**Cohnella** sp. A01 laccase: thermostable, detergent resistant, anti-environmental and industrial pollutants enzyme

Masoomeh Shafiei a, Farzaneh Afzali b, Ali Asghar Karkhane a, S. Mehdi Ebrahimic, Kamahldin Haghbeena,*, Saeed Aminzadeh ab, c, d

* Bioprocess Engineering Group, Institute of Industrial and Environmental Biotechnology, National Institute of Genetic Engineering and Biotechnology (NIGEB), Iran
b Institute of Medical Biotechnology, National Institute of Genetic Engineering and Biotechnology, Iran
c Department of Clinical Biochemistry, Faculty of Medical Sciences, Tarbiat Modarres University, Iran
d Institute of Agricultural Biotechnology, National Institute of Genetic Engineering and Biotechnology, Iran

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**ABSTRACT**

Laccase (EC 1.10.3.2; benzenediol; oxygen oxidoreductases) is a multi-copper oxidase that catalyzes the oxidation of phenols, polyphenols, aromatic amines, and different non-phenolic substrates with concomitant reduction of O2 to H2O. Enzymatic oxidation techniques have the potential of implementation in different areas of industrial fields. In this study, the Cohnella sp. A01 laccase gene was cloned into pET-26 (b+) vector and was transformed to E. coli BL21. Then it was purified using His tag affinity (Ni sepharose resin) chromatography. The estimated molecular weight was approximately 60 kDa using SDS-PAGE. The highest enzyme activity and best pH for 2,6-dimethoxyphenol (DMP) oxidation were recorded as 8 at 90 °C respectively. The calculated half-life and kinetic values including Km, Vmax, turn over number (kcat), and catalytic efficiency (kcat/Km) of the enzyme were 106 min at 90 °C and 686 μM, 10.69 U/ml, 20.3 S-1, and 0.029 s-1 μM-1, respectively. The DMP was available as the substrate in all the calculations. Enzyme activity enhanced in the presence of Cu2+, NaCl, SDS, n-hexane, Triton X-100, tween 20, and tween 80, significantly. The binding residues were predicted and mapped upon the modeled tertiary structure of identified laccase. The remaining activity and structural properties of Cohnella sp. A01 laccase in extreme conditions such as high temperatures and presence of metals, detergents, and organic solvents suggest the potential of this enzyme in biotechnological and industrial applications. This process has been patented in Iranian Intellectual Property Centre under License No: 91325.

**1. Introduction**

Laccase (benzodiol; oxygen oxidoreductase (EC.1.10.3.2)) is a multi-copper oxidase catalyzing the oxidation of phenols, poly-phenols, aromatic amines, and a variety of non-phenolic substrates [1]. The oxidation reaction happens through the Ping-Pong mechanism in which electron transference occurs in each step. The oxygen molecule (O2) first oxidizes the enzyme to produce the reactive intermediate (laccase-oxygen) along water; then laccase-oxygen removes one electron from the substrate [2]. This highly reactive substrate-driven radical is able to participate in a variety of reactions such as rearrangements and polymerizations. The initial laccase will be reformed through obtaining four dismissed electrons during oxygen reduction [3]. Laccase usually bears four copper atoms that are concentrated in three centers [4]. The type I copper has been suggested to have initial substrate oxidation role and its absence leads to enzyme inactivation. The copper type II is a single atom which binds to the nitrogen and oxygen donors to form a flat square. The type III contains two copper atoms that are responsible for oxygen reduction. The reduction of type I copper by an organic compound is the prerequisite of the oxygen attachment to type II/III copper [3].

Laccase is naturally produced by numerous organisms such as plants, fungi, some insects, and bacteria. Its roles in a variety of cellular processes such as biosynthesis of pigments, spore production, pathogenicity, repairing the tissue damages, and lignin production and degradation were mainly investigated in fungal and plants [5], while there is a limited information of them in bacteria. The first bacterial laccase was identified in *Azospirillum lipoferum* with its important functions in production of cellular pigments, consumption of the plant phenolic compounds, and electron transfer [6]. The environment-friendly oxidation technologies has been directed to oxidizing enzymes [7] and manifold studies were...
dedicated to discover applicable kinds of laccase for various industrial purposes. This new area of interest was expanded so much so that in September 2014, laccase was called “the green alternative for chemical oxidation” [8] and promised the likeliness of finding other novel biocatalysts for answering the green chemistry needs and rules. Some of the industrial applications of laccase are delignification of pulp, textile bleaching, waste water treatment, production of detergents and bleaches, modification of biopolymers, development of biosensors (detection of various phenolic compounds and azides), and oxidizing agent in hair dyes [5].

The usual chemical oxidation techniques require toxic materials and produce non-specific reactions thus the biological oxidation techniques have attracted plenty of attention lately [9]. Due to the importance of thermostable laccase in biological enzymatic oxidation [10], the thermophilic bacterium Cohnella sp. A01 (PTCC No: 1921) laccase was investigated in the current study. The recombinant enzyme was exogenously expressed in E. coli BL21, purified and characterized biochemically and structurally to evaluate its capabilities in industrial processes, especially the textile industry.

2. Materials and methods

2.1. Materials

DNA purification kit was obtained from PEQLAB. PCR Purification Kit was purchased from Bioneer (Seoul, Korea). Plasmid purification kit was purchased from Roche (Basel, Switzerland). Taq polymerase, Pfu DNA Polymerase, T4 DNA ligase, Xhol and Ncol enzyme, IPTG, and X-GAL were purchased from Fermentas (Glen Burnie, MD, USA). Ampicillin, kanamycin, and 2,6-dimethylphenol (DMP) were obtained from Sigma (St. Louis, USA). E. coli DH5α and E. coli BL21 (DE3) strains, and vector pET-26b (+) were obtained from Invitrogen (Carlsbad, CA, USA). Ni Sepharose resin was purchased from Qiagen. All other chemicals were provided from Merck (Darmstadt, Germany).

2.2. Tertiary structure prediction, refinement, and validation

The Translate (http://web.expasy.org/translate) and ProtParam (http://s:/www.expasy.org/protparam/) tools of Expasy server were used for translating the nucleotide sequence of the enzyme to amino acid sequence and physicochemical characterization, respectively. The three-dimensional structure of the enzyme was modelled through I-TASSER server (https://zhanglab.ccmb.med.umich.edu/I-TASSER/). The energy minimized structure of the enzyme was performed by SPDBV v4.10 (GROMOS 96 as the applied algorithm) and other structural refinements were done by GalaxyRefine server (http://galaxy.seoklab.org/). To estimate the accuracy of predicted model before and after refinements, Ramachandran plot was analyzed by Rampage server (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php) and then its z-score was calculated by ProSA-Web server (https://prosa.services.came.sbg.ac.at/prosa.php).

2.3. Active site and Ca2+ binding sites prediction

The active site was predicted by RaptorX Binding server (http://rapt orx.uchicago.edu/BindingSite/) according to the protein sequence. Then Ca2+ probable binding sites were predicted by lsolCom server (https://zh anglab.ccmb.med.umich.edu/IsonCom/). The provided data compared together to be validated and confirmed.

2.4. Cloning, expression and purification of Cohnella sp. A01 Laccase

Cohnella sp. A01 was grown in nutrient broth medium at 60 °C and 180 rpm orbital shaking for 72 hours. The grown bacteria were used to extract genomic DNA. The forward (5′ CTCCCATGGCGAGCGCAAT ACGGACAACTAGGAAG3′) and reverse (5′ TTCTTGAGCCTCTGGT TTTCGCGGTTGC3′) primer pairs with Ncol and Xhol restriction sites (the underlined residues) were implemented to amplify the laccase gene (1600 bp) in pT757R/T cloning vector. Then it was sent to Genfanavaran Company for sequencing regarding M13 primer pairs. After confirming the validity of laccase sequence, the amplified gene was inserted between Ncol and Xhol restriction sites on pET-26b (+) as the expression vector. Eventually, the recombinant expression vector transferred into E. coli BL21. A single colony of the transformed bacteria was grown overnight in LB medium at 37 °C with 30 μg/mL of kanamycin then was transferred into a fresh LB medium. In order to induce the protein expression and appropriate folding, 1 mM IPTG and 0.5 mM CuSO4 were added to the inoculated media, after reaching 0.6 as the desired OD600. The bacterial cells were pelleted (4 °C, 20 minutes, 4000 x g) after 16 hours incubation at 37 °C. The precipitate of bacteria was resuspended in lysis buffer (50 mM NaH2PO4, 300 mM NaCl, 10 mM Imidazole, pH = 8). The resulted suspension was subjected to ultrasonic waves (UP400S - Hielers Ultrasound 1MHz) incubating on ice for four sets of one minute pulses (70 mHz) with 20s intervals in order to break the cells’ walls. The suspension was subsequently centrifuged (4 °C, 30 minutes, 9000 x g). The supernatant was collected to be used for protein expression analysis and purification. The negative control was designed as same as previous sections except having the empty pET-26b (+) vector in E. coli BL21 instead of recombinant one. Laccase was designed to have His-tagged to allow its better purification through affinity chromatography using Nickel-sepharose column; the resulted fractions were visualized by SDS-PAGE. The purified enzyme was dialyzed against phosphate buffer (10 mM K2HPO4, pH = 8) and its concentration was estimated through Bradford’s assay.

2.5. Enzyme activity assay

The dialyzed laccase activity was measured based on its ability to oxidize DMP to 3,3′,5,5′-tetramethoxy-biphenyl-4,4′-diol [11]. The laccase activity was monitored in the presence of 4 mM DMP at 470 nm (Beckman DU 530 from US). The calculations were performed according to A = eε C (ε = 14,800 M⁻¹ cm⁻¹) formula [12]. The phosphate buffer (10 mM K2HPO4 in the presence of DMP was used as the control reaction.

2.6. Kinetic study

DMP concentrations of 0.3, 0.6, 1.25, and 2.5 mM were used to examine the kinetic properties of the laccase. Michaelis-Mentton plot was used to calculate the values of Vm and Km in GraphPad Prism 6.07. The Km value represents the enzyme affinity towards the substrate; the lower Km corresponds to higher affinity. The catalytic functionality can be considered as a criterion for determining the enzyme’s industrial applicability.

2.7. The effect of temperature and pH on the enzyme’s activity and stability

For determining the optimum temperature, the laccase activity was measured after incubation for 30 minutes at 20–100 °C. The optimum pH was then found by measuring the enzyme activity at 90 °C incubating with 50 mM substrate solutions in pH 3–10 (citrate phosphate buffer for pH 3–7, phosphate buffer for pH 6–8, glycine–sodium hydroxide for pH 9–10) [13].

The enzyme thermostability was determined by measuring the enzyme activity after 10, 20, 30, 40, 50, 60, 90, and 120 minutes incubation in 60, 90, and 100 °C and then incubation at optimal condition (pH = 8 and 90 °C) for 30 minutes. It was then confirmed by measuring the enzyme stability in the temperature range of 10–100 °C for 90 minutes and incubation at optimal condition (pH = 8 and 90 °C) for 30 minutes.

The pH stability of the enzyme was first measured by measuring the
enzyme activity in different pH ranges [5, 6, 9, 10] in four subsequent time sections with one-hour intervals and incubation at optimal condition (pH = 8 and 90 °C) for 30 minutes; then as a confirming test, the enzyme was incubated at pH 3 to 10 for 90 minutes and then incubation at optimal condition (pH = 8 and 90 °C) for 30 minutes.

2.8. The effect of metal ions, organic solvents, inhibitors, surfactants, and SDS on laccase activity

The enzyme activity was screened under the effect of 1 and 5 mM metal ions, including Cu²⁺, Na⁺, Ni²⁺, Zn²⁺, Fe²⁺, and Mn²⁺. The effect of organic solvents such as acetone, glycerol, methanol, ethanol, isopropanol, isobutanol, N-hexane, and acetic acid with concentrations of 25 and 50% (v/v) was also evaluated. The 1 and 5 mM of propanol, isobutanol, N-hexane, and acetic acid with concentrations of 0.1, 0.5, and 5 mM and SDS with the concentrations of 1.56, 3.12, 6.25, 12.5, 25, and 50 mM (as surfactants) were added to the enzyme's activity reactions to analyze their effects. All reactions were treated for 1 hour and the activity was determined at 90 °C and pH 8. The activity of the untreated enzyme was considered as the control.

2.9. Extrinsic and Intrinsic Fluorescence

Laccase (100 μg/ml) was incubated with SDS (25 and 50 mM) at 90 °C within the pH range of 3–10 (citrate phosphate buffer for pH 3–7, phosphate buffer for pH 6–8, glycine–sodium hydroxide for pH 9–10, 50mM) for 60 minutes to perform the fluorescence experiments using spectrophotometer (Varian Cary Eclipse Fluorescence Spectrophotometer, Santa Clara, USA).

The 8-Anilinonaphthalene-1-sulfonic acid (ANS) with the final concentration of 40 μM was added to the enzyme and then incubated at 25 °C for 10 minutes. The samples were excited at 360 nm and emission spectra were recorded from 400-600 nm. The spectral bandwidth was set at 10 nm. To perform the intrinsic fluorescence experiment, the samples were excited at 280 nm and emission spectra were recorded from 300-400 nm. The spectral bandwidth was set at 5 nm. The fluorescence graphs were plotted using SigmaPlot v12.5. Stored laccase in phosphate buffer (10 mM K₂HPO₄, pH = 8) at 4 °C was used as control.

2.10. Circular dichroism (CD)

Laccase (100 μg/ml) was incubated in two separate vials at 90 °C and with 25 mM SDS for 60 minutes. Then its secondary structure composition was recorded in the 180–240 nm wavelength range. The far UV circular dichroism (CD) spectroscopy was analyzed with spectropolarimeter 158 J-715 (Jasco, Tokyo, Japan). Stored laccase in phosphate buffer (10 mM K₂HPO₄, pH = 8) at 4 °C was used as control.

3. Result

3.1. Cloning, expression, purification, and kinetic properties calculation of Cohnella sp. A01 laccase

The alignment of amino acid sequence of Cohnella sp. A01 laccase with the protein sequences of Genbank revealed similarity to other related proteins; the most similar protein was Paenibacillus darwinianus laccase (Accession: E0X78783.1) with 68% identity. The laccase gene was amplified by PCR and then cloned in pTZ57R/T vector. The cloning was confirmed by sequencing. After confirming the gene sequencing, Cohnella sp. A01 laccase with the accession number of AKL79441.1 was recorded in GenBank database. Laccase gene was subcloned into pET-26b (+) as an expression vector and transferred into E. coli BL21 strains in order to be over expressed. The heterologous expression of the Cohnella sp. A01 laccase in E. coli BL21 was confirmed through SDS-PAGE analysis. The purity of the enzyme was examined and shown as a single band on acrylamide gel with an approximate size of 60 kDa by SDS PAGE after Ni Sepharose resin purification. The results are shown in Fig. 1. Yield of purification, purification fold, and specific activity of the purified enzyme were found to be 33.3, 4.7, and 20 (U·mg⁻¹), respectively. The kinetic properties of the purified enzyme were assessed through calculating the values of Kₘ, V_max, turn over number (kcat), and catalytic efficiency (kcat/Kₘ) in the presence of DMP. The values were respectively 686 μM, 10.69 U/ml, 20.3 S⁻¹, and 0.029 s⁻¹·μM⁻¹. The results of Cohnella sp. A01 laccase purification profile and kinetic parameters were summarized in Table 1.

3.2. Evaluation of temperature and pH on the laccase activity and stability

The optimum temperature of this enzyme was estimated to be 90 °C with a half-life of 1 hour and 46 minutes. The enzyme conserved more than 60% of its activity at 100 °C. The thermostability of the enzyme was approved since it maintained more than 60% of its activity after 90 minutes incubation at 60 to 90 °C. The enzyme still conserved 48% of its activity after the same duration of incubation at 100 °C.

The optimum pH of the Cohnella sp. A01 laccase was found to be 8. The relative activities of the enzyme were 62%, 97%, 91%, and 58% in the pH range of 6, 7, 9, and 10. The pH stability of the enzyme was analyzed and approved in the pH range of 5, 6, 7, and 8 regarding maintaining more than 50% of its activity after 90 minutes incubation. The results are shown in Fig. 2.

3.3. Evaluation of metal ions, inhibitors, organic solvents, and surfactants effects on the laccase activity

The examined effects of metal ions including Cu²⁺, Na⁺, Ni²⁺, Zn²⁺, Fe²⁺, and Mn²⁺ on the enzyme activity are presented in Fig. 3a. According to the results, 1 and 5 mM of Cu²⁺ and 1 mM of Na⁺ increased the activity and 1 mM of Zn²⁺, Fe²⁺, Mn²⁺, and Ni²⁺ reduced this value to 86, 68%, 48%, and 47%, respectively, considering the untreated enzyme as control (Fig. 3a).

The evaluated effects of organic solvents at concentrations of 25 and 50% (v/v) on Cohnella sp. A01 laccase activity are represented in Fig. 3b. The enzyme activity was increased 100% following 50% N-hexane treatment. The approximate lowered activity to 50% was observed following 25% and 50% acetone and glycerol treatments.
The inhibitors effects (1 and 5 mM of each inhibitor) in comparison with control reaction are provided in Fig. 3c. The EDTA inhibited most of enzyme activity and NaN₃ reduced the activity to 48% and 45%, respectively. The Cysteine dampened the enzyme activity to 27% and 12%, respectively. The activity of this enzyme was lowered to 77 and 7% when 1 and 5 mM β mercaptoethanol was added).

The Fig. 3d represents the surfactants’ effects on enzyme activity. The concentrations of 0.5 mM Tween 20, 0.1 mM Tween 80, and 0.1 mM Triton X100 increased the enzyme activity approximately 50%, 100%, and 100%, respectively. Nevertheless, the 5 mM concentration of the mentioned compounds decreased the activity to less than 50%. There was a surprising 41% increase in the enzyme activity under treatment of up to 25 mM SDS (Fig. 3e).

3.4. Fluorescence experiments

The laccase conformational changes were investigated under the influence of temperature and pH through intrinsic fluorescence. The fluorescence intensity was decreased in acidic condition (pH: 3, 5, 6), while similar fluorescence intensities were observed in the pH range of 7–10. The data at pH of 4 (assumed to be the enzyme’s pI) is not reliable due to the protein precipitation.

The laccase structural changes following exposure to different pH ranges in compare to untreated enzyme were evaluated through extrinsic fluorescence technique utilizing ANS. The fluorescence intensity was increased within the pH range of 3–6 and maintained constant within the pH range of 7–10. The fluorescence intensity was increased in the presence of 25 mM SDS (Fig. 4).

3.5. Circular dichroism (CD)

Analysis of the laccase secondary structure through Circular Dichroism spectra showed that it contains 7.4 % α-helix, 66% β-sheet, 7.5 % β-turn, and 18.8 % loop (random coil). The β-sheets were remained almost unchanged (67.2 %) at 90 °C, while the α-helix and β-turn were reduced to 2.2 % and 3.8 %, respectively. The amount of loops were increased to 26.8 %. In the presence of 25 mM SDS, the amount of α-helix reached zero and decreasing and increasing in the number of β-sheets and

| Steps | Volume (ml) | Protein (mg/ml) | Total protein (mg) | Total activity (units) | Specific activity (units/mg) | Purification fold | Yield (%) | K_m (μM) | V_max (U/ml) | k_cat (S⁻¹) | k_cat (S⁻¹ μM⁻¹) | Catalytic efficiency |
|-------|-------------|----------------|-------------------|-----------------------|-----------------------------|------------------|-----------|-----------|--------------|--------------|-------------------|------------------------|
| Crude | 4           | 1.4            | 5.6               | 24                    | 4.2                        | 1                | 100       | -         | -            | -            | -                 | -                      |
| 1     | 4           | 0.1            | 0.4               | 8                     | 20                         | 4.7              | 33.3      | 686       | 10.69        | 20.3         | 0.029             |                        |

Table 1

Purification profile and kinetic parameters of the Cohnella sp. A01 laccase.

Fig. 2. The effect of pH and temperature on the Cohnella sp. A01 laccase. (a) Temperature activity and stability. (b) Temperature stability. (c) pH activity and stability. (d) pH stability.
β-turns were observed, respectively (Fig. 5 and Table 2).

3.6. Tertiary structure prediction, refinement, and validation

According to ProtParam tool of Expasy, the laccase contains 557 amino acids and approximate molecular weight of 61.59 kDa. Among five predicted models resulted from I-TASSER, the first model by C-score = -0.83, TM-score = 0.61 ± 0.14, and RMSD = 9.4 ± 4.6Å was counted as the reliable one. The model was given to GalaxyRefine server for structural refinements and then experienced energy minimizations by SPDBV v4.10 software. The z-scores by ProSA-Web server were -2.97 and -4.75 before and after improvements, respectively. Although both values indicated the resemblance of the predicted model to native proteins but the improved value of -4.75 after refinements indicated the necessity of this step. The Ramachandran Plot which indicates the percentage of residues in favored, allowed, and disallowed regions resulted in respective values of 66.7%, 20.3%, and 13.0% before and 86.6% 8.9%, and 4.5% after structural improvements. The percentage of amino acids exist in the favored and allowed region were improved and meet 95.5% and is confident (Fig. 6a).

3.7. Active site and Cu²⁺ binding sites prediction

The predicted Cu²⁺ binding sites by IonCom server were Histidine (H) 140, H142, Tryptophan (W) 180, H182, H184, Asparagine (N) 426, H470, H473, H475, H477, Glutamate (E) 506, D514, H522, Cysteine (C) 523, H524, D525, H528, and Methionine (M) 533. There were three predicted binding regions from RaptorX Binding server that two of them specifically comprise Cu²⁺ ions. The first region includes Histidine (in 140, 142, 182, 184, 473, 475, 522, 524 residues) and the second one includes Histidine (in 470 and 528 residues), Cysteine 523, and Methionine 533. The resulted data were compared and confirmed each other (Fig. 6b).

4. Discussion

The Cohnella sp. A01 laccase was found to be thermophilic and thermostable, the main prerequisites for an industrial enzyme [14]. The high temperature increases the reaction rate through cutting down the reaction time and preventing the reactions contamination [15]. The comparison of optimum temperature and stability of Cohnella sp. A01 laccase and other kind of heterologously expressed bacterial laccase are shown in Table 3 [16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31].

Identification of the optimum pH is needed to manage the enzyme's purification buffers, storage, and industrial applications [32]. The Cohnella sp. A01 laccase had the optimum pH of 8 and it was appropriately stable in the pH range of 5–8. The optimum pH of laccase depends strictly on the implemented substrate, oxygen molecules, or its origin. The reductive potential between the substrate and the type I copper and then hydroxide anion attachment to the copper types II and III is an important factor in determining the optimum pH [33]. Table 4 shows the comparison of optimum pH of Cohnella sp. A01 laccase and other reported laccases in the presence of DMP substrate [34, 35, 36, 37, 38, 39, 40].

Metal ions are crucial factors for enzymes' structure and function. The stability of the enzyme in the presence of metal ions could be an asset considering its use in industry such as pulp and paper and bioremediation [41]. The reduction of laccase activity in the presence of metal ions could
be explained by the possible attachments of these ions to the copper types II and III that interfere with the internal electron transferring process affecting its activity [42]. For example, the activity reduction in the presence of Fe$^{2+}$ may be due to its reactions with the laccase electron transferring system [43]. The enhanced enzyme activity in the presence of some ions, especially copper, can be observed in the majority of laccases. During the expression of laccase, the unsaturated Cu$^{2+}$ residues lead to partial activation of the enzyme, hence reactivating process can be reached by Cu$^{2+}$ ions treatments. This procedure has been successful for improving the activity of Cohnella sp. A01 laccase, in line with previous studies [44].

According to our results, EDTA approximately inhibited most of the enzyme activity since it binds to type II copper ions. Its attachment to unstable type II copper ions facilitates their collection and in following inhibits the enzyme activity more than before [34]. The decreased activity of laccase that was exposed to NaN$_3$ may be due to electron transferring blockage in the types II and III copper binding sites [45]. Cysteine counts as a competitive inhibitor for polyphenol oxidases so the resulted decrease following Cysteine presence is due to this criterion. This amino acid binds to the enzyme's active site forming the enzyme-cysteine complex and reduces its activity [46]. The reduced enzyme's activity in the presence of β-mercaptoethanol may be related to the reduction of the oxidized substrate by the sulfhydryl group [41].

Using the organic solvents instead of natural aqueous environment of the enzyme could increase its applicability in industries. In fact the organic solvents alter the activity of the enzymes through various physicochemical interactions. It has been demonstrated in some cases that an impossible enzymatic reaction in water could be performed in organic solvents [47]. In addition, the majority of laccase substrates are insoluble in water and increasing their solubility in the presence of organic solvents and their concentrations could enhance the enzymatic reaction rate [9]. On the other hand, the organic solvents are able to denature the proteins through removing the water molecules around them which are necessary for the functional groups [48]. Thus, the activity and stability of enzymes in such solvents could be of high importance for their applications in industry. According to the obtained results, enzyme activity was doubled in presence of n-hexane assuring the usage of laccase in

![Fig. 4. Extrinsic and Intrinsic Fluorescence. (a) The analyzed intrinsic fluorescence at the temperature of 4, 25, and 90 °C. (b) The analyzed intrinsic fluorescence at the pH range of 3–10. (c) The analyzed intrinsic fluorescence in the presence of SDS. (d) The analyzed extrinsic fluorescence at the temperature of 4, 25, and 90 °C. (a) The analyzed extrinsic fluorescence at the pH range of 3–10. (f) The analyzed extrinsic fluorescence in the presence of SDS.](image)

![Fig. 5. The secondary structure evaluation by Circular Dichroism (CD) spectroscopy. The Cohnella sp. A01 laccase secondary structure was evaluated at 90 °C and 25mM SDS. The control sample is the stored laccase in 4 °C in phosphate buffer (10mM K$_2$HPO$_4$, pH = 8).](image)

| Table 2 | The secondary structure compartments of Cohnella sp. A01 laccase through CD spectroscopy. |
|----------------|--------------------------------------|
|             | Helix (%) | Beta sheet (%) | Turn (%) | Loop (%) | RMS       |
| CNT         | 7.4%      | 66.3%          | 7.5%     | 18.8%    | 122.74    |
| TEMP 90     | 2.2%      | 67.2%          | 3.8%     | 26.8%    | 67.485    |
| SDS 25      | 6.0%      | 57.4%          | 0.0%     | 36.7%    | 27.980    |


textile waste water treatment where n-hexane is abundantly available. According to the previous studies, there is positive relation between the enzyme thermostability and stability in the presence of organic solvents. Furthermore, the halophilic enzymes count as potential biocatalysts in aqueous–organic media due to the similarity between organic solvents and salt (both reduce water activity) [49]. Regarding the stability of Cohnella sp. A01 laccase against temperature and salt, it is expected to be stable against organic solvents too. It has been reported that 10, 30 and 50 % aceton treatment increased the laccase activity 137, 89 and 35%, respectively [50].

Surfactants can facilitate the elimination of hydrophobic contaminants such as PAHs (Polycyclic aromatic hydrocarbons) through increasing the solubility, bioaccessibility, and emulsifyability of hydrocarbons [51]. One application of laccase is the elimination of multi-cyclic aromatic hydrocarbons and xenobiotic compounds (the main contaminants of soil) [52]. SDS is an anionic detergent which disrupts almost all the noncovalent interactions in the proteins and increase the enzyme activity by its denaturing properties [53]. By exposing the Cohnella sp. A01 laccase to up to 25mM SDS, its activity enhanced by 41 percent which is impressive in comparison with other studies. For instance, in a study held by Badooei et al, the laccase activity was increased 4.3 fold when exposing to 0.05 mM SDS [53]. Increasing the enzyme activity was also observed in Azospirillum lipoferum laccase, while the inactivated laccase was reactivated by adding SDS and maximum activity was
and other kind of heterologously expressed bacterial laccase.

observed the increased emission of extrinsic fluorescence in treated Cohnella sp. A01 laccase with 25 mM SDS. As it was obtained through performing laccase Circular Dichroism, the amount of α-helix was reached to zero while decreasing and increasing in the amount of β-sheets and turns were observed, respectively. This can be due to conformational changes and flexibility enhancement of enzyme that lead to improvements in enzyme activity.

5. Conclusion

The laccase capabilities in oxidizing phenolic and nonphenolic compounds have made it a valuable enzyme for safe oxidizing technologies. The Cohnella sp. A01 laccase was exogenously expressed in bacterial host, purified, and characterized for its biochemical, thermodynamic, and structural features in the present study. This enzyme was naturally resistant to high temperatures, a wide pH range, and the presence of ions, surfactants, and organic solvents. The Cohnella sp. A01 laccase can be a candidate for industrial applications due to its high thermostability and maintained activity in harsh conditions. With reference to its distinctive features and the possibility of its production optimization during scale up studies, it can be a suitable alternative for the currently used laccases. This process has been patented in Iranian Intellectual Property Centre under Licence No: 91325.

Declarations

Author contribution statement

Masoomeh shafiei: performed the experiments; analyzed and interpreted the data; wrote the paper.
Farzaneh Afzali: analyzed and interpreted the data; wrote the paper.
Ali Ashgar Karkhan: analyzed and interpreted the data; wrote the paper.
Mehdi Ebribami: contributed reagents, materials, analysis tools or data.
Kamahldin Haghbeen: analyzed and interpreted the data.
Saeed Aminzadeh: conceived and designed the experiments; wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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References

[1] S.J. Moon, H.W. Kim, S.J. Jeon, Biochemical characterization of a thermostable cobalt- or copper-dependent polyphenol oxidase with dye decolorizing ability from Geobacillus sp. JS12 [Internet], Enzym. Microb. Technol. 118 (2018) 30–36. Elsevier Inc.
[2] F.J. Enguita, L.O. Martínez, A.O. Henríquez, M.A. Carroza, Crystal structure of a bacterial endopore coat component: a laccase with enhanced thermostability properties, J. Biol. Chem. 278 (21) (2003) 19416–19425.
[3] H. Claus, Laccases and their occurrence in prokaryotes, 2015) (April 2003). Epib. 2003 Feb 7, Arch. Microbiol. 179 (3) (2003 Mar) 145–150.
[4] N. Itoh, S. Takagi, H. Kuroka, Characterization and cloning of laccase gene from Hericium coralloides NBRC 7716 suitable for production of epinephelagillin 3-O-gallate, Enzyme Microbe Technol 82 (2016) 125–132. Elsevier Inc.
[5] S.R. Couto, J. Luís, T. Herrera, Industrial and biotechnological applications of laccases: A review, Biotechnol. Adv. 24 (2006) 500–513.
[6] G. Diamantidis, A. Aline, P. Potier, Purification and characterization of the first bacterial laccase in the rhizospheric bacterium Aspergillus lipoferum, Soil Biol. Biochem. 32 (2000) 919–927.
[7] R. Zhao, H. Yu, P. Yuan, J. Fan, L. Chen, Y. Li, et al., Heterologous Expression and Characterization of Three Laccases Obtained from Pleurotus Ostreatus HAUCC 162 for Removal of Environmental Pollutants, Elsevier B.V., 2017.
[8] C. Pezzella, L. Guarino, A. Piscitelli, How to Enjoy Laccases How to Enjoy Laccases, 2015 (January).
[9] V. Madhavi, S.S. Lele, Laccase: properties and applications, BioResources 4 (Thurston 1994) (2009) 1694–1717.
[10] R. Reis, J. Isachen, L. Thony-meyer, Bacillus pumilus laccase: a heat stable enzyme with a wide substrate spectrum, BMC Biotechnol. 11 (1) (2011) 9. BioMed Central Ltd.
[11] Y. Wan, Y. Du, T. Miyakoshi, Enzymatic catalysis of 2,6-dimethoxyphenol by laccases and products characterization in organic solutions, Sci. China Ser B Chem. 51 (7) (2008) 669–676.
[12] D. Slonezynski, J.P. Nakaw, S.S. Tanebaum, Production and characterization of laccase from Botrytis cinerea 61–34, Appl. Environ. Microbiol. 61 (3) (1995) 907–912.
[13] Richard, R. Burgers, Murray P. Deutcher, Methods in Enzymology, Guide to Protein Purification, second ed., Elsevier Inc, 2009, pp. 97–103.
[14] C. Vieille, G.J. Zeikus, C. Vieille, Hyperthermophilic Enzymes: sources, uses, and molecular mechanisms for thermostability hyperthermophilic Enzymes: sources, uses, and molecular mechanisms for thermostability, Microbiol. Mol. Biol. Rev. 65 (1) (2001) 1–43.
[15] A. Mukhopadhyay, A.K. Dasgupta, K. Chakrabarti, Thermostability, pH stability and dye degrading activity of a bacterial laccase are enhanced in the presence of G2O nanoparticles, Biosens. Bioelectron. 127 (2023) 25–36. Elsevier Ltd.
[16] J. Sun, M. Zheng, Z. Lu, F. Lu, C. Zhang, Heterologous production of a temperature thermostable and chloride-tolerant laccase from Thermus thermophilus SG0.5JP17–2279, J. Appl. Microbiol. 121 (5) (2016) 1335–1345.
[17] L.I. Ramírez-Cavazos, C. Junghanns, O. Ornelas-Soto, D.L. Cárdenas-Chávez, C. Hernández-Luna, P. Demarche, et al., Purification and characterization of two thermostable laccases from Pycnoporus sanguineus and potential role in degradation of endocrine disrupting chemicals, J Mol Catal B Enzym. Elsevier B.V. 108 (2014) 32–42.
[18] Z.K. Wang, Y.J. Cai, X.R. Liao, G.J. Tao, Y.Y. Li, F. Zhang, et al., Purification and characterization of two thermostable laccases with high cold adapted characteristics from Pycnoporus sp. SYBCL1, Process Biochem. 45 (10) (2010) 1720–1729. Elsevier Ltd.
[19] K. Koschorreck, S.M. Richter, A.B. Ese, E. Roduner, R.D. Schmid, V.B. Urlacher, Cloning and characterization of a new laccase from Bacillus licheniformis catalyzing dimerization of phenolic acids, Appl. Microbiol. Biotechnol. 79 (2) (2008) 217–224.
[20] K. Hildén, T.K. Hakala, P. Majaiä, T. Lundell, A. Hatakka, Novel thermostable laccases produced by the white rot fungus Physisporinus rutilans, Appl. Microbiol. Biotechnol. 77 (2) (2007) 301–309.
[21] R. Ulrich, M.H. Le, L.D. Nguyen, M. Hofrichter, Laccase from the medicinal mushroom Agaricus blazei: production, purification and characterization, Appl. Microbiol. Biotechnol. 67 (3) (2005) 357–365.
[22] D.M. Soden, J. O’Callaghan, A.D.W. Deacon, Molecular cloning of a laccase isoform from Pleurotus saporius-caju and expression in the heterologous Pichia pastoris host, Microbiology 148 (12) (2002) 4003–4014.
[23] S. Sonodhi, P. Sharma, S. Saini, P. Nuri, Gupta, Purification and characterization of an extracellular, thermo-alkali-stable, metal tolerant laccase from Bacillus tequilensis SN4, PLoS One 9 (5) (2014) 1–10.
[24] S. Shleev, A. Jarosz-Wilkolazka, A. Khalunina, O. Morozova, A. Yaropolov, T. Ruzgas, et al., Direct electron transfer reactions of laccases from different origins on carbon electrodes, Bioelectrochemistry 67 (1) (2005) 115–124.
[25] S. Goller, C.K. Peterbauer, J. Strauss, C. Galbaba, D. Haltrich, Characterization of the major laccase isoenzyme from Trametes pubescens and regulation of its synthesis by metal ions a, Microbiology 148 (7) (2011) 2159–2169.
[26] G. Grass, C. Rensing, C. Puri, Introduction of a trametes sanguineus laccase in trichoderma atroviride, PLoS One 11 (9) (2016) e0160770.
[27] I. Stoilova, A. Krastanov, V. Stanchev, Properties of crude laccase from Trametes versicolor produced by solid-substrate fermentation, Bioresour. Technol. 101 (2010) 206–215.
[28] A. Aultkanya, V. Gokmen, Effect of various inhibitors on enzymatic browning, antioxidant activity and total phenol content of fresh lettuce (Lactuca sativa), Food Chem. 107 (3) (2008) 1173–1179.
[29] M.N. GUPTA, Enzyme function in organic solvents, Eur. J. Biochem. 203 (1-2) (1992) 25–32.
[30] M. Khabiri, B. Minolar, J. Brezovský, J. Damborský, R. Ettich, Interaction of organic solvents with protein structures at protein-solvent interface, J. Mol. Model. 19 (11) (2013) 4701–4711.
[31] G.A. Sellek, J.B. Chaudhuri, Biocatalysis in organic media using enzymes from extremophiles, Enzyme Microb. Technol. 25 (6) (1999) 471–482.
[32] Q. Yang, M. Zhang, M. Zhang, C. Wang, Y. Liu, X. Fan, et al., Characterization of a novel, cold-adapted, and thermostable laccase-like enzyme with high tolerance for organic solvents and salt and potent dye decolorization ability, derived from a marine metagenomic library, Front. Microbiol. 9 (DEC) (2018) 1–9.
[33] X. Yao, R. Jiang, W. Xiao, J. Yu, Use of surfactants for the remediation of contaminated soils: a review, J. Hazard. Mater. 285 (2015) 419–435. Elsevier B.V.
[34] E. Balcázar-López, L.H. Méndez-Lorena, R.A. Batista-García, U. Esquivel-Naranjo, M. Ayala, V.V. Kumar, et al., Xenobiotic compounds degradation by heterologous expression of a trameetes sanguineus laccase in trichoderma atroviride, PLoS One 11 (2) (2016) 1–13.
[35] A.B. Dalfard, K. Khajeh, M.R. Soudi, H. Naderi-Manesh, B. Rainjahr, R.H. Sajedi, Isolation and biochemical characterization of laccase and tyrosinase activities in a novel melanogenic soil bacterium, Enzym. Microb. Technol. 39 (7) (2006) 1409–1416.