Bioleaching in batch tests for improving sludge dewaterability and metal removal using *Acidithiobacillus ferrooxidans* and *Acidithiobacillus thiooxidans* after cold acclimation

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**ABSTRACT**

Bioleaching is a promising technology for removal of metals from sludge and improvement of its dewaterability. Most of the previous studies of bioleaching were focused on removal of metals; bioleaching in cold environments has not been studied extensively. In this study, *Acidithiobacillus ferrooxidans* and *Acidithiobacillus thiooxidans* were acclimated at 15 °C and co-inoculated to explore the optimal conditions for improvement of sludge dewaterability and removal of metals by the sequencing batch reactors. The data show after 6 days of bioleaching at 15 °C, 89.6% of Zn, 72.8% of Cu and 39.4% of Pb were removed and the specific resistance to filtration (SRF) was reduced to ∼12%. In addition, the best conditions for bioleaching are an initial pH of 6, a 15% (v/v) inoculum concentration, and *A. thiooxidans* and *A. ferrooxidans* mixed in a ratio of 4:1. We found that bioleaching of heavy metals is closely related to final pH, while the sludge SRF is dominated by other factors. Bioleaching can be completed in 6 days, and the sludge dewaterability and removal of metals at 15 °C meet the requirements of most sewage treatment plants.

**Key words** bioleaching, cold acclimation, heavy metal, sewage sludge, specific resistance

**INTRODUCTION**

Cities produce large amounts of excess sludge, and the growth of sludge production is expected to increase in the next decade (Smith *et al*. 2009). Most municipal wastewater treatment plants use an activated sludge process, and data suggest that sludge disposal and treatment is seriously deficient in most developing countries (Pathak *et al*. 2009). Traditional methods such as composting, incineration and use of landfills are known to have inherent shortcomings (Liu *et al*. 2012a), and sludge disposal regulations are becoming more stringent. Sewage sludge is rich in organic matter and macronutrients especially nitrogen, phosphorus and potassium. However, high moisture and frequent high concentrations of heavy metals in sludge generated by mechanical dewatering restricts its agricultural application (Babel & Dacera 2006; Wong & Gu 2008). Bioleaching has recently emerged as a strategy with which to remove heavy metals and at the same time enhance the dewaterability of sludge. The practical application of the physical and chemical processes is limited due to the requirement of large amount of chemicals, the high operating cost, the operational difficulties and the associated secondary pollution problems. Compared with physical and chemical approaches, bioleaching with sulfur-oxidizing bacteria is eco-friendly and requires only a modest capital investment, demand for labor, and energy consumption (Mishra *et al*. 2005).

Bioleaching is defined as the solubilization of metals from solid substrates either directly by the metabolism of leaching bacteria or indirectly by the products of this metabolism. The bioleaching process uses the catalytic effect produced by the metabolic activities of iron-oxidizing and
sulfur-oxidizing microorganisms, and this results in acceleration of the chemical degradation of the sulfides (Pathak et al. 2009). The most widely used microorganism is Acidithiobacillus, and research on the mechanism of bioleaching has concentrated mainly on Acidithiobacillus ferrooxidans and Acidithiobacillus thiooxidans (Fang & Zhou 2006). Acidithiobacillus thiooxidans is a type of acidophilic aerobe that oxidizes sulfur to sulfuric acid, leading to acidification of the sludge (Liu et al. 2003). Acidithiobacillus thiooxidans directly oxidizes metal sulfides, producing soluble metal sulfates, and also oxidizes elemental sulfur, forming sulfuric acid (Wang et al. 2008). With similar biological characteristics, A. ferrooxidans is a Gram-negative acidophilic chemolithoautotroph, which uses CO₂ as a carbon source and obtains its energy for growth from the oxidation of sulfur, reduced sulfur compounds and Fe²⁺ (Zhou et al. 2005).

In the winter, sludge temperatures in municipal wastewater treatment plants are as low as 16.4 °C, a temperature at which A. ferrooxidans and A. thiooxidans lose their cellular activity (Liu et al. 2015). Most of the relevant research, however, has been conducted at temperatures optimum for growth, and studies of the cold acclimation of Acidithiobacillus are largely unknown. The objectives of the present study are to acclimate Acidithiobacillus to the cold and to attempt to shorten the low temperature bioleaching period. Bridging the gap between the duration of traditional methods and the bioleaching period in winter will improve the application range of bioleaching technology. It is well known that bioleaching plays an important role in the removal of sludge-borne metals as well as enhancing sludge dewaterability (Liao et al. 2009). Previously, sludge dewaterability and metals removal were studied individually, but most of the research was focused on the removal of sludge-borne heavy metals rather than sludge dewaterability (Peng et al. 2011; Zhou et al. 2014). Specific resistance to filtration (SRF), capillary suction time (CST) and sedimentation rate have all been used to assess sludge dewaterability (Feng et al. 2009). The physical meaning of SRF is the filtration resistance of a unit mass of sludge through a unit area of a filter under specified pressure. The SRF of the sludge represents the resistance caused by a unit mass of sludge under a given pressure filtration per unit of the filter area. Therefore, a lower SRF indicates a better sludge dewaterability. SRF values can reliably reflect sludge dewaterability in engineering applications because the same theory describes the use of the filter press in sewage treatment plants. Compared with CST, data are more available on sludge SRF (Xie et al. 2006). In the present study, bioleaching was performed to explore optimal conditions for sludge dewatering and metal removal efficiency using inoculation of both A. ferrooxidans and A. thiooxidans and the co-addition of Fe²⁺ and sulfur. The effects of conditional parameters such as pH, oxidation-reduction potential (ORP), concentrations of sulfate and ferrous compounds and operational parameters, including inoculation quantity and composition of the mixed strains. Initial pH values of the sludge were investigated in long-term continuous experiments.

**MATERIALS AND METHODS**

**Sludge properties**

The sewage sludge used in this study was collected from Wulongkou sewage treatment plant in Zhengzhou, Henan province, China. The sample was stored in polyethylene plastic buckets and stored at 4 °C in the laboratory prior to its use. The time interval between storage and use was less than 1 week. Sludge pH and SRF were determined immediately after collection, and sludge solid content was assessed by oven drying. Heavy metals were partitioned into five different fractions – exchangeable, bound to carbonates, bound to Mn-oxides, bound to Fe-oxides, and bound to organic matter – and were analyzed using a modified sequential extraction procedure (Tessier et al. 1979). The quantity of different metals in each fraction was determined by atomic absorption spectrometry (Shimadzu UV-2450, Japan). The total extractable amount of any specific heavy metal is defined as the sum of the amounts of that metal found in the five fractions. The major metals in sludge were found to be Zn, Cu and Pb. The dried sludge sample was measured for organic matter content (APHA 2005) and the physicochemical properties of the selected municipal sewage sludge samples are listed in Table 1.

**Microorganism and culture medium**

Acidithiobacillus ferrooxidans LX5 (CGMCC No. 0727) and A. thiooxidans TS6 (CGMCC No. 0759), obtained from China General Microbiological Culture Collection Center (CGMCC), were cultivated in a modified 9 K medium (Zhou et al. 2014). Nutrient elements in the sludge were analyzed in order to optimize the nutrient growth medium (9 K) and the composition of the modified medium was (NH₄)₂SO₄ (3 g), K₂HPO₄ (3 g), MgSO₄ (0.025 g), CaCl₂ (0.019)

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and 10 g of energy substrate (sulfur for *A. thiooxidans* and sulfur/FeSO₄·7H₂O (1:9) for *A. ferrooxidans*) per liter of substrate ([Bojinova & Velkova 2001]). In the cold acclimation tests, the composition of the nutrients was prepared based on 1 liter of deionized water, and in the bioleaching test, the composition of the nutrients was prepared based on 1 liter of sludge.

**Experimental design**

*Acidithiobacillus thiooxidans* and *A. ferrooxidans* were cultivated using respective modified 9 K media and were acclimated separately. Samples of 100 mL medium and sulfur/FeSO₄·7H₂O (1:9) were used for inoculation using the difference method, and the volume of complementary medium was adjusted to 4.0 and the amount used for inoculation of the successive cultures (10%) was the same for each of the three experiments. During the bioleaching process, water lost by evaporation and sampling was replenished daily with twice-distilled water using a weight difference method, and the volume of complementary water was nearly 2 mL per day in this experiment. All experiments were repeated three times.

**Analytical methods**

The pH in the reactor was monitored with a pH meter and the ORP with an ORP meter. Solids content was measured with a 105 °C oven-drying method and organic matter by an ignition gravimetric method (CJ/T 2005). The samples were centrifuged at 8,000 rpm for 10 min and the supernatant was filtered through a 0.45-μm membrane filter. The concentration of Fe²⁺ was determined by a spectrophotometric method using o-phenanthroline, as described previously ([Song & Zhou 2008]). The sulfate concentration in the filtrate was determined in a standard procedure using CI-100 ion chromatography. The SRF of the sludge, defined as the resistance caused by unit mass of sludge under a given pressure filtration per unit of the filter area, was tested using the Buchner funnel-vacuum filtration equipment shown in Figure 1. The space pressure and filter area of this equipment can be set to a stable value. The time required for a certain amount of sludge to pass through the filtration equipment can be measured and all of these values can be calculated from the formula: $\frac{dV}{dt} = PA^2/\mu(rCV + RmA)$ (dV/dt) represents filtration rate, m³/s; P represents filtration pressure, N/m²; A represents filtration area, m²; µ represents dynamic viscosity, N·s/m²; r represents filtration specific impedance, m/kg; C represents dry weight of filter cake from sludge filtrate per unit volume, kg/m³; V represents volume of the filtrate, m³; Rₘ represents resistance of the filter medium per unit area in the beginning of filtration, m²/m³. Samples were ground and digested with a mixture of HCl, HNO₃ and HClO₄ to dissolve heavy metals and the Cu, Zn and Pb levels were determined by atomic absorption spectrometry ([Bao 2005]).

**RESULTS AND DISCUSSION**

**Cold acclimation of the microorganisms**

The optimum temperature for growth of *Acidithiobacillus* is 30–38 °C. The adaptable phase of unacclimated
Acidithiobacillus will be prolonged at 15°C and this leads to a long buffering period in the bioleaching process. Pre-incubation at 15°C allows bioleaching to take place more rapidly, decreasing the low-temperature bioleaching period. The pH is widely known to be the most important parameter influencing solubilization of sludge-borne heavy metals during bioleaching (Chen & Lin 2001). Maintenance of the pH at around 2.0 as a result of bio-acidification is very important as most of the sludge-borne metals will remain in solution at this pH, and the pH value of 2.0 is commonly chosen as an end point in bioleaching studies (Liu et al. 2012b). The pH of the first generation A. thiooxidans solution decreased from 4.0 to 2.0 after 9 days, while the pH of the second and the third generations reached 2.0 in 6 days and 3 days respectively as shown in Figure 2(a). In A. ferrooxidans, with different acid-producing ability, the pH decreased from 4.0 to 3.0 in 9, 4 and 2 days in the three successive generations, respectively. These results clearly indicated that after an acclimation period of three generations, A. thiooxidans and A. ferrooxidans adapt to the cold environment. The whole preculture process proved the feasibility of Acidithiobacillus under three-generation cold acclimation and established the reliability of the subsequent experiments.

Conditions affecting the bioleaching

Effect of initial pH of the sludge on bioleaching result

As can be seen in Figure 3(a) and 3(b), pH changes in the initial 3 and 4 test groups fell slightly in days 1–4. The buffering period was extended to the fifth day in test group 3. The pH of the test group with the initial pH of 5 increased in the first two days and decreased subsequently. After 8 days of bioleaching, the final pH was close to 2.0 and ORP values were above 260 mV in all four test groups. In test groups with an initial pH of 5 and 6, the ORP increased by ~200 mV after 8 days while in the other two test groups the increase was ~100 mV. As can be seen in Figure 3(c), the concentration of sulfate ions in the test groups with an initial pH of 5 and 6 was more than three times that in the test group with an initial pH of 3. In the test group with an initial pH of 3, the concentration of sulfate ions was 1,200 mg/L, while it reached 3,700 mg/L in the test group with an initial pH of 6. The increase in the sulfate ion concentration was fairly rapid in the second half of the test, while the decrease in the concentration of ferrous ions was obvious at an early stage. The concentration of ferrous ions was just 200 mg/L in the test group with an initial pH of 3, compared to 800 mg/L in the test group with an initial pH of 6. In test groups with an initial pH of 5 and 6, the rate of increase of sulfate ions and the rate of decrease of ferrous ions were both significantly faster than in the test groups with the lower initial pH. As shown in Figure 3(e), the SRF in the test groups with the initial pH of 5 and 6 was reduced by ~70% before bioleaching, while in the test groups with an initial pH of 3 and 4 the corresponding reduction was ~50%. The minimum SRF was found in the test group with an initial pH of 6. As shown in Figure 3(f), removal of heavy metals was more effective in the test groups whose initial pH was 5 and 6 when compared to the other two test groups. The solubilization of heavy metals increased during the first 4 days, after which it remained constant. After 8 days of bioleaching, the solubilization ranges of Cu, Zn and Pb were 70.3–86.3%, 50.5–68.4% and 27.4–36.3% respectively when the initial pH was 3–6. The highest solubilizations of Cu and Zn were recorded at an initial pH of 6, while the lowest solubilizations of Cu, Zn and Pb were obtained when the initial pH was 3. The degree of solubilization of Pb was nearly the same at an initial pH of 5 or 6.
Figure 2 | Variations of pH (a) and (d), ORP (b) and (e) and sulfate (or ferrous) ions (c) and (f) during the acclimation process (9d, 6d, 5d for three generations respectively) for A. thiooxidans (a–c) and A. ferrooxidans (d–f).
Figure 3 | Dynamics of pH (a), ORP value (b), SO\(_2^-\) (c) and Fe\(^{2+}\) (d) concentrations between different initial pH (3, 4, 5 and 6 of 10% inoculation quantity with 1:1 composition ratio of A. thiooxidans and A. ferrooxidans) during bioleaching (8d) and changes of specific resistance (e) and metal solubility (f) after bioleaching.
Because pH plays an important role in bioleaching, the initial pH in the bioleaching system should be considered. The preferred environment of Acidithiobacillus is acidic, and inoculation of Acidithiobacillus in bioleaching decreases the sludge pH. The initial pH in the bioleaching system has a great influence on Acidithiobacillus (Zhou et al. 2005). The sludge system was acidified gradually in the bioleaching process with the growth and metabolism of Acidithiobacillus. Pre-acidification will change the pH of the system, making it more suitable for strong Acidithiobacillus. However, living space for weak Acidithiobacillus in this system was squeezed. Mutualism of strong and weak Acidithiobacillus could perform better in microbial groups of sludge environment. A small quantity of weak Acidithiobacillus makes an unfavorable environment for strong Acidithiobacillus and has a negative effect on the growing of Acidithiobacillus. The different efficiencies of heavy metal removal that have been reported can be explained by the final pH achieved in the system, as the solubility of metals is governed primarily by pH (Kumar & Nagendra 2007). Inoculation with Acidithiobacillus would acidify the sludge environment, and reduced the pH of the leaching system to ~6 in this experiment. In test groups with an initial pH of 3 and 4, pH and ORP changes in the first four days were not obvious. Although microbial activities of Acidithiobacillus in test groups with an initial pH of 3 and 4 produced increases in the SO$_2^{2-}$ concentration and decreases in the Fe$^{2+}$ concentration, the rate of change of the nutrient concentration was relatively lower than in test groups with an initial pH of 5 and 6. This indicates that pre-acidification, resulting in a more suitable pH, does not make it easier for Acidithiobacillus to adapt to the sludge environment. Changes of specific resistance and metal solubility after bioleaching in pre-acidification groups also indicated that pre-acidification failed to have a positive effect on bioleaching. Decreasing pH and increasing ORP in test groups with an initial pH of 6 showed the maximal variation in bioleaching process. Changes of SO$_4^{2-}$ and Fe$^{2+}$ concentrations indicated the test group with an initial pH of 6 utilizes the energy of the substrate more efficiently than the other test groups. The greatest improvement of sludge dewatering performance and maximum metal solubility were observed in non-acidification groups (initial pH of 6). These results demonstrate that chemical pre-acidification does not promote the bioleaching process. Acidithiobacillus is composed of weak acidophilic thiobacilli and strong acidophilic thiobacilli, which play a major role in the early stages (neutral to weak acid environment) and mid- to late-stages (weak acid environment to acid environment) (Zhou et al. 2005). Pre-acidification made the system more suitable for strong acidophilic thiobacilli, while the activity of weak acidophilic thiobacilli was inhibited. High RSF after bioleaching and low utilization of nutrients in test groups with an initial pH of 3 and 4 indicated that inhibition of weak acidophilic thiobacilli obstructs improvement of sludge dewatering performance and the bioleaching effect. Previous studies have revealed that sludge dewaterability is controlled by many factors, including sludge particle size, floc structure, and extracellular polymeric substances (EPS). Chemical acidification in the system will destroy the zoogloea structure and the flocculation morphology of the micropopulation in sludge (Huo et al. 2014). Disruption of floc structure and release of intracellular and extracellular materials lead irreversibly to significant deterioration of sludge dewaterability, increasing the difficulty of sludge dewatering (Smith et al. 2009). Pre-acidification has a significant effect on EPS content in sludge, and this is the reason for the different SRF decreases (Liu et al. 2012b; Huo et al. 2014). Pre-acidification in sludge bioleaching will not only expand the total cost but also increase the technological difficulty of the process. Previous reports generally agree that pre-acidification is superfluous to the industrial operation of bioleaching (Ren et al. 2009), and based on all these results, it can be concluded that an initial pH of 6.0 is beneficial for the bioleaching process. Wong et al. (2002) reported an initial pH of 6 as the optimum for Cu solubilization from anaerobically digested sludge employing A. ferrooxidans.

**Effect of inoculation quantity on bioleaching result**

As shown in Figure 4(a), the pH in the 0% test group was reduced by only 0.7 after buffering for 3 days and was relatively stable through the bioleaching process. After 7 days of bioleaching, test groups with inocula of 10% and 20% had pH < 2.5 and test groups with an inoculum of 15% had pH < 2.0. Thus an increase in the inoculum level leads to an increasing fall in pH when the inoculum concentration changes from 0% to 15%. In the first three days, the test group with inoculum of 20% performed better than the 10% test group, while the pH variations of the 10% and 20% test groups presented similar change tendencies in the last three days. The maximum rate of pH decrease appeared in the 15% test group. Figure 4(b) shows that the rate of increase of ORP in test groups with different inoculation quantities was 15% > 20% > 10% > 5% > 0% from high to low inoculation quantity. In Figure 4(c), an obvious increase in the concentration of sulfate ions can be seen to appear...
Figure 4 | Dynamics of pH (a), ORP value (b), $SO_4^{2-}$ (c) and $Fe^{2+}$ (d) concentrations between different inoculation quantities (0%, 5%, 10%, 15% and 20% with initial pH of 6.0 and 1:1 composition ratio of A. thiooxidans and A. ferrooxidans) in sludge during bioleaching (7d) and changes of specific resistance (e) and metal solubility (f) after bioleaching.
two days later and remained until the end of the bioleaching. Other test groups showed a similar tendency, and the test group with 15% inoculation quantity had the highest rate of increase of sulfate ions. In Figure 4(d), a greater rate of decrease of ferrous ions appeared early, and this rate of decrease was relatively lower in the final stage. The test group with 15% inoculation quantity showed the fastest rate of decrease of ferrous ion concentration in the five test groups. In Figure 4(e), the decrease of the SRF varied from 13% to 87% among the different test groups. The resistance in the test group with 15% inoculum was reduced to one-eighth of its original value, while in the test group without initial inoculum the specific resistance was reduced by just 12% and the decrease in SRF in test groups of 10% and 20% approached 60–70%. The greatest solubilization of heavy metals was observed in the 15% test group, and the removal efficiency of Cu, Zn and Pb was 79.5%, 73.8% and 34.6% respectively.

Inoculation quantity is an important factor which affects the initial adaptation of the microorganisms. A slight decrease in pH was observed in the test group with 0% inoculum indicating that a small amount of acid produced by microorganisms can be found in the sewage sludge. The bioleaching process involves complex mechanisms which are based mainly on the growth of Acidithiobacillus. In this study, the maximum efficiency of bioleaching was 15%. Figure 4(c) and 4(d) show that the rapid growth periods of A. thiooxidans and A. ferrooxidans were different. This is due to the massive replication of A. ferrooxidans, which oxidizes ferrous ions to ferric ions in the early stages of the process. Later in the bioleaching period, A. thiooxidans proliferates on a large scale and produces quantities of acid, completing the whole bioleaching process (Akcil et al. 2007). In 7 days of bioleaching all the metals reached a maximum level of solubilization which then remained relatively constant until the bioleaching was terminated. Higher concentrations of inocula within a given range accelerated sludge acidification, leading to increases in the bioleaching of heavy metals (Bayat & Sari 2010). The optimal inoculation quantity in bioleaching is limited by the resource supply from the sludge environment and competition of Acidithiobacillus. Excess inoculum however would provoke intensified competition of Acidithiobacillus which, with a limited environment and resources, would lead to an unreasonable use of nutrients. Based on these results, the optimal inoculation quantity of the mixed strains was determined to be 15%. Some published results indicate that leaching efficiency improves with inoculum from 0% to 10% (Tsaia et al. 2005; Wen et al. 2013). In consideration of the similar cell numbers, the results further showed that leaching efficiency improves as the inoculum concentration increases from 0% to 15% at 15 °C. As the inoculum reached 20%, the bioleaching effect was no better than in the 15% inoculum concentration test group in this study. It indicated that an inoculum concentration of 15% was appropriate for the bioleaching process and achieved a better bioleaching effect of improvement of sludge dewatering performance and removal of heavy metals.

Effect of different compositions of mixed Acidithiobacillus on the bioleaching

Figure 5(a) shows how different compositions affect pH variation during the microbial leaching process. In general, the rate of decrease in pH increases with increasing inoculum concentration of A. thiooxidans. Differences between the five test groups were minimal in the first two days, but after two days of bioleaching, the groups with composition ratios of 1:4 and 1:2 showed a slower pace of pH decrease and after five days, the final pH was >3.0. The final pH of test groups with composition ratios of 1:1 and 2:1 was <2.5. The largest rate of decrease in pH was observed in the test group with a composition ratio of 4:1, whose final pH was <2.0. With the increase of A. thiooxidans in the mixed strains, the ORP rise increased gradually, the greatest ORP rise appearing in the 4:1 test group. Changes of SO2−4 and Fe2+ concentrations in sludge solutions can be seen in Figure 5(c) and 5(d), which show that the sulfate ion concentration increased while the ferrous ion concentration showed a tendency to decrease gradually as the bioleaching process proceeded. Changes of sulfate concentration were different in the five test groups. In contrast to the other three test groups, the test groups of 1:2 and 1:4 had a similar variation in the sulfate concentration. As shown in Figure 5(e), the greatest SRF drop appeared in the test group with a ratio of 4:1, and in this test group the final SRF was 12% of the initial value. Sludge-borne metals dissolved gradually, with a continuous decrease in the pH in the bioleaching system. In Figure 5(f), metal solubilization increased with the increasing percentage of A. thiooxidans in the mixed bacteria. About 89.6% Cu, 72.8% Zn, and 39.4% Pb were dissolved in the test group with a composition ratio of 4:1, and the rates of the other test groups were lower.

When compared to a single culture of A. ferrooxidans, a mixed culture of A. ferrooxidans and A. thiooxidans resulted in about 10% higher solubilization of heavy metals. A batch bioleaching study showed that compared to sulfur-oxidizing bacteria, iron-oxidizing bacteria improved the sludge
Figure 5 | Dynamics of pH (a), ORP value (b), SO\textsubscript{2}\textsuperscript{2−} (c) and Fe\textsuperscript{2+} (d) concentrations between different compositions of mixed bacteria (A. thiooxidans : A. ferrooxidans = 4:1, 2:1, 1:1, 1:2 and 1:4 of 15% inoculation quantity with initial pH of 6.0) in sludge during bioleaching (6d) and changes of specific resistance (e) and metal solubility (f) after bioleaching.
dewatering performance more effectively. On the other hand, *A. thiooxidans* was reported to be highly effective in reducing the pH of the sludge (Falco et al. 2003; Ishigaki et al. 2005). Changes of pH and ORP both showed that mixed *Acidithiobacillus* with a ratio of 4:1 was better able to adapt to the sludge environment. The greatest increase of sulfate ions and the maximum decrease in the amplitude of ferrous ions showed that nutrient substance utilization was most efficient in the 4:1 test group, and mixed *Acidithiobacillus* can accelerate the whole bioleaching process most effectively in such conditions. In all the experiments related to removal of heavy metals, the order of metal ion solubility is Cu > Zn > Pb. The overall low Pb removal is mainly due to precipitation of PbSO₄, which has a very low solubility. The efficiency of Pb solubilization is generally lower than that of other metals in the bioleaching process, a result that is consistent with reports from other laboratories (Kumar & Nagendran 2009). In the test group with a ratio of 4:1, the increasing rate of SO₄²⁻ concentrations in the first two days was lower than in test groups with a ratio of 1:4 and 1:2, while in the later stages, the rate of increase of SO₄²⁻ concentrations in the test group with a ratio of 4:1 was the fastest among all the test groups. The rate of decrease of Fe²⁺ concentrations in the test group with a ratio of 4:1 was the highest among all the test groups in the whole process of bioleaching. Efficient usage of nutrients was observed when the ratio of *A. thiooxidans*: *A. ferrooxidans* was 4:1, and this mixed *Acidithiobacillus* adapts better to the sludge environment. Based all these results, when *A. thiooxidans* and *A. ferrooxidans* are mixed in a ratio of 4:1, the bioleaching period can be shortened and the efficiency can be maximally enhanced because, in the early stage of bioleaching, *A. ferrooxidans* has better survivability than *A. thiooxidans*. Increasing the proportion of *A. thiooxidans* assists *A. thiooxidans* to survive through the dormant period (Wang et al. 2008; Wen et al. 2012, 2015). Both *A. thiooxidans* and *A. ferrooxidans* can grow well when the pH of the system decreases to a value appropriate to *A. thiooxidans*. It supports the cooperative and symbiotic relationship between *A. thiooxidans* and *A. ferrooxidans* at the later stages of bioleaching.

**CONCLUSIONS**

Three-generation cold-acclimation ensures stable growth of *A. thiooxidans* and *A. ferrooxidans* at low temperatures. Maximum bioleaching can be achieved in 6 days with an 86.3% decrease in specific resistance and removal of 89.6% Cu, 72.8% Zn and 59.4% Pb in the optimal experimental conditions, an initial pH of 6, 15% inoculation quantity and *A. thiooxidans* and *A. ferrooxidans* mixed in a ratio of 4:1 at 15 °C. Compared with experiments without pre-culture, cold acclimation of *Acidithiobacillus* leads to a greatly shortened buffering period in the bioleaching process. This is a low cost, environmentally friendly technique that is cheaper in terms of chemical consumption compared to traditional chemical methods. These results suggest that bioleaching has an engineering application prospect for wastewater treatment plants in winter.

In the three experimental series, if the pH is decreased to ~2.5 for a contact time of at least 6 days, the removal efficiencies for Cu, Zn and Pb were found to be at least 80%, 60% and 30% respectively. At pH ~2, as much as 90% removal of Zn was obtained. These results indicated that better removal efficiencies were obtained at pH = 2 or less. For removal of heavy metals, the efficiency obtained was related to lower pH rather than to sludge dewaterability. In this study, lower final pH values were found not always to guarantee a better decrease in the SRF. Sludge dewaterability is determined by sludge structure and the final pH is simply an indication of the bioleaching stage. Although much research into sludge dewaterability has been reported, the mechanism of the process remains largely an open question.

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