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Enhanced expression of a recombinant bacterial laccase at low temperature and microaerobic conditions: purification and biochemical characterization

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Abstract Laccases (benzenediol oxygen oxidoreductase; EC 1.10.3.2) have many biotechnological applications because of their oxidation ability towards a wide range of phenolic compounds. Within recent years, researchers have been highly interested in the identification and characterization of laccases from bacterial sources. In this study, we have isolated and cloned a gene encoding laccase (CotA) from *Bacillus* sp. HR03 and then expressed it under microaerobic conditions and decreased temperature in order to obtain high amounts of soluble protein. The laccase was purified and its biochemical properties were investigated using three common laccase substrates, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), syringaldazine (SGZ) and 2,6-dimethoxyphenol (2,6-DMP). K_M and k_{cat} were calculated 535 μM and 127 s^{-1} for ABTS, 53 μM and 3 s^{-1} for 2, 6-DMP and 5 μM and 20 s^{-1} for SGZ when the whole reactions were carried out at room temperature. Laccase activity was also studied when the enzyme was preincubated at 70 and 80°C. With SGZ as the substrate, the activity was increased three-fold after 50 min preincubation at 70°C and 2.4-fold after 10 min preincubation at 80°C. Preincubation of the enzyme in 70°C for 30 min raised the activity four-fold with ABTS as the substrate. Also, L-dopa was used as a substrate. The enzyme was able to oxidize L-dopa with the K_M and k_{cat} of 1,493 μM and 194 s^{-1}, respectively.

Keywords CotA · Laccase · Expression · Microaerobic conditions · Biochemical characterization · Thermal activation

Introduction

The existence of environmental problems caused by industrial oxidation reactions has persuaded researchers to employ alternative biological systems, such as enzymatic oxidation, to avoid problems like undesirable side reactions and production of hazardous pollutants. Laccases (benzenediol oxygen oxidoreductase; EC 1.10.3.2), which belongs to a group of enzymes capable of oxidizing a broad range of aromatic compounds, are highly interesting for biotechnological and industrial applications [33]. Till now, laccases have been applied in food, textile, pulp and paper industries, and also have been used in many nanobiotechnology and bioremediation applications [10]. These enzymes are characterized according to three types of copper prosthetic groups, which differ in their light absorbance and EPR (electron paramagnetic resonance) signal [7]. Type-1 (T1) copper, which probably is the first oxidation site, has an absorption band at around 600 nm. The blue color of the enzyme is attributed to T1 copper as a result of severe electronic absorption by the covalent copper-cystein bond. Type-2 (T2) and type-3 (T3) coppers form a trinuclear group, where the reduction of molecular oxygen takes place. The T2 has weak absorption in the

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visible region but is EPR-active. The T3 copper is described by an absorption band at around 330 nm [26, 30].

Laccases are widespread among fungi, plants, insects and bacteria [1, 15]. Among them, fungal laccases have been currently used in biotechnological applications [35]. However, the presence of laccases also in bacterial sources has persuaded researchers to isolate and characterize other members of this family. To date, only a few bacterial laccases have been characterized [28], such as laccases from *E.coli* [36], *Bacillus halodurans* [27], *Bacillus subtilis* [22], *Thermus thermophilus* [23] and *Bacillus licheniformis* [18]. The best studied bacterial laccase is CotA, a 65-kDa protein belonging to the outer spore coat of *B. subtilis*, which participates in the biosynthesis of brown pigments [12]. We have recently reported laccase activity in *Bacillus* sp. HR03 that had been isolated from Iranian microflora [2]. Alignment and phylogenetic tree analysis of the 16S rDNA sequences showed that the HR03 strain is closely related to *Bacillus atrophaeus*. The brownish pigmented spores of *Bacillus* sp. HR03 showed remarkable resistance in exposure to H_2O_2, UV A and UV C when compared to CotA null mutant spores (ΔHR03). Therefore, it could be concluded that the spore pigments in *Bacillus* sp. HR03 are responsible for its resistance against harsh situations [2] and laccase is an effective enzyme in synthesis and accumulation of these spore pigments.

In the present study, the gene encoding laccase has been isolated, cloned and expressed in order to investigate its biochemical properties. As the active holoenzyme production is difficult under aerobic conditions and most of the over-expressed recombinant enzyme aggregates in inclusion bodies, we tried to gain large amounts of soluble and active holoenzyme through optimization of expression conditions. Due to the fact that the microaerobic condition favors copper accumulation in the cells, we prepared a low-oxygen situation and added copper supplement to the medium. High level soluble enzyme production was reached in this altered procedure of expression when the temperature was lowered to 18°C. Biochemical characterization showed that this recombinant enzyme differs from its family members in some catalytic properties. Moreover, due to the presence of thermal activation among fungal laccases, which has been reported in several publications [17, 20, 25], we tested the behavior of our bacterial laccase after preincubation at high temperatures.

Materials and methods

Taq DNA polymerase, restriction endonucleases and other modifying enzymes were obtained from Fermentas (Germany). 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), syringaldazine (SGZ), 2,6-dimethoxyphenol (2,6-DMP), L-dopa and tyrosine were purchased from Sigma (St. Louis, MO). All other chemicals were from Merck (Darmstadt, Germany) and reagent grades.

Isolation and cloning of the laccase gene

Genomic DNA was extracted from *Bacillus* sp. HR03 using the Genomic DNA Extraction Kit (Bioneer, Korea). The gene was amplified using the forward primer 5′-CGC*GGATCC*ATGACACTTGAAAAATTTGTG-3′ and the reverse primer 5′ -CGG*AAGCTT*TTATTTATGGCGA TCAGTTAT-3′. Restriction sites of *Bam*HI and *Hind*III were designed in forward and reverse primers, respectively. The PCR program was adjusted as: 5 min at 94°C, 30 cycles of 45 s at 94°C, 45 s at 45°C and 90 s at 72°C, and final extension at 72°C for 10 min. The DNA fragment was purified by the PCR purification Kit (Bioneer, Korea). The purified fragment was digested with *Bam*HI and *Hind*III, and subsequently cloned in the expression vector pET21a (+) (Novagen, USA) that had been previously digested with the same enzymes. Sequence analysis confirmed the correctness of the cloning procedure. The nucleotide sequence of the laccase gene was deposited in the GenBank database under the accession no. FJ663050.

Expression and purification

The laccase gene was cloned in pET21a (+) and transformed into *E.coli* BL21 (DE3) cells for expression. The overnight culture in Luria-Bertani (LB) medium was diluted 1:100 with fresh media containing 100 μg/ml ampicillin and incubated at 30°C with shaking (180 rpm). After cells grew to an OD_{600} of 0.5–0.6, the cells were induced with 0.1 mM isopropyl-β-D-thiogalactoside (IPTG) and supplemented with 2 mM $CuSO_4$. To obtain the best condition of expression with high amounts of soluble and active protein, cells were incubated at microaerobic conditions in different temperatures (18, 22, 25 and 30°C). For microaeration, the shaker was turned off 4 h after induction. Cells were harvested after 20 h by centrifugation (8,000 ×*g*, 15 min, and 4°C). The pellets were suspended in 50 mM potassium phosphate buffer, pH 7.6, which also contained protease inhibitor [phenylmethylsulphonyl fluoride (PMSF), 1 mM] then sonicated on ice. Disrupted cells were removed by centrifugation (18,000 ×*g*, 15 min, and 4°C). The supernatant was heated at 70°C for 15 min, and denatured proteins were removed by centrifugation (13,000 ×*g* for 10 min at 4°C). To gain purified protein, supernatant was loaded on a Q-Sepharose column (Amersham Biosciences) that had been equilibrated before with 20 mM potassium phosphate buffer, pH 7.6. Elution was performed with a linear NaCl gradient (0–1 M) in the potassium phosphate buffer at the same pH. Fractions

containing laccase activity were collected (activity was measured in the presence of 0.05 mM SGZ in phosphate buffer, pH 7) and concentrated by ultrafiltration (cutoff 10 kDa). The protein concentration was determined by the Bradford assay using BSA as standard [3]. The concentrated solution was dialyzed against 100 mM potassium phosphate buffer, pH 7.6, in order to remove NaCl. The purity of the enzyme and its molecular mass were determined by SDS-PAGE (12% acrylamide gel) according to the Laemmli method [19]. The gel was stained by Coomassie brilliant blue R-250 [32]. Zymogram analysis of laccase was performed after SDS-PAGE by activity staining [21]. The gel was soaked in 0.05 mM SGZ in 50 mM phosphate buffer, pH 7, at room temperature.

Laccase activity assay

Laccase activity was measured spectrophotometrically at room temperature using three common substrates, ABTS, SGZ and 2,6-DMP. In the assay mixture, oxidation of ABTS (2 mM) in 100 mM phosphate buffer (pH 4) was measured by the increase in absorbance at 420 nm ($\varepsilon = 36{,}000\ M^{-1}\ cm^{-1}$) [6]. Oxidation of 0.05 mM SGZ (dissolved in absolute methanol) in 100 mM phosphate buffer (pH 7) was monitored through the increase in absorbance at 525 nm ($\varepsilon = 65{,}000\ M^{-1}\ cm^{-1}$) [14]. The oxidation of 0.5 mM 2,6-DMP (dissolved in 10% ethanol) was measured at 468 nm ($\varepsilon = 49{,}600\ M^{-1}\ cm^{-1}$) in 100 mM phosphate buffer (pH 7.4) [31].

L-Dopa was also used as a laccase substrate. Oxidation of 8 mM L-dopa was monitored at 475 nm ($\varepsilon = 3{,}700\ M^{-1}\ cm^{-1}$) in 100 mM phosphate buffer, pH 7.4 [20]. Oxidation of 4 mM L-tyrosine (as a possible substrate) was also monitored at 475 nm ($\varepsilon = 3{,}700\ M^{-1}\ cm^{-1}$) in 100 mM phosphate buffer, pH 6. For this reaction, 25 μM of L-dopa was used as a cofactor, and 0.025% SDS was added to the assay mixture [4, 8, 29].

Biochemical characterization of laccase and tyrosinase activity

In order to find the pH optima, laccase activity was studied in mixed buffer (100 mM sodium citrate and 100 mM sodium phosphate) within the pH range of 3–8 using ABTS, SGZ and 2,6-DMP as substrates. The optimum pH of tyrosinase activity was determined using L-dopa as a substrate in 100 mM mixed buffer (pH 4.0–8.5). Kinetic parameters of the purified enzyme were determined at room temperature using different concentrations of ABTS, SGZ, 2,6-DMP and L-dopa. For thermal stability analysis, the enzyme was incubated for several time periods at elevated temperatures (70 and 80°C for SGZ and 70°C for ABTS) in 100 mM phosphate buffer, pH 7, then cooled on ice, and residual activity was measured at room temperature after addition of substrate. In all assays of thermal stability, 20 μl of enzyme (0.3 mg/ml) was added to an assay volume of 600 μl that contained 150 μl of substrate (0.05 mM of SGZ or 2 mM of ABTS). The activity of laccase, which was kept on ice without any thermal incubation, was considered as control (100%). Effect of NaCl on activity of recombinant laccase was examined in different concentrations (up to 0.8 M). The activity was measured in 100 mM phosphate buffer, pH 7, with SGZ as substrate.

Results and discussion

Previously, we have reported laccase activity in a *Bacillus* species that had been isolated from Iranian microflora [2]. Genomic DNA of this species (*Bacillus* sp. HR03) was used as the template, and primers were designed with respect to identified CotA genes of other *Bacillus* species annotated in the GenBank. After amplification of the gene, sequence analysis carried out, and the nucleotide sequence was submitted into GenBank (accession no. FJ663050).

Cloning, expression and purification of laccase

Amplification of the laccase (CotA) gene was performed from genomic DNA of *Bacillus* HR03 and cloned into the pET21a (+) expression vector. The plasmids containing CotA gene were transformed into the *E.coli* BL21 (DE3) and were grown in LB medium. As reported previously, only a minor part of the expressed enzyme was active under aerobic conditions, probably due to insufficient copper incorporation [18]. Durao et al. [11] mentioned that the copper content of CotA depends on copper supplementation and availability of oxygen in the culture media. Transition from aerobic to microaerobic conditions increases the intracellular copper load. Accumulation of intracellular Cu ions in addition to microaerobic conditions leads to an appropriate state for folding and formation of holoenzymes that are fully copper incorporated. Due to these results, induction of recombinant enzyme for expression was carried out with 0.1 mM IPTG, and the medium was supplemented with 2 mM $CuSO_4$, in a shaking incubator (120 rpm). After 4 h, oxygen availability was decreased by switching off the shaking function; this situation was maintained for 20 h at different temperatures (30, 25, 22 and 18°C). After incubation, cells were harvested and sonicated. Microaeration led to a reduced amount of contaminant proteins in the cell extract. The results also revealed that decreasing temperature from 30 to 18°C elevates the expression and specific activity of the enzyme (from 0.003 to 0.15 U/mg) (Fig. 1 and Table 1). To confirm laccase activity, zymography analysis carried

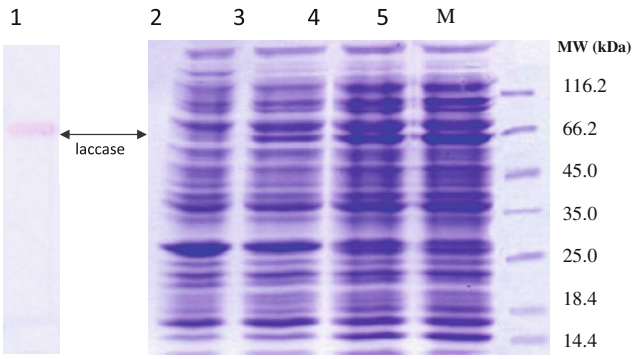

Fig. 1 SDS-PAGE analysis of enzyme expression at different temperatures and microaerobic condition. An increase in recombinant enzyme production is obvious as the temperature decreases from 30 to 18°C. The loaded samples on the gel were the soluble proteins released after sonication (for more details please see the Materials and methods section). Lane 1: laccase activity was detected by activity staining with syringaldazine in phosphate buffer, pH 7 (zymogram); lane 2: 30°C; lane 3: 25°C; lane 4: 22°C; lane 5: 18°C; M: size marker of protein

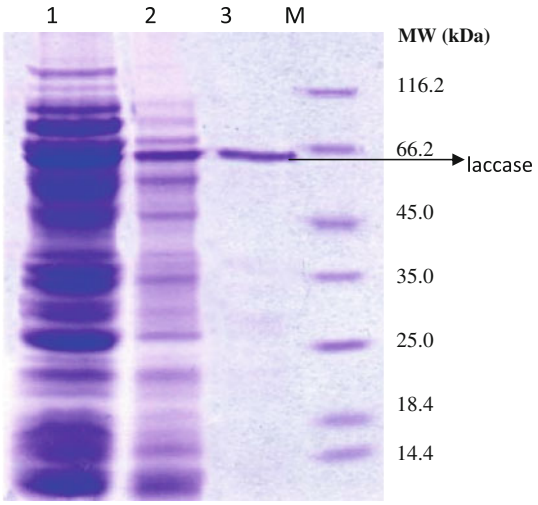

Fig. 2 SDS-PAGE analysis during purification procedure of laccase. Lane 1: cell extract; lane 2: temperature shock at 70°C indicating obvious removal of contaminating proteins; lane 3: purified protein by Q-Sepharose; M: size marker of protein

Table 1 Specific activity comparison at different temperatures and microaerobic conditions

Temperature (microaerobic conditions)	18°C	22°C	25°C	30°C
Specific activity (U/mg)	0.15	0.09	0.03	0.003

Table 2 Purification of laccase (CotA)

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yields (%)	Purification fold
Cell extract	62.50	9	0.14	100	1
Temp. shock (70°C)	5.32	7.4	1.39	82	10
Q-Sepharose column	0.16	3	18.75	33	134

After each step of purification, total protein (mg), total activity (U) and specific activity were determined using SGZ (0.05 mM) as substrate in phosphate buffer (pH 7)

out. After SDS-PAGE, the gel was soaked in phosphate buffer (pH 7) containing 0.05 mM SGZ. A protein band with laccase activity appeared in pink color (Fig. 1).

To simplify subsequent purification steps, the supernatant was heated at high temperature (70°C for 15 min). While most of the *E.coli* proteins precipitated, no significant reduction in activity was observed for the laccase. The enzyme was further purified on a Q-Sepharose column. The results of SDS-PAGE are shown in Fig. 2, and the different purification steps of laccase (CotA) are summarized in Table 2. The purification fold of laccase was determined to be 134 with a yield of 33%. Analysis of CotA by SDS-PAGE revealed a molecular mass of 65,000 Da. Therefore, it is suggested that the enzyme is a monomeric protein, with a high degree of tolerance to heat denaturation. The purified protein exhibited the typical blue color of the multicopper oxidases. The UV-visible spectrum of the purified enzyme showed a band at 600 nm (corresponding to the T1 or blue copper center).

Biochemical characterization of CotA

The primary structure of *Bacillus* sp. HR03 CotA is significantly similar to multi-copper oxidases, a protein family that includes the laccases [1, 24]. Moreover, the CotA from *Bacillus* sp. HR03 shows 98.2% identity and 98.8% similarity with CotA from *B. subtilis*, the structure of which has been already solved. Also, the comparison of the amino acid sequences showed that the copper-binding sites have remained conserved in *Bacillus* sp. HR03 CotA. Biochemical properties of *Bacillus* sp. HR03 CotA were investigated in the presence of three common laccase substrates, ABTS, SGZ and 2,6-DMP. The pH profile of the enzyme for each substrate is shown in Fig. 3. The optimum pH was studied in mix buffer in the range of pH from 3 to 8. The optimal pH for ABTS oxidation was 4, while the pH optimum for SGZ was determined to be 7. The pH activity profiles for these substrates are consistent with well-studied fungal laccases and CotA from *B. subtilis* [16, 22]. Finally, the optimum pH for oxidation of 2,6-DMP was 7.4.

Kinetic parameters of the enzyme were determined using various concentrations of ABTS, SGZ and 2,6-DMP in the reaction mixtures (supplementary material). K_M and k_{cat} values for three substrates were calculated and compared with catalytic constants of CotA from *B. subtilis* and

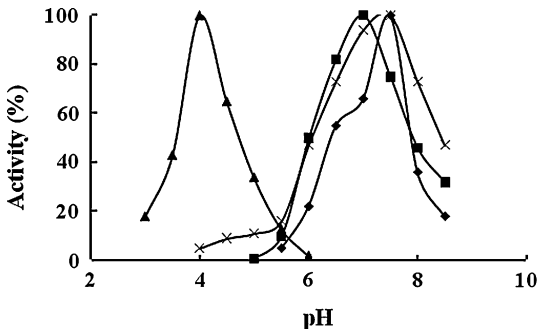

Fig. 3 The pH profiles for the purified laccase were tested in the mixed buffer, pH range of 3–8.5, for four different substrates: (*filled triangle*) ABTS, (*filled square*) SGZ, (*filled diamond*) 2,6-DMP, (*multiplication symbol*) L-dopa. Mixed buffer contained 100 mM sodium citrate and 100 mM sodium phosphate. Standard deviations were within 6% of the experimental values

B. licheniformis (Table 3). Substrate specificity of laccase towards three phenolic compounds indicates striking differences between this enzyme and its family members. The K_M value of laccase from *Bacillus* HR03 towards ABTS was significantly higher than the reported amounts for *B. subtilis* and *B. licheniformis*. The K_M for 2,6-DMP was lower than the reported amounts for other species.

Preincubation of laccases from a few fungal and bacterial origins in high temperatures remarkably increased their activity. This thermal promotion in enzyme activity was first reported for *Basidiomycete* PM1 in 1993 [9]. After that, similar effects were observed in other fungi like *Coriolus (Trametes) hirsutus*, *C. zonatus* and *Fomes sclerodermeus* [17, 25]. On the basis of these reports, the laccase from *Bacillus* HR03 was preincubated at 70 and 80°C for different periods, and residual activity was determined at room temperature while the enzyme was kept on ice. Enzyme activity accelerated after preincubation at 70 and 80°C compared to the control activity (Fig. 4). After 50 min preincubation at 70°C, the k_{cat} was determined to be $\sim 50\ s^{-1}$ for SGZ (see supplementary materials). For this substrate, the activity of laccase (CotA) increased above 300% after 50 min incubation in 70°C. Lengthening the incubation period decreased the activity. Incubation at 80°C promoted the activity to 241% after 10 min, but reduction was observed after this period. The half-life of laccase determined 250 and 45 min in 70 and 80°C, respectively. For ABTS, the enzyme showed a 400% increase in activity after 30 min of preincubation at 70°C (Fig. 4). Therefore, we can suggest that the thermostability of CotA from *Bacillus* sp. HR03 is almost comparable to *B. subtilis,* but higher than CueO [25] and CotA of *Bacillus licheniformis* [18] at 70°C. This intrinsic stability is a striking feature of this enzyme and is highly remarkable, especially when compared to fungal laccases. Laccases from fungal sources possess low thermostability with the optimal temperature variable between 30 and 60°C [5].

NaCl decreased the enzyme activity; the residual activity of laccase in exposure to increasing concentrations of NaCl is presented in Fig. 5. The effect of NaCl on the activity of laccases is not reported either for *Bacillus subtilis* or *Bacillus licheniformis*, but anion inhibition has been proved for both fungal and bacterial laccases when exposed to anions such as F^-, Cl^-, CN^- and also OH^- [27, 34]. Binding to T2/T3 coppers and subsequent interruption of internal electron transfer from the T1 to the T2/T3 centers inhibits the enzyme activity in the presence of these ions [27, 34]. Laccase of *Bacillus* sp. HR03 lost half of its activity at 0.7 M of NaCl, while 7.5 mM of NaCl inhibited 50% of laccase activity from a white-rot fungus, *Marasmius quercophilus* [13]. Furthermore, the enzyme from *Bacillus halodurans* was stimulated rather than inhibited with increasing concentrations of NaCl (up to 100 mM) [27]. The remarkable difference in laccase activity in the presence of NaCl is the result of its nature, native sensitivity and the source from which the enzyme is isolated.

We also tested the ability of recombinant laccase in L-dopa oxidation. The pH profile was determined in 100 mM mix buffer in a 4–8.5 pH range. The optimum pH value was 7.4 (Fig. 3). The K_M value for this oxidative activity was determined to be 1,493 μM in the reaction mixtures containing 0.213 to 7.6 mM of L-dopa (Fig. 4). The ability to oxidize L-dopa as a substrate has not been proved for CotA from *B. subtilis* and *B. licheniformis*. Further, tyrosinase (cresolase) activity was measured using 4 mM L-tyrosine as substrate, 25 μM L-dopa as a cofactor and 0.025% SDS [4, 8, 29], but no activity was detected.

Table 3 Kinetic properties of laccase (CotA) from *Bacillus* sp. HR03 compared to CotA of *Bacillus licheniformis* and *Bacillus subtilis*

Substrate	*Bacillus licheniformis*				*Bacillus subtilis*				*Bacillus HR03*			
	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M	pH	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M	pH	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M	pH
ABTS	6.5	83	12.76	4	124	322	2.60	4	535	127	0.24	4
SGZ	4.3	100	23.25	7	18	80	4.44	7	5	20	4	7
2,6-DMP	56.7	28	0.50	7	216	29	0.13	7	53	3	0.06	7.4

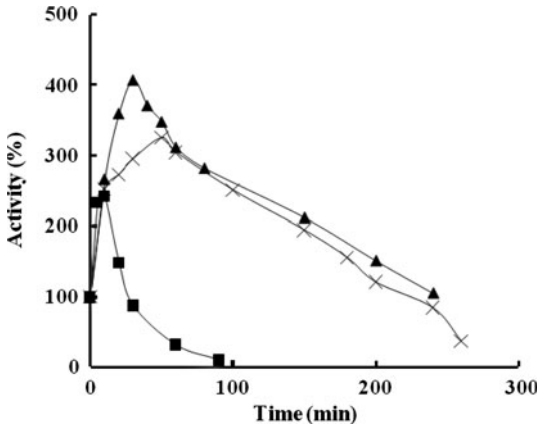

Fig. 4 Thermal activation of the isolated laccase was determined for ABTS at 70°C (*filled triangle*) and SGZ at 70°C (*multiplication symbol*) and 80°C (*filled square*) by incubating the enzyme in 30-µl aliquots for 5 to 260 min. After the incubation, tubes were chilled on ice, and the laccase activity was measured. Enzyme kept on ice without any thermal incubation was considered as control (100%). Highest elevation in enzyme activity observed after 50 and 30 min incubation at 70°C for SGZ and ABTS, respectively

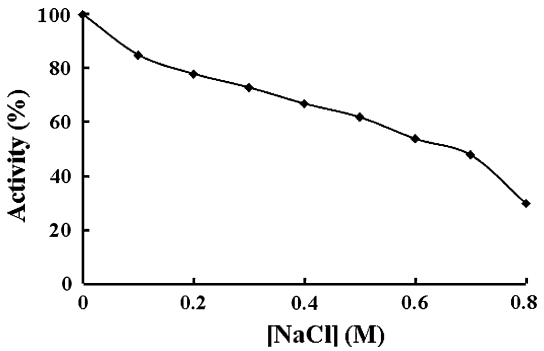

Fig. 5 Loss of laccase activity in exposure to NaCl. Standard deviations were within 4% of the experimental values. For more details, see the Materials and methods section

Conclusion

In the last decades, use of bacterial laccases in biotechnological applications was an important goal for researchers. We found that laccase from *Bacillus* sp. HR03 can be properly synthesized and folded in *E. coli.* After the expression and purification steps, its biochemical and kinetic properties (molecular mass, enzymatic activity, substrate specificity and activity dependence on pH) were studied. This enzyme, like all the blue oxidases, presents the typical blue color because of its absorbance at 600 nm. An absorption band at 330 nm is also obvious, presumably due to the T3 binuclear center. Due to these spectroscopic properties and the laccase-specific capability to oxidize SGZ, CotA from *Bacillus* sp. HR03 was identified as a laccase. Our systematic studies on the cloning, expression, purification and characterization of *Bacillus* sp. HR03 CotA permit the following conclusions:

- The laccase gene from *Bacillus* sp. HR03 was heterologously expressed in *E. coli.* Remarkably, only a minor part of the expressed enzyme was active, probably because of insufficient copper incorporation. As we have shown, owing to decreasing temperature and switching from aerobic to microaerobic conditions, noticeable amounts of soluble and active enzyme could be achieved.
- The enzyme was purified 134 fold with an overall yield of 33%, and the kinetic characteristics revealed that our recombinant laccase has distinctive affinity for 2,6 DMP when compared with CotA from *B. subtilis* and *B. licheniformis*.
- The results showed that the enzyme has high-level intrinsic stability, and the half lives of 250 and 45 min were obtained at 70 and 80°C for SGZ as substrate, respectively.
- In spite of significant similarity with its family members from other *Bacillus* species, our bacterial laccase shows thermal activation that is more common among fungal laccases. When the enzyme is heated up to 70°C, promotion in activity reaches 300% after 50 min of incubation, while activity increases 241% after 10 min at 80°C with SGZ as the substrate. Acceleration in enzyme activity reached 400% after 30 min incubation at 70°C when the enzyme was assayed with ABTS.
- The recombinant laccase with the source of *Bacillus* HR03 has the ability of L-dopa oxidation, which is not reported for its family members from other *Bacillus* species.

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