EFFECT OF TEMPERATURE ON CLOSTRIDIUM SP. STRAIN PXL2 AND REMOVAL OF ARSENIC AND NITRATE FROM GROUNDWATER

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Abstract. Anaerobic Fe(II) Oxidation DeNitrifiers (AFODN) have recently been proposed as an option to treat groundwater contaminated by arsenic and nitrates through oxidation of microbiological nitratedependent ferrous oxidation. In previous studies, a pure strain of AFODN bacteria, Clostridium sp. Strain PXL2, was isolated from anoxic activated sludge. In this paper, the growth curve of strain PXL2 is studied under different temperature conditions, along with its Fe(II) oxidation, nitrate reduction, and arsenic removal efficiency. The results showed that at 5°C, the growth of the strain was significantly inhibited. At 15°C, the metabolic rate of growth, denitrification and Fe (II) oxidation rate were optimum and the total arsenic removal rate was high at 51.1%. The strain PXL2 can remove nitrate and arsenic contamination at the same time under normal groundwater temperature conditions. These analyses provide the necessary data to support the application of AFODN in engineering projects.

Keywords: *denitrification, iron oxide minerals, heavy metal, bioremediation, synchronize*

Introduction

Section title Arsenic contamination in water bodies due to the release of untreated industrial wastewaters has been on the rise due to excessive mining and smelting. The prevalence of arsenic and its compound in water bodies grows more serious every day and currently threatens the lives of more than 140 million people around the world (Karim, 2000; Smedley and Kinniburgh, 2002; Singh et al., 2007; Song et al., 2017). In China alone, it is estimated that more than 3 million people are exposed to elevated levels of As (Sun, 2004). New endemic areas have been progressively identified, such as the Huhot Basins in Inner Mongolia (Guo et al., 2001) and the Datong Basin in Shanxi Province (Wang et al., 2007) since the first case of chronic As poisoning was observed in the Xinjiang autonomous region in 1980 (Wang and Huang, 1994). Populations in affected areas suffer from melanosis, keratosis in their palms and soles, gangrene, and even skin cancer.

Among the numerous reduction processes of the soluble matrix, NO₃⁻ reduction plays a key role in the nitrogen cycle and has important implications for agriculture, environmental health, and public health (Shen et al., 2010; Xing et al., 2010). Natural nitrate attenuation is driven by microbial enzyme reduction, although, the abiotic reduction of nitrate also occurs under some extreme conditions (Hansen et al., 1996; Ottley et al., 1997; Zhang et al., 2014). Oxidation of microbiological nitrate-dependent Fe(II) to Fe(III) has been recently described with ferrous iron as the only electron donor

and with nitrate as the electron acceptor (Straub et al., 1996; Hafenbradl et al., 1996; Straub et al., 2004; Weber et al., 2006; Chakraborty et al., 2011; Li et al., 2015). These microorganisms are called the Anaerobic Fe(II) Oxidation DeNitrifiers (AFODN) (Wu et al., 2010). Enrichments of AFODN were successfully established with a variety of marine, brackish, or freshwater sediment samples (Kappler, 2005). The metabolism of AFODN is affected by many external factors (Chen et al., 2018). The major environmental factors include temperature, salinity, carbon source, pH, dissolved oxygen, and iron source. The oxidation of ferrous oxide directly affects the removal efficiency of heavy metals. To achieve the highest removal efficiency of nitrate and heavy metals and the best governance effect, it is necessary to provide optimum conditions for the ferrous oxidation and nitrate reduction by AFODN, which will help activate the microorganisms and lay the foundation for its application.

The response of different microorganisms to heavy metals is dependent on their physiologies (Li, 2017). Various treatment technologies to mitigate the severe problem of arsenic tainted groundwater have been extensively studied during last few decades (Hu et al., 2015; Niazi et al., 2018; Cui et al., 2018). Previous studies showed that low concentrations of arsenic promoted the growth of heterotrophic microorganisms. However, the growth of heterotrophic microorganisms was inhibited with the increase of As(III) concentration. While under the same exogenous arsenic concentration, it has a stronger inhibitory effect on autotrophic microorganisms than on heterotrophic microorganisms.

In this study, a novel AFODN species, the *Clostridium* sp. strain PXL2, was isolated from anaerobic activated sludge (Li et al., 2016), and its growth curve was studied under different temperature conditions. Fe(II) oxidation, nitrate reduction, and arsenic removal efficiency by strain PXL2 were observed under different temperature conditions. The resistance of strain PXL2 to arsenic, as well as its ability to oxidize microbiological nitrate-dependent Fe(II) to Fe(III) in an arsenic contaminated environment made it ideal for application in practical engineering. These analyses can provide the data necessary to support the application of AFODN in engineering projects.

Experimental materials and methods

An anaerobic nitrate-reducing Fe(II) oxidizing bacteria *C. freundii* PXL2 could synchronously remove As(III) and nitrate from water, associated with Fe(II) oxidation. Batch experiments were conducted to study the effect of temperature on ferrous oxidation and denitrification of PXL2 strains. SEM-EDS analyzed the morphologies under different temperature conditions. The removal of arsenic under different temperature conditions was studied at the same time. The selection medium (Li et al., 2014) was used as the culture medium with three repetitions for each sample.

Three tubes of mediums inoculated with AFODN culture were set to determine the efficiency of Fe(II) oxidation and nitrate reduction. The whole experiment was carried out in the G250 anaerobic, insuring microbial metabolism in the absence of oxygen. The amount of inoculation was 4% (vol/vol). All the tubes were sampled every 24 hours to determine the nitrate, nitrite, and Fe(II) concentrations. The effect of temperature on the denitrification of AFODN was performed. The initial concentrations of $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ and KNO_3 were 1 mM and 20 mM, respectively.

Arsenic pollution was simulated by NaAsO₂ to study the removal of arsenic by strain PXL2 at different temperatures. the initial concentration of As (III) was 60-70 µM (about 5 mg L⁻¹). To improve arsenic removal rate, the experimental analysis was extended to seven days. In addition to the analysis of total arsenic concentrations, the concentrations of As (III) and As (V) were also analyzed separately.

Concentrations of nitrate, nitrite, and Fe(II) were measured by a spectrophotometer (DU 800 UV/Vis Spectrophotometer, Beckman coulter, United States). N-(α-naphthyl)-ethylenediamine spectrophotometry was used to determine the concentrations of nitrite at 540 nm (Geng et al., 1996). Nitrate concentration was measured by UV spectrophotometry at 220 nm and 275 nm (Veena and Narayana, 2009). Phenanthroline spectrophotometry was used to determine Fe(II) concentration at 510 nm (Gendel and Lahav, 2008). For the modified phenanthroline assay, the samples were mixed with 40 mM sulfamic acid (pH approximately 1.8) instead of 25 vol% HCl because the sulfamic acid reacted rapidly with nitrite and prevented Fe(II) oxidation by the nitrite at an acidic pH (Klueglein and Kappler, 2013). All the samples were measured within 2 h after sampling. Total As concentration was measured by an AFS-8X atomic fluorescent spectrophotometer (Jitian Instruments Co., Ltd., Beijing, China). Ten ml of 5% thiourea and 5% vitamin C mixture and 2.5 ml of concentrated HCl were added to 50 ml of sample as As(V) reducing agent and stabilizer, respectively, and 2.0% (W/V) KBH₄ and 0.5% (W/V) KOH were used to transform the ion state As(III) to AsH₃. The flow carrier of samples was 5% (V/V) HCl. The original As(III) concentration in the water samples was measured by the same method without the addition of the 5% thiourea and 5% vitamin C mixture. Microbial morphology was observed by a Scanning Electron Microscope (SEM) (Zeiss Super 55VP, Germany) equipped with an energy dispersive X-ray (EDX) (Bruker XFlash 5010, Germany) spectroscope. Accelerating voltages ranged from 15 to 35 kV.

All the experiments were performed in triplicates. Error bars on graphs show the standard deviation. The data were analyzed by analysis of variance and the means were compared by Tukey's test ($p < 0.05$).

Results and discussion

Growth curve of PXL2 strains at different temperatures

Temperature is a major environmental factor that affects the growth of microorganisms. The temperature at which fastest microbial growth and reproduction occur is called the optimum growth temperature. The reaction rate increases with rising temperature, however, increasing temperature leads to the destruction of intracellular enzymes and nucleic acids and eventually, microbial death. Lower temperatures decrease cellular metabolic activity and lead to slow growth. A temperature that is too low triggers the formation of intracellular ice crystals that would lead to dehydration of the microorganisms and ultimately microbial death.

Based on the variation of groundwater temperature in seasonal alternations, three temperature gradients of 5°C, 15°C and 30°C were selected to study the growth and reproduction ability of PXL2 strains at different temperatures. In the selected medium access, 2% bacterial suspension were placed in 5°C, 15°C, 30°C anaerobic environment static cultures for 72 h and the absorbance of the bacterial liquid was measured at 600 nm. The results are shown in *Figure 1*. As can be seen from the figure, 5°C obviously inhibited the growth of the strain, and the OD value of the 96 h bacterial

liquid continued to decline, indicating that the strain cannot complete its metabolism at 5°C. At 15°C, some microbes could not adapt to the low-temperature environment and died after about 8 h of inoculation, after which the OD of the bacteria began to increase, indicating that the surviving microbes gradually adapted to the ambient temperature and the growth and metabolism rates were consistent with those at 30°C. The growth, propagation and metabolism rate are the highest, the bacterial stagnation period is short, and the logarithmic phase can be quickly achieved at this temperature.

Figure 1. Growth curves of PXL2 strains at different temperatures

The optimal temperature for bacterial oxidization under ferrous anaerobic conditions is mainly related to the source of separation. Ehrenreich et al. (1994) isolated two strains of Chromatium strain L7 and Rhodobacter strain SW2 from the sediments of freshwater ditches whose optimal temperature range for growth was 18-20°C, and found that they did not thrive at temperatures above 24°C. Hafenbradl et al. (1996) isolated an extremely thermophilic archaeon named Ferroglobus Placidus gen. Sp. Nov. from a hydrothermal system. The optimal temperature range of this strain was 65°C to 95°C and its doubling time was about 2.8 h. The bacteria could not grow when the environment temperature was under 63°C or exceeded 96°C. AFODN strain HidR2, which was isolated from seabed sediments grew at temperatures of 5 to 40°C while the optimum temperature was 33°C (Benz et al., 1998). For two strains of photosynthetic non-ferrous anaerobic oxidative bacteria, strain N1 and strain N2, which were also separated from seafloor sediments, the optimal temperature ranges were similar to strain HidR2. The optimal growth temperature of strain N1 was 20-25°C, while the optimal growth temperature of strain N2 was 25-28°C (Straub et al., 1999). For AFODN strains BrG1 and BrG2, which were isolated from brackish water lakes and urban ditches, their growth rate at 15°C was greater than that at 28°C (Straub et al., 1996).

The strain PXL2 was isolated from the ditch sediments. It is a normal temperature living environment. So the strain PXL2 can grow at temperatures of 5 to 30°C. And the growth rate increases with temperature within the scope of the study.

Ferrous Oxidation and Nitrate Reduction of PXL2 Strains at Different Temperatures

The effect of temperature on the ferrous oxidation and nitrate reduction of AFODN is primarily due to the metabolic activity of the microorganism (Leahy and Colwell, 1990). The ferrous oxidation of AFODN and nitrate reduction is based on specific metabolic methods of microorganisms, and the catalytic effect of the enzyme is most efficient only within the optimum temperature range.

Figures 2A, B and C show the changes of Fe (II), NO_3^- and NO_2^- concentrations in the medium inoculated with PXL2 strains with time at different temperatures, respectively.

Figure 2. Time profile of Fe(II), NO_3^- and NO_2^- concentrations in the strain PXL2 culture at different temperatures

It can be seen from the figure that at the same oxidation rate, the temperature has little or no effect on the oxidation of ferrous ions within 4 h after inoculation. However, with time, the low temperature (5°C) sample significantly inhibits the oxidation of Fe (II). At 5°C, the ferrous oxidation rates were significantly lower than those at 15°C and 30°C. The oxidation rates of ferrous iron at 15°C and 30°C were almost the same as the initial Fe (II) concentration of 1 mM. About 90% of Fe (II) was oxidized after 24 h. Fe(II) oxidation was completed and nitrate degradation was also completed. As shown in Figure 2B, the nitrate degradation ability of PXL2 strains did not change significantly within 4 h after the completion of Fe (II) oxidation. However, with the duration of

inoculation, nitrate degradation ability was quite different. At 5°C, the biomass stagnated after 12 h of inoculation and no longer had the ability to degrade nitrate. The same degradation rate was maintained at 15°C and 30°C, but strain PXL2 completed 19.5% nitrate degradation at 15°C after 72 h of inoculation, While at 30°C, 24.8% nitrate degradation was completed, which is slightly higher than that at 15°C. The trend of accumulation of nitrite coincides with the trend of degradation of nitrate. Higher accumulation of nitrite, 1.7 mM, was observed in 30°C environments. The second highest concentration is the concentration of nitrite at 15°C reached 1.3 mM. No nitrite was accumulated in the environment at 5°C.

The PXL2 strains showed different rates of ferrous oxidation and nitrate degradation at different temperatures, which may affect the reproduction of microorganisms. At the same time, the oxidative degradation ability of AFODN at low temperature was greatly reduced. The low temperature inhibited the growth of microorganism's intracellular enzyme activity, so that it reduced the ability of oxidation and degradation.

SEM-EDS Analysis of Sediments at Low Temperatures

SEM analysis of microbial antioxidant sediments at 5°C, 15°C and 30°C (*Fig. 3*) shows that the morphology of the cells changed significantly at 5°C, the bacillus coarsened and shortened, and obvious dents generally appear, possibly as a response to the low temperature.

Figure 3. SEM analysis of microbial ioxidant sediments--strain PXL2 at different temperatures (The star indicated the point of EDS analysis in Figure 4. A: 5°C; B: 15°C; C: 30°C)

At 30°C, the cells were incrustated in nanosize Fe(III) oxides with only few cells being completely free of surface precipitates (Fig. 3c). The average diameter of the Fe(III) oxides was between 100~200 nm.

EDS analysis of ferrous oxide products at 5°C, 15°C and 30°C. Figure 4 shows that the sediments formed at 5°C mainly consist of C and O, with only a small amount of Fe. The highest C content was 62.41%, the content of O was 33.15% and the content of iron was only 0.35%. The contents of Fe in the sediments formed at 15°C were higher at about 9.64%, which far exceeded the content of Fe in the sediments formed at 5°C. At 30°C, EDS analysis showed that the precipitates were mainly composed of C (22.85%), O (40.68%) and Fe (22.41%). Iron content exceeds at the first two temperatures.

Figure 4. EDS analysis of strain PXL2 sediments at different temperatures (A corresponding Fig. 3A; B corresponding Fig. 3B; C corresponding Fig. 3C)

Based on the above analysis, we can see that low temperature environments had inhibitory effects on strain PXL2. At 5°C, the cell morphology changed and the Fe content in sediment was extremely low and mainly existed in pericellular region instead of the cell surface while Temperature of 15°C seemed to have less of an effect on the

metabolism of microorganisms, and the morphology of the cells remains the same, but the content of Fe was at least 50% lower than the sediment at 30°C. Thus, 30°C is most suitable for the growth and metabolism of strain PXL2. Under this temperature, microbial metabolism is the fastest, and the rate of Fe oxidation and the content of Fe in sediment are the highest.

Removal of arsenic by strain PXL2 at different temperatures

The removal of arsenic at different temperatures is shown in *Figure 5*. The different amounts of arsenic are removed at different temperatures over time. After 7 days of treatment, total arsenic concentrations decreased from 70 µM to 54.9 µM, 34.2 µM and 49.0 µM at 5°C, 15°C and 30°C, the removal rates were 21.6%, 51.1% and 30.0%, respectively. The highest rate was at 15°C.

Figure 5. Total arsenic removal efficiency at different temperatures

Strain PXL2 has the ability to remove arsenic by arsenic oxidation. As seen in *Figure 6*, as total arsenic is removed, some As (III) is oxidized to As (V). At 5°C and 30°C, with the removal of total arsenic, the concentration of As (III) decreased and the concentration of As (V) increased continuously. The percentage of As (III) in total arsenic dropped from the original 90% to 62.1% and 54.0%, respectively. The corresponding As (V) concentrations increased from 10% to 37.9% and 46.0%, respectively. The conversion of As (III) to As (V) at 15°C is less pronounced with respect to the other conversions at two temperature, but some of the As (V) production can be seen.

From the above analysis we can see that temperatures that are too high or too low are not suitable for strain PXL2 arsenic removal, with 15°C being the most suitable temperature. One of the reasons may be that enzyme catalysis in microbial metabolism is inhibited severely at extreme low and high temperatures. Compared with the medium temperature, microbial metabolism is active in high temperature environments, and therefore results in the fastest ferrous oxide oxidation rate. The rate of ferrous oxidation directly affects the type of oxidation product. Different types of iron oxides have different arsenic adsorption capacities. The environment of oxidative denitrification of AFODN anaerobic ferrous compounds is mainly for the pollution of nitrate and arsenic in groundwater. The perennial groundwater temperature is maintained at 15°C~17°C, which is very conducive to AFODN reproductive metabolism and enzyme catalysis. Therefore, strain PXL2 is suitable for applications to groundwater pollution control.

Figure 6. Time profile of As concentrations in the strain PXL2 culture at different temperatures

Conclusion

Growth of strain PXL2 was significantly inhibited at 5°C. At this temperature, morphological changes were also seen, where a rod-like shape changed to a spherical one, and dents appeared on the bacterial surface. Under this condition, the denitrification ability and Fe (II) capacity of the strain decreased significantly. The As removal ability was also affected. The total arsenic removal rate was at 21.6%. At 15°C, the metabolic rate of growth, denitrification and Fe (II) oxidation rate were slightly lower than 30°C, but the total arsenic removal rate was the highest at this temperature, at 51.1%. The total arsenic removal rate at 30°C was 30.0%. On the whole, the strain PXL2 metabolized well at 15°C, and was found to be suitable for treating nitrate and arsenic contamination in groundwater. This biological treatment has promising applications in future projects. This research promotes the research of ferrous anaerobic micro-organisms in this new field of research.

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