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DGGE diversity of manganese mine samples and isolation of a *Lysinibacillus* sp. efficient in removal of high Mn (II) concentrations

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HIGHLIGHTS

- Manganese oxidizing bacteria such as *Bacillus*, *Hyphomicrobiaceae* and *Erythrobacter* were identified.
- An efficient manganese oxidizing bacteria (total manganese removal reached 94.67%) MK-1 was isolated.
- Dynamic equations of manganese removal process were set up.
- Proved the existence of Mn(III) in the oxidation process.

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ABSTRACT

Manganese contamination has become a serious environmental problem in the world and bacterial removal plays an important role in global cycling of manganese. In this study, microorganism distribution within samples from a manganese mine was analyzed with PCR-DGGE technology. The results suggested that Manganese oxidizing bacteria (such as *Bacillus*, *Hyphomicrobiaceae* and *Erythrobacter*) were dominant in the soil. In addition, a *Lysinibacillus* sp. Isolate, strain MK-1, revealed robust growth at high Mn(II) concentrations up to 1 mM. At that concentration, 55.94% of added Mn(II) was oxidized and 36.23% of the Mn(II) was adsorbed by MK-1(total manganese removal reached 94.67%) after 7 days of culturing. By measuring its metabolic process, the great role of biological adsorption was found. Additionally, the spectroscopic result demonstrated that Mn(III) was an intermediate during the biological oxidation process. These findings increase the knowledge of biological manganese removal mechanisms and show some potentials to the operation of manganese treatment.

1. Introduction

Manganese (Mn) is an important trace element that plays a significant role in our lives. In groundwater, manganese is often present in its reduced Mn(II) form, which is more soluble compared to other forms. Mn(II) common oxide MnO_2 can cause unsightly black discoloration of water (Cerrato et al., 2010). In addition, excessive manganese in water can do great harm to our health. In a recent study, Mn has been shown to be linked to neurotoxic effects in children (Su et al., 2013). As a result, the World Health Organization (WHO) recommended a manganese level of 9×10^{-4} mM (Ormanci et al., 2013). Traditional manganese removal methods were mainly focused on physical and chemical approaches, such as free chlorine oxidation, granular activated carbon adsorption, and membrane filtration (Katsoyiannis and Zouboulis, 2004; Choo et al., 2005). These techniques, although effective, have several problems to be solved, such as high cost, complex processes and low economic benefit (Wang and Han, 2012).

Many recent studies have focused on a new manganese removal method, microbial manganese oxidation, which has higher efficiency and makes up for the shortcomings of conventional methods

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(Webb et al., 2005b; Burger et al., 2008; Tang et al., 2014a). It is reported that microbial processes, driven by enzymes, catalyze the oxidation of Mn(II) to Mn(IV) up to five orders of magnitude faster than natural chemical oxidation (Webb et al., 2005a). Thus, most manganese oxides existing in soil, sediment, and aquatic environments are believed to have resulted from biological oxidation. Studies on biological removal have found that many bacteria are capable of oxidizing manganese in nature. These bacteria are mainly distributed in *Bacillus*, *Leptothrix*, *Pseudomonas* and *Pedomicrobium* (Nelson et al., 1999; Bargar et al., 2005; Parikh and Chorover, 2005). Since the 1980s, biological removal of manganese has been successfully applied at many facilities (Qin et al., 2009; Li et al., 2013; Yang et al., 2014). Although many studies on biological oxidation have considered multicopper oxidase (MCO) type enzymes, such as MnxG, McoA and MofA, to be responsible for Mn(II) oxidation (Geszvain et al., 2012; Su et al., 2013), the specific mechanisms explaining the oxidation and deposition of manganese remain poorly understood (Dick et al., 2008; Nakama et al., 2014). In addition, the manganese removal process, from biological adsorption to oxidation, closely related to facility operations, has been less reported.

The overall goal of this study was to evaluate the roles of biological oxidation and adsorption in manganese removal efficiency and the specific oxidation process of manganese from Mn(II) to Mn(IV). We screened some samples from a manganese mine (China, Hunan Province) with PCR-DGGE technology and identified a manganese-oxidizing strain MK-1, identified as *Lysinibacillus*, whose removal rate was up to 94.67%. By studying its metabolic processes, we demonstrated enzymatic roles, both intracellular and extracellular. Moreover, Mn(III) was detected during manganese oxidation by MK-1. The results of this study explain the possible biological manganese oxidation mechanism and further perfect its removal theory.

2. Materials and methods

2.1. DNA extraction, PCR amplification and DGGE analysis

Solid samples (S1, S2, S3) were collected from a manganese mine in Hunan province, China, 27°42′north latitude, 111°58′east longitude. S1 sampled in manganese ore channel downstream silt, S2 in up stream silt, S3 sampled from manganese slag. The temperature range of the samples is 20–25 °C, The pH range is 7.0–7.4. DNA from the samples was extracted by using an Ezup Column Bacteria Genomic DNA Purification Kit (Sangon Biotech, China). The concentration and quality of the DNA were examined by agarose gel (1%) electrophoresis and UV spectrophotometry (Nano-Drop2000, USA).

The V3 region of the 16S rRNA gene was amplified using the primers 341f: 5′-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCGCCTACGGGAGGCAGCAG-3′ (Muyzer et al., 1993) and 534r: 5′-ATTACCGCGGCTGCTGG-3′. PCR experiments (ABI Veriti 96 Well, USA) were performed using the following program: initial denaturation at 95 °C for 3 min, followed by 30 cycles of denaturation at 98 °C for 10 s, primer annealing at 56 °C for 30 s and extension at 72 °C for 30 s. A final long extension was at 72 °C for 10 min. PCR products were detected by agarose gel (1%) electrophoresis.

The DGGE analyses were performed using the Bio-Rad DCode system (USA). PCR products were separated on polyacrylamide gels (8%) with a 30–60% linear gradient of denaturant (100% denaturant corresponds to 7 M urea plus 40% formamide). The gels were run at 120 V, 60 °C for 9 h, stained with SYBR Green I (Sangon Biotech, China) for 30 min and photographed on a UV transilluminator (Bio-Rad).

2.2. Cloning and sequencing

Specific gel bands were excised and placed into a 1.5 mL centrifuge tube. The DNA fragments were recovered using a San-Prep Column DNA Gel Extraction Kit (Sangon Biotech, China). The target DNA fragments were re-amplified and ligated into the pMD19-T plasmid vector (TaKaRa, Japan). Then, they were transferred into competent *Escherichia coli* DH5a (TaKaRa, Japan). Several positive colonies of each sample were randomly chosen and validated by colony PCR. Then, they were sent to a company (Sangon Biotech, China) for sequencing. Based on the 16S rRNA gene sequences, sequence homology analysis was performed by using the BLAST nucleotide program.

2.3. Strain screening and sequence alignment

We placed 1 g of sample (S1) into a sterilized conical beaker with 100 mL of sterilized water. The conical beaker was stirred for 2 h at 140 r/min at 37 °C and left to stand for 10 min. Then, 2 mL of supernatant was inoculated into sterilized liquid PYCM culture medium (Xu et al., 2009) containing peptone 0.5 g/L, glucose 0.3 g/L, yeast extract 0.2 g/L, $MnSO_4 \cdot H_2O$ 0.2 g/L, K_2HPO_4 0.1 g/L, $MgSO_4 \cdot 7H_2O$ 0.2 g/L, $NaNO_3$ 0.2 g/L, $CaCl_2$ 0.1 g/L, $(NH_4)_2CO_3$ 0.1 g/L, ammonium iron citrate 0.1 g/L and HEPES 2.38 g/L and incubated for 4 days (140 r/min, 37 °C).

We diluted the above culture medium into eight concentration gradients (10^{-2}-10^{-9}) then took 100 μL of each dilution and spread it evenly over the surface of a sterilized PYCM solid agar plate. We incubated these agar plates in a constant temperature incubator at 37 °C for three days. Colonies of different shapes, sizes and colors were examined carefully for isolation. Each type of colony was streaked across a Petri dish until a single strain was obtained.

The ability of manganese oxidation was determined qualitatively using the LBB method (Francis et al., 2001) and quantified using an atomic absorption spectrometer (AAS; Agilent, USA). To select the dominant strain, we selected a single colony into LBB (Sigma, USA) solution consisting of 0.2 mL of LBB (0.04%) and 3 mL of HAc (45 mM) and measured its absorbance (620 nm) 2 h later. These screened strains were cultured in sterilized liquid PYCM medium for 7 days and AAS was used to measure their manganese removal capacity. We compared the strains' removal efficiency and selected the most efficient strain, then we extracted its DNA by using the Ezup Column Bacteria Genomic DNA Purification Kit (Sangon Biotech, China). The 16S rRNA gene was amplified using the primers 27F and 1492R (Francis and Tebo, 2002). The PCR program used was the same as in 2.1. The DNA sequencing was performed at Sangon Biotech (Shanghai, China). We compared with sequences in the GenBank database and constructed a phylogenetic analysis with Mega 6.0 software using the neighbor-joining (N-J) algorithm. Bootstrap analysis was used to estimate the reliability of the phylogenetic reconstructions (1000 replicates).

2.4. Oxidation and adsorption experiments

The strain identified as Mk-1 was inoculated into sterilized liquid PYCM culture medium (1%) and incubated at 37 °C and 140 r/min for 8 days with daily oxidation and adsorption measurements.

Total removal: The medium (2 mL) in a centrifuge tube was centrifuged at 12,000 rpm for 10 min. The manganese concentration of the supernatant was measured by AAS, which was the remaining manganese content *Ce*. As we knew the original manganese concentration *Co*, the total removal of manganese was calculated as follows:

$$\mathrm{Mn}(100\%) = (Co - Ce)/Co * 100 \quad (1)$$