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Anaerobic nitrate reduction with oxidation of Fe(II) by *Citrobacter Freundii* strain PXL1 – a potential candidate for simultaneous removal of As and nitrate from groundwater

Baohua Li [b,c], Xiangliang Pan [c,*], Daoyong Zhang [a,c,**], Duu-Jong Lee [d], Fahad A. Al-Misned [e], M. Golam Mortuza [e,f]

[a] State Key Laboratory of Environmental Geochemistry, Institute of Geochemistry, Chinese Academy of Sciences, Guiyang 550002, China
[b] Xinjiang Institute of Engineering, Urumqi 830091, China
[c] Xinjiang Key Laboratory of Environmental Pollution and Bioremediation, Xinjiang Institute of Ecology and Geography, Chinese Academy of Sciences, Urumqi 830011, China
[d] Department of Chemical Engineering, National Taiwan University, Taipei 10617, Taiwan
[e] Department of Zoology, College of Science, King Saud University, Riyadh 11451, Saudi Arabia
[f] Department of Zoology, Faculty of Life and Earth Science, Rajshahi University, Rajshahi 6205, Bangladesh

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ABSTRACT

Nitrate and arsenic are common pollutants in groundwater. However, we are still lacking technologies that can remove nitrate and arsenic simultaneously using a single species of microorganism. In the present study, the potential of synchronous removal of nitrate and arsenite from water by a single anaerobic nitrate-reducing Fe(II) oxidizing *Citrobacter freundii* strain PXL1 was assessed. The strain PXL1 could grow well in arsenite concentration of 2.85–13.65 μM and efficiently oxidize Fe(II), reduce nitrate and remove As(III) from water. Scanning electron microscopy, X-ray diffraction spectroscopy, and Fourier transform infrared spectroscopy analysis showed that the strain PXL1 produced poorly crystalline Fe(III) oxides. As(III) was removed mainly by adsorption and co-precipitation of the biogenic Fe(III) oxides. The anaerobic nitrate-dependent Fe(II) oxidizing bacteria including *C. freundii* PXL1 are promising microbes for *in situ* remediation of nitrate and arsenite contaminated groundwater.

1. Introduction

Arsenic and nitrate are the most common pollutants in groundwater, especially in some rural endemic arsenic poisoning areas (Fytianos and Christophoridis, 2004). Arsenic is highly toxic to human being and can cause many serious diseases including cancer, respiratory system, digestive system, and skin diseases (Liu et al., 2003). The World Health Organization's maximum contaminant level (MCL) of arsenic is 10 $\mu g\,L^{-1}$. Arsenic pollution is usually caused by geological reasons and mining activities (Camacho et al., 2011) while nitrate pollution is mainly due to intense application of nitrogen fertilizers (Oenema et al., 1998). In many countries such as Bangladesh (Zahid et al., 2008), India, Nepal, (Singh, 2006) and China (Wang et al., 2007), thousands of people have been suffering from arsenicosis because of their long-term exposure to drinking water contaminated with high levels of arsenic (Nickson et al., 1998). In the past several decades, a number of technologies, including filtration (Devi et al., 2008), adsorption of arsenic species on waste materials, (Razmovski and Šćiban 2008; Baig et al., 2013; Shah et al., 2013) and iron or aluminum oxy-hydroxides (Arienzo et al., 2002; Biterna et al., 2010; Nagar et al., 2010), bioimmobilization (Achal et al., 2012), interception by permeable reactive barriers (Zouboulis and Katsoyiannis, 2005), have been developed to clean up the arsenic contaminated groundwater.

Nitrate is another common toxic pollutant that can be transformed to carcinogenic N-nitroso compounds in human digestive system. The MCLG (Maximum Contaminant Level Goal) of nitrate (in the term of nitrogen) for drinking water is set as 10 $mg\,L^{-1}$ in many countries. Nitrate can be leached from soil to groundwater as a consequence of extensive agricultural application of inorganic nitrate fertilizers and manures and irrigation with sewage. It has been frequently detected at hundreds of $mg\,L^{-1}$ in groundwater in many rural areas in China (Zhang et al., 1996). Nitrate is usually removed biologically from drinking water by reducing it to dinitrogen gas using denitrifying bacteria (Shin and Cha, 2008).

* Corresponding author. Tel.: +86 991 7885446; fax: +86 991 7885446.
** Corresponding author.
E-mail addresses: xlpan@ms.xjb.ac.cn (X. Pan), zhang-daoyong@163.com (D. Zhang).

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Although nitrate and arsenic have been frequently detected together in groundwater, there are biotechnologies that can only remove arsenic or nitrate separately. There are no technologies that can simultaneously remove nitrate and arsenic by a single species of microorganism. It is urgently necessary to seek cost-effective biological methods that can clean up groundwater contaminated with nitrate and arsenic.

It has been reported that some species of bacteria have the ability to oxidize Fe(II)–Fe(III) using nitrate as the electron acceptor under anoxic condition (Straub et al., 1996; Straub et al., 2004; Senko et al., 2005; Li et al., 2014). Because the anaerobic nitrate-reducing Fe(II) oxidizing bacteria simultaneously reduce nitrate to dinitrogen gas and produce ferric oxides that have great adsorption capacity for arsenite or arsenate (Nagar et al., 2010), these anaerobes provide a novel and promising solution to synchronously *in situ* remediate arsenic and nitrate contaminated groundwater. In other words, nitrate and arsenic can be removed by the anaerobic nitrate-reducing Fe(II) oxidizing bacteria by its denitrification and adsorption of arsenic to its biogenic iron oxides, respectively. Furthermore, remediation using a single bacterial species may be more cost-effective, efficient, and easily manipulated than multi-species. Therefore, this bioremediation method is expected to be used in the poverty-stricken arsenic poisoning areas.

In the present study, potential of simultaneous removal of nitrate and arsenite from groundwater by a nitrate-reducing Fe(II) oxidizing anaerobe, *Citrobacter freundii* strain PXL1, was investigated. The biogenic iron oxides and their adsorption of arsenite were characterized by scanning electron microscopy (SEM), X-ray diffraction (XRD) spectroscopy, and Fourier transform infrared (FTIR) spectroscopy. Our study showed that *C. freundii* strain PXL1 was an excellent bacterial species for bioremediation of nitrate and arsenite polluted groundwater.

2. Materials and methods

2.1. Isolation and identification of the bacteria

The anaerobic nitrate-reducing Fe(II) oxidizing bacterium, *C. freundii* strain PXL1, was isolated from anoxic activated sludge of Hedong sewage plant of Urumqi, China (Li et al., 2014). Briefly, to enrich the potential nitrate dependent Fe(II) oxidizing bacteria, 10 mM $FeCl_2{\cdot}4H_2O$ and 20 mM KNO_3 were added to the anaerobic activated sludge samples as electron donor and acceptor, respectively. One week after incubation of the sludge at 30 °C, some sludge samples were transferred to the fresh solid or liquid medium containing the following components (per liter): 20 g of potassium sodium tartrate ($C_4H_4O_6KNa{\cdot}4H_2O$), 1 g of K_2HPO_4, 1 g of $MgSO_4{\cdot}7H_2O$, 4 g of KNO_3, and 0.3976 g of $FeCl_2{\cdot}4H_2O$. The solid medium was prepared by mixing 2% agar with the above mentioned liquid medium. The bacterium was isolated by ten-fold serial dilution method using the solid medium plates. Colonies that showed brownish–red Fe(III) oxide precipitates around them were selected as the candidate bacteria and transferred into the liquid medium. Three days after anaerobic incubation at 30 °C, nitrite (NO_2^-) concentration was detected (Kleinbongard et al., 2002) in the medium to confirm reduction of nitrate by the bacteria.

Genomic DNA was extracted from the isolated bacterial cultures by alkaline lysis to identify the bacteria (Achal and Pan, 2011). Briefly, the universal 5′-AGAGTTTGATCCTGGCTCAG-3′ and 5′-AAGGAGGTGATCCAGCCGCA-3′ primers were used as the forward and the reverse primers, respectively (Hall et al., 1999). The PCR program started with an initial 5 min denaturation step at 94 °C, 36 cycles at 94 °C for 1 min, annealing of primers to the DNA template at 48 °C for 30 s, extension at 72 °C for 2 min, and final extension at 72 °C for 7 min. The sequences were detected using an Applied Biosystem automated sequencer (Biorad, USA) and submitted to GenBank. The possible sister groups found by BLAST research were edited and a phylogenetic tree was constructed using MEGA 4.0 software (Tamura et al., 2007).

2.2. Experiments for removal of As(III) and nitrate by strain PXL1

The strain PXL1 was incubated at 30 °C in the medium containing the following components: 20 g L^{-1} potassium sodium tartrate ($C_4H_4O_6KNa{\cdot}4H_2O$), 1 g L^{-1} K_2HPO_4, 1 $MgSO_4{\cdot}7H_2O$, 0.1 g L^{-1} $CaCl_2{\cdot}7H_2O$, 0.8 g L^{-1} KNO_3 (about 8 mM NO_3^-), 0.994 g L^{-1} $FeCl_2{\cdot}4H_2O$ (about 5 mM Fe(II)), 1 mL of vitamin solution (Widdel and Pfennig, 1981) and 1 mL of trace element solution (Tschech and Pfennig, 1984). After 2 weeks of incubation, the strain PXL1 culture was used for further experiments. As(III) solution was prepared by dissolving sodium arsenite ($NaAsO_2$) in deionized water. All the chemicals used were of analytical grade. All work was done in the DG250 anaerobic workstation (Don Whitley Scientific, West Yorkshire Shipley, England).

The above-mentioned liquid medium containing various concentrations of Fe(II), NO_3^-, and As(III) was inoculated with the strain PXL1 at a cell density of about 5×10^5 cells mL^{-1}. The cells were counted by MPN methods. The medium pH was set as 7 because ground water pH is neutral in most cases. The cells were incubated at 30 °C. The experiments were conducted without agitation. The actual initial concentrations of Fe(II) and NO_3^- were 4.8 and 8 mM, respectively. Various concentrations of As(III) were added to the serum bottles to get a series of final As(III) concentrations of 2.85 μM, 6.85 μM, and 13.65 μM (Zhu et al., 2008). The liquid medium samples without inoculation of the strain PXL1 were used as the control. 1 mL of medium samples were collected every 24 h to determine nitrate, nitrite, Fe(II) and As (III) concentrations. All experiments were done in triplicate in the DG250 anaerobic workstation.

2.3. Analytical methods

Concentrations of nitrate, nitrite and Fe(II) were measured by spectrophotometry using a UV/Vis spectrophotometer (Unico UV-2800, Shanghai, China). Nitrite concentration was measured at 540 nm by *N*-(α-naphthyl)-ethylenediamine spectrophotometry. Nitrate concentration was determined by UV spectrophotometry at 220 and 275 nm (Slanina et al., 1976). Nitrate absorbances equal to twice the absorbance at 275 nm minus the absorbance at 220 nm. Fe(II) concentration was determined by the modified phenanthroline spectrophotometry at 510 nm (Gendel and Lahav, 2008; Klueglein and Kappler, 2013). The samples were mixed with 40 mM sulfamic acid (pH approximately 1.8) instead of 3 M HCl because the sulfamic acid could react rapidly with nitrite and prevent Fe(II) oxidation by the nitrite at acidic pH (Klueglein and Kappler, 2013).

As(III) concentration was measured by an atomic fluorescence spectrophotometer (AFS-810, Jitian Instruments, Beijing, China) (Achal et al., 2012). A continuous flow hydride generation system was used for detection of arsenic concentration. Briefly, 10 mL of 5% thiourea and 5% vitamin C mixture and 2.5 mL of ultrapure HCl (37%) were added into 50 mL of water sample in order to reduce all the forms of arsenic to As(III). 2.0% (W/V) KBH_4, 0.5% (W/V) KOH, and 5% HCl solutions were used as the reducing agent, stabilizer and carrier, respectively. The AFS instrumental parameters were set as follows: lamp current (50 mA), negative high voltage of photomultiplier (280 V), carrier argon flow (400 mL min^{-1}), atomizer height (10 mm). The calibration curve of As concentration demonstrated good linearity ($r > 0.999$) (Achal et al., 2012). All the reagents used were of analytical grade or higher purity.

Bacterial cells and their Fe(III) oxides pellets were analyzed using scanning electron microscope-energy dispersive X-ray

spectroscope (SEM-EDS). The pellet samples were centrifuged at 12,000 rpm for 15 min and the pellets were collected and frozen at −80 °C for 4 h, and then freeze-dried at −60 °C for 48 h. The samples were examined using a SEM (Zeiss Super 55VP, Germany) at accelerating voltage from 15 to 35 kV. The elemental composition of sediments was analyzed using EDS (Bruker XFlash 5010, Germany). The freeze-dried sediment samples were further analyzed by XRD and FTIR. XRD spectra were obtained using a Bruker D8 diffractometer with a Cu anode (Bruker, Germany) scanning from 5 to 80 °. For FTIR analysis, about 1 mg of sediment was ground with 100 mg of KBr in an agate mortar. FTIR spectra of the sediment-KBr pellets were recorded from 4000 to 400 cm^{-1} with a vertex 70 FTIR spectrometer (Bruker, Germany).

3. Results

3.1. Fe(II) oxidation and nitrate reduction

C. freundii strain PXL1 grew well using Fe(II) and potassium sodium tartrate as the substrates and nitrate as the terminal electron acceptor under anoxic condition in the presence of various concentrations of arsenite. This suggests that the strain PXL1 had somewhat resistance to toxicity of arsenite. The strain PXL1 could efficiently oxidize aqueous Fe(II) in the medium containing about 2.85 to 13.65 μM arsenite (Fig. 1a). In the PXL1 inoculated medium with an initial Fe(II) concentration of 4.8 mM, almost all of the Fe (II) was oxidized on 6 d. Oxidation of Fe(II)–Fe(III) was also visually indicated by color change of bacterial cell suspension from whitish translucent to yellow and orange. The bacterially produced Fe(III) oxides eventually settled down and the cell suspension became transparent. Fe(II) oxidation was rapid on the first day and about 2.89 mM Fe(II) was oxidized. Fe(II) oxidation became slow during 2–6 d with an average Fe(II) oxidation rate of 0.38 mM d^{-1}. In the non-inoculated control, almost no Fe(II) oxidation were observed, confirming that the biological oxidation of Fe(II) was the dominant process.

For all treatments inoculated with the strain PXL1, nitrate was significantly reduced as Fe(II) was oxidized (Fig. 1b) in comparison with the slight decrease of nitrate for the control. The nitrate concentration decreased from 8 mM to about 4.1–4.5 mM on 6 d and thereafter. More than 44% of nitrate was removed by the strain PXL1. Similar to Fe(II) oxidation, the reduction of nitrate was also fast on the first day and became slow thereafter. The nitrate reduction rate was 2.05 mM d^{-1} on the first day and on an average 0.32 mM d^{-1} during 2–6 d. The high concentration of arsenite had negligible impact on the reduction rate of nitrate. Nitrite was formed concurrently with Fe(II) oxidation and nitrate reduction (Fig. 1c). The nitrite concentration reached the maximum (1.4 mM) on 4 d, implying the accumulation of nitrite during the initial stage of the reaction. The nitrite concentration began to decline after the fourth day and finally decreased to nearly zero. This change implies that nitrite was further reduced. No nitrite was detected for the control, indicating that it was the strain PXL1 that oxidized Fe(II) and reduced nitrate.

Nitrate and Fe(II) removal percentages by the strain PXL1 were not affected by arsenite in the concentration range of 2.85–13.65 μM, indicating the strain PXL1 viability was not inhibited by arsentite toxicity in the experimental concentration range.

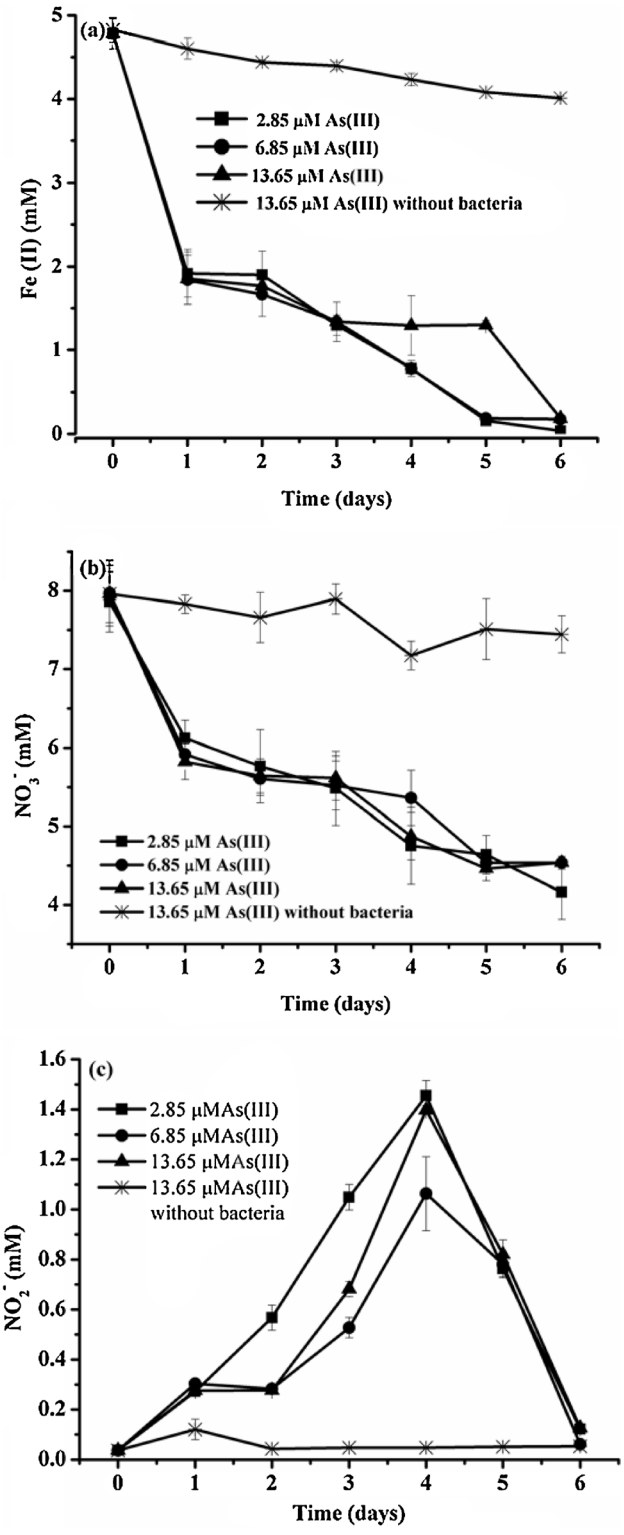

Fig. 1. Change of Fe(II), NO_3^- and NO_2^- concentrations in the medium inoculated or non-inoculated with the strain PXL1 as a function of incubation time in presence of various concentrations of arsenite. The initial concentrations of Fe(II) and nitrate were 4.8 mM and 8 mM, respectively. The cells were incubated at 30 °C in an anoxic workstation. (a) Fe(II) concentration, (b) NO_3^- concentration and, (c) NO_2^- concentration.

3.2. As(III) removal

As(III) was significantly removed from the PXL1 inoculated medium containing Fe(II) (Fig. 2). After 6 d remediation, As(III) concentration decreased from initial 2.85 μM to 2.11 μM. About 26% of As(III) was removed. Higher removal efficiency was obtained at higher initial As(III) concentrations. There was no significant difference in As(III) removal percentage among treatments with various concentrations (2.85–13.65 μM) of arsenite. The average removal percentage of As(III) was about 29% on 6 d.

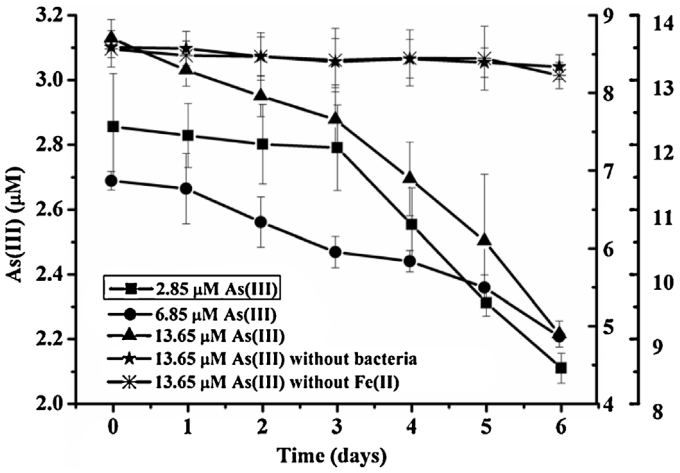

Fig. 2. Change of As(III) concentration in the medium inoculated or non-inoculated with the strain PXL1 as a function of incubation time in presence of various concentrations of arsenite. The initial concentrations of Fe(II) and nitrate were 4.8 mM and 8 mM, respectively. The adsorption of As(III) by strain PXL1 was studied in inoculated culture without Fe(II). The cells were incubated at 30 °C in an anoxic workstation.

However, the removal percentage of As(III) was about 3% for the control, which may be due to the adsorption of arsenic to Fe(III) oxides that was oxidized from Fe(II) by the trace oxygen in the medium. For the PXL1 inoculated medium without Fe(II), little As (III) was removed, indicating that PXL1 cells had little adsorption capacity for As(III). This further confirmed that As(III) was removed due to the adsorption of iron oxides produced by the strain PXL1.

3.3. Characterization of Fe(II) oxidation precipitates

Brown precipitates were observed for the strain PXL1 inoculated medium containing Fe(II), nitrate and various concentrations of arsenite. These precipitates were examined by SEM-EDS. The SEM image showed that most cells were encrusted with nanosize Fe(III) oxides (Fig. 3a). The average diameter of the Fe(III) oxides was 100–200 nm. EDS analysis showed that the precipitates were mainly composed of C (44.20%), O (33.39%), and Fe (13.48%) (Fig. 3b). The high proportion of C could be attributed to the cell biomass. Small amount of As was also found in the precipitates. Because the batch experiments showed that the bacterial cells did not adsorb As(III), the EDS results confirmed that As(III) was removed due to its adsorption to iron oxides produced by the strain PXL1.

No distinct diffractive peaks were found in the XRD spectrum of the precipitates (Fig. 4a) obtained, indicating that poorly crystalline and even amorphous Fe(III) precipitates were produced by the strain PXL1. The FTIR spectrum of precipitates was shown in Fig. 4b. The broad absorbance band around 3400 cm^{-1} was attributed to water molecular and/or —OH groups (Chua-anusorn and Webb, 2000). The bands at 1042 and 555 cm^{-1} could be assigned to Fe—O stretching vibration (Liu et al., 2006). These results indicate the presence of iron oxides.

4. Discussion

C. freundii strain PXL1 could efficiently remove As(III) from water, associated with Fe(II) oxidation and nitrate reduction. This suggest that the anaerobic nitrate-reducing Fe(II) oxidizing bacteria such as *C. freundii* strain PXL1 are promising microbes for remediation of nitrate and arsenic contaminated waters. A few anaerobic nitrate-reducing Fe(II) oxidizing bacteria, including *Thermomonas* BrG3 (Straub et al., 2004), *Chromobacterium violacens* Strain 2002 (Weber et al., 2006) and *Paracoccus ferrooxidans* strain BDN-1 (Kumaraswamy et al., 2006), have been found in various habitats such as activated sludge (Nielsen and Nielsen, 1998) and sediments (Kappler and Straub, 2005). All these isolated microorganisms may be expected to act as the remediating microbes for cleaning up water contaminated with nitrate and arsenic. These anaerobic nitrate-reducing Fe(II) oxidizing bacteria may also be used to treat wastewater contaminated with some heavy metals because the bacterially produced iron oxides usually have strong adsorption toward heavy metals (Lack et al., 2002).

XRD analysis showed that *C. freundii* strain PXL1 produced poor crystalline iron oxides. Some other anaerobic nitrate-reducing Fe (II) oxidizing bacteria such as *Acidovorax* sp. strain BoFeN1 (Miot et al., 2009) and some Fe(II) oxidizing bacteria (Liu et al., 2013) also produce poor crystalline iron oxides. In the present study, the bacterial cells had little arsenite adsorption ability and the biogenic amorphous iron oxides play a major role in removing As(III) due to their strong adsorption capacity for As(III) or their co-precipitation with As(III) (Aredes et al., 2012). Surfaces of the Fe(III) oxides are usually positively charged due to their high points of zero charge. Therefore, the Fe(III) oxides are good adsorbents for negatively charged oxyanions like arsenate (AsO_4^{3-}) and arsenite (AsO_3^{3-}). Biogenic Fe(III) oxides are known to have larger specific surface area and higher binding energy than chemically produced Fe(III) oxides and thus are expected to be more efficient adsorbents for heavy metals. The detected As(III) in the precipitates by SEM-EDS spectroscopy confirmed the major contribution of biogenic iron oxides to arsenite removal. A number of studies show that bacterial

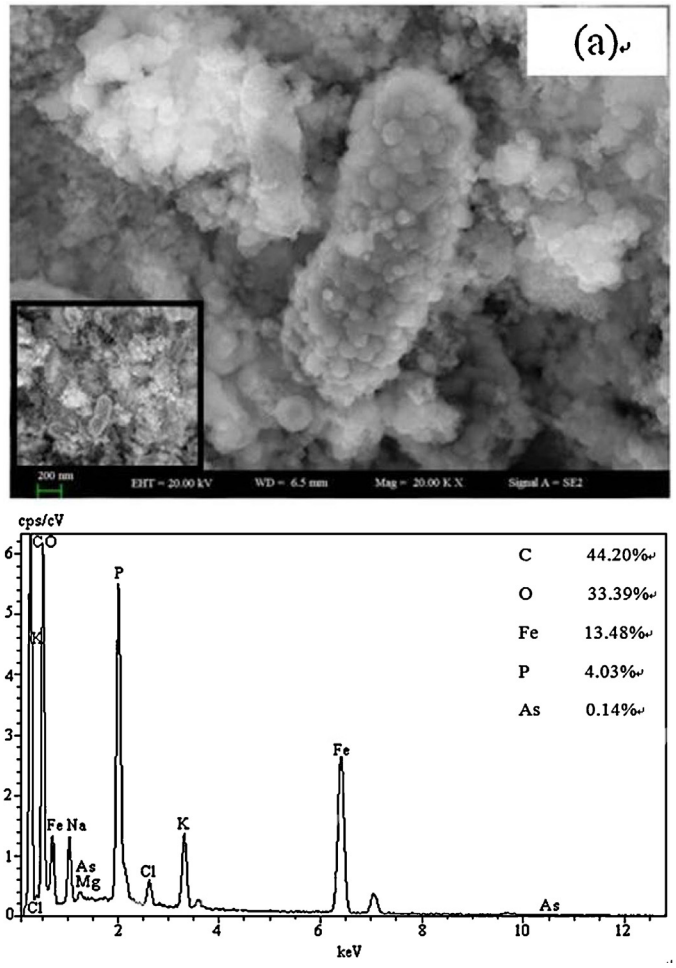

Fig. 3. SEM images (a) (magnification: ×20,000; insert magnification: ×5000) and EDS analysis (b) of pellets from the *C. freundii* strain PXL1.

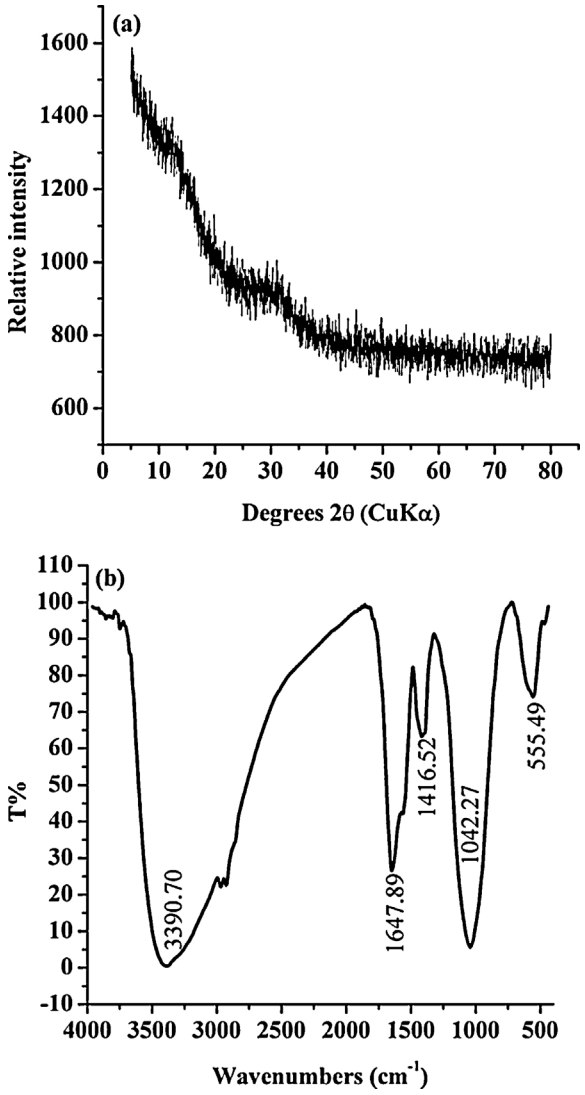

Fig. 4. X-ray diffractogram (a) and FTIR spectrum (b) of iron oxides produced by the strain PXL1.

oxidation of Fe(II) is a promising process for remediation of groundwater containing high-levels of arsenic (Mohan and Pittman, 2007). However, the *C. freundii* strain PXL1 is more versatile and has advantages over these reported bacterial Fe(II) oxidation because it can remove arsenic and nitrate concurrently.

5. Conclusions

It was demonstrated that the anaerobic nitrate-reducing Fe(II) oxidizing bacteria *C. freundii* PXL1 could synchronously remove As (III) and nitrate from water, associated with Fe(II) oxidation. Its cell metabolism, nitrate reduction and Fe(II) oxidation were not inhibited by arsenite in the concentration range that was usually found in water environments. As(III) was mostly removed by its adsorption to the iron oxides produced by the strain PXL1. The anaerobic nitrate-reducing Fe(II) oxidizing bacteria including *C. freundii* PXL1 are expected to be effective microbes for remediation of nitrate and arsenite polluted groundwater.

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