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ORIGINAL PAPER

Characterization of a thermophilic sulfur oxidizing enrichment culture dominated by a *Sulfolobus* sp. obtained from an underground hot spring for use in extreme bioleaching conditions

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Abstract A thermoacidophilic elemental sulfur and chalcopyrite oxidizing enrichment culture VS2 was obtained from hot spring run-off sediments of an underground mine. It contained only archaeal species, namely a *Sulfolobus metallicus*-related organism (96% similarity in partial 16S rRNA gene) and *Thermoplasma acidophilum* (98% similarity in partial 16S rRNA gene). The VS2 culture grew in a temperature range of 35–76°C. Sulfur oxidation by VS2 was optimal at 70°C, with the highest oxidation rate being 99 mg S^0 l^{-1} day^{-1}. At 50°C, the highest sulfur oxidation rate was 89 mg l^{-1} day^{-1} (in the presence of 5 g Cl^- l^{-1}). Sulfur oxidation was not significantly affected by 0.02–0.1 g l^{-1} yeast extract or saline water (total salinity of 0.6 M) that simulated mine water at field application sites with availability of only saline water. Chloride ions at a concentration above 10 g l^{-1} inhibited sulfur oxidation. Both granular and powdered forms of sulfur were bioavailable, but the oxidation rate of granular sulfur was less than 50% of the powdered form. Chalcopyrite concentrate oxidation (1% w/v) by the VS2 resulted in a 90% Cu yield in 30 days.

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Introduction

Microbes are applied in increasing numbers for metal recovery from various resources, such as mineral deposits, industrial residues, waste materials and incineration slag and ash [for a review, see 24]. Iron- and/or sulfur-oxidizing acidophilic microorganisms oxidize many sulfide minerals [e.g., 4, 12, 19, 35, 36]. Iron oxidizers produce ferric sulfate solvent and sulfur oxidizers use the leached sulfur compounds to produce protons that attack the acid-soluble minerals [38, 41, 42, for a review, see 37]. Proton production also maintains acidic bioleaching conditions that are essential for metal solubility [38, 41, 42, for a review, see 37].

Bioleaching depends upon various physicochemical, microbiological, and mineralogical factors such as available water sources, climate, availability of nutrients, metal tolerance of the organisms, the mineral type and composition, as well as the particle size [for reviews, see e.g., 4, 5, 18, 19]. Leaching reactions are generally highly exothermic and the temperature tends to rise with the metal dissolution (up to 80°C has been measured in heaps) [30]. Therefore, moderately and extremely thermophilic organisms may play an important role in high-temperature regions of the heaps.

The bioleaching micro-organisms need to be adapted to acidic conditions (pH below 3), the presence of certain heavy metals [30], local water, and possibly a wide range of inorganic ions. Consequently, these

micro-organisms are being sought from mining sites, acid mine drainage, and geothermally active areas, where these kinds of conditions exist [e.g., 3, 18, 21, 33]. Thermophilic and acidophilic sulfur/iron oxidizers dominating at a temperature range of 40–60°C are the typically rod-shaped, *Sulfobacillus* species [for reviews, see e.g., 12, 35], although other species, such as *Leptospirillum thermoferrooxidans, Acidimicrobium ferrooxidans, Acidithiobacillus caldus*, and *Hydrogenobacter acidophilus*, are also commonly present [12, 16]. Extremely thermophilic sulfur/iron oxidizers, which thrive above 60°C, are often *Archaea* from the genera *Sulfolobus*, *Acidianus*, or *Metallosphaera* [12, 35]. Other thermophilic and extremely acidophilic Archaea include *Thermoplasma* and *Picrophilus* species [e.g., 40]. These are mainly heterotrophs. Their role in the biomining community is to scavenge the organic material rather than leach the minerals [18, 19].

The aim of this study was to enrich and characterize moderately and extremely thermophilic microbial cultures that oxidize iron and/or sulfur compounds from an underground hot spring sediment (> 80°C) in a mine located on the island of Hokkaido, Japan. The iron and sulfur oxidation and the chalcopyrite leaching by the enriched culture were investigated. In addition, the sulfur bioavailability and effects of saline water, temperature, and yeast extract on sulfur oxidation were studied.

Materials and methods

Microbial enrichment

Four slurry samples were taken from underground hot spring run-off stream sediments located at an underground mine, in Japan. The pH of the two samples from a sediment stream was 1.8 and the temperature was about 60°C, while the other two were taken from a tunnel wall encrusted with ferric hydroxide and awash with hot spring water with a pH of 4.5 and a temperature of 80°C. Separate and combined samples were transferred to a nutrient salt medium (Table 1). Incubations were conducted in 20 ml volumes using nutrient salt medium with different substrates in a temperature gradient incubator (Test Tube Incubator, Terratec®, Australia) over a temperature range of 43–90°C. The pH of the nutrient salt medium was adjusted to 2.0 using concentrated sulfuric acid. Enrichments were first incubated using ferrous sulfate (4 g l^{-1}) as an energy source. In addition, incubations were conducted in 100 ml volumes in shake flasks at 55 and 85°C.

Growth was monitored by phase contrast microscopy (Leitz Diaplan, Germany) and in the case of the ferrous iron medium, the remaining ferrous iron was determined by a colorimetric 2,2-dipyridyl method [49]. Cultures where growth was detected were subcultured using 10% vol/vol transfers. Scanning electron microscopy was performed as reported by Kinnunen and Puhakka [21].

16S rRNA gene profiling

Total community DNA was extracted using an Ultra Clean Soil DNA Isolation Kit (MoBio Laboratories Inc.) and was used as a template in PCR. Bacteria were characterized by determining a partial sequence of the 16S rRNA gene using PCR-mediated denaturing gradient gel electrophoresis (DGGE) with primers GC-BacV3f [26] and 907R [27]. A PCR volume of 50 µl was used and both positive and negative controls were included. The contents of the reaction mixtures were as presented in Table 2. No DNA template was added to the negative control. The thermal amplification program was as follows: 95°C 5 min; 30 cycles: 94°C 0.5 min, 50°C 1 min, 72°C 2 min; final extension 72°C 10 min, held at 4°C.

Archaea were characterized using nested-PCR approach. First, a near-complete 16S rRNA gene fragment was amplified using primers called Ar4f [13] and Un1492r [6]. The inner PCR was conducted using the product from the former, outer PCR as a template, and primers called GC-ArchV3f [31] and Arcf915r [48]. The thermal amplification programs were as follows: outer PCR 95°C 4 min; 32 cycles: 92°C 1 min, 50°C 1 min, 72°C 2 min, and final extension 72°C 10 min after which was held at 4°C; inner PCR 94°C 1 min; 20

Table 1 Nutrient salt media and substrates used for enrichment and oxidation experiments and the composition of the artificial saline water of which dilutions were used to study the effect of salinity on sulfur oxidation of the VS2 culture

Nutrient salt medium			Saline water		
	Enrichment (g/l)	Oxidation (g/l)		mM	mg/l
$(NH_4)_2SO_4$	1.50	1.30	Na^+	265	6,100
KH_2PO_4	0.25	0.28	K^+	9.9	385
$MgSO_4{\cdot}7H_2O$	0.25	0.25	Ca^{2+}	5.9	235
$CaCl_2$	–	0.05	Mg^{2+}	29.0	695
Yeast extract	0.02	0.10	HCO_3^-	2.6	160
pH	2.0	1.8	Cl	268	9,500
Substrates			SO_4	21.9	2,100
$FeSO_4{\cdot}7H_2O$	4	4	NO_3	1.5	90
S^0	5	1–5			
$CuFeS_2$	10	>10			

Table 2 The contents of the PCR for VS2 culture profiling

Agent	Unit	Bacterial PCR	Archaeal outer PCR	Archaeal inner PCR
10× buffer IV	µl	5.0	5.0	5.0
$MgCl_2$	mM	1.75	1.75	1.75
dNTP	mM	0.1	0.1	0.1
RedHot polymerase	U	2.5	2.5	2.5
Primers	µM	0.5	1.0	1.0
BSA	µl	1.0	1.0	1.0
H_2O	µl	39.6	34.9	38.9
Template	µl	1.0	1.0	0.5
Total volume	µl	51.0	51	50.5

cycles: 92°C 1 min, 63–53°C 1 min (decrease of 0.5°C every cycle), 72°C 2 min, and 13 cycles: 92°C 1 min, 53°C 1 min, 72°C 2 min and 72°C 10 min, held at 4°C.

A gradient of 30–65% denaturant (urea-formamide) in an 8% wt/vol polyacrylamide gel was used for DGGE. Twenty-five microliter of PCR product was loaded onto the gel and separated electrophoretically using the DCode(system (BioRad Laboratories, USA). Electrophoresis was performed for 16 h at 100 V and 60°C. The gels were then stained with ethidium bromide (0.5 mg l^{-1}) and visualized under UV light at 302 nm.

Selected bands were excised from the gel using a sterilized surgical knife. The excised DNA fragments were melted in 20 µl of DNAase/RNAase-free water and re-amplified with PCR using the DGGE primers, but without the GC-clamp. The sequencing of the purified PCR products was performed at the DNA Sequencing Facility, Institute of Biotechnology, University of Helsinki, Finland. Basic Local Alignment Search Tool, BLAST [1], was used for the analysis of sequence data. Additional sequence data was obtained using the PCR of near-complete 16S rRNA gene of isolated strain and the Big Dye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), with electrophoresis of purified sequencing products performed at the Western Australian Genome Resource Centre (Royal Perth Hospital). Phylogenetic relationships were determined using ARB software [25] and distance matrix and neighbor joining methods.

Temperature range for growth

The temperature range for growth of the VS2 culture was determined using a temperature gradient incubator and application of the Ratkowsky equation [8, 34]. Nutrient salt medium at pH 1.8 supplemented with 5 g l^{-1} elemental sulfur was used as a growth medium in culture tubes containing 20 ml total volume. Sulfur oxidation was used as an indirect measure of cell growth rate. Twenty-four culture tubes containing a 10% (vol:vol) inoculum were incubated in the temperature gradient incubator initially over a temperature range from 30 to 75°C. A second experiment targeting the thermophilic microbes of the VS2 culture was conducted using the same culture medium over a temperature range from 40 to 95°C. These tubes were inoculated with the highest temperature tube culture of the first temperature gradient experiment, a culture dominated by *Sulfolobus*-like cell morphotypes. To measure sulfur oxidation, 0.5 ml was collected periodically from each culture tube and centrifuged at 13,000*g* for 10 min to pellet the sulfur particles. A 0.4 ml sample of supernatant was then added to 10 ml distilled water. This solution was analyzed for sulfate using ICP-OES by Ultra Trace Pty Ltd, Perth. Zeroth order plots of sulfate concentration versus time were constructed. From the plotted data, the square root of the inverse of the time taken for the sulfate concentration to increase to 3 g l^{-1} was plotted against temperature. The Ratkowsky equation [34] was fitted to the data to determine the theoretical minimum (T_{min}), optimum (T_{opt}) and maximum (T_{max}) growth temperatures:

$$y = b(x - T_{min})(1 - \exp(c(x - T_{max}))),$$

where b and c are model fitting parameters, y is the square root of the inverse of the time taken for the sulfate concentration to increase to 3 g l^{-1}, and x is temperature.

The effect of pH on growth

The effect of pH on growth was studied in the sulfur medium (Table 1, 5 g S^0 l^{-1}) using 50 ml tubes with a liquid volume of 20 ml (shaken 160 rpm). A 10% vol/vol inoculum was used. The pH of the growth media was adjusted with sulfuric acid and a range of pH 0.9–2.5 was studied. The increase of cell numbers was monitored with a phase contrast microscope (Zeiss Axioskop 2, Carl Zeiss, Germany).

Oxidation of elemental sulfur and chalcopyrite

Oxidation of sulfur and mineral sulfides was studied in 500 and 250 ml shake flasks (160 rpm) with a liquid volume of 200 and 100 ml, respectively. The nutrient salts media and ore concentrates were autoclaved, while sulfur was heat-sterilized at 110°C over night. The nutrient salts media (Table 1) were supplemented

with powdered elemental sulfur (2 or 5 g l^{-1}; WVR Prolabo) or chalcopyrite concentrate (1, 2, or 10% wt/vol), Granules of polished sulfur (diameter 1–3 mm) were used in bioavailability studies. Parallel experiments were conducted and a 10% (vol/vol) inoculum, pre-grown on the substrate to be studied, was used. In the abiotic control bottles, an equal amount of nutrient salts medium was used. The pH was adjusted to 1.8 with sulfuric acid. Sub-culturing was done at least three times on the new media prior to experiments. Samples, as well as inocula, were derived from well-shaken flasks. The chalcopyrite concentrate (74% chalcopyrite, 10% pyrite) consisted of 25.4% Cu, 27.9% Fe, 2.2% Zn, 0.75% Pb, 32.6% S, 9.6 ppm Au, and 260 ppm Ag and the particle size distribution was as follows: 97% < 149 μm, 89% < 105 μm, 75% < 74 μm, 60% < 53 μm, 46% < 37 μm and 33% < 20 μm particles. Sulfur oxidation was followed by measuring sulfate with ion chromatograph (Dionex DX–120 Ion Chromatograph, Dionex Corporation, Sunnyvale, CA, USA) and by monitoring the pH. Dissolved copper and iron concentrations were analyzed by atomic absorption spectrophotometer (Perkin Elmer 1100B, USA). The pH was measured using a WTW pH96 meter and the redox potential with a Hamilton Profitrode Pt-ORP electrode (Ag/AgCl as a reference system). Ferrous iron concentrations were determined by the colorimetric *ortho*-phenanthroline method, using UV 1601 spectrophotometer (Shimadzu, Europe) according to 3500-Fe-method [2], which was modified as follows: 2 ml of 1,10-phenanthroline (10 g l^{-1}) and 1 ml of ammonium acetate buffer were added to 3 ml of sample.

The leaching experiments were additionally conducted in stirred tank reactors (2 l) with a working volume of 1.5 l [23]. The solids concentration in the first inoculated stirred tank reactor was gradually increased to 20% in 2 months. The number of the cells was monitored with a phase contrast microscope (Zeiss Axioskop 2). The reactors with chalcopyrite concentration of 10 and 20% were directly inoculated (10% vol/vol) from the first reactor and pH, redox, Fe^{2+}, dissolved Fe and Cu concentrations were analyzed as in shake flask experiments.

Chloride inhibition and the influence of yeast extract

The effects of chloride and yeast extract on the sulfur oxidation by VS2 at 50°C was investigated using a concentration range of 0–15 g l^{-1} of chloride as sodium chloride and 0–0.1 g l^{-1} yeast extract. Experiments were conducted in 500 ml shake flasks (160 rpm) with a liquid volume of 200 ml in the presence of one g l^{-1} of elemental sulfur. Sulfur oxidation was followed by measuring sulfate in the medium by ion chromatography (Dionex DX–120 Ion Chromatograph) and by measuring the pH. Microbial growth was monitored with a phase contrast microscope (Zeiss Axioskop 2). Parallel experiments were conducted and 10% inoculum was used.

In order to avoid the traces of yeast extract that had been used during the sub-culturing (0.1 g l^{-1}), the inocula for yeast extract studies were prepared as follows: the cells were first recovered by centrifuging at 1,315*g* or 1,424*g* for 10 min and the supernatant was discharged. Cells were then resuspended to 150 ml of nutrient salt medium that excluded yeast extract. Centrifugation was repeated and after the second resuspension, the solution was used as the inoculum.

Effects of saline water

The local water available at many mine sites is saline. Therefore, the influence of the salty water on sulfur oxidation with the VS2 culture was studied in 250 ml shake flasks with a total volume of 100 ml, with 1 g l^{-1} of elemental sulfur and 0.02 g l^{-1} yeast extract at 50°C. Water from the other mine site was analyzed and used as a template for making the artificial salt water (Table 1). Dilutions from 5 to 95% were studied and 5% inoculum was used. Cell growth was measured by monitoring the produced sulfate and pH of the media as above and by a phase contrast microscope (Zeiss Axioskop 2).

Results

In this study, thermophilic organisms were enriched from underground mine samples on sulfur, ferrous iron and chalcopyrite in an acidic nutrient salt medium using a temperature gradient incubator and shake flasks. A thermophilic enrichment culture, VS2, was obtained in a ferrous iron medium at 59°C. Rod-shaped microbes were detected in the tubes below 50°C in the ferrous iron medium in the temperature gradient incubator, but those were not chosen for further testing. No growth was detected on any of the other tested substrates.

The VS2 contained two different cell morphotypes; the majority of the cells were large, lobed cells resembling typical *Sulfolobus*-like morphology (Fig. 1) accompanied by small cocci. Lobed cells formed clusters of two, three, or more cells, and they became dominant during subsequent culturing.

The VS2 culture grew on elemental sulfur, ferrous iron, chalcopyrite, and nickel concentrate [39]. The VS2 culture originally enriched at 59°C was later incubated at 50°C due to the available incubation equipment. At 50°C, the iron oxidation rate of VS2 when grown on ferrous iron was about 0.10 g Fe^{2+} l^{-1} day^{-1}. Elemental sulfur was oxidized at a rate of 0.071 g l^{-1} day^{-1} equaling to 0.214 g of SO_4^{2-} produced l^{-1} day^{-1} at 50°C, with initial concentration of 5 g S^0 l^{-1} concentration (Fig. 2). The sulfur was oxidized at a constant rate and was not affected by the decrease of the pH from 1.8 to 0.9 (Fig. 2). Cell numbers did not, however, increase during incubation at pH below 1.1 (results not shown).

With 2 g S^0 l^{-1}, the yeast extract supplementation was decreased from 0.1 to 0.02 g l^{-1} and the resulting sulfur oxidation rate was 0.044 g S^0 l^{-1} day^{-1} at 50°C (Fig. 3a). The sulfur oxidation rate at 70°C with 2 g S^0 l^{-1}, 0.02 g l^{-1} yeast extract, and VS2 was 0.099 g S^0 l^{-1} day^{-1} (Fig. 3a). The pH decreased from 1.6 to 1.1 (Fig. 3a).

The bioavailability of sulfur granules (1–3 mm diameter) to the micro-organisms was also studied. The granular sulfur (2 g l^{-1}) was oxidized at 50 and 70°C with rates of 0.015 and 0.044 g l^{-1} day^{-1}, respectively (Fig. 3a).

The effect of yeast extract

To evaluate the contribution of yeast extract supplementation on sulfur oxidation in more detail and to minimize its supplementation, the sulfur oxidation by the VS2 culture was studied in the presence of 0–0.10 g l^{-1} yeast extract. One gram per liter powdered sulfur was oxidized in 31 days and the pH decreased from 1.8 to 1.3 (Fig. 3b). The highest sulfur oxidation rate, 0.066 g S^0 l^{-1} day^{-1}, was obtained with 0.05 g l^{-1} yeast extract ($P < 0.05$). Significantly lower sulfur oxidation rates were observed with yeast extract concentrations 0 and 0.01 g l^{-1} than with the higher amounts ($P < 0.05$) (Fig. 4).

Chloride inhibition and the effect of saline water source

Many existing unexploited mineral deposits are located in areas where only highly saline water sources are available. Therefore, the influence of chloride and general saline water (Table 1) on sulfur (1 g l^{-1}) oxidation by the VS2 culture was investigated. The sulfate production and pH in the presence of 0–15 g l^{-1} of chloride and VS2 at 50°C was as presented in Fig. 3c. Chloride enhanced sulfur oxidation at below 5 g l^{-1} concentrations, but was inhibitory at 15 g l^{-1} (Fig. 4). Chloride at 10 g l^{-1} partially inhibited sulfur oxidation. The highest sulfur oxidation rate was 0.089 g S^0 l^{-1} day^{-1} in the presence of 5 g l^{-1} of chloride; but no statistically significant difference between 0 to 10 g Cl l^{-1} was observed ($P < 0.05$).

Artificial saline water simulating the salty mine site water was used in dilutions of 0, 25, 50, 75, and 95% with the VS2. Sulfur oxidation rates did not significantly differ ($P < 0.05$) (Fig. 4). The pH decreased from 1.8 to 1.2 during incubation in 0–50% saline water and to 1.1 in 75–95% saline water (results not shown).

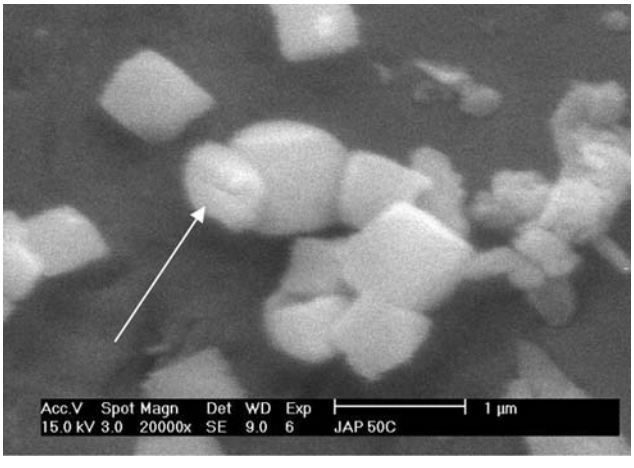

Fig. 1 Scanning electron micrograph of a VS2 lobed cell (shown with the *arrow*)

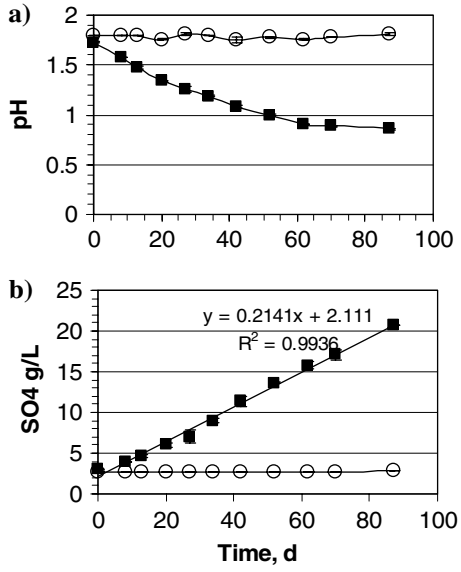

Fig. 2 The pH (**a**) and sulfate production (**b**) by VS2 culture on 5 g l^{-1} elemental sulfur at 50°C. The linear fit of sulfur oxidation is also shown in **b**. *Error bars* show standard deviations *open circles* Control *filled squares* VS2

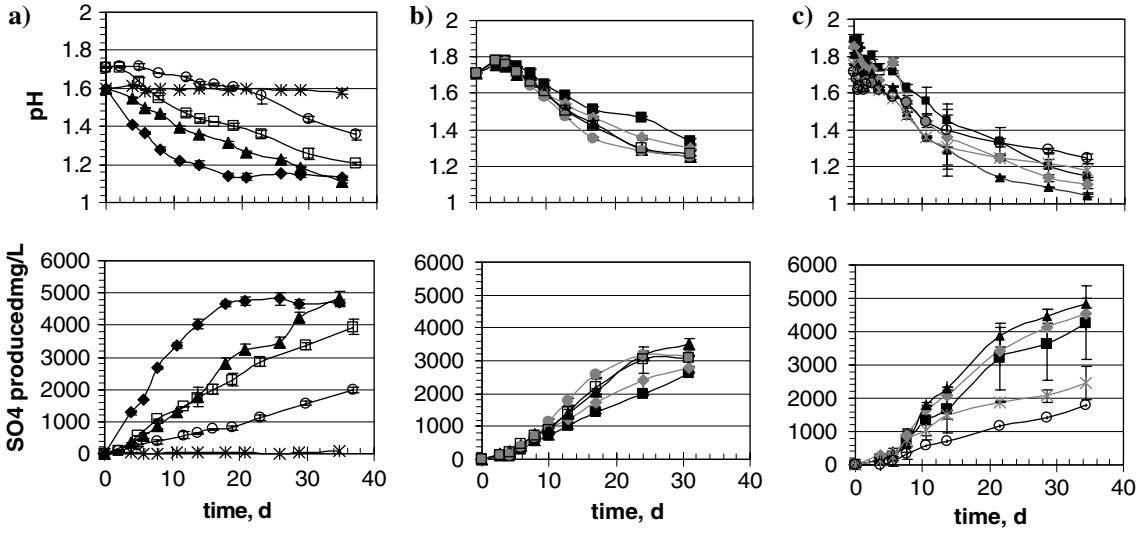

Fig. 3 The pH (*upper panels*) and sulfate production (*lower panels*) during culturing of VS2 on (**a**) 2 g l^{-1} powdered and granular sulfur at 50 (*open squares* powdered, *open circles* granular sulfur) and 70°C (*filled diamonds* powdered, *filled triangles* granular sulfur, *asterisks* abiotic control); (**b**) 1 g l^{-1} powdered sulfur in the presence of 0–0.1 g l^{-1} yeast extract (YE) at 50°C (*filled squares* 0, *filled diamonds* 0.01, *filled triangles* 0.02, *filled circles* 0.05, and *open squares* 0.1 g l^{-1} YE) and (**c**) 1 g l^{-1} powdered sulfur in the presence of 0–15 g l^{-1} chloride at 50°C (*filled squares* 0, *filled diamonds* 2, *filled triangles* 5, *asterisks* 10, and *open cirlces* 15 g Cl^{-} l^{-1}). *Error bars* show standard deviations

Chalcopyrite bioleaching at different solids concentrations

The ability of the VS2 to oxidize chalcopyrite concentrate was studied, first with 1% wt/vol solids and the results were as shown in Fig. 5. Copper was dissolved at a rate of 0.32 g l^{-1} day^{-1}. Biological and chemical Cu leaching yields (%) were 90 and 35 in 30 days, respectively. Biological iron dissolution was continuous and the dissolved iron remained mainly in the ferrous state. Supplementation with 15 g H_2SO_4 kg^{-1} of ore concentrate maintained the pH at around 2 (Fig. 5). The redox-potential first increased and later leveled to + 425 mV in the presence of VS2, whereas in the chemical control it remained between + 350 and + 375 mV.

The effect of chalcopyrite solids concentration on bioleaching by VS2 was investigated at 2%, and 10% wt/vol in shake flasks, and with 10 and 20% wt/vol in a stirred tank reactor, both at 50°C. Copper was dissolved almost linearly with both solid concentrations in shake flasks (Fig. 6). A 25% copper yield was obtained in 28 days in 2% w/v chalcopyrite leaching, while the same recovery from the 10% wt/vol chalcopyrite leaching took almost 80 days. The copper dissolution rates were 0.054 and 0.078 g $l^{-1}day^{-1}$ in 2 and 10% solids concentration, respectively. The pH remained below 2.3 and was not adjusted during the leaching.

Growth of the VS2 in the stirred tank reactor was studied as the solids were gradually increased in concentration to 20%. According to light microscopic observations, dense cell suspensions were observed. When the reactors with chalcopyrite concentration of 10 and 20% were directly inoculated from this reactor, no growth or copper dissolution occurred even after 17 days of operation (results not shown). The results

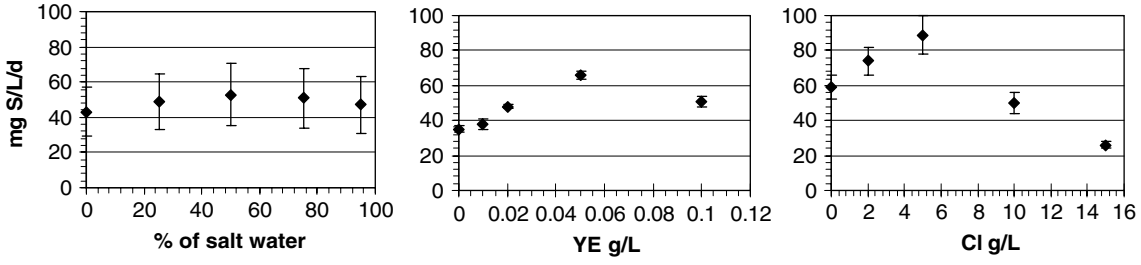

Fig. 4 Effects of salinity, yeast extract and chloride ions on sulfur oxidation rate by VS2. *Error bars* show standard errors

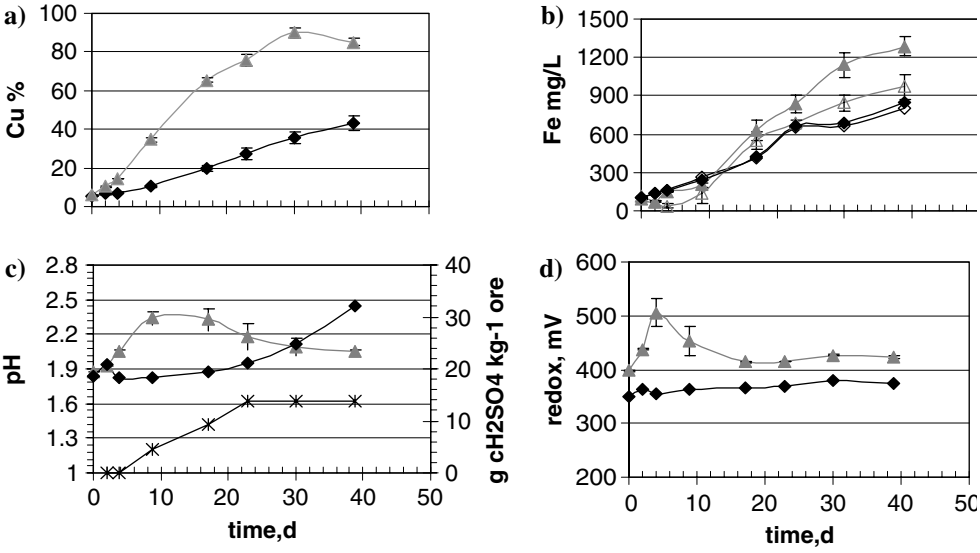

Fig. 5 Copper (**a**) and iron (**b**) dissolution, pH (**c**), the redox potential (**d**), and the acid additions as g concentrated H_2SO_4 added kg^{-1} chalcopyrite concentrate (**c**) during the bioleaching of chalcopyrite concentrate with VS2 culture at 50°C with 1% wt/vol solids concentration. Total dissolved iron is marked with *solid symbols* and Fe^{2+} with *open symbols*. *Error bars* show standard deviations.*Filled triangles* VS2, *filled diamonds* abiotic control, and *asterisks* acid addition

indicated that shear forces caused by the agitation and high concentration of solids hindered the growth.

Phylogenetic profiling of the VS2 culture

The two different cell morphotypes in the VS2 culture, i.e., pleomorphic cocci and regular cocci were further characterized. At first, PCR-mediated DGGE-analysis (results not shown) resulted in a detection of only one archaeal species (universal bacterial and archaeal primers were used). That partial 16S rRNA gene sequence was 98% similar to *Thermoplasma acidophilum*, which is a heterotroph and therefore, does not take part in the elemental sulfur oxidation. The PCRs were then repeated in parallels and again only archaeal amplification was obtained. This archaeal PCR-product was analyzed using DGGE (30–65% gradient of denaturants), but no separate bands were obtained. Therefore, attempts were made to separate the two different morphotypes by dilution series and overlay plates described by Johnson [17], but no isolate was obtained. The culture was then grown in the temperature gradient incubator at a temperature range of 35–75°C. All the tubes grew well. However, based upon observations using a phase contrast microscope, only the larger coccus-morphotype resembling S*ulfolobus*-like species grew at the highest temperature of above 70°C. Based on analysis of partial 16S rRNA gene sequence (679 bp corresponding to positions 51–788 according to *E. coli* numbering), the thermophilic culture contained only one species closely related to *Sulfolobus metallicus* (96% similarity based on pairwise sequence homology). Phylogenetic analysis of this and related species is shown in Fig. 7.

The temperature range of growth for this thermophilic culture was profiled using a temperature range of 40–95°C. A temperature-dependent growth profile of the culture was as presented in Fig. 8. The optimal growth temperature for this culture was 69.7°C with minimum and maximum growth temperatures of 34.2 and 75.8°C, respectively.

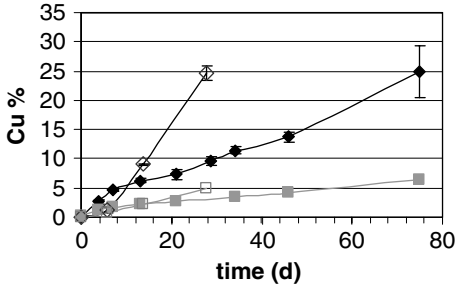

Fig. 6 Copper yields in 2 and 10% wt/vol chalcopyrite leaching in shake flasks with VS2 at 50°C. *Error bars* show standard deviations.*Filled diamonds* VS2 10% $CuFeS_2$, *filled squares* control 10% $CuFeS_2$, *open diamonds* VS2 2% $CuFeS_2$ and *open squares* control 2% $CuFeS_2$

Discussion

A thermoacidophilic enrichment culture that oxidized ferrous iron, elemental sulfur, and mineral sulfides was obtained from wall sediments of an underground geothermal mine. The culture included a *S. metallicus*-like micro-organism which oxidized elemental sulfur at a pH above 1.1 and a temperature range of 34–76°C. The sulfur oxidation was generally not affected by the decrease of the pH in the media (to as low as pH 0.9),

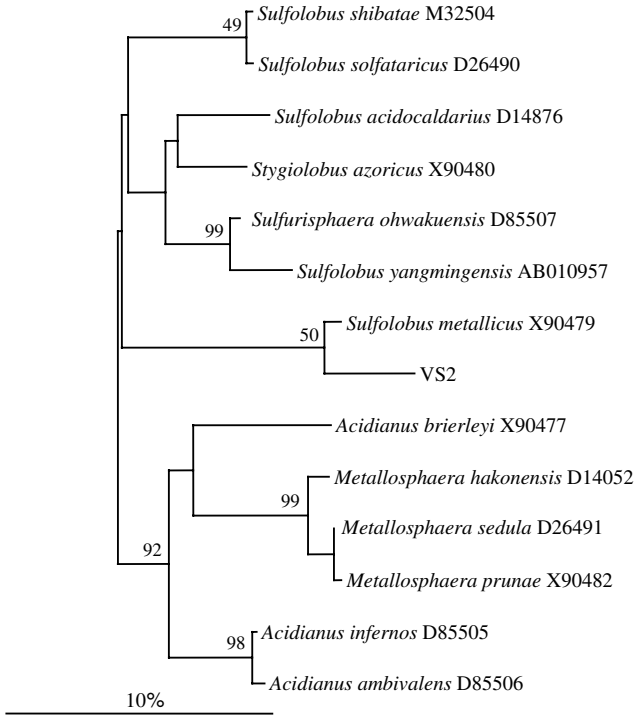

Fig. 7 Phylogenetic tree based on 16S rRNA gene sequences of members of the *Sulfolobales* showing the taxonomic position of the *Sulfolobus metallicus*-like strain in the VS2 culture (679 bp corresponding to positions 51–788 according to *E. coli* numbering). The tree was generated using neighbor joining methods and distance matrix. The bootstrap values (shown at the nodes) are calculated from 1,000 trees. *Pyrodictium occultum* M21807 (not shown) was used as an outgroup for construction of the tree. The *scale bar* represents sequence dissimilarity of 10%

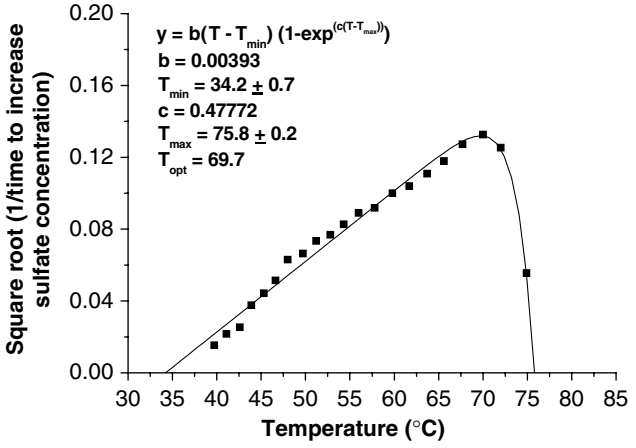

Fig. 8 Ratkowsky plot showing the relationship between temperature and the square root of 1/time (time as hours) taken to increase sulfate concentration to a selected value, representing growth of the culture through the oxidation of sulfur to sulfate. Theoretical predictions of T_{min}, T_{opt} and T_{max} are 34.2, 69.7 and 75.8°C, respectively

although the culture did not grow at a pH below 1.1. Sulfur oxidation by VS2 was optimal at 70°C, and the highest observed sulfur oxidation rate was 99 mg S^0 l^{-1} day^{-1}. The VS2 remained alive for over a year without any sub-culturing at 50°C, which indicates good durability in field.

The growth of *Sulfolobus* species in the VS2 enrichment culture was not restricted by a hyperthermophilic regime. Generally, the limits of growth of *Sulfolobus* species are at a pH range of 1–6 and a temperature range of 50–95°C, varying slightly depending upon the species [12, 14, 15, 43, 50]. *S. metallicus* is an obligate autotroph that oxidizes both iron and sulfur typically at a temperature range of 50–75°C and pH range of 1.0–4.5 [14, for reviews, see 12 or 18]. *S. yangmingensis* is a sulfur-oxidizing facultative chemolithotroph [15], while most of the other *Sulfolobus* species (such as *S. acidocaldarius* and *S. solfataricus*) are obligate heterotrophs and consequently incapable of sulfur oxidation [12].

Sulfur oxidation by the VS2 was not affected by the presence of the saline water, indicating that marine or other saline water source could be used in the leaching applications. Inorganic ions, such as ammonium, sulfate, potassium, and sodium, inhibit the growth of *S. solfataricus* at as low as a 50 mM concentration [32], and here, at 250 mM Na^+ (5.7 g l^{-1}) concentration in addition to the presence of other ions had no effect on sulfur oxidation by VS2. However, 10 g l^{-1} chloride (Cl^- was added as NaCl; consequently, the Na^+ concentration in the solution was 6.5 g l^{-1}) reduced sulfur oxidation and is, therefore, a concern since the mine water may contain such concentrations of chloride. A similar inhibitory concentration of chloride (11.3 g l^{-1}) was reported by Grogan et al. [11] for five different *Sulfolobus* species, including *S. solfataricus* and *S. acidocaldarius*. Huber and Stetter [14] reported optimal sulfur oxidation of *S. metallicus* at below 0.74% NaCl (4.5 g Cl^- l^{-1}), whereas growth took place up to 3% NaCl (18 g Cl^- l^{-1}).

The VS2 culture oxidized elemental sulfur at a rate of 71 mg l^{-1} day^{-1} at 50°C when 5 g l^{-1} of elemental sulfur was provided. With 2 g l^{-1} sulfur, the oxidation rates were 99 and 44 mg l^{-1} day^{-1} at 70 and 50°C, respectively. In addition, the VS2 culture oxidized sulfur at a similar rate (63 mg S^0 l^{-1} day^{-1}) in the presence of low-grade nickel ore [39] as it did on pure sulfur. The sulfur oxidation rates in our experiments were also alike with the rates reported for pure cultures of *S. metallicus* (appr. 90–156 mg l^{-1} day^{-1}) [14]. The form of sulfur, i.e., granular or powdered, significantly affected the sulfur oxidation rates. The powdered-form was oxidized over two times faster than

Table 3 Moderate thermophilic or thermophilic chalcopyrite leaching experiments in shake flasks

Microbial culture	Experimental conditions			Rate of Cu dissolution g l^{-1} day^{-1}	Biological leaching recovery (%)	Comments	Reference
	Temp (°C)	Solids (%)	Duration (days)				
VS2 (dominated by *Sulfolobus metallicus*-like Archaeum)	50	1	30	0.32	90	pH kept at 2.0	This study
	50	2	28	0.054	25	pH stayed at 2.3. No adjustment	This study
	50	10	80	0.078×10^{-3}	25	pH < 2.3 No adjustment	This study
''Sulfobacillus yellowstonensis'' enrichments (Ind2)	50	2	90	0.26	>90	pH maintained 1.8–2.7	[22]
Sb. acidophilus enrichment (SA2)	50	2	90	0.19–0.22	18	No pH adjustment	[22]
Sb. thermosulfidooxidans	50	3.5	21	~0.1	22	Retardation of leaching after 50 h due to precipitation	[45]
Sb. thermosulfidooxidans (DSM 9295)	50	3.2	21	0.21	< 20	Retardation of leaching after 50 h	[47]
Sb. acidophilus (DSM 10332)	45	3.2	21	0.14	< 20		
Acidiaus brierley (DSM 1651)	70	3.2	24	0.62	70		
S. metallicus (DSM 6482)	65	3.2	24	NA	58		
S. metallicus (DSM 6482)	65	3.2	25	0.18 (after 48 h) 1.03 (before 48 h)	61	Retardation of leaching after 48 h	[46]
S. rivotincti	68.5	3	21–30	Not reported	60–90	3 different concentrates	[9]
Moderate thermophilic enrichment cultures	50	3	~15	0.37–0.57	Not reported		[20]
Sulfolobus BC-enrichment	70	3	Not reported	0.38	Not reported		
A. brierleyi-enrichment		3	Not reported	0.30	Not reported		

granular-sulfur at both temperatures, although the granular-sulfur was also oxidized. In the field conditions, granular sulfur would be easier to manage, and therefore, the bioavailability in comparison to required oxidation rate should also be addressed at heap leaching conditions.

The copper dissolution rate from chalcopyrite by the VS2 culture was dependent upon the solids concentration and adjustments of pH of the leaching solution, which has also been observed in other bioleaching experiments [21]. Copper dissolution rate and the overall yield observed with VS2 compares favorably with most of the other reported chalcopyrite leaching experiments in shake flask conditions at 50°C (Table 3). Higher chalcopyrite leaching rates have been reported with, e.g., moderately thermophilic enrichment culture at 50°C [20] and *Acidianus brierleyi* at 70°C [47]. The VS2 culture was highly sensitive to shear forces in the stirred tank conditions. *S. metallicus* as well as many other thermophilic Archaea are sensitive to hydrodynamic conditions such as attrition and shear forces caused by smaller particle size or increased pulp density in stirred tanks [3, 5, 28, 29]. However, the shear forces do not prevail in heap leaching conditions.

Possible applications benefiting from the VS2-like cultures include sulfur-oxidation and/or acid requiring processes, such as metal recovery from mineral deposits, incineration slag, or waste water sludge [44], acid generation to overcome high acid demand of marginal ore deposits [39], and bioremediation (e.g., sulfur rich sediments or metal contaminated soils or sludge) [10]. Sulfur oxidizers could also be used to scavenge the sulfur-rich layers formed on top of the chalcopyrite surfaces to prevent the formation of the diffusion hindrance [7] that has been observed in many leaching experiments [e.g., 45–47]. The on-going studies concerning the VS2 enrichment culture focus on the removal of the precipitates from chalcopyrite surfaces during leaching with ferric iron, and the capability of the culture to be used in heap leaching applications.

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