Effect of Cu(II) and Conserved Copper Binding Sites on Multicopper Oxidase CopA and Characterization of the BioMnO_x

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Abstract

The microbial manganese removal process is believed to be the catalytic oxidation of Mn(II) by manganese oxidase. In this study, the multicopper oxidase CopA was purified and found to have high manganese oxidation activity in vitro and Cu(II) can significantly enhance its manganese oxidation activity. The gene site-directed mutagenesis was used to mutate four conserved copper binding sites of CopA and then obtain four mutant strains. The manganese removal efficiency of the four strains was determined to find that H120 is the catalytic active site of the CopA. Protein modification analysis of CopA obtained under different conditions by mass spectrometry revealed that the loss of Cu(□) and the mutation of the conserved copper binding site H120 resulted in the loss of modification of ethoxyformyl and quinone, the number of modifications was reduced and the position of modification was changed, eventually causing a decrease in protein activity. It reveals that Cu(II) and H120 play an indispensable role in the manganese oxidation of the multicopper oxidase CopA. The Mn valence state of BioMnOx was analyzed by XPS, finding that both the strain-mediated product and the CopA-mediated product were composed of MnO_2 and Mn_3O_4 and the average valence of Mn is 3.2.

Introduction

The average abundance of manganese in the earth's crust is 950 ppm, which is the most abundant among trace elements. Manganese is an essential nutrient for the human body and has important industrial applications. With the development of manganese industry, pollution has occurred in soil, atmosphere and water, leading to problems for not only the environment but also human health owing to its toxicity. Excessive manganese can damage the nervous system and reproductive system, causing liver and lung damage (Nieuwenhuijsen et al. 2000). Therefore, the content of manganese should be controlled at a level that is harmless to human body.

Biogenic Mn(□) oxidation mediated by microorganisms dominates the biomineralization of Mn oxides in natural systems. But sewage treatment plant mainly use physicochemical methods for manganese removal. Biological manganese oxidation processes are much faster than chemical catalysis five orders of magnitude in a neutral environment and it has the advantages of high safety, low introduction of chemicals, low cost, etc (Zeiner et al. 2012). Biogenic Mn(□) oxidation is due to Mn(□)-oxidizing bacteria producing enzymes which has the ability of oxidizing Mn(II) to Mn(III/□) oxides. Manganese oxidase mainly includes the multicopper oxidase (MCO), manganese peroxidase, lignin peroxidase and lignin degrading enzyme. MCOs are named for their conserved Cu sites, containing four copper atoms. These copper atoms are classified into type 1, type 2, and type 3 coppers (T1Cu, T2Cu, and T3Cu, respectively) based on their electronic and magnetic properties. T1Cu participates in the oxidation of one-electron transfer of substrate, and T2Cu and T3Cu participate in the reduction of four electrons to reduce molecular oxygen to water (Solomon et al. 1996). The most reported MCOs are MnxG (Dick et al. 2008), CueO (Su et al. 2014), CumA (Francis and Tebo 2001), MoxA (Ridge et al. 2007), MofA (Corstjens et al. 1997), CotA (Su et al. 2013).

It is reported that many metal ions could affect the oxidizing activity of MCO, one of which is Cu(Ⅱ) (Butterfield and Tebo 2017; El Gheriany et al. 2009; Lee et al. 2014). Many results have been observed that MCOs binds much more Cu than necessary to fill the four Cu sites (Butterfield and Tebo 2017; Musci et al. 1993; Roberts et al. 2003). The proclivity to bind so much Cu may suggest that the solubility, activity, and complex formation of MCOs are particularly rely on Cu, even without the addition of Cu to the strain growth medium, the protein is inactive (Butterfield and Tebo 2017).

Previous studies have demonstrated the potential of several bacteria for efficiently promoting Mn(II) removal. Several strains with manganese oxidizing capacity have been reported including *Bacillus* sp. SG-1 (Vanwaasbergen et al. 1993), *Pedomicrobium* ACM 3067 (Ridge et al. 2007), *Leptothrix discophora* SS-1 (Corstjens et al. 1997), and *Pseudomonas putida* GB-1 (Brouwers et al. 1999). Most of these studies involved bacterium or consortia that were able to remove Mn(II) by oxidation and/or adsorption mechanisms. The investigation of direct removal of manganese by enzymes and the effect of Cu(II) and conserved copper ion binding sites on biooxidation were much less (Butterfield et al. 2013; Butterfield et al. 2015;(Durao et al. 2008;(Komori et al. 2014; Solomon et al. 1996; Vanwaasbergen et al. 1993; Xiao and Wedd 2011). How do these conserved copper ion binding sites affect the oxidative capacity of the microorganism? How much influence of Cu(Ⅱ) on the oxidation ability of copper oxidase?

In the previous study, the isolated manganese-oxidizing bacterium was identified as *B.panacihumi* and was named *Brevibacillus. panacihumi* MK-8. The *copA* gene was successfully amplified from MK-8 by designing primers from a homologous strain and then overexpressed in *Escherichia coli* strain BL21(DE3). Thus, the highly efficient manganese oxidation recombinant strain BL21-pET-*copA* has been successfully constructed and the target gene *copA* deletion recombinant plasmid has also been constructed by overlapping PCR. The *copA* gene-deleted strain (MK-8-Δ*copA*) barely oxidized manganese, further demonstrating that the *copA* gene is the manganese oxidase gene (Zeng et al. 2018).

In this study, BL21-pET-*copA* was used as the research object, and the role of conserved copper ion binding site of multicopper oxidase CopA studied by gene-directed mutagenesis. The Liquid chromatography-mass spectrometer was used to analyze the changes of protein modification of CopA obtained under different conditions. The morphology of strain-mediated and CopA-mediated $BioMnO_x$ was characterized by SEM and TEM. Finally, the composition and valence of $BioMnO_x$ was analyzed by XPS.

Materials And Methods

Materials

Kanamycin, isopropyl-b-D-thiogalactoside (IPTG), Anti-His antibody were obtained from Tiangen Biotech Co., Ltd (Beijing, China). Ni Focurose 6FF and DEAE Focurose 6FF were purchased from HUIYAN Bio (Wuhan, China). Fast Mutagenesis system was bought from TRANSGEN BioTECH (Beijing, China).

Sequencing services provided by TSINGKE (Beijing, China) and Primer synthesis is supported by Sangon Biotech (Shanghai, China).

Strain, media and growth conditions

The recombinant strain BL21-pET-*copA* has been successfully constructed in previous experiment (Zeng et al. 2018). The strain was cultured in LB medium containing 1% (w/v) tryptone, 0.5% (w/v) yeast extract and 1% (w/v) sodium chloride and grown at 37℃ to an OD_{600}~0.6-0.8 supplemented with 10 mM Tris-HCl pH7.2 and 50 μg/mL kanamycin. The temperature was then lowered to 28℃ and then 1mM IPTG was added to induce transcription of the *copA* genes. The growth of BL21-pET-*copA* was performed under two conditions, in the presence and absence of Cu(Ⅱ).

Protein extraction and purification

The cells were centrifuged at 10,000×g for 15 min, suspended in buffer (20 mM Tris-HCl pH 7.9, 0.5 M NaCl) amended with phosphatase inhibitor cocktail and lysozyme, and lysed by sonication for 30min at 80% amplitude with 6 s on/off pulses on ice. The lysate remove cell debris by centrifugation (12,000×g, 15 min) at 4℃. The N-terminal His-tagged recombinant protein was purified by Ni Focurose 6FF column and DEAE Focurose 6FF column. Protein assays were performed using the BCA methodology with bovine serum albumin (BSA) as the standard following the manufacturer's instructions of BCA Protein Assay Kit (Tiangen, Beijing, China).

SDS-PAGE and western blot assay

The soluble extracts and the purity of the isolated target protein was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were run at 120 V for 120 min and stained with Coomassie Brilliant Blue. Finally, the gel was decolorized overnight with deionized water, and the protein band size was observed.

To investigate the expression of CopA in BL21-pET-*copA*, a western blot assay was performed. After electrophoresis on a 12% sodium dodecyl sulphate-polyacrylamide gel, proteins were transferred to polyvinylidene fluoride membranes. Membranes were incubated overnight at 4°C with antibodies against His-tag. After rinsing three times, membranes were incubated with antibody IgG horseradish peroxidase-linked antibodies for 1 h at room temperature. Blots were visualized using an enhanced chemiluminescence luminescent substrate (Thermo Fisher Scientific, Waltham, MA, USA) and exposed to X-ray film.

Activity analysis of enzyme

Crude enzyme, flow-through and purified CopA in different culture conditions were tested on their Mn(Ⅱ)-oxidizing activities in liquid culture system consisting of 2.3 mg/mL enzyme, 1 mM $MnCl_2$ and 1 mM $CuCl_2$ in 10 mM HEPES (pH 7.0). The reaction mixture was incubated at room temperature for 24 h and the produced Mn oxides were then quantified spectrophotometrically with leucoberbelin blue (LBB) reagent (620 nm).

Sequence analysis of copper ion conserved sites

The amino acid sequences of CopA was compared with other copper oxidases by software MEGA7.0 (http://www.megasoftware.net/download_form). The MCOs include MnxG (Francis et al. 2002) (AAB06489.1), MofA (Zhang et al. 2002) (CAA81037.2), MoxA (Ridge et al. 2007) (CAJ19378.1), CotA (Su et al. 2013) (AFL56752.1) and CueO (Su et al. 2014) (NP_414665.1). The amino acid sequence of CopA is in the supplementary information.

Construction of copA mutant strains

In this study, we used site-directed mutagenesis to perform mutagenesis of four copper conserved binding sites in the manganese oxidation gene *copA*. Using the plasmid of strain BL21-pET-*copA* as a mutation template, we designed four sets of primers (Table S1) to respectively mutate the conserved sites H120, H162, H442, H492 to N120, N162, N442, N494. The plasmid was subjected to PCR according to the Fast Mutagenesis System operating instructions and transferred to competent cells. The plasmid extracted from cells and send to the sequencing to obtain the mutant strains.

Comparison the manganese removal efficiency of strains

To compare Mn(Ⅱ) oxidation ability of recombinant strain BL21-pET-*copA* and mutant strains BL21-pET-*copA*$^{120H-N}$, BL21-pET-*copA*$^{162H-N}$, BL21-pET-*copA*$^{442H-N}$, BL21-pET-*copA*$^{494H-N}$, the manganese removal efficiency was tested respectively. Culture conditions were divided into two group: in group Ⅰ strains grown in LB medium with 1 mM Mn(Ⅱ) and 10 mM Tris-HCl(pH 7.0); group Ⅱ is to supplement 0.25 mM Cu(Ⅱ) to the group Ⅰ; and the original strain BL21-pET-*copA* is the control group. The strains were grown at 37℃ in LB medium supplemented with 50 mg/L Kanamycin Sulfate. When the OD_{600} reached to 0.6-0.8, 1 mM IPTG was added to induce the protein, and then system was shaken at 28℃ for eight days. The concentrations of residual Mn(Ⅱ) in the culture medium were measured by ICP-OES. The reason why the initial concentration of manganese is 1 mM is that the content of manganese wastewater is generally 10 to 200 mg/L. The manganese removal target we studied is manganese wastewater.

Protein digestion and LC-MS/MS analyses

Since the manganese removal efficiency of the strain BL21-pET-*copA*$^{120H-N}$ was low, the multicopper oxidase CopA (condition c) produced by the strain was purified by a Ni Focurose 6FF column. Similarly, the multicopper oxidase CopA produced by the strain BL21-pET-*copA* in the copper-containing (condition a) and copper-free conditions (condition b) were also purified by a Ni Focurose 6FF column, and the CopA under the three conditions were subjected to SDS-PAGE electrophoresis.

The single bands of CopA were cut and then added 100 μL SDT lysate(4% SDS, 100mM DTT, 100mM Tris HCl) and appropriate DTT with a final concentration of 100mM, and the mixture was boiled for 5 min, and the supernatant was centrifuged at 4°C. The protein sample was subjected to digestion using the FASP method, and the enzymatically decomposed peptide was desalted using C18 Stage Tip, and finally

vacuum dried. The dried peptide was reconstituted with 0.1% FA, and the peptide concentration was determined by OD_{280}, and LC-MS analysis was performed.

The prepared three samples were subjected to chromatography using an Easy nLC 1200 chromatography system(Thermo Scientific). After peptide separation, the Q-Exactive Plus mass spectrometer(Thermo Scientific) was used for DDA mass spectrometry, and the Mascot 2.3.02 software(WWW.matrixscience.com) was used to perform protein search on the database composed of the *Brevibacillus panacihumi* W25 protein database downloaded by UNIPROT and the known CopA sequence.

Mineral identification of biogenic Mn oxidase ($BioMnO_X$)

Scanning electron microscopy (SEM, Hitachi-S4800, Japan), transmission electron microscopy (TEM, JEM-1230, Japan) and X-ray photoelectron spectroscopy(XPS, AXIS Ultra DLD, Japan) were used to tentatively characterize $BioMno_X$ by comparison with standard analytical reagent-grade MnO_2 (Aladdin, Shanghai, China). For SEM observation, reaction mixtures were centrifuged at 12000 rpm for 15 min, and a final concentration of 2.5% glutaraldehyde solution was added and fixed at 4°C for 24 h. Then the precipitate dehydrated with 50%, 70%, 80%, 90%, 100% ethanol solution for 10 min each time, and freeze-dried for 2 h in a vacuum freeze dryer. On the other hand, the precipitate was ultrasonically dispersed in ethanol and 10 μL of suspension was pipetted onto a copper grid and dried at 37℃ for TEM observation. The dried $BioMnO_x$ was uniformly coated on the conductive adhesive on the copper sheet, and pressed tightly, and then was subjected to manganese valence analysis by XPS. Software(https://srdata.nist.gov/xps/selEnergyType.aspx#opennew-window) is used to analyze the valence and composition of $BioMnO_x$.

Results

Enzyme activity of CopA

As shown in Fig. 1, a purified band (lane 5) at approximately 60.0 kDa was observed by SDS-PAGE analysis. The molecular mass of CopA is 58.3 kDa calculated by software ExPASy (https://web.expasy.org/cgi-bin/compute_pi/pi_tool) and the vector pET-28a(+) has 21 amino acids about 2.3 kDa. Therefore, the fusion protein mass is about 60.6 kDa, which is consistent with the experimental results. By comparing lane 5 with lane 2 and lane 4, the target protein CopA was successfully enriched and purified by Ni Focurose 6FF column and DEAE Focurose 6FF column. The WB analysis results of the protein samples are shown in FIG S1, the band of lane3 is significantly larger than lane1, indicating that the protein concentration is greatly improved after purification, which is consistent with the results of the BCA test. The lane2 test was negative, and lane1 and lane3 were positive, indicating that the fusion protein CopA is immunologically active.

We put CopA purified from BL21-pET-*copA* which cell grew in two conditions without Cu(Ⅱ) and with 0.25 mM Cu(Ⅱ) into reaction systems which contain 1 mM Cu(Ⅱ). As shown in FIG. 2A, lots of brown precipitates generated in tube 2 and tube 4 and they all colored upon addition of LBB reagent (FIG 2B). Surprisingly, in addition to tube 2 and tube 4, tube 5 also turned blue, indicating that the BL21-pET-*copA* in copper-free culture condition produced CopA which still has manganese oxidation activity although weakened. This is inconsistent with the report Mnx (Butterfield and Tebo 2017). Without the addition of extra Cu(Ⅱ) to the *mnx*-expressing *E. coli* growth medium, Mnx will precipitate during dialysis and is inactive. The produced Mn oxide were quantified spectrophotometrically using Microplate reader (Saputra et al. 2013). The manganese oxide yield of tube 4 is 11.2 times that of tube 2 and 114 times that of tube 5. This shows that Cu(Ⅱ) has a great influence on the manganese oxidation ability of CopA.

Sequence analysis of CopA

The similarity of amino acid sequence is not high (less than 31%) between CopA and known manganese oxide protein (Zeng et al. 2018), which may lead to different oxidation mechanism. But MCOs almost all contain four highly conserved copper ion binding sites and a Cys and 10 His and its surrounding amino acid ligands linked to copper atoms are relatively conservative as shown in Fig. 3a, suggesting that they may have similar biological functions, that is, they all have manganese oxidizing ability. The copper-binding domain of the copper oxidase is usually in the form of HXH (H is histidine) as shown in Fig. 3a. In Fig. 3b, the A and B sites of CopA, are located near the N-terminus of the protein like other MCOs (MofA, MoxA, CotA, CueO), and the C and D sites are located at the C-terminus. However, the sequence feature of MnxG are reversed, with the A and B sites at the C-terminus and the C and D sites at the N-terminus (Dick et al. 2008).

Manganese removal efficiency of mutant strains

Figure 4 shows manganese removal efficiency of BL21-pET-*copA* and mutant strains BL21-pET-*copA*$^{120H-N}$, BL21-pET-*copA*$^{162H-N}$, BL21-pET-*copA*$^{442H-N}$, BL21-pET-*copA*$^{494H-N}$ in LB medium. The culture conditions of the strain in Figure 4A were free of copper, and the strain in Figure 4B contained 0.25 mM Cu(Ⅱ). The reason why the manganese removal efficiency is negative is mainly the evaporation of water. By weighing the daily quality of the medium, it was found that its quality would decrease by 0.8 g to 1.7 g in one day. In the negative control group containing no microorganisms, the manganese concentration was increased by 10.4% after 8 days of culture. This should be due to evaporation of water. In addition, the growth and metabolism of bacteria also require water, so manganese removal efficiency has negative value. BL21-pET-*copA*$^{120H-N}$ and BL21-pET-*copA*$^{494H-N}$ exhibit manganese oxidizing ability faster than BL21-pET-*copA*$^{162H-N}$ and BL21-pET-*copA*$^{442H-N}$. All strains showed maximum manganese oxidation rate from day 3 to day 6. With the increase of culture time, although 10 mM Tris-HCl(pH 7.0) was added to the system, the pH value of the culture system was gradually increased. Microorganisms also increase manganese oxidation efficiency by changing the pH of the system. In contrast, in the reaction system of CopA, the pH does not change. On the 8th day, the Mn(Ⅱ) removal efficiency of BL21-pET-*copA*$^{494H-N}$ reached 91.5%, and the Mn(Ⅱ) removal efficiency of the other strains was 89.9% (BL21-pET-*copA*), 70.1%(BL21-pET-*copA*$^{120H-N}$), 87.1%(BL21-pET-*copA*$^{162H-N}$), 84.4%(BL21-pET-*copA*$^{442H-N}$),

respectively(Table S2). Interestingly, comparing the two graphs 4A and 4B, the manganese removal efficiency of the strains was greatly reduced under the condition without Cu(Ⅱ). The manganese removal efficiency of each strain was 55.60% (BL21-pET-*copA*), -4.16% (BL21-pET-*copA*$^{120H-N}$), 47.5% (BL21-pET-*copA*$^{162H-N}$),57.5% (BL21-pET-*copA*$^{442H-N}$), and 50.2% (BL21-pET-*copA*$^{494H-N}$), respectively(Table S2).

The manganese removal efficiency of the mutant strains was inconsistent, indicating that the four conserved copper ion binding sites have different effects on the manganese oxidation process. By comparing the removal efficiency of two conditions, it is known that the Cu(Ⅱ) greatly promotes the manganese oxidation activity of strains. Interestingly, the manganese removal efficiency of strain BL21-pET-*copA*$^{120H-N}$ was the lowest in both copper-containing and copper-free conditions, indicating that H120 is the catalytically active site of the multicopper oxidase CopA.

Compared with the strain BL21-pET-*copA*, the other mutant strains showed no significant increase or decrease in manganese removal efficiency, indicating that the HXH (H means histidine; X means other amino acids) structure of the conserved copper binding site is coordinated and coordinated to maintain the stability of the oxidized structure. In addition, a mutant strain BL21-pET-*copA*$^{494H-N}$ with higher manganese oxidation activity was obtained in this study.

Global analysis of the LC-MS/MS results

The mass spectrometry data of CopA under three different conditions(condition a: multicopper oxidase CopA produced by strain BL21-pET-*copA* under copper-containing conditions; condition b: multicopper oxidase CopA produced by strain BL21-pET-*copA* under copper-free condition; condition c: multicopper oxidase CopA produced by mutant strain BL21-pET-*copA*$^{120H-N}$ under copper-free condition) were introduced into the software for analysis, the modification information of each sample was obtained.

There are overall 165 modifications in different peptides, and the specific modification information can be found in the supporting information(Table S3).

The main modification are carbamidomethyl, oxidation, deamidated, methyl, carboxymethyl, carbofuran, acetyl, ammonium, propionyl, gly, formyl, ethoxyformyl, in addition to sulfide, quinone, amidine, dihydroxyimidazolidine, propionamide, homocysteic acid, carbamyl, diethylphosphate modification. The sample of condition a has 117 modifications, the sample of condition b has 106 modifications, and the sample of condition c has 74 modifications. Comparison of the modification of sample a with sample b found that ethoxyformyl and quinone are modifications specific to sample a. Ethoxyformyl occurs on the histidine of the peptide MHLPGHSFTIVSTDGQPIHNPPETQDQLLNIAPGER. It is reported in the literature that when histidine is modified by ethoxyformyl, it will lose its ability to bind to metal ions, but will not change the conformation of the protein (Costa et al. 2009). Therefore, it is speculated that ethoxyformyl modifies the histidine of the peptide to allow Cu(II) to bind correctly to the histidine of the conserved copper ion binding sites.

Quinone is modified on the tryptophan of TAWTYNGTVPGPQLR. Quinone has a strong tendency to recover into a benzene ring structure and is highly oxidizing, so quinone is often used as an electron acceptor in oxidation reactions (Zengin et al. 2019). Therefore, it is speculated that the modification of quinone can increase the oxidation activity of CopA. The sample b was compared with the sample c, and no special modification was found, but the modification of sample c was greatly reduced. It has been reported that the presence of protein modifications can increase gene expression and is an important means of epigenetic regulation, so a reduction in modification results in incomplete structure and function of the protein (Veenstra 2003).

Peptide modification analysis

This section selects some peptides for detailed analysis. A total of 19 modifications were detected in the peptide MHLPGHSFTIVSTDGQPIHNPPETQDQLLNIAPGER, as shown in FIG 5A.The protein under a condition has 16 modifications, the protein under b condition has 10 modifications, and the protein under c condition has 10 modifications, mainly modified on histidine, threonine and methionine. Through the protein modification analysis of the same peptide segment, it can be seen that the change of the condition can reduce the modification of the amino acid, the position of the modification changes, and causing the activity of the protein decreases.

Since the multicopper oxidase CopA is an oxidizing enzyme, the peptides MPGHDMSK and MPGHDMSKMDSASTAEHENLK with more oxidation modifications were selected for analysis, as shown in FIG 5B.

Oxidation modifications in the peptide occur on methionine, and oxidative modification on methionine protects methionine from the dual effects of irreversible oxidative modification and regulation of protein function. This modification allows the protein to exert physiological functions under appropriate conditions, and can act as an antioxidant against various oxidative stresses in the environment. Under the conditions of a, b, and c, the sample a has more oxidation modification, indicating that under condition a, the protein has stronger oxidative resistance, can better protect the activity of the protein, and can avoid irreversible oxidation modification. Therefore, Cu(II) in culture conditions can increase the oxidation activity of protein CopA, which is consistent with the experimental results in 3.1.

Predicting the secondary structure of the protein before and after the mutation, it was found that the mutation of H120 caused its secondary structure to change, and the IT in the ITKY sequence was more obvious, as shown in the FIG S2. This sequence is present in the peptide sequence obtained by mass spectrometry, and therefore the peptides SESMDLPVVDITK and SESMDLPVVDITKYGEAAK containing this sequence are analyzed, as shown in Fig. 5C. It was found that the mutated protein, ie, the protein under the condition of c, had only one modification, and the other four modifications were lost due to the mutation, indicating that the mutation of the conserved copper binding site H120 would result in the loss of protein modification and affect the gene expression of the protein making the structure and function of the protein incomplete. The manganese oxidation activity of the H120 mutant strain was significantly

reduced, which indicated that the protein modification of the multicopper oxidase CopA played an indispensable role in the manganese oxidation process.

Characterization of produced $BioMnO_x$

The $BioMnO_x$ formed by multicopper oxidase CopA and recombinant strain BL21-pET-*copA* were characterized. The oxide formed by CopA shows a uniform layered structure(FIG 6b). This is different from the granulated and irregular polyhedral $BioMnO_x$ produced by the multicopper oxidase CueO (Su et al. 2014). It was found by the energy dispersive spectrometer(EDS) sweep these oxide that the oxidized particles were mainly composed of Mn and oxygen in the FIG S3.

The morphologies and structures of the oxide were further characterized with TEM. TEM confirm that the $BioMnO_x$ has a multi-layered mesh structure with irregularly distributed holes and many connection points (FIG 6e). In contrast, the analytical reagent-grade MnO_2 particles were generally much larger crystalline particles and obviously needle structure(FIG 6c and 6f). The $BioMnO_x$ has a specific surface area and has a porous structure as compared with the chemically synthesized manganese oxide. Many studies have now used $BioMnO_x$ for the removal of heavy metal ions and organic contamination, and have achieved good results (Forrez et al. 2010; Sabirova et al. 2008).

SEM revealed finely grained, nanosize, amorphous, round manganese oxide particles produced by BL21-pET-*copA* (FIG 6a). Scanning these aggregates with EDS revealed that these aggregates were mainly composed of Mn, C and O elements in the FIG S3. Among them, C and O are the main elements of bacteria, and Mn and O are constituent elements of oxides. TEM observed that black small particles were formed on the surface of the cells, and as the manganese oxidation progresses, these particles become larger and larger, gradually covering the cell surface. (FIG 6d). This may be related to the physiology of microbial manganese oxidation, which oxidizes Mn(II) forming manganese oxide which surrounds the entire cell and forms a mesh-like structure. This can effectively protect cells from toxic substances, ultraviolet rays, reactive oxygen species, ionizing radiation, viruses and predation (Archibald and Fridovich 1981; Daly et al. 2004).

The valence electron configuration of the manganese atom is $3d^5\ 4s^2$, so that polyvalent oxides (+2, +3, +4, +6, +7) can be formed. EDS can only determine the elemental composition of manganese oxide, but can't measure its chemical valence. Therefore, the chemical valence of the $BioMnO_x$ were further determined by XPS. The XPS original map and fitting map of manganese oxides produced by CopA are shown in Fig. 7A. Four peaks were obtained by XPS peaking software with binding energies at 653.8 eV, 653.1 eV, 642.6 eV and 641.1 eV, respectively. The peaks of 653.8 and 642.6 are in agreement with MnO_2; the peaks of 653.1 and 641.1 are in agreement with Mn_3O_4The XPS original map and fitting map of manganese oxides produced by strain BL21-pET-*copA* are shown in Fig. 7B. Four peaks were obtained by XPS peaking software with binding energies at 653.8 eV, 653.1 eV, 642.2 eV and 641.1 eV, respectively. Given the literature comparison, the peaks of 653.8 and 642.2 are in agreement with MnO_2; the peaks of 653.1 and 641.1 are in agreement with Mn_3O_4. Thus $BioMnO_x$ contain three different valence states,

respectively Mn(II), Mn(III) and Mn(IV). By calculating the peak area, it is understood that the ratios of Mn(II), Mn(III), and Mn(IV) are 26.5%, 26.5%, and 47.0%, respectively, and the average oxidation state is 3.2. The average oxidation state of biological manganese oxides is generally lower than 4 because Mn(II) oxidation undergoes two successive one-step electron transfer processes, i.e., Mn(II) is first oxidized to Mn(III) and then Mn(III) oxidized to Mn (VI).

Discussion

Cu(□) is an essential factor for manganese oxidation of CopA

The manganese oxidation activity of CopA under copper-containing conditions is much higher than without copper, and CopA was able to oxidize of Mn(□) both in vitro and in vivo. In addition, the manganese removal efficiency of the strain under copper-containing conditions is much higher than that of the copper-free strain, due to the effect of Cu(□) on CopA. Especially for mutant strain BL21-pET-*copA*$^{120H-N}$, the effect of Cu(□) is more significant. It is speculated that the mutation destroys the HXH structure, and if there is Cu(□) in the system, the coordination with histidine can restore its stereostructure to some extent. The significant increase of Mn(□) oxidase activity was stimulated by Cu(□) show that Cu(□) was the essential cofactor and may enhanced the folding efficiency of CopA. The modification analysis of CopA by mass spectrometry showed that the loss of Cu(□) in the culture condition reduced the CopA modification, the position of the modification changed, and finally the protein activity decreased. One explanation is that the addition of Cu(□) induced further conformational changes in the R-loop and methionine-rich helix as a result of the new Cu-binding sites on the enzyme's surface (Wang et al. 2018). These observations well explain the copper dependence of CopA oxidase activity.

CopA has a higher manganese oxidation activity than other reported copper oxidases in the presence of Cu(□) (Su et al. 2013;(Su et al. 2014). CopA has weaker manganese oxidation activity even in the absence of copper. This result indicates that BL21-pET-*copA* can maintain its structural stability when induced to express CopA even if the culture system does not contain Cu(□).This is inconsistent with some MCO experimental results (Butterfield and Tebo 2017; Su et al. 2014), indicating that CopA is more stable and more application value than other MCOs.

H120 is the catalytic active site of CopA.

The known sequence similarity of multicopper oxidase is between 20% and 31%, but 1 Cys and 10 His and its surrounding amino acid ligands linked to a copper atom are relatively conservative (Hakulinen et al. 2002; Komori et al. 2014; Su et al. 2013; Zeng et al. 2018). This shows the importance of copper ion binding conserved sites. However, the effect of single site-directed mutation on the manganese oxidation ability of the strain was not expected to be large. Further experiments will perform simultaneous mutations at multiple sites. Based on this observation, it was proposed that coordination and cooperation between histidine at conserved sites and the HXH structure has high stability.

The manganese removal efficiency of the mutant strains is inconsistent, indicating that the four copper ion conserved binding sites have different effects on the manganese oxidation process. Under the condition of no copper, the mutant strain BL21-pET-*copA*$^{120H-N}$ almost lost the manganese oxidation ability, indicating that H120 is the catalytic active site of CopA.

The mutation of a single histidine has a little effect, but a single amino acid mutation can change the secondary structure of CopA. Protein modification analysis of CopA produced by the mutant strain BL21-pET-*copA*$^{120H-N}$ by mass spectrometry revealed that the modification was significantly reduced after the mutation. The presence of protein modifications can increase gene expression and is an important means of epigenetic regulation, so a reduction in modification results in incomplete structure and function of the protein. These results proved that the role of H120 site in manganese oxidation of CopA is critical.

In addition, a more efficient manganese oxidation engineering strain was obtained. The BL21-pET-*copA*$^{494H-N}$ strain is superior to the BL21-pET-*copA* strain. Recent structural and functional studies found the activity of MCO mutant is higher than wild-type MCO. The reason is that dramatic conformational changes in methionine-rich helix and the relative regulatory loop (R-loop) have occurred (Kataoka et al. 2007; Wang et al. 2018). Amino acid changes affect the redox potential and steric hindrance of copper ion binding conserved sites (Li et al. 2007). Further investigation is needed before real application of recombinant strains can take place.

The average valence of Mn in BioMnOx is 3.2..

In this study, The Mn valence state of $BioMnO_x$ was analyzed by XPS, and it was found that both the strain-mediated product and the CopA-mediated product were composed of MnO_2 and Mn_3O_4. By calculating the peak area, it is understood that the ratios of Mn(II), Mn(III), and Mn(IV) are 26.5%, 26.5%, and 47.0%, respectively, and the average oxidation state is 3.2. The average oxidation state of biological manganese oxides is generally lower than 4 because Mn(II) oxidation undergoes two successive one-step electron transfer processes, ie, Mn(II) is first oxidized to Mn(III) and then Mn(III) oxidized to Mn (VI).

The $BioMnO_x$ generated by BL21-pET-*copA* is granular, which is consistent with $BioMnO_x$ formed by *Bacillus* sp strain SG-1, which have irregular shapes and polyhedral nanocrystals (Su et al. 2014), and the $BioMnO_x$ generated by CopA is layered. One possible explanation for strain-mediated oxidation was that microorganisms remove heavy metals from the environment, not only by direct pathways involving enzymes but also by indirect mechanisms such as biosorption. The bacteria adsorb Mn(□) in the system and then oxidize the Mn(□) on the cell surface to form smaller nanoparticles, and many newly formed oxide colloids aggregated together and formed amorphous particles having a diameter of about 10 nm. The layered structure of CopA-mediated oxidation was that the formation of $BioMnO_x$ might be caused by a direct oxidation that catalyzed by CopA enzyme in a biotic process. Additionally, the layered MnO_2 exists in $BioMnO_x$ was also found in the natural environments (Mandernack et al. 1995). The $BioMnO_x$ formed by CopA has layered structure with irregularly distributed holes and many connection points.

Such a structure has high surface-area-to-volume ratio, the enzymatic synthesis of Mn oxide mineral might offer great potentials in biotechnological and industrial applications (Luo et al. 2019).

Conclusion

Four mutant strains were obtained by mutation of the conserved copper binding site of CopA by gene site-directed mutagenesis. Then the manganese removal efficiency of the four mutant strains was determined to understand the different effect of the conserved copper binding site on the manganese oxidation. It was found that H120 is the catalytic active site of the multicopper oxidase CopA, and the strain BL21-pET-*copA*$^{494H-N}$ with higher manganese oxidation activity was obtained. Protein modification analysis by mass spectrometry revealed that the loss of Cu(Ⅱ) and the mutation of the conserved copper binding site H120 resulted in the loss of modification of ethoxyformyl and quinone, the modification of amino acids was reduced, the position of modification was changed, and the activity of the protein was caused decline. It can be seen that the protein modification of the copper oxidase CopA plays an indispensable role in the oxidation of manganese.

These findings are of importance in production of highly Mn(Ⅱ) removal efficiency recombinant strain for industrial and/or environmental applications. Both CopA enzyme and its overexpression strain had high capacities to oxidize and remove Mn(II), offering potential applications for Mn(II) decontamination and heavy metal scavenging in water.

Declarations

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Conflict of interest

The authors declared that they have no conflicts of interest to this work. We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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Figures

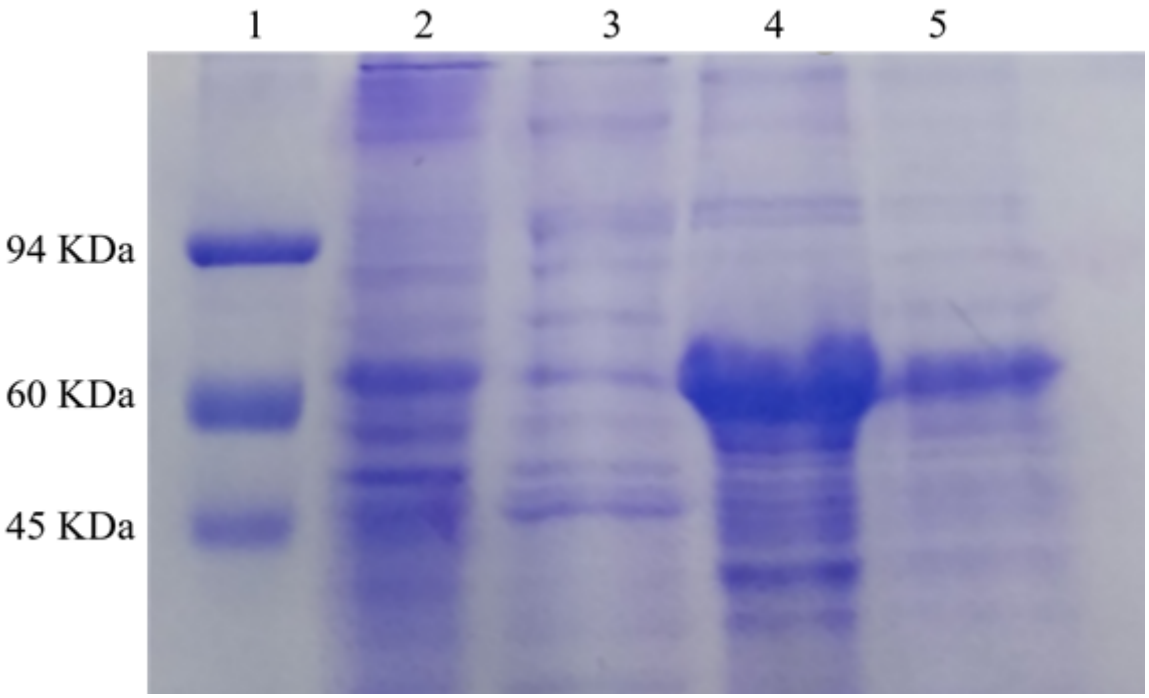

Figure 1

SDS-PAGE analysis results for protein stain. Lane 1, pre-stained protein marker (18-94 kDa); lane 2, crude enzyme; lane 3, flow-through; lane 4, purified protein by Ni Focurose 6FF column; lane 5, purified protein by Ni Focurose 6FF column and DEAE Focurose 6FF column.

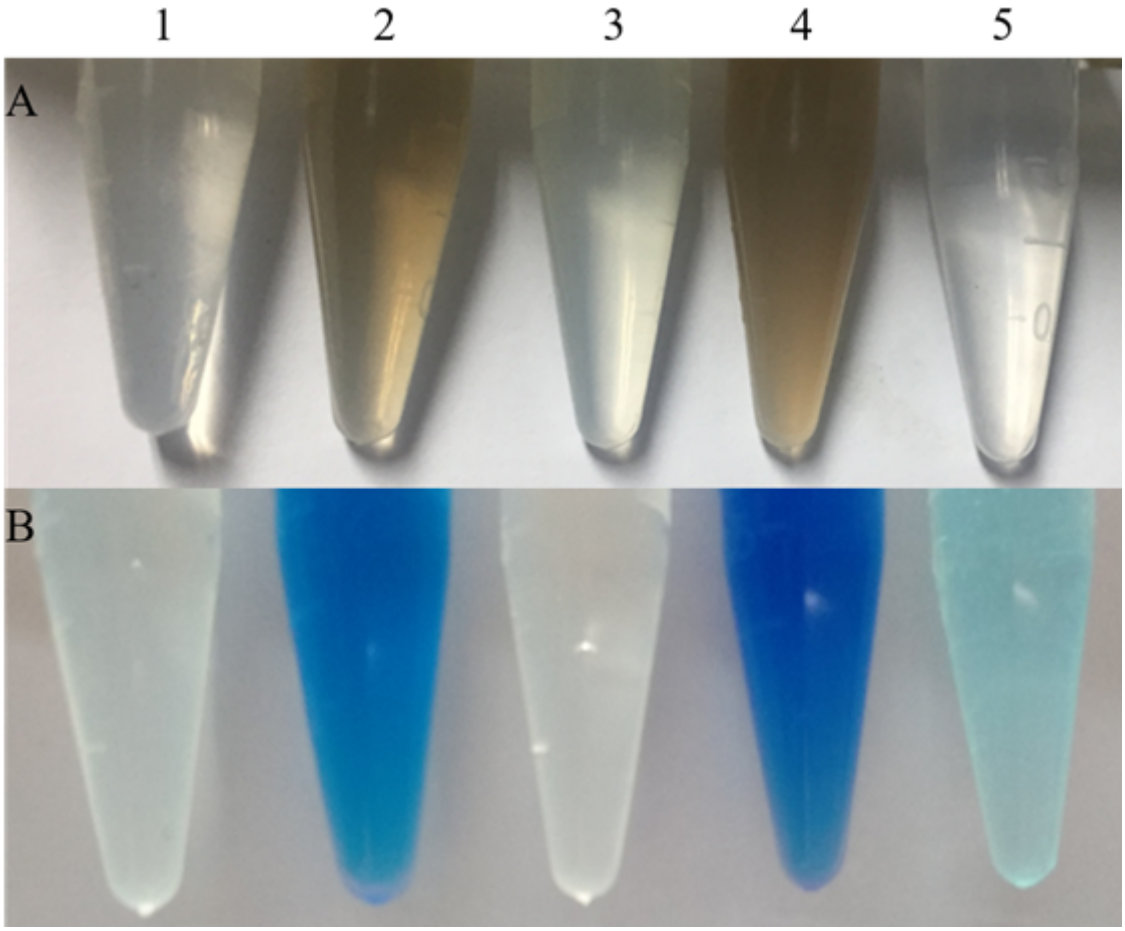

Figure 2

Mn(Ⅱ) oxidase activities of enzyme. A. Reaction mixtures contained 10 mM HEPES, 1 mM $MnCl_2$ and 1 mM $CuCl_2$ in the absence (tube 1) and presence (tube2-5) of 200 μL 2.3 mg/ml enzyme. B. Take 1 ml reaction mixtures from A and add 0.2ml 0.04%(w/v) LBB solution and 3 ml 45 mmol/L acetic acid solution. These tubes placed in the dark at room temperature for 2 h color reaction. Lane 1, Control group; lane 2, crude enzyme; lane 3, flow-through; lane 4, CopA (culture conditions containing 0.25 mM Cu(Ⅱ)); lane 5, CopA (culture conditions not containing Cu(Ⅱ).

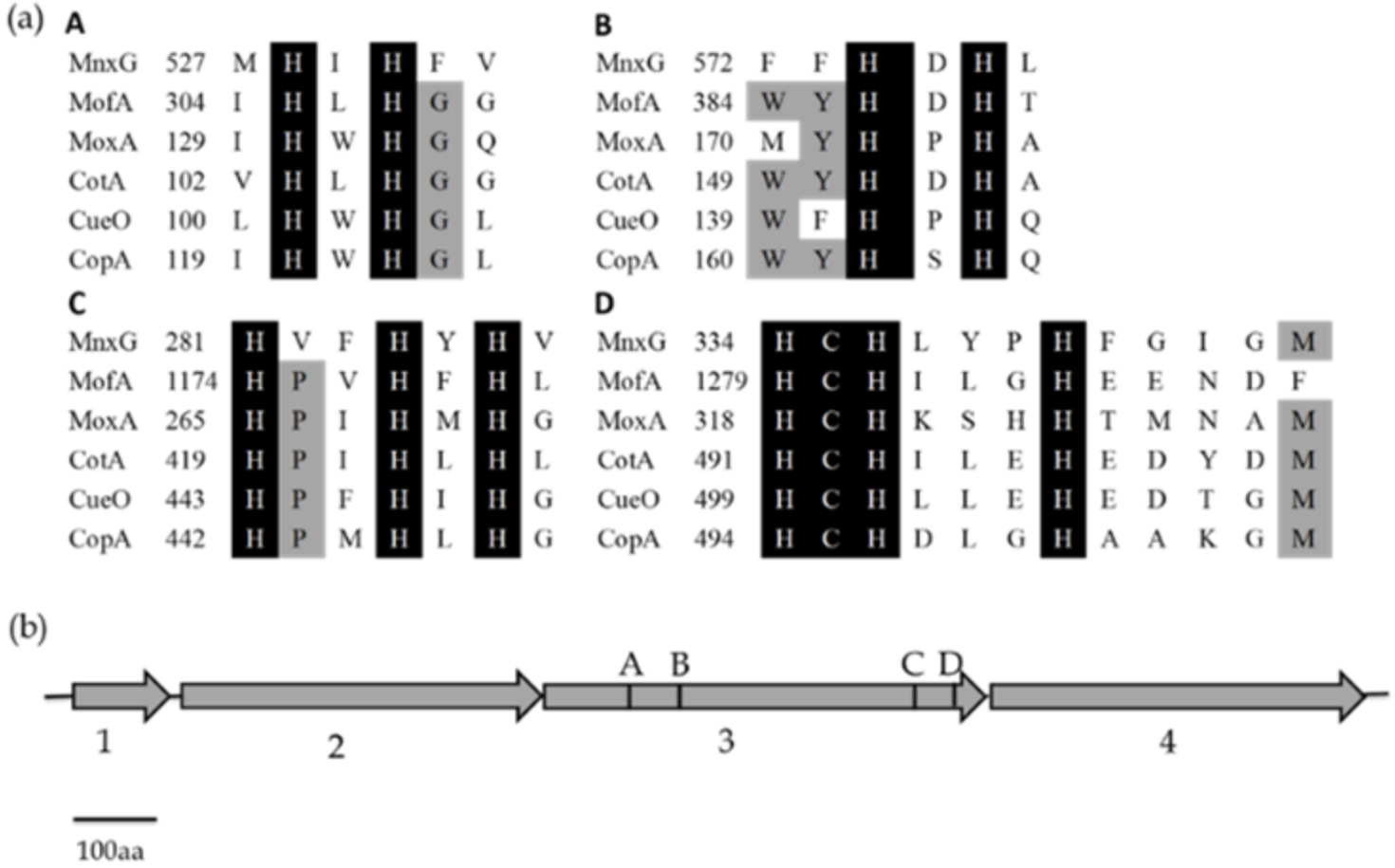

Figure 3

Comparison of amino acid sequences of four conserved copper binding sites in MCOs from different organisms.

Note: (a) black means 100% similarity, gray means 66.7% similarity; (b) 1: copper-sensing transcriptional repressor CsoR, 116aa; 2: transposase, 431aa; 3: copper oxidase, 529aa; 4: two-component sensor histidine kinase, 448aa.

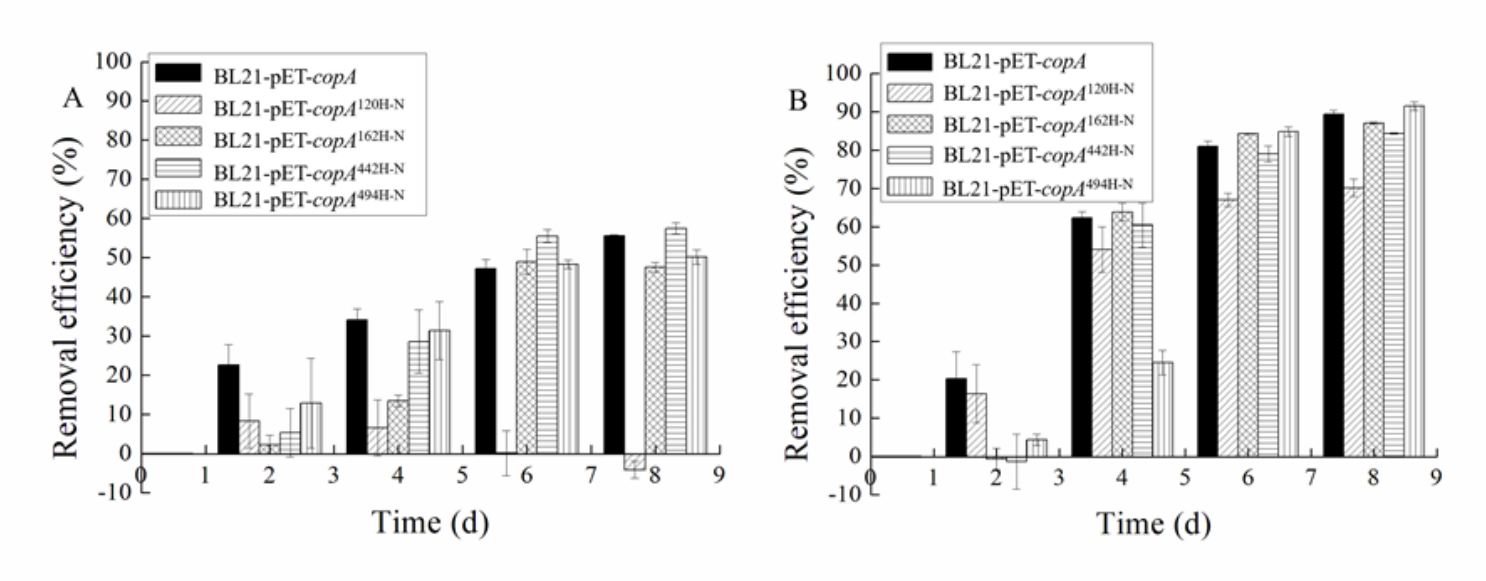

Figure 4

Mn(Ⅱ) removal efficiency of strains. BL21-pET-*copA*, BL21-pET-*copA*$^{120H-N}$, BL21-pET-*copA*$^{162H-N}$, BL21-pET-*copA*$^{442H-N}$, and BL21-pET-*copA*$^{494H-N}$ were grown at LB liquid medium containing 10 mM Tris-HCl(pH 7.0), 1 mM $MnCl_2$ and without 0.25 mM $CuCl_2$ (A) and with 0.25 mM $CuCl_2$ (B) for 8 days. The values were means ± standard deviations for triplicate assays.

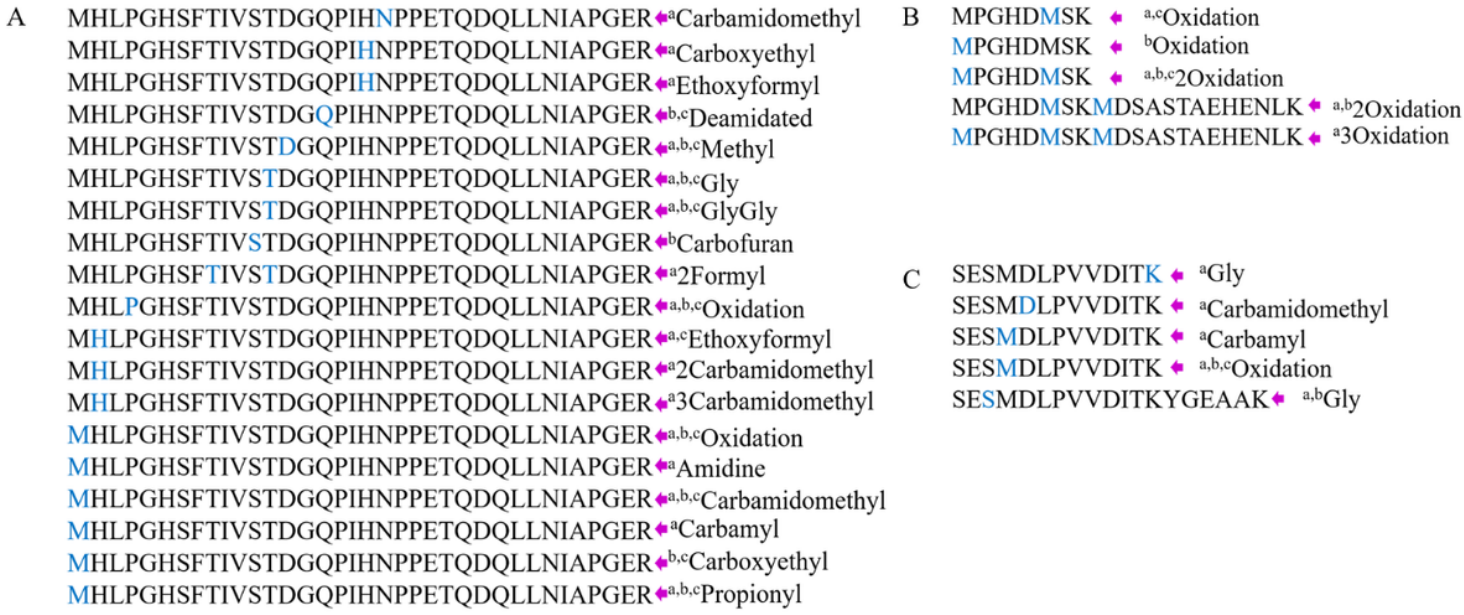

Figure 5

Protein modification information of peptide. A. MHLPGHSFTIVSTDGQPIHNPPETQDQLLNIAPGER; B. MPGHDMSK and MPGHDMSKMDSASTAEHENLK; C. SESMDLPVVDITK and SESMDLPVVDITKYGEAAK

Note: a: multicopper oxidase CopA produced by strain BL21-pET-*copA* under copper-containing conditions; b: multicopper oxidase CopA produced by strain BL21-pET-*copA* under copper-free condition; c: multicopper oxidase CopA produced by mutant strain BL21-pET-*copA*$^{120H\text{-}N}$ under copper-free condition.

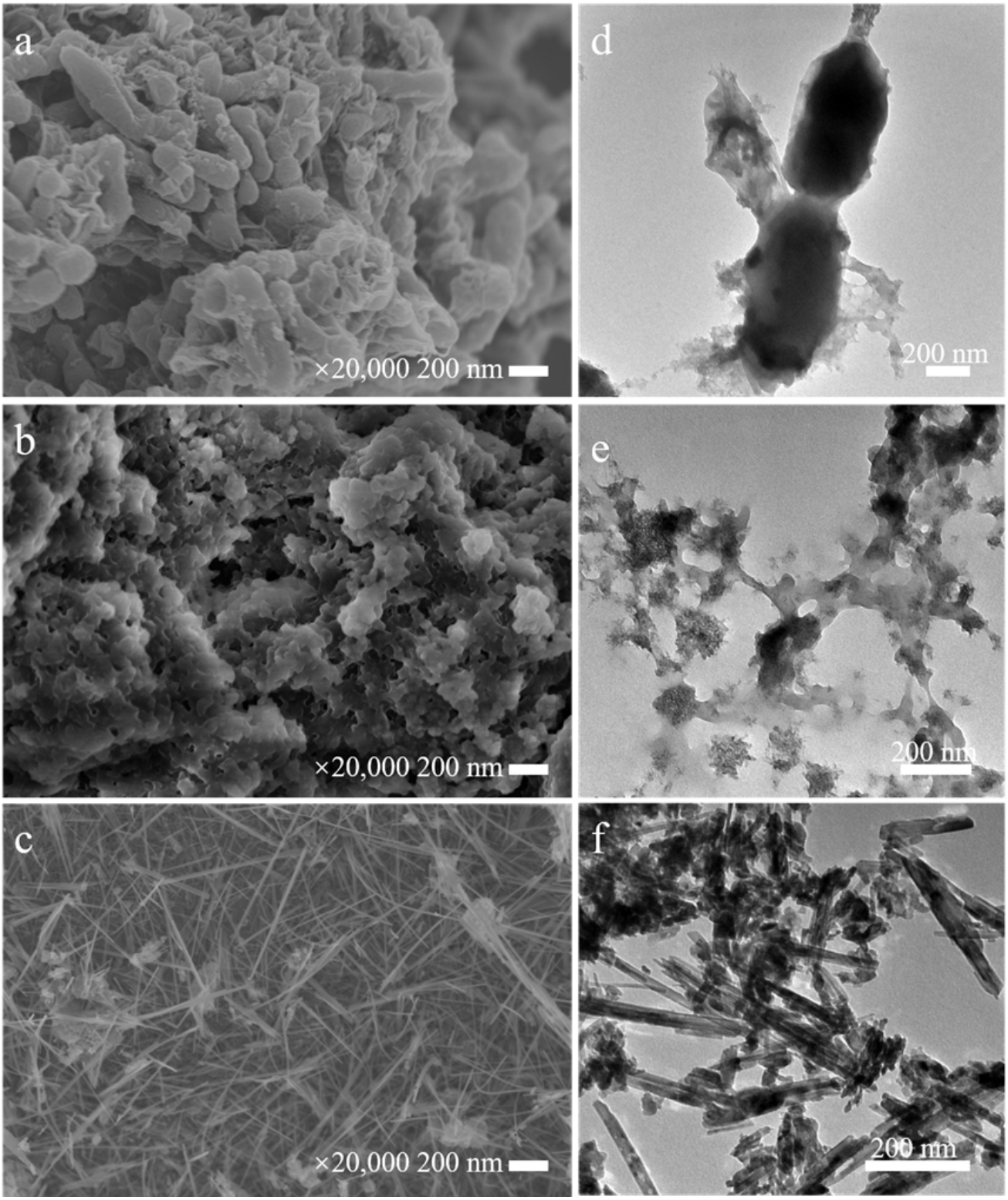

Figure 6

SEM and TEM photographs of BioMnO_x, and analytical reagent-grade MnO_2. (a, d) SEM/TEM image showing the morphologies of BL21-pET-*copA* cultivated with Mn(II) after 7 days. (×20,000); (b, e) SEM/TEM image of biogenic Mn oxides formed by CopA; (c, f) SEM/TEM image of analytical reagent-grade MnO_2.

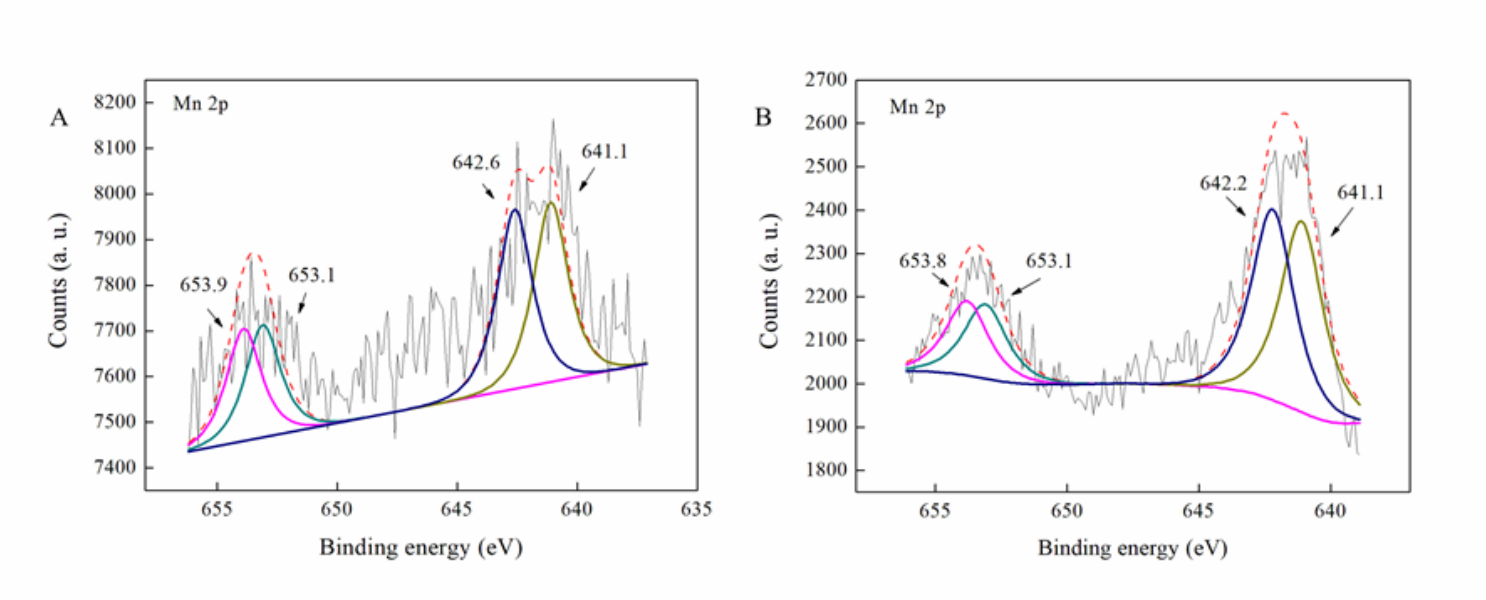

Figure 7

XPS photographs of BioMnO_x. A. BioMnO_x formed by multicopper oxidase CopA; B. BioMnO_x formed by recombinant strain BL21-pET-*copA*.

Supplementary Files

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