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Isolation of a non-traditional sulfate reducing-bacteria *Citrobacter freundii* sp. and bioremoval of thallium and sulfate

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ABSTRACT

A novel non-traditional sulfate reducing bacterium (Sr 10) was isolated from an up-flow anaerobic sludge bed for acid mine drainage treatment which contains thallium (Tl) and sulfate. Phylogenetic analysis of partial 16S rRNA gene sequence of isolate Sr 10 revealed that it was identified to *Citrobacter freundii* species. Sr 10 was visually rod-shaped and very motile with *peri*-flagellum according to transmission microscopy. The Fourier transform infrared spectroscopy analysis indicated that the stains had various functional groups for Tl and sulfate removal, including hydroxyl, carboxyl, amide and phosphate. Under anaerobic conditions, the optimized growth conditions for the stain were obtained at temperature of 35 °C and initial pH value of 7.0. Sr 10 was able to remove both Tl(I) and SO_4^{2-} simultaneously with the removal efficiency up to 99.60% and 89.80%, respectively. This strain might be used for Tl and sulfate removal in the process of bioremediation restoration.

1. Introduction

As a rare and widely dispersed element, thallium (Tl) was discovered by William Crookes in 1861(Smith and Carson 1977), which usually existed in nature environment in monovalent thallium (Tl^+) and trivalent thallium (Tl^{3+}) states. Thallium is one of the 13 priority pollutant metals, which is reported to have higher toxicity to human health than mercury, cadmium, lead, copper or zinc (Keith and Telliard, 1979; Marchiol et al., 2013). Thallium ion is similar to potassium (K^+) in ionic radius and electrical charge. It can alter heme metabolism, compete with potassium for membrane transport systems, inhibit mitochondrial oxidative phosphorylation and disrupt protein synthesis (Rodríguezmercado and Altamiranolozano, 2013). The United States Environmental Protection Agency (USEPA) set the maximum contaminant levels of thallium in drinking water and wastewater (effluent) at 2 and 140 $\mu g\,L^{-1}$, respectively. And 1.7 $\mu g\,L^{-1}$ was the environmental safe dose of thallium for human (John Peter and Viraraghavan, 2008). Tl is a quite mobile metal and readily transported through aqueous routes into environment for the high solubility of thallium compounds. It has been known that Tl might be responsible for many accidental, occupational, and therapeutic poisonings since its discovery in 1861 (Nriagu 2003; Wang 2013).

As a highly toxic element, in comparison with heavy metals, such as Cd, Pb, Cu and Zn, the knowledge for removal of thallium from wastewater is far from enough. Physical-chemical processes were used to remove thallium from wastewater, such as reductive precipitation, adsorption, ion exchange, filtration, electrodialysis, reverse osmosis (Catherine and Twidwell 2003; Kajitvichyanukul et al., 2003; Twidwell and Williams-Beam, 2002). However, these processes were complex facilities or extremely expensive for thallium removal. And they may lead to secondary pollution. For example, there were lots of oxidant under alkaline conditions needed in oxidative precipitation process. And also, low density sludge produced in this process was hard to be treated (Davies et al., 2016). Reverse osmosis was an energy-condensed process. The restoration of membranes were hard after Tl removal (Fu and Wang 2011). Meanwhile, Tl bearing wastewater usually accompanied with high concentration sulfate, which would affect the adsorption effect of activated alumina. Anaerobic techniques with sulfate reducing bacteria (SRB) have been considered as the most promising technology for the treatment of industrial wastewaters due to the advantage of low operation cost and environmental safety (Benner et al., 1997; Singh et al., 2011; Uster et al., 2015). Under

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strictly anaerobic conditions, SRB can convert sulfate to highly reactive hydrogen sulfide and produce bicarbonate in the presence of a suitable electron donor and carbon source (Guo et al., 2014). Then, bicarbonate alkalinity can neutralize acidity and hydrogen sulfide can react with metal. These metals can be easily removed in the form of very insoluble metal sulfide from the water at neutral pH. The biological transformation process is described in Eqs. (1)–(3) (Mueller 2001; Qiu et al., 2009).

$$2CH_2O + SO_4^{2-} \rightarrow H_2S + 2HCO_3^- \quad (1)$$

$$H_2S + 2Tl^+ \rightarrow Tl_2S_{(S)} + 2H^+ \quad (2)$$

$$HCO_3^- + H^+ \rightarrow H_2O + CO_2 \quad (3)$$

Where CH_2O represents the electron donor. It had showed that thallium could be effectively removed from wastewater to <2.5 ppb by reductive precipitation of thallium sulfide. SRB played an important role in this biological sulfate reduction process (Mueller 2001). However, there had no SRB strains being reported for thallium removal.

In this work, we reported isolation of a sulfate-reducing stain of *Citrobacter freundii* sp. from an up-flow anaerobic sludge bed for treating acid mine drainage of YunFu mining area of Guangdong province, China. The strain does not belong to the traditional SRB. Although the ability of *Citrobacter freundii* for sulfate reduction has been reported, the ability for thallium removal had not been studied (Sitte et al., 2013). In this paper, a strain of *Citrobacter freundii* (Sr 10) had been isolated and investigated. Moreover, the functional groups of the strain for thallium removal were studied by Fourier Transform infrared spectroscopy (FTIR). The effects of temperature and pH value on the growth of Sr 10 were investigated and the activity of Sr10 for thallium and sulfate removal were also tested with the goal of evaluating its suitability for thallium removal.

2. Materials and methods

2.1. Growth media and conditions

A modified Postgate growth medium was used in all experiments. The medium was composed of (per liter of deionized water): 0.5 g Na_2SO_4, 2.0 g $MgSO_4$, 1.5 g KH_2PO_4, 0.1 g NH_4Cl, 0.5 g cysteine hydrochloride, 1 g yeast extract, 0.1 g $CaCl_2$, 0.3 g sodium citrate, 0.1 g ascorbic acid, 2.0 g sodium lactate (Postgate 1949). The pH value of culture medium was adjusted to the range of 7.0–7.5. Culture medium was purged with nitrogen gas to achieve anaerobic condition and autoclaved 30 min at 121 °C. And then, 0.05 g $(NH_4)_2Fe(SO_4)_2$ and 0.1 g vitamin C was added into culture medium after cooling down to room temperature. All chemicals were analytical grade, and solutions were prepared with sterile deionized water.

2.2. Source of inoculum

The inoculum for the enrichment cultures were collected from an up-flow anaerobic sludge bed for treating acid mine drainage in the YunFu Pyrite Mining area, a site located in YunFu City, Guangdong province, China. This area is the largest production base for pyrite in China and is abundant in Tl-rich ores, which poses high risks to water safety in the catchment of Pearl River (Xiao et al., 2012). Concentrations of the Tl-bearing acid mine drainage have been observed to be at the range from 8 to 56 $\mu g\,L^{-1}$ of Tl. Now this area is designated as a provincially funded bioremediation site.

2.3. Enrichment and isolation

The raw sludge was filtered through a 2 mm sieve, then it was fed with culture medium to form a mixture with 2% sludge. 100 mL mixture was added into a 250 mL anaerobic flask with several sterile glass beads. The medium in anaerobic flask was purged with nitrogen gas for 10 min to remove dissolved oxygen (DO). Then it was sealed and transferred into an anaerobic workstation (YQX-II, Yuejing, China) for enrichment. 1 mL culture mixture was inoculated into next flask with 100 mL culture solution. This step was repeated thrice. The enrichment culture was diluted into different gradient concentrations and inoculated on solid culture medium with above reagents by conventional spread plate techniques in anaerobic workstations for 48 h. Black colonies were picked and then purified thrice by streak dilution plating. At the same time, colonies were transferred into solution to determine sulfate reduction activity again. When colonies growing on plates have the same appearance, they were sent to Guangdong Institute of Microbiology for identification. All incubations were done at 35 °C in the dark. Stock cultures were stored at 4 °C and regularly transferred to fresh medium to maintain viability. A filter-sterilized solution of $TlNO_3$ (1000 mg L^{-1}, Guangdong Analysis and Testing Center, AR Grade) was used as a source of Tl (I). 2 mm sieve was supplied by Laboratory Equipment Co., Ltd, China.

2.4. Analytical methods

The wavelength used for monitoring the growth of Sr 10 bacteria was optimized by detecting the absorbance with wavelength scanning spectrometer (HITACHI U2910, Japan), and the highest absorbance was achieved under the wavelength of 600.45, thus, wavelength of 600 nm was used for monitoring the growth of Sr 10 in the follow study.

In order to determine the growth curve of the strain, three autoclaved flasks filled in 100 mL of enrichment medium with pH value of 7, and then inoculated with 1 mL of freshly prepared culture of bacterial isolate, respectively.

The flasks were incubated at 35 °C and shaken at 150 rpm for 8 d. The absorbance of occasional sampling was measured during the incubation period.

Effect of temperature on growth of bacterial isolates was studied by using 6 sets with 3 flasks for each set. Each flask was autoclaved and filled in 100 mL of enrichment medium with pH value of 7 separately, then inoculated with 1 mL of freshly prepared culture of each bacterial isolate growth at 35 °C. The bacteria culture temperatures were varied from 20, 25, 30, 35, 40–45 °C in 6 sets, respectively. After an incubation period of 2 d, the absorbance of medium was measured at 600 nm using the spectrophotometer to determine the optimum growth temperature of the strains.

The original pH values were adjusted from 4.0, 5.0, 6.0, 7.0, 8.0 to 9.0 in another 6 sets obtained as described before. These flasks were also autoclaved and filled with 100 mL of enrichment medium, then each flask was inoculated with 1 mL freshly prepared isolated culture. The absorbance of occasional sampling was measured at 600 nm during the incubation period of 48 h to determine the optimum growth pH value of the strains.

In order to determine the ability of bacterial isolates resistance to Tl (I), test flasks with 100 mL LB broth (pH value of 7) containing Tl (I) were prepared in 6 sets with 3 test flasks for each set. The Tl (I) concentrations were varied from 5.0, 10.0, 20.0, 30.0, 40.0 and 50.0 mg L^{-1}, separately, and each test tube was autoclaved. These tubes were inoculated with 10 mL freshly prepared culture of each bacterial isolate. Samples (10 mL) were taken from cultures after 96 h, then centrifuged at 12000 r min^{-1} for 6 min and supernatants were used to estimate Tl (I) and sulfate remained in medium.

Tl concentration was determined by using the flame atomic absorption method with a flame atomic absorption spectrometer (FAAS, TAS990, Beijing, China) (Rice et al., 2012). The sulfate concentration in the filtrate was analyzed by using the turbid metric method with the UV spectrophotometer as outlined in Standard