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ORIGINAL PAPER

A sulfate-reducing bacterium with unusual growing capacity in moderately acidic conditions

L. R. Rampinelli · R. D. Azevedo · M. C. Teixeira · R. Guerra-Sá · V. A. Leão

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Abstract The use of sulfate-reducing bacteria (SRB) is a cost-effective route to treat sulfate- contaminated waters and precipitate metals. The isolation and characterization of a SRB strain from an AMD in a Brazilian tropical region site was carried out. With a moderately acidic pH (5.5), the C.1 strain began its growth and with continued growth, modified the pH accordingly. The strain under these conditions reduced sulfate at the same rate as an experiment performed using an initial pH of 7.0. The *dsr*B gene-based molecular approach was used for the characterization of this strain and its phylogenetic affiliation was similar to genus *Desulfovibrio* sp. The results show an SRB isolate with unexpected sulfate reducing capacity in moderately acidic conditions, bringing new possibilities for the treatment of AMD, as acid water would be neutralized to a mildly acidic condition.

L. R. Rampinelli · R. D. Azevedo · M. C. Teixeira · R. Guerra-Sá · V. A. Leão (✉)
Núcleo de Valorização de Materiais Minerais, Federal University of Ouro Preto, Campus Morro do Cruzeiro, Ouro Preto, MG CEP 35400-000, Brazil
e-mail: versiane@demet.em.ufop.br

L. R. Rampinelli
e-mail: leticiarony@gmail.com.br

M. C. Teixeira
e-mail: teixeira@ef.ufop.br

R. Guerra-Sá
e-mail: rguerra@iceb.ufop.br

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Introduction

Acidic wastewaters from mining and other industrial activities constitute a worldwide environmental problem. Acid mine drainage (AMD) water is highly acidic (pH < 4). It also contains high concentrations of sulfate and dissolved heavy metals which are very toxic to many living organisms. Many biotechnological processes of AMD remediation using sulfate-reducing bacteria (SRB) have been investigated throughout the world (García et al. 2001; Kappler and Dahl 2001; Kaksonen et al. 2003). SRB are a group of anaerobic microorganisms that use sulfate as a terminal electron acceptor. One of the greatest challenges for the use of SRB to minimize the environmental impacts of AMD is that the optimum pH for most known SRB is close to 7.0. As a consequence, a neutralization step is necessary before those effluents are submitted to the SRB treatment (Tsukamoto et al. 2004). The acidity of the AMD provides an unusual habitat for SRBs, and the occurrences of these organisms in such habitats have received little attention (Johnson 1998; Loy et al. 2004).

Molecular techniques have provided alternative approaches to study the diversity of SRB in natural and engineered ecosystems. Most of these studies have focused on the presence, rather than on the activity, of the SRB in the samples, and have analyzed the functional

genes to identify SRB (Pérez-Jiménez et al. 2001). Dissimilatory sulfite reductase encodes β subunits (*dsr*B) of an enzyme that catalyzes the six-electron reduction of sulfite to sulfide. Due to a remarkably high degree of conservation observed in *dsr*B across SRB and archaea, this enzyme is a potential candidate for phylogenetic studies of these organisms (Wagner et al. 1998). The *dsr*B gene-based molecular approach has been used to describe SRB in diverse environments (Joulian et al. 2001; Leloup et al. 2006; Dar et al. 2007a).

The study is part of a larger project aiming to treat AMD with SRB in continuous reactors. It addresses the isolation and molecular characterization of SRB strains capable of growing in acidic conditions so that AMD could be treated with minimum pH adjustment before being fed to an anaerobic reactor containing SRB. Here, we characterize a SRB strain with unusual sulfate reducing capacity at moderately acidic pH.

Material and methods

Source, enrichment and isolation of bacteria

Sediment samples were collected from an AMD site (pH 2.4) in Brazil. Enrichment of acid-resistant bacteria was carried out immediately after transporting the sample into laboratory. The enrichment was processed in a modified Postgate C liquid medium (Cheung and Gu 2003) containing lactate as a carbon source at pH 5.5 ± 0.2. Serial dilutions of enrichment culture were plated (*pour plate* method) in a solid Postgate C modified medium, pH 5.5 ± 0.2, solidified with agar 1.5% and incubated at 35°C for isolation. The isolates were morphologically characterized using Scanning Electron Microscopy images. To obtain these images, 100 µl of the isolate culture (C.1) was placed on slides and metallized with carbon.

Bacterial growth

Bacterial growth was determined with an optical density of 600 nm ($OD_{600\ nm}$) (Hitachi-U2800A spectrophotometer). Iron was removed from the growth medium to avoid the formation of black iron sulphide precipitates, which affects the optical density determinations. The results are an average of at least two determinations.

Sulfate reduction

Sulfate reduction was assessed by measuring sulfate concentration before and after SRB inoculation. Sulfate was determined by ion chromatography (Metrohm) in a 75 mm polymethacrylate-quaternary ammonium column with a carbonate (2.0 mmol/l $NaHCO_3$ and 1.3 mmol/l Na_2CO_3) eluent and conductivity detection.

DNA extraction

Genomic DNA was extracted from 5 ml of pure SRB culture by the CTAB method (Sambrook et al. 1989). The quality of DNA was analyzed in 0.6% (wt/vol) agarose gel. For PCR purposes, the DNA concentration was measured in SHIMADZU UV-1601 spectrophotometer at 260 nm and adjusted to a concentration of 100 ng/µl.

PCR amplification of *dsrB* gene

*dsr*B gene was amplified with a primer pair, DSRp2060F/DSR4R (Geets et al. 2006). PCR amplification was performed under standard conditions. Thermal cycling was carried out by using an initial denaturation step of 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and elongation at 72°C for 1 min. Cycling was completed by a final elongation step of 72°C for 6 min. PCR products were examined with ethidium bromide stained 1.2% agarose gels.

Cloning and sequencing *dsrB* gene fragments

The *dsr*B amplicons were purified and ligated into the pGEMT-Easy plasmid vector and transformed into *Escherichia coli* DH5α cells according to manufacturer's instructions (Invitrogen). Three independent clones were sequenced in an ABI 3100 automated sequencer (Applied biosystem), using a dye terminator kit.

Computer analysis for sequence alignments

The partial *dsr*B gene sequences obtained were first compared to sequences stored in GenBank by using the BLAST algorithm. This sequence was edited using Artemis to translate *dsr*B sequences into protein sequences. The COG and Pfam were used for the determination of protein domain and its position. The ClustalX 2.0 was used for the alignment of nucleotides and amino acids sequences and MEGA 4.0 was used for the drawing phylogenetic tree (Thompson et al. 1997).

Total RNA extraction and RT-PCR

Total RNA was isolated using Trizol reagent (GIBCO-BRL) and quantified spectrophotometrically. The integrity of RNA was evaluated by gel electrophoresis. Total RNA was reverse-transcribed using the gene-specific primers DSRp2060F/DSR4R through the Thermoscript RT System (Invitrogen). RT-PCR was then performed under the same methods described previously and analyzed in acrylamide gel and stained by silver.

Results and discussion

Three different mixed SRB cultures were obtained from the AMD and named A, B and C. All of them were able to grow on Postgate C medium, producing a black iron sulfide precipitate as a result of H_2S formation. Nevertheless, only the mixed culture C reduced sulfate in acidic conditions and, after serial dilution, 17 pure cultures were obtained and only one was able to grow and reduce sulfate at a significant level. This strain is referred to as C.1 throughout the paper. The morphological characterization with Scanning Electron Microscopy image (Fig. 1) showed that C.1 cells were bacillus-like and after staining was determined Gram negative.

The C.1 strain, despite being able to grow at pH 4.3, showed a very slow growth rate (4 weeks was required for the formation of iron sulfide to be detected) and, therefore, the sulfate reduction could not be determined at this pH. As a result, sulfate reduction was assessed in batch experiments with the initial pH of the growth medium (Postgate C) adjusted

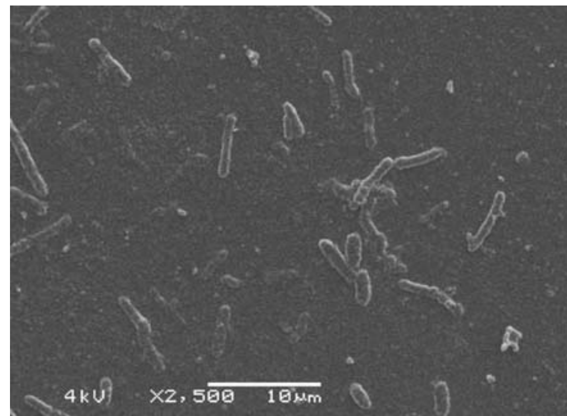

Fig. 1 Scanning Electron Microscopy image of C.1 strain cells. An aliquot of 100 µl of the isolate culture (C.1) was placed on slides and the metallized with carbon

to 5.5 and 7.0. The C.1 strain showed similar growth rate at both pHs, which was not expected since the pH 5.5 is not as good as pH 7.0 for growing most known SRB strains (Fig. 2a and b). Figure 3 shows that this isolate can start growth in moderately acidic conditions (pH 5.5), increasing the pH with time.

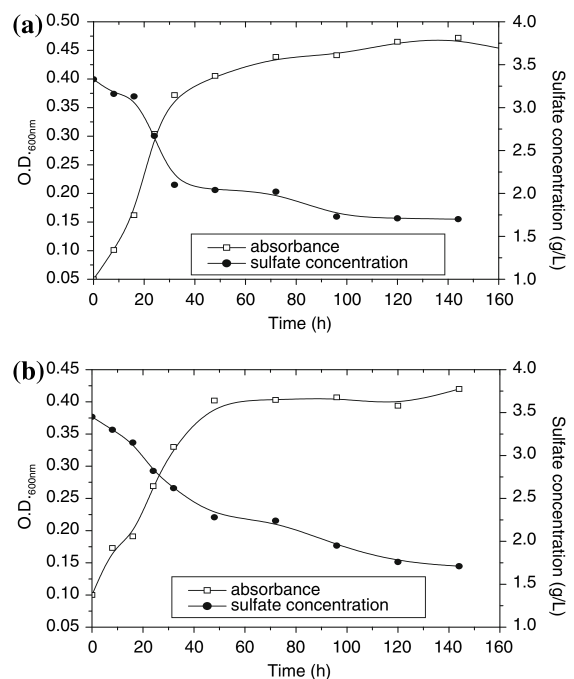

Fig. 2 Bacterial growth and sulfate reduction of strain C.1 in bath condition. (**a**) pH 7.0 and (**b**) pH 5.5. Temperature: 35°C, growth medium: Postgate C

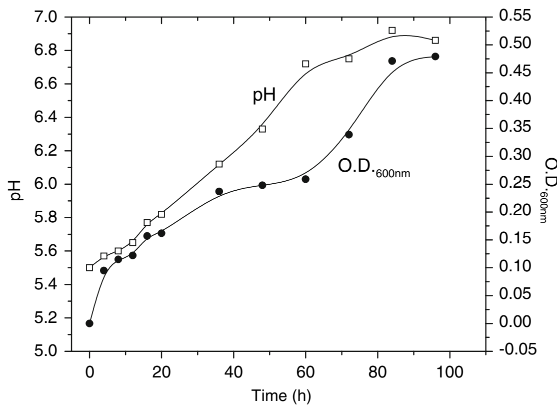

Fig. 3 Evaluation of the pH increase during bacterial growth. The left axis (empty squares) represents the pH values. The right axis (filled squares) represents the OD (600 nm) values

A maximum $OD_{600\ nm}$ of 0.44 ± 0.01 was observed in both pH conditions, after 144 h.

Sulfate reduction followed the behavior of the OD as the C.1 strain reduced sulfate at approximately the same rate at both initial pHs (5.5 and 7.0), i.e. 50% reduction was observed after 144 h. This suggests that the bacterial growth at this initial pH 5.5 did not affect the sulfate reduction. Many studies have used a consortium of SRB in reactors for AMD bioremediation. There are only a few studies, however, addressing sulfate reduction using pure cultures, especially in batch conditions. Medircio et al. (2007) have observed 26% sulfate reduction in the presence of manganese while Cabrera et al. (2006) noticed around 40% sulfate reduction with a *Desulfovibrio vulgaris* strain. However, the latter reduced sulfate at pH 7.0, which is within the optimal pH range for most known SRB. Furthermore, continuous sulfate reduction studies have reported SRB growth at pH 3.0 (Kolmert and Johnson 2001; Johnson et al. 2006). Elliott et al. (1998) adapted a mixed SRB culture for increasing acidity (pH 4.5; 4.0; 3.5 and 3.25) and observed a progressive decline in sulfate reduction as the pH dropped, with 38.3% reduction at pH 3.5. Notwithstanding, there are no citations in other literature to SRB isolates with the ability to reduce sulfate at moderately acidic pHs as presented in the present work. Therefore, SRB isolation from AMD sites represents an effective way for selecting acid-tolerant SRB, since these are habitats for microorganisms with specific physiology to grow in extreme pH conditions.

As acidity is a drawback for the use of SRB in AMD remediation, several engineering approaches have been proposed, generally in continuous operations. Kaksonen et al. (2003) treated AMD (pH 2.5–5.0, 1.0–2.2 g/l sulfate) in a fluidized bed reactor (FBR), at 35°C. Their approach was to recycle the FBR effluents, which have a pH of around 7.0, mixing it with the acidic water so that the pH was increased before entering the FBR. This procedure permitted treatment of waters at pH 2.5. Another approach, used by Tsukamoto et al. (2004), was to adapt SRB to increasing acidity (pH was progressively dropped to 3.0) in columns. The authors observed that the pH affected the efficiency of SRB bioreactors, and a source of alkalinity could be necessary to raise the influent pH, particularly when the pH is low. Regardless of the type of reactor used for sulfate reduction, acidic waters

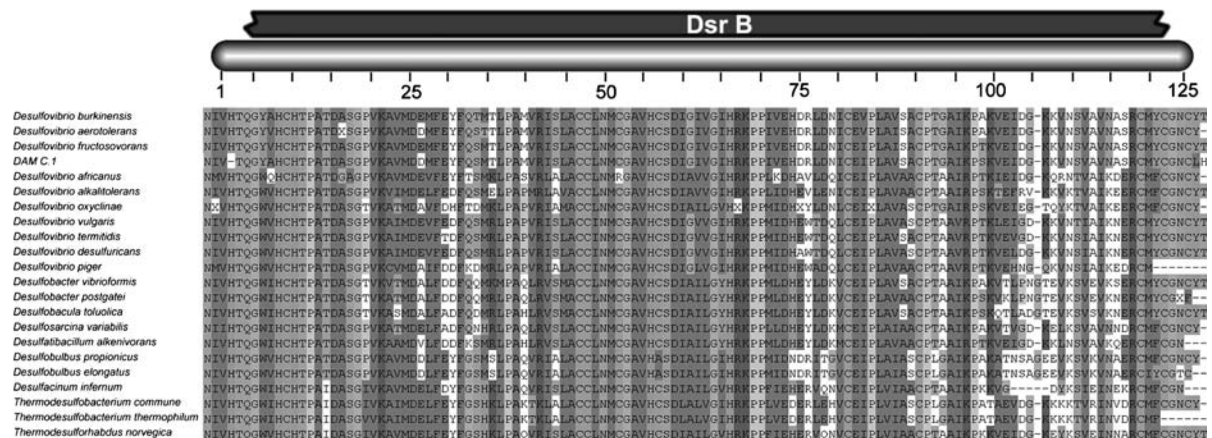

Fig. 4 The amino acid sequence alignment with sequences of orthologs in the domain of the *dsr*B enzyme

are diluted when entering the reactor and if the alkalinity production rate is larger than the increasing acidity rate, pH does not drop and the SRB activity is not affected. Notwithstanding, the isolation of a SRB strain able to start growing at pH 5.5 enables a more direct approach for an anaerobic AMD treatment, without a previous neutralization step to pH 7.0 as carried out in some industrial applications. The findings of the present work reinforce that AMD microbiology should be studied in detail so that sulfate reducing bacteria, able thrive in acid pH, can be identified and isolated from these environments.

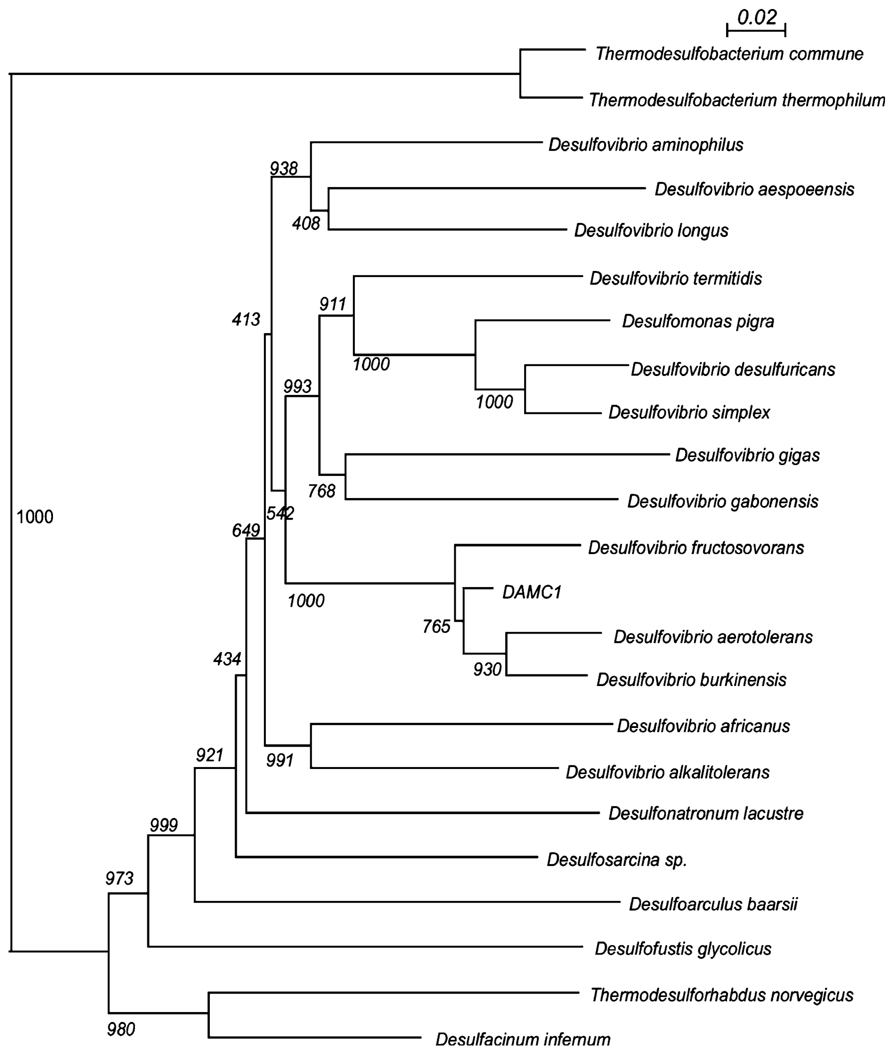

Fig. 5 Phylogenetic tree consensus based on nucleotide sequences of the *dsr*B gene with 376 bases. The tree was built with the neighbour-joining method by using MEGA 4.0 software. Bootstrap values are indicated at the nodes. The scale bar represents two nucleotide substitutions per 100 nucleotides. The accession number in Genbank of orthologs are *Desulfovibrio burkinensis* (AB061536.1), *Desulfovibrio aerotolerans* (AY749039.1), *Desulfovibrio fructosovorans* (AB061538.1) *Desulfovibrio alkalitolerans* (AY864856.1), *Desulfosarcina* sp. (AF360646), *Desulfovibrio africanus* (AF271772.1), *Desulfovibrio longus* (AB061540.1), *Desulfovibrio aespoeensis* (AF492838.1), *Desulfovibrio aminophilus* (AY626029.1), *Desulfovibrio gabonensis* (AY626027.1), *Desulfovibrio gigas* (DGU80961), *Desulfovibrio simplex* (AB061541.1), *Desulfovibrio desulfuricans* (DDE249777), *Desulfomonas pigra* (AB061534.1), *Desulfovibrio termitidis* (AB061542.1), *Desulfonatronum lacustre* (AF418189), *Desulfoarculu baarsii* (AF334600), *Desulfofustis glycolicus* (AF482457.1), *Desulfacinum infernum* (AF482454.1), *Thermodesulfobacterium thermophilum* (AF334598.1), *Thermodesulfobacterium commune* (AF334596.1), *Thermodesulforhabdus norvegicus* (AJ277293.1)

Aspects of the C.1 strain are currently being characterized along with its performance in a continuous anaerobic reactor.

DNA was isolated from a C.1 strain and used as a template for PCR. Agarose gel electrophoresis of the PCR products revealed the size of amplified fragments, which was as expected (Geets et al. 2006). The *dsr*B gene fragments, defined by the primer pair DSRp2060F/DSR4R from the C.1 strain, were sequenced and 376 nucleotides were determined. This sequence was deposited in GenBank with access number EU086051. The sequence was translated into protein sequence using Artemis software. Total of 125 amino acid residues were obtained, although 119 amino acids belonged to the conservation domain *dsr*B (COG2221.2). The result of BLASTN showed high similarities with the genera *Desulfovibrio*. The species *D. fructosovorans* (access number AB061538.1) and *D. burkinensis* (access number AB061536.1) had the same similarity of 93%, while *D. aerotolerans* (access number AY749039.1) had a similarity of 92%. The amino acid sequence was aligned with various sequences of orthologs in the domain of the DSR enzyme. The result demonstrated a region of high conservation inside the *dsr*B gene (Fig. 4).

The phylogenetic relationships of *dsr*B nucleotide sequences are shown in Fig. 5. The *dsr*B sequence of the C.1 strain formed an independent branch within a monophyletic group consisting of *D. fructosovorans*, *D. burkinensis* and *D. aerotolerans*. The results of bootstrap values, growth medium specific for *D. fructosovorans*, (fructose replaced lactate in the Postgate C medium, data not shown) for instance, did not support an appreciable growth rate. Also, morphological analysis did not indicate the presence of flagellum, which is characteristic of *D. fructosovorans*, *D. burkinensis* and *D. aerotolerans* (Ollivier et al. 1988; Ouattara et al. 1999; Mogensen et al. 2005). In summary, this suggests that the C.1 strain can be a new *Desulfovibrio* species. In sequence, new studies are being carried out with the 16S rRNA gene to confirm this hypothesis.

*dsr*B, a key enzyme in dissimilatory sulfate reduction, occurs in all SRB. This enzyme is very important in the metabolism of the SRB. It catalyzes the six-electron reduction of sulfite to sulfide (Zverlov et al. 2005; Dar et al. 2007b). The expression profile of the *dsr*B was investigated with the initial pHs of the growth medium (Postgate C) adjusted to 5.5 and 7.0 aiming to determine the metabolic activity of the C.1 strain at these pHs. As observed in Fig. 6, at both pHs and during two different growth times, the expression profile is equivalent, suggesting that pH 5.5 did not adversely affect the transcription of this gene. This investigation is essential for the comprehension of metabolic activity of SRB in changing conditions. Because of the potential for AMD treatment of this strain, a series of experiments will be carried out to establish an ideal growth medium, aiming at maximizing sulfate reduction capacity. Furthermore, a biotechnological application involving anaerobic continuous reactors will be studied.

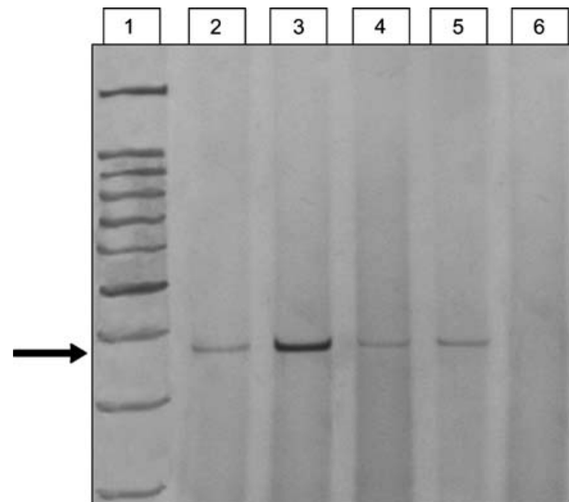

Fig. 6 Expression of *dsr*B gene, visualized in acrylamide gel stained with silver. In each lane 10 μl of the PCR product were applied. (1) Molecular weight marker at 100 bp (invitrogen); (2) after 30 h in pH 5.5; (3) after 100 h in pH 5.5; (4) after 30 h in pH 7.0; (5) after 100 h in pH 7.0 and (6) negative control

Conclusions

A *Desulfovibrio* strain was isolated from an AMD in a Brazilian tropical region, showing an unexpected sulfate reducing capacity. The isolate reduced sulfate at similar rates at both initial pHs 5.5 and 7.0. This brings new possibilities for the treatment of AMD, as acid water would be neutralized to mildly acidic conditions.

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