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ORIGINAL PAPER

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Characterization of iron- and sulphide mineral-oxidizing moderately thermophilic acidophilic bacteria from an Indonesian auto-heating copper mine waste heap and a deep South African gold mine

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Abstract Iron- and chalcopyrite-oxidizing enrichment cultures were obtained at 50°C from acidic, high-temperature, copper/gold mine environments in Indonesia and South Africa. Over 90% copper yield was obtained from chalcopyrite concentrate with the Indonesian enrichment in 3 months with 2% solids concentration, when pH was maintained at around 2. Neither addition of silver cations nor an enhanced nutrient concentration influenced chalcopyrite leaching. Excision and sequencing of bands from denaturing gradient gel electrophoresis of the amplified partial 16S rRNA gene showed that the enrichment cultures from different environments in South Africa and Indonesia were very simple, and similar. Chalcopyrite concentrate supported a simpler and different community than Fe^{2+}. The members of the enrichment cultures were closely related to *Sulfobacillus yellowstonensis* and *Sulfobacillus acidophilus*.

Keywords Acidophiles · Bioleaching · Moderate thermophiles · *Sulfobacillus* spp.

Introduction

Biohydrometallurgical processes are often considered more environmentally friendly and economical than conventional pyrometallurgical processes [4, 11, 19, 29]. Bioleaching is based on the iron- and sulphur-oxidation abilities of a wide range of mesophilic, moderately thermophilic, and thermophilic acidophiles [for a review, see 10]. Microbes regenerate ferric ions and protons, which then chemically leach sulphide minerals such as chalcopyrite [25, 30]. The leaching microbes are widely utilized in the processing of metal ores, but they also create environmental problems by generating acid mine drainage [13].

High temperatures are often generated inside the leaching tank, or heap, due to the exothermic mineral oxidation reactions. Moderately and extremely thermophilic organisms are being considered for bioleaching as a means to improve mineral sulphide oxidation rates [23, 27, 28], and to reduce the costs associated with the cooling of this exothermic process [12, 29]. Therefore, biomining is increasingly focusing on the use of high temperatures and novel microorganisms need to be screened from suitable acidic high-temperature environments to improve the bioleaching process [14, 25].

Acidic metal-rich environments hosting thermophilic acidophiles are associated with geothermal activities in volcanic regions and mining activities [14]. The heterogeneous conditions at, for example, mining sites with temperature and acidity gradients support a wide diversity of acidophiles [13, 23]. At temperatures above 60°C, archaea tend to predominate, while in moderately thermal environments archaea and Gram-positive bacteria may co-exist [13]. Lower temperature environments are generally dominated by Gram-negative bacteria [13]. Thermophiles growing at temperatures above 60°C, and currently applied in leaching processes, belong to the genera *Acidianus*, *Sulfolobus* and *Metallosphaera* [for reviews, see 4, 12]. Moderate thermophiles ($T_{optimum}$ 40–60°C) include *Sulfobacillus* sp., *Leptospirillum thermoferrooxidans*, *Acidimicrobium ferrooxidans* [10, 15], *Acidithiobacillus caldus* and *Hydrogenobacter acidophilus* [15].

In the present work, iron- and chalcopyrite-oxidizers from acidic copper/gold mines at elevated temperatures in Indonesia and South Africa were enriched. The gold mines in South Africa belong to the deepest excavations in the world, thus having constantly high temperature, and the mineral waste piles in Indonesia auto-heat despite the cold temperature in the environment. The ability of the cultures to oxidize iron and sulphide minerals was determined and the possibility of enhancing chalcopyrite leaching rates under different growth

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conditions was evaluated. Further, the microbial community structures were characterized with denaturing gradient gel electrophoresis (DGGE) followed by partial 16S rRNA gene sequencing.

Materials and methods

Sampling sites

Samples for enrichment of iron- and sulphide mineral-oxidizing microbes were collected from the Target Avgold gold mine in South Africa and the Freeport Grasberg copper/gold mine in Indonesia. The workings of the acidic Target mine are 2.5 km below the surface at high rock temperature (52°C). The mineral-containing waste piles in Grasberg mine auto-heat to temperatures above 70°C.

Substrate oxidation

Microbes were enriched in $CuFeS_2$ (chalcopyrite) concentrate, S^0 and Fe^{2+} growth media at 50 and 70°C. The ability of the cultures to oxidize iron and $CuFeS_2$ was determined and compared in batch experiments. The composition of the mineral medium was as follows (in g l^{-1}): $(NH_4)_2SO_4$, 1.3; KH_2PO_4, 0.28; $MgSO_4{\cdot}7H_2O$, 0.25; $CaCl_2$, 0.05; yeast extract, 0.02 or 0.2. The pH was adjusted with H_2SO_4 to 1.8. Either 2% (w/v) $CuFeS_2$ concentrate, 0.5% w/v S^0 or 4 g l^{-1} $FeSO_4{\cdot}7H_2O$ was added to the medium. The mineralogical composition of the chalcopyrite concentrate originating from Pyhäsalmi in Finland was 73.7% chalcopyrite, 10.4% pyrite, 3.7% sphalerite, 0.9% galena and 10.7% silicates. Substrate oxidation experiments were performed in 500 ml shake flasks with a liquid volume of 200 ml. Inoculum was added to 10% except in control bottles. A natural evolution of the pH was allowed. The progress and conditions of bioleaching were monitored by measuring soluble metals, Fe^{2+}, pH and redox potential.

In further chalcopyrite leaching experiments, conditions were modified in order to enhance chalcopyrite leaching rate and yield. When the pH rose above 2 at the sampling time, it was adjusted to 1.9 with H_2SO_4. A natural evolution of the pH was allowed below pH 2. In addition, a 4-fold base medium concentration was used in order to ensure that leaching was not limited by lack of nutrients. Since catalytic silver cations have been shown to enhance the rate and yield in chalcopyrite leaching [9], the effect of 2 mg Ag^+/g concentrate on leaching rate and yield was studied.

Analytical procedures

Ferrous iron concentrations were determined by the colorimetric *ortho*-phenanthroline method using a UV 1601 spectrophotometer (Shimadzu, Europe) according to 3500-Fe [2] modified as follows: 2 ml 1, 10-phenanthroline (10 g l^{-1}) and 1 ml ammonium acetate buffer were added to 3 ml sample. Dissolved copper (Cu_{tot}) and total iron (Fe_{tot}) concentrations were analysed by atomic absorption spectrophotometry (Perkin Elmer 1100B, Foster City, Calif.). pH and redox potential were measured using a WTW pH96 meter and a Hamilton Pt-ORP electrode, respectively. Microbial growth was visualized with a Zeiss Axioskop 2 microscope (Zeiss, Jena, Germany).

Polymerase chain reaction-denaturing gradient gel electrophoresis

Decimal dilutions of the samples were used for DGGE analyses. The most numerous microbes in the mixed cultures were obtained using decimal dilution series repeated three times. The most diluted microbial solution in which growth was still observed was selected for further dilutions. In the most diluted samples, bands are expected to represent the most abundant species in the system. The microbial community was investigated by PCR-DGGE followed by partial sequencing of 16S rRNA genes. Samples were filtered and washed [17] before nucleic acid extraction [16]. Crude DNA samples were used as templates for PCR. Archaeal 16S rRNA gene was amplified using primers Ar3f [6] and Ar958r [16]. Bacterial fragments corresponding to nucleotide positions 341–926 of the *Escherichia coli* 16S rRNA gene sequence were amplified with the forward primer 341fGC to which a GC clamp was added at the 5′ end to stabilize the melting behavior of the DNA fragments in DGGE, and the reverse primer 907r [21]. PCR mixtures contained 5 µl 10x PCR buffer IV (200 mM $(NH_4)_2SO_4$, 750 mM Tris-HCl, 0.1% (v/v) Tween, pH 8.8), 1.75 mM $MgCl_2$, 0.5 µM each primer, 100 µM each deoxynucleoside triphosphate, 1.25 U Red Hot DNA polymerase (ABgene, Advanced Biotechnologies, Epsom, UK), 400 ng μl^{-1} bovine serum albumin [18], and sterile water to a final volume of 50 µl, to which 1 µl template was added. PCR, DGGE and 16S rRNA gene sequencing were performed as described by Kinnunen and Puhakka [17] except that the DGGE electrophoresis was run for 16 h. Sequence data were initially analysed using basic local alignment search tool (BLAST) and further phylogenetic analyses were conducted using MEGA version 2.1 software [20].

Nucleotide sequence accession numbers

The sequences obtained in this study were deposited in GenBank under accession numbers AY519475–AY519482.

Results

Enrichments

Enrichment on $CuFeS_2$ concentrate and Fe^{2+} at 50°C resulted in growth of up to 5 µm long rod-like mor-

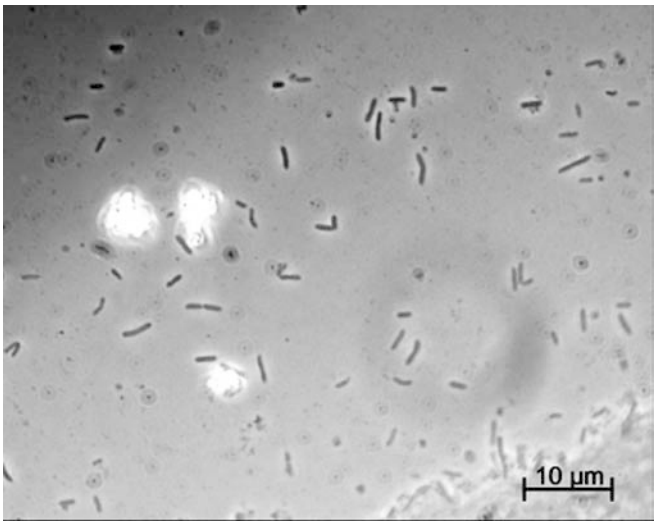

Fig. 1 Phase contrast micrograph showing *Sulfobacillus*-like long rods from Ind1 enrichment culture

photypes from both the Indonesian and South African samples (Fig. 1). The enrichments on the same substrates at 70°C and on elemental sulphur at 50 and 70°C were not successful, based on chemical analyses or microscopy. Ind1 and SA1 were enrichments on ferrous iron, and Ind2 and SA2 on chalcopyrite of Indonesian and South African material, respectively. Cultures enriched on a certain growth substrate were used for the oxidation experiments of that particular substrate. Ind1 and SA1 oxidized between 1.5 and 2 g l^{-1} Fe^{2+} after 50 h incubation. Ind2 and SA2 enrichments oxidized $CuFeS_2$ concentrate as described in more detail in the following section.

Effect of solids concentration on $CuFeS_2$ oxidation

The influence of solids concentration on $CuFeS_2$ oxidation by Ind2 and SA2 was studied. Copper leaching was slightly faster and the yield higher with Ind2 than with SA2 (Fig. 2). However, the bioleaching yield remained relatively low for both cultures at both 2 and 5% solids concentration. With an increase of solids concentration from 2% to 5%, the percent copper yield decreased from 22 to 12 and from 18 to 9 by Ind2 and SA2, respectively. Conversely, the soluble copper concentrations were greater in 5% than in 2% solids concentration. Fe^{2+} concentrations decreased during leaching, indicating biological iron oxidation. Fe_{tot} concentration was similar to the Fe^{2+} concentration. The pH increased in the course of leaching from 2 to almost 3, and was likely due to the consumption of H^+ by the iron oxidation and protonic attack of the chalcopyrite.

Effect of silver cations, pH and enhanced nutrient concentration on $CuFeS_2$ oxidation

Since $CuFeS_2$ leaching at different solids concentrations was slightly faster with Ind2 than with SA2, Ind2 was selected for further leaching experiments. The leaching conditions were modified in order to determine whether incomplete $CuFeS_2$ leaching was due to the formation of a passivation layer and whether the changes in solution conditions could enhance the Cu leaching rate and yield. Soluble copper yield increased from 22 to >90% when the pH was adjusted and maintained at 1.8–2.7 during leaching (Fig. 3). Addition of silver cations together with pH adjustment did not increase the leaching rate further. A 4-fold concentration of the mineral medium also had no further effect on chalcopyrite leaching. The results indicate that maintenance of pH at around 2 was needed to obtain high Cu yields. The results also show that the growth medium concentration was adequate, as the 4-fold growth medium in addition to pH adjustment had no effect on $CuFeS_2$ leaching compared to pH adjustment alone.

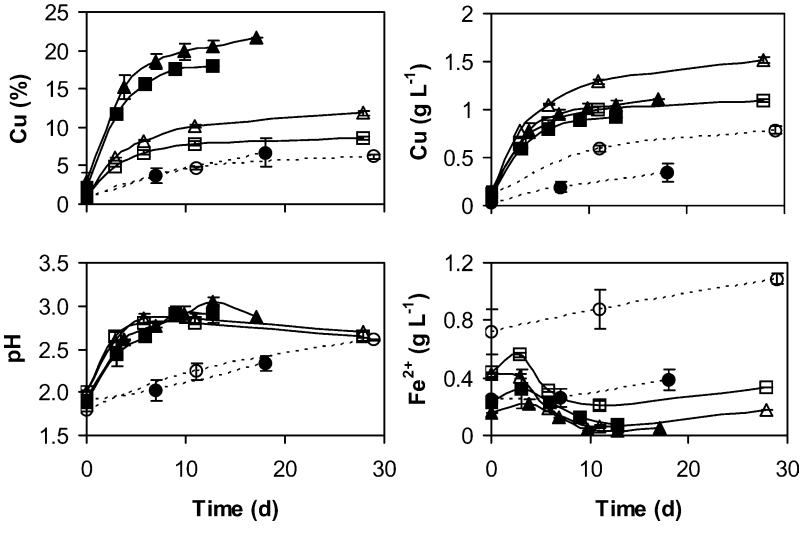

Fig. 2 Chalcopyrite leaching at 2% (*filled symbols*) and 5% (*hollow symbols*) solids concentration with Ind2 (*triangles*) and SA2 (*squares*) enrichment cultures and in controls (*circles*)

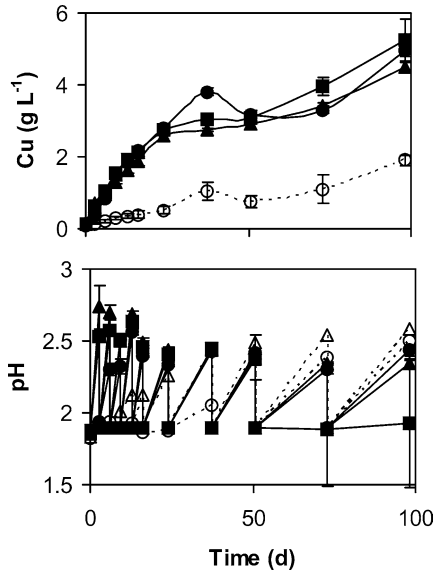

Fig. 3 Chalcopyrite leaching at 2% solids concentration with Ind2 without pH adjustment (*crosses*), with pH adjustment (*squares*), pH adjustment and Ag addition (*triangles*), pH adjustment and enhanced nutrient concentration (*circles*) and in controls (*hollow symbols*)

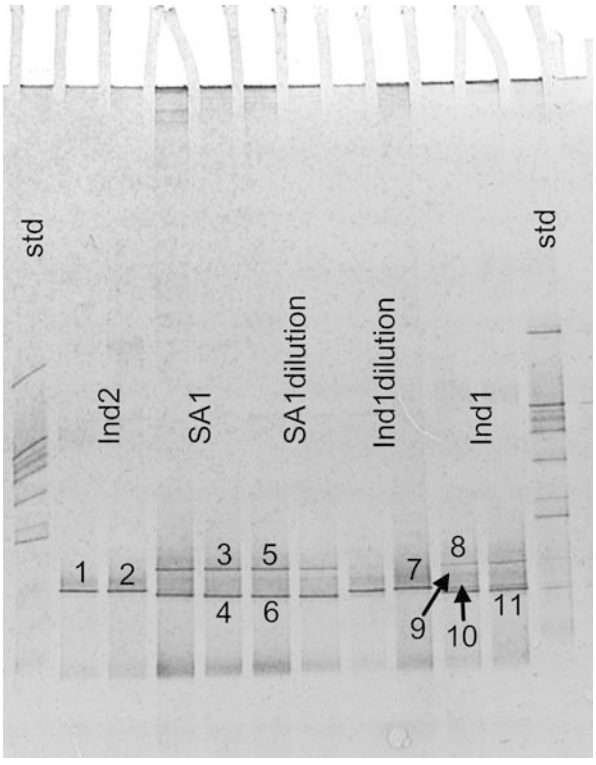

Fig. 4 Duplicate denaturing gradient gel electrophoresis (DGGE) profiles of Ind1, Ind2 and SA1 enrichment cultures and Ind1 and SA1 dilutions

16S rRNA gene sequence analysis

No product was obtained using archaeal primers for DNA amplification. Bacterial 16S rRNA gene fragments obtained by PCR amplification of the enrichment cultures were analysed by DGGE. Bacterial DGGE profiles showed a fairly simple community structure in all enrichment cultures, independent of the growth substrate or the geographical origin (Fig. 4). Bands were excised from the DGGE gel and amplified by PCR and the nucleotide sequences were determined. The Ind2 enrichment on $CuFeS_2$ concentrate produced only one DGGE band showing 98% similarity with *Sulfobacillus yellowstonensis* in a BLAST search. The Ind1 enrichment on ferrous iron produced several bands, which were all related to *Sb. yellowstonensis* (98–99% similarity), but differed slightly from each other and from the band obtained from the Ind2 enrichment. The dominant population in Ind1 enrichment based on dilution series (band 7) differed from the dominant population based on the intensity of the DGGE band. Bands obtained from SA1 were related to *Sb. acidophilus* (99% similarity). Both bands present in the SA1 enrichment were also present in the $SA1_{dilution}$ culture, thus indicating that their abundances were similar. The phylogenetic dendrogram based on 16S rRNA gene sequence comparison (Fig. 5) shows the relationships among DGGE sequences obtained from Ind1, $Ind1_{dilution}$, Ind2, SA1 and $SA1_{dilution}$ and reference strains. The members of the enrichment cultures from South African and Indonesian sources were similar. The number of DGGE bands of the chalcopyrite-grown enrichment culture was lower than that of Fe^{2+}-grown enrichment cultures. This indicates that chalcopyrite supported a simpler and different microbial community than Fe^{2+}. These results show that the microbial enrichment culture was determined more by growth substrate than the origin of the microbes.

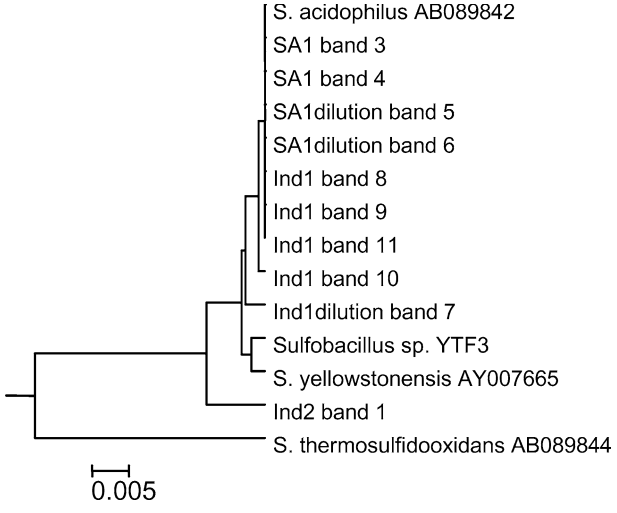

Fig. 5 Phylogenetic dendrogram of DGGE sequences obtained from Indonesian and South African cultures and reference strains. The dendrogram was constructed using MEGA software based on the comparison of 545 nucleotides. *Bar* 0.005 substitutions per nucleotide position

Discussion

Moderately thermophilic bioleaching microbes enriched and characterized from different acidic, high-temperature, metal-rich mine environments in Indonesia and South Africa were dominated by the genus *Sulfobacillus*. At pH 2, these enrichment cultures oxidized $CuFeS_2$ concentrate with >90% Cu yields.

Both Ind2 and SA2 enrichments oxidized $CuFeS_2$, which is one of the most economically valuable and recalcitrant copper minerals. Copper leaching was slightly faster, and the yield higher, with Ind2 than with SA2. Copper release from chalcopyrite (900–1,300 mg l^{-1} Cu in 300 h) was similar to leaching by *Sb. thermosulfidooxidans* (1,250 mg l^{-1} in 300 h at 3.5% solids concentration) [31] in shake flasks. The increase of the pH to almost 3 during the course of leaching, likely due to the consumption of H^+ during the iron oxidation and protonic attack of the chalcopyrite, resulted in low copper yields (9–22%). The maintenance of pH at the optimal growth pH for *Sulfobacillus* sp. of approximately 2 [8, 22] was essential in obtaining high copper yields, but did not affect the copper leaching rate. Although silver cations have been shown to enhance the rate and yield in chalcopyrite leaching [9], no positive effect was observed in this study. On the other hand, the moderate thermophiles from Indonesia tolerated silver concentrations as high as 40 mg l^{-1}, even though microbial activity is generally inhibited above 1 mg l^{-1} Ag [5, 24]. The use of Ag as a catalyst may depend on the temperature, as Ag catalyst has been shown to increase copper extraction yield with mesophiles, but not with thermophiles [1]. A 4-fold increase in the nutrient concentrations of the growth medium also had no effect on $CuFeS_2$ leaching. Therefore, the amount of nutrients in the original growth medium was adequate for the activity of moderate thermophiles.

DGGE followed by partial 16S rRNA gene sequencing demonstrated that the iron- and chalcopyrite-oxidizing cultures at 50°C enriched from different acidic, high-temperature copper/gold mines in Indonesia and South Africa were of low diversity, were similar, and were related to *Sb. yellowstonensis* and *Sb. acidophilus*, respectively. All sequences shared greater than 98% identity with sequences in the databases. The microbial community was more influenced by the growth substrate than the origin of the microbes. The addition of yeast extract tends to select for *Sulfobacillus*-like bacteria. Yeast extract was however added to the medium, since many thermophiles require yeast extract for their growth. No archaea were enriched. As DGGE has weaknesses in analysing populations because of potential differences in lysing or amplification efficiency among different populations, dilution series were performed from enrichment cultures showing several bands in DGGE to determine the predominant strain. The numbers of the two South African iron-oxidizing strains were likely similar with each other, whereas among Indonesian iron-oxidizers, *Sb. yellowstonensis* predominated, corresponding to band 7 in Fig. 4. Although previous studies of the microbial ecology in extremely acidic environments by culture-independent nucleic acid methods have revealed considerable biodiversity [19], enrichment methods tend to select for a narrower range of acidophiles [14]. Sources of moderately thermophilic iron- and sulphur-oxidizers have been thermal springs in Iceland, Yellowstone National Park, the island of Montserrat, geothermal sites in New Zealand, mineral sulphide ore deposits in Armenia and eastern Kazakhstan, coal heaps in the United Kingdom and Australia, and copper leach dump and sites of acid mine drainage production in the United States [3, 7, 8, 22, 26]. Even though the method used for the enrichment of the moderate acidophiles may have favored *Sulfobacillus*, this study demonstrated the wide distribution of *Sulfobacillus* sp. in mining environments.

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