

## A NOVEL ACIDOPHILIC, THERMOPHILIC IRON AND SULFUR-OXIDIZING ARCHAEON ISOLATED FROM A HOT SPRING OF TENGCHONG, YUNNAN, CHINA

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### ABSTRACT

A novel thermoacidophilic iron and sulfur-oxidizing archaeon, strain YN25, was isolated from an in situ enriched acid hot spring sample collected in Yunnan, China. Cells were irregular cocci, about 0.9-1.02  $\mu\text{m} \times 1.0-1.31 \mu\text{m}$  in the medium containing elemental sulfur and 1.5-2.22  $\mu\text{m} \times 1.8-2.54 \mu\text{m}$  in ferrous sulfate medium. The ranges of growth and pH were 50-85 (optimum 65) and pH 1.0-6.0 (optimum 1.5-2.5). The acidophile was able to grow heterotrophically on several organic substrates, including various monosaccharides, alcohols and amino acids, though the growth on single substrate required yeast extract as growth factor. Growth occurred under aerobic conditions or via anaerobic respiration using elemental sulfur as terminal electron acceptor. Results of morphology, physiology, fatty acid analysis and analysis based on 16S rRNA gene sequence indicated that the strain YN25 should be grouped in the species *Acidianus manzaensis*. Bioleaching experiments indicated that this strain had excellent leaching capacity, with a copper yielding ratio up to 79.16% in 24 d. The type strain YN25 was deposited in China Center for Type Culture Collection (=CCTCCZNDX0050).

**Key words:** isolation, identification, *Acidianus manzaensis*, bioleaching

### INTRODUCTION

It is well known that bioleaching, recovering metals from low-grade sulfidic ores by microorganisms, has developed into a successful commercial biotechnology. In many cases, it offers environmental and technical advantages over other available technologies (13, 21). This process is driven by

consortia of chemolithotrophic iron and sulfur-oxidizing bacteria and archaea that are ubiquitous at sites of mineral oxidation. The most studied acidophilic metal sulfide oxidizing microorganisms belong to the mesophilic and moderately thermophilic bacteria. Thermophilic and acidophilic sulfur/iron oxidizers dominating at a temperature range of 40–60 are the typically rod-shaped, *Sulfobacillus* species (15, 22), although

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other species, such as *Leptospirillum ferriphilum*, *Acidimicrobium ferrooxidans*, *Acidithiobacillus caldus*, and *Hydrogenobacter acidophilus*, are also commonly present (15, 19, 33, 34). Thermophilic sulfur/iron oxidizers, which thrive above 60, usually belong to Archaea domain, genera *Sulfolobus*, *Acidianus*, or *Metallosphaera* (15, 22, 32).

Biological regeneration of  $\text{FeP}^{3+\text{P}}$  from  $\text{FeP}^{2+\text{P}}$  is the key to chemical attack of metal sulfides. However, biooxidation of reduced inorganic sulfur compounds (RISCs) is also important to prevent the accumulation of passivating sulfur particulate on metal surfaces that can limit metal mobilization rates. Thermoacidophiles grow at temperatures (60-85°C) where mesoacidophilic or moderate biocatalysts (or contaminants from nonsterile substrates) are not able to exist, and where passivation from RISCs is nearly eliminated, lead to more effective leaching rates (23).

Sulfur-oxidizing thermoacidophilic archaea *Acidianus brierleyi* was firstly isolated from an acidic thermal spring in Yellowstone National Park, Wyoming, USA (2, 26), whose excellent ferrous oxidation ability revealed a potential in industrial application. The archaeon demonstrated a remarkable capacity in chalcopyrite bioleaching (the most refractory primary copper sulfide), which possessed a significant advantage over mesophilic leaching process.

This study focused on screening of thermophilic microorganisms that oxidize iron and/or sulfur compounds from hot acidic water samples. Strain YN25, a novel thermophilic iron and sulfur-oxidizing archaeon, from a hot spring (> 80°C) located on Tengchong, Yunnan, China, was identified as *Acidianus manzaensis* and its iron and sulfur oxidation and chalcopyrite leaching ability has been further investigated.

## MATERIALS AND METHODS

### Microbial enrichment and Isolation

The water samples from a hot spring located in

Tengchong were in situ enriched by medium A with an initial pH 1.8 at 70°C. The medium A contains (g·LP<sup>-1</sup>):  $(\text{NHB}_{4\text{B}})\text{B}_{2\text{B}}\text{SOB}_{4\text{B}}$  (1.5),  $\text{MgSOB}_{4\text{B}}\cdot 7\text{HB}_{2\text{B}}\text{O}$  (0.25),  $\text{KHB}_{2\text{B}}\text{POB}_{4\text{B}}$  (0.25), Yeast Extract (0.1),  $\text{FeSOB}_{4\text{B}}\cdot 7\text{HB}_{2\text{B}}\text{O}$  (5-20), pH 1.8. Two thermophilic microbial cultures (one of which is Strain YN25) were obtained. by serial dilution method.

### Growth conditions

Strain YN25 was cultivated in basic salts (medium A with  $\text{FeSOB}_{4\text{B}}$  as energy source and modified 9K with  $\text{SP}^{0\text{P}}$ ,  $\text{NaB}_{2\text{B}}\text{SB}_{2\text{B}}\text{OB}_3$  or  $\text{KB}_{2\text{B}}\text{OB}_{6\text{B}}\text{SB}_4$  as energy source), trace elements (7) and 0.02% YE. Modified 9K contains (g·LP<sup>-1</sup>):  $(\text{NHB}_{4\text{B}})\text{B}_{2\text{B}}\text{SOB}_{4\text{B}}$  (3.0),  $\text{KHB}_{2\text{B}}\text{POB}_{4\text{B}}$  (0.5), KCl (0.1),  $\text{MgSOB}_{4\text{B}}\cdot 7\text{HB}_{2\text{B}}\text{O}$  (0.5) and  $\text{Ca}(\text{NOB}_{3\text{B}})\text{B}_2\text{B}$  (0.01). Trace elements (mg·LP<sup>-1</sup>) compose:  $\text{FeClB}_{3\text{B}}\cdot 6\text{HB}_{2\text{B}}\text{O}$  (11.0),  $\text{CuSOB}_{4\text{B}}\cdot 5\text{HB}_{2\text{B}}\text{O}$  (0.5),  $\text{NaB}_{2\text{B}}\text{SOB}_{4\text{B}}$  (50),  $\text{HB}_{3\text{B}}\text{BOB}_3$  (2.0),  $\text{MnSOB}_{4\text{B}}$  (2.0),  $\text{NaB}_{2\text{B}}\text{MoOB}_{4\text{B}}\cdot 2\text{HB}_{2\text{B}}\text{O}$  (0.8),  $\text{CoClB}_{2\text{B}}\cdot 6\text{HB}_{2\text{B}}\text{O}$  (0.6),  $\text{ZnSOB}_{4\text{B}}\cdot 7\text{HB}_{2\text{B}}\text{O}$  (0.9) and  $\text{NaB}_{2\text{B}}\text{SeOB}_{4\text{B}}$  (0.1). The pH of medium 9K-S<sup>0</sup> and medium A- $\text{FeSO}_4$  were adjusted with 1 M sulfuric acid to 2.5 and 2.0, respectively. Cultures were incubated in rotary shakers at the indicated temperatures.

The optimum temperature and pH were detected by temperature- and pH- controlled experiments in 250 mL shaking flasks with 100 mL medium. The purified strain was inoculated into liquid medium with a final cell density of  $1 \times 10^6$  cells mL<sup>-1</sup>. The temperature optimization was conducted under, initial pH 1.5 in medium A- $\text{FeSOB}_{4\text{B}}$  and pH 2.5 in 9K- $\text{SP}^{0\text{P}}$ , while the initial pH test was carried out at a constant temperature at 65°C, which was given in the former one. The cell densities of cultures in the flasks were monitored by a cell counting chamber directly.

To determine the optimal concentration of YE, a series of tests with the following different concentration of YE (w/v): 0.005%, 0.01%, 0.015%, 0.02%, 0.025%, 0.03%, 0.035%, 0.04%, 0.045%, 0.05%, 0.06%, 0.07%, 0.08%, 0.085%, 0.09%,

0.1%, were implemented.

### Microscopy

The morphology and motility of the strain YN25 were observed with optical microscope (Olympus CX-31). Surface micro-structural features of the cells in the logarithm growth phase were examined with scanning electron microscope (SEM, JEOL JSM-6360 LV), following fixation, dehydration and critical point drying of samples (12).

### 16S rRNA gene profiling

Chromosomal DNA was purified in accordance with the manufacturer's instructions by DNA extraction kit (Tiangen Biotech), and was used as a template in PCR. The 16S rRNA genes of YN25 were amplified by polymerase chain reaction (PCR) using the forward primer 21F: 5'-TTCCGGTTGATCCTG-3' and reverse primer Ar958R: 5'-TCCGGCGTTGAGTCC-3' (4). The PCR program was 94° C for 3 min, followed by 30 cycles of 94° C for 30 s, 52° C for 30 s, and 72° C for 60 s, and finally 72° C for 10 min. PCR products of the expected size (approximately 1.5 kb) were excised from 1.0% agarose gels and purified with the purification columns (Promega), following the manufacturer's recommendations. The PCR products were ligated to the pGEM-T vector and transformed into *Escherichia coli* DH5 $\alpha$ . The white colonies on the Luria-Bertani (LB) plates containing ampicillin (100  $\mu$ g·mLP<sup>-1</sup>), X-gal (20 mg·mLP<sup>-1</sup>) and IPTG (16 mg·mLP<sup>-1</sup>) (31) were selected and sent to Sunbiotech Co.Ltd for sequencing. The nucleotide sequence of the inserts was determined by cycle sequencing with an ABI PRISM Big Dye terminator cycle sequencing Ready Reaction kit (Applied Biosystems, Foster City, California) and run in an ABI PRISM 3700 DNA analyzer (Applied Biosystems).

The phylogenetic tree showing the relationship of YN25 to other *Acidianus* species was elaborated with related sequences obtained from public databases (<http://www.ncbi.nlm.nih.gov/>). The sequences were aligned

with YN25 sequence using Clustal X 1.80, which was used to make a distance matrix, followed by a neighbor-joining tree. Bootstrap analysis was carried out on 1000 replicates input data sets, and Phylogenetic trees were generated by Treeview software MEGA 3.1.

### Nutrition growth

In Chemoorganotrophic growth tests, the following organic compounds were tested without FeSOB<sub>4B</sub> or SP<sup>OP</sup>, which were: Yeast extract (YE), Casamino acids, Tryptone, D-glucose, Maltose, Sucrose, Lactose, Starch, Sorbitol, Galactose, Fructose, Xylose, Mannitol, Serine, Cysteine, Tyrosine, Arginine, Tryptophan, Phenylalanine, Histidine, Methionine, Leucine, Threonine, Glycine, Valine, Glutamine. The concentrations of YE, Casamino acids and Tryptone were 0.2, 0.1 and 0.2 g·LP<sup>-1P</sup>, respectively. The other test substrates concentration was 1.0 g·LP<sup>-1P</sup>. The isolate was grown in 9K-base medium with the culture maintained at pH 2.5, 65°C. Growth was estimated by detecting the total protein concentration after incubation for 72 h (8).

Medium A-FeSOB<sub>4B</sub> and 9K-base medium were used for chemomixotrophic growth. 30 g·LP<sup>-1P</sup> FeSOB<sub>4B</sub>·7HB<sub>2B</sub>O for medium A, 10 g·LP<sup>-1P</sup> SP<sup>OP</sup>, NaB<sub>2B</sub>SB<sub>2B</sub>OB<sub>3</sub> B or 2 g·LP<sup>-1P</sup> KB<sub>2B</sub>OB<sub>6B</sub>SB<sub>4B</sub> for 9K-base medium, were added as energy. The only organic source was 0.2 g·LP<sup>-1P</sup> YE.

Chemoautotrophic growth tests were performed in the same way without YE.

### Anaerobic growth

Anaerobic growth was tested in anaerobic tank with of 21 kPa COB<sub>2B</sub> and 79 kPa NB<sub>2B</sub> containing basic salts, trace elements and YE (0.2 g·LP<sup>-1</sup>) with elemental sulfur (10 g·LP<sup>-1P</sup>) or ferric iron (10 mmol·LP<sup>-1</sup>)<sub>B</sub>. The initial inoculation concentration was adjusted to 1×10<sup>6P</sup> cells·mLP<sup>-1</sup> with cells washed in basal salts medium thoroughly. To assure the anaerobic circumstance, the media were sprinkled with NB<sub>2B</sub> for 5 min and oxygen indicators were placed in the anaerobic

tank. To monitor the sulfate reduction reaction lead acetate papers was used in the anaerobic tank. Concentration of total iron was analyzed by atomic absorption spectrometry, and that of ferric iron was calculated by the concentrations of total iron and ferrous iron (34). Cell growth was determined by the protein concentration started from the stationary phase.

#### Sensitivity to antibiotics and tolerance to heavy metals

The antibiotic sensitivity to antibiotics and tolerance to several heavy metals of strain YN25 were monitored in sulfur-containing media (as described above) different concentrations of ampicillin, chloramphenicol, kanamycin, rifampin, tetracycline and gentamicin (35) and varying concentrations of  $\text{CuSO}_{4\text{B}} \cdot 5\text{HB}_{2\text{B}}\text{O}$ ,  $\text{NiSO}_{4\text{B}} \cdot 6\text{HB}_{2\text{B}}\text{O}$ ,  $\text{ZnSO}_{4\text{B}} \cdot 7\text{HB}_{2\text{B}}\text{O}$ ,  $\text{AlB}_{2\text{B}}(\text{SOB}_{4\text{B}})\text{B}_{3\text{B}} \cdot 18\text{HB}_{2\text{B}}\text{O}$ ,  $\text{CoSO}_{4\text{B}} \cdot 7\text{HB}_{2\text{B}}\text{O}$  and  $3\text{CdSO}_{4\text{B}} \cdot 8\text{HB}_{2\text{B}}\text{O}$  (33). Growth was estimated by detecting the total protein concentration in 72 h of incubation (8).

#### Whole-cell lipid fatty acid analysis

Cells of strain YN25 were harvested by centrifugation, and then transferred directly to a screw-cap vial. The fatty acid methyl esters (FAMES) were obtained by methylation, saponification and extraction, as described previously (18). The separation of FAMES was performed by a gas chromatography (model DNAI 6500-HR) equipped with a flame ionization detector (FID) and a 25-m fusedsilica column cross-linked with an SE-30 liquid phase (SGE; Ringwood, Victoria, Australia), in a carrier gas ( $\text{HB}_{2\text{B}}$ ) flow rate of  $1.2 \text{ mL} \cdot \text{min}^{-1\text{P}}$ , an injector temperature of 250, a detector temperature of 300, and an oven temperature program of 120 to 280 at  $4 \text{ min}^{-1\text{P}}$ . The FAMES were identified by comparison with the retention times of standards and their proportions were calculated on a Chromatography Data System.

#### Bioleaching of chalcopyrite

Chalcopyrite used in this experiment was provided by Institute of Mineral Processing Engineering, School of

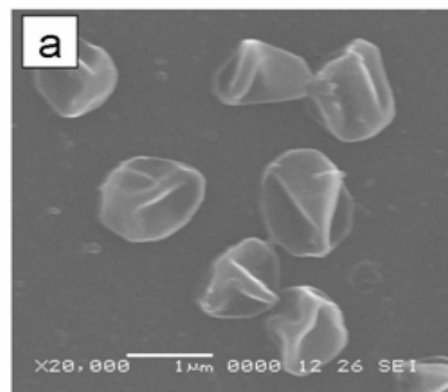
Resources Processing and Bioengineering, Central South University, China. The chalcopyrite contains 30.6% of Cu, 22.64% of Fe, 29.6% of S, 1.72% of Zn and 8.95% of Pb.

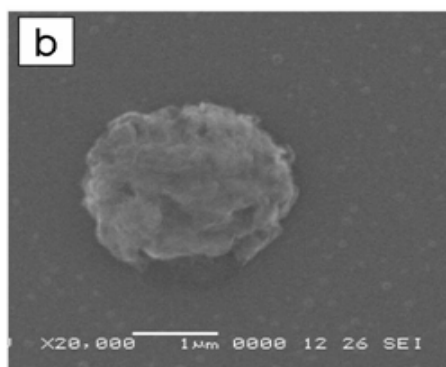
Bioleaching tests were carried out in 250 mL flasks with 100 mL 9K-base medium, in a mineral concentration of 3% (w/v). The initial concentration of YN25 was  $2\text{-}4 \times 10^6 \text{ cells} \cdot \text{mL}^{-1}$ , and all the experiments were carried out in triplicates. Abiotic controls were also implemented at same conditions by replacing the bacterial inoculum with an equal volume of medium. Aliquots of solution were sampled, and the concentration of  $\text{CuP}^{2+ \text{P}}$  was determined by atomic absorption spectrometry (Hitachi Z-8000). The evaporated water in the medium was supplemented with sterilized deionized water.

## RESULTS AND DISCUSSION

#### Morphological characterization

The cell morphology was similar to other members in the order of *Sulfolobales*, with slightly aspherical shape (Figure 1). The cell shape and size were significantly varied in different energy resource. The dimension of strain YN25 was about  $0.9\text{-}1.02 \mu\text{m} \times 1.0\text{-}1.31 \mu\text{m}$  in the medium containing elemental sulfur and  $1.5\text{-}2.22 \mu\text{m} \times 1.8\text{-}2.54 \mu\text{m}$  in the medium containing ferrous sulfate. The cell surface was crimply but smooth in medium 9K-SP<sup>0</sup> while its counterpart in medium A-FeSOB<sub>4B</sub> was round and, rough.





**Figure 1.** Scanning electron micrographs of strain YN25 cultivated chemoautotrophically on liquid medium at 65 with Sulfur (a) and Ferrous iron (b).

### Growth conditions

The strain YN25 was able to grow on several organic

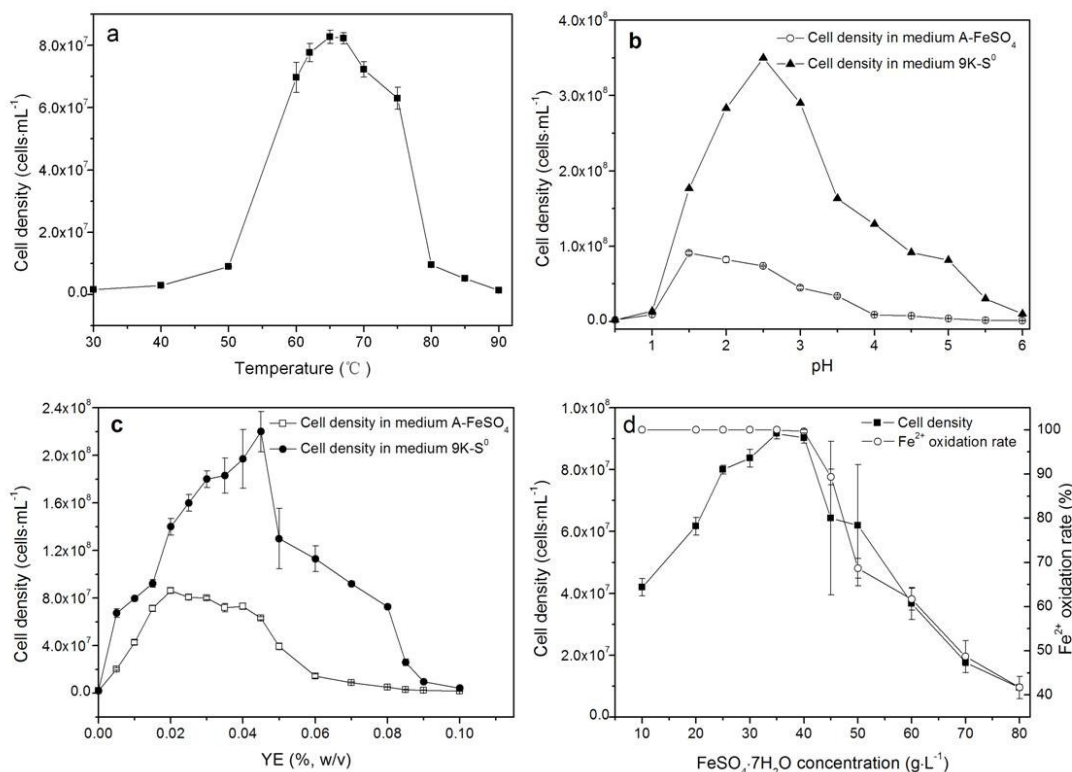
substrates, including various monosaccharides, alcohols and amino acids (Table 1), while no growth was observed when it was cultivated in inorganic substrates alone. However, in the presence of YE, peptone or other organics, the strain YN25 could grow well in inorganic substrates. These results suggested that strain YN25 is a facultative autotrophic microorganism, in absence of the evidence from carbon isotope experiment.

Bacterial growth was observed at temperatures between 50 and 85, with an optimum status at 65 (Figure 2a). No growth occurred when temperature was below 30 or above 90 (data not shown). Growth occurred at initial pH of 1.0 to 5.0, with an optimum pH of 1.5 in the medium A-FeSOB<sub>4</sub>, and, at initial pH of 1.0 to 6.0 with an optimum pH of 2.5 in 9K-SP<sup>OP</sup> medium (Figure 2b).

**Table 1.** Growth characteristics of strain YN25 under different nutritional conditions

Growth condition or substrate	Protein concentration (μg·mLP <sup>-1P</sup> )	Growth condition or substrate	Protein concentration (μg·mLP <sup>-1P</sup> )
<b>Chemoorganotrophic</b>		Glycine	1.06 ± 0.18
Yeast extract	2.95 ± 0.23Pa	Valine	0.97 ± 0.25
Casamino acids	0.26 ± 0.14	Glutamine	0.32 ± 0.21
Tryptone	3.02 ± 0.33	<b>Chemomixotrophic</b>	
Glucose	2.97 ± 0.36	SPOP plus organic substrate	
Sucrose	2.33 ± 0.39	Yeast extract	2.26 ± 0.23PbP
Sorbitol	0.00 ± 0.17	Casamino acids	0.34 ± 0.22
Galactose	2.10 ± 0.17	Tryptone	2.46 ± 0.32
Mannitol	0.15 ± 0.23	FeP2+P plus organic substrate	
Xylose	0.00 ± 0.13	Yeast extract	1.95 ± 0.29
Lactose	1.06 ± 0.25	Casamino acids	0.18 ± 0.20
Starch	1.82 ± 0.26	Tryptone	1.79 ± 0.30
Maltose	2.19 ± 0.30	NaB2BSB2BOB3B	
Fructose	0.20 ± 0.13	Yeast extract	0.35 ± 0.39PcP
Serine	0.00 ± 0.18	Casamino acids	0.10 ± 0.12
Cysteine	0.00 ± 0.14	Tryptone	0.37 ± 0.23
Tyrosine	0.00 ± 0.22	KB2BOB6BSB4B	
Arginine	0.33 ± 0.15	Yeast extract	1.33 ± 0.22
Tryptophan	0.00 ± 0.20	Casamino acids	0.13 ± 0.10
Phenylalanine	0.16 ± 0.23	Tryptone	1.41 ± 0.25
Histidine	0.13 ± 0.15	<b>Chemoautotrophic</b>	
Methionine	0.18 ± 0.14	SP0	0.14 ± 0.08
Leucine	0.86 ± 0.19	NaB2BSB2BOB3	0.03 ± 0.11
Threonine	0.13 ± 0.11	KB2BOB6BSB4	0.19 ± 0.24

<sup>aP</sup>Growth was measured as the protein concentration after 72 h. Values are means ± SD (n≥3).



**Figure 2.** Effect of temperature (Figure 2a), pH (Figure 2b), YE (Figure 2c) and  $\text{Fe}^{2+}$  (Figure 2d) on growth of strain YN25. Each data point shows the mean of triplicate tests

The growth of strain YN25 at different YE concentrations was determined. The optimum concentration of YE for YN25 in medium A- $\text{FeSO}_4$  and 9K- $\text{S}^0$  was 0.02% and 0.45%, respectively. Growth was strongly inhibited by the presence of YE in concentration above 0.10% (Figure 2c), which may act as growth factors in the process.

It can be seen from Figure 2d,  $\text{Fe}^{2+}$  was completely oxidized after 35 h when  $\text{FeSOB}_{4\text{B}} \cdot 7\text{HB}_{2\text{B}}\text{O}$  concentration varied between 10-40  $\text{g} \cdot \text{LP}^{-1}$ , and the largest cell density was gained with a ferrous iron concentration of 35  $\text{g} \cdot \text{LP}^{-1}$ . Cell density and  $\text{Fe}^{2+}$  oxidizing activity significantly decreased when  $\text{FeSOB}_{4\text{B}} \cdot 7\text{HB}_{2\text{B}}\text{O}$  concentration was above 45  $\text{g} \cdot \text{LP}^{-1}$ , while cells reproduction was reduced in a  $\text{FeSOB}_{4\text{B}} \cdot 7\text{HB}_{2\text{B}}\text{O}$  concentration below 25  $\text{g} \cdot \text{LP}^{-1}$ . Generally, compared with other iron-oxidizing bacteria, strain YN25 had higher ferrous iron oxidizing activity (2, 8, 11, 33, 34).

The sulfur oxidizing activity of strain YN25 was determined in the optimum conditions (initial pH 2.5, 10  $\text{g} \cdot \text{L}^{-1}$   $\text{S}^0$ , 0.2  $\text{g} \cdot \text{L}^{-1}$  YE), where the concentration of  $\text{SOB}_{4\text{PB}}^{2-}$  reached 25  $\text{g} \cdot \text{L}^{-1}$  after 70 hours' cultivation.

### Anaerobic Metabolisms

The biggest differences in characteristics of genus *Acidianus* were the two chemolithotrophic cultivations. Under aerobic conditions, *Acidianus* grew aerobically with  $\text{OB}_{2\text{B}}$  as the final electron acceptor. At extremely anaerobic conditions, *Acidianus* grew anaerobically, forming  $\text{HB}_{2\text{B}}\text{S}$  or  $\text{FeP}^{2+\text{P}}$ , in which  $\text{HB}_{2\text{B}}$  or organic components served as electron donor, and  $\text{SP}^0$  or  $\text{FeP}^{3+\text{P}}$  served as the final electron acceptor (16, 20, 26). Electron donor and acceptor were important basis for this archaea system classification.

YN25 has high elemental sulfur reducing capacity at

extremely anaerobic conditions, generating a significant amount of HB<sub>2B</sub>S. In the anaerobic experiment, the lead acetate filter paper suspended in the experimental bottle began to change color after 40 h cultivation, and became completely black after 60 h. Meanwhile, we found strain YN25 can not reduce the ferric iron. At anaerobic conditions, YN25 shared the same electron donor and acceptor, HB<sub>2B</sub> and SP<sup>0P</sup>,

respectively, with *A. infernus*, *A. ambivalens*, *A. brierleyi* and *A. tenchongensis*. *A. manzaensis*, and had the same electron acceptor of ferric iron as *A. sulfidivorans*, but different electron donor, which were HB<sub>2B</sub> and HB<sub>2B</sub>S, respectively (Table 2). Based on the characterization of anaerobic metabolism, strain YN25 had the same electron donor with *A. manzaensis* ATCC BAA 1057 but clear differences in electron acceptor (32).

**Table 2.** Main physiological, biophysical and phylogenetic traits and of isolate YN25 and members of the genus *Acidianus*

	YN25	<i>A. manzaensis</i>	<i>A. infernus</i>	<i>A. ambivalens</i>	<i>A. brierleyi</i>	<i>A. tenchongensis</i>	<i>A. sulfidivorans</i>
Growth temperature (optimum)	50-85 (65)	60-90 (80)	65-96 (90)	70-87 (80)	45-75 (70)	60-75 (70)	45-83 (74)
Growth pH (optimal pH)	1.0-6.0 (1.5, 2.5)	1.0-5.0 (1.2-1.5)	1.0-5.5 (2.0)	1.0-3.5 (2.5)	1.0-6.0 (1.5-2.0)	1.0-5.5 (1.5-2.0)	0.35-3.0 (0.8-1.4)
Electron donor	HB <sub>2B</sub> , SP <sup>0P</sup> , FP <sup>2+P</sup> and organic compounds (Yeast extract, peptone, tryptone, cassamino acid)	HB <sub>2B</sub> , SP <sup>0P</sup> , FeP <sup>2+P</sup> and organic compounds (Yeast extract, peptone, tryptone, cassamino acid)	HB <sub>2B</sub> , SP <sup>0P</sup>	HB <sub>2B</sub> , SP <sup>0P</sup>	HB <sub>2B</sub> , SP <sup>0P</sup> , FP <sup>2+P</sup> and organic compounds (Yeast extract, peptone, tryptone, cassamino acid)	HB <sub>2B</sub> , SP <sup>0P</sup>	SP <sup>0P</sup> , FP <sup>2+P</sup> and HB <sub>2B</sub> S
Electron acceptor	SP <sup>0P</sup> and OB <sub>2B</sub>	FeP <sup>3+P</sup> and OB <sub>2B</sub>	SP <sup>0P</sup> and OB <sub>2B</sub>	SP <sup>0P</sup> and OB <sub>2B</sub>	SP <sup>0P</sup> and OB <sub>2B</sub>	SP <sup>0P</sup> and OB <sub>2B</sub>	FeP <sup>3+P</sup> and OB <sub>2B</sub>
Autotrophy	Facultative autotroph	Facultative autotroph	Obligate autotroph	Obligate autotroph	Facultative autotroph	Obligate autotroph	Obligate autotroph
16S rDNA similarity	1.00	0.99	0.93	0.93	0.90	0.90	0.97

### Antibiotic sensitivity and heavy metal tolerance

Strain YN25 was sensitive to all antibiotics except gentamicin in a limited concentration (Table 3). It presented little sensitivity to gentamicin (100 mg·L<sup>-1</sup>) and high sensitivity to rifampin (5 mg·L<sup>-1</sup>), tetracycline (20 mg·L<sup>-1</sup>), Kanamycin (50 mg·L<sup>-1</sup>), (Chloramphenicol 50 mg·L<sup>-1</sup>) and Ampicillin (50 mg·L<sup>-1</sup>).

Generally, the strain YN25 growth was inhibited with the increase of heavy metal concentrations. CoP<sup>2+P</sup>, NiP<sup>2+P</sup> and CuP<sup>2+P</sup> were deleterious for the strain growth. However, YN25

showed tolerance in different levels to a range of heavy metals and showed strongest resistance to AIB<sub>2B</sub>(SOB<sub>4B</sub>)B<sub>3B</sub>·18HB<sub>2B</sub>O (Table 4). Growth was significantly enhanced with 20-40 mM AIB<sub>2B</sub>(SOB<sub>4B</sub>)B<sub>3B</sub>·18HB<sub>2B</sub>O and the biomass increased by 61% in the presence of 40 mM AIB<sub>2B</sub>(SOB<sub>4B</sub>)B<sub>3B</sub>·18HB<sub>2B</sub>O. In addition, low concentration of ZnSOB<sub>4B</sub>·7HB<sub>2B</sub>O (<4 mM) and 3CdSOB<sub>4B</sub>·8HB<sub>2B</sub>O (<0.4 mM) could slightly promote the growth of YN25. The heavy metals tolerance granted strain YN25 a special advantage in bioleaching.

**Table 3.** Sensitivity of strain YN25 to antibiotics

Antibiotic	Concentration ( $\mu\text{g}\cdot\text{mLP}^{-1\text{P}}$ )	Growth of strain YN25 ( $\mu\text{g}\cdot\text{mLP}^{-1\text{P}}$ ) <sup>aP</sup>	Antibiotic	Concentration ( $\mu\text{g}\cdot\text{mLP}^{-1\text{P}}$ )	Growth of strain YN25 ( $\mu\text{g}\cdot\text{mLP}^{-1\text{P}}$ ) <sup>aP</sup>
No antibiotic	0	3.36 ± 0.23	No antibiotic	0	3.36 ± 0.23
Ampicillin	0.2	3.42 ± 0.38	Rifampin	0.2	3.38 ± 0.29
	1.0	3.50 ± 0.42		1.0	3.00 ± 0.46
	5.0	3.39 ± 0.44		5.0	1.08 ± 0.32
	20	2.72 ± 0.35		20	0.96 ± 0.49
	50	2.02 ± 0.46		50	0.86 ± 0.37
	100	1.53 ± 0.55		100	0.65 ± 0.59
Chloramphenicol	0.2	3.39 ± 0.32	Tetracycline	0.2	3.32 ± 0.38
	1.0	3.35 ± 0.36		1.0	3.01 ± 0.33
	5.0	3.12 ± 0.28		5.0	2.51 ± 0.48
	20	2.08 ± 0.53		20	1.19 ± 0.35
	50	1.51 ± 0.39		50	0.65 ± 0.24
	100	1.41 ± 0.47		100	0.53 ± 0.42
Kanamycin	0.2	3.44 ± 0.31	Gentamicin	0.2	3.78 ± 0.55
	1.0	3.41 ± 0.29		1.0	3.76 ± 0.39
	5.0	3.25 ± 0.51		5.0	3.68 ± 0.53
	20	3.20 ± 0.33		20	3.52 ± 0.49
	50	2.96 ± 0.46		50	3.36 ± 0.63
	100	2.82 ± 0.55		100	3.27 ± 0.63

<sup>aP</sup>Growth was measured as a function of total protein concentration after 72 h. Values are means ± SD (n≥3)

**Table 4.** Tolerance of the strain YN25 to some heavy metals

Heavy metal	Concentration (mM)	Growth of strain YN25 ( $\mu\text{g}\cdot\text{mLP}^{-1\text{P}}$ ) <sup>aP</sup>	Heavy metal	Concentration (mM)	Growth of strain YN25 ( $\mu\text{g}\cdot\text{mLP}^{-1\text{P}}$ ) <sup>aP</sup>
No heavy metal	0	3.41 ± 0.26	No heavy metal	0	3.41 ± 0.26
CuSOB <sub>4B</sub> ·5HB <sub>2B</sub> O	5	3.03 ± 0.32	AlB <sub>2B</sub> (SOB <sub>4B</sub> )B <sub>3B</sub> ·18HB <sub>2B</sub> O	20	4.54 ± 0.43
	10	2.70 ± 0.43		40	5.51 ± 0.66
	20	1.84 ± 0.39		80	3.08 ± 0.73
	40	0.95 ± 0.45		160	2.59 ± 0.38
	80	0.42 ± 0.26		320	2.16 ± 0.47
	20	3.11 ± 0.54		1	1.80 ± 0.58
NiSOB <sub>4B</sub> ·6HB <sub>2B</sub> O	40	2.73 ± 0.66	CoSOB <sub>4B</sub> ·7HB <sub>2B</sub> O	2	1.20 ± 0.32
	80	1.87 ± 0.30		4	1.00 ± 0.51
	160	1.08 ± 0.33		8	0.91 ± 0.28
	320	0.51 ± 0.37		16	0.49 ± 0.33
	2	3.66 ± 0.51		0.2	4.50 ± 0.65
	4	3.42 ± 0.69		0.4	3.57 ± 0.49
ZnSOB <sub>4B</sub> ·7HB <sub>2B</sub> O	6	2.95 ± 0.51	3CdSOB <sub>4B</sub> ·8HB <sub>2B</sub> O	0.8	1.49 ± 0.63
	8	1.53 ± 0.46		1.6	0.35 ± 0.29
	32	0.81 ± 0.19		3.2	0.25 ± 0.19

<sup>aP</sup>Growth was measured as the protein concentration after 72 h. Values are means ± SD (n≥3).

### Fatty acid analysis

Thermophilic archaea possess unique fatty acid composition which enable it to grow in high temperature (17), which is also an important method for phylogenetic study such

as archaea identification and classification of (3).

Whole-cell fatty acid compositions were shown in Table 5. There were five groups of fatty acids found in strain YN25 by gas chromatograph. The predominant one was C16:0,



accounting for 41.36% of the total fatty acids. The other four groups included C14:0, C18:0, C18:1 $\omega$ 7c and C18:1 $\omega$ 9c, which account for 31.94%, 8.1%, 8.32% and 10.27% respectively. Strain YN25 had similar composition of membrane fatty acid with strain *A. ambivalens* JCM 9191 and *A. brierleyi* JCM 8954. However, compared with YN25, *A. ambivalens* JCM 9191 does not contain C18:1 $\omega$ 7c monounsaturated fatty acid while *A. brierleyi* JCM 8954 possesses a unique fatty acid-- C16:1  $\omega$ 7c/15 iso 2OH.

According to the characteristics of *Alicyclobacillus*,  $\omega$ -alicyclic acids are the predominant membrane fatty acids in this genus (14, 29). Membrane lipid composition of *Metallosphaera sedula* YN23 (accession number of GenBank: EF142855) was more complex than YN25. As shown in Table 5, strain YN25 was substantially different from *Alicyclobacillus sendaiensis*, *Sulfolobus metallicus* and *Metallosphaera sedula*. According to the analysis of fatty acid, strain YN25 was closed related to the genus *Acidianus*.

**Table 5.** Membrane fatty acid composition of strain YN25, related *Acidianus* species and three archaea (Data in each column are percentages of membrane fatty acids in a species, %)

Fatty acid	1	2	3	4	5	6	7
c14 : 0	31.94				19.33	27.37	18.42
c15 : 0			0.2				
c15 : 0 iso		0.88	1.1	1.4			
c15 : 0 anteiso			1.5				
c16 : 0	41.36	3.45	4.9		16.26	50.15	43.56
c16 : 0 iso		6.69	1.6	1.4			
c17 : 0			0.6				
c17 : 0 iso		5.55	4.5	1.9			
c17 : 0 anteiso		4.80	4.5	2.3	10.27		
c18 : 0	8.1	0.69	0.9			14.21	12.63
c18 : 0 iso		1.87					
c18:1 $\omega$ 7c	8.32	76.07					6.96
c18:1 $\omega$ 9c	10.27					8.26	7.96
$\omega$ -Cyclohexane C17: 0			44.1	78.0			
$\omega$ -Cyclohexane C19: 0			30.2	16.0			
unknown 13.565					12.21		
unknown 13.957					4.08		
15:0 3OH					28.39		
16:1 $\omega$ 7c/15 iso 2OH					5.47		10.46

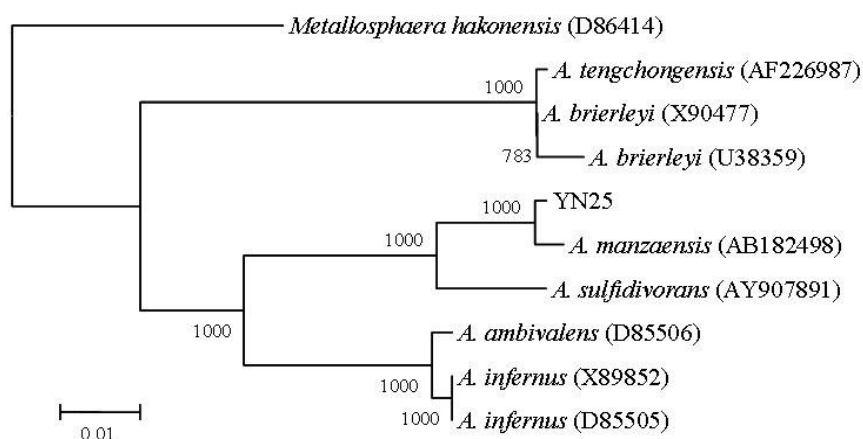
1. Strain YN25; 2. *Alicyclobacillus sendaiensis* YNTC-1 (6); 3. *Alicyclobacillus sendaiensis* (29); 4. *Ab. acidocaldarius* ATCC 27009P<sup>TP</sup> (29); 5. *Metallosphaera sedula* YN23; 6. *Acidianus ambivalens* JCM 9191; 7. *A. brierleyi* JCM 8954

### Molecular phylogenetic analysis

The phylogenetic position of the new isolate was evaluated by 16S rRNA gene sequence information, in which a total of 1416 nucleotides were sequenced (accession number of GenBank EF522787). The nearest phylogenetic relative to the isolate was *Acidianus manzaensis* ATCC BAA 1057 with exactly 99% of similarity. A neighbour-joining phylogenetic tree was constructed based on the distance matrix data of the

isolate and several reference archaea. As described in Figure 3, the species of *Acidianus* chosen were divided into six groups based on the 16S rRNA gene sequences, and strain YN25 clustered with *Acidianus manzaensis* ATCC BAA 1057.

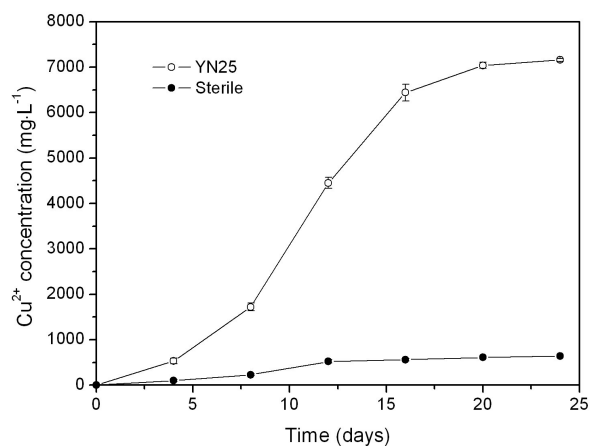
Based on morphological, biochemical, physiological characteristics and the molecular biology analysis, strain YN25 could be classified into *Acidianus manzaensis*.



**Figure 3.** Distance-matrix tree showing phylogenetic affiliations of the new isolate YN25 and referenced archaea based on 16S rRNA sequences. Bootstrap values obtained with 1000 bootstrap resamplings are given at branching points of interest. The DDBJ/EMBL/GenBank accession numbers of the 16S rRNA sequences used is shown in parentheses. Bar, 1 nucleotide substitutions per 100 nucleotides

### Bioleaching experiments

The bioleaching results by *Acidianus manzaensis* YN25 were as shown in Figure 4. In the leaching process, where more than 70% copper was extracted during the first 16 days, the copper extraction ratio continuously increases with a final concentration of  $7.16 \text{ g}\cdot\text{L}^{-1}$  after 24 days. Comparatively, almost no soluble copper was detected in sterile controls. This was the first report in bioleaching by the an *Acidianus* species.



**Figure 4.** Chalcopyrite leached by *Acidianus manzaensis* YN25

As a thermophilic microbe, *Acidianus manzaensis* YN25 showed strong capacity of chalcopyrite bioleaching compared with moderate thermophiles (*Acidithiobacillus caldus*, *Sulfobacillus thermosulfidooxidans*, *Sulfobacillus acidophilus*, *Leptospirillum ferriphilum* and *Ferroplasma thermophilum*) (11, 30, 33, 34, 35) or mesophiles (*Leptospirillum ferrooxidans*, *Acidithiobacillus ferrooxidans*, *Acidithiobacillus thiooxidans*, *Acidithiobacillus albertensis*) (5, 9, 27, 31). The sulfur particles formed during the bioleaching were readily to accumulate on the surface of solid phase and form passivation layer, which can significantly inhibit the contact of solid and liquid phases thus hinder the leaching process (10, 24, 25, 28). *Acidianus manzaensis* YN25 showed strong capacity of oxidizing  $\text{FeP}^{2+\text{P}}$  and  $\text{SP}^{0\text{P}}$  in our previous study. It can be inferred that, *Acidianus manzaensis* YN25 thriving at temperatures of  $65^\circ\text{C}$ , which is lethal to moderate thermophiles and mesophiles, could effectively limit passivation, consequently, leading to a better leaching kinetics.

Pure culture of *Acidianus manzaensis* was first discovered in 2007 by Yoshida et al, named *Acidianus manzaensis* strain NA-1<sup>T</sup> (strain NA-1<sup>T</sup> = NBRC 100595 = ATCC BAA 1057).

The strain isolated from a hot fumarole in Manza, Japan is a facultative autotrophic archaeon in both anaerobic and aerobic conditions (32). *Acidianus manzaensis* YN25 was the second strain of this species. Strain YN25 have new features in cell morphology, growth conditions and anaerobic metabolisms compared with *Acidianus manzaensis* ATCC BAA 1057, which could be relevant to its excellent leaching ability and prosperous prospective in industrial use.

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