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Original Article

Recombinant CotA-Laccase from Bacillus Licheniformis: Characterization and Overexpression in Bacillus Subtilis WB600

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Abstract

Background: Laccases are a class of multi-copper oxidases that can catalyze the oxidation of various phenolic substrates while reduacing molecular oxygen to water. Although only a few bacterial laccases have been studied to date, recent advances in genome research suggest that these enzymes are widespread in bacteria. Due to their ability to oxidize a broad range of phenolic compounds, laccases have numerous biotechnological applications. The aim of this study was to isolate the gene encoding laccase (CotA) from recombinant *Escherichia coli* BL21 (DE3) containing the *Bacillus licheniformis LS04 CotA-laccase* gene and investigate its properties.

Methods: The bacterial strains, vectors, and growth conditions were used in the study, and also the recombinant and expression host strain construction was described. Plasmid isolation, PCR amplification, gel electrophoresis, and protein purification were also carried out. SDS-PAGE was used to visualize the protein bands and plasmid stability was analyzed. In addition, this study characterized the *CotA laccase* by evaluating its optimum temperature, pH, thermal stability, and activity after bathing at 50 °C for 10 min.

Results: The results showed that the CotA laccase produced a protein with a molecular weight of 65 kDa, and the plasmid was stable in the absence of antibiotic pressure for 200 generations. The pH profile for laccase activity showed a peak at pH 7.4, and the optimal temperature was found to be 45 °C. However, the pH and temperature stability of the CotA laccase was lower than that of the spore laccase.

Conclusion: The purified recombinant CotA-laccase showed high stability towards alkaline pH, high temperatures, and a broad pH range for catalyzing substrates. Nevertheless, the study demonstrates that CotA-laccase has the potential for industrial use due to its high stability and broad substrate range.

Keywords: *Bacillus subtilis*, *Bacillus licheniformis*, Cloning, CotA, Laccase.

1. Introduction

Laccases are multi-copper oxidases that may catalyze the oxidation of various phenolic substrates while also

reducing molecular oxygen to water [1, 2].

These enzymes have a catalytic fraction of four copper ions per molecule and are divided into several categories based on spectroscopic characteristics and copper coordination [3]. Laccases have been identified as promising prospects for a variety of biotechnological and industrial applications due to their ability to interact with a wide range of substrates. They can, for example, be employed in the biosensors development to detect phenolic contaminants, biological bleaching in the pulp and paper sector, and textile dye de-colorization, as well as the detoxification of long-term environmental pollutants and bioremediation [4].

Laccases are found in bacteria, fungi, and plants [5]. Fungal laccases participate in biosynthesis, lignin degradation, pigment production, and pathogenicity. Plant laccases, on the other hand, are predominantly involved in the lignification process. High heat and alkaline environments make fungal laccases unstable [2].

The physiological purpose of bacterial laccases is unknown, but they are thought to be involved in spore coat resistance, copper detoxification, morphogenesis, and melanin synthesis. Despite the fact that fungal laccases have garnered more attention, bacterial laccases have a lower redox potential and are more stable at high temperatures and across a wider pH range.

They are also less sensitive to inhibitors and rely less on metal ions. Bacterial laccases offer enormous potential in a wide range of industrial applications [6]. Furthermore, unlike their fungal counterparts, bacterial laccases can be overproduced in a host and their catalytic characteristics, stability, and expression level can be considerably increased through directed evolution [7].

Only a few bacterial laccases have been researched so far. However, recent advances in genome research indicate that these enzymes are common in bacteria [5]. CotA is the most essential bacterial laccase responsible for *Bacillus subtilis* endospore coating composition. Laccases have also been discovered in *E. coli* (CueO) [8], *Bacillus halodurans* (Lbh-1) [9], *Thermus thermophilus* (TTC1370) [10], and various streptomycetes [11]. CotA participates in the manufacture of a melanin-like substance protecting the spore coat against hydrogen peroxide and the UV light [12].

CotA laccase in *B. subtilis* have several applications in biotechnology and bioremediation [13].

The structure, catalytic mechanisms, and substrate specificity of *CotA laccase* can aid in enzyme engineering efforts. By modifying specific amino acids or domains, potentially enhance the enzyme's activity, stability, or substrate range, making it more suitable for various biotechnological applications [14].

CotA laccase can serve as a component in biosensors designed to detect phenolic compounds or other substrates it can oxidize. By integrating *CotA laccase* with appropriate transducers, biosensors can be developed for environmental monitoring, food safety, and medical diagnostics [15].

CotA laccase has the ability to degrade various pollutants, including synthetic dyes, aromatic compounds, and phenolic pollutants.

It can be employed in bioremediation processes to detoxify contaminated soil, water, and industrial effluents.

CotA laccase-mediated degradation offers a more sustainable and environmentally friendly approach compared to the traditional chemical methods [16].

CotA laccase's role in lignin degradation can have implications in biorefineries and biofuel production. *CotA laccase*, can be used to selectively delignify biomass and improving the accessibility of cellulose and hemicellulose for biofuel production [17].

CotA laccase can be used as a biocatalyst in various industrial processes. Its ability to oxidize a wide range of phenolic compounds makes it valuable for the production of high-value chemicals, pharmaceutical intermediates, and fine chemicals [18].

We looked for candidate genes for putative multi-copper oxidases in different bacterial genomes, focusing on *pseudomonads*, streptomycetes, and bacilli, and then from recombinant *E. coli* BL21 (DE3) containing the *CotA-laccase* gene from *B. licheniformis* LS04, we extracted and cloned a gene encoding laccase (CotA). Under microaerobic circumstances, we expressed this gene in *B. subtilis WB600*.

2. Materials and Methods

2.1. Bacterial strains, vectors, and growth conditions

The *CotA laccase* gene was obtained as a gift from a previous study [12] and expressed in Recombinant *E. coli* BL21 (DE3). The host bacteria were *B. subtilis WB600*, and the laccase expression vector was the pWB980 plasmid. The bacteria were grown in Luria-Bertani (LB) medium supplemented with 10 g/mL kanamycin as needed.

The Ethics Committee of Damghan Branch of Islamic Azad University approved the study with No. 14230507922035.

2.2. Construction of the recombinant and the expression host strain

A plasmid DNA extraction kit (Miniprep, Pouya gene, Iran) was used to obtain plasmids from recombinant DE3 isolates containing the *CotA-laccase* gene. The *laccase* gene was amplified using polymerase chain reaction (PCR) with particular primers (forward: 5′-GGATGCTCTCCCAATCCCAGATA 3′ and reverse:

5′- TTATGGCGATCAGTTATATCCATCG -3′). The PCR programme was configured as follows: 3 minutes at 95 °C, 30 cycles of 30 seconds at 94 °C, 30 seconds at 50 °C, and 120 seconds at 72 °C, with a final 10-minute extension at 72 °C. The amplified DNA fragment was electrophoretically separated and purified using a gel extraction kit (Pouya gene, Iran). The purified *CotA-laccase* PCR fragment was then digested with *BamHI*/*HindIII* (Thermofisher Scientific, USA) and subcloned into digested *pWB980* before being electroporated into competent *B. subtilis WB600* cells [19].

The control strain was *B. subtilis WB600* (*pWB980/ WB600*), which lacked an insert in the vector. The recombinant bacteria and the control strain were put on triplicate LB/agar plates with 10 g/mL kanamycin and grown at 37 °C for 36 hours to allow the plasmid-carrying strain to develop. Colony PCR and sequencing analysis revealed the presence of recombinant B. subtilis *WB600* strains containing plasmid *cotA-pWB980* (*WB600-cotA-pWB980*) or control (*WB600-cotA-pWB980*). The chosen recombinant strain was then cultured for 24 hours in a liquid medium before being extracted by centrifugation at 8,000 g for 10 minutes.

The protein concentration was determined using the Bradford Protein Assay Kit (Tiangen, Beijing, China) using bovine serum albumin as the standard [20]. Protein purification was performed according to Liu *et al.* [21].

2.3. SDS–PAGE protein gel

To perform SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel

electrophoresis), a 12% polyacrylamide gel was used, and the protein bands were stained with Coomassie Brilliant Blue R-250. Following the manufacturer's instructions, purified laccase underwent deglycosylation with PNGase F (New England Biolabs, Ipswich, MA). To determine the molecular mass, a low molecular weight protein ladder (SinaClon, Iran) was utilized.

2.4. Plasmid stability analysis

To begin, a single recombinant strain colony was inoculated on LB medium containing 10 g/mL kanamycin and cultured at 37 °C for roughly 20 generations. After that, the cultures were diluted 105 times in LB medium without kanamycin and incubated at 37 °C.

This dilution process was performed ten times at 20-generation intervals. To measure the frequency of plasmid loss, 100 colonies were chosen at random and transferred to LB plates with and without 10 g/mL kanamycin. Plasmids were also isolated from the samples at 100 and 200 generations.

These plasmids were subjected to restriction mapping tests to investigate their structural stability and confirm their presence.

2.5. Characterization of CotA laccase

Using syringaldazine (SGZ), the best temperature range (0-100 °C) for pure laccase was determined. The effect of pH on *CotA laccase* activity relative to SGZ was also investigated using a 0.1 M citrate-phosphate buffer in the pH range of 5.6-8.0.

The enzyme solution was incubated at 60 °C and 80 °C in 0.1 M citrate-phosphate buffer, pH 7.2, to evaluate thermal stability. Samples were obtained at appropriate times, and residual activity was evaluated using SGZ as the substrate. Laccase activities were studied following a 10-minute soak at 50 °C. All investigations were carried out in duplicate.

3. Results and Discussion

3.1. Cloning and Expression of CotA-Laccase Gene in B. subtilis

A Mini-prep plasmid extraction kit (Qiagen) was used to isolate plasmid DNA from recombinant *E. coli*. To confirm the presence of the *CotA-laccase* gene acquired on the plasmid, PCR was performed (Figure 1a).

To create *pWB980-CotA*, a DNA fragment containing the *CotA-laccase* gene was ligated into the *BamHI* and *HindIII* sites of the *pWB980* vector. After enzymatic digestion with *BamHI* and *HindIII*, as well as PCR, the expected product sizes were achieved. The competent *B. subtilis WB600* was transformed with the recombinant plasmid *pWB980-CotA* and cultured overnight in LB medium containing 10 g/mL kanamycin. On each plate, twelve bacterial colonies were produced, as depicted in Figure 2a. The identification for the *pWB980-CotA* plasmid was accomplished by the use of restriction enzyme digestions (Figure 2b).

The *CotA* gene was successfully expressed in *B. subtilis*, resulting in the production of active laccase. The successful expression of the *CotA* gene in *B. subtilis* highlights the feasibility of utilizing this bacterium as a host for laccase production, offering promising avenues for further research and development in the field.

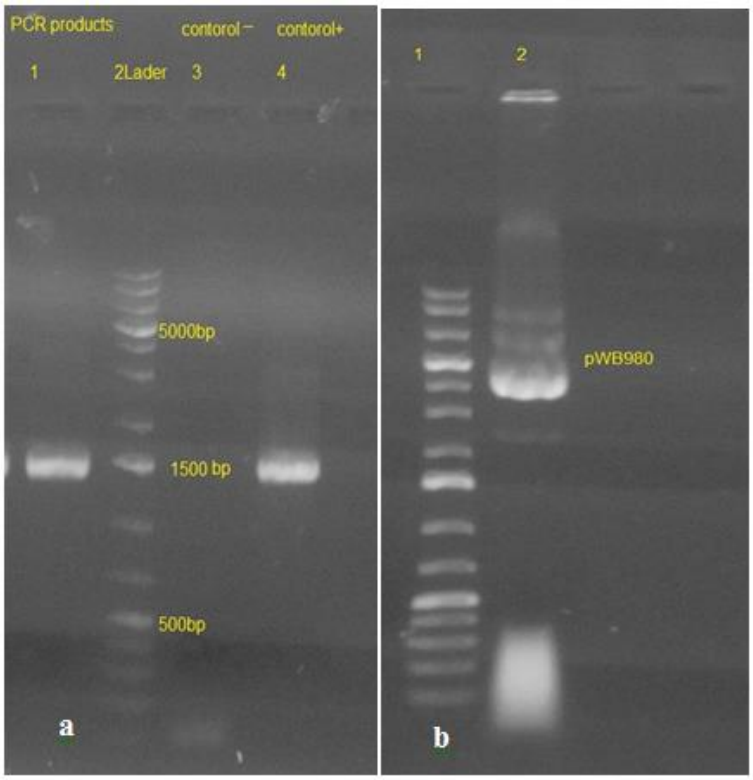

Figure 1. (a) The Electrophoresis of PCR product with specific primers designed for *CotA-laccase* gene; lane 1: PCR product; lane 2: ladder; lane 3: negative control; and lane 4: positive control and (b) Preparation of *pWB980* for laccase *gene* ligation

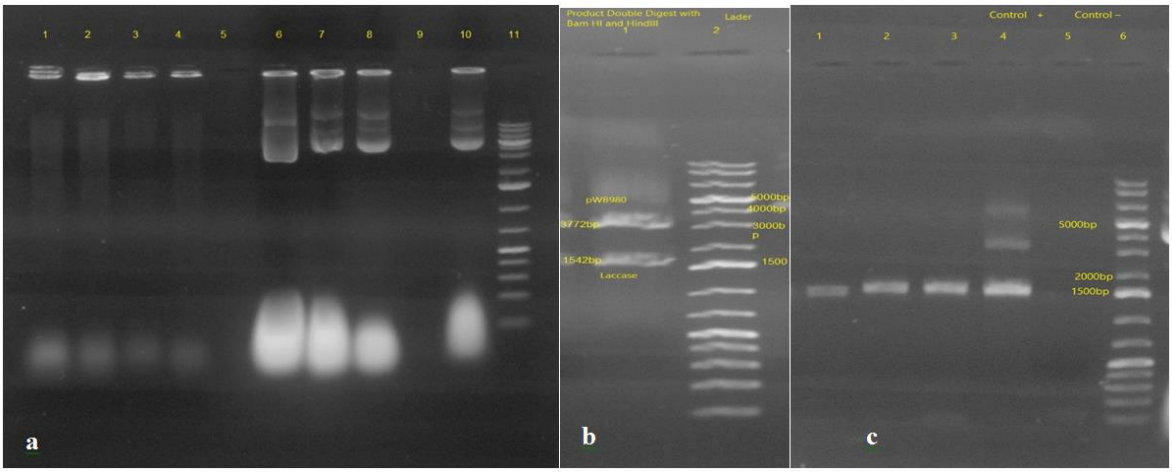

Figure 2. (a) Extraction of *pWB980* plasmid carrying the laccase gene from *Bacillus subtilis WB600*. Wells 1-5 and 7-9: the extracted plasmids; well 6: *pWB980* plasmids without gene; well 11; Ladder 1 kb Plus, (b) Two-enzymatic cleavage of *pWB980* plasmid containing laccase gene by enzymatic digestion by *BamHI* and *HindIII* restriction enzyme. Lane 1: plasmid *pWB980* contains double digested gene (one band 1542 bp and ther band 3772bp [plasmid *pWB980*]; lane 2: Ladder 1 kb Plus, and (c) The results of PCR reaction by specific primers designed in this study. Lane 1-3 contains PCR products, Lane 4 contains positive controls, Lane 5 contains negative controls, and Lane 6 contains Ladder 1 kb Plus.

3.2. PCR Amplification of CotA-Laccase Gene

PCR amplification of the cotA primers from *B. subtilis* WB600 genomic DNA resulted in a 1.5 kb product, as displayed in Figure 2c. The amplified gene fragment matched our expected results.

Upon DNA sequencing, the PCR product sequence was found to be identical to the reported *B. subtilis WB600* gene sequence.

3.3. SDS-PAGE analysis

The laccase protein produced from the recombinant crude extract of *B. subtilis WB600* in LB medium was analyzed by loading it onto an SDS-PAGE gel. A band of approximately 65 kDa was detected, which corresponds to the expected size of the *B. licheniformis* laccase enzyme (Figure 3).The protein concentration was determined to be 50 mg/L using the Bradford assay.

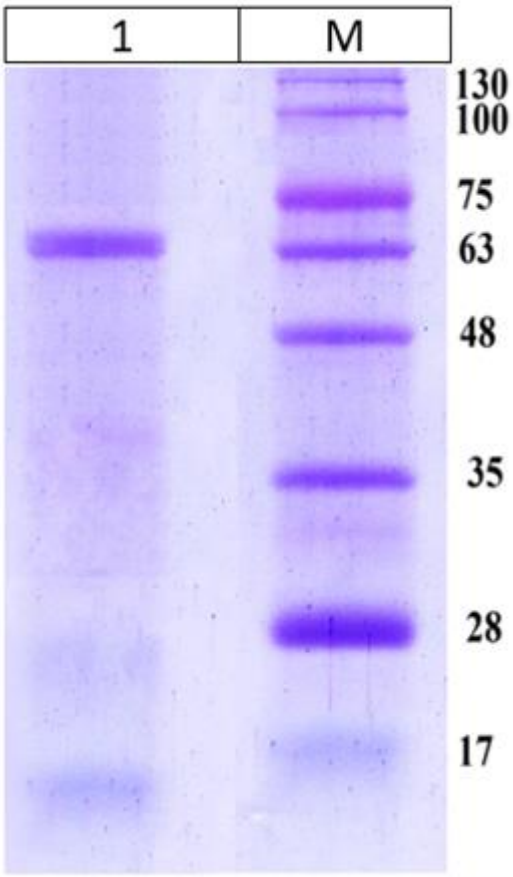

Figure 3. Expression and purification of recombinant *CotA-Laccase*. Lane M: protein molecular weight marker and Lane 1, purified recombinant Laccase

3.4. Stability of the recombinant plasmid

Plasmid loss was not identified after 200 generations without selective pressure, demonstrating that *pWB980-CotA* was stable in *B. subtilis* even in the absence of antibiotic pressure. The cleavage patterns of restriction endonucleases on plasmid DNA were identical to those of the original *pWB980-CotA* at 100 and 200 generations, showing that no deletions or rearrangements had occurred throughout serial replication. Because the use of antibiotics is rarely practicable

in commercial fermentation, the durability of plasmid in the absence of selective pressure is critical for practical applications. As a result, our findings show that *pWB980-CotA* and B. subtilis *pWB980-CotA/WB600* may be acceptable for industrial fermentation.

3.5. Effects of pH and Temperature on Laccase Stability and Activity

At pH 7.4, purified *CotA laccase* had the highest activity against SGZ. 45 °C was determined to be the best temperature for pure laccase. The laccase half-life at 80 °C was 1.5 hours at optimum pH. The *CotA laccase* exhibited high alkalinity and thermal stability. During an 8-hour pre-incubation of the enzyme at the ideal temperature and pH of 9.0, however, half of the laccase activity was lost. The pH and temperature stability of the *CotA laccase* were lower than those of the spore laccase.

Laccases find application in various sectors, including the pulp and paper industry, textile industry, bioremediation, and food and beverage industry, among others. While fungal enzymes are commonly used, bacterial laccases have also been identified, although their number remains limited. In terms of recombinant production, the bacterial expression system offers advantages over fungal expression systems, allowing for faster and more cost-effective production of laccases [22, 23].

Most recombinant prokaryotic laccases generated in *E. coli*, however, congregate in inclusion bodies, limiting their utility. To get over this limitation, we used *B. subtilis* as an expression host to generate a novel laccase secretion from *B. licheniformis* LS04. The cloned *CotA laccase* gene was expressed in recombinant *E. coli*, and the enzyme produced was studied. The isolated enzyme showed an optimal pH of 7.4 for SGZ oxidation, which was consistent with prior findings on laccases that *oxidise* DMP and ABTS. The spectroscopic characteristics and specificity of recombinant CotA laccase for SGZ oxidation recognized it as a laccase with properties similar to the other bacterial laccases discovered so far. The molecular mass of *B. licheniformis* LS04 laccase (about 65 kDa) was similar to other laccases from M. mediterranea (59 kDa) [24], *P. putida* F6 59 kDa) [25], A. hydrophila WL-11 (59 kDa) [26], and *Klebsiella* sp. 601 (58 kDa). Our findings emphasise the potential of using *B. subtilis* as an expression host to produce stable and active recombinant *laccases*.

In a study investigated the optimizing production of laccase, a multicopper oxidase enzyme with various applications, using *B. subtilis* MTCC 2414 [13].

In another study, *Bacillus* spores were utilized to showcase laccases, blue multicopper *oxidases*, for the purpose of decolorizing indigo carmine, a commonly used textile dyestuff. The *laccase*-encoding gene, cotA, from *B. subtilis* was cloned and expressed in *B. subtilis* DB104, resulting in the enzyme being naturally localized on *Bacillus* spores. The findings showed the potential of laccase displayed on *B. subtilis* spores as an effective environmental tool for treating textile dye effluent [27].

The researchers investigated the ability of laccase to catalytically degrade glyphosate, isoproturon, lignin polymer, and parathion for detoxification purposes.

The results indicated successful interactions between laccase, glyphosate, lignin polymer, isoproturon, and parathion, and also presented a novel approach for understanding the molecular-level biodegradation of xenobiotic compounds using laccase and highlights its potential for contaminant removal [16].

5. Conclusion

The expression of bacterial *CotA laccase* in an appropriate host offers a straightforward approach, enabling the possibility of enhancing its activity and selectivity through directional evolution. This is a distinct advantage over fungal laccases, which are less amenable to such modifications. The purified CotA laccase has shown remarkable stability even under high temperatures and alkaline pH conditions. These favorable properties make it a promising candidate for a range of industrial applications, including but not limited to wastewater treatment. Further exploration of its potential in sectors such as pulp and paper, textile dyeing, and bioremediation unveils its full industrial utility.

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