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Differential utilization and speciation transformation of orthorhombic α-S_8 and amorphous μ-S by substrate-acclimated mesophilic *Acidithiobacillus ferrooxidans*

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Abstract: The utilization and speciation transformation of α-S_8 and μ-S by the typical mesophilic acidophilic strain *Acidithiobacillus ferrooxidans* ATCC 23270 were investigated. *A. ferrooxidans* cells first acclimated to the energy source α-S_8 or μ-S, respectively. The results of cell growth and sulfur oxidation behavior showed that the strain grown on α-S_8 entered slowly (about 1 d later) into the exponential phase, while grew faster in the exponential phase and attained higher maximal cell density and lower pH value than that on μ-S. After bio-corrosion, both of the two sulfur samples were evidently eroded and modified by *A. ferrooxidans* cells. After growth of *A. ferrooxidans*, the surface composition of amorphous μ-S became 63.1% μ-S and 36.9% α-S_8, and that of orthorhombic α-S_8 became 68.3% α-S_8 and 31.7% μ-S, while the surface compositions of α-S_8 and μ-S in sterile experiment were not changed, indicating that these two elemental sulfur species can be interconverted by *A. ferrooxidans*.
Key words: sulfur utilization; sulfur speciation transformation; α-S_8; μ-S; *Acidithiobacillus ferrooxidans*

1 Introduction

Elemental sulfur (S^0) oxidation by sulfur-oxidizing microbes (SOMs) takes an important role in bio-hydrometallurgy of metals from sulfide minerals [1]. In bioleaching process, S^0 may accumulate on the mineral surface as a passivation layer, while the existence of SOMs can eliminate the S^0 layer by oxidizing S^0 into sulfuric acid [2].

S^0 can exist in a wide variety of allotropic forms. Among them, orthorhombic cyclo-octasulfur α-S_8 and amorphous polymeric sulfur μ-S are the two major species. The observed S^0 produced during bioleaching of metal sulfide minerals has been reported mostly as orthorhombic form [1,3] and in few cases as short chain polysulfides (S_n^{2-}) and long sulfur chains terminated with organic groups [4,5]. The α-S_8 form was probably chemically transformed by the unstable S_n^{2-} [6] which was considered as a polymerization process from monosulfide (S^{2-}) of bulk chalcopyrite via surface S_n^{2-} to α-S_8. On the other hand, the bio-oxidation of the produced α-S_8 was suggested to be activated firstly by reacting with reactive thiol groups (—SH) of outer membrane proteins, forming —S_nH (n≥2) complexes, and then transported into periplasmic space for further oxidation. This indicated that there might exist transformation between these two S^0 allotropes. Therefore, it is very valuable to study the differential utilization and sulfur speciation transformation of orthorhombic α-S_8 and polymeric μ-S by SOMs.

Up to now, microbial sulfur metabolism has been widely studied [7], but there have been few publications focused on differential utilization and sulfur speciation transformation of sulfur allotropes [8−14]. These studies indicated that the utilization of sulfur allotropes by

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different SOMs is quite different. The inherent clue to that, however, is still unclear because of the scarceness of knowledge on utilization of S^0 allotropes by SOMs. Recently, according our previous description [12], extreme thermophilic *Acidianus manzaensis* cultured on μ-S grew faster and had higher sulfur oxidation activity than that on α-S_8. Further study showed that μ-S was mostly converted into α-S_8 after *A. manzaensis* cell growth while α-S_8 was not transformed into another allotrope. After growth of *A. manzaensis* the surface composition of μ-S was composed of 92.1% α-S_8 and 7.9% μ-S, while no change in composition for α-S_8 was found. However, PENG et al [14] found that μ-S could be more easily bio-adapted and activated than α-S_8 by the Fe^{2+}-grown *Acidithiobacillus ferrooxidans* cells, but had a lower sulfur oxidation activity when this strain has acclimated to the new energy substrate. Meanwhile, HE et al [11] suggested that α-S_8 was activated first and then a part of it might be converted to polymeric sulfur before it was further utilized by the cells. These indicate that not only microbial species but also the culture condition can affect the utilization and sulfur speciation transformation of allotropic forms of S^0.

In the present study, the utilization of α-S_8 and μ-S by the typically mesophilic strain *A. ferrooxidans* ATCC 23270, which has acclimated to each energy substrate, was comparatively studied, and the sulfur transformation of these two sulfur allotropes was also investigated by combining the Fourier transforming infrared spectrum (FT-IR) and sulfur K-edge X-ray absorption near edge structure (XANES) spectroscopy.

2 Experimental

2.1 Strain and culture conditions

The mesophilic strain *A. ferrooxidans* ATCC 23270 was purchased from American Type Culture Collection (ATCC) and stored at the Key Laboratory of Biometallurgy of Ministry of Education, Central South University, Changsha, China. The basal medium used for cell cultivation was composed of the following components: 3.0 g/L $(NH_4)_2SO_4$, 0.5 g/L $MgSO_4{\cdot}7H_2O$, 0.5 g/L K_2HPO_4, 0.1 g/L KCl, 0.01 g/L $Ca(NO_3)_2$, adding with α-S_8 (10 g/L) or μ-S (10 g/L) as the energy substrate. The initial pH of the culture medium was adjusted to 1.95 with sulfuric acid. Before the experiment, *A. ferrooxidans* cells first acclimated to the energy source α-S_8 and μ-S, respectively for several generations' cultivation, and then inoculated respectively on the corresponding energy substrate α-S_8 and μ-S with initial cell density of 2×10^7 cell/mL. The culture was incubated in 250 mL Erlenmeyer flasks containing 100 mL culture medium on a rotary shaker at 30 °C and 170 r/min. The abiotic controls were carried out under the same conditions. All experiments were performed in triplicate.

The α-S_8 and μ-S samples used in the present study were prepared according to the previous description [12]. Briefly, for the preparation of μ-S, the purchased polymeric μ-S IS-60 was first continuously stirred in CS_2 (~1 g/mL) for 20 min, let stand for 10 min and then the CS_2 phase was discarded. For the preparation of α-S_8, the purchased analytically pure sublimed orthorhombic sulfur was first sufficiently dissolved in CS_2 (~0.5 g/mL), then the insoluble part was filtered off, and at last the yellow solution was evaporated and crystallized in a fume hood. The purified α-S_8 and μ-S were ground to fine powder with the size of 38−75 μm and stored at 4 °C. The purified α-S_8 and μ-S were characterized by X-ray diffraction and Raman spectroscopy. The XRD patterns and Raman spectra of the original α-S_8 and μ-S were well consistent with the orthorhombic α-S_8 and amorphous μ-S as previously described [12].

2.2 Growth and sulfur oxidation determination

The growth and sulfur oxidation of *A. ferrooxidans* on α-S_8 or μ-S were evaluated by monitoring the cell density, pH and sulfate concentration of the culture solution at 1 d interval during the cultivation. The cell density was determined by direct counting with a blood corpuscle counter (XB-K−25), the pH was measured with a pH meter (PHS−3C), and the concentration of sulfate ion was determined by barium sulfate turbidimetry [15].

2.3 Sulfur surface morphology and bacterial modification analysis

The surface morphologies of α-S_8 and μ-S samples after utilization by *A. ferrooxidans* were observed by the scanning electron microscopy (SEM) (Nova™ NanoSEM 230, FEI, USA). Briefly, the samples were first pre-fixed by 25% formaldehyde overnight, then dehydrated using a graded ethanol series, and at last coated with gold and introduced into the SEM chamber for observation. The bacterial modification of sulfur samples was determined by Fourier transform-infrared (FT-IR) spectroscopy in the range of 4000−500 cm^{-1} using an FT-IR spectrometer (Nexus 670, Nicolet, USA).

2.4 Sulfur speciation transformation analysis

The sulfur speciation transformation was analyzed by S K-edge X-ray absorption near edge structure (XANES) spectroscopy, which was performed at soft X-ray beam-line (4B7A beam-line) in Beijing Synchrotron Radiation Facility, China. S K-edge XANES data were recorded in the total electron yield (TEY) model in vacuum and scanned at a step width of 0.3 eV in the region from 2.460 to 2.490 keV. In all cases, the XANES spectra were first normalized to the maximum of the absorption spectrum, and then fitted for

their linear combinations using known spectra from "reference samples" (i.e. standard α-S_8 and μ-S) with IFEFFIT program [16,17], where the errors of the fitting results in the software were provided to evaluate the quality of a single fitting.

3 Results and discussion

3.1 Growth and sulfur oxidation of *A. ferrooxidans* grown on α-S_8 and μ-S

The growth, pH and [SO_4^{2-}] curves of bacterial cells cultured on α-S_8 and μ-S, and the corresponding growth rate, pH decreasing rate and SO_4^{2-} production rate curves are shown in Figs. 1 and 2, respectively.

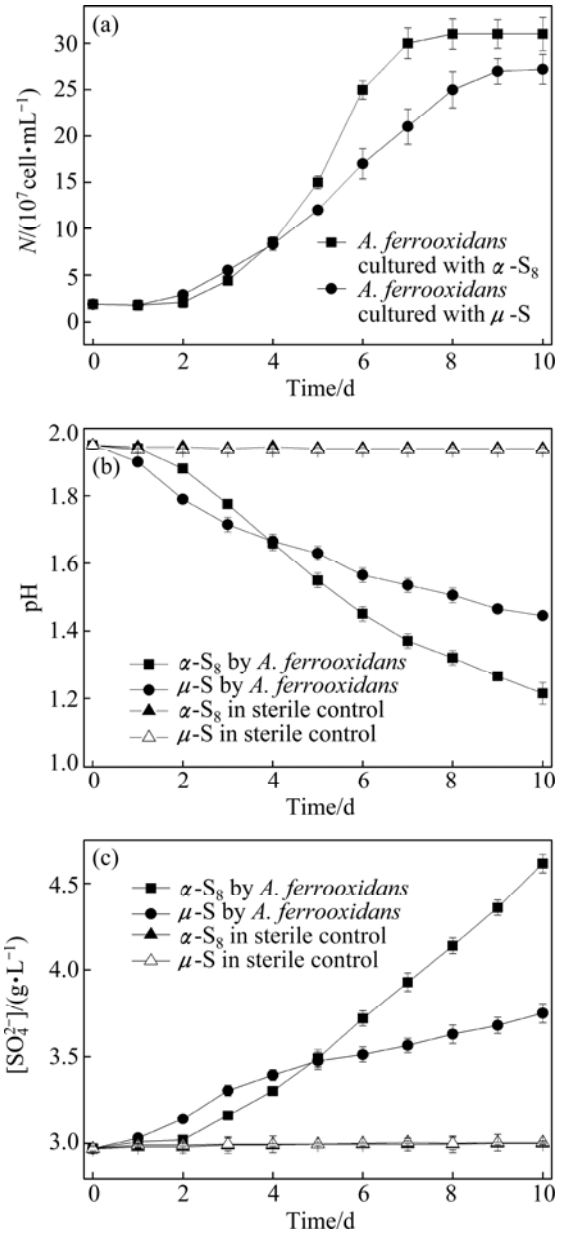

Fig. 1 Growth (a), pH (b) and sulfate concentration (c) curves of *A. ferrooxidans* cultured with α-S_8 and μ-S, respectively

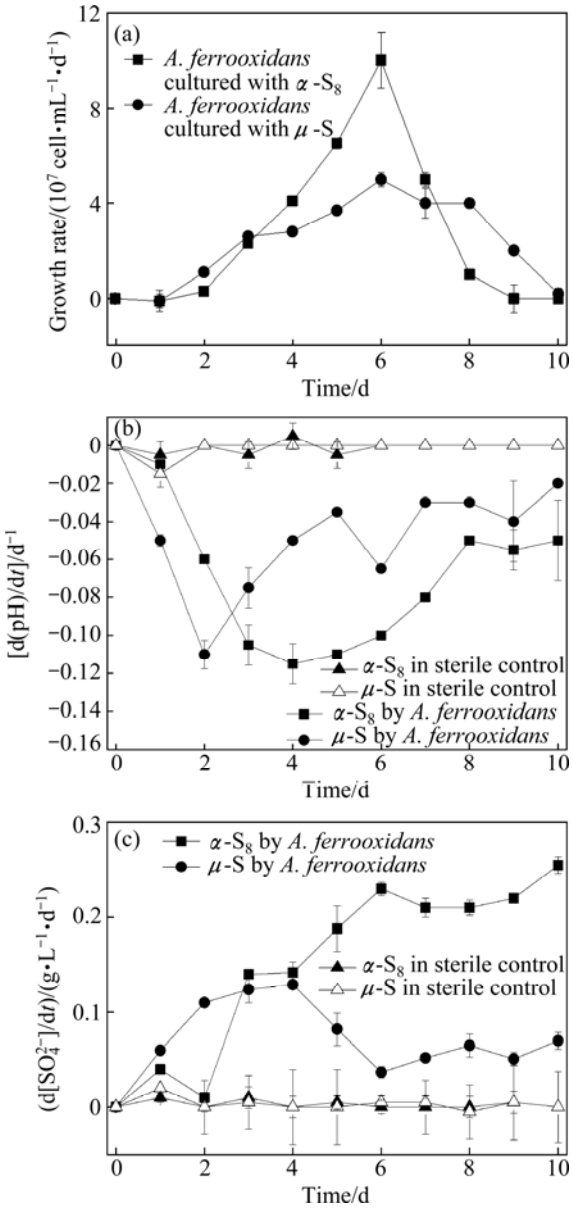

Fig. 2 Growth rate (a), pH decreasing rate (b) and SO_4^{2-} production rate (c) curves of *A. ferrooxidans* cultured with α-S_8 and μ-S, respectively

Results in Fig. 1(a) showed that *A. ferrooxidans* grown on α-S_8 had longer (about 1 d) lag phase, but reached the earlier (about 1 d) stationary stage and attained the higher maximal cell density than that on μ-S. Results in Fig. 2(a) further showed that cells grown on α-S_8 had a lower growth rate from 1−3 d and a higher growth rate from 3−7 d than that on μ-S, indicating that *A. ferrooxidans* had a better adaptability on μ-S than that on α-S_8, while better growth on α-S_8 than that on μ-S. S^0 can be oxidized by sulfur-oxidizing bacteria into SO_4^{2-} with producing H^+, resulting in the decrease of pH and the increase of [SO_4^{2-}]. Results in Figs. 1(b) and (c) showed that, after growth of *A. ferrooxidans*, the pH value of the culture solution containing α-S_8 was significantly lower and the [SO_4^{2-}] value of the solution

containing α-S_8 was significantly higher than those of the solution containing μ-S, respectively. The pH decreasing rate (Fig. 2(b)) and SO_4^{2-} production rate (Fig. 2(c)) also showed that, from the 3rd day, the bacterial culture solution containing α-S_8 had higher pH decreasing rate and SO_4^{2-} production rate than those of the solution containing μ-S. This indicated that after adaption for the energy substrates, *A. ferrooxidans* cultured on α-S_8 had a higher sulfur oxidation rate than that on μ-S.

The initial stage of the growth and sulfur oxidation of mesophilic *A. ferrooxidans* grown on α-S_8 and μ-S showed similar results with PENG et al [14]. The latter used the Fe^{2+}-grown *A. ferrooxidans* as the initial incubated cells, and also found that the Fe^{2+}-grown *A. ferrooxidans* cells grew on μ-S faster at initial stage comparing with that on α-S_8.

3.2 Surface morphology and modification of α-S_8 and μ-S after growth of *A. ferrooxidans*

The surface morphologies (Fig. 3) and the surface modification (Fig. 4) of α-S_8 and μ-S before and after the growth of *A. ferrooxidans* were examined by SEM and FT-IR spectroscopy, respectively.

Figures 3(b) and (e) showed that the sulfur surfaces for α-S_8 and μ-S in the sterile controls were very slight corrosion by comparison with the original α-S_8 and μ-S (Figs. 3(a) and (d)). However, the surfaces for α-S_8 and μ-S after growth of *A. ferrooxidans* suffered serious

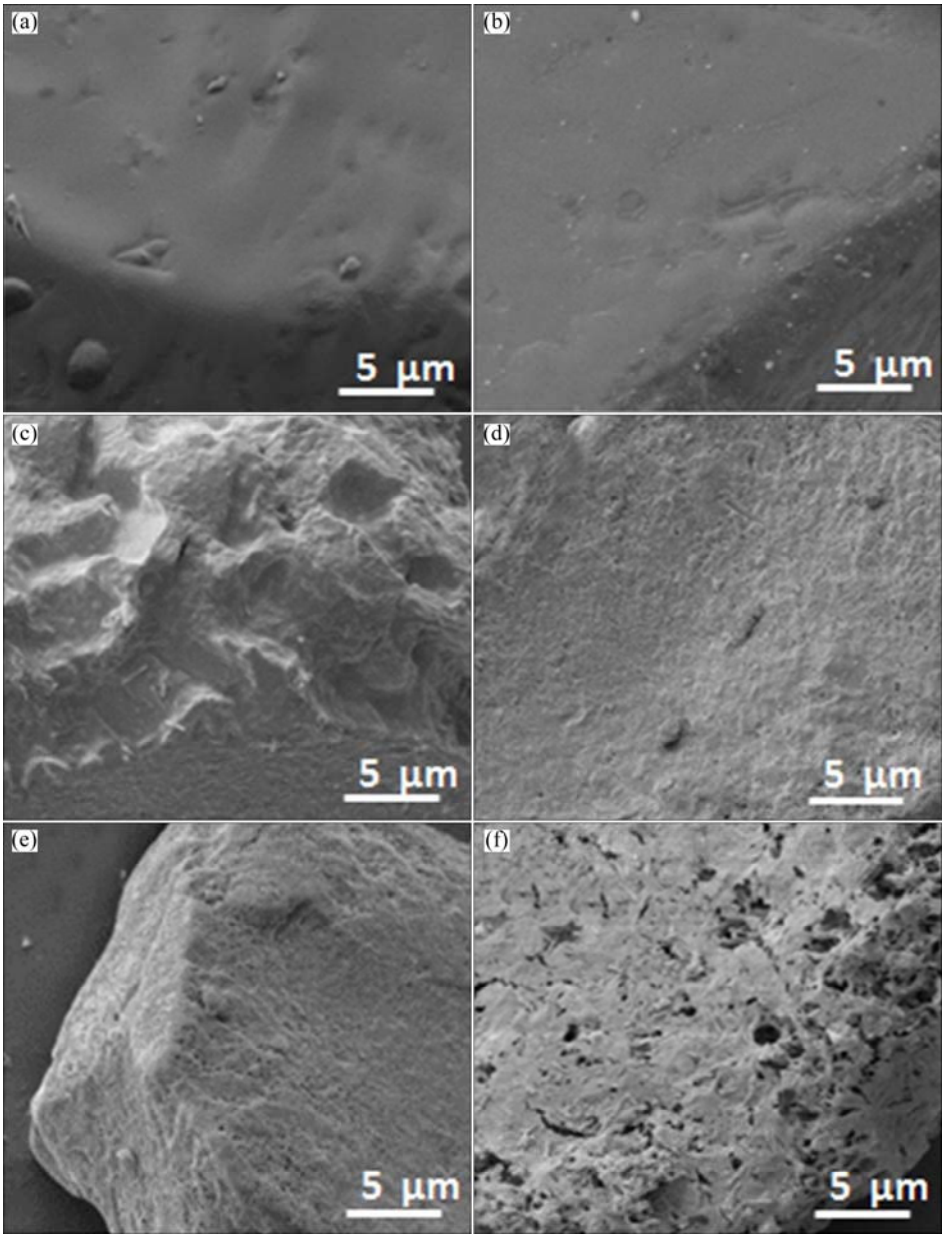

Fig. 3 Surface morphologies of α-S_8 (a−c) and μ-S (d−f) before and after growth of *A. ferrooxidans*: (a) Original α-S_8; (b) α-S_8 in sterile control; (c) α-S_8 after growth of *A. ferrooxidans*; (d) Original μ-S; (e) μ-S in sterile control; (f) μ-S after growth of *A. ferrooxidans*

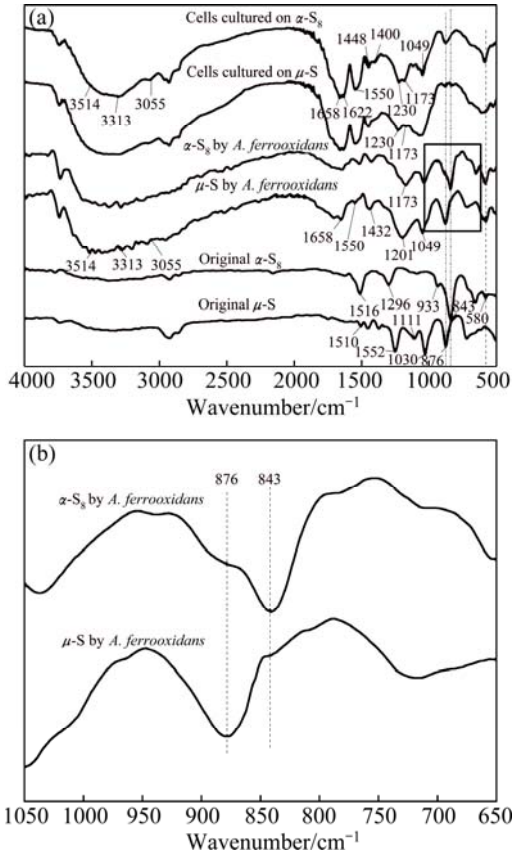

Fig. 4 FT-IR spectra of original α-S_8 and μ-S before and after growth of *A. ferrooxidans* and cells grown on α-S_8 and μ-S, respectively (a), and spectra of α-S_8 and μ-S after growth of *A. ferrooxidans* at specific wavelength from 1050 to 650 cm^{-1} (b)

corrosion (Figs. 3(c) and (f)). In addition, the bio-corroded α-S_8 revealed apparently eroding pits on the sample's surface (Fig. 3(c)), while bio-corroded μ-S was loose and porous. The results indicated that the utilization of α-S_8 by *A. ferrooxidans* was mostly at sulfur surface level, while the cells produced perforation of μ-S and the interaction between *A. ferrooxidans* cells and μ-S could be both on the surface and in the interior of this sulfur species.

The bands of the FT-IR spectra of *A. ferrooxidans* cells (Fig. 4(a)) were assigned according to the previous descriptions [18,19]. The band at 3055−3514 cm^{-1} reflected —OH, and protein —NH and —NH_2 groups. The bands at 1622−1658 cm^{-1} and near 1550 cm^{-1} represented —C═O stretching and —NH_2 vibration, respectively, which always suggested the presence of protein amide group (—CONH—). The bands near 1448 cm^{-1} are assigned to —CH_3 and —CH_2 bending vibration. The bands at 1200−1250 cm^{-1} and 1040−1220 cm^{-1} are due to C═S and S═O groups, respectively. Compared with the FT-IR spectra of standard α-S_8 and μ-S, the spectra of α-S_8 and μ-S isolated from culture media became much more complex and showed the absorption bands described above, indicating that the surfaces of both α-S_8 and μ-S were modified by the bacterial cells. In addition, according to the previous description [12], the bands at 843 cm^{-1} and 876 cm^{-1} can be described as the characteristic peaks of α-S_8 and μ-S, respectively. It is worthy to note that the peak at 876 cm^{-1} was also found in the spectra of both α-S_8 after growth of *A. ferrooxidans* and *A. ferrooxidans* cells cultured on α-S_8, and that the new (shoulder) peaks at 843 cm^{-1} appeared in the spectra of both μ-S after growth of *A. ferrooxidans* and *A. ferrooxidans* cells cultured on μ-S (Figs. 4(a) and (b)). This indicated that these two S^0 species can be interconverted by the mesophilic *A. ferrooxidans*.

3.3 Sulfur speciation transformation of α-S_8 and μ-S after growth of *A. ferrooxidans*

The sulfur speciation transformation was analyzed by S K-edge XANES spectroscopy, such results are shown in Fig. 5. The S K-edge XANES spectra of original α-S_8 and μ-S showed clear difference in the width and intensity at 2.4727 and 2.4800 keV. The S K-edge XANES spectra of α-S_8 and μ-S in sterile controls and after growth of *A. ferrooxidans* were analyzed by the linear composition fitting using the spectra of original α-S_8 and μ-S. The fitting results (Table 1) showed that after growth of *A. ferrooxidans*, the surface composition of amorphous μ-S became 63.1% μ-S and 36.9% α-S_8, while the surface composition of orthorhombic α-S_8 became 68.3% α-S_8 and 31.7% μ-S, which supported the FT-IR results (Fig. 4(b)). In contrast, the surface compositions of α-S_8 and μ-S in the sterile experiment were not changed.

In the present study, these two S^0 allotropes were found to be interconverted by the mesophilic *A. ferrooxidans*. However, only μ-S was found to be converted to another species after growth of extremely

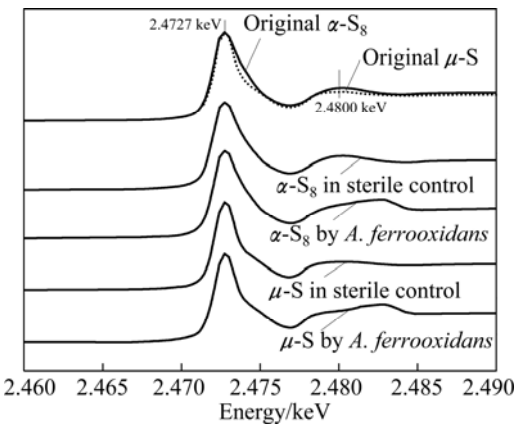

Fig. 5 Normalized sulfur K-edge XANES spectra of standard α-S_8 and μ-S, α-S_8 and μ-S in sterile controls and after growth of *A. ferrooxidans*

Table 1 Fitted results of S K-edge XANES spectra of measured sample with standard α-S_8 and μ-S spectra (Values in brackets represent errors of fitting results)

Sample	Contribution of standard spectra/%	
	α-S_8	μ-S
α-S_8 in sterile control	100.0 (0.00)	0.0 (0.00)
α-S_8 treated by *A. ferrooxidans*	68.3 (3.72)	31.7 (1.43)
μ-S in sterile control	0.0 (0.00)	100.0 (0.00)
μ-S treated by *A. ferrooxidans*	36.9 (1.55)	63.1 (3.21)

thermophilic *A. manzaensis* [12] and moderately thermophilic *Sulfobacillus thermosulfidooxidans* [20]. Results of *A. manzaensis* showed that bio-corroded μ-S was composed of 92.1% α-S_8 and 7.9% μ-S, while no change in composition for bio-corroded α-S_8 was found [12]. Results of *S. thermosulfidooxidans* also showed that bio-corroded μ-S began to gradually convert into α-S_8 from the 2nd day when it entered the exponential phase, and the composition finally became 62.3% μ-S and 37.7% α-S_8 on the 4th day at the stationary phase, while no transformation of bio-corroded α-S_8 into μ-S occurred [20]. This indicated that the transformation of sulfur allotropes was also different with different microorganisms. It should be noted that, in the sterile groups, the surface compositions of α-S_8 and μ-S were not changed in the present study (30 °C), which is consistent with the sterile groups at 50 °C. However, at 65 °C the natural transformation of μ-S to the α-S_8 was found. This suggests that different environmental temperatures adopted can also influence the transformation of sulfur allotropes, because α-S_8 is more stable than μ-S and some of μ-S can be converted slowly into α-S_8 at high temperatures [21], as shown in Eq. (1) (short-chain like S_n^{2-} is a unstable transition state). Furthermore, previous studies demonstrate that this process can be accelerated with the existence of SOMs [12, 14], which is assured by the transformation from μ-S to α-S_8 by *A. ferrooxidans* even if the ambient temperature (30 °C) is lower than 65 °C.

$$\mu\text{-S}\rightarrow S_n^{2-}\rightarrow\alpha\text{-}S_8\ (n\geq1) \quad (1)$$

According to the previous studies concerned on the sulfur metabolism of SOMs [2,22], the utilization of S^0 can be divided into three steps: 1) extracellular adsorption and activation of sulfur, 2) outer membrane transportation of sulfur, and 3) oxidation of sulfur in the periplasmic space. The extracellular activation was considered to be the rate-limiting step. Previous studies [23,24] indicated that S^0 (mainly α-S_8) was probably activated by thiol group containing protein(s) by forming —SS_nH (Eq. (2)).

$$S_8+\text{P—SH}\rightarrow(\text{P—}SS_8\text{H})\rightarrow\text{P—}SS_n\text{H},\ n\geq1$$
(P represents protein, $n\geq1$) (2)

Bacterial utilization of μ-S may take the similar mechanism to Eq. (2). With the interaction between μ-S and SOMs, the unstable short-chain like S_n^{2-} formed in Eq. (1) can be transformed to P—SS_nH by protein —SH group (Eq. (3)). Under the conditions of the growth of *A. ferrooxidans*, these activated polysulfide compounds can be stored as sulfur globules, which were located either inside or outside the cells with organic groups, containing long sulfur chains terminated with organic groups, sulfur rings (S_8), polysulfides or polythionates, etc [4,25]. Thus, we speculated that α-S_8 could be transformed into μ-S by SOMs as Eq. (2), which could explain why μ-S was detected after utilization of α-S_8 by *A. ferrooxidans* in the present study. However, the transformation from μ-S to α-S_8 was not found for moderate thermophilic *S. thermosulfidooxidans* and extreme thermophilic archaea *A. manzaensis* [12]. This indicated that these different environmental temperatures adopted SOMs might have different activation mechanisms for μ-S and α-S_8.

$$S_n^{2-}+\text{P—SH}\rightarrow\text{P—}SS_n\text{H} \quad (3)$$

4 Conclusions

1) The results of cell growth and sulfur oxidation behavior showed that the strain grown on α-S_8 entered slowly (about 1 d later) into the exponential phase, while grew faster in the exponential phase and attained higher maximal cell density and lower pH value than those on μ-S.

2) After bio-corrosion, both the two sulfur samples were eroded and modified by *A. ferrooxidans* cells, but α-S_8 was mostly on sulfur surface, while μ-S surface was loose and porous.

3) After growth of *A. ferrooxidans*, the surface composition of amorphous μ-S became 63.1% μ-S and 36.9% α-S_8, and that of orthorhombic α-S_8 became 68.3% α-S_8 and 31.7% μ-S, indicating that these two elemental sulfur species can be interconverted by the mesophilic *A. ferrooxidans*.

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Acidithiobacillus ferrooxidans 适应底物后对两种不同形态单质硫(α-S_8 和 μ-S)的利用和硫形态转化

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摘 要：研究典型中温嗜酸菌株 *Acidithiobacillus ferrooxidans* ATCC 23270 对两种不同形态单质硫(α-S_8 和 μ-S)的利用和硫形态转化。*A. ferrooxidans* 分别在 α-S_8 或者 μ-S 中培养以适应能源底物。*A. ferrooxidans* 在这两种单质硫中的生长和硫氧化行为的结果表明，*A. ferrooxidans* 在 α-S_8 中生长时比在 μ-S 中生长晚 1 d 进入对数期，但是 *A. ferrooxidans* 在 α-S_8 中生长时在对数期生长较快且培养液具有较高的细胞浓度和较低的 pH 值。经过 *A. ferrooxidans* 的培养，这两种单质硫都被明显腐蚀和细胞修饰。此外，经过 *A. ferrooxidans* 的生长，无定型态的 μ-S 表面组分变为 63.1% μ-S 和 36.9% α-S_8，正交晶系的 α-S_8 表面组分变为 68.3% α-S_8 和 31.7% μ-S；而无菌对照组 μ-S 和 α-S_8 表面组分都没有变化，表明这两种不同形态的单质硫在 *A. ferrooxidans* 的作用下发生了相互转化。

关键词：硫利用；硫形态转化；α-S_8；μ-S；*Acidithiobacillus ferrooxidans*

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