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RESEARCH ARTICLE

Relationship between microbial community dynamics and process performance during thermophilic sludge bioleaching

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Abstract Heavy metals can be removed from the sludge using bioleaching technologies at thermophilic condition, thereby providing an option for biotreatment of wasted sludge generated from wastewater treatment. The purposes of this study were to establish a molecular biology technique, real-time PCR, for the detection and enumeration of the sulfur-oxidizing bacteria during the thermophilic sludge bioleaching. The 16S rRNA gene for real-time PCR quantification targeted the bioleaching bacteria: *Sulfobacillus thermosulfidooxidans*, *Sulfobacillus acidophilus*, and *Acidithiobacillus caldus*. The specificity and stringency for thermophilic sulfur-oxidizing bacteria were tested before the experiments of monitoring the bacterial community, bacterial number during the thermophilic sludge bioleaching and the future application on testing various environmental samples. The results showed that *S. acidophilus* was identified as the dominant sulfur-oxidizing bacteria, while *A. caldus* and *S. thermosulfidooxidans* occurred in relatively low numbers. The total number of the sulfur-oxidizing bacteria increased during the thermophilic bioleaching process. Meanwhile, the decrease of pH, production of sulfate, degradation of SS/VSS, and solubilization of heavy metal were found to correlate well with the population of thermophilic sulfur-oxidizing bacteria during the bioleaching process. The real-time PCR used in this study is a suitable method to monitor numbers of thermophilic sulfur-oxidizing bacteria during the bioleaching process.

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Introduction

Recently, the management of waste sludge produced from the municipal sewage treatment plants (STPs) becomes the most important issue of environmental protection in Taiwan. After the marine dispersal has been banned and the disposal to landfill becomes more limited, land application of sewage sludge has been widely practiced for many areas, including Taiwan. Due to high contents of nutrients and organic matter in sewage sludge, using sewage sludge as soil conditioners or fertilizers has the benefits of resource recycling and waste minimization for STPs. Besides, for sustainable development, utilization of waste sewage sludge as feedstocks for production of bioresources (e.g., short-chain fatty acid) and bioenergy (e.g., methane and hydrogen) by anaerobic digestion process has attracted increasing attention (Wang et al. 2013; Wang et al. 2015; Zhao et al. 2015). However, the presence of hazardous contaminants such as heavy metals affects the land application of sewage sludge and the performance of anaerobic digestion (Babel and del Mundo 2006; Mudhoo and Kumar 2013). For minimization of environmental risk or human health, it is very important to develop an appropriate and economically feasible technology for removing heavy metals from sewage sludge.

Various physical or chemical technologies such as chemical extraction (Ito et al. 2000; Naocum et al. 2001; Zaleckas et al. 2013), ion exchange resins (Evaristo et al. 2013), electrokinetic (Gao et al. 2013), ultrasonication (de La Rochebrochard et al. 2013), and supercritical fluid extraction (Yabalak and Gizir 2013), have been extensively applied for removal of heavy metals from sludge. However, several disadvantages, such as

high consumption of chemicals and energy, high operating cost, or complexity of process, limit their application in practice (Babel and del Mundo 2006). Recently, the bioleaching process has been considered as a promising, cost-effective, and environmentally friendly approach for removing heavy metals from sludge, soil, and sediment (Wong et al. 2004; Park et al. 2007; Chen and Lin 2009; Chen and Lin 2010; Chen and Huang 2014).

Microorganisms performing the bioleaching process can be categorized as mesophiles and thermophiles based on growth condition. The most domain mesophiles generally used in the bioleaching process are sulfur-oxidizing bacteria including *Acidithiobacilus thiooxidans*, *Acidithiobacilus ferrooxidans*, and *Thiobacillus thioparus* (Chen and Lin 2009). However, the bioleaching process operated at ambient temperature has been shown to have the disadvantages of slow rate and incomplete efficiency of metal leaching (d'Hugues et al. 2002). Since thermophilic microorganisms possess the characteristics of high metal tolerance capacity and metabolic activity, recent studies found that the metal bioleaching could proceed much more rapidly and efficiently at higher temperatures (Mousavi et al. 2005; Zhou et al. 2009). Some sulfur-oxidizing bacteria, such as *Sulfobacillus thermosulfidooxidans*, *Stephanodiscus yellowstonensis*, *Sulfobacillus acidophilus*, *A. caldus*, *Sulfolobus metallicus*, and *Sulfolobus solfataricus* have been found to play important roles in the solubilization of heavy metals during the thermophilic bioleaching process (Plumb et al. 2002; Robertson et al. 2002; Salo-Zieman et al. 2006; Zhou et al. 2009). However, the bioleaching efficiency is generally affected by several chemical and biological parameters including solids concentration, pH, temperature, and substrate concentration (Gerayeli et al. 2013; Watling et al. 2014). Pina et al. (2010) investigated the oxidation kinetics of ferrous iron (substrate) using *S. thermosulfidooxidans*. Their study observed that the ferrous iron oxidation rate and the microbial-specific growth rate are a function of the initial ferrous iron concentration. Shiers et al. (2010) compared the behavior of bioleaching bacteria with the ability to oxidize both ferrous iron and sulfur oxyanions in bioleaching environments. It is interesting to note that substrate utilization in batch cultures varied between four *Sulfobacillus* species (*Sulfobacillus acidophilus*, *S. thermosulfidooxidans*, *Sulfobacillus thermotolerans*, and *Sulfobacillus sibiricus*). No inhibition in sulfur oxidation by ferrous ions was observed for these *Sulfobacillus* species. Ilyas et al. (2014) showed that an increase in sulfur dosage increased the removal efficiency of heavy metals from recycling industry electronic waste with a consortium of *S. thermosulfidooxidans* and *Thermoplasma acidophilum*.

Besides understanding the effects of the chemical and biological parameters, it is also very important to monitor the population dynamics of bioleaching microorganisms in response to these affecting parameters for maximizing the bioleaching efficiency. However, there are relatively few studies reporting the changes of microbial population during the thermophilic sludge bioleaching process. Generally, the conventional cultivation methods are laborious and time consuming, which are not applicable for monitoring the presence of microbial population in the thermophilic bioleaching systems. The application of culture-independent techniques can overcome the shortcomings of conventional cultivation methods and reveals far more complex bacterial communities in the environmental samples (Schabereiter-Gurtner et al. 2001; Rompre et al. 2002; Kirk et al. 2004). Recently, several nucleic acid-based techniques have been developed and applied for investigation of microbial population in bioleaching of mineral ores (Dopson and Lindstrom 2004; Okibe and Johnson 2004; Zammit et al. 2008; Schippers et al. 2008). Among the above molecular biological techniques, the real-time PCR exhibits the advantages of speed, sensitivity, accuracy, reproducibility, and high throughput over other detection techniques (Zammit et al. 2008; Babu et al. 2011). Therefore, the main objectives of this study were: (1) to establish a real-time PCR technique for detection and enumeration of the sulfur-oxidizing bacteria during the thermophilic sludge bioleaching, (2) to monitor the population dynamics during the sludge bioleaching using the real-time PCR technique, and (3) to investigate the relationships between microorganisms and bioleaching of heavy metal from the sludge at different substrate concentrations.

Materials and methods

Sludge sampling

The waste activated sludge (WAS) was collected from the municipal wastewater treatment plant located in the City of Tainan, Taiwan. The sludge was then passed through a 20-mesh (0.84 mm) screen and stored at 4 °C before the experiment. The characteristics of the WAS are shown in Table 1. These characteristics include pH (USEPA 2004), total solids (TS) volatile solids (VS), suspended soilds (SS), volatile

Table 1 The characteristics of sludge used in this study

Property	Value[a]
pH	6.9 ± 0.1
TS (%)	1.75 ± 0.01
VS (%)	1.00 ± 0.01
SS (%)	1.46 ± 0.07
VSS (%)	0.81 ± 0.03
Cu (mg/kg)	221 ± 5
Zn (mg/kg)	2020 ± 34
Mn (mg/kg)	3427 ± 46
Pb (mg/kg)	206 ± 5

[a] Mean ± standard deviation ($n = 8$)

suspended soilds (VSS) (APHA 2005), and heavy metal contents (USEPA 1995).

Acclimation of thermophilic sulfur-oxidizing bacteria

Ten liters of waste-activated sludge (1 % (*w*/*v*) of total solids) mixed with 2 kg of pre-prepared sulfur tablets were poured into a completely mixed batch (CMB) reactor. The sulfur tablets (1 cm in diameter by 0.5 cm in thickness) used in this study were recoverable and prepared following the procedures in Chen et al. (2003). The reactor was agitated at a speed of 200 rpm, aerated at a rate of 4 L/min, and maintained at the temperature of 55 °C. The activation of indigenous thermophilic sulfur-oxidizing bacteria in the sludge was determined based on sludge pH. The acclimation procedure was completed when sludge pH dropped to approximately 2.0, and the sulfur tablets were recovered from the acclimation reactor. Then, 1 L of the pre-acclimated sludge and the recovered sulfur tablets were transferred to another reactor to mix with 9 L of fresh sludge. The aforementioned acclimation procedure was repeated at least three times. The acclimated sludge was then used as an inoculum for the subsequent thermophilic bioleaching experiment. The indigenous thermophilic sulfur-oxidizing bacteria in the acclimated sludge were identified and characterized by the real-time PCR method developed in this study. The initial bacterial populations in the inoculum were found to comprise approximately 5.9×10^{11} cells/mL *S. acidophilus*, 3.6×10^{11} cells/mL *A. caldus*, and 2.0×10^{10} cells/mL *S. thermosulfidooxidans*, respectively.

Thermophilic sludge bioleaching experiments

The thermophilic sludge bioleaching experiments were carried out in 12-L CMB reactor containing 10 L of WAS and 10 % (*v*/*v*) of inoculum obtained from the above acclimation experiments of thermophilic sulfur-oxidizing bacteria. In order to evaluate the effects of sulfur dosage on the thermophilic sludge bioleaching process, 10 and 20 % (*w*/*v*) of sulfur tablets recovered from the acclimation experiment were added into two bioleaching reactors, respectively. The bioreactor was aerated with unmodified air at a rate of 4 L/min, and then incubated at 55 °C with 200 rpm for 8 days. During the bioleaching experiments, the pH of sludge was measured daily and sludge samples were periodically taken from the bioreactor for chemical (SS and VSS) and bacterial analyses. In addition, the sludge samples were centrifuged and filtered by 0.45 μm filter membrane, and the filtrate was used to determine the amounts of soluble sulfate and heavy metals (Zn, Mn, Pb, and Cu). The analysis of soluble sulfate was carried out by the turbidimetric method (APHA 2005). The concentration of soluble heavy metals was measured with an atomic absorption spectrophotometer (Shimadzu AA-6200). All samples were analyzed in triplicate and the mean values were calculated. The relative standard deviation of measurements was always below 5 %.

Microorganisms and growth conditions

The pure bacterial strains for creating standard curves in real-time PCR quantification were *Acidithiobacillus caldus* DSM 8584, *S. thermosulfidooxidans* DSM 9393, *Sulfobacillus acidophilus* DSM 10332, and *Escherichia coli* BCRC 11634. *A. caldus* DSM 8584, *S. thermosulfidooxidans* DSM 9393, and *S. acidophilus* DSM 10332 were cultivated according to the protocols provided by Deutsche Sammlung von Mikroorgaismen unnd Zellkulturen (DSMZ). The culture condition for *Escherichia coli* BCRC 11634 was described by Bioresource Collection and Research Center (BCRC), Hsinchu, Taiwan.

DNA extraction and primers design

Two milliliters of sludge sample was centrifuged at 10,000×*g* for 3 min; 0.25 g (wet weight) of pellet taken from the 2-mL microtube was used for DNA extraction by using a PowerSoil DNA isolation kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA). DNA was extracted from pure cultures in shake flasks by using an illustra Bacteria Genomic Prep Mini Spin Kit (GE Healthcare, Pittsburgh, PA, USA). The purity of the extracted DNA was assessed spectrophotometrically by calculating A_{260}/A_{280} ratios in a ChromTech UV3100 spectrophotometer (ChromTech Co., Ltd., Singapore). The primers used in the study for detection of thermophilic sulfur-oxidizing bacteria are listed in Table 2. The specific primers used in the PCR reactions for *A. caldus* (Acaldus-F/NR-R) and *S. acidophilus* (Sacid-F/Sacid-R) were obtained from previously published sequences, respectively (Liu et al. 2006; Zhang et al. 2009). A set of new primers (DQ-F/DQ-R) for *S. thermosulfidooxidans* was designed by using Primer Express version 2.0 software (Applied Biosystems, Foster City, CA) based on the 16S rRNA gene sequence obtained from the GenBank database. In addition, the primer set of NR-F/NR-R was used for detection of total bacteria (Liu et al. 2006). All PCR primers used in this study were synthesized by Mission Biotech (Mission Biotech Co., Ltd., Taipei, Taiwan). To confirm the specificity of the primer sets, the PCR products were analyzed by either 1.5 or 2 % agarose gel electrophoresis stained with 0.01 % ethidium bromide. These primers were also analyzed for the requirements imposed by real-time quantitative PCR by using Primer Express version 3.0 software.

PCR and real-time PCR quantification

In this study, the conventional PCR was carried out with a MultiGene Gradient Thermal Cycler (Labnet International

Table 2 Primers used for PCR and real-time PCR in this study

Target species	Primer	Sequences 5′ → 3′	References
Bacteria	NR-F	GTAGTCCMSGCYSTAAACGATG	Liu et al. (2006)
	NR-R	AGCTGRCGACRRCCATGCA	
Sulfobacillus acidophilus	Sacid-F	ACGTAGCGGTTTTCAGCC	Zhang et al. (2009)
	Sacid-R	GACACCTCGTATCCATCGTTTAC	
Acidithiobacillus caldus	Acaldus-F	TTGGCGCCTTAGGTGCTGA	Liu et al. (2006)
	NR-R	AGCTGRCGACRRCCATGCA	
Sulfobacillus thermosulfidooxidans	DQ-F	GCCTTGATGTTCTGGGCTACA	This study
	DQ-R	CGTCGCATCCCGTTGTC	

Inc., Woodbridge, NJ). The amplification programs of PCR for bacteria detected in this study are shown in Table 3. The PCR products were then examined by agarose gel electrophoresis, and then purified by using an illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, UK) if necessary.

The real-time PCR was performed in an Applied Biosystems StepOne Real-time PCR system (Applied Biosystems, Forster City, CA). The reaction occurred in a 20-μL solution containing 10 μL Fast SYBR® Green Master Mix (Applied Biosystems, Forster City, CA), 0.4 μL of each primer, 2 μL of template DNA, and 7.6 μL of PCR-grade water. The negative controls were also designed. The cycling parameters were 10 min at 95 °C, and followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The real-time PCR products were confirmed through melting curves analysis. The standard curves were calculated following the procedures described in the study by Liu et al. (2006). The 10-fold serial-diluted 16S ribosomal DNA (rDNA) from the pure bacterial strains was used to obtain the standard curve for each primer set. The threshold cycle (C_t) value was determined by the software of the StepOne Real-time PCR system. The standards and samples were performed in triplicate.

Table 3 Amplification programs of PCR in this study

Primer pairs	Program	Cycles	Reference
NR-F/NR-R	95 °C, 5 min	1	Liu et al. (2006)
Sacid-F/Sacid-R	95 °C, 15 s; 55 °C, 30 s; 72 °C, 30 s	40	Zhang et al. (2009)
	72 °C, 7 min	1	
A.caldus-F/NR-R	95 °C, 5 min	1	Liu et al. (2006)
	95 °C, 15 s; 55 °C, 30 s; 72 °C, 30 s	45	
	72 °C, 7 min	1	
DQ-F/DQ-R	95 °C, 5 min	1	This study
	95 °C, 15 s; 60 °C, 1 min	40	
	72 °C, 7 min	1	

To assess the validity and reliability of real-time PCR quantification in this study, some spiked samples with known quantity of DNA were also quantified by the real-time PCR to calculate recovery rates.

Results and discussion

Performance of thermophilic sludge bioleaching

The variations of pH, sulfate production, solids degradation and metal solubilization in thermophilic sludge bioleaching experiment with 10 % of sulfur dosage are shown in Fig. 1. In general, nitrification might cause a slight decrease in pH at the beginning of the experiment. However, the pH mainly decreased with time due to the sulfuric acid production, resulting from the oxidation of sulfur tablets by thermophilic sulfur-oxidizing bacteria. As revealed in Fig. 1a, the pH dropped from 8.3 to 2.8 after 8 days of bioleaching experiment with 10 % of sulfur dosage. Furthermore, sulfate increased from 130 to 345 mg/L as sulfur was oxidized by bacteria. Figure 1b depicts the variation of the solids degradation during the thermophilic sludge bioleaching experiment under 10 % of sulfur dosage. Previous studies have reported that the degradation of organic matter by indigenous heterotrophic microorganisms, and the metal solubilization reaction happens simultaneously when the acclimated indigenous microorganisms are inoculated for bioleaching reaction. Besides, the acidification of sulfur-oxidizing bacteria will also cause the degradation of solids during the sludge bioleaching process (Chen and Pan 2010). After 8 days of reaction, it was observed that 59 % of SS and 66 % of VSS were degraded in thermophilic sludge bioleaching experiment under 10 % of sulfur dosage. Figure 1c shows the result of metal solubilization during the thermophilic sludge bioleaching experiment under 10 % of sulfur dosage. It was found that the leaching efficiencies for Zn, Mn, Pb, and Cu from the sludge under 10 % sulfur dosage after 8 days were 80, 79, 41, and 22 %, respectively.

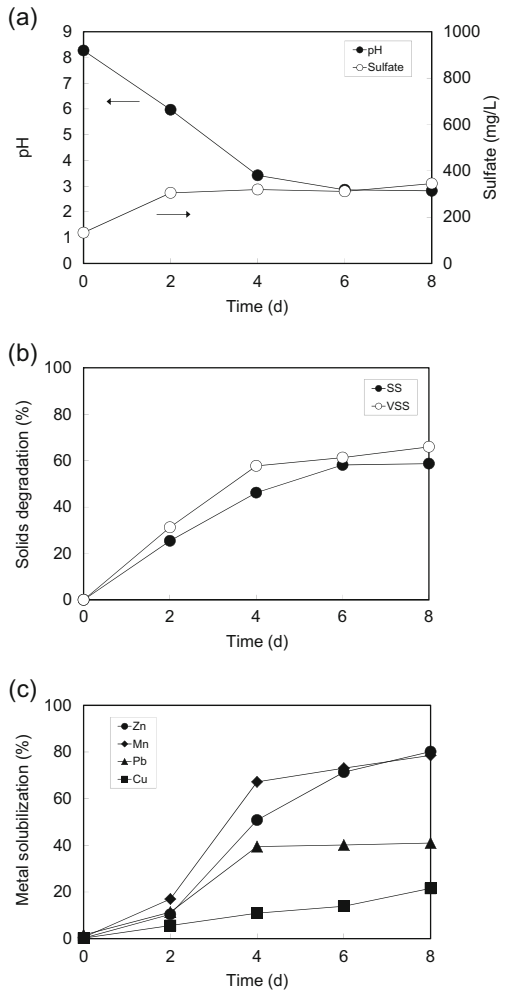

Fig. 1 Variations of **a** pH/sulfate, **b** solids degradation, and **c** metal solubilization in the thermophilic sludge bioleaching process (sulfur dosage = 10 %)

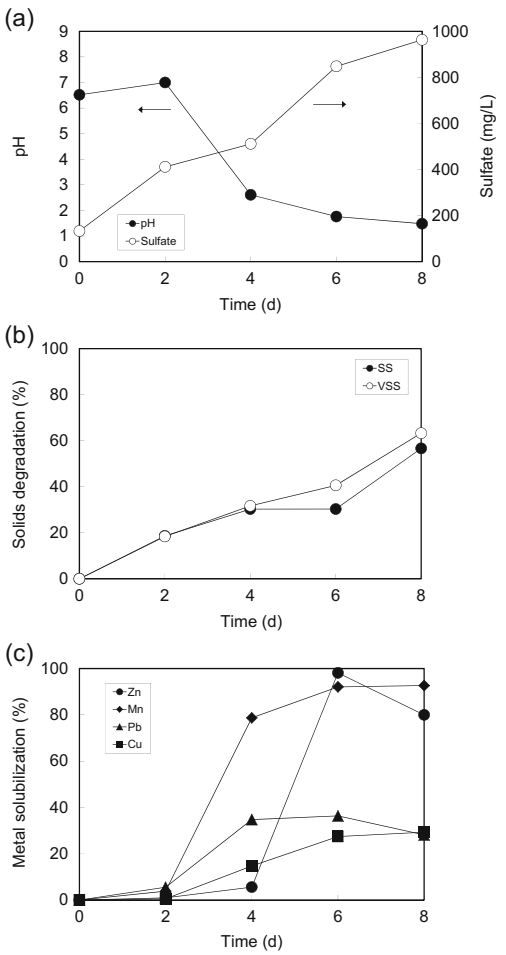

Fig. 2 Variations of **a** pH/sulfate, **b** solids degradation, and **c** metal solubilization in the thermophilic sludge bioleaching process (sulfur dosage = 20 %)

Because of high affinity of Cu to organic matters in the sludge (Wang et al. 2008), the lowest efficiency of Cu solubilization was observed in the thermophilic sludge bioleaching experiment. Additionally, it was found that Pb was not efficiently solubilized from the sludge due to the formation of poorly soluble $PbSO_4$ ($k_{sp} = 1.62 \times 10^{-8}$) with sulfate.

Figure 2 indicates the variations of pH, sulfate production, solids degradation, and metal solubilization under 20 % of sulfur dosage. After 8 days of reaction, the pH decreased from 6.8 to 1.5, whereas the sulfate concentration increased from 133 to 963 mg/L (Fig. 2a). In addition, it was found that 57 % of SS and 65 % of VSS were degraded after 8 days of bioleaching (Fig. 2b). Meanwhile, the efficiencies of metal solubilization for Zn, Mn, Pb, and Cu were 82, 93, 28, and 30 %, respectively (Fig. 2c). Comparing with the results under 10 % of sulfur dosage (Fig. 1), it is apparent that the sulfur oxidation and metal solubilization increased with increasing sulfur dosage, except for the solubilization of Pb. However, increasing sulfur dosage did not significantly influence the solid degradation.

Verification of specificity and sensitivity of real-time PCR

The specificity of primers for the target bacteria was verified by traditional PCR and real-time PCR. Results of electrophoresis of PCR product revealed that the unique band of the expected length was found for each set of primers. Meanwhile, after inspecting the amplification specificity of real-time PCR assay by melting curve analysis, the single peaks were observed on melting curves for all primer sets, and there were no non-specific amplifications with the primers used (data not shown). From the average C_t values obtained with serial 10-fold dilutions of 16S rDNA of pure bacterial strains, four standard curves were established as shown in Fig. 3. It was found that the determination coefficient (R^2) were higher than 0.99 for all standard curves. In addition, the amplification efficiency (E) for real-time PCR was between 80 and 97 % for all primer sets. The linear dynamic range for the standard curves of total bacteria and

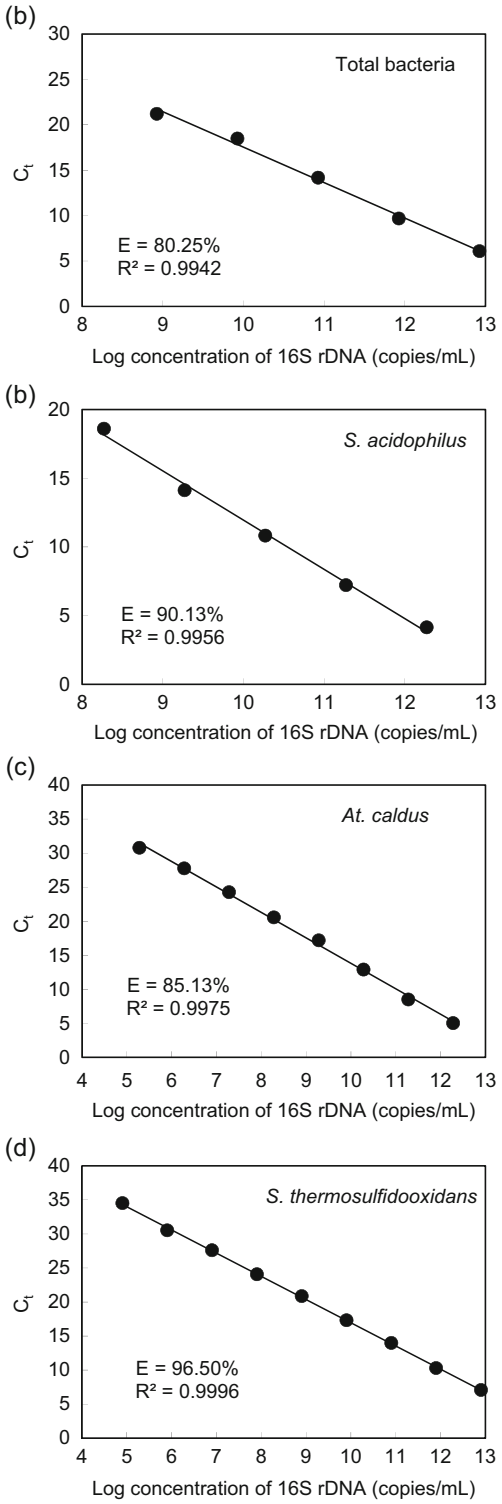

Fig. 3 Standard curves used in the real-time PCR quantification for **a** total bacteria, **b** *S. acidophilus*, **c** *A. caldus*, and **d** *S. thermosulfidooxidans*

S. acidophilus covered at least five logs from 8.5×10^8 to 8.5×10^{12} and 1.9×10^8 to 1.9×10^{12} copies/mL, respectively. For the standard curves of *A. caldus* and *S. thermosulfidooxidans*, the wide linear dynamic ranges were obtained from 1.9×10^5 to 1.9×10^{12} and 8.0×10^4 to 8.0×10^{12} copies/mL, respectively. To determine the sensitivity of real-time PCR assays, several spiked samples with known quantity of DNA were amplified and quantified. The results revealed that the copy numbers determined by the assay fitted in with the actual copies at a recovery rate of 65–133 % (Table 4), which indicated that the real-time PCR assay was suitable for quantification of total and individual 16S rDNA in the sludge samples. Table 5 shows the summary of triplicate spiked samples in 10-fold serial dilutions measured by the real-time PCR assay. The results indicate that the recovery rates and the relative standard deviation (RSD) were in the scope from 63 to 234 and 2 to 26 %, respectively. This describes the real-time PCR assay used in this study had high accuracy and low variability.

Variation of microbial population during thermophilic sludge bioleaching

Figure 4 shows the variations of bacterial number during the thermophilic sludge bioleaching process. After 8 days of bioleaching, the total bacteria number slightly decreased from 6.7×10^{12} to 2.3×10^{12} copies/mL under 10 % of sulfur dosage (Fig. 4a). Also shown in Fig. 4a, the number of *S. acidophilus* increased from 5.3×10^{11} to 1.4×10^{12} copies/mL, whereas there are no significant changes for *A. caldus*. Although the number of *S. thermosulfidooxidans* decreased in the first 4 days during the bioleaching, it increased to 7.1×10^{10} copies/mL after 8 days of bioreation. In addition, the ratios of *S. acidophilus*, *A. caldus*, and *S. thermosulfidooxidans* to total bacteria significantly increased from 8 to 61, 9 to 25, and 0.4 to 3 %, respectively (Fig. 5a). The pH for the growth of *S. acidophilus*, *A. caldus*, and *S. thermosulfidooxidans* were in the range of 1.6–2.3 (optimum 1.7–2.0), 0.5–5.5 (optimum 1.5–2.5), and 1.0–5.5 (optimum 1.5–2.4), respectively (Plumb et al. 2008; Watling et al. 2008). Therefore, the increases of cell numbers for sulfur-oxidizing bacteria were correlated with the decrease of pH during thermophilic sludge bioleaching (Fig. 1a). Overall, the percentage of sulfur-oxidizing bacteria in the total bacterial count was 89.0 ± 9.9 % after 8 days of bioleaching. In

Table 4 Recovery rates of spiked samples for the real-time PCR quantification

Strain	Added (copies/mL)	Measured (copies/mL)	Recovery (%)[a]
Total bacteria (*E. coli*)	8.45E+09	5.80E+09	69
S. acidophilus	1.87E+09	2.48E+09	133
A. caldus	1.91E+09	1.24E+09	65
S. thermosulfidooxidans	8.00E+09	7.84E+09	98

[a] Recovery = measured/added × 100 %

Table 5 Summary of spiked samples in 10-fold serial dilutions for the real-time PCR quantification

Added (copies/mL)	Measured[a] (copies/mL)	SD[b] (copies/mL)	Recovery (%)	RSD[c] (%)
7.63E+11	8.9E+11	2.08E+10	116.29	2.35
7.63E+10	8.5E+10	5.73E+09	110.63	6.78
7.63E+9	8.3E+09	1.89E+09	108.17	22.86
7.63E+8	6.6E+08	8.56E+07	86.85	12.91
7.63E+7	6.0E+07	1.53E+07	78.30	25.57
7.63E+6	4.8E+06	6.01E+05	62.96	12.50
7.63E+5	7.9E+05	1.73E+05	103.52	21.89
7.63E+4	1.8E+05	1.54E+04	234.17	8.60

[a] Mean ($n = 3$)

[b] Standard deviation

[c] Relative standard deviation = SD/mean × 100 %

particular, *S. acidophilus* was the most dominant species during the thermophilic sludge bioleaching process.

Similar results were obtained in Fig. 4b. The numbers of total bacteria did not change apparently and it remained in between 4.8×10^{12} and 7.7×10^{12} copies/mL under 20 % of sulfur dosage. After 8 days of bioleaching, the numbers of *S. acidophilus* and *A. caldus* slightly increased from 5.3×10^{11} to 1.8×10^{12} copies/mL and 1.0×10^{10} to 3.9×10^{10} copies/mL, respectively. Additionally, the cell count of *S. thermosulfidooxidans* drastically increased from 9.5×10^{9} to 1.7×10^{12} copies/mL. With the increase of sulfur dosage, the numbers of total bacteria did not change significantly (Fig. 4a, b). However, the population remarkably increased for *S. thermosulfidooxidans* and decreased for *A. caldus* when the sulfur dosage was increased. The results in Fig. 5b also reveal that the proportion of each species of sulfur-oxidizing bacteria in total bacteria increased with time under 20 % of sulfur dosage. The sum of these three sulfur-oxidizing bacteria counts also increased to 74 % at the end of bioleaching. *S. acidophilus* was also found to be the most dominant species during the bioleaching process, accounting for up to 37 % of the total bacteria. The previous study reported that *S. acidophilus* and *A. caldus* were both the dominant microbial populations in bioleaching of chalcopyrite (Zhang et al. 2009). Wang et al. (2014) indicated that the pH decreased to less than 2.0 when bioleaching of chalcopyrite was in the progress and then *S. acidophilus* gained an advantage to outcompete with other species during bioleaching. Paivi et al. (2004) reported that over 90 % of copper was extracted from chalcopyrite concentrate by *S. acidophilus* and *S. yellowstonenis* after 3 months of bioleaching. Therefore, *S. acidophilus* cooperating with other moderate thermophilies could attain good performance in the bioleaching of chalcopyrite. Watling et al.

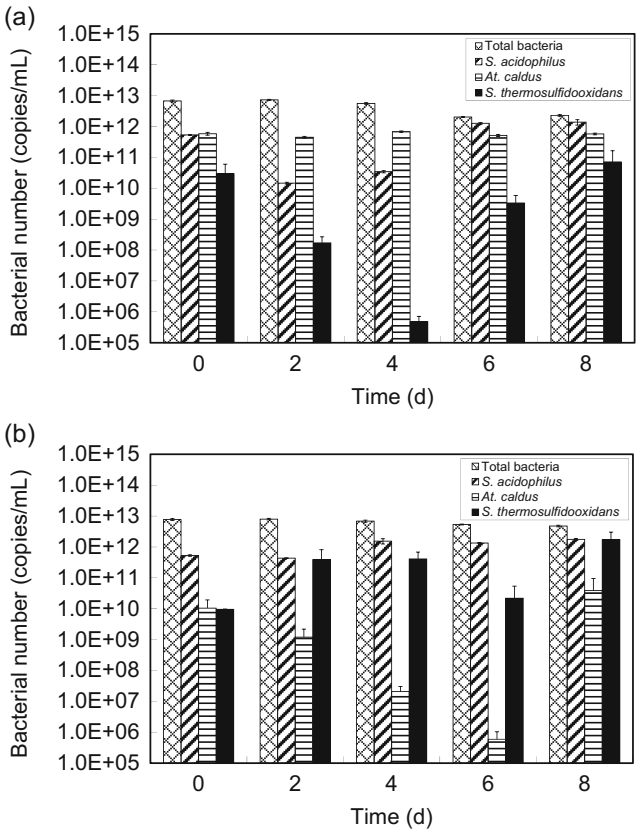

Fig. 4 Variations of bacterial number during the thermophilic sludge bioleaching process **a** sulfur dosage = 10 % and **b** sulfur dosage = 20 %

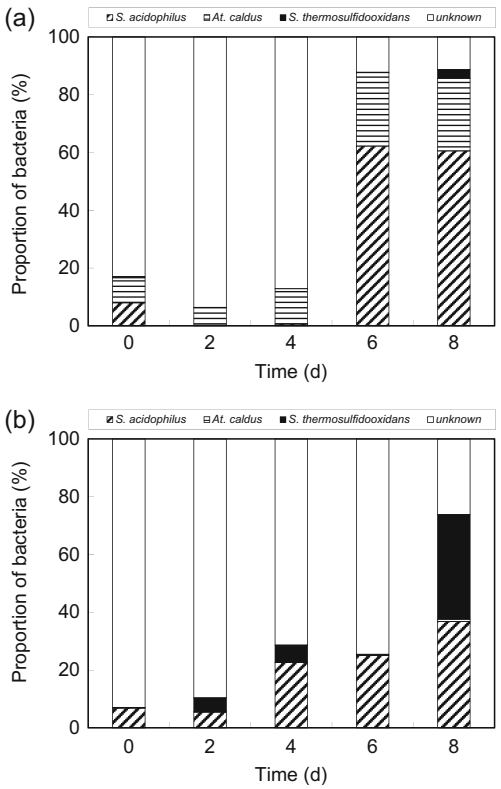

Fig. 5 Proportion of bacterial numbers during the thermophilic sludge bioleaching process **a** sulfur dosage = 10 % and **b** sulfur dosage = 20 %

Table 6 Correlation coefficients between bioleaching performance and microbial population

	Total bacteria	*S. acidophilus*	*A. caldus*	*S. thermosulfidooxidans*	Total sulfur-oxidizing bacteria
pH	0.65[a]	−0.71[a]	0.14	−0.34	−0.64[a]
Sulfate	−0.17	0.64[a]	−0.54	0.56	0.58
Solids degradation					
SS	−0.80[a]	0.42	0.26	−0.08	0.32
VSS	−0.78[a]	0.39	0.28	−0.02	0.35
Metal solubilization					
Zn	−0.82[a]	0.60	0.13	0.39	0.69[a]
Mn	−0.68[a]	0.73[a]	−0.04	0.35	0.70[a]
Pb	−0.78[a]	0.57	0.23	0.23	0.64[a]
Cu	−0.64[a]	0.78[a]	−0.16	0.43	0.72[a]

[a] Correlation is significant at the 0.05 level (two-tailed)

(2008) also found *Sulfobacillus* spp. (*S. acidophilus*, *S. thermosulfidooxidans*, *S. sibiricus*, and *S. thermotolerans*) with the characteristics of versatility and resilience could accelerate metal extraction rate in the thermophilic bioleaching.

Table 6 shows the matrix of correlation coefficients between bioleaching performance and microbial population. It was found that pH value positively correlated with total bacteria concentration in statistical significance ($p < 0.05$). Meanwhile, the total bacteria concentration had a significantly negative correlation with the bioleaching performance (solids degradation and metal solubilization). Since sulfur-oxidizing bacteria played a significant role in the thermophilic bioleaching process, the total concentration of *S. acidophilus*, *A. caldus*, and *S. thermosulfidooxidans* highly correlated with metal solubilization. *S. acidophilus* was observed to be the most dominant species in the thermophilic sludge bioleaching (Fig. 5). Therefore, the correlation coefficients of solids degradation and metal solubilization were relatively greater than those for *A. caldus* and *S. thermosulfidooxidans* (Table 6).

Conclusions

The thermophilic sludge bioleaching process conducted in this study could simultaneously achieve metal removal and solids degradation. Increasing the sulfur dosage resulted in higher efficiency of metal removal, while the degradation of solids was not significantly affected by the sulfur dosage. The molecular biology techniques established in this study accurately identified and quantified the sulfur-oxidizing bacteria in the thermophilic sludge bioleaching. *S. acidophilus* was observed to be the main species during the thermophilic sludge bioleaching. Although the populations of *A. caldus* and *S. thermosulfidooxidans* were less, their concentration slightly increased during the bioleaching. In addition, the population of *S. thermosulfidooxidans* increased, but decreased for *A. caldus* when the sulfur dosage was increased. Overall, the total concentrations of *S. acidophilus*, *A. caldus* and *S. thermosulfidooxidans* increased throughout the thermophilic sludge bioleaching process. The results of statistical analysis indicated that the performance of thermophilic sludge bioleaching highly correlated with the concentration of the three sulfur-oxidizing bacteria.

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References

APHA (2005) Standard methods for examination of water and wastewater, 21st edn. American Public Health Association, Washington

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

Babu BK, Mesapogu S, Sharma A, Somasani SR, Arora DK (2011) Quantitative real-time PCR assay for rapid detection of plant and human pathogenic Macrophomina phaseolina from field and environmental samples. Mycologia 103:466–473

Chen SY, Huang QY (2014) Heavy metals recovery from wastewater sludge of printed circuit board industry by thermophilic bioleaching process. J Chem Technol Biotechnol 89:158–164

Chen SY, Lin JG (2009) Enhancement of metal bioleaching from contaminated sediment using silver ion. J Hazard Mater 161:893–899

Chen SY, Lin PL (2010) Optimization of operating parameters for the metal bioleaching process of contaminated soil. Sep Purif Technol 71:178–185

Chen SY, Pan SH (2010) Simultaneous metal leaching and sludge digestion by thermophilic microorganisms: effect of solids content. J Hazard Mater 179:340–347

Chen SY, Chiu YC, Chang PL, Lin JG (2003) Assessment of recoverable forms of sulfur particles used in bioleaching of contaminated sediments. Water Res 37:450–458

d'Hugues P, Foucher S, Galle-Cavalloni P, Morin D (2002) Continuous bioleaching of chalcopyrite using a novel extremely thermophilic mixed culture. Int J Miner Process 66:107–119

de La Rochebrochard S, Naffrechoux E, Drogui P, Mercier G, Blais JF (2013) Low frequency ultrasound-assisted leaching of sewage sludge for toxic metal removal, dewatering and fertilizing properties preservation. Ultrason Sonochem 20:109–117

Dopson M, Lindstrom EB (2004) Analysis of community composition during moderately thermophilic bioleaching of pyrite, arsenical pyrite, and chalcopyrite. Microb Ecol 48:19–28

Evaristo AAA, Santos KCR, Costa LC, Marques MRC (2013) Evaluation of ion exchange resins for recovery of metals from electroplating sludges. Polymer Bull 70:2239–2255

Gao J, Luo QS, Zhang CB, Li BZ, Meng L (2013) Enhanced electrokinetic removal of cadmium from sludge using a coupled catholyte circulation system with multilayer of anion exchange resin. Chem Eng J 234:1–8

Gerayeli F, Ghojavand F, Mousavi SM, Yaghmaei S, Amiri F (2013) Screening and optimization of effective parameters in biological extraction of heavy metals from refinery spent catalysts using a thermophilic bacterium. Sep Purif Technol 118:151–161

Ilyas S, Lee J, Kim B (2014) Bioremoval of heavy metals from recycling industry electronic waste by a consortium of moderate thermophiles: process development and optimization. J Clean Prod 70:194–202

Ito A, Umita T, Aizawa J, Takachi T, Morinaga K (2000) Removal of heavy metals from anaerobically digested sewage sludge by a new chemical method using ferric sulfate. Water Res 34:751–758

Kirk JL, Beaudette LA, Hart M, Moutoglis P, Klironomos JN, Lee H, Trevors JT (2004) Methods of studying soil microbial diversity. J Microbiol Methods 58:169–188

Liu CQ, Plumb J, Hendry P (2006) Rapid specific detection and quantification of bacteria and archaea involved in mineral sulfide bioleaching using real-time PCR. Biotechnol Bioeng 94:330–336

Mousavi SM, Yaghmaei S, Vossoughi M, Jafari A, Hoseini SA (2005) Comparison of bioleaching ability of two native mesophilic and thermophilic bacteria on copper recovery from chalcopyrite concentrate in an airlift bioreactor. Hydrometallurgy 80:139–144

Mudhoo A, Kumar S (2013) Effects of heavy metals as stress factors on anaerobic digestion processes and biogas production from biomass. Int J Environ Sci Technol 10:1383–1398

Naocum C, Fatta D, Haralambous KJ, Loizidou M (2001) Removal of heavy metals from sewage sludge by acid treatment. J Environ Sci Health A 36:873–881

Okibe N, Johnson DB (2004) Biooxidation of pyrite by defined mixed cultures of moderately thermophilic acidophiles in pH-controlled bioreactors: significance of microbial interactions. Biotechnol Bioeng 87:574–583

Paivi HM, Kinunen J, Puhakka A (2004) Characterization of iron- and sulphide mineral-oxidizing moderately thermophilic acidophilic bacteria from an Indonesian auto-heating copper mine waste heap and a deep South African gold mine. J Ind Microbiol Biotechnol 31:409–414

Park HS, Lee JU, Ahn JW (2007) The effects of *Acidithiobacillus ferrooxidans* on the leaching of cobalt and strontium adsorbed onto soil particles. Environ Geochem Health 29:303–312

Pina PS, Oliveira VA, Cruz FLS, Leão VA (2010) Kinetics of ferrous iron oxidation. Biochem Eng J 51:194–197

Plumb JJ, Gibbs B, Stott MB, Robertson WJ, Gibson JAE, Nichols PD, Watling HR, Franzmann PD (2002) Enrichment and characterization of thermophilic acidophiles for the bioleaching of mineral sulphides. Min Eng 15:787–794

Plumb JJ, Muddle R, Franzmann PD (2008) Effect of pH on rates of iron and sulfur oxidation by bioleaching organisms. Miner Eng 21:76–82

Robertson WJ, Kinnunen PHM, Plumb JJ, Franzmann PD, Puhakka JA, Gibson JAE, Nichols PD (2002) Moderately thermophilic iron oxidizing bacteria isolated from a pyritic coal deposit showing spontaneous combustion. Min Eng 15:815–822

Rompre A, Servais P, Baudart J, de-Roubin MR, Laurent P (2002) Detection and enumeration of coliforms in drinking water: current methods and emerging approaches. J Microbiol Methods 49:31–54

Salo-Zieman VLA, Kinnunen PHM, Puhakka JA (2006) Bioleaching of acid-consuming low-grade nickel ore with elemental sulfur addition and subsequent acid generation. J Chem Technol Biotechnol 81:34–40

Schabereiter-Gurtner C, Piñar G, Lubitz W, Rölleke S (2001) An advanced molecular strategy to identify bacterial communities on art objects. J Microbiol Methods 45:77–87

Schippers A, Nagy AA, Kock D, Melcher F, Gock ED (2008) The use of FISH and real-time PCR to monitor the biooxidation and cyanidation for gold and silver recovery from a mine tailings concentrate (Ticapampa, Peru). Hydrometallurgy 94:77–84

Shiers D, Ralph D, Watling H (2010) A comparative study of substrate utilization by Sulfobacillus species in mixed ferrous ion and tetrathionate growth medium. Hydrometallurgy 104:363–369

USEPA (1995) Microwave assisted acid digestion of sediments, sludge, and oils. Test methods for evaluating solid waste, method 3052. USEPA, USA

USEPA (2004) Physical/chemical methods: soil and waste pH. Test methods for evaluating solid waste, method 9045D. USEPA, USA

Wang X, Chen L, Xia S, Zhao J (2008) Changes of Cu, Zn, and Ni chemical speciation in sewage sludge co-composted with sodium sulfide and lime. J Environ Sci 20:156–160

Wang Q, Ye L, Jiang G, Jensen PD, Batstone DJ, Yuan Z (2013) Free nitrous acid (FNA)-based pretreatment enhances methane production from waste activated sludge. Environ Sci Technol 47:11897–11904

Wang Y, Zeng W, Qiu G, Chen X, Zhou H (2014) A moderately thermophilic mixed microbial culture for bioleaching of chalcopyrite concentrate at high pulp density. Appl Environ Microbiol 80:741–750

Wang D, Zeng G, Chen Y, Li X (2015) Effect of polyhydroxyalkanoates on dark fermentative hydrogen production from waste activated sludge. Water Res 73:311–322

Watling HR, Perrot FA, Shiers DW (2008) Comparison of selected characteristics of Sulfobacillus species and review of their occurrence in acidic and bioleaching environments. Hydrometallurgy 93:57–65

Watling HR, Collinson DM, Fjastad S, Kaksonen AH, Li J, Morris C, Perrot FA, Rea SM, Shiers DW (2014) Column bioleaching of a polymetallic ore: Effects of pH and temperature on metal extraction and microbial community structure. Min Eng 58:90–99

Wong JWC, Xiang L, Gu XY, Zhou LX (2004) Bioleaching of heavy metals from anaerobically digested sewage sludge using FeS_2 as an energy source. Chemosphere 55:101–107

Yabalak E, Gizir AM (2013) Subcritical and supercritical fluid extraction of heavy metals from sewage sludge. J Serb Chem Soc 78:1013–1022

Zaleckas E, Paulaukas V, Sendzikiene E (2013) Fractionation of heavy metals in sewage sludge and their removal using low-molecular-weight organic acids. J Environ Eng Landsc Manag 21:189–198

Zammit CM, Mutch LA, Watling HR, Watkin ELJ (2008) Evaluation of quantitative real-time polymerase chain reaction for enumeration of biomining microorganisms in culture. Hydrometallurgy 94:185–189

Zhang R, Wei M, Ji H, Chen X, Qiu G, Zhou H (2009) Application of real-time PCR to monitor population dynamics of defined mixed cultures of moderate thermophiles involved in bioleaching of chalcopyrite. Appl Microbiol Biotechol 81:1161–1168

Zhao J, Wang D, Li X, Yang Q, Chen H, Zhong Y, Zeng G (2015) Free nitrous acid serving as a pretreatment method for alkaline fermentation to enhance short-chain fatty acid production from waste activated sludge. Water Res 78:111–120

Zhou HB, Zeng WM, Yang ZF, Xie YJ, Qiu GZ (2009) Bioleaching of chalcopyrite concentrate by a moderately thermophilic culture in a stirred tank reactor. Bioresour Technol 100:515–520