pH on the activity (**a**) and stability (**b**) of the purified recombinant laccase at 37 °C. **a** ABTS, pH 3.0–6.5; SGZ, pH 6.0–8.5; 2,6-DMP, pH 6.5–9.0; **b** substrate: pH 3.0, ABTS; pH 7.0, SGZ; pH 9.0, 2,6-DMP

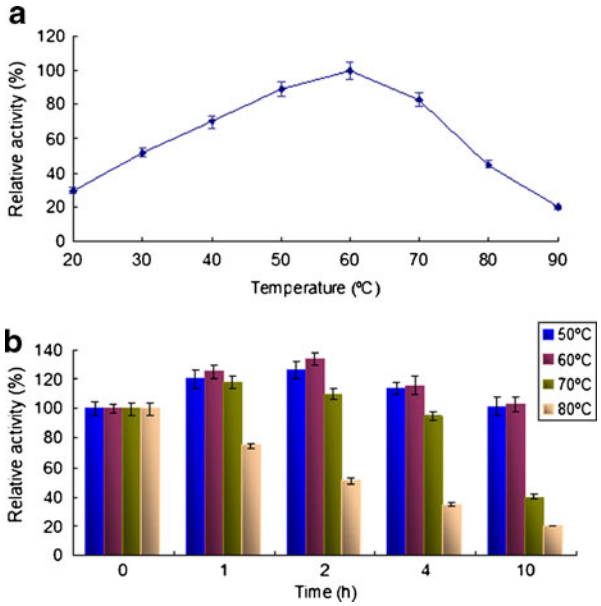

Fig. 4 Effect of temperature on the activity (**a**) and stability (**b**) of purified laccase with SGZ as the substrate at pH 6.9. **a** Laccase activity was measured at different temperatures (20 °C to 90 °C); **b** residual activity was measured after incubation at 50 to 80 °C for 0 to 10 h

proceeded slowly. Only 36 % of reactive yellow brown, 28 % of reactive red 11, and 31 % of reactive brilliant orange were decolorized after 3 h; and the highest decolorization rate after 10 h was approximately 59 % for reactive blue 4 (Fig. 5a). Adding 0.1 mM methylsyringate clearly increased the decolorization efficiency for all synthetic dyes, and more than 80 or 90 % decolorization was observed after incubation for 1 or 10 h, respectively (Fig. 5b). The promoting effect in dye decolorization by the laccase mediator has also been demonstrated by other researchers [22, 28, 29]. To test the decolorization ability of laccase under alkaline conditions, dye decolorization was also conducted at pH 9.0 in the presence of methylsyringate. The recombinant laccase retained high efficiency in decolorization, and more than 80 or 90 % of decolorization toward all dyes was observed after incubation for 1 or 10 h, respectively (Fig. 5c). The final decolorization rate even exceeded 95 % for the anthraquinonic dyes at alkaline conditions. Given that most textile effluents are characterized by a high

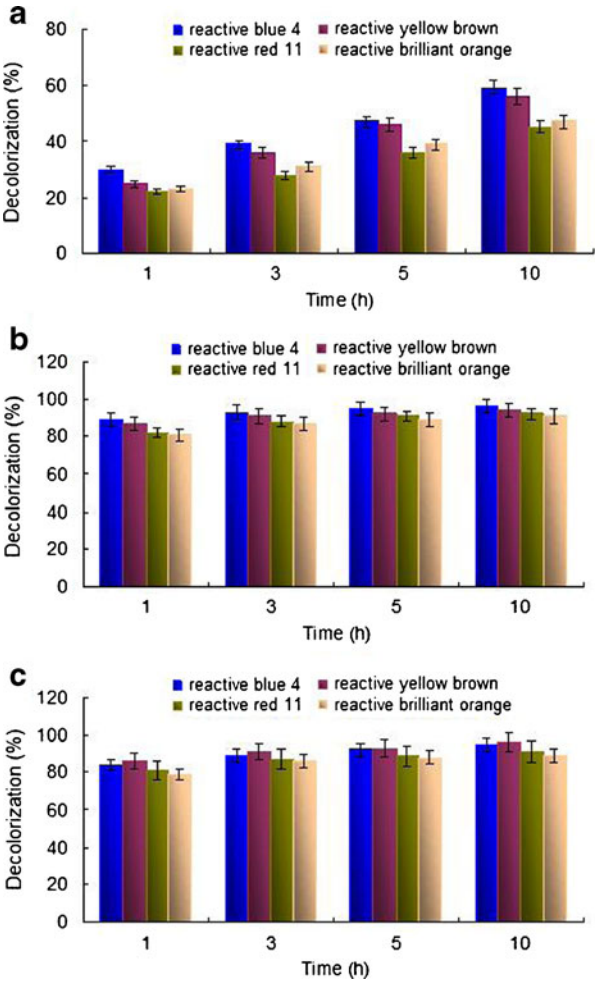

Fig. 5 Decolorization of synthetic dyes (5 mM) by the purified laccase (0.12 U) at 50 °C. **a** No mediator in 0.1 M citrate–phosphate buffer (pH 7.0); **b** 0.1 mM methylsyringate as mediator in 0.1 M citrate–phosphate buffer (pH 7.0); and **c** 0.1 mM methylsyringate as mediator in 0.1 M Tris–HCl buffer (pH 9.0)

temperature and an alkaline pH [30], most fungal laccases tend to lose their activities under such extreme conditions [31]. Therefore, the high efficiency in dye decolorization indicated the promising application of the *B. subtilis* X1 laccase mediator system in treating dye effluents.

Conclusion

In summary, a bacterial strain exhibiting laccase activity was isolated and identified as *B. subtilis* X1. The CotA-laccase gene was cloned from strain X1, and efficiently expressed in *E. coli* in a biologically active form. The purified recombinant laccase exhibited a high degree of stability toward alkaline pH and high temperatures. This laccase could efficiently decolorize anthraquinonic and azo dyes when combined with a mediator under neutral-to-alkaline conditions. These unusual properties make this recombinant laccase a suitable candidate for an extensive range of industrial applications.

Acknowledgments This work was supported by the National Natural Science Foundation of China (31101331), the Open Project Program of the Key Laboratory of Industrial Biotechnology, Ministry of Education, China (KLIB-KF201001), and New Teachers' Fund for Doctor Stations, Ministry of Education (20110093120002).

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