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Short Communication

Characterization of manganese oxidation by *Brevibacillus* at different ecological conditions

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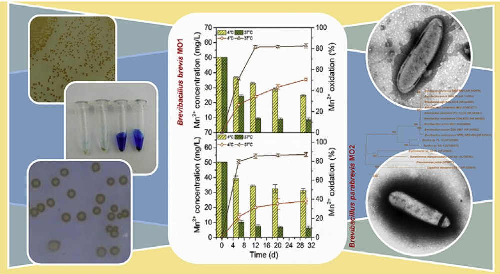
HIGHLIGHTS

- Two species of *Brevibacillus* have the capability of manganese oxidation.
- *Brevibacillus* can oxidize 1 mM concentration of Mn(II).
- *Brevibacillus* has capability of manganese oxidation at low temperature (4 °C).

GRAPHICAL ABSTRACT



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ABSTRACT

Bacterial Mn(II) oxidation plays an important role in the biogeochemical cycling of manganese and many trace metals. This study describes Mn(II) oxidation by two isolated manganese (Mn)-oxidizing strains that were identified and assigned as *Brevibacillus brevis* MO1 and *Brevibacillus parabrevis* MO2 based on physiochemical and phylogenetic characterizations. The ecological conditions influenced Mn(II) oxidation by both strains. Mn(II) stimulated the growth of strain MO2 while slightly inhibiting strain MO1. Mn(II)-oxidizing activity of two strains was enhanced with increase of initial pH, and maximum Mn(II)-oxidizing activity occurred at pH 8 for both strains (93.5%–94.0%). *Brevibacillus* showed the capability of mesophilic and psychrophilic Mn(II) oxidation. X-ray photoelectron spectroscopy (XPS) analysis indicated that the biogenic manganese oxides had an intermediate valence between 3 and 4. These results demonstrated that *Brevibacillus*, which is capable of oxidizing dissolved Mn(II), will be a suitable strain for exploring the mechanism of manganese oxidation in engineered and natural environments.

1. Introduction

Manganese-oxidizing microorganisms play an important role in the biogeochemical cycling of manganese, iron, carbon, nitrogen, sulfur, and several nutrients and trace metals since produced Mn(IV) oxides behaved as strong oxidants (Murray et al., 2007; Chinni et al., 2008; Spiro et al., 2010; Wang et al., 2013). Manganese (Mn)-oxidizing bacteria (MOB), isolated from various environments, such as soil, natural waters, rivers and sediments, have been identified in divergent phylogenetic lineages in the bacterial domain (Tebo et al., 2004). Although many MOB including *Leptothrix*, *Hyphomicrobium*, *Pseudomonas*, *Arthrobacter*, *Bacillus*, and *Citrobacter* (Douka, 1977; de Vrind et al., 1986; Boogerd and de Vrind, 1987; Francis et al., 2001; Geszvain et al., 2012) have been

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isolated and investigated, the knowledge about Mn oxidation and MOB still remains limited.

The possible pathways including indirect and direct oxidation of Mn(II) have been proposed (Gounot, 1994). Indirect oxidation mainly happens through modification of redox environments by increasing pH and Eh, free radicals (hydroxyl radical), reactive oxygen species (ROS) such as superoxide, and hydrogen peroxide degradation mediated by catalase (Nealson, 2006; Learman et al., 2013). A marine *Roseobacter* bacterium indirectly oxidizes Mn(II) via superoxide production mediated by extracellular haem peroxidases (Andeer et al., 2015). Direct oxidation is an enzymatic reaction catalyzed by Mn-oxidizing proteins such as multicopper oxidase (MCO) and heme-containing manganese peroxidases (MnPs). MCO enzymes have been identified in bacterial genera such as *Bacillus*, *Leptothrix*, *Pedomicrobium*, and *Pseudomonas* (Nealson, 2006; Nakama et al., 2014). MnPs have been identified in *Erythrobacter* sp., *Aurantimonas manganoxydans*, and *Pseudomonas putida* (Anderson et al., 2009; Nakama et al., 2014; Geszvain et al., 2016). Although bacterial multicopper oxidase specificity has broadened, the low abundance of copper-binding enzymes at translation level results in identification problems of the Mn(II) oxidase (Brouwers et al., 2000; Tebo et al., 2004; Geszvain et al., 2013). A recent study reported that co-culture of two non-Mn(II)-oxidizing strains showed Mn(II)-oxidizing activity by stimulating the production of bilirubin oxidase A (BoxA), which is similar to MCOs (Liang et al., 2016), implying a complicated mechanism of manganese oxidation. Moreover, previous studies showed that ecological factors (pH, temperature, and dissolved oxygen) affected manganese removal in engineered bioreactors (Pacini et al., 2005; Hoyland et al., 2014; Cao et al., 2015). The mechanism of manganese oxidation under ecological factors should be further investigated to enhance manganese oxidation in drinking water utilities through MOB bioaugmentation.

Soluble iron and manganese are widely found in groundwater (Qin et al., 2009; Patil et al., 2016), which can result in discolored water, stains on plumbing fixtures, unpleasant metallic taste, and rusty odor of the drinking water (Sharma et al., 2005; Funes et al., 2014). Because biological manganese oxidation is faster than abiotic processes by several orders of magnitude (Hastings and Emerson, 1986; Tebo et al., 1997), biological treatment methods for the removal of manganese has been more commonly used in drinking water supply plants than physicochemical methods (Hoyland et al., 2014; Cai et al., 2015). The potential benefits of biofiltration treatment systems are simple low-cost operation and high efficiencies of iron and manganese removal. However, there are still some challenges that need to be solved, particularly the long start-up time for the enrichment of manganese (Mn)-oxidizing bacteria (MOB) (Li et al., 2005; Cao et al., 2015). Therefore, insights into the mechanisms of microbial oxidation and reduction of manganese will be significant for Mn removal from drinking water utilities. The aim of this study was to isolate Mn(II)-oxidizing bacteria and reveal effects of ecological conditions on manganese oxidation.

2. Materials and methods

2.1. Isolation and cultivation of manganese-oxidizing bacteria

Mn(II)-oxidizing bacteria were isolated from activated sludge that was obtained from Wenchang municipal wastewater treatment plants (Harbin, China), and were cultured in K medium with 1 mM $MnSO_4$ solution in a 250-mL flask. K medium (L) was composed of (g/L): 0.1516 $MnSO_4 \cdot H_2O$, 0.001 $FeSO_4 \cdot 7H_2O$, 2 peptone, 0.5 yeast extract, 10 mM HEPES buffer, and 15 agar for plates (pH~7.2) (Nealson, 2006). The flask was then incubated at 37 °C for 14 days. The enriched culture of 10 ml was transferred to another flask for a second cycle of enrichment and cultured for 10 days. The suspension of 100 μL at appropriate dilutions was spread on agar plates for culture. Single brown-colored colonies were screened for the presence of Mn oxides via Leucoberbelin Blue (LBB, 0.04%) (Krumbein and Altmann, 1973). The colonies changed to blue color were re-incubated and then spread on agar plates in serial solutions. The procedure was repeated three times for isolation until pure cultures were obtained.

2.2. Identification of morphology and physicochemical characteristics

Strain morphology was examined using transmission electron microscopy (TEM) (JEOL JEM-1400) and atomic force microscopy (AFM) (Veeco Di, America). For TEM and AFM analysis, bacteria were pre-cultured using K medium. Prior to TEM analysis, the carbon support films on copper were socked in fresh cultures for about 20 min, and then stained using 1% phosphotungstic acid for about 40 s (Akob et al., 2014). A single colony was evenly spread on a fresh slide and dried naturally for AFM detection using tapping mode (Fotiadis et al., 2002). Cellular fatty acids were extracted, methylated, and analyzed using the standard Sherlock Microbial Identification System (MIS) (Miller, 1982; Sasser, 1990).

2.3. Phylogenetic analysis of isolates based on 16S rRNA gene

Single-colonies were collected and DNA extraction was processed according to the manufacturer's instructions (TIANGEN, Beijing, China). 16S rRNA gene was amplified by PCR using the universal bacterial primers of 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACCTTGTTACGACTT-3′), corresponding to base positions 8–27 and 1477–1492, respectively, of the 16S rRNA gene of *Escherichia coli* (Lane, 1991). Cloning, ligation, and transformation of amplified 16S rRNA gene products were conducted as previously described (Xing et al., 2006). Sequencing was performed on an ABI Prism 3730 genetic analyzer (Perkin-Elmer Applied Biosystems). A neighbor-joining phylogenetic tree was constructed using MEGA 7 software (Kumar et al., 2016). The tree topologies were evaluated by performing a bootstrap analysis with 1000 replicates. 16S rRNA gene sequences determined in this study have been deposited in the NCBI GenBank database under the accession nos. KX832899 and KX853077.

2.4. Detection of Mn(II)-oxidizing activity at different ecological conditions

Bacterial growth at different culture conditions was measured by optical density (OD) curve at 600 nm using the Bioscreen C automatic growth analyzer (Lab systems, Helsinki, Finland). The sterile medium of 300 ml with 10% (V/V) bacterial inoculum from different test conditions were distributed into each well of the Bioscreen C plates under continuous shaking at 37 °C. The turbidity at OD_{600} was automatically acquired every 15 min until the bacteria had reached the stationary stage of growth. A bacteria-free K culture medium was used as a control. All tests were conducted in triplicate.

Manganese oxidation by strains was detected at different ecological conditions including pH (4–8), initial concentrations of Mn(II) (0.01–1 mM) and temperatures (4 °C and 37 °C). Mn(II) oxidation by both strains under different conditions was detected for 10 days. To verify Mn(II) oxidation by the cell-free solution of pre-cultured inocula at 4 °C, the pre-cultured solution filtered through Millex-GS syringe filters (mixed cellulose esters, 0.22 μm of pore size) was used as inoculum. The K medium supplemented with $MnSO_4 \cdot H_2O$ of 1 mM was used for the manganese oxidation