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Effect of surfactant Tween-80 on sulfur oxidation and expression of sulfur metabolism relevant genes of *Acidithiobacillus ferrooxidans*

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Abstract: The effects of surfactant Tween-80 on the growth, sulfur oxidation, and expression of selected typical sulfur metabolism relevant genes of *Acidithiobacillus ferrooxidans* ATCC 23270 were investigated. The results showed that in the presence of 10^{-2} g/L Tween-80, the growth of *A. ferrooxidans* and its metabolism on the insoluble substrate S^0 and $CuFeS_2$ were promoted. After 24 d of bioleaching, the copper extraction yield of chalcopyrite at 10^{-2} g/L Tween-80 increased by 16% compared with the bioleaching experiment without Tween-80. FT-IR spectra analysis revealed that the result was probably caused by the extracellular polymeric substances whose composition could be changed by the surfactant addition. RT-qPCR was used to analyze the differential expressions of 17 selected sulfur metabolism relevant genes in response to the addition of Tween-80. Down-regulation of the extracellular protein genes indicated the influence of Tween-80 on bacteria-sulfur adsorption. Variation of the expression level of the enzymes provided a supplement to sulfur metabolism investigation.

Key words: *Acidithiobacillus ferrooxidans*; sulfur metabolism; surfactant Tween-80; RT-qPCR

1 Introduction

Acidithiobacillus ferrooxidans is an acidophilic chemolithoautotrophic bacterium. It obtains energy from the oxidation of ferrous iron, elemental sulfur or reduced inorganic sulfur compounds (RISCs), and it is the most extensively studied bioleaching microorganism for understanding the mechanisms of bioleaching [1,2]. It is generally recognized that metal sulfide minerals are chemically attacked by Fe^{3+} (and H^+) through the thiosulfate and/or polysulfide pathway with the assistance of sulfur/ferrous iron-oxidizing micro-organisms. These microorganisms oxidize ferrous iron into ferric iron and sulfur, via a series of reduced sulfur compounds, to sulfate, providing ferric iron and proton [3,4]. Elemental sulfur (S^0) as one of sulfur intermediates may be accumulated during bioleaching when S^0 is not oxidized efficiently by the sulfur- oxidizing bacteria (SOB) [5,6], implying the important roles of the oxidation of elemental sulfur by SOB in the bioleaching process.

Elemental sulfur is hydrophobic and inert at room temperature and ambient pressure. Although the sulfur oxidation biochemistry is very complex, various models for the oxidation of elemental sulfur and other RISCs in *Acidithiobacillus* spp. and related gram-negative sulfur-oxidizing bacteria have been developed [7,8]. According to these models, the oxidation of S^0 by acidophilic SOB could be considered a three-stage process: 1) extra-cellular adsorption and activation, 2) outer membrane transportation, and 3) oxidation in the periplasmic space. The extracellular adsorption and activation stage of S^0 is often considered the rate-limiting stage. Extracellular polymeric substances (EPS) involved in the process of bioleaching play a role of "bridge" in connecting minerals and cells. It was found that the EPS of S^0 -grown cells contain considerably less neutral sugar and uronic acids but much more fatty acids, which assure the contact between hydrophobic S^0 and SOB [9−11]. GOVENDER and GERICKE [12] suggested that the flotation of chalcopyrite ($CuFeS_2$) could be significantly

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increased in the presence of bio-generated EPS.

Tween-80, formally named as polysorbate 80, is a viscous, water-soluble nonionic surfactant. It is a gentle surfactant because of the low toxicity and often used in the medical and physiological researches. Tween-80 has been investigated for orpiment and chalcopyrite bioleaching [13,14]. Addition of Tween-80 in the culture media may improve the hydrophilicity of the sulfide minerals as well as elemental S^0, thus it may enhance the contact between the cells and the minerals. ZHANG et al [15] found that lower than a certain concentration of Tween-80 was conducive to growth and energy metabolism of the SOB *Acidithiobacillus albertensis* BY-05. But it is still unclear how the addition of Tween-80 affects the growth and sulfur oxidation of bioleaching bacteria. In order to evaluate the application potential of Tween-80 in bioleaching of metal sulfide minerals, the authors investigated the impact of Tween-80 on the growth and sulfur oxidation of the cells and the differential expression of the sulfur metabolism relevant genes of the type strain *A. ferrooxidans* ATCC 23270.

2 Experimental

2.1 Bacterial strain and culture media

A. ferrooxidans ATCC 23270 was purchased from American Type Culture Collection (ATCC). The media used in this work were composed by 9K basal medium added with S^0 (10 g/L), $FeSO_4·7H_2O$ (44.7 g/L), $Na_2S_2O_3·5\ H_2O$ (20 g/L), or chalcopyrite (10 g/L) as the energy substrate. The 9K basal medium contained the following components: 0.5 g/L $MgSO_4·7H_2O$; 0.5 g/L K_2HPO_4; 3.0 g/L $(NH_4)_2SO_4$; 0.1 g/L KCl, 0.01 g/L $Ca(NO_3)_2$. The main components of the chalcopyrite were (mass fraction): 34.63% Cu, 25.35% Fe and 30.45% S. The S^0 powder was pretreated with the method described by KONISHI et al [16]. The initial pH for thiosulfate-containing medium was adjusted to 4.0 and the initial pH for other media was adjusted to 2.0.

2.2 Cultivation of *A. ferrooxidans*

A. ferrooxidans was cultivated in 250 mL Erlenmeyer flask containing 100 mL of medium on a rotary shaker at 30 °C and 170 r/min. To examine the effect of Tween-80 on the cell growth on S^0, Fe^{2+}, $S_2O_3^{2-}$ and $CuFeS_2$, respectively, Tween-80 in concentrations from 10 to 10^{-3} g/L (in 10-fold serial dilutions) was added to the culture media. Assays free of Tween-80 were used as control. Triplicate experiments were performed under identical conditions. The water evaporation was compensated with sterilized distilled water and the loss due to sampling for analyses was compensated with sterilized fresh medium. Cell number was determined by the blood corpuscle count method. The a pH values of the culture media were measured with pH meter (PHS-3C). The concentration of copper ions was determined by atomic adsorption spectrophotometry.

2.3 FT-IR spectrometry

A. ferrooxidans cells and S^0 for FT-IR spectrometric analysis were prepared as follows. *A. ferrooxidans* cells grown on S^0 (with or without 10^{-2} g/L Tween-80) or on Fe^{2+} were collected at the late exponential growth phase. The cultures were filtered through Waterman No.1 filter paper. The cells in each filtrate were obtained by centrifugation at 10000 r/min for 10 min, and the S^0 residue on the filter paper was re-suspended in di-distilled H_2O and then centrifuged at 3000 r/min for 1 min. Both the cells and the S^0 were thoroughly washed with di-distilled H_2O, centrifuged, dried in vacuum, and then daubed into the KBr pellet slice, and analyzed by FT-IR spectrometer (Nexus 670, Nicolet, USA) in the range of 500−4000 cm^{-1}.

2.4 Quantitative real-time PCR

Cells were harvested at the late exponential phase. The extraction and purification of genomic DNA were performed using TIANamp genomic DNA extraction kit (Tiangen Biotech Ltd.) and E.Z.N.A® Gel extraction kit (Omega Bio-Tek, Inc.). The extraction and purification of total RNA were performed using total RNA extraction kit (Tiangen Biotech Ltd., China). The quality of total RNA was checked by agarose gel electrophoresis and ethidium bromide staining. The purity and the yield of total RNA were assessed by the A_{260}/A_{280} ratio with a Thermo Scientific NanoDrop® ND-1000 spectrophotometer. Then the total RNA was immediately served as the template in reverse transcription reactions to synthesize cDNA with ReverTra Ace-α-first stand cDNA synthesis kit (Toyobo co., LTD., Osaka, Japan).

Nine putative sulfur-activation relevant protein genes derived from our results of comparative proteomics study including two outer membranes (unpublished work) and seven extracellular proteins [17] and eight genes annotated to play important roles during sulfur oxidation process were selected [8,18]. 16S rRNA (*Afe*_2854) was chosen as control gene for RT-qPCR analysis. In addition, *Afe*_3146 for ferrous iron oxidation was selected as a reference gene. The selected genes with their annotated functions, primer sequences are listed in Table 1. The primers for RT-qPCR were designed by primer premier 3.0 on-line design system (http://frodo.wi.mit.edu/primer3/), based on the genome of *A. ferrooxidans* ATCC 23270 from NCBI GenBank, and then synthesized by Sangon Biotech, Shanghai, China. The PCR amplification of the specific fragments was performed with Veriti 96-well fast thermal cycler

Table 1 Primers for RT-qPCR detection of genes related to sulfur metabolism, and reference genes (16S rRNA and Rusticyanin)

Locus		NCBI annotated function	Primer sequence	Amplicon length/bp
Outer membrane protein gene	*Afe*_1991	Conserved hypothetical protein	F: TTCTGGGGCAGGCTTATCTA R: GTATCGCTACCGCCTGAAAG	135
	*Afe*_2542	Outer membrane protein, OMPP1/FadL/TodX family	F: AGGATTGTCCTCCATTGCAC R: GATGGTGTTGTTGCTGATGG	142
Extracellular protein gene	*Afe*_0168	Hypothetical protein	F: AATGGGTTACGACTGCTTGG R: GATCACCAGAAGCAGCAACA	208
	*Afe*_0258	Hypothetical protein	F: CACCGGCTTTTACTCGGATA R: AGGCCAGACTCAACTCCAGA	134
	*Afe*_0416	Pilin, putative	F: GGGTAATCCTGCCTACACGA R: TATCCGGTCGTGGTTAAAGC	207
	*Afe*_0500	Conserved hypothetical protein	F: AAAGAGGTTTCTGCCCGAAT R: GCTTTGTGCTGACCCTTTCT	197
	*Afe*_1306	Hypothetical protein	F: ATCATTTCCGGTTGTGGGTA R: TCTGAATGACATCCCCATGA	219
	*Afe*_2051	Hypothetical protein	F: CCCGGGACTTGACTTTTTC R: GTCCTGGTACCGGAATGATG	103
	*Afe*_2239	Hypothetical protein	F: CACCTTCACCCAGAACACCT R: GAAGCCGTCATTCCAGGATA	247
Sulfur metabolism related gene	*Afe*_0267	Sulfide quinone reductase, putative	F: TCCGGTCTTTGAACTGCTCT R: GCCTTCGGTCATCACTTTGT	223
	*Afe*_1792	Sulfide-quinone reductase, putative	F: GTGAGCCCTACATCGGTCAT R: TTCTCGTCCACCTGGGTTAC	157
	*Afe*_3121	Sulfite reductase (NADPH) flavoprotein, alpha component	F: TTCTATACCAGCGGGAGTGG R: CCACCAGGGATTGATGTACC	213
	*Afe*_0029	Tetrathionate hydrolase	F: TGCTGTTCCAATGGATTCAA R: TGATACGCCGTTCCTTATCC	181
	*Afe*_0044	DoxD-like family protein	F: TCGTCCAGGGATGGATCTAC R: CGGGTATACAAGCCGACAAT	247
	*Afe*_0045	Sulfur/pyrite/thiosulfate/sulfide-induced protein	F: TTCCCTACGGCACCTATCAG R: CCCTGTGCATAGCCGTAAAT	184
	*Afe*_1088	Thiol:disulfide interchange protein, DsbA family	F: TTTTCTCTGGTGCAAAAGCA R: CGTCTTGACGATCTGGGTCT	177
	*Afe*_2947	Thiol:disulfide interchange protein DsbG, putative	F: GAGCATCTGGTGCAAGACAA R: TGATATCCTGGCCCTTATCG	184
Reference gene	*Afe*_2854	16S rRNA	F: CAGCTCGTGTCGTGAGATGT R: GGACATAAAGGCCATGAGGA	153
	*Afe*_3146	Rusticyanin	F: GAAAGACACCGGGAAAGTCA R: ATCTCCAAGGTCGGGTTCTT	138

and the specificity of primers and the size of PCR products were checked. The product sequencing was carried out by Sagon Biotech, and BLAST analysis was carried out with NCBI online service.

Quantitative real-time PCR was performed in triplicate with an iCycler iQ™ Real-time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, USA) using THUNDERBIRD™ SYBR® qPCR Mix (Toyobo co., LTD., Osaka, Japan). The qPCR thermal cycling program was: 95 °C for 3 min, 40 cycles of 95 °C for 15 s, 58 °C for 30 s, and 72 °C for 30 s, with fluorescence measurements recorded during each annealing step. A melting curve analysis was followed by raising the temperature from 55 °C to 95 °C with a step width of 0.5 °C to verify the specificity of the reactions. Efficiency of amplifications was determined by running a standard curve with serial dilutions of purified 16S rDNA amplification product. Relative quantification was used

to detect the changes in the expression of the genes of interest relative to 16S rDNA and the results were expressed as $lg_2(S/Fe)/lg_2(S_T/S)$ of three independent experiments.

3 Results

3.1 Effect of Tween-80 on growth and sulfur oxidation of *A. ferrooxidans* ATCC 23270 grown on different substrates

The growth curves of *A. ferrooxidans* ATCC 23270 grown on S^0 with 10−10^{-3} g/L Tween-80 are shown in Fig. 1(a). Compared with the state in the absence of Tween-80, the cell growth was enhanced by 10^{-1}–10^{-2} g/L Tween-80. The cell growth was not affected when the concentration of Tween-80 was less than 10^{-3} g/L, but was obviously inhibited when the concentration of Tween-80 was higher than 1 g/L. pH curves (Fig. 1(b)) further show that 10^{-1}–10^{-2} g/L Tween-80 led to apparent decrease in pH values, while 10 g/L Tween-80 led to no change in pH values. Decrease in pH could be mainly due to the sulfur oxidation. This suggests that 10^{-1}–10^{-2} g/L, with the optimal of 10^{-2} g/L, of Tween-80 could promote the cell growth and sulfur oxidation of *A. ferrooxidans* ATCC 23270 grown on S^0.

The growth curves and pH curves of *A. ferrooxidans* ATCC 23270 grown on $S_2O_3^{2-}$ and ferrous iron with 10−10^{-3} g/L Tween-80 are shown in Fig. 2 and Fig. 3, respectively. The results show that the cell growth in these two culture media was inhibited when the concentration of Tween-80 was higher than 1 g/L, but was not affected by 10^{-1}–10^{-3} g/L Tween-80.

The growth curves and pH curves of *A. ferrooxidans* ATCC 23270 grown $CuFeS_2$ with 10−10^{-3} g/L Tween-80 are shown in Fig. 4. The results show that the cell growth on $CuFeS_2$ could be significantly promoted by addition of 10^{-2} g/L Tween-80, while growth was inhibited by Tween-80 higher than 10^{-1} g/L, and was little affected by Tween-80 less than 10^{-3} g/L. And the variation of pH was inversely associated with bacterial counts. It is worthy to mention that the recovery of copper from chalcopyrite with 10^{-2} g/L Tween-80 was higher than that without Tween-80 from the 5th day of bioleaching (Fig. 4(c)). After 24 d of bioleaching, the copper extraction yield was about 70 % when 10^{-2} g/L Tween-80 was added to the bioleaching medium, while 54% was found in the control in which no Tween-80 was added. This indicated that the addition of a certain

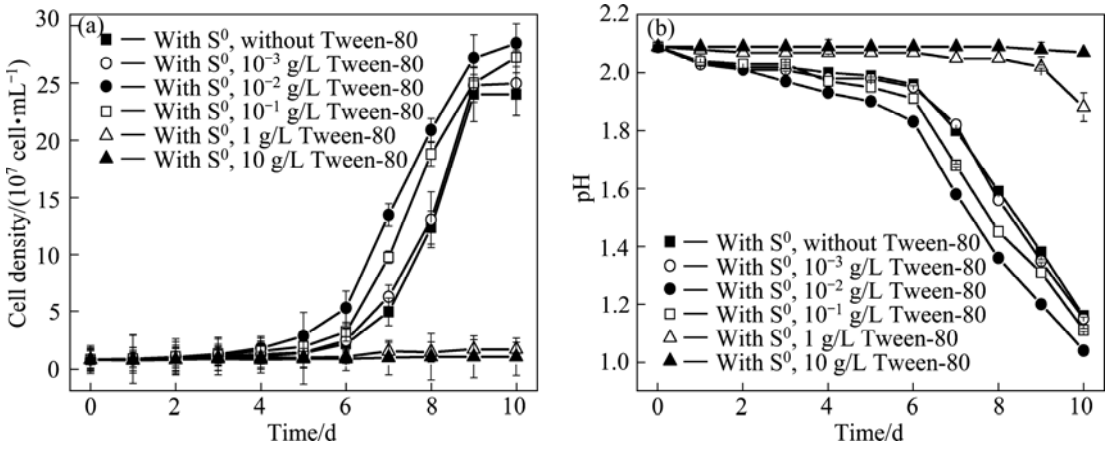

Fig. 1 Growth curves (a) and pH curves (b) of *A. ferrooxidans* ATCC 23270 grown on S^0 with 10−10^{-3} g/L Tween-80

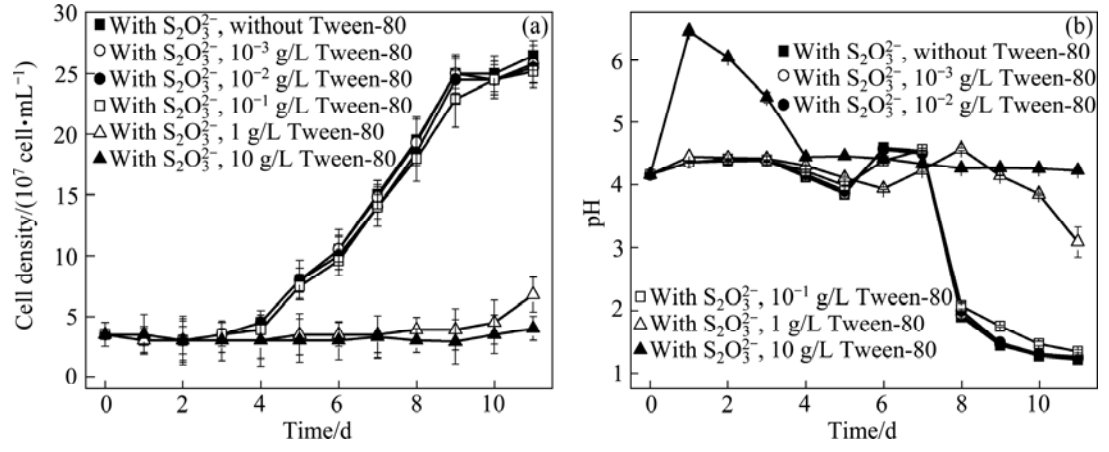

Fig. 2 Growth curves (a) and pH curves (b) of *A. ferrooxidans* ATCC 23270 grown on $S_2O_3^{2-}$ with 10−10^{-3} g/L Tween-80

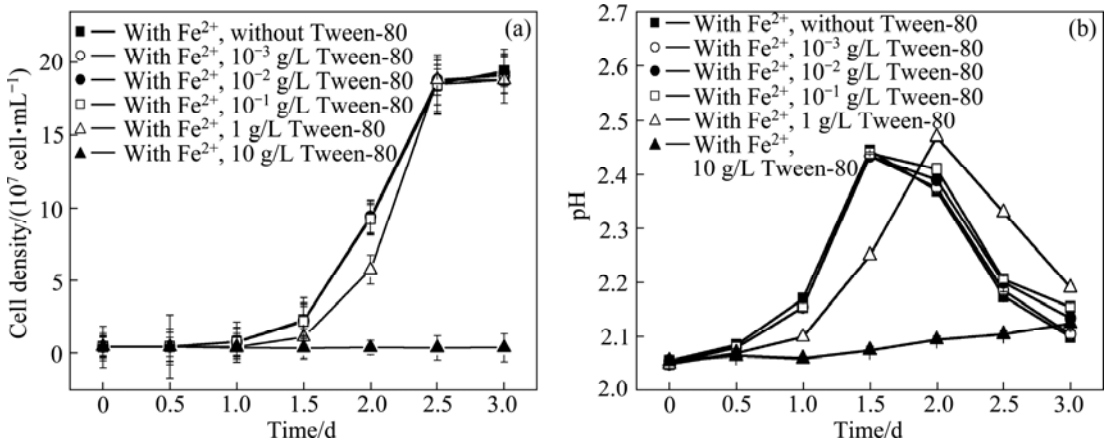

Fig. 3 Growth curves (a) and pH curves (b) of *A. ferrooxidans* ATCC 23270 grown on Fe^{2+} with 10−10^{-3} g/L Tween-80

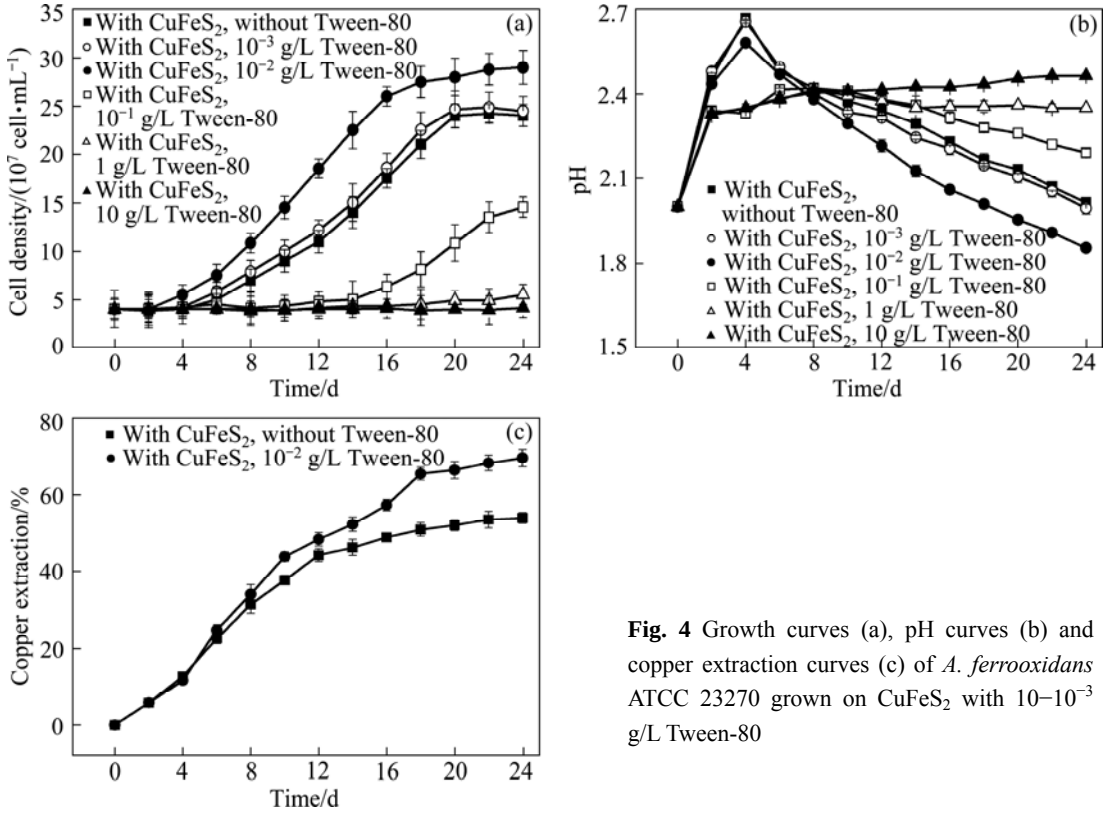

Fig. 4 Growth curves (a), pH curves (b) and copper extraction curves (c) of *A. ferrooxidans* ATCC 23270 grown on $CuFeS_2$ with 10−10^{-3} g/L Tween-80

concentration range of Tween-80 may be an effective way to improve the bioleaching rate of chalcopyrite.

3.2 FT-IR spectra analysis

The FT-IR spectra of standard S^0, the S^0 isolated from culture without Tween-80, and the S^0 isolated from the culture with 10^{-2} g/L Tween-80 are shown in Figs. 5(a)−(c). The FT-IR spectra of the *A. ferrooxidans* cells grown on S^0 without and with 10^{-2} g/L Tween-80 are shown in Figs. 5(d) and (e), respectively. The FT-IR spectra of *A. ferrooxidans* cells grown on Fe^{2+} are shown in Fig. 5(f). The bands were assigned according to the comparison with previous publications [19,20]. The broad and strong band zone at 3100−3500 cm^{-1} could be assignable to —OH, and protein —NH and —NH_2 groups. The sharp bands near 3068, 2942 and 2925, and 2868 cm^{-1} could characterize asymmetrical stretching vibration of fatty acids ═C—H, —CH_3 and —CH_2 groups, respectively. The sharp bands at 1650−1850 cm^{-1} and near 1543 cm^{-1} were due to —C═O stretching and —NH_2 bending vibration, respectively, and these two bands indicate the presence of protein amide group (—CONH—). The bands near 1450 cm^{-1} could be assigned to bending vibration of —CH_3 and —CH_2 groups, which are probably included in polysaccharide and lipids. The sharp bands at 1207−1247 cm^{-1} and

1040−1220 cm^{-1} represented stretching vibration of C—O group and S═O group, respectively. The sharp band around 845 cm^{-1} could be the characteristic peak of orthorhombic sulfur (S_8), the most stable form of elemental sulfur (S^0) existence [15].

The FT-IR spectra of S^0 treated by *A. ferrooxidans* ATCC 23270 without and with 10^{-2} g/L Tween-80 (Figs. 5(b) and (c)) showed the similar absorbance bands to the cellular surface (Figs. 5(d) and (e)), indicating that the surface of S^0 was mainly modified by the bacteria. It is worthy, however, to note that the relative intensity of most FT-IR peaks of S^0 treated by bacterial cells plus 10^{-2} g/L Tween-80 (Fig. 5(c)) was significantly different from that of the S^0 treated only by bacterial cells (Fig. 5(b)), indicating the contribution of Tween-80 to the modification of the sulfur surface. The FT-IR spectra of the cells grown on sulfur (Figs. 5(d) and (e)) show obvious difference from those on ferrous iron (Fig. 5(f)), indicating the dependence of the chemical properties of the cell surface on the energy substrates.

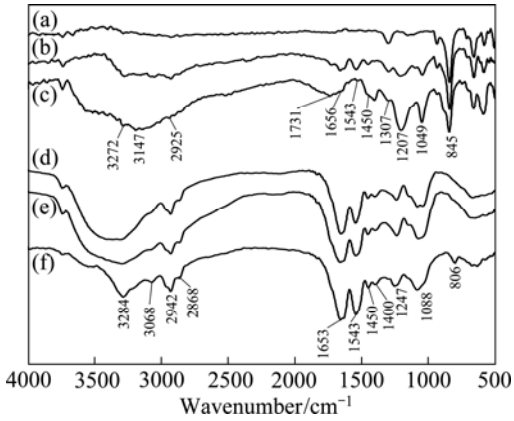

Fig. 5 FT-IR spectra of standard S^0 (a), S^0 isolated from culture without 10^{-2} g/L of Tween-80 (b), S^0 isolated from culture with 10^{-2} g/L of Tween-80 (c), *A. ferrooxidans* cells grown on S^0 without 10^{-2} g/L of Tween-80 (d), *A. ferrooxidans* cells grown on S^0 with 10^{-2} g/L of Tween-80 (e), *A. ferrooxidans* cells grown on Fe^{2+} without 10^{-2} g/L of Tween-80 (f)

3.3 Differential expressions of genes related to sulfur metabolism of *A. ferrooxidans* ATCC 23270 grown with or without Tween-80

Table 2 shows the differential expressions of the selected genes between the cells grown on S^0 with and without Tween-80 (in terms of $lg_2(S_T/S)$), and differential expressions between the cells grown on S^0 and ferrous iron (in terms of $lg_2(S/Fe)$). 16S rRNA was used as the internal control to adjust the systematic and random errors during the operation procedure. Statistical significance was assessed by t-test probability value *p*.

Table 2 RT-qPCR expression data for relevant validated genes

Gene locus		NCBI annotated function	$lg_2(S/Fe)$	$lg_2(S_T/S)$
Outer membrane protein gene	*Afe*_1991	Conserved hypothetical protein	6.54±0.38[a]	−0.32±0.25[b]
	*Afe*_2542	outer membrane protein, OMPP1/FadL/TodX family	2.77±0.29[a]	0.55±0.42[a]
Extracellular protein gene	*Afe*_0168	Hypothetical protein	1.02±0.28[b]	−1.01±0.72[b]
	*Afe*_0258	Hypothetical protein	2.24±0.23[a]	2.44±0.15[a]
	*Afe*_0416	Pilin, putative	8.22±0.58[b]	−1.95±0.55[a]
	*Afe*_0500	Conserved hypothetical protein	4.26±0.23[a]	−0.50±0.19[a]
	*Afe*_1306	Hypothetical protein	3.24±0.26[a]	−2.38±0.38[b]
	*Afe*_2051	Hypothetical protein	1.08±0.41[b]	−1.07±0.42[b]
	*Afe*_2239	Hypothetical protein	1.04±0.39[b]	−0.30±0.54[b]
Sulfur metabolism related gene	*Afe*_0267	Sulfide quinone reductase, putative	1.37±0.17[a]	1.26±0.15[b]
	*Afe*_1792	Sulfide-quinone reductase, putative	1.94±0.19[a]	3.09±0.11[a]
	*Afe*_3121	Sulfite reductase (NADPH) flavoprotein, alpha component	2.58±0.12[a]	−1.39±0.14[a]
	*Afe*_0029	Tetrathionate hydrolase	7.48±0.15[a]	−0.68±0.17[a]
	*Afe*_0044	DoxD-like family protein	1.86±0.12[a]	1.68±0.19[b]
	*Afe*_0045	Sulfur/pyrite/thiosulfate/sulfide-induced protein	3.68±0.15[a]	1.69±0.19[b]
	*Afe*_1088	Thiol:disulfide interchange protein, DsbA family	4.12±0.18[a]	−0.64±0.20[a]
	*Afe*_2947	Thiol:disulfide interchange protein DsbG, putative	4.64±0.39[a]	−1.25±0.33[b]
Reference gene	*Afe*_2854	16S rRNA	0	0
	*Afe*_3146	Rusticyanin	−1.63±0.28[a]	−0.27±0.22[b]

$lg_2(S/Fe)$ and $lg_2(S_T/S)$ represent the logarithm to base 10 of the differential expressions of the selected genes betueen the cells grown on S^0 with and without Tween-80, respectively. SD. is an abbreviation for standard deviation. Superscript letters represent *p*-value based on t-test, *p*-value <0.05 is considered significant (indicated with b), and *p*-value <0.01 is considered very significant (indicated with a). Genes with the value of $lg_2(S/Fe)$ and $lg_2(S_T/S)$ ratio of median larger than |1.0| (corresponding to genes induced more than 2.0 fold) are considered differentially expressed (indicated with bold numbers).

Compared with the down regulation of the rusticyanin (*Afe*_3146, rus), the expressions of all the genes selected, especially the hypothetical and putative protein genes from outer membrane and extracellular, were significantly up regulated when the culture was transformed from Fe^{2+} substrate to S^0 substrate, indicating the relevance of them to sulfur metabolism [17].

Gene expressions of the cells grown on S^0 were significantly different when 10^{-2} g/L Tween-80 was added into the culture media. The surfactant has an obvious effect on the expressions of the extracellular proteins and the sulfur metabolism related genes indicated from Table 2. Hypothetical extracellular protein gene *Afe*_2170 and four sulfur metabolism relevant genes (*Afe*_0267, *Afe*1792, *Afe*_0044, *Afe*_0045) were significantly up regulated. Four genes of extracellular proteins (*Afe*_0168, *Afe*_0416, *Afe*_1306, *Afe*_2051), and two genes (*Afe*_3121, *Afe*_2947) encoding sulfur metabolism related proteins were significantly down regulated. The expression of the outer membrane protein genes were not significantly influenced by Tween-80 at this concentration.

4 Discussion

It is well known that elemental sulfur could exist as one of the intermediates during bioleaching of chalcopyrite, and it could form a passivation layer when it is not oxidized efficiently by SOB [6,21]. The addition of the surfactant Tween-80 (10^{-2} g/L) shortened the adaption stage of bacterial growth (Fig. 4(a)), promoted sulfur oxidation (i.e., decrease of pH as shown in Fig. 4(b)) and enhanced the copper extraction yield of chalcopyrite (Fig. 4(c)). This demonstrates the addition of a certain concentration of surfactants may be an effective way to improve the bioleaching rate of chalcopyrite.

The adsorption of SOB on the hydrophobic surface is generally thought to be an essential step in sulfur oxidation process. Electrostatic interaction does not play an important role in the bacteria-sulfur interaction according to the evidence of TAN and CHEN [22]. So the affinity is affected by the wetting behavior of the hydrophobic substrates. According to the microbial population dynamics and the pH variations in this study, 10^{-1}−10^{-2} g/L Tween-80 showed no sign of toxicity for *A. ferrooxidans* ATCC 23270 grown on insoluble substrate (S^0) and soluble substrates ($S_2O_3^{2-}$, Fe^{2+}), and about 10^{-2} g/L Tween-80 could promote the cell growth on insoluble sulfur substrates (S^0, $CuFeS_2$). This is because suitable concentration of Tween-80 can efficiently modify the contact between the minerals and the cells.

EPS is thought to be amphiphilic and it can mediate the contact of the hydrophilic cells and the hydrophobic sulfur or minerals, though so far little is known about the EPS composition [10]. In this work, addition of Tween-80, as one of the typical surfactants or wetting agents, altered the wettability of elemental sulfur. FT-IR spectra analysis reflected the wetting of the hydrophobic elemental sulfur (Fig. 5) by the cells, because of the appearance of the absorption brands of the organic groups, such as —OH, —NH, —NH_2, —CH_2, —CH_3, ═C—H, C—O, —C═O, and —CONH— in the FT-IR spectra of the sulfur treated by cells.

The cells grown on S^0 demonstrated a different FT-IR spectra from that on Fe^{2+} substrates (Fig. 5), indicating the substrate specificity of EPS formation. This can be supported by the significant down-regulation of the expression of the extracellular proteins *Afe*_0416, *Afe*_0168, *Afe*_1306, *Afe*_2051 and the significant up-regulation of the extracellular protein *Afe*_0258 (Table 2). Most of the extracellular proteins, except *Afe*_0258 and *Afe*_2239, are cysteine-rich proteins. This indicates clearly the important role of the thiol groups of cysteine in EPS in the present study.

Pilin as the appendix of the cell surface has been considered to play an important role in the attachment of bioleaching microbes to mineral surface [23]. As shown in Table 2, the pilin relevant gene *Afe*_0416 was down-regulated, when 10^{-2} g/L Tween-80 was added to the sulfur containing medium. But this gene expression in sulfur-grown cells was significantly higher than that in ferrous iron-grown cells, indicating the important role of the pilin of *A. ferrooxidans* in sulfur adsorption, which enables *A. ferrooxidans* to attach and colonize to solid sulfur [23,24].

Extracellular conserved hypothetical protein *Afe*_0500 contains CXXC domains, belonging to the characteristics domain relating to oxidation and reduction of sulfur. This is assured by the fact that *Afe*_0500 was highly expressed in the sulfur grown *A. ferrooxidans*, but it seems to be not affected by 10^{-2} g/L Tween-80.

The effect of Tween-80 on the expression of the known proteins and enzymes involved in the sulfur oxidation process was also observed. The genes *Afe*_0267 and *Afe*_1792 encode sulfide/quinone oxidoreductase (SQR), and *Afe*_0044 and *Afe*_0045 encode thiosulfate: quinone oxidoreductase (TQR). They were highly expressed in S^0 grown cells and further up-regulated in expression when 10^{-2} g/L Tween-80 was supplemented. This indicates the improvement of bio-oxidation of sulfur by addition of 10^{-2} g/L Tween-80. Afe_3121 is a flavoprotein, subunit of sulfite reductase, which is speculated to be involved in the assimilation of sulfur into cysteine in *A. ferrooxidans* [23]. *Afe*_2947 and *Afe*_1088 (thiol: disulfide interchange proteins,

DsbG and DsbA) are used for protein folding and stabilization by resolving incorrectly formed disulfide bonds and were considered to be related to stress response [18,24]. Down regulation of the genes *Afe*_3121, *Afe*_2947 and *Afe*_1088 was observed when Tween-80 was added into S^0 medium. The reason for this phenomenon needs to be further investigated.

No comprehensive understanding of biochemical mechanism of the sulfur oxidation process in bioleaching system has been achieved yet. However, surfactant Tween-80 in this study has been proven its important effect on the cell adhesion, sulfur activation and the sulfur metabolism. Further investigation could not only help us establish a more effective bioleaching system but also shed light on the understanding of the regulation of sulfur-oxidizing process.

5 Conclusions

1) The growth and sulfur oxidation of *A. ferrooxidans* ATCC 23270 grown on S^0, $S_2O_3^{2-}$, Fe^{2+}, and chalcopyrite with $10-10^{-3}$ g/L Tween-80 showed that 10^{-2} g/L Tween-80 promoted the growth of *A. ferrooxidans* and its metabolism on the insoluble substrates S^0 and $CuFeS_2$.

2) After 24 d of bioleaching, the copper extraction yield of chalcopyrite increased by 16 % with addition of 10^{-2} g/L Tween-80.

3) The results of FT-IR spectrometry and RT-qPCR demonstrated the significant effect of Tween-80 on the cell adhesion, sulfur activation and the sulfur metabolism.

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表面活性剂 Tween-80 对 *Acidithiobacillus ferrooxidans* 硫氧化和硫代谢相关蛋白质基因表达的影响

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摘 要：研究了表面活性剂 Tween-80 对 *Acidithiobacillus ferrooxidans* ATCC 23270 生长、硫氧化和硫代谢相关典型基因表达的影响。结果表明，当培养基中含有 10^{-2} g/L Tween-80 时，*A. ferrooxidans* 的生长以及其对不溶性底物（S^0 和 $CuFeS_2$）的代谢得到了促进。在该条件下，经过 24 d 的生物浸出，黄铜矿的铜离子浸出率比对照组(不含 Tween-80)高 16%。FT-IR 光谱分析表明，这可能是由于 Tween-80 的存在而导致胞外多聚物成分发生变化而引起的。用 RT-qPCR 来分析 17 个硫代谢相关基因在 Tween-80 存在时的表达差异。胞外蛋白质基因表达下调表明了 Tween-80 对细菌−硫吸附作用的影响。硫代谢相关酶基因表达水平的变化为硫代谢的研究提供了参考。

关键词：*Acidithiobacillus ferrooxidans*；硫代谢；表面活性剂 Tween-80；RT-qPCR

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