African Journal of Biotechnology Vol. 11(97), pp. 16336-16341, 4 December, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB12.607
ISSN 1684–5315 ©2012 Academic Journals

Full Length Research Paper

Isolation of *Thiobacillus* spp. and its application in the removal of heavy metals from activated sludge

Qiang Li[1], Cong Wang[1,2], Baobin Li[1], Cunmin Sun[2], Fei Deng[1], Cunjiang Song[1]*, and Shufang Wang[2]

[1]Key Laboratory of Molecular Microbiology and Technology for Ministry of Education, Nankai University, Tianjin 300071, People's Republic of China.
[2]Key Laboratory of Bioactive Materials for Ministry of Education, Nankai University, Tianjin 300071, People's Republic of China.

Accepted 29 November, 2012

Two strains of *Thiobacillus* isolated from native excess activated sludge were identified as *Acidithiobacillus ferrooxidans* and *Acidithiobacillus thiooxidans* by 16S rRNA gene sequencing and physiological-biochemical characteristics. Single and mixed cultures of the strains were used to carry out bioleaching for 9 days in order to remove heavy metals from activated sludge. The changes in pH, oxidation-reduction potential, and contents of heavy metals were measured. The results show that the bioleaching effect of the mixed culture was best in all runs, and that the final removals of As, Cr, Cu, Ni, and Zn were 96.09, 93.47, 98.32, 97.88, and 98.60%, respectively, whereas the removals of Cd and Pb decreased rapidly after six days. In addition, we demonstrate for the first time that bioleaching can reduce the pathogenicity of sludge by detecting fecal coliforms before and after bioleaching in order to ensure that the sludge was suitable for agricultural land application.

Key words: *Acidithiobacillus ferrooxidans*, *Acidithiobacillus thiooxidans*, excess activated sludge, removing heavy metals, sludge pathogenicity.

INTRODUCTION

Sewage sludge, which is produced during sewage disposal, must be disposed in order to maintain a sufficient oxygen supply and a fixed concentration of sludge (Kosobucki et al., 2008). Currently, landfill, incineration, sea disposal, and agricultural land application are used to dispose off sewage sludge (Kim et al., 2005). Among these techniques, land application is considered to be the most attractive because the high content of organic matter in sewage sludge can provide nutrients to crops (Tyagi and Couillard, 1987). However, the high content of toxic heavy metals in the sludge often causes severe environmental problems, and this significantly restricts the land application of sludge (Bruce and Davis, 1989; Burton, 1991; McGhee, 1991). Therefore, pretreatment of sewage sludge to remove heavy metals before land application becomes an indispensable part of adequate sludge management (McGhee, 1991).

Chemical methods, including chlorination, ion exchange, complexation, and acidification, have generally been used to remove heavy metals from dewatered sludge. However, these techniques have many disadvantages, such as high cost, operational difficulty, high energy requirements, and sometimes unsatisfactory metal solubilization (Xin et al., 2009). As an alternative means of removing heavy metals, bioleaching has several advantages, including low cost, easy operation, low energy requirement, a high degree of metal solubilization, and non-hazardous byproducts (Mercier et al., 1996).

Bioleaching refers to the direct and indirect reactions of certain microorganisms in the natural environment, including oxidation, reduction, chelation, adsorption, and dissolution, which can dissolve some of the insoluble substances (heavy metals, sulfur, and other metals) from solid substances (Bosecker, 1997).

Acidithiobacillus ferrooxidans and *Acidithiobacillus thiooxidans* are considered to be the most effective bacteria

*Corresponding author. E-mail: songcj@nankai.edu.cn. Tel/Fax: 86-22-2350-3866.

Table 1. The characteristics of the sludge sample.

Characteristic	Te content	Method
Solid content (%)	5	Drying in 105°C
pH (H_2O)	6.87	Potentiometric
ORP (mV)	-38	Potentiometric
TN (mg/kg DS)	4950	UV–vis
TP (mg/kg DS)	3259	UV–vis
SO_4^{2-} (mg/kg DS)	3592	UV–vis
Cu (mg/kg DS)	2740	ICP
Zn (mg/kg DS)	1765	ICP
Cd (mg/kg DS)	5.213	ICP
Cr (mg/kg DS)	181.9	ICP
Pb (mg/kg DS)	101.2	ICP
Ni (mg/kg DS)	117.0	ICP
As (mg/kg DS)	21.47	ICP
Fecal coliforms (mpn/g)	14600	the National Standard (GB/T 19524.1-2004)

TN, Total nitrogen; TP, total phosphorus; DS, dry sludge; ORP, oxidation-reduction potential.

for bioleaching (Solisio et al., 2002). They are both chemoautotrophic bacteria, using CO_2 as a carbon source and iron or sulfur as an energy source, without a requirement for organic nutrients. In addition, strict aseptic conditions are not necessary when these bacteria are employed for bioleaching because the growth of most other bacteria is markedly inhibited by the low pH (Rousk, 2009). Thus, there are prospective applications of these two bacteria in the removal of heavy metals and comprehensive utilization of excess activated sludge. The reactions of bioleaching by *Acidithiobacillus ferrooxidans* and *Acidithiobacillus thiooxidans* also include the direct and indirect reactions (Babel and del Mundo, 2006): The elemental sulfur can be oxidized into sulfate through the direct reaction by *Acidithiobacillus thiooxidans* and the dissolution of heavy metals takes place concomitantly. In indirect way, Fe (III) oxidized by *Acidithiobacillus ferrooxidans* take part in the dissolution of heavy metals and the formation of H_2SO_4 during the process further enhances the overall efficiency.

In this study, two native bacteria, identified as *Acidithiobacillus ferrooxidans* and *Acidithiobacillus thiooxidans*, were isolated from excess activated sludge. Single and mixed cultures of these bacteria were used to carry out bioleaching for 9 days to remove heavy metals from activated sludge. The changes in pH, oxidation-reduction potential (ORP), and the contents of heavy metals were measured and analyzed to compare the efficiency of the two bacteria and to investigate suitable conditions for bioleaching. Furthermore, fecal coliforms in the sludge before and after bioleaching were determined in order to ensure that the sludge was suitable for land application.

MATERIALS AND METHODS

Excess activated sludge was obtained from Dongli Sewage Treatment Works, Tianjin, China. It was stored at 4°C before use. The characteristics of the sludge sample are shown in Table 1. The content of heavy metals in the sludge generally exceeded the National Standard (GB18918-2002).

A. ferrooxidans was isolated from the activated sludge using 9K selective culture medium. The composition of 9K medium was as follows: $(NH_4)_2SO_4$, 3.0 g; K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KCl, 0.1 g; $Ca(NO_3)_2 \cdot 4H_2O$, 0.01 g; $FeSO_4 \cdot 7H_2O$, 44 g; distilled water, 1000 mL; pH 2.0.

1 to 2 g of sludge was added to 100 mL culture medium, and the culture was incubated in a shaker at 150 rpm and 30°C for 7 to 10 days. The *A. ferrooxidans* obtained by this method was transferred to fresh medium and cultured under the same conditions for enrichment.

A. thiooxidans was also isolated from the activated sludge by using 9K selective culture medium. 44 g $FeSO_4 \cdot 7H_2O$ was changed to 10 g sulfur. The process and conditions are the same as those described above.

Total DNAs of the two strains were extracted. Freeze-thawing was used to break the cell walls. The cells were frozen at –20°C for 10 min and then thawed at 70°C for 10 min. This procedure was repeated three times. Total DNA was then extracted using bacterial genome kits (TIANGEN DP302-02). The 16S rRNA gene was extended by PCR using special extending primers 27F/1492R and PCR program was carried out as Yang (2011), and subsequently sequenced by a commercial company. The sequences were blasted on NCBI (National Center for Biotechnology Information) and submitted to GenBank. Phylogenetic tree for two bacteria was conducted by ClustalX version 1.83 and MEGA version 3.0, using neighbour-joining method and Kimura 2 parameter distance with 1000 replicates to produce Boot-strap values (Kumar et al., 2004).

Bioleaching experiments were conducted in 500 mL Erlenmeyer flasks. The bioleaching systems, made to a volume of 200 mL with distilled water, comprised the following: 5% (w/v) sludge; $(NH_4)_2SO_4$, 0.6 g; K_2HPO_4, 0.1 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; KCl, 0.02 g; and $Ca(NO_3)_2 \cdot 4H_2O$, 0.002 g. To the *A. ferrooxidans* bioleaching system, 8.8 g $FeSO_4 \cdot 7H_2O$ and 10% (v/v) *A. ferrooxidans* were added, whereas to the *A. thiooxidans* bioleaching system, 2.0 g sulfur and 10% (v/v) *A. thiooxidans* were added. To the system bioleached by a mixed culture of *A. ferrooxidans* and *A. thiooxidans*, 8.8 g $FeSO_4.7H_2O$ and 2.0 g S, together with 5% (v/v) *A. ferrooxidans* and 5% (v/v) *A. thiooxidans* were added. The control included 5% (w/v) sludge and 200 mL water without bacteria. The initial pH for bioleaching was adjusted to 4.0 by adding H_2SO_4.

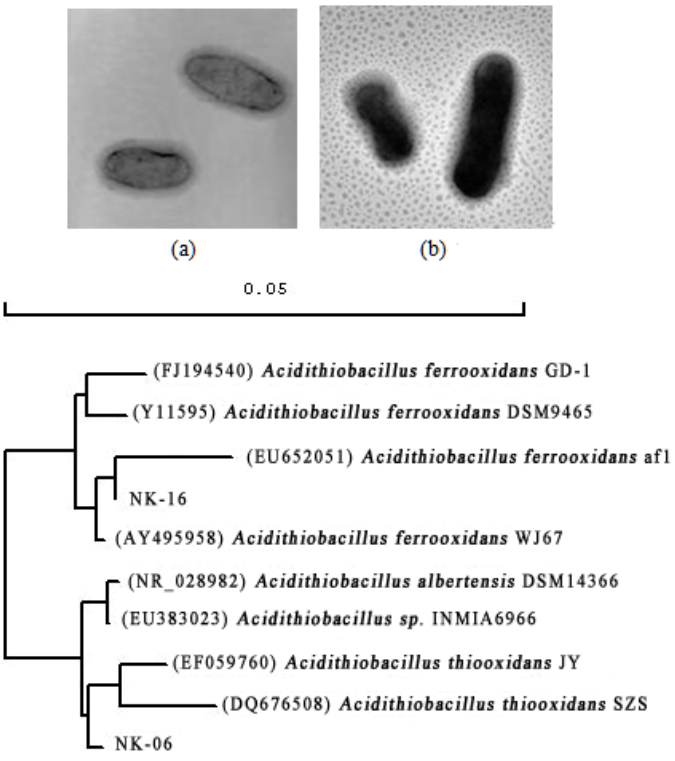

Figure 1. TEM images (×18000) and Phylogenetic tree of *A. ferrooxidans* and *A. thiooxidans*. **(**a**)**: *A. ferrooxidans* NK-16; (b): *A. thiooxidans* NK-06. Bacterial suspension dyed by phosphortungstic acid was observed by transmission electron microscope (Philips EM400ST).

Each of the systems had three replicates. The flasks were incubated in a shaker at 150 rpm and 30°C for 9 days.

The pH of the bioleaching systems was measured every 48 h using a pH meter. The ORP of the bioleaching systems was measured every 48 h using an ORP meter (Mettler SG2-T). The content of heavy metals in the bioleaching systems was determined every 72 h by inductively coupled plasma (ICP) analysis (Thermo IRIS Intrepid II XSP). The resolution rate of heavy metals = (A–B)/A × 100% (A is the content of heavy metals in sludge before bioleaching; B is the content of heavy metals in sludge after bioleaching). Fecal coliforms in the sludge were determined according to the National Standard (GB/T 19524.1-2004).

RESULTS AND DISCUSSION

$FeSO_4 \cdot 7H_2O$ was the only energy source in the culture, without an organic carbon source, and the pH of the culture was 2. Only *A. ferrooxidans* could survive in these conditions. $FeSO_4 \cdot 7H_2O$ was transformed to a jarosite precipitation by the bacteria, which indicated the metabolic process of *A. ferrooxidans*. Furthermore, sequence analysis of the 16S rRNA gene revealed that it had 99% similarity with the corresponding sequences of *A. ferrooxidans* in GenBank. Therefore, the strain isolated was identified as *A. ferrooxidans*, and was named *Acidithiobacillus ferrooxidans* NK-16.

Identification of NK-16 was difficult because jarosite attached to the cell walls made it difficult to break the cell walls using traditional methods. Alkaline lysis, enzyme digestion, and boiling methods all proved ineffective because of the stability of jarosite. In contrast, freeze-thawing efficiently destroyed the jarosite and broke the cell walls.

Sulfur was the only energy source in the culture and the initial pH of the culture was 3.5 to 4. Only *A. thiooxidans* could survive in these conditions. The bacteria used sulfur to yield H_2SO_4, indicating the biochemical characteristics of *A. thiooxidans*. In addition, the 16S rRNA gene of the strain and the corresponding GenBank sequences of *A. thiooxidans* had similarities of 99%. Therefore, the strain isolated was identified as *A. thiooxidans*, and was named *Acidithiobacillus thiooxidans* NK-06.

Sulfur attached to the cell walls also made it difficult to break the cell walls. Therefore, freeze-thawing was also used for *A. thiooxidans*.

The 16S rRNA gene sequences of the two bacteria were submitted to GenBank (the serial numbers are FJ598321 and FJ946877).

The phylogenetic tree of the two bacteria is shown in Figure 1, which also indicates that NK-16 and NK-06 are strains of *Acidithiobacillus ferrooxidans* and *Acidithiobacillus thiooxidans*, respectively.

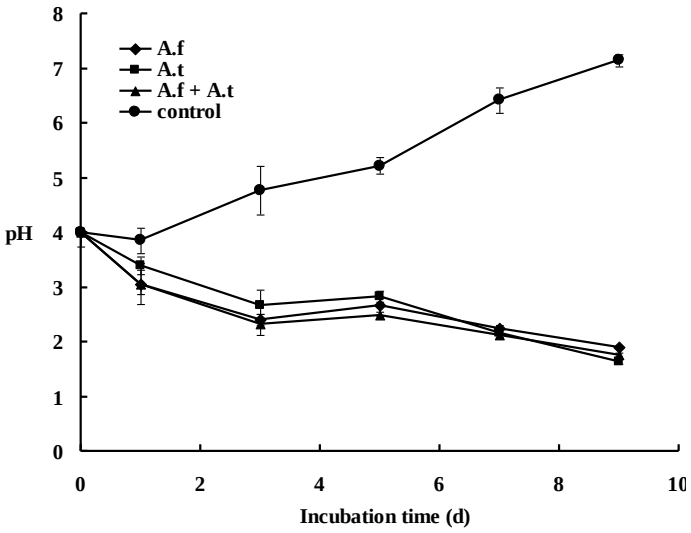

Figure 2. Changes of pH during the bioleaching. A.t, *Acidithiobacillus ferrooxidans*; A.f, *Acidithiobacillus thiooxidans*.

The pH of the bioleaching systems dropped during the bioleaching process, whereas that of the control gradually increased (Figure 2). Such variation in pH can indicate the activity of *Thiobacillus* (Zhang et al., 2009).

After bioleaching, the pH of the solutions that were bioleached by *A. ferrooxidans*, *A. thiooxidans*, and the mixed culture of *A. ferrooxidans* and *A. thiooxidans*

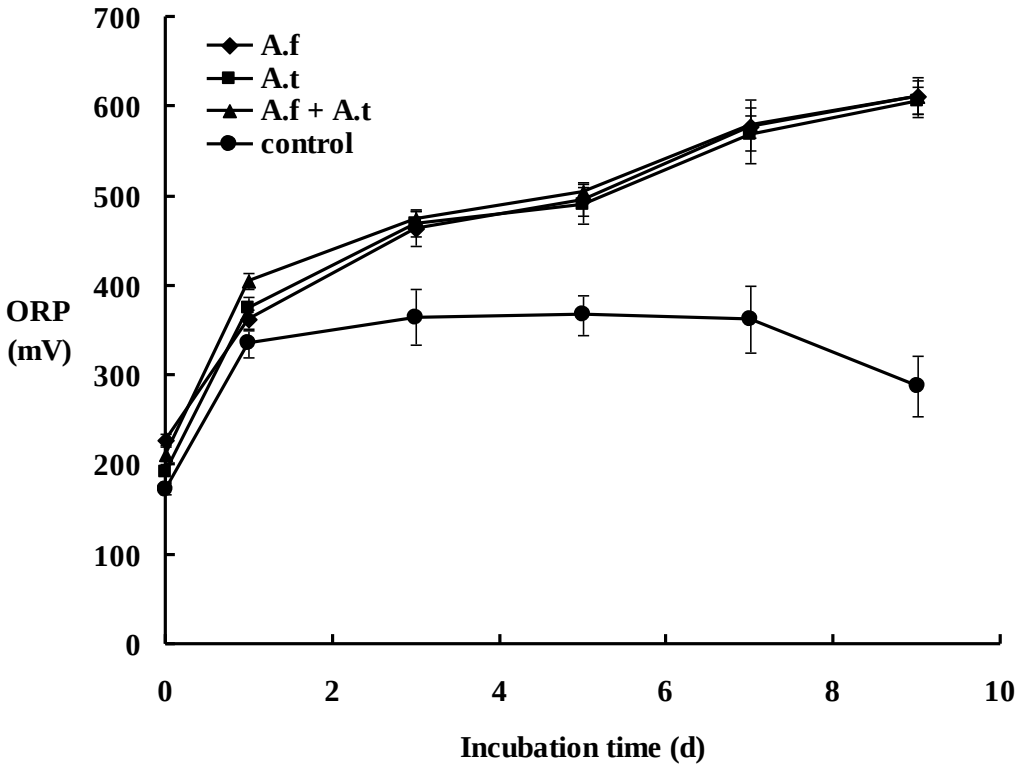

Figure 3. Changes of oxidation-reduction potential (ORP) during the bioleaching. A.t, *Acidithiobacillus ferrooxidans*; A.f, *Acidithiobacillus thiooxidans*.

decreased from the initial value of 4.0 to 1.9, 1.64, and 1.75, respectively. However, the pH of the control increased to 7.15 from 4.0. The pH between mixed culture system and single culture system was not significant.

A. ferrooxidans caused a decrease in pH because the Fe^{3+} produced from Fe^{2+} by *A. ferrooxidans* could hydrolyze and yield jarosite precipitation, during which H^+ was produced. *A. thiooxidans* caused a decrease in pH because it used sulfur to produce H_2SO_4. Variation in ORP can also indicate the activity of *Thiobacillus* (Zhang et al., 2009).

On the 1St day of bioleaching, the ORP of all the solutions increased. From the 2nd to the 9th day, the ORP of the control decreased after a slight increase, whereas that of the others continued to increase (Figure 3). At the end of bioleaching, the ORP of the solutions bioleached by *A. ferrooxidans*, *A. thiooxidans*, and the mixed culture increased to 612, 605, and 611 mV from the initial values of 228, 191, and 212 mV, respectively. In contrast, the ORP of the control increased from 173 mV to just 288 mV. During bioleaching, the ORP between mixed culture system and single culture system was also not significant. The increase in ORP was attributable to the oxidation of Fe^{2+} and S.

As shown in Table 2, the removal of As, Cr, and Ni increased with time. The effect of bioleaching by the mixed culture was better than that by either single culture in most case. Dissolution increased concomitant with a decrease in pH and increase in ORP, because the high acidity can help the dissolution of heavy metals and high ORP can dissociate the complicated sludge solid to set the heavy metals free (Bosecker, 1997). After 9 days of bioleaching, the removal of As, Cr, and Ni in the system of the mixed culture was 96.09, 93.47, and 97.88%, respectively.

The results show that bioleaching by *A. ferrooxidans* and the mixed culture dissolved over 90% of Cu and Zn until the 3rd day, whereas bioleaching by *A. thiooxidans* needed 6 days to have a similar effect (Table 2). The pH of the solution bioleached by *A. ferrooxidans* and the mixed culture was 2.33 and 2.4, respectively, whereas that of the solution bioleached by *A. thiooxidans* was 2.66. Thus, the dissolution of Cu and Zn was sensitive to a pH value of approximately 2.5, and most of the Cu and Zn were dissolved below pH 2.4. The dissolution rates of Cu and Zn remained relatively constant or increased slightly. After bioleaching for 9 days, the mixed culture had the highest removal of Cu (98.32%) and Zn (98.60%), whereas *A. ferrooxidans* dissolved 95.98% of Cu and 96.49% of Zn, and *A. thiooxidans* dissolved 95.87% of Cu and 96.83% of Zn.

As indicated in Table 2, the removal of Cd and Pb increased sharply during the first 3 days, remained essentially unchanged or increased slightly from the 3rd day to the 6th day, but then decreased. This may be due to the fact that the dissolved heavy metals combined with the

Table 2. The removal of heavy metals during the bioleaching by single and mix bacteria.

Removal (%)	Time (day)	A.t	A.f	A.f+A.t
Cu	3	62.86±2.51	89.71±6.55	94.28±1.99
	6	92.01±2.08	93.95±1.09	97.71±1.21
	9	95.87±1.23	95.98±0.22	98.32±0.05
Zn	3	85.10±0.83	94.26±1.16	97.72±0.75
	6	95.46±1.67	96.54±2.03	97.58±1.85
	9	96.83±0.24	96.49±1.28	98.60±0.43
Cr	3	1.38±0.65	53.49±3.29	75.77±6.08
	6	61.64±5.44	80.90±3.36	85.29±3.89
	9	80.36±3.66	92.09±0.25	93.47±1.14
Ni	3	63.54±4.05	75.21±2.37	91.59±2.27
	6	82.46±3.54	85.55±1.72	95.26±1.35
	9	93.51±0.55	92.83±0.88	97.88±0.27
As	3	53.81±1.36	75.05±4.43	65.00±5.69
	6	64.47±2.98	86.76±2.16	86.72±0.88
	9	68.29±3.73	94.51±0.48	96.09±1.04
Pb	3	97.56±2.05	75.55±4.22	96.95±1.33
	6	98.70±0.78	84.96±3.78	94.71±2.81
	9	91.37±3.55	0±0	28.62±4.48
Cd	3	97.27±0.42	85.08±0.13	99.90±0.02
	6	98.75±0.12	100±0	100±0
	9	86.62±0.05	0±0	0±0

Value ± SD, n=3. A.t, *Acidithiobacillus ferrooxidans*; A.f, *Acidithiobacillus thiooxidans*.

sludge again. The highest removal of Cd and Pb in the solution bioleached by the mixed culture was 100 and 94.71% on the 6th day. However, on the 9th day, these rates decreased to 0 and 28.62%, respectively. The highest removals of Cd and Pb by *A. ferrooxidans* also occurred on the 6th day (100 and 84.96%, respectively) but both decreased to 0% on the 9th day. *A. thiooxidans*, however, played an important role in bioleaching Cd and Pb, removing 97.56, 98.70, and 91.37 on the 3rd, 6th, and 9th days, respectively.

The removal of bioleaching appears different between several heavy metals. Nareshkumar (2008) found that solubilization of Cr, Zn, Cu, Pb and Cd from the contaminated soil was in the range of 11 to 99% using *A. thiooxidans*. Xiang (2000) used *A. ferrooxidans* to deal with anaerobically digested sludge and obtained the removal of Cr, Cu, Zn, Ni and Pb which were between 16.2 and 91.5%. Moreover, Qiu (2006) found similar results with *A. thiooxidans* and *Acidithiobacillus ferrooxidans*. However, in this study, the removal of As, Cr, Cu, Ni, and Zn bioleaching by the mixed culture were all above 90%. The high removal may be due to the effect of indigenous bacteria. The activated sludge which the strains were isolated from and the bioleaching carried out with came from the same bioreactor. So NK-06 and NK-16 should be indigenous bacteria to the sludge sample. Exogenous species often fail to compete with the indigenous population due to a lack of suitability and may also cause process inhibition. In addition to the potential for lower cost, indigenous bacteria also have a better environmental friendliness, which show a bright prospect for environmental improvement. Such *in-situ* action could promote bacterial metabolism and strengthen bioleaching effectiveness (Mikkelsen et al., 2009).

In addition, few studies have paid attention to the effect of bioleaching time. However, in this study, we found that several kinds of metals could be absorbed by sludge as the reaction time increased, which could cause the removal to decreased. Therefore, reaction time is an important factor of bioleaching.

The number of fecal coliforms in the sludge before bioleaching was 14600 mpn/g, as determined by the most probable number technique. In contrast, the number after bioleaching was 0. This showed that pathogenicity of the

sludge was markedly reduced after bioleaching, and that the sludge was suitable for land application.

Conclusions

The pH of sludge decreased to below 2 after bioleaching for 9 days. After the same period, the ORP of the sludge increased to over 600 mV. In terms of As, Cr, Cu, Ni, and Zn removal, the mixed culture of *A. ferrooxidans* and *A. thiooxidans* was more effective in bioleaching than the single cultures of *A. ferrooxidans* or *A. thiooxidans*, and the effect improved with time. While, for Cd and Pb, the removal by the mixed culture or *A. ferrooxidans* single culture decreased after 6 days, whereas with *A. thiooxidans* single culture the removal rate remained high. Therefore, an appropriate bioleaching time and proportion of the two bacteria should be employed to achieve an optimal removal of heavy metals.

ACKNOWLEDGEMENTS

This work was supported by the National 863 High-Tech R&D Program of China (2007AA06Z323), The Scientific and Technological Project of Tianjin, China (09JGZDJC18400, 09ZCKFSH00800).

REFERENCES

Babel S, del Mundo DD (2006). Heavy metal removal from contaminated sludge for land application: A review. Waste Manage. 26: 988-1004.

Bosecker K (1997). Bioleaching: metal solubilization by microorganisms. FEMS. Microbiol. Rev. 20: 591-604.

Bruce AM, Davis RD (1989). Sewage sludge disposal: current and future options. Water Sci. Technol. 21: 1113-1122.

Burton FL (1991). Wastewater Engineering: Treatment, Disposal and Reuse, third ed. McGraw-Hill, Singapore.

Kim IS, Leem JU, Jang A (2005). Bioleaching of heavy metals from dewatered sludge by *Acidithiobacillus ferrooxidans*. J. Chem. Technol. Biot. 80: 1339-1348.

Kosobucki P, Kruk M, Buszewski B (2008). Immobilization of selected heavy metals in sewage sludge by natural zeolites. Bioresource Technol. 99: 5972-5976.

Kumar S, Tamura K, Nei M (2004). MEGA3 integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform. 5: 150–163.

McGhee TJ (1991). Water Supply and Sewage, sixth ed. McGraw-Hill, Singapore.

Mercier G, Chartier M, Couillard D (1996). Strategies to maximize the microbial leaching of lead from metal- contaminated aquatic sediments. Water. Res. 30: 2452-2464.

Mikkelsen D, Kappler U, McEwan AG, Lindsay IS (2009). Probing the archaeal diversity of a mixed thermophilic bioleaching culture by TGGE and FISH. Syst. Appl. Microbiol. 32: 501–513.

Nareshkumar R, Nagendran R, Parvathi K (2008). Bioleaching of heavy metals from contaminated soil using *Acidithiobacillus thiooxidans*: effect of sulfur/soil ratio. World. J. Microbiol. Biotechnol. 24: 1539–1546.

Qiu MQ, Xiong SY, Zhang WM (2006). Efficacy of chalcopyrite bioleaching using a pure and a mixed bacterium. J. Univ. Sci. Technol. Beijing. 13: 7-10.

Rousk J, Brookes PC, Bååth E (2009). Contrasting soil pH effects on fungal and bacterial growth suggest functional redundancy in carbon mineralization. Appl. Environ. Microbiol. 75(6):1589-1596.

Solisio C, Lodi A, Veglio F (2002). Bioleaching of zinc and aluminum from industrial waste sludges by means of *Thiobacillus ferrooxidans*. Waste Manage. 22: 667-675.

Tyagi RD, Couillard D (1987). Bacterial leaching of metal from sewage sludge. Process. Biochem. 22: 114-117.

Xiang L, Chan LC, Wong JWC (2000). Removal of heavy metals from anaerobically digested sewage sludge by isolated indigenous iron-oxidizing bacteria. Chemosphere 41: 283-287.

Xin BP, Zhang D, Zhang X, Xia YT, Wu F, Chen S, Li L (2009). Bioleaching mechanism of Co and Li from spent lithium-ion battery by the mixed culture of acidophilic sulfur-oxidizing and iron-oxidizing bacteria. Bioresource. Technol. 100: 6163-6169.

Yang C, Zhang W, Liu R, Li Q, Li B, Wang S, Song C, Qiao C, Mulchandani A (2011). Phylogenetic diversity and metabolic potential of activated sludge microbial communities in full-scale wastewater treatment plants. Environ. Sci. Technol. 45(17): 7408-7415.

Zhang P, Zhu Y, Zhang GM, Wu Z (2009). Sewage sludge bioleaching by indigenous sulfur-oxidizing bacteria: Effects of ratio of substrate dosage to solid content. Bioresource Technol. 100: 1394-1398.