Catalytic oxidation of manganese(II) by multicopper oxidase CueO and characterization of the biogenic Mn oxide

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Abstract

Manganese(II) contamination is naturally occurring in many groundwater and surface water sources. Moreover, industrial wastewater is also responsible for much of the Mn(II) contamination. Nowadays, Mn(II) contamination has become a serious environmental problem in some regions of the world. To explore a biological approach for removing excessive amounts of aqueous Mn(II) from water, we found a new biocatalyst multicopper oxidase CueO, which was firstly proved to catalyze the oxidation of Mn(II) both in vitro and in vivo. Subsequently, we established a CueO-mediated catalysis system to prepare biogenic Mn oxide (BioMnOx), which was confirmed to be $\gamma$-Mn$_3$O$_4$ by X-ray diffraction. This newly prepared BioMnOx consisted of 53.6% Mn(II), 18.4% Mn(III) and 28.0% Mn(IV) characterized by X-ray photoelectron spectroscopy. It exhibited distinct polyhedral structure with nanoparticles of 150–350 nm diameters observed by transmission electron microscopy. Importantly, CueO could remove 35.7% of Mn(II) after a seven-day reaction, and on the other hand, the cueO-overexpressing Escherichia coli strain (ECueO) could also oxidize 58.1% dissolved Mn(II), and simultaneously remove 97.7% Mn(II). Based on these results, we suggest that ECueO strain and CueO enzyme have potential applications on Mn(II) decontamination in water treatment.

Keywords:
- Multicopper oxidase CueO
- Biogenic Mn oxide
- $\gamma$-Mn$_3$O$_4$
- Mn(II) removal
- Mn(II) oxidation

1. Introduction

Manganese (Mn) is one of the most abundant metals in Earth’s crust. Mn can exist in 11 oxidative states, but the most widely occurring forms in the environment are soluble Mn(II) when reduced and insoluble Mn(III/IV) when oxidized (Tebo et al., 2004). Naturally occurring Mn(II) is commonly found in drinking water sources and is essential for human health at low concentrations (Roth, 2009; Rivera-Mancia et al., 2011). On the other hand, excessive ingestion of Mn(II) can result in respiratory, reproductive, and especially neurological adverse effects for human beings (Roth, 2009; Rivera-Mancia et al., 2011). According to the World Health Organization (WHO), a...
Mn(II) concentration of <100 μg/L in drinking water is usually acceptable to consumers (Zeiner et al., 2012).

Groundwater is a main source of drinking water and the soluble Mn(II) often exceeds WHO standard (Miyata et al., 2007). Conventional chemical and physical treatments for Mn(II) removal from contaminated effluent consist of precipitation, depth filtration, ion exchange, adsorption and oxidation (Katsiyannis and Zouboulis, 2004). In recent years, biological Mn(II) oxidation, generally carried out by microorganisms (Katsiyannis and Zouboulis, 2004; Zhu et al., 2010), has been nowadays considered as a promising technique for aqueous Mn(II) decontamination (Miyata et al., 2007; Zeiner et al., 2012). Although Mn(II) is oxidized to form precipitates on exposure to oxygen or at higher pH values, the microbial oxidation of Mn(II) is faster than abiotic oxidation process at several orders of magnitude in natural systems (Miyata et al., 2007). Moreover, the resulting Mn oxides also have high capacities to adsorb other metals and toxic compounds, contributing to the biogeochemical cycles of various elements in natural environment (Tebo et al., 2004). Despite the fact that the structures and compositions of biogenic Mn oxides have been gradually identified (Villalobos et al., 2003; Webb et al., 2005; Zhu et al., 2012), the catalytic mechanism of microbial Mn(II) oxidation is not fully understood (Mann et al., 1988; Mandernack et al., 1995; Tebo et al., 2004).

So far, studies on biogenic manganese oxides have mainly focused on some model organisms (Mandernack et al., 1995; Nelson et al., 1999; Villalobos et al., 2003; Bargar et al., 2005; Parikh and Chorover, 2005; Zhu et al., 2010). In these strains, multicopper oxidase (MCO) type enzymes MnxC, McOA and MofA are identified to be responsible for Mn(II) oxidation (Geszvain et al., 2012). Recently, it has been proven that CtaO can oxidize Mn(II) in vitro and the ctaA-overexpressing strain also possesses the ability to remove Mn(II) (Su et al., 2013).

CueO (formerly known as YacK) is a periplasmic MCO that is associated with the copper efflux system and implicated in intrinsic copper resistance (Grass and Rensing, 2001). As a sole cupric oxidase, it can oxidize cuprous ion to less toxic cupric ion and therefore reduce the toxicity of copper (Grass et al., 2004; Kataoka et al., 2007). CueO also has the ability to oxidize a wide variety of substrates including ferrous iron, 2,2’-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), p-phenylenediamine, 2,6-dimethoxyphenol (DMP), and enterobactin, in the presence of copper (Grass and Rensing, 2001; Grass et al., 2004; Kataoka et al., 2007). However, none of the research has demonstrated the ability of CueO to oxidize Mn(II) (Tebo et al., 2004). In the present study, we speculated that CueO might have the ability to oxidize Mn(II) and to produce Mn oxides, simultaneously.

To prove the hypothesis, we cloned cueO gene from Escherichia coli and examined the Mn(II)-oxidizing activity with both purified CueO (in vitro) and recombinant strain (in vivo). Moreover, we characterized the generated Mn oxides using scanning electron microscopy (SEM), transmission electron microscope (TEM), X-ray diffraction (XRD) and X-ray photoelectron spectroscopy (XPS) techniques to complement the physicochemical data with morphological and structural information. These results provide compelling evidences that CueO possesses high Mn(II) oxidase activity and generates biogenic Mn oxide γ-Mn₃O₄, shedding new light on the removal of toxic metals from contaminated waters.

2. Materials and methods

2.1. Materials

Taq DNA polymerase, restriction endonucleases and T4 DNA ligase were purchased from TaKaRa Biotechnology Co., Ltd. (Dalian, Liaoning, China) or Fermentas (St. Leon-Rot, Germany). Isopropyl-β-D-thiogalactoside (IPTG), Ampicillin (Amp) and N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES) were obtained from Amresco (Solon, Ohio, USA). Nickel-chelating nitrilotriacetic acid (Ni-NTA) agarose column were bought from Genescript (Piscataway, NJ, USA). Leuco-berbelin blue I (LBB) was obtained from Sigma–Aldrich (St. Louis, MO, USA). KMnO₄ and pyrulosate β-MnO₂ were purchased from Chemical Reagent Corporation (Shanghai, China). All other chemicals were of analytical reagent grade.

2.2. Bacterial strains and culture conditions

E. coli strains K-12 substr. MG1655 and BL21(DE3) were routinely grown in Luria–Bertani (LB) broth or on agar plates at 37 °C. For Mn(II) oxidation assays, E. coli strains were cultured at 28 °C in LB, supplemented with 10 mM HEPES (pH 7.5) and 5 mM MnCl₂, Amp (100 μg/mL), IPTG (0.5 mM) and CuCl₂ (0.25 mM) were added as required.

2.3. Cloning, expression and purification of CueO

Genomic DNA from E. coli str. K-12 substr. MG1655 was employed to amplify cueO by PCR with the forward primer 5’-GAAAACATGGGGCAAGCTGCTGATTTCCTT-3’, and the reverse primer 5’-GTCTAGATTCTGGAACGTCGTGATTTCTT-3’. After purifying and digesting, the PCR product was ligated into the digested expression vector pET-22b(+) (Novagen, Madison, WI, USA) by T4 DNA ligase via the NcoI and XhoI restriction sites. Subsequently, the ligated product pET-cueO was transformed into E. coli BL21(DE3) (named EBL21) to obtain recombinant strain EcueO. The sequence of inserted gene was then confirmed by DNA sequencing.

EcueO was grown overnight and then transferred to fresh LB medium containing 100 μg/mL Amp (1% inoculation). After growing at 37 °C to an OD₆₀₀ of 0.6, IPTG and CuCl₂ were added to the final concentrations of 0.5 mM and 0.25 mM, respectively (Ueki et al., 2006; Kataoka et al., 2007). The culture was then incubated at 25 °C for 10–16 h (Hiall et al., 2008; Djokic et al., 2010). The C-terminal His-tagged recombinant protein was purified using a Ni-NTA resin with buffers as previously reported (Su et al., 2013). The purity of the isolated target protein was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentration was determined by Coomassie (Bradford) protein assay kit (Jiancheng Bioengineering Institute, Nanjing, China).
2.4. UV–visible spectroscopy and copper content of CueO

UV–visible spectrum of CueO (~50 µM) was acquired at room temperature in 20 mM Tris–HCl buffer (pH 8.0) using a DU 800 UV–Vis spectrophotometer (Beckman Coulter, Inc., Fullerton, CA, USA) (Grass and Rensing, 2001; Grass et al., 2004). The total copper content of CueO was measured by an Optima 8000 ICP-OES (inductively coupled plasma optical emission spectrometry, PerkinElmer, Norwalk, CT, USA).

2.5. Characterization of Mn(II) oxidase activity in vitro

Two assay systems were applied to verify the Mn(II)-oxidizing activity of CueO in vitro: (i) detection of the Mn-oxidizing activity by native PAGE, the in situ “in-gel oxidation assay”, and (ii) detection of the conversion reaction of Mn(II) to Mn oxide in liquid culture system.

For the native PAGE, a sufficient quantity of purified recombinant CueO was loaded in 12% polyacrylamide gels. After electrophoresis, the gels were treated with the previously described approach (Su et al., 2013).

Standard enzyme assay in liquid culture system was carried out as described below. The reaction mixture, consisting of 0.013 mg/mL purified recombinant CueO, 5 mM MnCl₂ and 1 mM CuCl₂ in 10 mM HEPES (pH 8.0), was incubated static at 37 °C for about 24 h. The produced Mn oxides were then quantified spectrophotometrically with LBB reagent (620 nm) according to the standard curve of KMnO₄ as described previously (Francis et al., 2001). Here, the Mn average valence in Mn oxide (Mn₃O₄) was 8/3, so the Mn (IV) oxidation on long-term exposure to oxygen. Simultaneously, we decreased the CuCl₂ concentration to avoid copper incorporation within the Mn oxide. The same liquid culture system without the addition of enzyme was set as a negative control to monitor the auto-oxidation. 5 mL aliquots were withdrawn every day to determine the residual Mn(II) by an ICP-OES.

After incubating at 37 °C under static condition for seven days, the produced brown precipitate was washed with distilled deionized water, and freeze-dried for further analyses.

2.8. Mineral identification of biogenic Mn oxides (BioMnOₓ)

To identify the crystalline phases of BioMnOₓ, the powder X-ray diffraction (XRD) patterns were recorded on a Bruker D8 Advance (Bruker AXS, Karlsruhe, Germany) equipped with a LynxEye detector using Ni-filtered Cu Kα radiation (λ = 0.15418 nm) (Yin et al., 2011). The diffractometer was operated at a tube voltage of 40 kV and a tube current of 40 mA with 0.12 s counting time per 0.02° 2θ step from 5° to 80°. Two control Mn oxides, the chemically synthesized Mn₃O₄ (Ardizzone et al., 1998) and commercial β-MnO₂ were assayed in parallel. Particle morphologies and sizes were characterized with a JSM-6390/LV scanning electron microscope (SEM, JEOL, Tokyo, Japan) (Su et al., 2013) and an H-7000 FA transmission electron microscope (TEM, Hitachi Co., Tokyo, Japan). For TEM observation, a sample was ultrasonically dispersed in ethanol, and then a drop of the suspension was pipetted onto a holy carbon-coated copper grid and dried at room temperature. The enlarged TEM micrographs were taken at an accelerating voltage of 75 kV and at magnification of ×10⁵ (Zhang et al., 2004).

The elemental valence states of samples were obtained by a VG MicroLab 2000 X-ray photoelectron spectrometer (XPS, Thermo VG Scientific, UK), using a Mg Kα X-ray source (1253.6 eV) and a base pressure of 3 × 10⁻⁹ Torr in the analytical chamber. The survey scans were collected with a pass energy of 100 eV and an energy step size of 1.0 eV, whereas the narrow scans had a pass energy of 25 eV and an energy step size of 0.1 eV (Yin et al., 2011). Each spectrum was calibrated using the C (1 s) binding energy (BE) at 284.8 eV, and the Shirley-type background was subtracted before deconvolution and fitting. The parameters (Santos et al., 2010; Yin et al., 2011, 2012) for the multiplet peaks of Mn (2p₃/2) were adopted in spectra fitting. Data were analyzed with the Avantage software version 4.75 (Thermo Fisher Scientific, East Grinstead, UK), using Gaussian–Lorentzian peak fitting.

2.9. Mn(II) oxidation tested in recombinant E. coli strain

Mn(II) oxidation was tested by mother strain EBL21 and recombinant strain ECueO, respectively. Culture conditions
were divided into three groups: strains grown in LB broth with 10 mM HEPES (pH 7.5) (Group I, it can be considered as a negative control group), in LB supplemented with 10 mM HEPES (pH 7.5), 5 mM MnCl₂ (Group II), and Group II appending 0.25 mM CuCl₂ (Group III). The recombinant strain ECueO was additionally supplemented with 100 μg/mL Amp to maintain the stability of recombinant plasmid. The strains in three groups were grown at 37 °C until the OD₆₀₀ reached to 0.6–0.8, then 0.5 mM IPTG was added to all of the three groups. The cultures were then shaken at 28 °C for eight days, because a lower temperature can slow the bacterial growth and maintain the Mn(II) oxidation activity for longer time during the slow bio-oxidation process. The Mn(II) oxidation abilities in Group II and Group III were calculated by subtracting the data of Group I, respectively. The residual Mn(II) concentrations in the supernatants of consumed media were measured by the ICP-OES. All assays were performed in triplicate. SEM technique was used to observe the morphologies of the microorganisms and Mn oxides, as described previously.

3. Results

3.1. CueO is a typical MCO

CueO protein was expressed with an intact 28-residue N-terminal signal for periplasmic translocation, which was removed during export through the TAT translocation pathway, and with a C-terminal his-tag for affinity purification (Hall et al., 2008; Djoko et al., 2010). According to the SDS-PAGE analysis (Fig. S1), CueO was mainly produced as a processed form (mature protein) with a molecular weight of 57.6 kDa, while the unprocessed form of CueO (precursor protein, 59.9 kDa) was also detectable in the periplasm (Grass and Rensing, 2001; Graubner et al., 2007). Therefore, the purified recombinant CueO displayed two overlapping protein bands with the two forms of proteins in the gels (Fig. S1 and Fig. 1). The total protein concentration was approximately 0.124 mg/mL as determined by Bradford method.

Amino acid sequence analysis revealed that CueO protein contained four conserved copper-binding motifs (Fig. S2) like other MCOs (Grass and Rensing, 2001). ICP-OES analysis showed that copper content was 4.05 ± 0.05 copper atoms per CueO molecule. Moreover, the UV–visible spectrum of CueO showed a maximum near 610 nm (Fig. S3). These data demonstrated that CueO possessed the typical features of MCOs.

3.2. CueO is a highly active Mn(II) oxidase

As shown in Fig. 1a, two brown Mn oxide bands were visible in gel. The active bands were coincident with the Coomassie blue-staining gel and turned blue upon addition of LBB reagent. On the other hand, lots of brown or black precipitates were present at the bottom of tube 3 in the liquid culture system containing Mn(II) and it was colored (tube 4) upon addition of LBB reagent (Fig. 1b). Both assays confirmed that the purified CueO had the ability to oxidize Mn(II) in vitro.

The enzymatic properties of CueO are described in Fig. 2. The pH-activity profile shows a high Mn(II) oxidase activity in the range 7.6–8.2 and a negligible activity at pH values below 7.0, while no auto-oxidation occurs in the control sample (Fig. 2a). Considering the fact that autocatalytic abiotic Mn(II) oxidation is kinetically inhibited at pH values lower than 8.5 (Rosson and Nealson, 1982; Larsen et al., 1999), pH 8.0 was therefore selected for further study. The maximal activity of Mn(II) oxidase was observed at 55 °C (Fig. 2b). The thermal stability assay indicated that CueO was quite stable ranging from 37 °C to 55 °C, and retained more than 90% of the maximum activity after incubation for 1 h (Fig. 2c). However, the activity decreased rapidly at temperatures above 64 °C, and only 19% of the original activity remaining after 1 h of incubation at 73 °C. Moreover, the activity of CueO showed a sharp decline when incubated at 46 °C and 55 °C longer than 5 h, respectively (data not shown). In view of the long-term reaction of Mn(II) oxidation, 37 °C was chosen as the moderate reaction temperature in the subsequent experiments.

As can be seen in Table S1, CueO typically preferred Cu²⁺ as its cofactor. Furthermore, the enzyme activity increased simultaneously with elevated concentrations of CuCl₂ at first. However, no further effect was observed at higher CuCl₂ concentrations from 1 to 2 mM (Fig. 2d). Therefore, 1 mM CuCl₂ was selected as the optimum concentration.

Besides, the activity of CueO was strongly inhibited by the organic compounds including SDS and EDTA, and was affected by o-phenanthroline and DTT at different degrees. While it was not influenced by guanidine-HCl (Table S2).

The specific activity of purified CueO towards Mn(II) was 1.46 U mg⁻¹. The kinetic parameters Kₘ, Vₘₐₓ and kcat of CueO, using Mn(II) as the substrate, were 17.33 ± 3.63 mM, 9.33 × 10⁻⁶ ± 0.23 M min⁻¹ and 2.09 ± 0.20 s⁻¹, respectively.

3.3. Removal of Mn(II) by CueO and characterization of produced BioMnOₓ

For the large-scale synthesis of Mn oxides, the reaction volume was eventually upscaled to 500 mL. As displayed in Fig. 3,
The effects of pH (a), temperature (b), thermal stability (c) and Cu²⁺ concentration (d) on Mn(II)-oxidizing activities of CueO. Standard enzyme assay system: 0.013 mg/mL CueO in 10 mM HEPES buffer (pH 8.0) containing 5 mM MnCl₂ and 1 mM CuCl₂ was incubated at 37 °C for 24 h. (a) Enzyme reaction in experimental group (■) was performed in the standard enzyme assay system except for pH ranging from 6.8 to 8.2, while the control group (▲) was the same as experimental group except without CueO. (b) Enzyme reaction was set at the standard enzyme assay system except for temperature. (c) Enzyme reaction was carried out at the standard enzyme assay system except that CueO was pretreated at different temperatures for 1 h. (d) Enzyme reaction was measured at the standard enzyme assay system except for Cu²⁺ concentration. The values were means ± standard deviations for triplicate assays.

Removal of Mn(II) from liquid culture system by purified CueO. The initial Mn(II) concentration was 5 mM and the residual Mn(II) concentrations in experimental group (■) and control group (▲) were measured by ICP-OES. The values were means ± standard deviations for triplicate assays.

Powder XRD patterns of typical Mn oxides: (a) BioMnOₓ, (b) chemically synthesized Mn₃O₄ and (c) commercial β-MnO₂.
the percentage of Mn(II) removal increased with the increase of contact time, reaching 35.7% after seven days by CueO enzyme. Correspondingly, the auto-oxidation was only 8.3% from the control group.

Fig. 4 illustrates the powder XRD patterns of BioMnOx, whose positions of reflections and relative intensities matched well with those of standard hausmannite \( \gamma \)-Mn_3O_4 (JCPDS 089-4837), indicating that they were the same mineral. Moreover, the characteristic diffraction peaks of this product at 0.490, 0.308, 0.275, 0.249, 0.204, 0.179, 0.154 and 0.144 nm were very similar to the chemically synthesized Mn_3O_4 (JCPDS 018-0803), although the intensity of latter peak was much stronger. However, the XRD peak intensity of BioMnOx was quite different from commercial \( \beta \)-MnO_2 (JCPDS 002-0567), a well-crystallized control, which exhibited detectable sharp peaks at 0.311, 0.241, 0.212, 0.163, 0.156 and 0.131 nm. Additionally, the big humps at about 2\( \theta \) 20\( ^\circ \) existed in the XRD patterns of BioMnOx and pyrolusite \( \beta \)-MnO_2 were caused by the glass slides that supported the sample.

The TEM micrographs of Mn oxide nanoparticles reveal that both BioMnOx (Fig. 5d) and chemically synthesized Mn_3O_4 (Fig. 5e) have the irregular shapes and polyhedral nanocrystals. By contrast, the commercial pyrolusite \( \beta \)-MnO_2 shows a uniform layered structure, with homogeneous nanoparticles of about 50,000 nm diameters (Fig. 5c). The morphologies and structures of the BioMnOx and Mn_3O_4 were further characterized with TEM. Particle size estimates for these oxides were hindered by particle aggregation, but the mean particle diameters might be safely assumed to be 150–350 nm for BioMnOx (Fig. 5d) and 15–60 nm for Mn_3O_4 (Fig. 5e). On the basis of these results, the product of biotic Mn(II) oxidation can be attributed with no specific shape, variable particle size and weak crystalline nature.

The Mn chemical states of BioMnOx and control Mn_3O_4 were next investigated by XPS. The result shows that the samples consist of Mn, O and C elements, yet no Cu signal is present in the spectra (Fig. S4). Detailed narrow scan spectra of Mn (2p) were also analyzed (Table S3). The binding energy value of Mn (2p_3/2) is 641.33 eV, and the spin orbit splitting
between the Mn 2p3/2 and Mn 2p1/2 level is 11.41, which perfectly matches the previously reported values for hausmannite (Dubal et al., 2010; Moses Ezhil Raj et al., 2010; Yang et al., 2013). Since Mn2+, Mn3+ and Mn4+ have essentially the closest and overlapped BEs, it is difficult to directly identify the oxidation state of manganese only by the BEs of Mn (2p) (Santos et al., 2010; Yin et al., 2012). Therefore, Curve-fitting of Mn (2p3/2) was taken and the results are described in Fig. 6 and Table S4. The percentages of Mn2+, Mn3+ and Mn4+ in Bio-MnOx are 53.6%, 18.4% and 28.0%, respectively, whereas the fitting data of chemically synthesized Mn3O4 are 24.1%, 73.3% and 2.6% respectively, which contains lower concentration of Mn4+.

3.4. Oxidation and removal of Mn(II) by the recombinant E. coli strain

ECueO possesses robust Mn(II)-oxidizing ability in the presence of Mn(II) after an eight-day treatment, especially when supplemented with CuCl2. The Mn(II)-oxidizing abilities of both E. coli strains cultivated in LB medium with different treatments are plotted in Fig. S5. After eight days in Group III medium, strains EBL21 and ECueO could oxidize 3.5% and 58.1% of Mn(II), respectively. While in Group II medium, the Mn(II)-oxidizing activities by both strains were a little less (1.2% and 52.7%, respectively).

The residual soluble Mn(II) contents of the eight-day cultivated supernatant were determined accurately by ICP-OES. 97.7% dissolved Mn(II) was removed by ECueO in Group III, while only 3.7% soluble Mn(II) was removed by EBL21 (Fig. S6). When cultivated with MnCl2 but without CuCl2 (Group II), the values were a little smaller (96.3% and 2.3%, respectively).

The SEM images of the Mn oxides and cell surfaces are shown in Fig. S7. In the presence of MnCl2, there are very few crystalline structures resembling Mn oxides on the strain surfaces of the EBL21, whereas many Mn oxides are deposited outside the cells of ECueO. Similar observations were obtained in ECueO when cultivated with CuCl2 and MnCl2, where the cell surfaces were also coated by some aggregated nonuniform nano-sized particles.

4. Discussion

4.1. CueO from E. coli is a periplasmic MCO with special characters

Recently, there has been considerable progress towards understanding of the properties of CueO, including substrate specificity, crystal structure and its essential function (Kataoka et al., 2007). Beside the cuprous oxidase and ferroxidase activity, the present study is the first time to present evidence that CueO from E. coli is a new Mn(II) oxidase. It can also oxidize ABTS and 2,6-DMP (Fig. S8) and therefore possesses laccase activity.

So far, only the CotA from Bacillus pumilus WH4 has been purified as an active Mn(II) oxidase by heterologous overexpression (Su et al., 2013). However, the currently well-defined MCOs like MnxG, MofA, and MoxA are unable to oxidize Mn(II) in vitro when produced from an expression vector in E. coli (Tebo et al., 2004; Su et al., 2013; Toyoda and Tebo, 2013). Fortunately, the recombinant CueO from E. coli in this study was able to oxidize of Mn(II) both in-gel and in liquid culture system. The significant increase of Mn(II) oxidase activity was only stimulated by Cu2+, as it was the essential cofactor and enhanced the folding of CueO (Brouwers et al., 2000). When compared the kinetic constant kcat/Km and specific activity of CueO with those of CotA (Su et al., 2013), CueO had remarkably higher (5.6 fold and 5.4 fold) Mn(II) oxidase activity than CotA did.

4.2. CueO-mediated Mn(II) oxidation produced hausmannite γ-Mn3O4

To date, there is no unequivocal evidence directly linking the Mn(II) oxidation with CueO in the laboratory, nor study on biological Mn oxides that have been synthesized by purified multicopper oxidases (Mann et al., 1988; Mandernack et al., 1995; Toyoda and Tebo, 2013).

In this study, the obtained BioMnOx by CueO was confirmed to be hausmannite γ-Mn3O4 by XRD. There were considerable conflicting evidences regarding the existence of hausmannite in previous studies by the marine Bacillus sp. strain SG-1.
Hastings and Emerson (1986) interpreted the occurrence of hausmannite (Mn3O4) as an intermediate in the oxidation of Mn(II) by spores. The formation of Mn3O4 was thermodynamically favored at a pH of 7.5–7.6 and Mn(II) concentrations over 30 μM. Mann et al. (1988) also observed hausmannite as an SG-1 oxidation product at 25 mM Mn(II). In contrast to these findings, Mandernack et al. (1995) suggested that SG-1 spores were capable of direct oxidation of Mn(II) to Mn(IV) without a hausmannite intermediate. Because hausmannite was very stable at high pH (>8) and Mn(II) concentrations >10–7 M, the further oxidation to Mn(IV) might be impeded due to relatively high Mn(II) concentrations (Greene and Madgwick, 1993; Mandernack et al., 1995; Bargar et al., 2005). Given that the Mn(II) oxidation was an extremely sophisticated process, it might involve both the biotic and abiotic reactions. However, the mechanism of hausmannite (γ-Mn3O4) production in the present study was not clear. One possible explanation was that the formation of γ-Mn3O4 might be caused by a direct oxidation that catalyzed by CueO enzyme in a biotic process. Another likely interpretation was that γ-Mn3O4 could be produced as a secondary product by the autocatalytic (abiotic) oxidation of Mn(II) which occurred at the surfaces of primary biogenic Mn(III,IV) oxides. Moreover, Bargar et al. (2005) pointed out that the reaction of Mn(II) with the primary δ-MnO2 resulted in the production of abiotic secondary products, feitknechite or a 10 Å Na phyllosilicate. Similarly, hausmannite (γ-Mn3O4) in the present study might also be a secondary products that caused by conproportionation reaction. Further understanding of the complex reaction steps in the transformation of Mn oxides and the concomitant changes of Mn states required the in-situ studies at different contacting time (Bargar et al., 2000, 2005). Additionally, the biogenic γ-Mn3O4 (hausmannite) identified in this study, was also found in the natural environments (Mandernack et al., 1995).

The particle size of BioMnOx was much bigger than chemically synthesized Mn3O4. The most possible reason was that BioMnOx nanoparticles were in fact constructed by smaller nanoparticles, and many newly formed Mn3O4 colloids aggregated together and formed nuclei (Li et al., 2011; Yang et al., 2013). This could be further confirmed by a high resolution TEM analysis. This anisotropic crystal growth followed a self-assembly mechanism and then experienced an Ostwald ripening dominated process (Li et al., 2011; Yang et al., 2013). Finally, the size of BioMnOx grew bigger and it exhibited distinct polyhedral nanocrystals with smooth surface as the reaction time was extended.

### 4.3. It is possible that layered MnO2 exists in BioMnOx

An improved understanding of the process of BioMnOx formation requires determination of the oxidation state of the product (Adams and Ghiorse, 1988), which can reflect the composition of BioMnOx to a certain extent.

In this study, the actual valence states of Mn in BioMnOx and the chemically synthesized Mn3O4 were fitted by XPS, the result showed that BioMnOx consisted of 53.6% Mn²⁺, 18.4% Mn³⁺ and 28.0% Mn⁴⁺. The high content of Mn⁴⁺ probably hinted at the existence of layered MnO₂, which might be reasonably interpreted as a primary product of biotic oxidation process by CueO enzyme. However, the formation of layered MnO₂ always occurred in a short time (Bargar et al., 2005), so it might not be detected on the seventh-day. Alternatively, as the layered MnO₂ (birnessite or vernadite) was usually XRD-poorly crystalline, its phase might not be detected by XRD analysis. Besides, BioMnOx also had a higher percentage of Mn²⁺ compared with the chemically synthesized Mn3O4, demonstrating a lower average oxidation state (AOS) in BioMnOx (2.67) than Mn3O4 (2.79).

### 4.4. CueO can oxidize and remove Mn(II) both in vitro and in vivo

The results revealed that CueO could remove 35.7% of Mn(II) after seven days, and the ability tended to increase slightly after the fourth day in liquid culture system, which was confirmatory to the observations of Mandernack et al. (1995). Importantly, the recombinant strain ECueO also displayed robust Mn(II)-oxidizing ability. It should be noted that the Mn oxide formed by CueO in vitro was γ-Mn3O4, as a result, it was reasonable to speculate that the valence states of Mn oxides produced by ECueO strain might be +8/3. If so, the Mn oxide concentration could be calculated by multiplying with a factor of 7.5 according to the KMnO4 concentration in standard curve just as the oxidation by CueO in vitro. Therefore, ECueO could oxidize 58.1% dissolved Mn(II), and meanwhile remove 97.7% Mn(II) when cultivated in LB medium with MnCl₂ and CuCl₂ after eight days. Two likely explanations could be put forward considering the discrepancy between the Mn(II) oxidizing and removal percentages. Firstly, Mn(II) was probably transported into the bacterial cells, lowering the concentration of soluble Mn(II) in the medium. Secondly, Mn oxide was a strong adsorbent for metal ions, and many of Mn(II) in medium were absorbed by the Mn oxides (Dashtban Kenari and Barbeau, 2013) that surrounding the bacterial cellular surfaces. Thus, the ingestion and adsorption might lead to a higher removal percentage.

### 4.5. CueO has many potential applications

The present study highlights the significant role of ECueO strain and purified CueO enzyme on removal of aqueous Mn(II), conducing to the development of efficient, economical and sustainable bioreactors with immobilized microorganisms or enzymes for Mn(II) decontamination in water treatment. The concomitantly generated Mn oxides, in turn may further remove Mn(II) (Dashtban Kenari and Barbeau, 2013) and many other toxic metals like As(III) (Saleh et al., 2011) or oxidize various kinds of organic compounds (Einaga et al., 2013; Saputra et al., 2013). The need to deepen our understanding of these important biogeochemical cycles demands greater knowledge of bacterial Mn(II) oxidation processes, their mechanisms, and products (Bargar et al., 2005). In addition, various methods for synthesis of Mn₃O₄ have attracted a great deal of attention due to its interesting magnetic and electrochemical properties, but most of the methods need high-temperature calcination (over 1000 °C) or decomposition (Dubal et al., 2010). Here, we propose a feasible enzymatic synthesis of Mn oxide minerals under environmentally favorable conditions, exploring promising applications in catalysis, chemical sensing devices, energy storage...
and rechargeable battery technologies (Li et al., 2011; Saputra et al., 2013).

5. Conclusions

To our knowledge, our study provided the first evidence that multicopper oxidase CueO could catalyze the oxidation of Mn(II) both in vitro and in vivo. The generated biogenic Mn oxide was confirmed to be γ-Mn3O4 by XRD, which consisted of 53.6% Mn3+, 18.4% Mn4+ and 28.0% Mn4+ characterized by XPS. Both CueO 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.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2014.03.013.

REFERENCES


