



A non-blue laccase of *Bacillus* sp. GZB displays manganese-oxidase activity: A study of laccase characterization, Mn(II) oxidation and prediction of Mn(II) oxidation mechanism

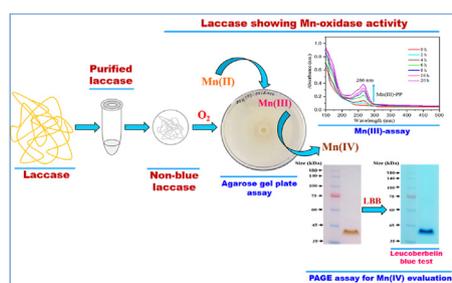
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HIGHLIGHTS

- The recombinant laccase of *Bacillus* sp. GZB is a non-blue laccase.
- The non-blue laccase shows some unique features in enzyme activity and stability.
- The non-blue laccase exhibits Mn(II)-oxidase activity.
- Agarose gel plate assay was developed to examine Mn-oxidase activity of LACREC3.
- Mn(II) was transformed to Mn(IV) by recombinant laccase via Mn(III) as intermediate.

GRAPHICAL ABSTRACT



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ABSTRACT

Laccase, a unique class of multicopper oxidase, presents promising potential as a biocatalyst in many industrial and biotechnological applications. Recently, it has been significantly applied in many metal-polluted sites due to its Manganese (Mn)-oxidation ability. Here, we demonstrate the Mn(II)-oxidase activity of laccase obtained from *Bacillus* sp. GZB. The *CotA* gene of GZB was transformed in *E. coli* BL21 and overexpressed. The purified laccase (LACREC3-laccase) displayed the absence of a peak at 610 nm that is usually found in blue-laccase. Further, the LACREC3-laccase exhibited high activity and stability at different pH and temperatures with substrates 2, 2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonate) and syringaldazine, respectively. It also functioned in the presence of various metals and enzyme inhibitors. Most notably, LACREC3-laccase formed insoluble brown Mn(III)/Mn(IV)-oxide particles from Mn(II) mineral, exhibiting its Mn(II)-oxidase activity. In addition to native polyacrylamide gel electrophoresis and buffer test, we developed an 'agarose gel plate' assay to evaluate Mn(II) oxidation activity of laccase. Furthermore, using the leucoberberin blue assay, a total of $44.45 \pm 0.45\%$ Mn(IV)-oxides were quantified, in which $5.87 \pm 0.61\%$ autoxidized after 24 h. The Mn(II) oxidation mechanisms were further predicted by trapping Mn(III) using pyrophosphate during Mn(II) to Mn(IV) conversion by LACREC3-laccase. Overall, the laccase of GZB has excellent activity and stability plus an ability to oxidize Mn(II). This study is the first report on a non-blue laccase, exhibiting Mn(II)-oxidase activity. Thus, it offers a novel finding of the Mn(II) oxidation processes that can be a valuable way of Mn(II)-mineralization in various metal-polluted environments.

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1. Introduction

Manganese (Mn) is an essential micronutrient commonly used by all living organisms in the Earth (Horning et al., 2015). It plays some important elemental roles in human health, including development, reproduction, immune function, energy metabolism, and the antioxidant system. However, excessive Mn intake might cause neural disorders, primarily known as Mn-induced Parkinsonism or manganism (Herrero Hernandez et al., 2006; Horning et al., 2015). In the Earth's crust, Mn abundantly presents at multiple transition phases, among which Mn(IV), in the form of insoluble Mn-oxides, is the most stable and thermodynamically favorable state under oxic condition (Tebo et al., 2010). Thus, the existing structures of Mn present in most soils, waters, and sediments always participate in different biogeochemical cycles via chemical and biological reactions (Lu et al., 2012; Tebo et al., 2004; Zhang et al., 2015). More importantly, it reacts with various elements, including S, C, N, P, Fe, other trace elements and radionuclides generated through scavenging and oxidation processes to maintain the essential elements balances in the environments (Butterfield et al., 2013; Geszvain et al., 2012).

In both natural and engineered environments, Mn-oxides are profoundly conceded by various microbial groups, notably bacteria and fungi (Liang et al., 2016; Su et al., 2013; Tebo et al., 2004). The bacterial enzymes, mostly the multicopper oxidases (MCOs) are known to be the significant contributors in the Mn(II) oxidation process (Butterfield et al., 2013; Soldatova et al., 2017b). For example, proteins like MnxG, MofA, CumP, and MoxA are well-known MCOs found in several model microorganisms, including *Bacillus* sp. SG-1 and PL-12, *Leptothrix discophora* SS-1 and SP-6, *Pseudomonas putida* strains GB-1, and *Pedomicrobium* sp. ACM306 (Butterfield and Tebo, 2017; Geszvain et al., 2013). Intriguingly, CotA has been recognized recently, an essential Mn-oxidizing protein that enables to produce Mn-oxides from Mn(II) minerals (Su et al., 2013; Zhang et al., 2019b).

Among MCOs, laccases are an extraordinary group of metalloenzymes that readily oxidize numerous compounds by reducing molecular oxygen to water (Atalah et al., 2019; Das et al., 2018; Reiss et al., 2013). To date, the most elaborated sources of laccase that mainly apply in many industrial, biotechnological and environmental practices are bacteria and fungi (Atalah et al., 2019; Copete et al., 2015; Rodríguez Couto and Toca Herrera, 2006). A typical laccase contains four copper atoms that are catalytically distributed in three distinct copper centers as Type 1 (T1), Type 2 (T2), and Type 3 (T3) (Chen et al., 2015; Copete et al., 2015). The catalytic actions of laccases are mainly involved in the coordination of amino acids (particularly, histidine and cysteine residues) and copper atoms, present in the copper-binding sites (Enguita et al., 2003; Reiss et al., 2013). The transition of charge molecules from cysteine residue to Cu^{2+} at the T1 site is also engaged to produce the color of blue-laccase plus an absorption peak around 600 nm (Daroch et al., 2014; Lee et al., 2002). In contrast, the yellow or white laccase (mostly found in fungi) is notably different due to amino acid residues present in the copper centers and their synchronization with Cu^{2+} sphere at the T1 site, usually aiding different colors and spectral characteristics to laccase (Mate et al., 2013). As compared to fungal sources, the bacterial laccases display more pH and temperature stability due to distinct protein structure and interaction with Cu^{2+} (Gianfreda et al., 1999; Hildén et al., 2009).

Indeed, most of the MCOs are reactive to low-valence metal ions, like Fe(II), Cu(I), and Mn(II); those enzymes included ferroxidases, cuproxidases, and Mn-oxidase or laccases (Boonen et al., 2014; Schlosser and Höfer, 2002; Soldatova et al., 2012; Su et al., 2013,

2014). However, their physiological roles and biochemical mechanisms towards Mn(II) are still in the thought of the research. The central process occurs through binding of Mn(II) at the T1 site on the enzyme, then a sequential transmission of electrons from T1 to T2/T3 binuclear sites and ensuing the transformation of Mn(II) into Mn(IV)-oxides by reducing molecular O_2 to H_2O (Butterfield et al., 2013; Soldatova et al., 2017b; Su et al., 2013). Similar to MCOs, laccases are actively functioned on Mn(II) and transform it to Mn(IV)-oxides (Schlosser and Höfer, 2002). Previously, Su et al. (2013) established a CotA in *Bacillus pumillus* WH4, presenting Mn-oxidase activity, but the reported laccase was a typical blue copper oxidase. Here, we demonstrate a new non-blue laccase, which displays Mn-oxidase activity.

This study implemented a previously identified spore coat protein (CotA) from *Bacillus* sp. GZB, inserted into *E. coli* BL21 (DE3) for the overexpression. The expressed enzyme was purified and characterized in the presence of various physicochemical parameters. For instance, the purified laccase (LACREC3-laccase) was monitored in the UV-visible absorption spectrum (200–800 nm). The activity and stability profiles of the LACREC3-laccase were tested in a wide range of pH and temperatures with multiple substrates. The enzyme activity was further estimated in the presence of different enzyme inhibitors and heavy metals. Additionally, the LACREC3-laccase was examined the Mn-oxidase test. We developed an agarose gel plate assay and encompassed a native polyacrylamide gel electrophoresis (PAGE), and a liquid buffer assay to evaluate the Mn(II)-oxidase activity. Leucoberbelin blue (LBB) assays confirmed all the tests and was also applied for the quantification of Mn(IV)-oxides in a separate reaction. The Mn(II)-oxidase activity of laccase also assessed at different pH, temperatures, and various concentrations of Cu^{2+} . Finally, the Mn(II) oxidation mechanisms were predicted using a trapping agent (pyrophosphate) in buffer assay. This study, for the first time, reports a highly active and stable non-blue laccase having Mn(II)-oxidizing ability that it can be applied as a biocatalyst for denominating Mn in metal-polluted sites.

2. Materials and methods

2.1. Chemicals, media, and microorganisms

All chemicals used in this study are analytical grade, and details are described in the supporting information (SI). A putative *cotA* gene identified previously from *Bacillus* sp. GZB was further applied for recombinant protein expression (Das et al., 2018). Expression was carried out using the pET30a (+) vector and *Escherichia coli* BL21 (DE3) strain. The clones of *E. coli* BL21 were routinely maintained on Luria-Bertani (LB) agar medium supplemented with kanamycin antibiotic ($30 \mu\text{g mL}^{-1}$).

2.2. Expression and purification of recombinant laccase

The expression of the *cotA* gene was achieved in *E. coli* BL21 (DE3) host after ligating with the pET30a (+) vector. One positive clone (LACREC3) was selected for protein expression. The full expression and purification of LACREC3-laccase were described in our previous report (Das et al., 2018). Furthermore, the effects of Cu^{2+} on CotA expression were studied at a graded concentration of CuCl_2 (0–0.6 mM). SDS-PAGE was used to check the expression levels of the recombinant protein.

2.3. Laccase activity assay

The activity of purified laccase (0.85 mg mL^{-1}) was assayed in

0.1 M Mcllvaine buffer (pH 3.5 and 6.8) at 50 °C using 2, 2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonate) (ABTS), syringaldazine (SGZ) as substrates, respectively. The oxidations of ABTS and SGZ by laccase were measured by the previously mentioned process (Das et al., 2018). One unit of laccase activity is defined as the amount of enzyme required to oxidize one μmol of substrate per min under a standard assay condition. Relative activity was calculated by observing maximum activity in samples or using a control sample set as 100%. All the assays were performed in triplicate.

2.4. Sequence alignment and protein modeling

Protein sequence alignments were performed using the ClustalW2 web services' tools (Li et al., 2015). The three-dimensional protein structures of GZB laccase were predicted with the SWISS-MODEL program using CotA of *Bacillus subtilis* (PDB: 1GSK_A) as a protein template. The three-dimensional cartoon model was visualized using PyMol 1.7. All other essential analyses for protein modeling are demonstrated in SI.

2.5. Characterization of recombinant laccase

The purified laccase was employed to record the UV–visible absorption spectrum at a range of 200–800 nm in 20 mM Tris–HCl buffer (pH 7.6). The copper content of the recombinant laccase was quantified using atomic absorption spectroscopy (Z2000, Hitachi, Japan).

Furthermore, the effects of pH and temperatures on recombinant laccase activity and stability were evaluated using two canonical substrates, ABTS and SGZ, in 0.1 M Mcllvaine buffer. The detailed information is provided in SI.

2.6. Manganese oxidase activity assays

The Mn(II)-oxidase activity of LACREC3-laccase was established using three different methods: an agarose gel plate assay, 12% native PAGE, and a buffer assay. Here, we described the agarose gel plate assay; while the details of the other two methods are found in SI.

We developed for the first time the “agarose gel plate assay” to estimate the Mn-oxidase activity of laccase. In detail, an agarose gel plate was made by 1% agarose in 10 mM HEPES buffer (pH 8.0) supplemented with 10 mM MnCl_2 and 0.8 mM CuCl_2 . The molten agarose mixture was poured into a Petri plate and solidified. After that, a diluted purified laccase (20 μL) was applied to solid agarose at the center of the plate and stored it at 4 °C for 15 min to allow the enzyme absorption on the agarose surface. A wetted filter paper (90 mm diameter) was adjusted to the lid of the plate and sealed with parafilm. Then the plate was incubated overnight at 37 °C. The Mn-oxidase activity of laccase on agarose was observed and assessed using the LBB solution.

2.7. Quantification of Mn-oxide minerals and effects of different physicochemical factors on Mn(II) oxidation

The Mn(IV)-oxides formation by laccase was quantified using the reaction mixtures prepared in a 10 mM HEPES buffer (pH 8.0) with 5 mM MnCl_2 and 0.8 mM CuCl_2 . The reaction was initiated by adding 4.25 $\mu\text{g mL}^{-1}$ of purified laccase and maintained it at 37 °C under dark condition. At the specific time intervals, the reaction mixtures sampled and reacted with the LBB solution. The colored formation was measured using a microtiter plate reader at 620 nm (Varioskan Flash, Thermo Scientific, USA). A standard curve of KMnO_4 was prepared to determine the Mn(IV)-oxides equivalent concentration. In the separate reactions, Mn(II)-transformation was

carried out in a 1 cm quartz cuvette containing MnCl_2 (500 μM) and Na-pyrophosphate (a trapping agent, 100 μM). The reaction was started by the addition of purified laccase and incubated at 37 °C under shaking conditions. Samples were scanned at the regular time intervals using a UV–visible absorption spectrum at a range of 150–700 nm. Furthermore, the effects of pH, temperature, and Cu^{2+} on Mn(II)-oxidizing activity were measured under the standard assay conditions as described elsewhere (Su et al., 2013). All the experimental protocols applied in this study are described in SI.

2.8. Determination of kinetic parameters of recombinant laccase

Kinetic parameters of the purified laccase (0.21 $\mu\text{g mL}^{-1}$) were studied at 50 °C in 0.1 M Mcllvaine buffer using ABTS (10–100 μM ; pH 3.5) and SGZ (5–70 μM ; pH 6.8) as the substrates, respectively. The kinetic measurements of laccase ($E = 5.4 \times 10^{-4}$ mMol) during Mn(II) oxidation were also performed in 10 mM HEPES (pH 8.0) with MnCl_2 (2.5–25 mM) at 37 °C. The kinetic constants: K_m , V_{max} , and k_{cat} were calculated using OriginPro Software (OriginLab Corp., USA), by the typical settings for non-linear regression curve fitting in Michaelis-Menten equations. All the assays were performed in triplicate. The details are demonstrated in SI.

3. Results and discussion

3.1. Expression, purification, and characterization of recombinant laccase

A spore coat protein (CotA) with 513 amino acids corroborated earlier as a laccase in *Bacillus* sp. GZB. The spore-laccase exhibited a high degree of activity and stability at multiple pH and temperatures. It was active in the presence of various heavy metals, enzyme-inhibitors, and alcohol. Furthermore, the spore-laccase could degrade bisphenol A (BPA) and different synthetic dyes (Das et al., 2018, 2019).

Thus, it engrossed us in expressing the enzyme in *Escherichia coli* system and characterize the purified protein for further study. We used *E. coli* host for expression because it is the most commonly used heterologous system for recombinant laccase production (Guan et al., 2014; Safary et al., 2016; Su et al., 2013; Sun et al., 2017). Besides, a large volume of expressed protein is obtained at a short time as compared to fungal host (Chen et al., 2015). The expressed protein was produced and purified using a CotA-recombinant clone (LACREC3). The purified protein exhibited a single band of approximately 63 kDa in SDS–PAGE. As well, the activity staining of native protein showed a colored band of 40 kDa with SGZ or ABTS (Das et al., 2018).

Laccases are a group of MCOs, whose activities are primarily depending on copper atoms. A full copper loaded purified laccase exhibits an intense blue color, i.e., also called blue-laccase (Safary et al., 2016; Su et al., 2013). Compared with blue-laccase, purified LACREC3-laccase displayed a colorless complex. The UV–visible absorption spectrum of purified laccase conformed a lack of visible peak around 610 nm (Fig. 1). Furthermore, the A_{280}/A_{610} absorbance ratio (use to differentiate blue and white or yellow laccases) of LACREC3-laccase was 74.3, which was also higher than many typical blue-laccases; those only presented at the range of 15–30. To date, most of the bacterial sources are blue-laccase, which are primarily characterized by blue color and producing a 600 nm peak on the UV–visible spectrum (Lee et al., 2002; Su et al., 2013). In contrast, both yellow and white laccases, mostly found in fungi, are easily distinguished by the color, absorbance peak (600 nm), and a higher ratio of A_{280}/A_{610} (Daroch et al., 2014; Mot et al., 2012). Hence, our data were most comparable with yellow or white laccase rather than a blue type, which clarified further by the

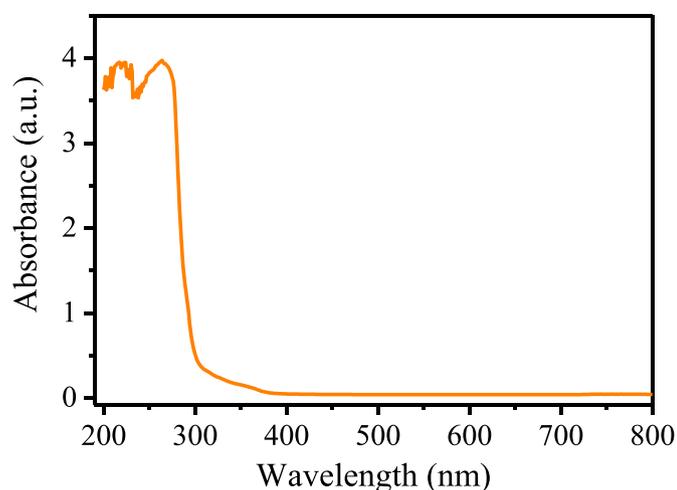


Fig. 1. UV–visible spectrum analysis of purified recombinant laccase. The absorption spectrum was recorded at a range of 200–800 nm in 20 mM Tris-HCl buffer (pH = 7.6) at room temperature.

measuring of copper content. The LACREC3-laccase contained $2.01 \pm 0.04 \text{ Cu}^{2+}$ per molecule, suggesting a non-blue variety. Earlier, only [Chen et al. \(2015\)](#) have been reported a non-blue laccase from *Bacillus amyloliquefaciens* having $2.00 \pm 0.08 \text{ Cu}^{2+}$ per molecule as well as an A_{280}/A_{610} ratio of 88.5, and lacking a visible peak at 610 nm. Thus, the current results were relatively closed to the ascribed data of non-blue laccase from *B. amyloliquefaciens* (see [Fig. 2](#)).

Moreover, the whole genome data of *Bacillus* sp. GZB (MTQG00000000) showed the closest genome match to *B. amyloliquefaciens* FZB742, CC178, and UCMB5036, with $\geq 98\%$ of average nucleotide identity (ANI) values ([Das et al., 2019](#)). The *cotA* gene further displayed $>99\%$ identity to *B. amyloliquefaciens* (WP_109566752). Thus, strain *Bacillus* sp. GZB was most closely related to *B. amyloliquefaciens*, which could produce a non-blue laccase, as earlier reported by [Chen et al. \(2015\)](#). Importantly, the

laccase of GZB carried some distinctive features in its structure that assisted in predicting the protein functions.

3.2. Protein structure and functions prediction

The amino acid sequence of laccase from GZB was aligned with a blue-laccase of *B. subtilis* (1GSK_A), and a non-blue laccase of *B. amyloliquefaciens* (ADZ57285.1) to establish a likelihood with blue or non-blue varieties. The alignment data displayed four conserved histidine-rich copper-binding motifs, including Cu I (HLH), Cu II (HDH), Cu III (HPIHLH), and Cu IV (HCHILEH) ([Fig. S1](#)). In addition, the result showed amino acid substitutions in the sequences that indicated an evolution of blue to non-blue laccase in the genus of *Bacillus*. A total of 118 in 513 amino acids was found to be substituted in laccases between GZB and *B. subtilis* ([Fig. S1](#)); conversely, only one amino acid (E510) replaced in GZB with a non-blue laccase of *B. amyloliquefaciens* ([Chen et al., 2015](#)). Thus, the conserved amino acid sequences in both GZB and *B. amyloliquefaciens* specified that the CotA in GZB was a non-blue type. However, it was unclear how the enzyme switch from blue to non-blue or yellow laccase. Meanwhile, the amino acid substitutions might shift the protein conformation by changing protein folding that might be directly switched blue to non-blue or yellow laccase ([Daroch et al., 2014](#)). The molecular mechanisms and functions of laccase were further predicted using a three-dimensional model (3D) of laccase from GZB.

A protein 3D model of GZB laccase was built using a template structure of CotA from *B. subtilis* (PDB ID: 1GSK_A). The interactions among catalytic amino acids and ligand were further projected through superimposition. It was found that the mononuclear T1-copper center was coordinated by five amino acids, including His419, His492, His497, Cys492, and Met502, with a non-bonding residue Ile494 ([Figs. 2 and S2](#)). Besides, the T2 and T3 were connected with the essential residues, such as His105, His107, His422, His424, and His151, His153, His491, His493, respectively. Notably, it has been reported that the coordination of Cu^{2+} and amino acid residues on the T1 site is the primary cause of the catalytic functions of the enzymes ([Daroch et al., 2014](#)). The reliability of the

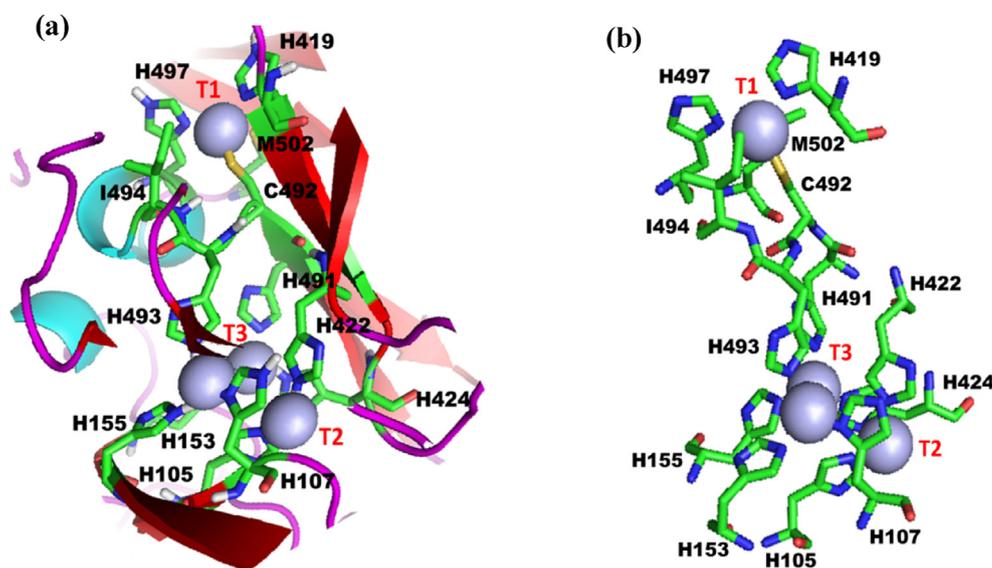


Fig. 2. Protein Homology model of laccase of *Bacillus* sp. GZB, generated by Swiss-Model using a template of CotA from *Bacillus subtilis* (PDB ID: 1GSK_A) with a 77.25% sequence identity. (a) Three-dimensional cartoon model with β -strands (red), α -helices (cyan), and catalytic amino acid residues. (b) The residues of GZB laccase are shown in stick model and labeled with amino acid residue numbers. In both figures, Cu^{2+} is represented as light blue spheres. The models are visualized using PyMol 1.7. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

developed model was further validated by PROCHECK tool analysis. The Ramachandran plot exhibited that 88% of amino acids were in the favored region, and 11.5% in the allowed area (Fig. S3a). The model was also analyzed by the Z-score value and energy profiling. The Z-score assessment was -8.59 , and it positioned the protein related to X-Ray (Figs. S3b and S3c). Compared with the template 1GSK_A (Z-score: -8.07), the Z-score value supported that the generated model was reliable. However, the structural consistency of laccase was further highlighted of its functions that observed clearly in LACREC3-laccase when it tested in the presence of different physicochemical parameters.

3.3. Effects of different physicochemical factors on recombinant laccase

3.3.1. Effects of pH and temperature

The LACREC3-laccase was characterized at various pH (2.0–8.0) and temperatures (30–80 °C) using ABTS and SGZ as substrates. The optimal pH activities of LACREC3-laccase were pH = 3.5 and pH = 6.8 with ABTS and SGZ, respectively (Fig. 3a). The pH stability profile exhibited that the LACREC3-laccase was highly stable at pH = 6.8, showing $144.52 \pm 2.43\%$ of activity after 5-day incubation at 25 °C. However, it was unstable at pH = 3.5; only $11.18 \pm 0.75\%$ of the activity was found under the same conditions (Fig. 3b), suggesting that LACREC3-laccase was stable at neutral pH. A similar kind of result was previously observed in a recombinant laccase of *B. subtilis* X1 (Guan et al., 2014). Additionally, Chen et al. (2015) demonstrated a non-blue laccase displaying optimal activity at

pH = 3.8 and pH = 6.6 with ABTS and SGZ and further established its stability at neutral to alkaline pH. The changes in laccase activity and stability at different pH mainly occurred by modification of substrate specificity, available oxygen, and potential redox differences between the substrate and the enzyme (Dwivedi et al., 2011).

The thermal activity of LACREC3-laccase exhibited the optimal oxidation profile at 60 °C with both ABTS and SGZ as substrates. However, it reduced when the temperatures changed from the optimal level (Fig. 3c). The LACREC3-laccase displayed a similar activity profile to the native spore laccase (Das et al., 2018). The temperature stability profile showed that the activity was steadily decreased from $71.88 \pm 2.07\%$ to $39.52 \pm 2.49\%$ to change of 50 °C–70 °C after 8 h, and then remarkably downed at temperature raised to 80 °C (Fig. 3d). Overall, LACREC3-laccase showed a high degree of thermostability than other laccases isolated from *Bacillus* spp. (Safary et al., 2016). This further advocated that the LACREC3-laccase had a high potential to function at different industrial hardness. What is more, we observed that LACREC3 activity was enhanced by pre-incubation at 50–70 °C (Fig. 3d), suggesting that the enzyme was thermally activated. This condition might change the protein structure that was entropically favored at high temperatures (Brander et al., 2014). More importantly, laccases are MCO with three different copper-binding centers. The interactions between Cu^{2+} with copper centers, including multiple bonding networks in protein structure, may influence the thermal activity and stability of the laccases (Hildén et al., 2009).

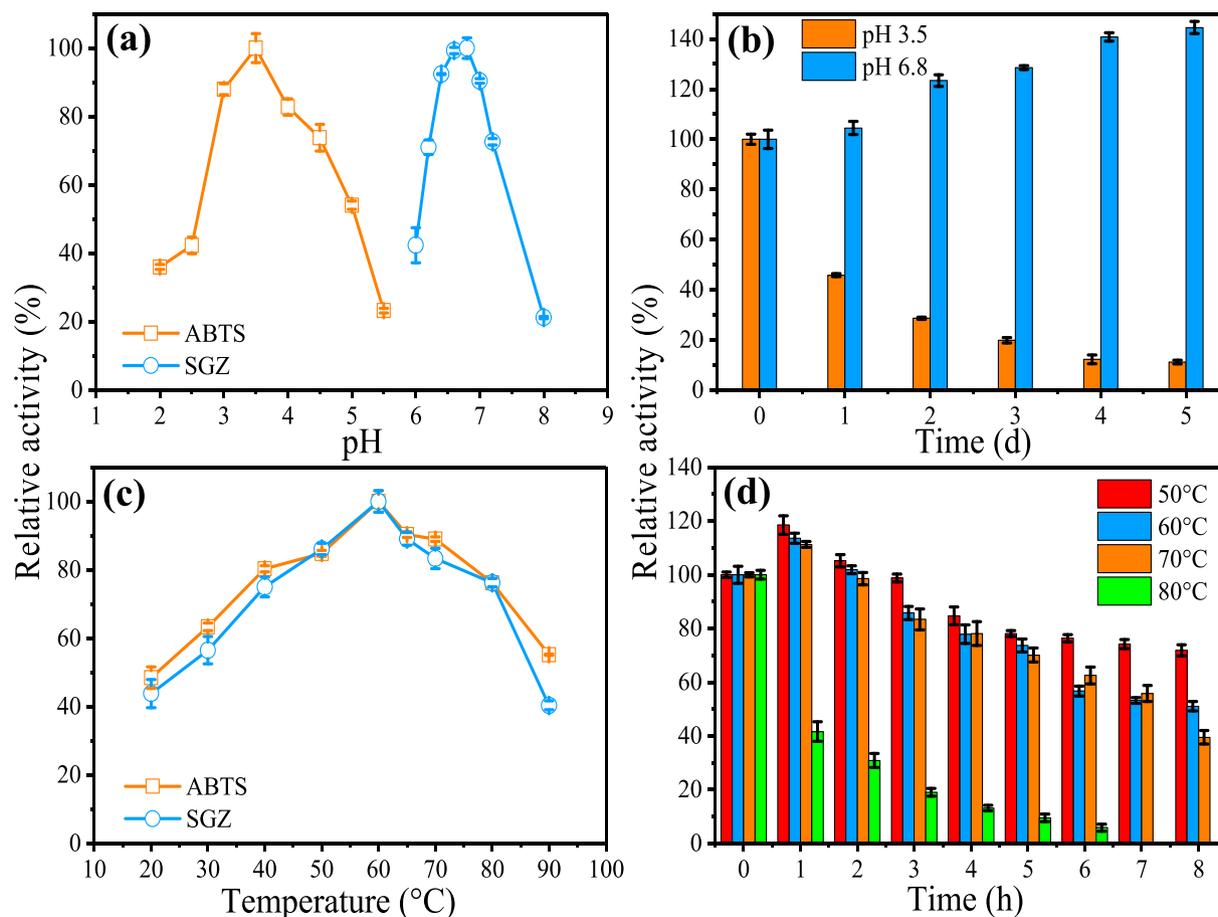


Fig. 3. Effect of pH and temperature on recombinant laccase (a) pH activity with ABTS (pH: 2.0–5.5), and SGZ (pH: 6.0–8.0), as substrates; (b) pH stability using ABTS (pH = 3.5), and SGZ (pH = 6.8); (c) temperature activity in the presence of ABTS and SGZ; (d) temperature stability using SGZ.

3.3.2. Effects of enzyme inhibitors and heavy metals

The stability of LACREC3-laccase was further tested with various enzyme inhibitors (Table S1). The LACREC3-laccase exhibited a significant tolerance of EDTA (0.5 mM) and showed more than 90% of activity. In contrast, it was partially suppressed by SDS (0.1 mM) and NaN_3 (0.1 mM) and fully inhibited by l-cysteine and dithiothreitol. A similar data of enzyme inhibitors were also revealed previously in *Bacillus vallismortis* fmb-103 and *B. amyloliquefaciens* LC02 (Sun et al., 2017; Chen et al., 2015). Generally, EDTA hindered the activity by chelating copper atoms in the enzyme (Afreem et al., 2017). Thus, the lesser effectiveness of EDTA might be indicated a limiting number of Cu^{2+} presence in the laccase. However, SDS (as a detergent) denatured the protein and impeded the activity (Johannes and Majcherczyk, 2000). Earlier, it revealed that NaN_3 had a strong ability to inhibit the laccase by binding of N_3^- at the laccase-trinuclear copper center and restricted the electron transfers during the reaction process (Dwivedi et al., 2011; Safary et al., 2016). Similarly, the reducing agents, l-cysteine , and dithiothreitol were strongly inhibited the laccase activity by reducing the oxidized substrate in their sulfhydryl groups (Johannes and Majcherczyk, 2000). These results were consistent with our previous study of spore-laccase and a laccase reported from *B. amyloliquefaciens* (Chen et al., 2015; Das et al., 2018).

Furthermore, the effects of different metal ions (1–5 mM) on laccase activity were also measured. Results showed that at a concentration (1 mM), Cu^{2+} and Zn^{2+} had slightly increased the laccase activity ($103.12 \pm 2.76\%$ and $101.66 \pm 1.85\%$) as compared to the control sample (100%; without metal ion). However, most of the metal ions decreased the enzyme activity at 5 mM concentration (Table S2). A similar trend of Cu^{2+} and Zn^{2+} on recombinant laccase was also found in *B. vallismortis* fmb-103 (Sun et al., 2017). It is reported that the catalytic functions of laccase in the presence of metals are primarily involved by interactions between metal ions and copper centers of the enzyme. In contrast, the inhibition of laccase activity by metals is mainly arisen by chelating of copper atoms or changing of protein structure through amino acid modification (Gianfreda et al., 1999).

3.3.3. Effects of Cu^{2+} on laccase expression and activity

This study demonstrated that laccase from GZB was a non-blue, which further proved by laccase expression study in the presence of graded concentrations of Cu^{2+} . Results showed that Cu^{2+} had a limited extension on the laccase expression. The LACREC3-laccase was expressed only at a range of 0.2–0.5 mM of Cu^{2+} (Fig. S4a). Previous studies reported that 0.25 mM of Cu^{2+} was sufficient to stimulate the expression of recombinant laccase (Callejon et al., 2017; Su et al., 2013). Cu^{2+} played a vital role in protein folding and yielding as well as enhancement of soluble protein, which further aided to color and enzyme activity (Reiss et al., 2013; Sun et al., 2017; Xia et al., 2019). As a result, a purified blue-laccase produced a dense blue color complex (Callejon et al., 2017; Safary et al., 2016). However, LACREC3-laccase exhibited a colorless compound; surprisingly, it maintained all aspects for copper loading. Thus, this data supported that LACREC3-laccase was not a usual blue-laccase. Moreover, this finding was further evidenced by activity assay with Cu^{2+} (0–5 mM). As shown in Fig. S4b, the alleviated concentrations of Cu^{2+} , did not increase the laccase activity; a maximum of $102.55 \pm 1.71\%$ activity was found at 1 mM of CuSO_4 . In contrast, most studies exposed that blue-laccase was directly influenced by Cu^{2+} (Callejon et al., 2017; Sondhi et al., 2014). Early, Sun et al. (2017) established a laccase from *B. vallismortis* that showed high activity up to $132.6 \pm 2.7\%$ at 5 mM of Cu^{2+} . It hypothesized that the positive impacts of Cu^{2+} on laccase were mainly caused by the filling of Cu^{2+} at the copper-binding sites (Gianfreda et al., 1999). Thus, the laccase from GZB was a

non-blue, and the activity was less influenced by Cu^{2+} .

3.4. Kinetic study of the recombinant laccase

The LACREC3-laccase showed an excellent oxidation ability towards SGZ with the calculated K_m and k_{cat} of $15.8 \mu\text{M}$ and 22.8S^{-1} , respectively. For ABTS, the values were $187.79 \mu\text{M}$ and 77.9S^{-1} (Table S3). This discrepancy in K_m values suggested that SGZ was more favorably binding to laccase than ABTS. The preference of SGZ as a substrate of laccase was also observed formerly in both *Bacillus* sp. SL-1 and *B. subtilis*, while their estimating K_m values were higher than GZB (Safary et al., 2016).

As a biocatalyst, laccases demonstrate a wide range of substrate specificity and a high degree of redox potential. They also display an extend robustness against different physicochemical parameters, which allow mostly the enzyme to apply in many industrial and biotechnological processes. The spore-laccase from GZB had the capability to degrade BPA and many synthetic dyes. Here, we developed a recombinant laccase and tested it for Mn(II) oxidation activity.

3.5. Mn(II) oxidase activity of recombinant laccase

The LACREC3-laccase of GZB exhibited Mn(II)-oxidase activity by producing insoluble brown Mn-oxides in the presence of Mn(II) salt. The activity was measured using three different ways: agarose gel plate assay, native-PAGE analysis, and buffer assay and confirmed by the LBB reactions.

3.5.1. Agarose gel plate assay

In this assay, we observed a circle of brown Mn(IV)-oxides' deposition surrounding the enzyme applied on the solid surface of the agarose plate. This brown colored band was formed via conversion of Mn(II) to Mn(IV)-oxides by LACREC3-laccase (Fig. 4a). The band was further turned into a blue color with the addition of LBB that proved the presence of Mn-oxides in the gel (Fig. 4b). Here, we developed for the first time the 'agarose gel plate assay' to examine Mn(II)-oxidase activity of laccase. The agarose gel plate assay is an essential *in-vitro* development that simplifies the rigorous estimation of Mn(II)-oxidase activity of numerous enzymes like laccases or various MCOs.

3.5.2. Native-PAGE assay

Simultaneously, the Mn(II)-oxidase activity of laccase was also observed in native-PAGE. This study applied the purified laccase first to run in a native gel and then reacted with MnCl_2 in HEPES buffer (pH 8.0). A brown color band ~40 kDa was formed in gel, indicating the formation of Mn(IV)-oxides by laccase (Fig. 4c). The Mn(IV)-oxides further reacted to the LBB solution, leading the change of the band from brown to blue (Fig. 4d). The applied method of Mn(II) oxidation by laccase was also illustrated the biochemical activity of Mn-oxidase. They also established a brown colored band of MnO_2 in SDS-PAGE reacted with MnCl_2 (Butterfield et al., 2013).

3.5.3. Mn(II) oxidation in a buffer system

Successively, in this assay, we found insoluble brown or black particles of Mn(IV)-oxides, when laccase reacted with Mn(II) in the oxygenated buffer. The reaction further produced a blue color mixed with the LBB solution (Fig. 4e). The Mn(IV)-oxides' formations by laccase in buffer were also reported elsewhere (Su et al., 2013). All the above three tests were necessarily proven the Mn(II)-oxidase activity of LACREC3-laccase, which further characterized in the presence of different physicochemical parameters including pH, temperatures, and various concentrations of Cu^{2+} .

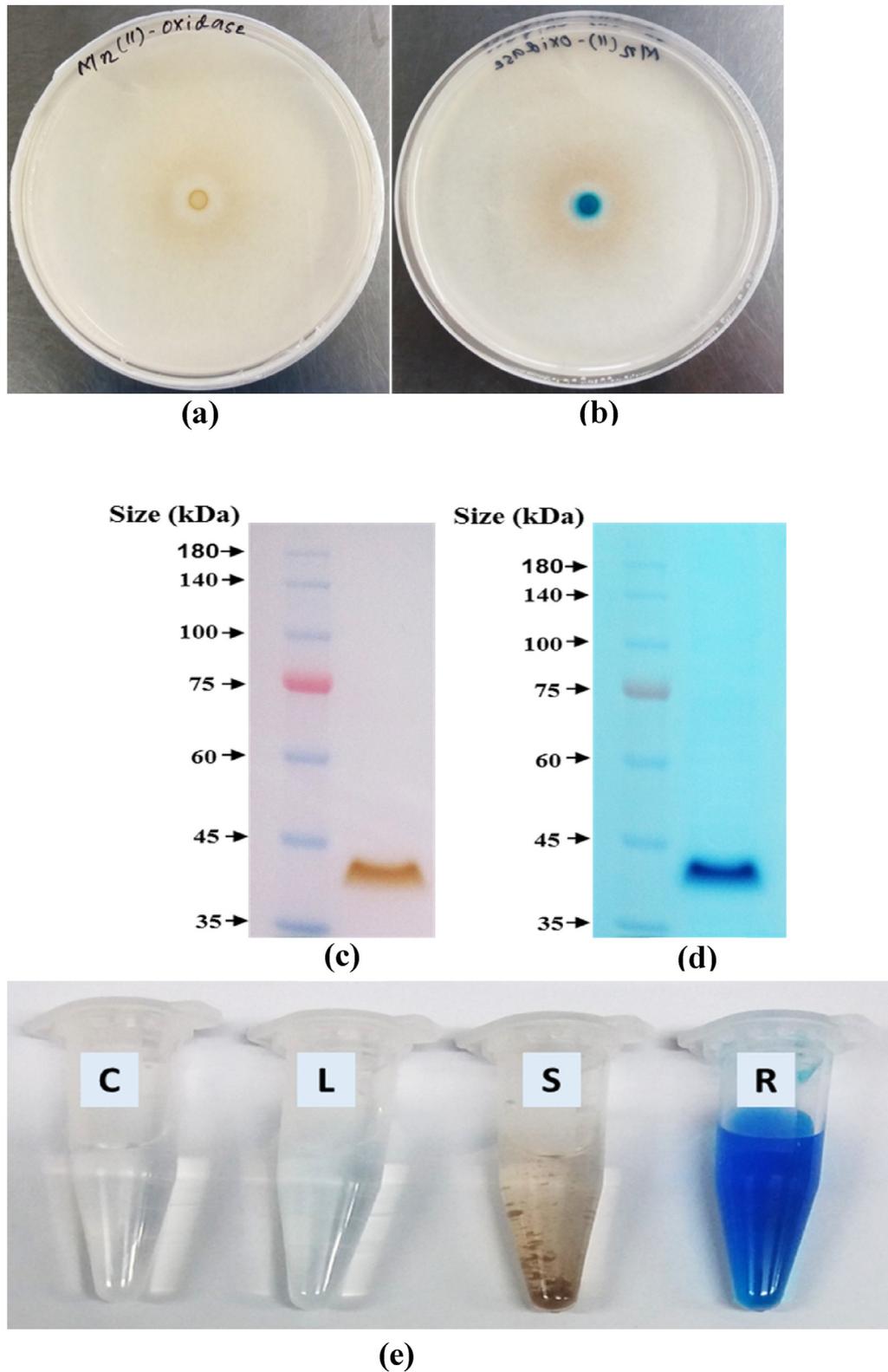


Fig. 4. Mn-oxidase assays of recombinant laccase. (a) Mn(II)-oxidase activity assessed using an agarose gel plate assay. (b) Mn(IV)-oxides formation evaluated with LBB assay. (c) Mn(II)-oxidase activity was analyzed by native PAGE. Lane 1: pre-stain protein marker, Lane 2: Mn(IV)-oxides formation by laccase as a brown colored band. (d) Mn(IV)-oxides assayed using LBB stain, Lane 1: pre-stain protein marker; Lane 2: brown Mn(IV)-oxides turned into a blue colored complex. (e) Mn(II)-oxidase activity test in liquid buffer system; Tube C: 10 mM HEPES (pH 8.0), 5 mM MnCl_2 , and 0.8 mM CuCl_2 (positive control); Tube L: LBB solution (negative control); Tube S: Sample after 24 h of enzymatic transformation; Tube R: reaction mixture reacted with LBB stain. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.6. Influences of pH, temperature, and Cu^{2+} on Mn(II)-oxidase activity

The pH activity profile of LACREC3-laccase during Mn(II) oxidation is illustrated in Fig. 5a. Laccase displayed high Mn(II)-oxidase activity at pH 8.0–8.6. The maximum activity was obtained at pH = 8.2 in HEPES buffer, without autooxidation of Mn(II). Moreover, pH = 8.6 showed optimal activity in CHES buffer followed by the reduction of autooxidized Mn(IV)-oxides in the control samples. The autooxidation of Mn(II) is a natural process that is kinetically inhibited at pH less than 8.5 (Su et al., 2014); therefore, pH 8.0 was selected for further experiments.

Moreover, the effect of temperature on Mn(II)-oxidase activity of laccase was monitored at 30 °C–75 °C. We found a maximum Mn(II)-oxidase activity at 55 °C (Fig. 5b). The Mn(II)-oxidase activity dropped rapidly at high temperatures. Only $36.17 \pm 1.07\%$ and $28.48 \pm 2.66\%$ of relative Mn(II)-oxidase activities were found at 65 °C, and 75 °C, respectively. A similar trend of pH and temperature activities during Mn(II) oxidation was also observed in a Cota from *Bacillus* sp. WH4 (Su et al., 2013), and a multicopper oxidase CueO of *E. coli* K12 (Su et al., 2014). As mentioned above, the stability of laccase declined at higher temperatures; conversely, Mn(II) autooxidized at this condition. For that reason, we performed all other experiments at 37 °C, as a moderate reaction temperature.

Mn(II)-oxidase activity was then examined in the presence of various levels of Cu^{2+} . Similar to laccase, Mn(II)-oxidase activity was also not effectively enhanced in the presence of Cu^{2+} (Fig. 5c). As compared to the control (100%; absence of Cu^{2+}), LACREC3-laccase showed an optimal $103.75 \pm 1.46\%$ of relative Mn(II)-oxidase activity in the presence of 0.2 mM Cu^{2+} . In addition, it maintained near to 100% of activity up to 0.8 mM Cu^{2+} . The relative activity was negatively impacted when the levels of Cu^{2+} increased from 1.0 to 1.6 mM. Only $80.30 \pm 3.43\%$ of relative activity was found with 1.6 mM Cu^{2+} . Copper is an essential cofactor for MCOs, and elevated levels could increase the manganese or laccase activity, which was also established in many studies (Butterfield and Tebo, 2017; Su et al., 2013). Thus, these results also suggested that LACREC3-laccase was a non-blue laccase, and Cu^{2+} had a limited effect on Mn(II)-oxidase activity.

3.7. Mn(II) oxidation by recombinant laccase

The formation of Mn(IV)-oxides by LACREC3-laccase was measured using the LBB solution. Data showed that the Mn(II) oxidation gradually increased, and up to $43.93 \pm 0.67\%$ of Mn(IV)-oxides were measured after 16 h of the experiment (Fig. 5d). Moreover, we also found a total of $44.45 \pm 0.45\%$ Mn(IV)-oxides after 24 h, in which $5.87 \pm 0.61\%$ were autooxidized that observed

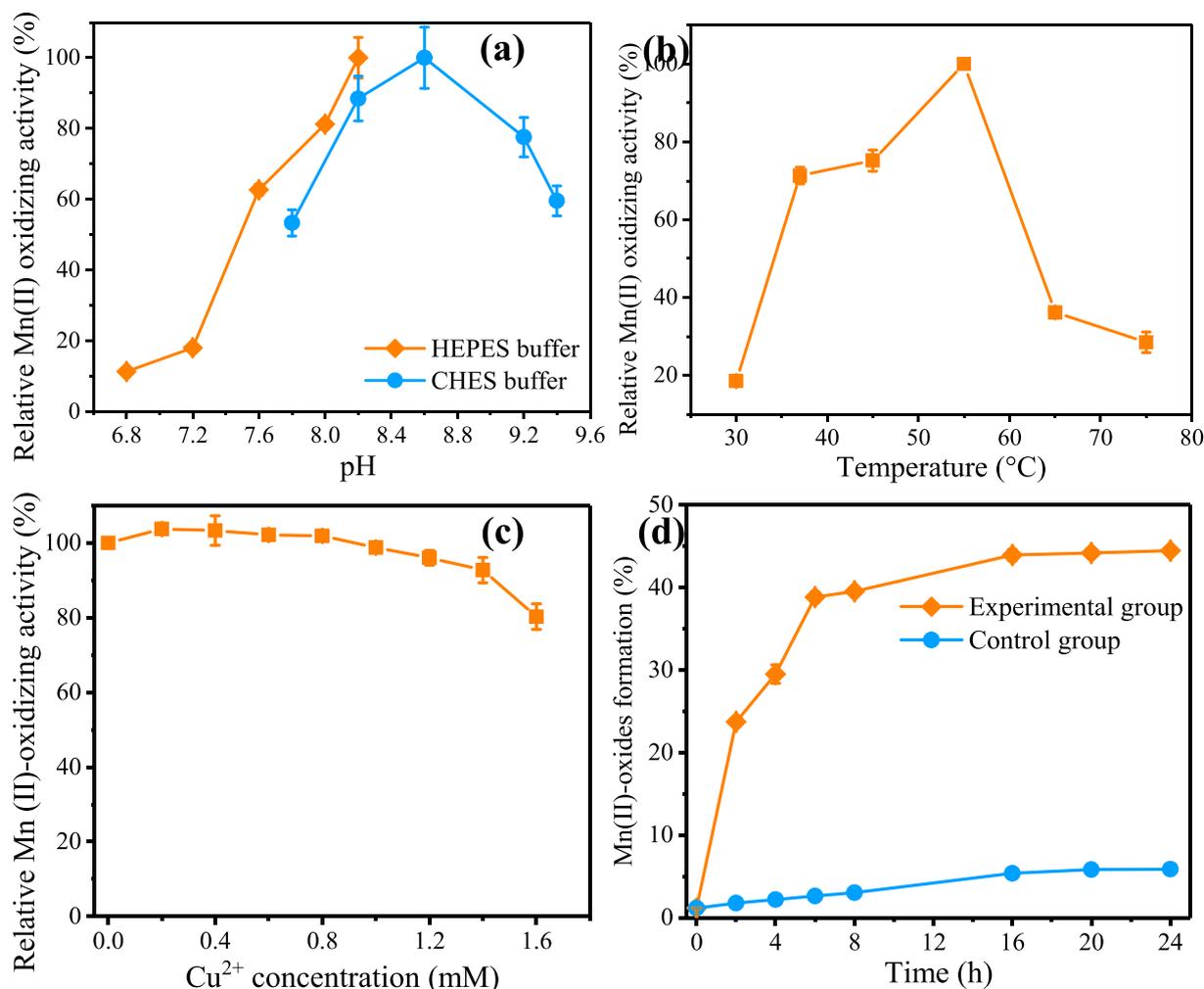


Fig. 5. Mn(II)-oxidase activity assays by purified laccase, at different (a) pH, (b) temperatures, and (c) Cu^{2+} concentrations. (d) Quantification of Mn(IV)-oxides at different time intervals. All the experiments were carried out using 10 mM HEPES buffer.

in the control samples. Recently, Zhang et al. (2019a) have reported a 49.55% of biogenic Mn-oxide formation using *Aeromonas hydrophila* DS02 strain after 144 h of incubation. Thus, the laccase from *Bacillus* sp. GZB established an efficient oxidation ability towards Mn(II). The estimated kinetic values such as $K_m = 35.28$ mM, $V_{max} = 0.62$ $\mu\text{M min}^{-1}$, and $k_{cat} = 19.14$ S^{-1} also supported that Mn(II) could be a suitable substrate for laccase of GZB (Table S4). Besides, these results were also comparable with a blue-laccase in *Bacillus pumilus* WH4 and a CueO enzyme in *E. coli* (Su et al., 2013, 2014).

As evident from the previous studies, the oxidation of manganese in natural or engineered environments is a microbial process. Both microbes and their enzymes (particularly MCOs) are dynamically transformed Mn(II) to Mn(III)/(IV)-oxide minerals (Brouwers et al., 2000; Liang et al., 2016; Tebo et al., 2004). MCOs are a group of enzymes that are well-known for the oxidation of numerous organic and inorganic compounds (Su et al., 2014; Xia et al., 2019; Zhang et al., 2019b). The protein sequence data of MCOs also suggest that the signature motifs in protein and Cu^{2+} are interacting with each other to function the catalytic reactions (Butterfield and Tebo, 2017; Soldatova et al., 2012). Our sequence alignment data (laccase of GZB with other MCOs (Mn-oxidase, and MCOs and laccase with/without Mn-oxidase activity)) also supported that the MCOs contained four conserved histidine-rich copper-binding motifs, which might help the catalytic activity of laccase towards manganese (Fig. S5). However, the precise mechanisms are still unclear due to the difficulty of isolation of Mn(II)-oxidase enzymes. To date, only, Tebo and co-workers have been successfully expressed Mn_xG from a marine *Bacillus* sp. PL-12 and proposed the possible mechanisms for Mn(II) oxidation (Butterfield et al., 2013; Soldatova et al., 2017a). Hence, the development of effective methods for the expression and purification of Mn(II)-oxidizing protein would be the main scope of future research. Luckily, we expressed a *CotA* gene in *E. coli*; more vitally, it showed Mn(II) oxidation activity *in-vitro*. Until now, the *CotA* from *B. pumilus* WH4 and *B. pumilus* A56 were reported for Mn(II) oxidation; however, both the enzymes were blue-laccase (Su et al., 2013; Zhang et al., 2019b). Hence, the elucidation of Mn(II) oxidation by a non-blue laccase is one of the essential steps in this research that essentially helps to draw the unknown mechanisms of Mn(II) oxidation by laccase.

3.8. Prediction of Mn(II) oxidation mechanisms

Similar to previous research outcomes (Geszvain et al., 2012; Soldatova et al., 2017a), this study also established an outline of the Mn(II) oxidation process. To elucidate the Mn(II) oxidation mechanisms, we monitored a reaction in the UV–visible absorption spectrum. The spectra upraised a peak around 266 nm, which notably predicted as Mn(III)-PP derived from Mn(III)-oxides trapping by pyrophosphate (Fig. 6a). At starting point, the peak formed by Mn(III)-PP showed a sigmoidal curve; however, when the Mn(III)-oxides eroded to Mn(IV)-oxides, the peak level reduced and appeared around 350–400 nm as a broad peak area (Fig. 6a and b). Previous studies had also been recognized that Mn(III) was an intermediate product of Mn(II) to Mn(IV) transformation. In the reaction, Mn(III) is very unstable and mainly observed after trapping by pyrophosphate (Butterfield et al., 2013; Geszvain et al., 2012; Soldatova et al., 2012). In time progress, Mn(III) was rapidly transformed into Mn(IV) as the colloidal particles of Mn(IV)-oxides that shifted a broad peak area around 350–400 nm (Soldatova et al., 2017b). Even so, we did not acquire a precise elevation of 400 nm peak, which might be due to larger particle size of Mn(IV)-oxides that conquered by an extensive peak area rather than a prominent ridge. Previously, Soldatova et al. (2012) delineated the

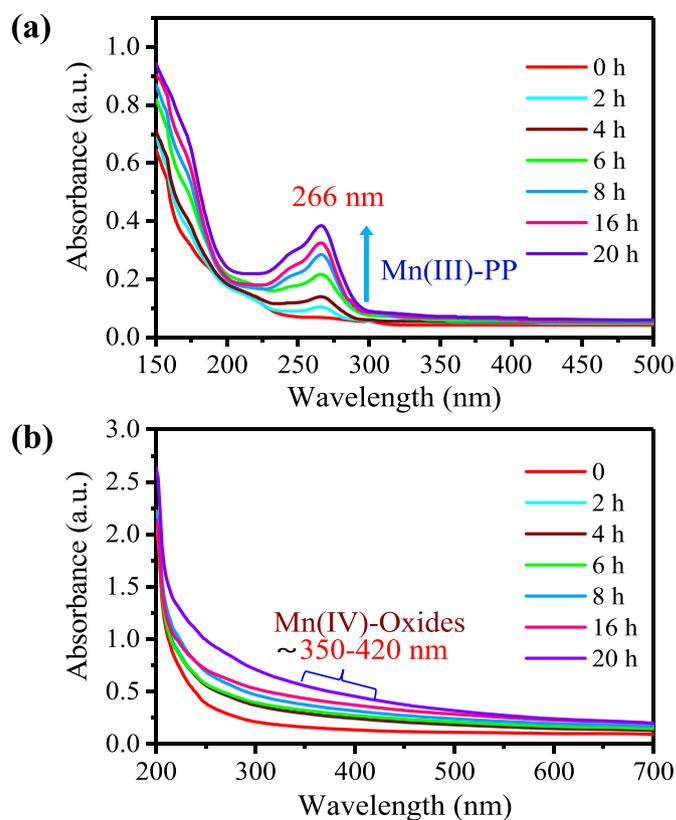


Fig. 6. Time-resolved UV–visible absorption spectral measurements of Mn(II) oxidation by purified laccase. (a) The absorption spectra were recorded in the presence of 10 mM HEPES buffer (pH 8.0), 100 μM MnCl_2 , 500 μM of Na-pyrophosphate, and purified laccase. The formation of Mn(III)/(IV)-oxides was assayed at room temperature in selected time intervals. A growing of the 266 nm peak indicated the formation of Mn(III)-oxides trapping by pyrophosphate. (b) Mn(IV)-oxides was measured without adding pyrophosphate. A broad absorption band at around 350–420 nm designated the formation of Mn(IV)-oxides.

Mn(II) oxidation ability of an exosporium from *Bacillus* sp. SG-1. They further ascribed that Mn(II) to Mn(IV) was a two-electron process, in which Mn(III) was an intermediate, which detected by a peak at 265 nm using pyrophosphate. However, other studies demonstrated a band of Mn(III)-PP at 258 nm (Butterfield et al., 2013; Tran et al., 2018). From this study, it was difficult to describe, the enzymatic transformation of Mn(II) to Mn(IV) by LACREC3-laccase was either a one-step or two-step reaction. To predict the specific mechanism of Mn(II) oxidation by laccase of GZB, we need further study.

4. Conclusions

As a biocatalyst, recently, laccase has been attracted more attention to the industry, biotechnology, and environmental applications due to its high activity and durability. Consequently, it can be quickly produced at laboratory scale in a larger volume. However, the most crucial step is to identify an efficient laccase that can be competent with different physiochemical hardness. Therefore, this study revealed a non-blue laccase withstanding the high degree of pH and temperatures, as well as active in the presence of various metals and enzyme inhibitors. More importantly, *Bacillus* sp. GZB laccase oxidized Mn(II) to Mn(III)/Mn(IV)-oxides that notably indicated a possible way of Mn-decontamination in many environments, especially at metal-polluted sites. Therefore, our study highlights a novel finding of Mn-oxidation by laccase that can

be applied as a suitable biocatalyst in green technology.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Ranjit Das: Conceptualization, Investigation, Methodology, Data curation, Writing - original draft, Writing - review & editing. **Zhishu Liang:** Validation. **Guiying Li:** Conceptualization, Supervision. **Taicheng An:** Supervision.

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Appendix B. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.chemosphere.2020.126619>.

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