

Article

Influence of *Sulfobacillus thermosulfidooxidans* on Initial Attachment and Pyrite Leaching by Thermoacidophilic Archaeon *Acidianus* sp. DSM 29099

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Abstract: At the industrial scale, bioleaching of metal sulfides includes two main technologies, tank leaching and heap leaching. Fluctuations in temperature caused by the exothermic reactions in a heap have a pronounced effect on the growth of microbes and composition of mixed microbial populations. Currently, little is known on the influence of pre-colonized mesophiles or moderate thermophiles on the attachment and bioleaching efficiency by thermophiles. The objective of this study was to investigate the interspecies interactions of the moderate thermophile *Sulfobacillus thermosulfidooxidans* DSM 9293^T and the thermophile *Acidianus* sp. DSM 29099 during initial attachment to and dissolution of pyrite. Our results showed that: (1) *Acidianus* sp. DSM 29099 interacted with *S. thermosulfidooxidans*^T during initial attachment in mixed cultures. In particular, cell attachment was improved in mixed cultures compared to pure cultures alone; however, no improvement of pyrite leaching in mixed cultures compared with pure cultures was observed; (2) active or inactivated cells of *S. thermosulfidooxidans*^T on pyrite inhibited or showed no influence on the initial attachment of *Acidianus* sp. DSM 29099, respectively, but both promoted its leaching efficiency; (3) *S. thermosulfidooxidans*^T exudates did not enhance the initial attachment of *Acidianus* sp. DSM 29099 to pyrite, but greatly facilitated its pyrite dissolution efficiency. Our study provides insights into cell-cell interactions between moderate thermophiles and thermophiles and is helpful for understanding of the microbial interactions in a heap leaching environment.

Keywords: bioleaching; initial attachment; biofilm formation; *Sulfobacillus*; *Acidianus*; interspecies interactions

1. Introduction

Bioleaching is applied at an industrial scale (biomining) for extraction of valuable metals from mineral concentrates and low-grade ores [1,2], for instance copper, zinc, cobalt, and so on [3,4]. Bioleaching can occur in nature and accelerate acidity of ground water and cause serious environmental problems, like acid mine/rock drainage (AMD/ARD) [5,6]. Microorganisms involved in the dissolution of metal sulfides are mainly extremely acidophilic archaea and bacteria, which are able to oxidize reduced inorganic sulfur compounds (RISCs) and/or iron(II) ions [7,8].

Sulfobacillus sp., Gram-positive bacteria, are moderately thermophilic acidophiles that can utilize a broad range of energy substrates, including iron(II) ions, RISCs, and mineral sulfides in environments with high metal concentrations [9,10]. Since spore formation enables *Sulfobacillus* to survive long periods of time under unfavorable, or even deleterious, growth conditions, these bacteria are often

found in heap leaching operations and acidic geothermal springs [10]. *Acidianus* sp. are facultatively anaerobic thermophilic archaea capable of growth at high temperature and at low pH with iron(II) ions, RISCs, metal sulfides, or complex organic compounds [11]. These thermophiles can be used to bioprocess recalcitrant minerals, e.g., chalcopyrite [12].

At the industrial scale, the main technologies used for metal recovery include dumps, heaps, in situ and in stope leaching, vat leaching, and stirred tank leaching [3]. Mesophilic, moderately thermophilic, and thermophilic microbes are involved in heap leaching [13]. The dominant microorganisms in both tank and heap leaching environments are *Sulfobacillus*, *Acidithiobacillus caldus*, *Ferroplasma* spp, *Leptospirillum ferriphilum*, and thermophiles *Sulfolobus* sp. and *Acidianus* sp. [13–16]. Fluctuations in temperature can have a pronounced effect on the growth of individual microbes and also on the composition of mixed microbial populations. Mesophilic and moderately thermophilic microbes are present in heaps at the beginning of heap leaching. Reactions involved in the biological oxidation of mineral sulphides (exothermic reactions) result in heat generation and the increased temperature provides favourable conditions for the growth of thermophilic microorganisms [13,17]. Mineral sulphide oxidation is compromised at elevated temperatures, unless an appropriate thermophilic consortium is present in the process [13].

Microbial attachment to and biofilm formation on mineral sulphides are of great importance for mineral bioleaching [18]. During the succession of microbial communities caused by temperature fluctuations, attached populations may subsequently change. It has been shown that the presence of *L. ferriphilum* in the biofilm can affect the initial attachment of *S. thermosulfidooxidans* to pyrite [19]. Additionally, the presence of an active biofilm of iron oxidizers may influence the subsequent cell attachment of other species [20]. It was shown that *At. thiooxidans* cells showed a 40% increased attachment to pyrite pre-colonized by cells of *At. ferrooxidans* or *L. ferrooxidans*. In addition, the attachment of *At. thiooxidans* was more rapid if the pyrite was already colonized by *L. ferrooxidans*, but not by *At. ferrooxidans* [21].

This study focused on how pyrite pre-colonized by moderately thermophilic microorganisms affected the attachment, colonization and pyrite bioleaching efficiency of thermophiles. In this study, we have chosen the moderate thermophile *S. thermosulfidooxidans* DSM 9293^T and a thermophile *Acidianus* sp. DSM 29099 to study their interspecies interactions with respect to the pyrite-leaching process. The effect of pyrite pre-colonized with cells of *S. thermosulfidooxidans*^T on initial attachment and bioleaching by *Acidianus* sp. DSM 29099 were evaluated in a simulated heap leaching environment.

2. Experimental Section

2.1. Strains and Media

Acidianus sp. DSM 29099 (GeneBank accession number of 16S rRNA: KJ921703) was isolated from one of the geothermal hot springs at Caviabue–Copahue, Neuquen, Argentina [22]. *S. thermosulfidooxidans* DSM 9293^T was purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). The cultures were cultivated in Mackintosh (MAC) medium [23] with an initial pH of 1.7. Cultures were grown on pyrite (10% *w/v*) with yeast extract (0.2 g/L). Cells of *Acidianus* sp. DSM 29099 and *S. thermosulfidooxidans*^T were cultivated at 65 and 45 °C, respectively, with shaking at 120 rpm.

2.2. Pyrite Preparation

Pyrite grains with a diameter of 200–500 µm were selected after grinding and sieving of pyrite cubes (origin Navajun, Spain). These were used for the attachment and leaching experiments. Pyrite slices with a size of approx. 1 cm × 1 cm × 2 mm were cut from pyrite cubes and were used for visualization of biofilms. Both slices and grains were washed with boiling 6 M HCl for 30 min, and rinsed with deionized water until a neutral pH. Afterwards, pyrite samples were washed three times with acetone. These treatments were done in order to remove impurities such as iron ions and sulphur

compounds on pyrite surface. After cleaning, pyrite was dried at 80 °C for 12 h and sterilized at 120 °C in a nitrogen atmosphere for 24 h [24].

2.3. Iron and pH Determination

Iron ions were determined according to German standard method for the examination of water, wastewater, and sludge (DIN 38406-1). The determination was performed in 96-well-plate and scanned at 492 nm with a spectrophotometer equipped with a microplate reader (Infinite[®]200 PRO, TECAN, Männedorf, Switzerland). The iron standard solutions used for calibration curve were prepared in the range of 0.8–6 mg/L.

For iron(II) ion determination, each sample was mixed with 2 mL of master solution consisting of ammonium acetate solution (16 g/L in glacial acetic acid) and (1,10)-phenanthroline chloride monohydrate (0.2 g/L). The mixtures were incubated for 15 min in the dark. After the measurement, hydroxylammonium chloride (4 g/L) was added into the samples for total iron determination. After 15 min incubation in the dark, samples were measured again at 492 nm. Iron(III) ion concentration can be determined by subtracting the one of iron(II) ions from the one of total iron ions.

A WTW (pH 537) pH meter with a Mettler Toledo electrode (InLab[®] 422 semimicro pH electrode; Mettler Toledo GmbH, Urdorf, Switzerland) was used for pH determination. The pH meter was calibrated before measurements by using pH calibration solutions (Roth, Germany).

2.4. Preparation of Pyrite Pre-Colonized by *S. thermosulfidooxidans*^T or of *S. thermosulfidooxidans*^T Exudates

Cells of *S. thermosulfidooxidans*^T with an initial cell number of 5×10^8 cells/mL were inoculated in 100 mL Erlenmeyer flasks containing 10% (*w/v*) pyrite grains and 50 mL MAC medium. The cultures were incubated at 45 °C with shaking at 120 rpm for seven days. Pyrite grains colonized with active or inactivated biofilm cells of *S. thermosulfidooxidans*^T were prepared according to the previous report [21]. Briefly, pyrite grains colonized with active cells were obtained by washing the grains twice with 50 mL sterile MAC medium. To prepare pyrite grains colonized by inactive cells, pyrite grains attached with cells were subsequently incubated at 80–100 °C for 2 h.

The efficiency of this inactivation procedure was evaluated. Briefly, the inactive cells and active cells were both inoculated in the fresh MAC medium at 45 °C for three days. Cell growth from both inactive cells and active cells was compared.

The culture supernatants were filtered into a new sterilized 100 mL Erlenmeyer flask through a filter with the pore-size of 0.2 µm. The exudates were determined to be contained 0.5 g/L iron(III) ions. Thus, supplementation with 0.5 g/L iron(III) ions was tested to evaluate their influence on cell initial attachment and leaching efficiency by *Acidianus* sp. DSM 29099.

2.5. Initial Attachment

Initial attachment tests were carried out in 100 mL Erlenmeyer flasks containing 50 mL MAC medium, 0.2 g/L yeast extract, and 10% (*w/v*) pyrite or pyrite pre-colonized by cells of *S. thermosulfidooxidans*^T. Cell attachment of *Acidianus* sp. DSM 29099 to clean pyrite in the presence of *S. thermosulfidooxidans*^T exudates was also tested.

Surface area may be a limiting factor for cell attachment. Based on surface area of a pyrite grain ($= 4\pi r^2$) and a cell ($= 2\pi rh + 2\pi r^2$) used in the experiments, 10% of pyrite was used for the attachment experiments. This provided sufficient surface area for cell attachment (Supplementary Figure S1). Briefly, cells were cultivated at 65 °C (pure culture of *Acidianus* sp. DSM 29099) or 45 °C (pure culture of *Acidianus* sp. DSM 29099, *S. thermosulfidooxidans*^T and mixed cultures) and shaking at 120 rpm. Cell numbers were monitored by direct counting using a Thoma chamber within the first 6 h. Control experiments without adding pyrite were also included. Each experiment was performed in triplicate. The initial cell number was 1×10^8 cells/mL for pure cultures. For mixed cultures, the initial total cell number was 1×10^8 cells/mL (Mix 1) or 2×10^8 cells/mL (Mix 2) with equal cell numbers for each species.

The attached cells were calculated by subtracting the number of planktonic cells from the initial total cell number. The theoretical attachment rate of mixed cultures (A_t) was calculated by averaging the sum of the attachment rate of each pure culture [25]. The formula is as follows:

$$A_t = (A_a + A_{S,t})/2$$

where A_t = theoretical attachment rate of mixed cultures, a = *Acidianus* sp. DSM 29099, and $S.t$ = *S. thermosulfidooxidans*^T.

2.6. Leaching Experiments

Leaching experiments were carried out in 100 mL Erlenmeyer flasks containing 50 mL MAC medium, 0.2 g/L yeast extract, and 10% (*w/v*) of pyrite or pyrite pre-colonized by cells of *S. thermosulfidooxidans*^T. Leaching activity of *Acidianus* sp. DSM 29099 with clean pyrite in the presence of *S. thermosulfidooxidans*^T exudates was also tested. Briefly, leaching experiments were carried out for three weeks. In addition, pyrite leaching experiments in MAC medium without adding cells of *S. thermosulfidooxidans*^T as an abiotic control were included. To determine the bioleaching efficiency, 1 mL of leach solutions were withdrawn from each flask at specific intervals and used for determining planktonic cell number, pH, and total iron concentration. The increase of total iron was used to estimate the pyrite dissolution efficiency. Each experiment was performed in triplicate.

2.7. Visualization of Biofilm Formation by Confocal Laser Scanning Microscopy

For visualization of cells and biofilms on pyrite, samples were stained and observed by confocal laser scanning microscopy (CLSM) according to the previous report [26]. Briefly, pyrite samples colonized with cells were rinsed by 1 mL deionized, particle-free water three times. Nucleic acid staining was done by covering the samples with 6 μ M SYTO 9 (Invitrogen, Carlsbad, CA, USA) for 15 min. After staining, a washing step was carefully performed by using 1 mL particle-free deionized water to remove the unbound stain. Pyrite grains were fixed on diagnostic microscope slides (Gerhard Menzel, Braunschweig, Germany, GmbH) by using an anti-fading agent (Citifluor, Ltd., London, UK, AF2) and covered by coverslips. All staining was performed in the dark. Examination of stained samples was performed by CLSM using a laser scanning module (LSM 510 Carl Zeiss[®], Jena, Germany) coupled to an inverted Axiovert100MBP microscope (Zeiss[®], Hallbergmoos, Germany). The laser excitation wavelength (Argon laser) 488 nm was used to detect fluorophore signals. Surface topography and texture of the pyrite surface were recorded by using the CLSM in reflection mode. Fluorescence images were analysed by an extended version of the software ImageJ (NIH, Bethesda, MD, USA) [27].

3. Results and Discussion

3.1. Initial Attachment of Cells to Pyrite

3.1.1. Pure and Mixed Cultures

Pure cultures of *S. thermosulfidooxidans*^T exhibited the lowest attachment to pyrite grains, with 17% of total cells attached to pyrite after 6 h incubation (Figure 1), which coincides with previous reports [19,28]. In case of *Acidianus* sp. DSM 29099, cells showed the highest attachment, with 46% of total cells attached to pyrite when cultivated at 65 °C. Both species did not show a clear tendency to attach to the glass wall of Erlenmeyer flasks (Supplementary Figure S2). In a previous study, it was found that up to 60% and 35% of cells adhered to pyrite, and chalcopyrite surfaces within the first 2 h [29]. In case of *Acidianus copahuensis* 29038, maximum initial cell attachment to pyrite and chalcopyrite were 43% and 69%, respectively [30,31]. These data suggest that cell attachment strongly depends on the nature of the species and the pre-cultivation conditions [18,32]. In addition, it has

been reported that even within the same species, different strains have a different affinity for the same mineral [33].

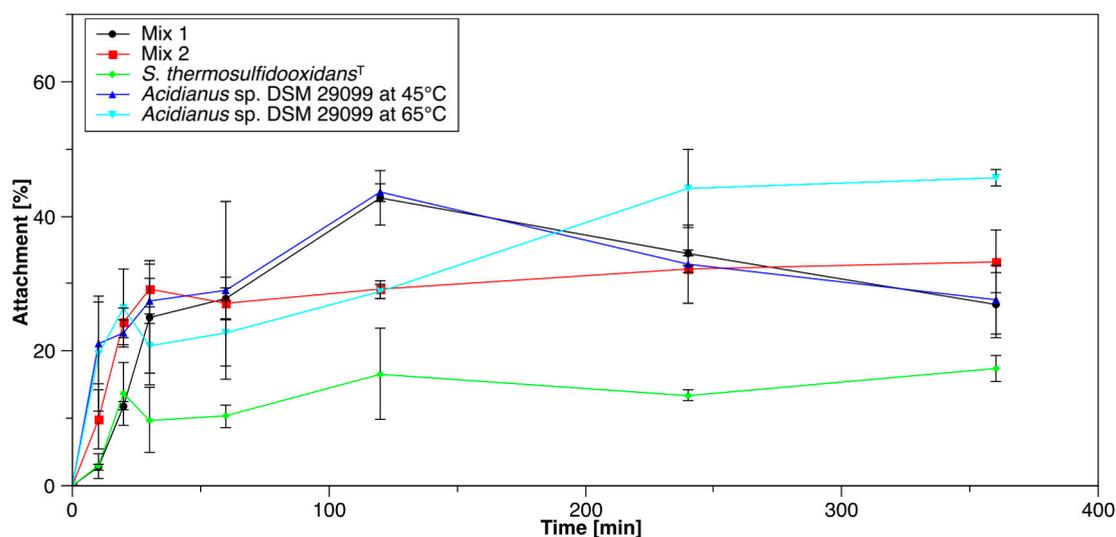


Figure 1. Initial attachment of cells of *Acidianus* sp. DSM 29099 and *S. thermosulfidooxidans*^T to pyrite in pure and mixed cultures. The initial cell number was 1×10^8 cells/mL for each pure culture and Mix 1, and 2×10^8 cells/mL for Mix 2, respectively. Cells were cultivated in 50 mL MAC medium (pH 1.7) with 0.2 g/L yeast extract containing 10% pyrite grains (200–500 μm) at 45 °C and 120 rpm.

Attachment of *Acidianus* sp. DSM 29099 at 45 °C was around 28% (Figure 1). It was 65% less than when cultivated at 65 °C (its optimal growth temperature). Obviously, the cultivation temperature influences the initial attachment of cells of *Acidianus* sp. DSM 29099. This is in agreement with the previous report that attachment and biofilm formation by *Metallosphaera hakonensis* showed the highest attachment at the optimal growth temperature and less cells attached to minerals at reduced temperature [34].

The attachment of mixed cultures was compared to each pure culture with the theoretical value (for the pure culture): $(23\% = (28\% + 17\%)/2)$. In this test, 27% of the cells in sample Mix 1 (initial total cell number: 1×10^8 cell/mL) and 33% of cells in sample Mix 2 (initial total cell number: 2×10^8 cell/mL) were detected to attach to pyrite, respectively (Figure 1). Both attachment rates of mixed cultures showed an increase compared to the theoretical attachment of pure cultures. This suggests that cells of *Acidianus* sp. DSM 29099 and *S. thermosulfidooxidans*^T may have some interactions influencing the attachment. Although no quorum-sensing molecules from acidophilic archaea have been detected, specific interactions like cell-cell communication in the form of quorum sensing between the two species may exist. Cells in Mix 2 showed a considerably increased attachment (24%) if compared to Mix 1, indicating that initial cell density could influence the initial attachment in mixed cultures. The attachment dependency on initial cell number has been reported for *Acidithiobacillus ferrooxidans* to refractory gold concentrate [27]. It might be that cell-cell interactions between *Acidianus* sp. DSM 29099 and *S. thermosulfidooxidans* are cell density-dependent. In the case of *Leptospirillum ferrooxidans* DSM 2391 and *Acidithiobacillus caldus* DSM 8584, a mixed culture of the two species showed 37% increased attachment ratio to pyrite compared to the theoretical attachment value for pure cultures [25].

3.1.2. *Acidianus* sp. DSM 29099 to Pyrite Pre-Colonized with *S. thermosulfidooxidans*^T

The efficiency of the inactivation procedure was confirmed due to the fact that no cell growth was observed in the fresh MAC medium with inactive cells on pyrite after three days incubation. By contrast, with active cells on pyrite cell numbers reached 3×10^8 cells/L (not shown). Attached cells of *Acidianus* sp. DSM 29099 increased over time within 6 h under all test conditions (Figure 2).

A notable increase of the attached cells to clean pyrite was noticed and the attachment reached 28% within the first 20 min. By comparison, only 5% of inoculum attached to the pyrite pre-colonized by active cells of *S. thermosulfidooxidans*^T and almost no cells attached to the pyrite colonized with dead *S. thermosulfidooxidans*^T cells within the first 20 min. There is no notable difference between the attachment of *Acidianus* sp. DSM 29099 to pre-colonized pyrite with active cells or inactive cells during the early stages of the experiments. They do not diverge until about 2 h of incubation. After 6 h, more than 46% of the total cells attached to the clean pyrite, while approximately 44% and 31% of the total cells attached to the pyrite covered with active and inactivated *S. thermosulfidooxidans*^T cells, respectively. The presence of active and inactive cells of *S. thermosulfidooxidans*^T on the surface of pyrite grains both inhibited the attachment of *Acidianus* sp. DSM 29099 at the beginning of the experiment. The pre-colonization by cells of *S. thermosulfidooxidans*^T may change the surface properties (e.g., hydrophobicity) in a way which is inhibitory to cell attachment of cells of *Acidianus* sp. DSM 29099. Another assumption is that the sites favourable for cell attachment have been occupied by *S. thermosulfidooxidans*^T cells, resulting in a reduced number of active sites available for cells of *Acidianus* sp. DSM 29099. In addition, the release of some antagonistic compounds by *S. thermosulfidooxidans*^T cells may be possible.

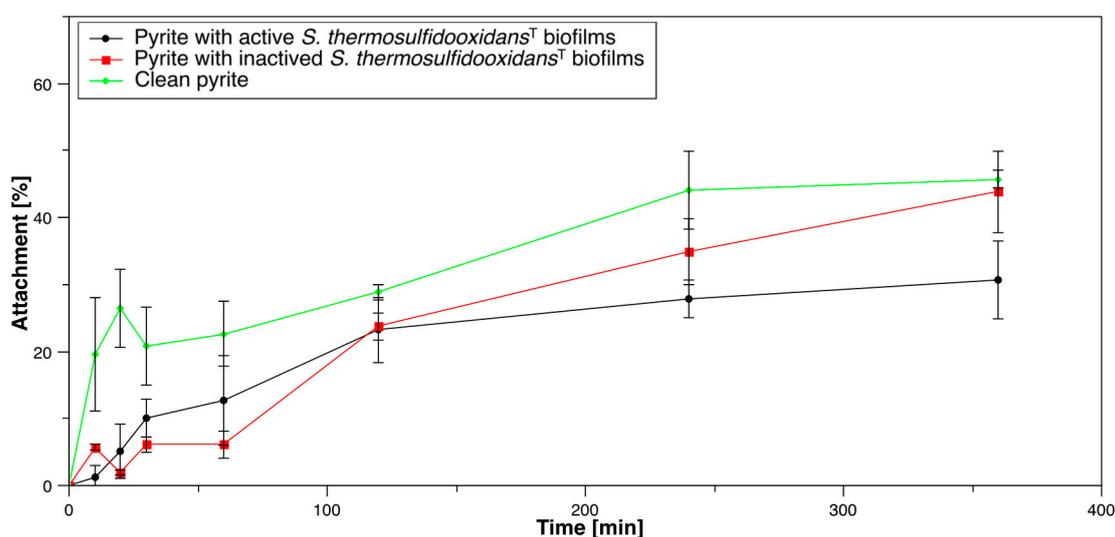


Figure 2. Initial attachment of cells of *Acidianus* sp. DSM 29099 to pyrite pre-colonized by cells of *S. thermosulfidooxidans*^T. Cells of *Acidianus* sp. DSM 29099 (1×10^8 cells/mL) were incubated in 50 mL MAC medium (pH 1.7) containing 0.2 g/L yeast extract and 10% (*w/v*) pyrite grains (200–500 μ m) at 65 °C and 120 rpm.

3.1.3. Influence of *S. thermosulfidooxidans*^T Exudates on Initial Attachment of *Acidianus* sp. DSM 29099

After 6 h incubation, 46% and 48% of total cells attached to pyrite with and without 0.5 g/L iron(III) ion supplementation, respectively (Figure 3). Thus, it seems that iron(III) ions had no significant effect on initial cell attachment of *Acidianus* sp. DSM 29099. This is, however, in contrast to the previous study indicating that attachment of *Acidithiobacillus* was promoted by addition of 1 mM iron(III) ions, especially when cells were pre-grown on pyrite [35]. When incubated with *S. thermosulfidooxidans*^T exudates, 57% of the cells of *Acidianus* sp. DSM 29099 attached to pyrite within 6 h (Figure 3). However, there was no significant difference before 4 h incubation. Thus, *S. thermosulfidooxidans*^T exudates did not show clear stimulation on the initial attachment of *Acidianus* sp. DSM 29099. The organic compounds in the *S. thermosulfidooxidans*^T exudates, probably humic acids, proteins, and polysaccharides [36], may be relevant to the cell attachment of *Acidianus* sp. DSM 29099. It has been reported that surfaces immersed in an aquatic environment adsorb dissolved organic matter [37,38]. This is called the conditioning film. The EPS from acidophiles grown on minerals can be partly

considered as surface active compounds [39]. These can create a conditioning film at the interface and thus affect the cellular attachment [40]. Such compounds have already been detected on surfaces of cells of *Acidianus*, *Sulfolobus metallicus*, *Ferroplasma acidiphilum*, and *Acidithiobacillus* sp. and they may be involved in the initial stages of adhesion to mineral or sulphur surfaces [22,41,42]. This conditioning film formed by *S. thermosulfidooxidans*^T exudates may have an effect on the cellular attachment to surfaces [43,44].

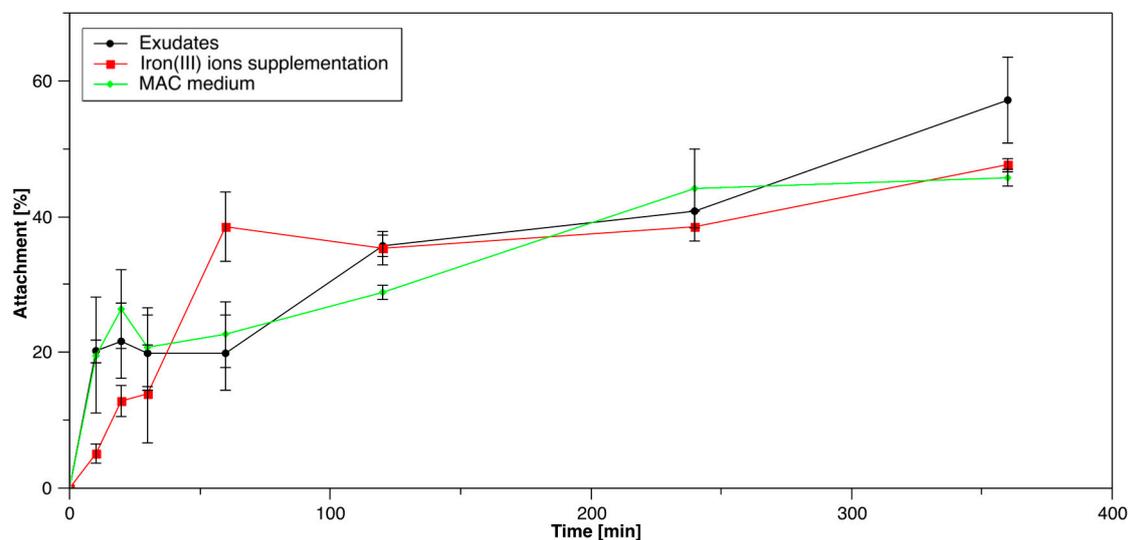


Figure 3. Influence of *S. thermosulfidooxidans*^T exudates on the initial attachment of cells of *Acidianus* sp. DSM 29099 to pyrite. Cells of *Acidianus* sp. DSM 29099 with an initial cell number of 1×10^8 cells/mL were incubated at 65 °C and 120 rpm.

3.2. Biofilm Formation

3.2.1. *Acidianus* sp. DSM 29099 on Pyrite Pre-Colonized by *S. thermosulfidooxidans*^T

We first observed the morphology of biofilm cells on pyrite. Cells of *Acidianus* sp. DSM 29099 show a spherical appearance, but *S. thermosulfidooxidans*^T cells are rod-shaped. Thus, the two strains in mixed cultures are distinguishable by cell morphology (Supplementary Figure S3). Biofilm formation by *S. thermosulfidooxidans*^T cells on pyrite decreased after seven days of incubation [45]. The biofilm formation of *Acidianus* sp. DSM 29099 on pyrite pre-colonized with active/inactivated *S. thermosulfidooxidans*^T biofilms is shown in Figure 4. Biofilm cells of *Acidianus* sp. DSM 29099 increased over time on all kind of pyrite grains. On day 6, biofilm cells of *Acidianus* sp. DSM 29099 were found on clean pyrite as well as on pyrite pre-colonized by cells of *S. thermosulfidooxidans*^T. Pre-colonized *S. thermosulfidooxidans*^T cells were also observed (Figure 4, upper row). In addition, pyrite surfaces showed more *S. thermosulfidooxidans*^T cells after inactivation treatment than the surfaces without treatment. It could be that active cells of *S. thermosulfidooxidans*^T detached from the pyrite surface because of the unfavourable conditions (65 °C). On the pyrite surface with inactivated *S. thermosulfidooxidans*^T cells, *Acidianus* sp. DSM 29099 and pre-established *S. thermosulfidooxidans*^T cells showed comparable amounts on day 6. Due to the constant increase of attached *Acidianus* sp. DSM 29099 cells, the proportion of *S. thermosulfidooxidans*^T cells was reduced on pyrite (Figure 4). However, there were no notable differences in the biofilm formation by *Acidianus* sp. DSM 29099 on the grains as a result of the pyrite pre-treatment.

S. thermosulfidooxidans^T cells were detected on pyrite grains, which were pre-colonized either by active or inactivated cells during the whole test. However, in both cases no growth of cells of *S. thermosulfidooxidans*^T were observed (not shown). Cells of *Acidianus* sp. DSM 29099 were

heterogeneously distributed over the pyrite surface. No physical contact between the two species was observed (Figure 4).

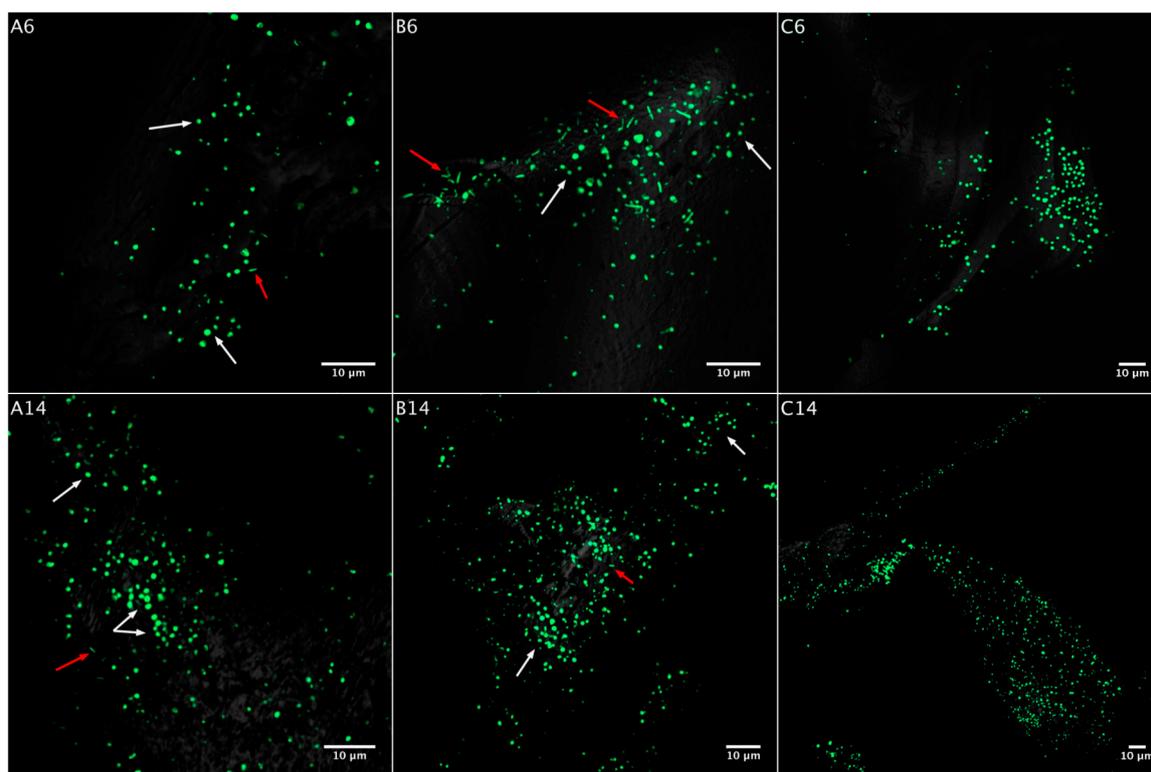


Figure 4. Biofilm formation of *Acidianus* sp. DSM 29099 on pyrite pre-colonized by *S. thermosulfidooxidans*^T cells. Cells of *Acidianus* sp. DSM 29099 with an initial cell number of 1×10^8 cells/mL were incubated at 65 °C and 120 rpm. A: Biofilms on pyrite pre-colonized by active *S. thermosulfidooxidans*^T. B: Biofilms on pyrite pre-colonized by inactivated *S. thermosulfidooxidans*^T. C: Biofilms on clean pyrite. Numbers behind the letters: days of incubation. Red arrows indicate *S. thermosulfidooxidans*^T cells and white arrows indicate *Acidianus* sp. DSM 29099 cells. All samples were stained by SYTO 9.

3.2.2. Influence of *S. thermosulfidooxidans*^T Exudates on Biofilm Formation by *Acidianus* sp. DSM 29099

Cells of *Acidianus* sp. DSM 29099 grown with iron(III) ion supplementation were often found on pyrite grains on day 3. In contrast, cells were rarely observed on pyrite grains, when grown in *S. thermosulfidooxidans*^T exudates (Supplementary Table S1). Attached cells on pyrite grains increased in all tested conditions over time (Figure 5). On day 6, biofilms were much denser with additional iron(III) ions than in the other growth conditions. This phenomenon was also observed on day 14 (Supplementary Table S1). Thus it seems that biofilm formation by cells of *Acidianus* sp. DSM 29099 is stimulated by additional iron(III) ions. Also, *S. thermosulfidooxidans*^T exudates seem to promote its biofilm development (Figure 5). It has been reported that addition of iron(III) ions promotes *A. ferrooxidans* attachment on pyrite [32,46]. Although addition of iron(III) ions showed no significant effects on cell initial attachment of *Acidianus* sp. DSM 29099 (Figure 3), biofilm cells of *Acidianus* sp. DSM 29099 on pyrite surfaces were greatly increased. Thus, iron(III) ions did not enhance the cell attachment at early stage (Figure 3) but may be relevant in the persistence of attachment and biofilm maintenance (Figure 4).

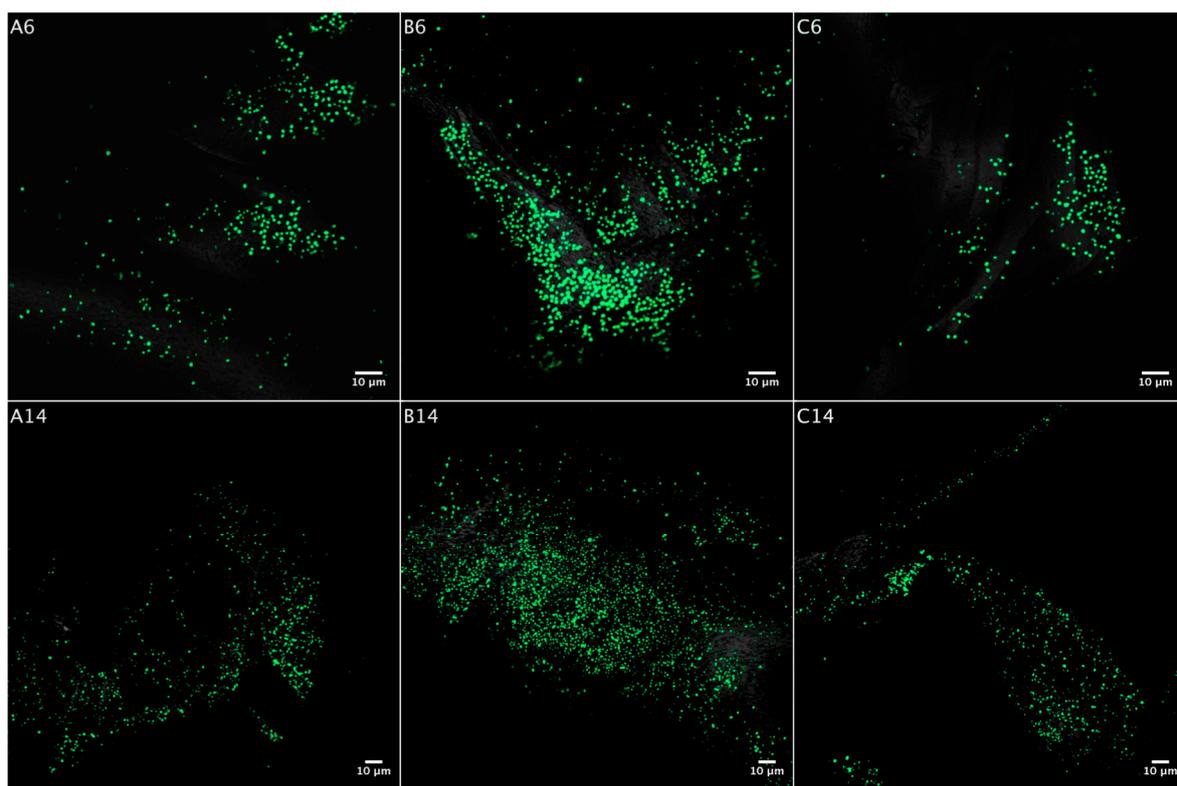


Figure 5. Influence of *S. thermosulfidooxidans*^T exudates on biofilms formation of *Acidianus* sp. DSM 29099 on pyrite. Cells of *Acidianus* sp. DSM 29099 with an initial cell number of 1×10^8 cells/mL were incubated at 65 °C and 120 rpm. A: Biofilms on pyrite in the presence of *S. thermosulfidooxidans*^T exudates. B: Biofilms on pyrite in the culture with additional iron(III) ions (0.5 g/L). C: Biofilms formation with no additions. Numbers behind the letters: days of incubation. All samples were stained by SYTO 9.

3.3. Pyrite Leaching

3.3.1. Pure and Mixed Cultures

Pure culture of *S. thermosulfidooxidans*^T showed a low leaching efficiency (Figure 6A), which is in agreement with the previous reports that pyrite oxidation by *Sulfobacillus* strains was slow [47,48]. *Acidianus* sp. DSM 29099 showed the highest pyrite leaching efficiency at 65 °C according to the highest concentration of total iron (5.0 g/L), the highest cell number (6.8×10^8 cells/mL) and pH changes (0.56). However, at 45 °C cell number or pH did not change notably (Figure 6B) and no significant leaching was noticed (Figure 6A). Pyrite leaching at 45 °C in both mixed cultures (Mix 1 and 2) was similar with that in the pure culture of *S. thermosulfidooxidans*^T. In abiotic controls at 65 °C around 190 mg/L total iron was detected, which was much higher than for the assay at 45 °C (30 mg/L, Supplementary Figure S4). In any case, abiotic leaching at 65 °C was negligible compared to bioleaching by *Acidianus* sp. DSM 29099. This agrees with the fact that pyrite can be oxidized by iron(III) ions but not proton [8]. A formation of flocs was observed for *Acidianus* sp. DSM 29099 in pure and mixed cultures with cells of *S. thermosulfidooxidans*^T if cultivated at 45 °C (Supplementary Figure S5).

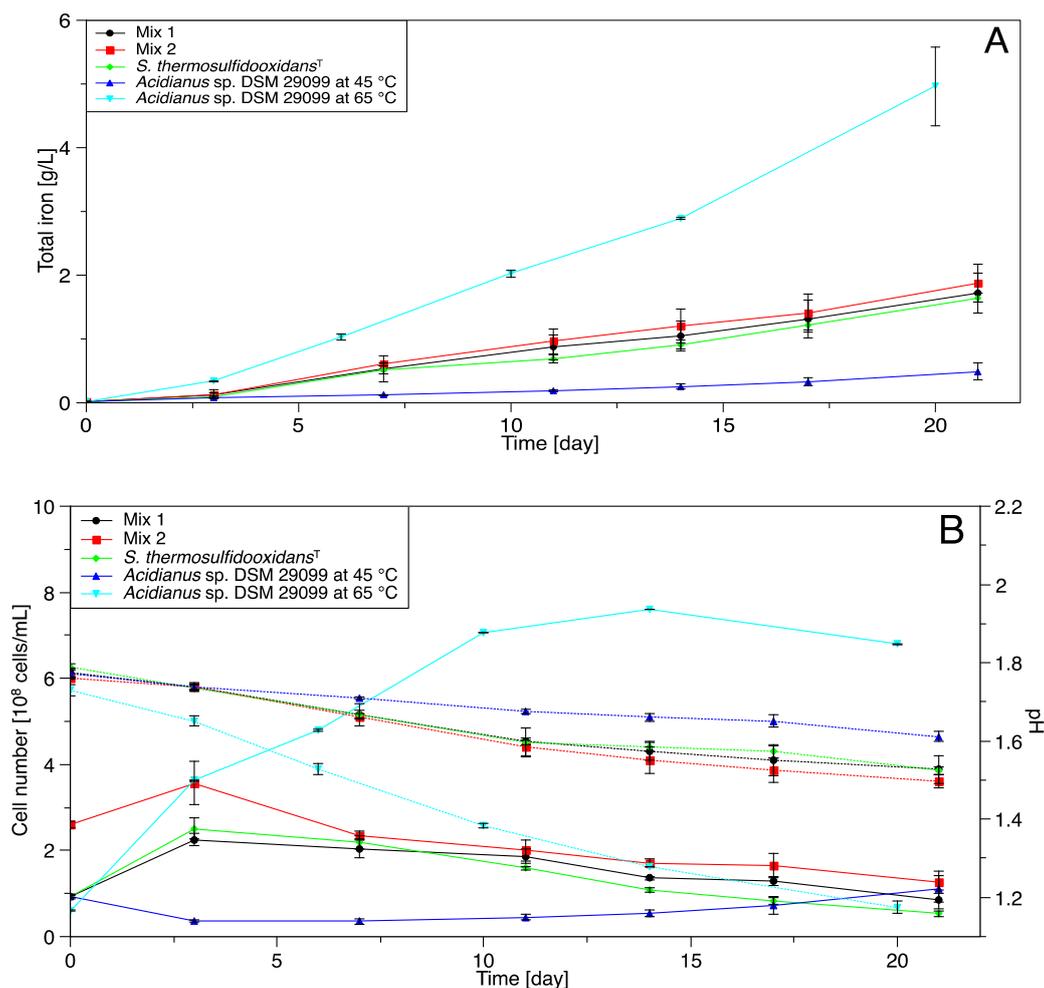


Figure 6. Pyrite leaching by cells of *Acidianus* sp. DSM 29099 and *S. thermosulfidooxidans*^T in pure and mixed cultures. (A) total iron concentrations; and (B) planktonic cell numbers (solid lines) and pH changes (dot lines).

3.3.2. Bioleaching by *Acidianus* sp. DSM 29099 with Pyrite Pre-Colonized by *S. thermosulfidooxidans*^T

The cell number of *Acidianus* sp. DSM 29099 grown on clean pyrite increased from 1×10^8 to 8×10^8 cells/mL after 14 days (Figure 7B). Then it slightly decreased to 7×10^8 cells/mL until the end of the incubation. When grown on pyrite pre-colonized with active or inactivated cells of *S. thermosulfidooxidans*^T, the numbers of planktonic cells kept increasing and reached 11×10^8 and 12×10^8 cells/mL, respectively (Figure 7B). Meanwhile, the decline of pH in all tested cultures from around 1.7 to 1.1 was correlated with the increased cell numbers of *Acidianus* sp. DSM 29099. The drop of pH in *Acidianus* sp. DSM 29099 cultures grown on pyrite pre-colonized by both active and inactivated cells of *S. thermosulfidooxidans*^T (0.7 ΔpH) was slightly higher than the one for clean pyrite (0.6) (Figure 7B). Total iron concentrations in all samples were continuously increasing during the experiments. The highest total dissolved iron concentration (~7.8 g/L) was detected in the culture with pyrite pre-colonized by both active and inactivated cells of *S. thermosulfidooxidans*^T (Figure 7A). Bioleaching by *Acidianus* sp. DSM 29099 with pyrite pre-colonized by either active or inactivated *S. thermosulfidooxidans*^T cells was ~55% higher than that with clean pyrite. This clearly shows that pyrite leaching by *Acidianus* sp. DSM 29099 was enhanced by pre-established biofilms of *S. thermosulfidooxidans*^T. However, no significant difference in leaching efficiency of *Acidianus* sp. DSM 29099 were detected with clean pyrite or pyrite pre-colonized by *S. thermosulfidooxidans*^T cells before 10 days of incubation. Although EPS are playing a key role in bioleaching, killed cells of *A. ferrooxidans*

with EPS caused no significant pyrite dissolution [46]. We observed that both, the active biofilm cells of *S. thermosulfidooxidans*^T on pyrite and the inactivated *S. thermosulfidooxidans*^T cells, showed no pyrite oxidation activity at 65 °C (not shown). The yeast extract was most probably exhausted in the medium after 10 days' incubation and EPS of *S. thermosulfidooxidans*^T may have facilitated bioleaching by *Acidianus* sp. DSM 29099. As mentioned above, the main components of *S. thermosulfidooxidans*^T EPS, such as humic acid, proteins, and polysaccharides [36] can be utilized by *Acidianus* sp. DSM 29099. Considering the reduced initial cellular attachment of *Acidianus* sp. DSM 29099 to pre-colonized pyrite by active cells of *S. thermosulfidooxidans*^T (Figure 2), it seems that the initial attachment to pyrite is not always correlated with pyrite dissolution.

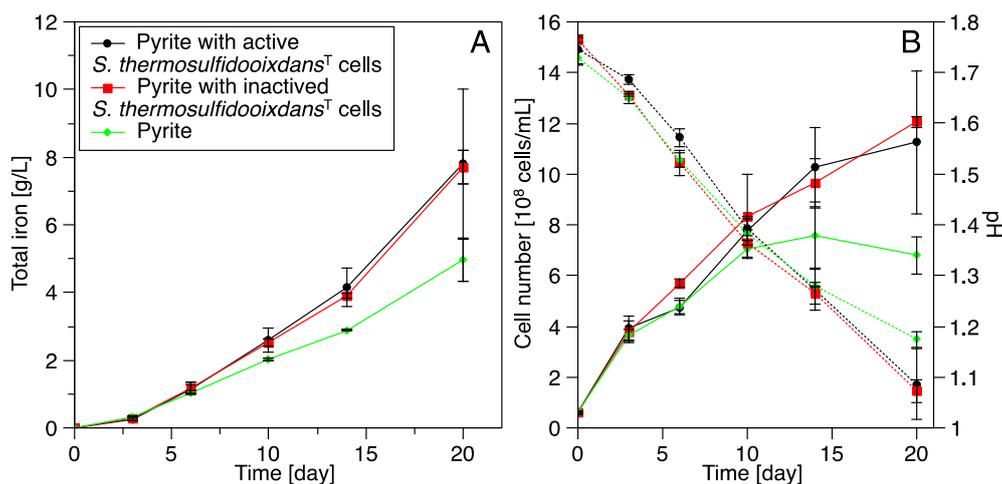


Figure 7. Influence of pre-established biofilms of *S. thermosulfidooxidans*^T on pyrite leaching by *Acidianus* sp. DSM 29099. (A) Total iron concentration; and (B) planktonic cell (solid lines) and pH changes (dot lines). Cells of *Acidianus* sp. DSM 29099 with an initial cell number of 1×10^8 cells/mL were incubated with 10% (w/v) pyrite grains (200–500 μ m) and cultivated at 65 °C and 120 rpm.

3.3.3. Influence of *S. thermosulfidooxidans*^T Exudates on Pyrite Leaching by *Acidianus* sp. DSM 29099

Cell numbers of *Acidianus* sp. DSM 29099 under all growth conditions increased gradually in the first 10 days (Figure 8). The maximum was reached in the culture with no supplementations (MAC medium) and in the one with iron(III) ion supplementation on day 10. However, cells in the culture with exudates of *S. thermosulfidooxidans*^T kept increasing and reached 1.5×10^9 cells/mL after 14 days, slightly decreasing to 1.3×10^8 cells/mL at the end of the experiments. Obviously, cell growth of *Acidianus* sp. DSM 29099 was promoted by *S. thermosulfidooxidans*^T exudates. The highest pH drop was noticed in the culture with *S. thermosulfidooxidans*^T exudates. The lowest pH change was found in the culture with no supplementation (MAC medium). The pH decrease was obviously correlated with cell growth and pyrite oxidation activity.

The highest total iron of 15.4 g/L until the end of the study, was measured in the culture with *S. thermosulfidooxidans*^T exudates. In the culture with supplementation of iron(III) ions, 7.3 g/L of total iron was detected. By contrast, only around 5 g/L of total iron was released in the culture with no supplementation (Figure 8). These data clearly show that exudates of *S. thermosulfidooxidans*^T or supplementation of iron(III) ions significantly enhance pyrite dissolution by *Acidianus* sp. DSM 29099 cells if compared to the ones grown in MAC medium with no supplementations. Due to metabolic versatility of *Acidianus* [49], exudates of *S. thermosulfidooxidans*^T could be used as energy source and promote cell growth. In addition, it is possible that certain molecules in *S. thermosulfidooxidans*^T exudates may influence pyrite leaching by *Acidianus* sp. DSM 29099. However, this needs further investigations.

It has been shown that cell attachment to ores as well as biofilm formation is correlated with pyrite leaching by acidophiles [18,50]. A recent study shows that biofilm populations of *At. ferrooxidans*^T are responsible for pyrite dissolution at the early stage (first 4–5 days). However, planktonic cells are contributing substantially later on in the bioleaching process [35]. Thus, it is not surprising that the initial attachment of *Acidianus* sp. DSM 29099 was not enhanced (Figure 3) or even inhibited (Figure 2) but pyrite leaching was promoted (Figures 7 and 8).

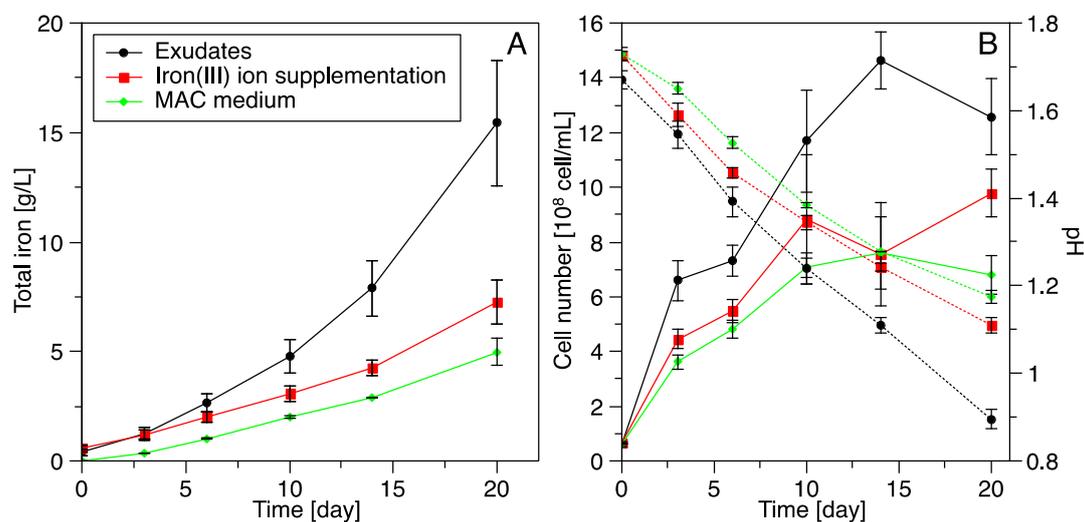


Figure 8. Influence of *S. thermosulfidooxidans*^T exudates on pyrite leaching by *Acidianus* sp. DSM 29099. (A) total iron concentration; and (B) planktonic cell (solid lines) and pH change (dot lines). Cells of *Acidianus* sp. DSM 29099 with an initial cell number of 1×10^8 cells/mL were incubated with 10% (*w/v*) pyrite grains (200–500 μ m) and cultivated at 65 °C and 120 rpm.

4. Summary and Conclusions

We studied the interspecies interactions of *S. thermosulfidooxidans*^T and *Acidianus* sp. DSM 29099 during pyrite leaching. Pre-colonized biofilms of *S. thermosulfidooxidans*^T promoted pyrite leaching by *Acidianus* sp. DSM 29099, while the initial attachment of *Acidianus* sp. DSM 29099 was not positively influenced. *S. thermosulfidooxidans*^T exudates did not enhance the initial attachment but significantly facilitated the pyrite leaching by *Acidianus* sp. DSM 29099. Our data suggest the presence of interactions between *S. thermosulfidooxidans*^T and *Acidianus* sp. DSM 29099 during pyrite dissolution. Further investigations on the interaction mechanisms between these acidophilic bacteria and archaea may allow to design and optimize microbial consortia to be used in bio-heap leaching applications.

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