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An Acid-Tolerant Heterotrophic Microorganism Role in Improving Tannery Sludge Bioleaching Conducted in Successive Multibatch Reaction Systems

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The bioleaching technique with *Acidithiobacillus* species has been shown to be an efficient and cost-effective means in removing heavy metals from tannery sludge. However, tannery sludge dissolved organic matter (DOM) is toxic to *Acidithiobacillus* species and results in decreasing the efficiency of Cr removal from sludge. Here we report the role of an acid-tolerant DOM-degrading heterotrophic microorganism *P. spartinae* D13 successfully isolated from local tannery sludge in improving activities of *A. ferrooxidans* LX5 and *A. thiooxidans* TS6. In tannery sludge DOM-rich liquid culture medium coinoculated with *P. spartinae* D13 and *Acidithiobacillus* species, the activities of *A. ferrooxidans* LX5 and *A. thiooxidans* TS6 are increased by 33- and 12-fold, respectively. In four successive batches of tannery sludge bioleaching trials by circulating 10% of acidified bioleached sludge, the addition of *Pichia spartinae* D13 could shorten bioleaching time by 3 days in the first batch and obtained more than 90% of Cr removal efficiency within 6 days. However, the effectiveness of *P. spartinae* D13 in improving bioleaching maintain only four successive recycle or batches until *P. spartinae* D13 inoculum is replenished in the fifth batches. Therefore, for enhancing the activity of *Acidithiobacillus* species in bioleaching system, *P. spartinae* D13 should be added periodically at a given batch interval.

Introduction

Tanning process using Cr(III) compounds is one of the most common methods in leather industry for processing hides to protect leather against microbial degradation, moisture, sweat, etc (*1*). In this process, only 60−70% of Cr(III) reacts with hides and the rest of chromium remains in the tannery effluent and consequently goes into the tannery sludge during sewage treatment process (*1*−*3*). In accord with the shift of the international leather industry toward Asia, China is being developed as a center for leather manufacture and production in the world (*4*). About 300 000 tons tannery sludge (dry matter basis) containing extreme high content of Cr(III) is generated annually in China (*5*). In the absence of proper handling or disposal, the chromium accumulated in tannery sludge tends to be released to the environment (*6*). However, Cr(III) is not biodegradable, and its uptake by plants and subsequent accumulation along the food chain is a potential threat to animal and human health (*7*). Thus, tannery sludge is classified as a hazardous waste by many nations due to its high content of Cr(III) in the sludge (1−4%, wt/wt) (*8*), and the urgency has also been raised to dispose tannery sludge economically and safely.

Traditional disposal methods including incineration and landfills have some limitations in both practical application and environmental consideration (*9*). One promising long-term solution appears to be recycling of tannery sludge and using it for beneficial purposes after the removal or the recovery of Cr(III), such as land application of the detoxified sludge on forest and disturbed lands. On the other hand, the recovery of Cr(III) and its reuse in the tanning process has the economical interest considering the high content of Cr(III) in tannery sludge and annual astonishing consumption in the Cr(III)-containing tanning agent around the world. More important, this process can greatly reduce Cr(III) release to the environment (*10*). Therefore, the extraction of Cr(III) from tannery sludge is indispensable and vital for sludge land utilization and/or the Cr (III) recovery purposes.

Bioleaching technique, applied successfully and commercially in biohydrometallurgy for extracting metals from low-grade ores, has been studied mainly in Canada for removal of toxic metals from sewage sludge without seriously affecting its soil conditioning and fertilizing properties (*11*−*14*). Generally, two *Acidithiobacillus* species, *Acidithiobacillus ferrooxidans*, and *Acidithiobacillus thiooxidans* are the most significant species used in bioleaching process (*15, 16*). During bioleaching process, heavy metals can be dissoluted from sewage sludge by acidification of sludge through both direct mechanisms and indirect mechanisms by sulfur-oxidizing bacteria *A. thiooxidans* (*17*−*19*) or iron-oxidizing bacteria *A. ferrooxidans* (*12, 16*).

The maximum efficiency of metal solubilization could be achieved only when the conditions of bioleaching consistent with the optimum growth conditions of the applied bacteria (*20*). Many operation parameters including type of substrate (*19*), temperature (*21*), sulfur concentration (*18, 22*), solid content (*10, 11*), and dissolved oxygen (*23*) have been extensively studied to achieve higher metal solubilization efficiency. Our previous studies have demonstrated that 96% of Cr could be recovered from tannery sludge through 6−8 days bioleaching with 4 g L^{-1} of elemental sulfur added as the energy source, and the recovered Cr could be reused in leather industry which meets the desired quality requirements (*5, 24*).

Besides, some other inhibitory factors also have possibilities to affect the activity of *A. ferrooxidans* and *A. thiooxidans*, as a result, influence the performance of bioleaching process. For example, Tuovinen and Kelly (*25*) reported that *A. ferrooxidans* did not tolerate organic acids, making it difficult to grow on solid media. Other studies also found that some organic acids including formic, propionic, hexanoic, and succinic acids could disturb iron-oxidizing activity of *A. ferrooxidans* by reacting abiologically with ferrous iron outside the bacteria cell (*26*). Unfortunately, sewage sludge contains many organic compounds such as low molecular weight organic acids (*27*) especially for tannery sludge (*28*). In fact, previous studies have demonstrated that both municipal sludge DOM and tannery sludge DOM significantly inhibited ferrous iron and sulfur oxidation by *Acidithiobacillus* species, especially when the concentration was higher than 150 mg DOC L^{-1} (*28*). Also, Gu and Wong

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(*29*) found that the presence of 10.8 mM acetic and 9.88 mM propionic acid in sewage sludge led to long lag periods of 6 and 7 days for solubilization of Cu and Cr from sludge, respectively. However, it is very interesting that the *Acidithiobacillus* species could survive in sludge habitat during bioleaching process even though the concentrations of organic acids in sludge were very high. This may be attributed to the complex biodiversity of sewage sludge, which contains many other acidophilic or acid-tolerant microbes besides the *Acidithiobacillus* species (*30*). Furthermore, Gu and Wong (*29, 31*) isolated two strains of heterotrophic acidophiles, *Blastoschizomycetes capitatus* and *Pichia* sp. from anaerobically digested municipal sludge to degrade acetate and propionate acids. Also, Flournier et al. (*30*) reported that the introduction of *Rhodotorula* sp. into the solid medium could shorten the incubation period required for the numeration of *A. ferrooxidans*. However, the concentration of DOM or the lower molecular weight organic acids mainly formic and acetate in tannery sludge is much higher than that in municipal sewage sludge (*28*). The relationship between DOM from tannery sludge and heterotrophic microorganisms in bioleaching process is still poorly understood. Especially, little information on the growth dynamics of heterotrophic microorganism during sludge bioleaching and the duration for the improvement of the leaching activity of *Acidithiobacillus* spp. by heterotrophic microorganisms are available.

Therefore, the purposes of this study are to (1) evaluate the commensalism effect of the heterotrophic microorganism isolated from bioleached tannery sludge and *Acidithiobacillus* species on the tannery sludge bioleaching process, (2) investigate the role of the isolated strain in degrading sludge DOM and improving the growth of *Acidithiobacillus* species in both liquid medium and tannery sludge bioleaching system, and (3) demonstrate the growth dynamics of isolated strain and its duration in improving sludge bioleaching efficiency in the successive multibatch bioleaching systems with acidified sludge circumfluence.

Materials and Methods

Tannery Sludge Sample. The tannery sludge was collected from Tannery Sewage Treatment Plant from Fubang Leather Co. Ltd., Zhejing, China and stored at 4 °C until use. Dried sludge samples were first digested according to standard methods and then measured for soluble metals, total N, S, and organic matter content (*32*). The original sludge with pH 7.92 contained 5.1% of total solids, 43.1% of organic matter, 1.68% of total N, 5.45% of total S, 2.11% of total Fe, 2.10% of Fe(II), and 2.34% of total Cr(III). Cr(VI) is undetectable in tannery sludge.

Isolation and Identification of Sludge DOM-Degrader. Heterotrophic microorganisms capable of degrading sludge DOM were isolated from the bioleached tannery sludge (pH 1.62) by plating on potato dextrose agar (PDA) medium. After two days cultivation on PDA, 13 isolates including bacteria and yeasts were obtained. Dissolved organic matter (DOM) derived from the sludge sample was obtained through shaking original sludge samples in a gyratory shaker at 25 °C and 180 rpm for 16 h followed by centrifuging at 12 500*g* for 20 min and filtered through 0.45 μm membrane (*33*), and low molecular weight organic acids in DOM samples were determined by high performance liquid chromatography (Waters 600−2487, U.S.) (*28*). The concentrations of formic acid, acetic acid, and propionic acid present in 100 mg DOC L^{-1} of sludge DOM were 61.6, 33.5, and 2.32 mg DOC L^{-1}, respectively, and no butyric acid was detected. Degradation of sludge DOM by isolated strains was determined in 250 mL conical flasks containing 100 mL autoclaved tannery sludge DOM as a sole carbon source and 0.1 mL of these actively growing cultures introduced as inoculum. After two days cultivation on a gyratory shaker at 28 °C and 180 rpm, sludge DOM concentrations were determined by TOC analyzer (TOC-5000A, Shimaduz) to assess the abilities of the isolated cultures on the degradation of sludge DOM. A strain with white pigmented colony on PDA medium could degrade tannery sludge DOM from 983 mg DOC L^{-1} to 322 mg DOC L^{-1} in 2 days compared to no DOM reduction in the control inoculated with autoclaved cells, and other strains could only degrade 10−30% of sludge DOM within the same incubation period. Thus, it was considered as sludge DOM-degrader (hereinafter named by D13). The morphology of the isolate was observed by microscopic examination and its identification was performed through both physiological analysis (*34*) and molecular phylogenetic analysis based on the 26S rRNA gene D1/D2 region and internal transcribed spacer (ITS) (*35*) (see Supporting Information).

Iron (Sulfur)-Oxidizing Bacterium and Inoculum Preparation. *Acidithiobacillus ferrooxidans* LX5 (CGMCC No. 0727) and *Acidithiobacillus thiooxidans* TS6 (CGMCC No. 0759) obtained from China General Microbiological Culture Collection Center (CGMCC) were cultivated in modified 9K and SM liquid medium (*28*), respectively. The modified 9K and SM mediums autoclaved at 121 °C for 15 min were adjusted to pH 2.5 and 3.0 with sulfuric acid and then spiked with 44.2 g L^{-1} of 0.21 μm membrane-filtered $FeSO_4 \cdot 7H_2O$ and 10 g L^{-1} of elemental sulfur as the energy source, respectively. The inoculums were prepared by growing these bacteria in 500 mL Erlenmeyer flasks each containing 250 mL of these 9K or SM medium on a gyratory shaker at 200 rpm and 28 °C.

Sludge DOM-Degrader D13 Effect on the Activities of *Acidithiobacillus* spp. in the DOM-Rich Medium. The experiments were carried out in 500 mL Erlenmeyer flasks containing 30 mL, 5 times concentrated 9K and/or SM synthetic medium, 210 mL of distilled water, and 30 mL of sludge DOM as described above with final concentration of DOM in the flask being ~100 mg DOC L^{-1}. The 5% (v/v) of viable *A. ferrooxidans* LX5 or *A. thiooxidans* TS6 and the 5% (v/v) of viable DOM-degrader D13 as the inoculums, 44.2 g L^{-1} of $FeSO_4 \cdot 7H_2O$ for *A. ferrooxidans* LX5 or 10 g L^{-1} of S^0 for *A. thiooxidans* TS6 as the energy source were added to the flasks. The controls were also performed through inoculating 5% (v/v) autoclaved DOM-degrader D13 instead of viable DOM-degrader D13 inoculum. The pH of all flasks was adjusted to 3.0 for the treatment inoculating *A. ferrooxidans* LX5 or 2.5 for the treatment inoculating *A. thiooxidans* TS6, and total volume in each flask was maintained to 300 mL. Then all flasks were incubated in a gyratory shaker at 28 °C and 180 rpm. The loss of water in each flask due to evaporation was compensated by adding distilled water based on weight loss. During incubation, samples were withdrawn everyday from flasks and measured for the strain D13 density by plate count method on PDA plates, for aqueous Fe^{2+} or SO_4^{2-} concentration according to the standard methods (*32*), and for dissolved organic carbon (DOC) with TOC analyzer (TOC-5000A, Shimaduz).

Effect of Sludge DOM-Degrader D13 on Tannery Sludge Bioleaching. The bioleaching was conducted in 500 mL Erlenmeyer flasks, each containing 270 mL of tannery sludge, 1.2 g of S^0 (*5, 24*), 15 mL of viable the strain D13 and 15 mL of viable growing cultures of *A. ferrooxidans* LX5 and *A. thiooxidans* TS6 (1:1). The control with the same volume of the autoclaved strain D13 inoculum instead of viable D13 was also performed. The flasks were incubated in a gyratory shaker at 28 °C and 180 rpm. During the incubation, 10 mL of sludge samples were withdrawn from the flasks at 1 day intervals and determined for pH, the strain D13 density, and Cr solubilization efficiency. The strain D13 counts were done by plate count method as described above. Subsequently these samples were centrifuged at 12 000 rpm for 15 min, filtered through 0.45 μm membrane filter, and analyzed for

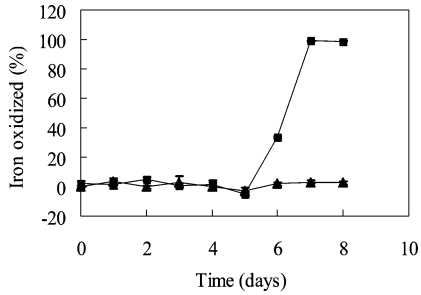

FIGURE 1. Effect of coinoculation of *A. ferrooxidans* LX5 with *P. spartinae* D13 on the iron oxidation in synthetic 9K medium containing tannery sludge DOM: pure culture of *A. ferrooxidans* LX5 (▲); mixed cultures of *A. ferrooxidans* LX5 and *P. spartinae* D13 (■).

Cr using inductively coupled plasma-atomic emission spectrometry (ICP-AES). Cr removal efficiency was calculated as the ratio of the soluble Cr in the filtrate to total Cr in the primary sludge.

After 90% of Cr removal efficiency or pH < 2 in sludge was achieved, which is regarded as the termination of each batch bioleaching (*5*), 30 mL acidified bioleached sludge was withdrawn and added to new flasks containing the same sludge and S^0 as previously described except viable *A. ferrooxidans* LX5, *A. thiooxidans* TS6, and D13. Then these new flasks were incubated again in a gyratory shaker at 28 °C and 180 rpm. During the incubation process, sludge pH, the D13 density and Cr removal efficiency were determined according to the methods as previously described. The recycle of acidified bioleached sludge were performed for successive four batches of bioleaching experiments (see Supporting Information Figure S4).

Results and Discussion

Isolation and Identification of Sludge DOM-Degrader D13. A strain degrading sludge DOM was isolated from bioleached tannery sludge. The colonies on PDA medium were large, unstained, white, and round to convex-shaped. The morphological and biochemical characteristic of the isolate are shown in Supporting Information Table S1. Neighbor-joining tree depicting the phylogenetic relationship of the isolated strain with related species based on the 26S rRNA gene D1/D2 domain sequence are shown in Supporting Information Figure S1. It was identified as *Pichia spartinae* species based on both physiological characterization and molecular characterization. The isolate (hereinafter called as *P. spartinae* D13) grew well between pH 2.0 and 9.0, with an optimum pH 5.0∼7.0, however, the lag phase of *P. spartinae* D13 could be prolonged when the pH of medium was 2.0 and its growth was totally inhibited when the pH of medium was 1.0. Besides, it had an optimum temperature of 28∼35 °C, but was unable to oxidize iron or sulfur.

Effect of *P. spartinae* D13 on the Growth of *A. ferrooxidans* LX5 and *A. thiooxidans* TS6 in DOM-Rich Medium. As shown in Figure 1 and Figure 2, the presence of about 100 mg DOC L^{-1} of tannery sludge DOM in the medium drastically inhibited the activities of *A. ferrooxidans* LX5 and *A. thiooxidans* TS6, as indicating that only 3% of ferrous iron and 1.38% of S^0 was oxidized by *A. ferroxidans* LX5 and *A. thiooxidans* TS6 in the first 7 days of incubation, respectively, in pure culture of *Acidithiobacillus* spp. without viable heterotrophic microorganism *P. spartinae* D13. These results were consistent with those reported by other researchers (*28, 29, 36*). In our previous study, it was identified that the main inhibitory substances in sludge DOM were formic and acetic acids, especially in tannery sludge DOM (*28*). In contrast, for the mixed cultures of *Acidithiobacillus* spp. with *P. spartinae* D13, more than 98% of ferrous iron oxidation by *A. ferrooxidans* LX5 was achieved in the same incubation time as that in the pure culture system with *A. ferrooxidans* LX5 only, and more than 16% of S^0 was bio-oxidized by *A. thiooxidans* TS6 in 5 days. Obviously, the activities of *A. ferrooxidans* LX5 and *A. thiooxidans* TS6 were enhanced by 33- and 12-fold, respectively, in the tannery sludge DOM-rich culture medium by *P. spartinae* D13. Undoubtedly, *P. spartinae* D13 played a key role in eliminating DOM toxicity to *Acidithiobacillus* spp. It was worthily noted that tannery sludge DOM inhibition effect on *A. thiooxidans* TS6 growth led to the prolongation of the lag phase of *A. thiooxidans* TS6 (see Figure 2).

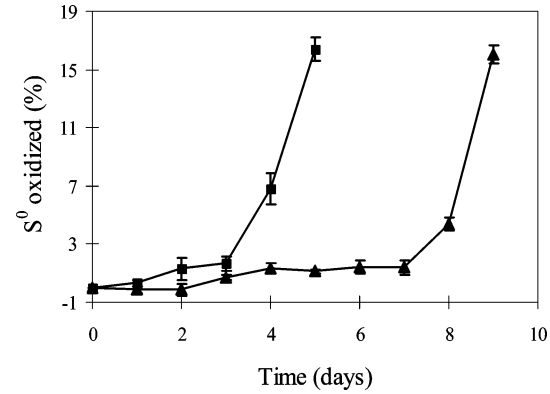

FIGURE 2. Effect of coinoculation of *A. thiooxidans* TS6 with *P. spartinae* D13 on the S^0 oxidation in synthetic SM medium containing tannery sludge DOM: pure culture of *A. thiooxidans* TS6 (▲); mixed cultures of *A. thiooxidans* TS6 and *P. spartinae* D13 (■).

Dynamic changes of *P. spartinae* D13 density and sludge DOM concentrations during the pure incubation of *A. ferrooxidans* LX5 only and the mixed incubation of *A. ferrooxidans* LX5 with *P. spartinae* D13 in the culture medium containing 100 mg DOC L^{-1} of initial sludge DOM were given in Supporting Information Figure S1. In the first two days of incubation, DOM in liquid medium inoculated with the mixed culture of *A. ferrooxidans* LX5 and *P. spartinae* D13 was decreased dramatically by 64% from initial 96 mg DOC L^{-1} to final 35 mg DOC L^{-1}. Correspondingly, the counts of *P. spartinae* D13 in the medium increased slightly from 9.14×10^6 cells mL^{-1} to 2.70×10^7 cells mL^{-1} in the first two days of incubation. However, the concentration of sludge DOM in the control inoculated only with *A. ferrooxidans* LX5 still maintained more or less at the initial level (i.e., approximately 100 mg DOC L^{-1}). With the decrease of DOM in the medium coinoculated with *A. ferrooxidans* LX5 and *P. spartinae* D13 after two days of incubation, the counts of viable *P. spartinae* D13 dropped linearly (see Supporting Information Figure S1). It implied that, in the first two days of incubation, *P. spartinae* D13, a heterotrophic microorganism taking organic carbon as C source and energy substrate for its growth, assimilated sludge DOM and improved the growth for itself. After two days of incubation, the reduction of viable *P. spartinae* D13 was contributed partly to DOM decrease in the medium and partly to lower pH effect as mentioned later.

The similar phenomenon was also observed in the mixed culture of *A. thiooxidans* TS6 and *P. spartinae* D13 (see Supporting Information Figure S2). Nearly 51% of DOM was assimilated in the first two days, and 10^7-10^8 cells mL^{-1} of *P. spartinae* D13 were detected in the period. Especially, the counts of *P. spartinae* D13 reached the maximum in the second day accompanied by the drastic decrease of DOM. Likewise, the concentration of DOM in the control inoculated with *A. thiooxidans* TS6 only but without *P. spartinae* D13 always maintained at an initial level (∼100 mg DOC L^{-1}) throughout the experiment. Again, it indicated that most of the sludge DOM toxic to both the strain LX5 and TS6 in the liquid medium was assimilated readily by heterotrophic strain

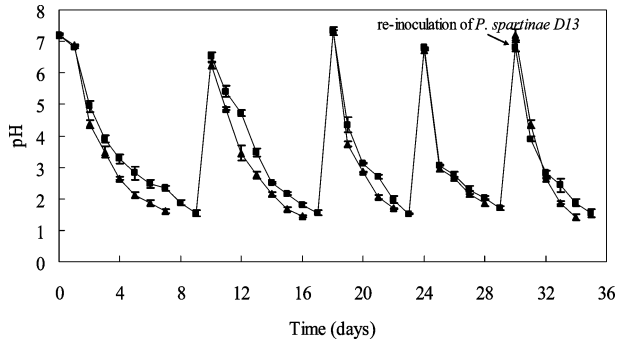

FIGURE 3. Dynamics of pH in the consecutive multibatch bioleaching processes: tannery sludge with inoculation of *P. spartinae* D13 and *Acidithiobacillus* species (▲); tannery sludge without inoculation of *P. spartinae* D13 (control) (■).

P. spartinae D13 for its growth in the first few days of incubation. After the drastic decrease, the concentration of DOM was stable due to the rest DOM probably being hard to be assimilated by *P. spartinae* D13. Therefore, a slight decrease of *P. spartinae* D13 counts was observed after two days incubation.

It was noted that *P. spartinae* species could assimilate a wide range of organic compounds including low molecular weight organic acids which were common metabolites of anaerobic digestion of sewage sludge (*27*). Johnson and McGinness (*37*) also reported that the presence of heterotrophic species in solid media could improve the plate efficiencies and the growth rates of iron-oxidizing acidophiles, and the incorporation reduced the concentration of monosaccharide causing the inhibition of *A. ferrooxidans*, which was produced by acid-hydrolysis of agar or agarose.

Therefore, the enhanced bio-oxidation activities of *A. ferrooxidans* LX5 and *A. thiooxidans* TS6 in sludge DOM-rich modified 9K medium or SM medium could be attributed, to a great extent, to DOM elimination by *P. spartinae* D13.

Successive Tannery Sludge Bioleaching in the Presence and Absence of *P. spartinae* D13. In previous studies, it was found that a consecutive bioleaching process by circulating pH < 2 acidified bioleached sludge was a feasible method to overcome the buffering capacity of tannery sludge, and also a very convenient way to inoculate *Acidithiobacillus* species in practical operation (*5*). However, unlike *Acidithiobacillus* species, *P. spartinae* D13 only was an acid-tolerant heterotrophic microorganism, which grew in the pH range from 2 to 7 with optimum pH 5.5. Thus, it was very important to investigate the ability of *P. spartinae* D13 in eliminating DOM during successive multibatch sludge bioleaching process because pH of bioleached sludge at the final phase of each batch bioleaching often reached below 2, which was not favorable for the growth of *P. spartinae* D13. In this trial, the successive four batches of tannery sludge bioleaching trials were performed by circulating 10% ($V_{\text{bioleached sludge}}$ / V_{total}) of pH < 2 acidified bioleached sludge into next batch bioleaching system as inoculums. As shown in Figure 3, both single (only inoculated with *Acidithiobacillus* species) and combined (simultaneously inoculated with *Acidithiobacillus* species and *P. spartinae* D13) systems had a similar decreasing trend but with different rates and levels of changes in pH. For the first batch trial, the combined system resulted in a sharp drop of pH from 7.2 to 2 within 6 days. However, it took 9 days for the single system to reduce the pH from initial 7.2 to ~2. Three days of bioleaching time was shorten for combined system in comparison with single system. The differences of pH decrease rate between single and combined systems tended to be minimized with the increase of the batches of bioleaching trials. No difference in pH change was observed in the fourth batch. But pH reduction rate was enhanced markedly and pH difference between single and combined system occurred again when *P. spartinae* D13 was readded or re-inoculated to the fifth batch of bioleaching system. In other words, the coinoculation of *P. spartinae* D13 and *Acidithiobacillus* species in the first batch of tannery sludge trial will not exhibit significant role in improving bioleaching efficiency of the subsequent fourth batch of tannery sludge until the fresh *P. spartinae* D13 inoculums was replenished in the subsequent fifth batch.

Consequently, Cr removal efficiency for the combined system was much higher than for the single system in the first batch trial (Figure 4). For example, more than 90% of Cr removal efficiency for the combined system was achieved in day 6, whereas only 50% for the single system was observed in the same time. Likewise, the differences tended to be minimized with the increase of number of subsequent batch trials until fresh inoculums of *P. spartinae* D13 was replenished.

The differences between single and combined systems in the dynamic change of the pH or Cr removal efficiency in various batches of sludge bioleaching could be interpreted partly by the change of viable *P. spartinae* D13 density during successive multibatch bioleaching (Figure 5). With the drastic decrease of pH during bioleaching, some heterotrophic *P. spartinae* D13 would die because of extremely low pH value (pH 1.6 ~ 1.8) and high Cr concentration (1013 ~ 1176 mg L^{-1}) in sludge in the final phase of each batch bioleaching. It was noted that the counts of *P. spartinae* D13 decreased with the increases

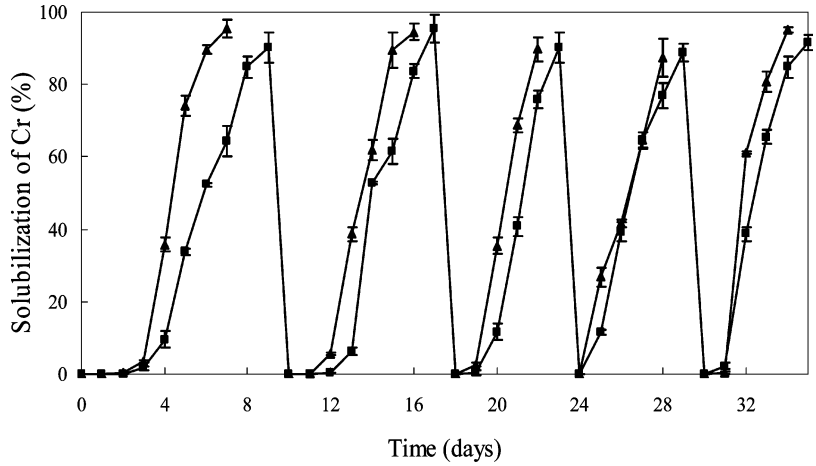

FIGURE 4. Solubilization of Cr during the consecutive multibatch bioleaching processes: tannery sludge with inoculation of *P. spartinae* D13 and *Acidithiobacillus* species (▲); tannery sludge without inoculation of *P. spartinae* D13 (control) (■).

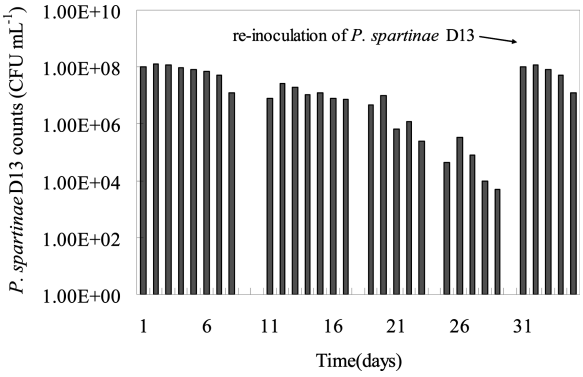

FIGURE 5. Dynamics of *P. spartinae* D13 population in the sludge with inoculation of *P. spartinae* D13 during the consecutive multibatch bioleaching processes.

of bioleaching time for each batch, especially for batch 3 and 4. Density of *P. spartinae* D13 declined to only between 10^3 and 10^4 cells mL^{-1} in batch 4 from about $\sim 10^8$ cells mL^{-1} in initial phase of batch 1. In contrast to *Acidithiobacillus* species, *P. spartinae* D13 in unfavorable acid environment (pH < 2 in the final phase of each batch bioleaching trial) was not renewable or did not restore to normal growth even when subsequent pH was raised to neutral condition in the first days of next bioleaching trial due to fresh sludge incorporation (see Figure 3 and Figure 5). In fact, the single system only inoculated with *Acidithiobacillus* species also exhibited a similar density level ($10^3 \sim 10^4$ cells mL^{-1}) of indigenous *P. spartinae* D13 in the four batches of bioleaching (data no shown). Expectedly, after readded in subsequent fifth batch bioleaching medium, *P. spartinae* D13 was able to be restored to $\sim 10^8$ cells mL^{-1} (see Figure 5), and pH decrease rate and Cr removal efficiency were obviously improved once again (see Figures 3 and 4). It demonstrated that enhancing bioleaching efficiency facilitated by the addition of acid-tolerant heterogenic microorganism *P. spartinae* D13 only could maintain four successive recycle or four batch experiments.

In fact, some works have indicated that the bioleaching was actually initiated by various kinds of heterotrophic microorganisms (*38*, *39*). Gamache et al. (*40*) observed that several acid-tolerant heterotrophic microorganisms such as the yeast *Blastoschizomyces capitatus* and a nonidentified fungus did persist and could be involved in the whole bioleaching process of sewage sludge. Hence, the acid-tolerant heterotrophic microorganism was replenished periodically in DOM-rich tannery sludge bioleaching system perhaps be a good way to increase bioleaching efficiency.

Acknowledgments

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Supporting Information Available

One table and four figures showing additional details of our analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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