Abstract of Dissertation
学位論文全文の要的

Title Novel Methods to Enrich Manganese Oxidizing Bacteria for Removal and Recovery of Minor Metals by bio-MnO_2
（バイオ MnO_2 によるレアメタル除去・回収のためのマンガン酸化細菌の新規集積培養方法）

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Minor metals are essential to modern technologies; however, their use and manufacture inevitably result in a portion of the metal being discharged into the environment. Heavy metals have negative effects on aquatic systems, even when present at low concentrations; accordingly, the removal and recovery of minor metals from wastewater streams is necessary. Recently, biosorption technology for the removal and recovery of metals has received a great deal of attention. Biomineralization has also been investigated as a promising method. Specifically, the application of biogenic manganese oxides (bio-MnO_2), which are products of biologically mediated Mn(II) oxidation processes, is very attractive because these compounds adsorb significant amounts of minor metals owing to their structural features. The key points for bio-MnO_2 application are to facilitate enrichment of manganese oxidizing bacteria (MnOB) and clarify how and why MnOB oxidize Mn(II) to produce bio-MnO_2.

To date, no autotrophic bacteria with the ability to oxidize Mn(II) have been identified. All currently known MnOB are heterotrophs. As a result, organic carbon is supplied during cultivation of MnOB. However, cultivation is not easily accomplished by simply adding substrate to open mixed cultures owing to competition with other fast-growing heterotrophs. Accordingly, a rich medium might not be favorable for enrichment of MnOB in engineering processes, and a better approach may be the use of very low concentrations of organic substrate. Despite its potential, this method may not enable organic loading rates to reach levels sufficient to enhance MnOB without interfering with the overall wastewater treatment processes.

Therefore, I have conceived a unique strategy to continuously provide low concentrations of organic substrate during cultivation of MnOB. During autotrophic nitrification, a small amount of organic carbon is excreted. Accordingly, cultivation of nitrifiers on ammonium (NH_4^+) without any added organic substances might result in the generation of SMP that could serve as a substrate for heterotrophic MnOB. Even if the NH_4^+ loading rate were increased, by decreasing hydraulic retention time (HRT) with maintaining low NH_4^+ concentration, a low concentration of SMP would be kept in the reactor. Indeed, nitrification activity was shown to stimulate Mn(II) oxidation in a rapid sand filter system.

In this study, long-term operations were conducted:

- To determine whether heterotrophic MnOB could be cultivated in combination with nitrification, and investigate the removal rates for Mn(II) and minor metals.
- To investigate how ammonium loading influences Mn(II) oxidation rate.
- To determine whether other particular organic carbon sources like activated sludge or dead cells could be used to replace nitrification in cultivation of MnOB.
- To determine whether MnOB could be cultivated at low pH range wastewater by employing the new method.

To achieve above objectives, this thesis was presented in 6 chapters:

✧ In **Chapter 1** the background, purposes and outline of the thesis are introduced.

- ✧ **Chapter 2** focuses on the diversity of MnOB, current methods for isolation and enrichment of MnOB, mechanism of Mn oxidation, biogenic Mn oxides (bio-MnO_2) and its features, the application of biological Mn oxidation (or its product bio-MnO_2) on removal of minor metals from wastewater.
- ✧ **Chapter 3** describes the idea and performance of the new method to enrich MnOB by coupled with nitrification using downflow hanging sponge reactor. Nickel and Cobalt were added during operation to investigate the ability of bio-MnO_2 produced in the reactor to adsorb minor metals.
- ✧ **Chapter 4** describes more about the cultivation of MnOB coupled with nitrification by investigating the influences of ammonium loading to MnOB. At ammonium loading equals zero, Mn oxidation was not stopped but stably increased, evokes a new insight about the substrate utilization of MnOB when coupled with nitrification.
- ✧ **Chapter 5** describes the cultivation of MnOB fed on activated sludge, an available abundant source of substrate for MnOB.
- ✧ **Chapter 6** describes the cultivation of MnOB at low pH range wastewater by employing the new method.
- ✧ **Chapter 7** shows the results obtained in this study.

The experimental outcomes and conclusions drawback from this study are briefly described below:

Biological oxidation of Mn(II) coupled with nitrification

In **Chapter 3** a new method to enrich MnOB by coupled with nitrification using downflow hanging sponge reactor (DHS) is described. Synthetic substrate contained Mn^{2+}, NH_4^+ and minerals without organic carbon matters, was continuously fed to the DHS reactor, a kind of trickling filter, has been developed for cost-effective wastewater treatment without imposing external aeration. The substrate contained no organic carbon matters to avoid a nuisance from other fast-growing heterotrophs. The only inoculation source of bacteria is activated sludge from a municipal sewage treatment. Nitrification was easily accomplished during operation since slow-growing nitrifiers were well cultivated in DHS reactor, which provided a continuously low concentration of organic carbon matters (SMP) for MnOB. During a long-term operation, Mn(II) oxidation was successfully established at a rate of 48 g Mn m^{-3} d^{-1} (based on sponge volume), indicating a successful in cultivation of MnOB in coupled with nitrification. The string of sponges in DHS reactor was covered with black particulates. The black particulates were also fall down and precipitated in the bottom of reactor, which were confirmed bio-MnO_2 by chemical indicator Leucoberbeline Blue I and through microscopic observation which showed microorganisms embedded in the black fine particles of Mn oxide. Ni and Co added to the influent were simultaneously removed via adsorption to bio-MnO_2. The removal molar ratios of Ni(II) and Co(II) to Mn(II) were 9 % and 45 %, respectively, which are comparable to the values reported by previous studies on the adsorption of minor metals to bio-MnO_2. Microbial 16S rRNA gene clone analysis identified nitrifiers supporting MnOB growth and showed that only one clone of *Bacillus subtilis*, which was affiliated with a known MnOB cluster, was present, suggesting the existence of other novel bacteria with the ability to oxidize Mn(II).

While an attempt to enrich MnOB to produce bio-MnO_2 failed under high organic substrate loading conditions by using K medium, it was effective when the strategy coupled to nitrification was employed. This result suggested oligotrophic conditions would be favorable for MnOB and would explain why MnOB have been detected and isolated from oligotrophic environments such as drinking water treatment biofilms and riverbeds, where nitrifiers also exit. This is the first report of cultivation of MnOB coupled with nitrifiers under open mixed cultures without external organic carbon matters supplied. The method described herein will be effective for wastewater contaminated with bacteria. The continuous cultivation of MnOB with nitrifiers in a DHS reactor amended with ammonium holds great potential for the removal and recovery of minor metals such as Ni and Co from wastewater.

Chapter 4 describes more about the cultivation of MnOB coupled with nitrification by investigating the influences of NH_4^+ loading to MnOB. The experiment demonstrated that

MnOB are much enriched when increasing nitrification strategy was employed, suggesting MnOB utilized more SMP from increasing nitrification via the increase of ammonium loading to DHS reactor. High Mn(II) oxidation rate of 0.7 kg Mn m^{-3} d^{-1} was successful established. Interestingly, MnOB were still enriched during a long-term operation without NH_4^+ addition, which in combination with former results evidenced the hypothesis that MnOB either utilized SMP by nitrifiers or dead cells to oxidize Mn(II) and formed bio-MnO_2 in the reactor because they were the unique substrate under this condition. Microbial 16S rRNA gene clone analysis in combination with 454 pyrosequencing technology identified different kind of MnOB under different operation: *Bacillus cereus* at no NH_4^+ supplied and *Hyphomicrobium*, coexisting with nitrifiers when NH_4^+ was supplied. The physiological functions of these MnOB species were not investigated in this study, but based on the literature, their existences in such environments showed physiologically relevance.

Promotion of Mn(II) oxidation and production of bio-MnO_2 in a bioreactor fed on activated sludge

Following chapter 4, a hypothesis that dead cells can be an alternative substrate for MnOB was investigated (**Chapter 5**). An attempt to enrich MnOB using activated sludge was failed, but once MnOB was inoculated, they can be enriched rapidly on activated sludge, a unique substrate under studied environment. A high Mn(II) oxidation rate of 1.7 kg Mn m^{-3} d^{-1} was successful established via the enrichment of MnOB fed on activated sludge from a municipal sewage treatment using a DHS reactor. The continuously promotion of Mn(II) oxidation in a DHS reactor periodically fed on activated sludge holds great potential for the removal and recovery of minor metals from wastewater because activated sludge substrate is abundant and no costly.

Biological Mn(II) oxidation under low pH condition

Chapter 6 describes the cultivation of MnOB at low pH wastewater by employing the new method described in above sections. The cultivation of MnOB coupled with methane oxidation and nitrification was employed. Since methane also produced organic carbon source, the coupling with both methane and nitrification was successful in cultivated MnOB under low pH as 5.0. However, Mn oxidation occurred only at pH 5.5 or higher even though the oxidation rate was fairly low (by 23 g m^{-3} d^{-1}). The microbial bacteria analysis showed representatives of MnOB, *Hyphomicrobium* (8 % of bacterial community) and methane oxidizing bacteria (*Methylosinus* and *Methylocaldum*), supporting growth of MnOB. Nitrification was appeared not suitable for the coupling cultivation at low pH because nitrification often results in the lower of pH which is thermodynamically unfavorable for Mn(II) oxidation.

In summary, this thesis provides novel methods to enrich MnOB, which can be applied for the removal and recovery of minor metals from contaminated water by bio-MnO_2.