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(Bio) chemistry of bacterial leaching—direct vs. indirect bioleaching

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Abstract

Bioleaching of metal sulfides is effected by bacteria, like *Thiobacillus ferrooxidans*, *Leptospirillum ferrooxidans*, *Sulfolobus / Acidianus*, etc., via the (re)generation of iron(III) ions and sulfuric acid.

According to the new integral model for bioleaching presented here, metal sulfides are degraded by a chemical attack of iron(III) ions and/or protons on the crystal lattice. The primary iron(III) ions are supplied by the bacterial extracellular polymeric substances, where they are complexed to glucuronic acid residues. The mechanism and chemistry of the degradation is determined by the mineral structure.

The disulfides pyrite (FeS_2), molybdenite (MoS_2), and tungstenite (WS_2) are degraded via the main intermediate thiosulfate. Exclusively iron(III) ions are the oxidizing agents for the dissolution. Thiosulfate is, consequently, degraded in a cyclic process to sulfate, with elemental sulfur being a side product. This explains, why only iron(II) ion-oxidizing bacteria are able to oxidize these metal sulfides.

The metal sulfides galena (PbS), sphalerite (ZnS), chalcopyrite ($CuFeS_2$), hauerite (MnS_2), orpiment (As_2S_3), and realgar (As_4S_4) are degradable by iron(III) ion and proton attack. Consequently, the main intermediates are polysulfides and elemental sulfur (thiosulfate is only a by-product of further degradation steps). The dissolution proceeds via a H_2S^{*+}-radical and polysulfides to elemental sulfur. Thus, these metal sulfides are degradable by all bacteria able to oxidize sulfur compounds (like *T. thiooxidans*, etc.). The kinetics of these processes are dependent on the concentration of the iron(III) ions and, in the latter case, on the solubility product of the metal sulfide.

Keywords: Chemolithotrophic bacteria; Bioleaching; Extracellular polymeric substances

1. Introduction

The bacterial dissolution of metal sulfides, termed bioleaching, is effected by bacteria like *Thiobacillus ferrooxidans*, *Leptospirillum ferrooxidans*, *T. thiooxidans*, *Metallogenium*, *Acidianus/Sulfolobus* spp. and some others. Most work with regard to the mechanisms of dissolution has been done with *T. ferrooxidans* [1]. Almost since the discovery of this bacterium in acid mine drainage [2], two dissolution mechanisms are discussed: the direct one and the indirect one.

According to the definition(s), which are to some extent imprecise and equivocal, the direct mecha-

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nism assumes the action of a metal sulfide-attached cell oxidizing the mineral by an enzyme system with oxygen to sulfate and metal cations. The sulfur moiety of the mineral is supposed to be biologically oxidized to sulfate without any detectable intermediate occurring.

In contrast, the indirect mechanism basically comprises the oxidizing action of iron(III) ions dissolving a metal sulfide. In the course of this chemical reaction, iron(II) ions and elemental sulfur (S_8) shall be generated. These compounds then are biologically oxidized to iron(III) ions and sulfate. This mechanism does not require the attachment of cells to the sulfide mineral.

The following equations summarize the two mechanisms: (a) Direct:

$$FeS_2 + 3.5O_2 + H_2O \rightarrow Fe^{2+} + 2H^+ + 2SO_4^{2-}, \quad (1)$$

$$2Fe^{2+} + 0.5O_2 + 2H^+ \rightarrow 2Fe^{3+} + H_2O; \quad (2)$$

(b) Indirect:

$$FeS_2 + 14Fe^{3+} + 8H_2O \rightarrow 15Fe^{2+} + 16H^+ + 2SO_4^{2-}, \quad (3)$$

$$MS + 2Fe^{3+} \rightarrow M^{2+} + S^0 + 2Fe^{2+}, \quad (4)$$

$$S^0 + 1.5O_2 + H_2O \rightarrow 2H^+ + SO_4^{2-}. \quad (5)$$

In addition, two other mechanisms exist contributing to bioleaching, namely acid leaching and galvanic leaching. Both will not be considered in this context, since the biologically dominated direct and/or indirect ones are considered to be most important.

Especially the hypothesis of the direct mechanism remained under question, and many workers have reported about experiments either confirming or rejecting that hypothesis [3–8]. Consequently, up to now this discussion is still pending. New insights may, however, be derived from recent research, which integrated for the first time advanced techniques for the unequivocal analysis of degradation products occurring in the course of metal sulfide dissolution and the analysis of extracellular polymeric substances, EPS, allowing for cell attachment and biofilm formation. A combination of this new evidence with previous knowledge, obtained from scientific areas like sulfur chemistry, mineralogy, and solid state physics [9–14], resulted in the new, integral model for bioleaching, which will be described and discussed in the following paragraphs.

The main characteristic of this model is the hypothesis that iron(III) ions and/or protons are the only (chemical) agents dissolving a metal sulfide. The mechanism is, thus, sensu strictu an indirect one. The bacteria have the functions to (i) regenerate the iron(III) ions and/or protons, and to (ii) concentrate them at the interface mineral/water or mineral/bacterial cell in order to enhance the degradation/attack. The determining factor is, thus, the tiny exopolymer layer, the glycocalyx, with a thickness in the nanometer range, surrounding the cells. In this layer, the chemical processes take place, which cause metal sulfide dissolution. Due to the concentration of the degradative agents at the interface, the acceleration of the dissolution in the presence of bacteria over the chemical attack becomes explainable. Furthermore, the integral model does not need hypothetical assumptions of enzymes, factors, etc., which up to now have never been detected. In contrast, it allows without any contradiction to chemistry or physics to integrate all known facts into a "natural bioleaching model". Based on key intermediates, two indirect leach mechanisms need to be differentiated: the thiosulfate and the polysulfide mechanism, both of which will be described in detail. Since the electronic structure of a metal sulfide, explained by valence bond and molecular orbital theories, is a decisive factor for the (bio)leaching mechanism, some background information is given in the next chapter.

2. Electronic structure and solubility of metal sulfides

Most metal sulfides are semiconductors. The metal and sulfur atoms are bound in the crystal lattice. According to molecular orbital and valence bond theories, the orbitals of single atoms or molecules form electron bands with different energy levels. The

band with the highest energy level, which is still filled with electrons, is the valence band. In the case of pyrite, molybdenite, and tungstenite the valence bands are only derived from orbitals of metal atoms, whereas the valence bands of all other metal sulfides are derived from both, metal and sulfur orbitals. This is illustrated for pyrite and chalcopyrite in Fig. 1.

Consequently, the valence bands of pyrite, molybdenite, and tungstenite do not contribute to the bonding between metal and sulfur moiety of the metal sulfide. This bonding can, thus, only be broken by several oxidation steps with the attacking agent iron(III) hexahydrate ion. In case of the other metal sulfides, in addition to iron(III) ions, protons can remove electrons from the valence band, causing a break of the bonding between the metal and the sulfur moiety of the sulfide. Consequently, these metal sulfides are more or less soluble in acid,

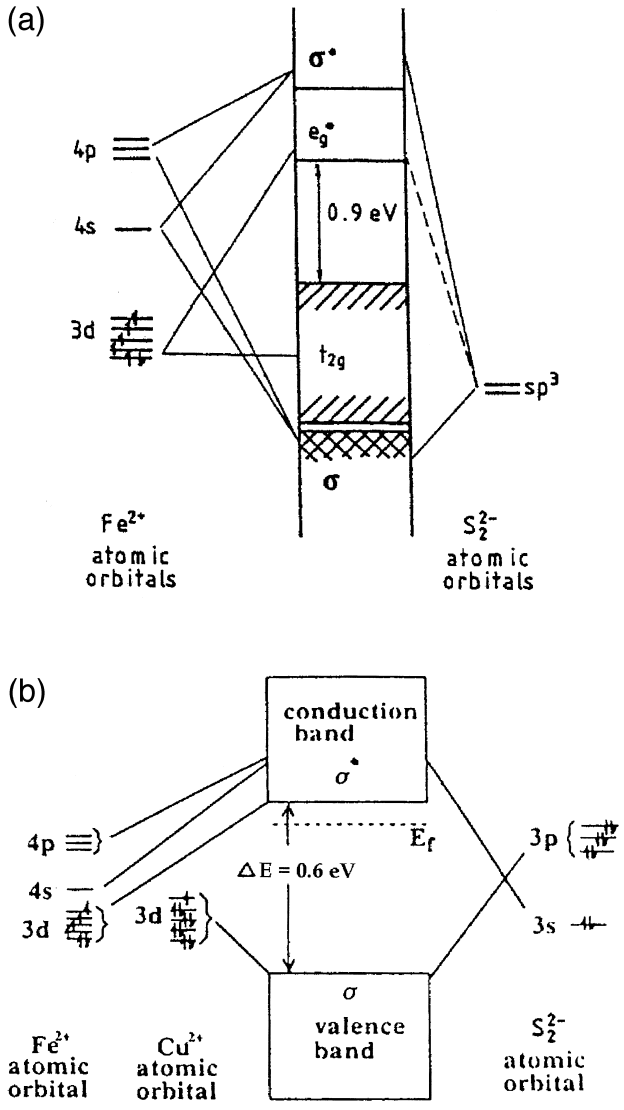

Fig. 1. Electron band diagrams for pyrite a and chalcopyrite b (reprinted from Crundwell [11] and Torma [15]). a: The non-bonding valence band t_{2g} is derived from Fe^{2+} atomic orbitals only. b: The bonding valence band is derived from Cu^{2+} and S_2^{2-} atomic orbitals.

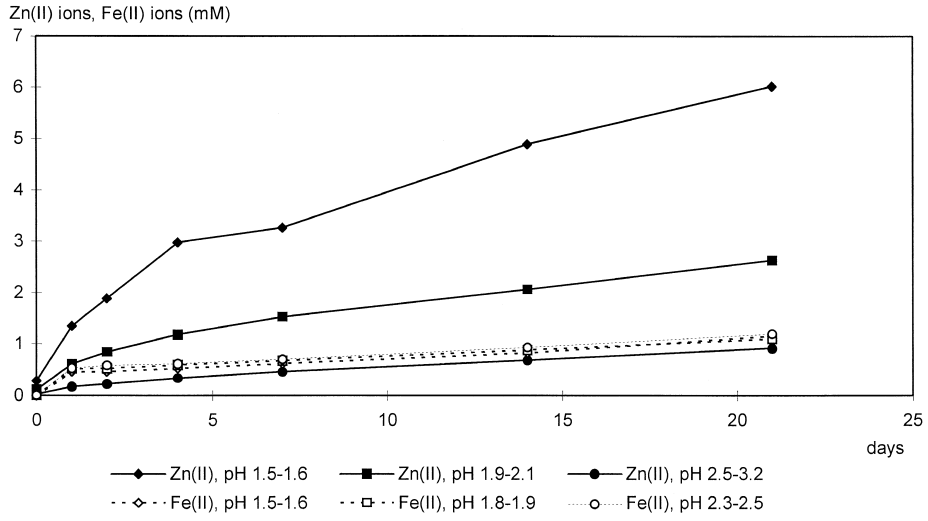

Fig. 2. Acid insolubility of FeS_2 and acid dissolution of ZnS. 1 g of each mineral (grain size 36–50 μm) was added to 50 ml H_2SO_4 at pH 1.5, 1.9, or 2.5 in shake flasks. Concentrations of Zn(II) ions for ZnS, and Fe(II) ions for FeS_2, and pH at the beginning and the end of each experiment were measured. Dissolution of ZnS increases with decreasing pH, whereas FeS_2 remained, independent of pH, almost insoluble (curves are upon each other).

whereas pyrite, molybdenite, and tungstenite are insoluble. This is demonstrated for pyrite and sphalerite in Fig. 2.

3. Thiosulfate mechanism in (bio)leaching

Studies on molybdenite, tungstenite, and pyrite degradation indicated that these metal sulfides are only degradable by an oxidizing attack, e.g. by iron(III) ions [1,9,10,16]. Pyrite was chosen as model substance to elucidate the oxidation mechanism and the intermediary sulfur compounds.

3.1. Pyrite oxidation

Pyrite is the most frequently occurring and for the sulfur cycle most important metal sulfide. In contrast to most metal sulfides the complete oxidation of pyrite causes an acidification of leach biotopes and a formation of acid rock drainage. Countermeasures have been developed to protect the environment [17,18]. Pyrite is also of economic interest, because uranium and gold are often closely associated with pyrite in the ore. Furthermore, pyrite is one of the main sulfur compounds in coal [3,4] and, thus, needs to be removed.

Generally, dissolved oxygen or iron(III) ions are oxidizing agents for pyrite in leaching operations and in the environment. In the literature (see reviews [3–5,8,17,19–21]), chemical or biological pyrite oxidation by molecular oxygen or by iron(III) ions is described by Eqs. (1)–(5).

At low pH, the chemical pyrite oxidation rate is controlled by iron(III) ions and not by molecular oxygen [21–24]. Based on molecular orbital considerations, Luther [25] explains, why at low pH iron(III) ions preferentially react with the pyrite surface. Accordingly, hydrated iron(III) ions are, in contrast to dissolved oxygen, connected with the pyrite surface via σ-bondings. These bondings shall facilitate an electron transfer from the sulfur moiety of the pyrite to the iron(III) ions. On the other hand, based on the valence bond theory, electrons shall be extracted from the t_{2g} valence band, formed by the iron atoms, and not directly from the sulfur valence band. Crundwell [11] proposes that in this process holes are initially injected into the t_{2g} valence band by the oxidizing agent, e.g. iron(III) ions. These holes are able to form hydroxyl radicals by splitting water. The strongly oxidizing hydroxyl radicals can now react with the sulfur valence band causing the sulfur moiety to become oxidized. Tributsch [26] proposes that iron hydroxides or oxides, formed at the pyrite

surface, accumulate charges by extraction of electrons from the t_{2g} valence band. This accumulation shall cause a shift of the electronic states to such positive potentials that the sulfur moiety will be oxidized. Whereas the latter two explanations for the oxidation of the pyritic sulfur moiety by iron(III) ions are similar, the first one is fairly different. Up to now, the detailed mechanisms have not been clarified yet. Nevertheless, all theories are congruent in the fact that pyrite can only be solubilized by an oxidizing attack, namely by iron(III) ions.

Furthermore, even at neutral pH iron(III) ions are the preferred electron acceptor in comparison to molecular oxygen [27]. At neutral pH, the iron(II) ions remain adsorbed at the pyrite surface and are reoxidized by dissolved oxygen. Because of kinetic data and molecular orbital considerations it became obvious that iron(III) ions instead of dissolved oxygen are the decisive pyrite attacking agents at low and also at high pH. Thus, Eq. (1) is an inadequate description of pyrite oxidation.

The formation of sulfate or elemental sulfur as products of iron(III) ion mediated pyrite dissolution is described by Eqs. (3) and (4). However, these are summarizing equations and cannot explain the underlying mechanisms. Especially the formation of polythionates, detected in chemical and biological pyrite oxidation, remains unclear from these equations [22,28]. Consequently, the leach equations have to be revised.

3.2. (Bio)leaching of pyrite

Shake flask leaching experiments were performed to study the degradability of pyrite by different lithotrophic bacteria. The results are shown in Fig. 3.

Pyrite dissolution was shown for pure cultures of the lithotrophic, acidophilic iron(II) ion oxidizing bacteria *T. ferrooxidans*, *L. ferrooxidans* [30,31], and a thermophilic archaea of the genus *Sulfolobus / Acidianus*. *L. ferrooxidans*, lacking sulfur/compound oxidizing activity, is nearly as effective in pyrite oxidation as is *T. ferrooxidans.* This is in agreement with results of Sand et al. [32], and also with calorimetric reaction energy measurements of pyrite oxidation [33,34].

In contrast, *T. thiooxidans*, lacking iron(II) oxidizing activity, cannot dissolve pyrite. This finding is in agreement with results of Norris and Kelly [35] and Norris [36], but contradicts the results of Lizama and Suzuki [37], who concluded from oxygen consumption measurements that *T. thiooxidans* is able to oxidize pyrite. Lizama and Suzuki did not remove the elemental sulfur, which is formed on the pyrite surface in the course of grinding [38–40], by washing their pyrite with an organic solvent. Thus, the detected oxygen consumption resulted probably from sulfur and not from pyrite oxidation.

The finding that only iron(II) ion oxidizing bacteria are able to dissolve pyrite elucidates the importance of iron(III) ions as the pyrite attacking agent

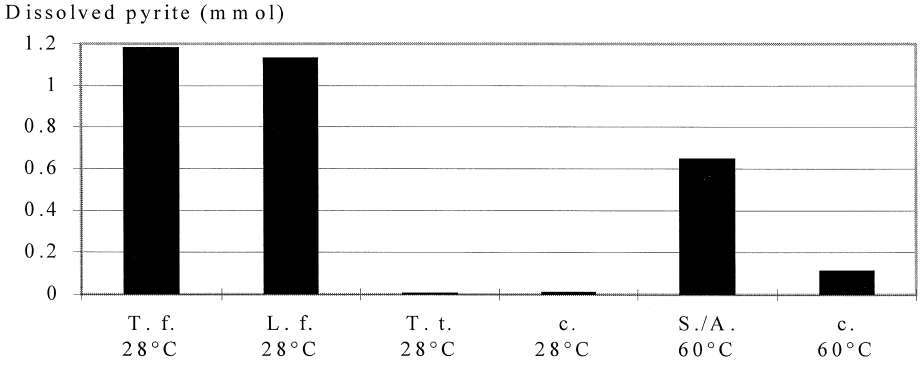

Fig. 3. Pyrite dissolution by *T. ferrooxidans* (T.f.), *L. ferrooxidans* (L.f.), *T. thiooxidans* (T.t.), *Sulfolobus / Acidianus* sp. (S./A.), and in sterile control assays (c.) determined as dissolved iron after one week of incubation. Assay conditions: 1 g pyrite, grain size 36–50 μm, 50 ml salt solution, pH 1.9. Assays at 28°C were inoculated with 1×10^9 cells and shaken at 150 rpm, assays at 60°C were inoculated with 2×10^8 cells and not shaken (reprinted from Schippers and Sand [29]).

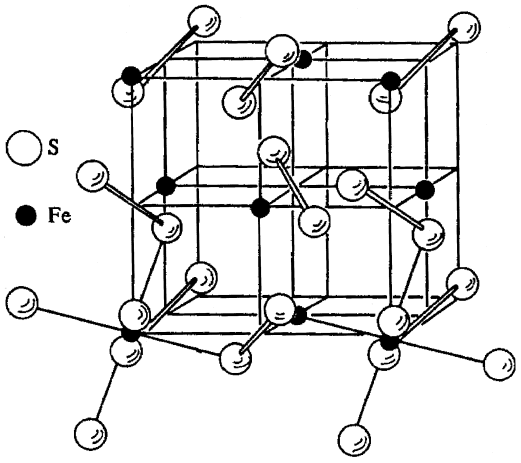

Fig. 4. Crystal structure of the disulfide pyrite (reprinted from Vaughan and Craig [13]).

and, consequently, supports the hypothesis of the indirect leaching mechanism as the basic one being active in bioleaching. To explain the importance of iron(III) ions, mineralogy, molecular orbital, and valence bond theories need to be considered. In the crystal lattice of pyrite, the sulfur moiety occurs as a disulfide. The structure is shown in Fig. 4.

According to molecular orbital considerations, iron(III) hexahydrate ions shall cleave the chemical bonding between the iron and the disulfide in the pyrite lattice, after the disulfide group has been oxidized to a thiosulfate group. As a consequence, thiosulfate and iron(II) hexahydrate ions occur as dissolution products [7,22,25,28]. Whereas the iron(II) hexahydrate ions are oxidized by *T. ferrooxidans*, *L. ferrooxidans*, *Sulfolobus / Acidianus*, or other iron(II) ion oxidizing bacteria to regenerate iron(III) ions for further attack, thiosulfate is oxidized via tetrathionate, disulfane-monosulfonic acid, and trithionate to mainly sulfate in a cyclic mechanism. Besides, minor amounts of elemental sulfur and pentathionate occur as by-products [28,41]. Because thiosulfate is the key compound in the oxidation of the sulfur moiety of pyrite, the mechanism has recently been defined as thiosulfate mechanism [1]. A simplified scheme is presented in Fig. 5.

All reactions, comprising the thiosulfate mechanism, have been shown to occur on a purely chemical basis. However, sulfur compound oxidizing enzymes, like the tetrathionate hydrolase of *T. ferrooxidans*, *T. thiooxidans*, or *T. acidophilus*, may be involved [42–46]. It still needs to be elucidated, to what extent these enzymes catalyze the reactions in competition with chemistry. If research in this field would allow to manipulate the flux of intermediary sulfur compounds, the accumulation of elemental sulfur in bioleaching and coal desulfurization processes could be prevented [47], or sulfate formation in bioleaching plants could be enhanced (e.g. sulfur formation in gold recovery increases costly cyanide consumption and lowers leaching rates [48–50]). Thus, considerable environmental and economical benefits would result.

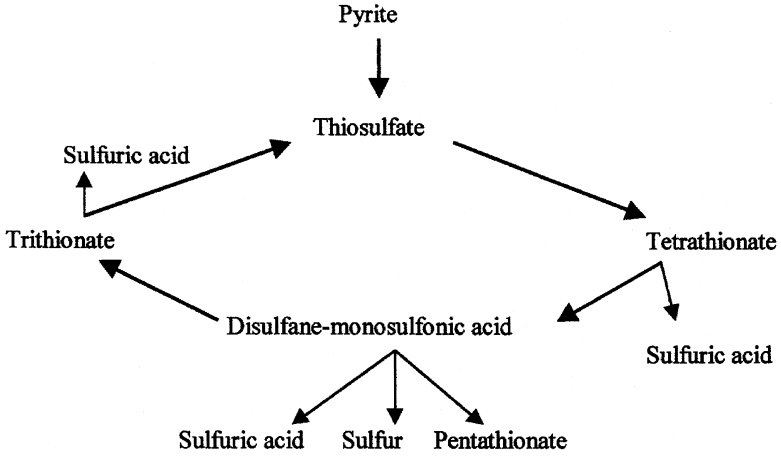

Fig. 5. Simplified scheme of the thiosulfate mechanism in pyrite oxidation (adapted from Schippers et al. [28]).

The thiosulfate mechanism is also valid for chemical pyrite oxidation at neutral pH, e.g. in carbonate and pyrite containing mine waste [51,52]. At neutral pH, the chemical pyrite oxidation rate is about 10 times higher than the one under acidic conditions [20,21]. Thiosulfate, trithionate, and tetrathionate are the main products of pyrite oxidation in carbonate buffered solutions. These substances are suitable substrates for moderately acidophilic, sulfur compound oxidizing bacteria [53]. Consequently, *T. neapolitanus*, *T. novellus*, and *Thiomonas intermedia* are able to grow with the dissolution products of pyrite, but are not able to increase pyrite dissolution, because of the lack of iron(II) ion oxidizing activity [52]. These bacteria live from the "energy gap" between the incomplete chemical oxidation of the sulfur moiety of pyrite at neutral pH values and its complete oxidation to sulfuric acid. In addition, by acid production the pH is lowered, allowing acidophilic leaching bacteria, like *T. ferrooxidans*, to grow [54] and to oxidize pyrite.

3.3. (Bio)leaching of molybdenite (MoS_2) and tungstenite (WS_2)

Chemical leach experiments have been performed with pyrite and molybdenite. The results are shown in Table 1. Because with molybdenite the same end-products were obtained as with pyrite and because of the same electronic structure, it is obvious that molybdenite is degraded by the same mechanism. Tungstenite is, because of the identical electronic structure, included in this group of metal sulfides, which are degraded via the key intermediate thiosulfate. Accordingly, the main end-product of the sulfur moiety of molybdenite and tungstenite degradation is sulfate.

4. Polysulfide mechanism in (bio)leaching

4.1. Oxidation of metal sulfides with different crystal and electronic structure

Based on molecular orbital and valence bond theories, the previously discussed metal sulfides, like pyrite, are unique in their structure, because they can only be degraded by an oxidizing attack. Most other metal sulfides are, however, amenable to a proton attack too. Thus, six metal sulfides differing in crystal and electronic structure from pyrite were selected for dissolution experiments. These metal sulfides were sphalerite (ZnS), chalcopyrite ($CuFeS_2$), galena (PbS), hauerite (MnS_2), orpiment (As_2S_3), and realgar (As_4S_4). The structures of sphalerite, chalcopyrite, and galena are shown in Fig. 6.

Table 1
Formation of sulfur compounds resulting from chemical metal sulfide oxidation

Metal sulfide	Formula	Structure[a]	Purity (%)[b]	S_8 (%)[c]	SO_4^{2-} (%)[c]	$S_4O_6^{2-}$ (%)[c]	$S_5O_6^{2-}$ (%)[c]
Pyrite	FeS_2	disulfide	> 99	16.1	81.7	1.3	0.9
Molybdenite	MoS_2	layer	93	8.4	90.4	0.6	0.6
Hauerite	MnS_2	disulfide	> 99	93.6	3.7	1.2	1.5
Sphalerite	ZnS	sphalerite	95	94.9	4.8	0.1	0.2
Chalcopyrite	$CuFeS_2$	sphalerite	> 99	92.2	7.3	0.3	0.2
Galena	PbS	halite	> 99	99.9	0.1	0.0	0.0
Orpiment	As_2S_3	layer	> 99	94.8	5.2	0.0	0.0
Realgar	As_4S_4	ring	> 99	92.5	7.5	0.0	0.0

Oxidizing agent 10 mM Fe(III) chloride, pH 1.9, 28°C. Reprinted from Schippers and Sand [1].

[a] Mineralogical structure type [13].

[b] Purity calculations base on ICP measurements of elemental composition. Impurities were not detected by X-ray diffraction except some geerite (Cu_8S_5) in case of chalcopyrite.

[c] Percentage values were calculated after 24 h incubation except for galena (1 h), hauerite (5 h), and realgar (168 h), due to the different reaction rates. In case of hauerite, traces of hexathionate were detectable, too. Experiments with iron(III) sulfate instead of iron(III) chloride under anaerobic conditions in a glove-box with four selected metal sulfides gave similar results (data not shown).

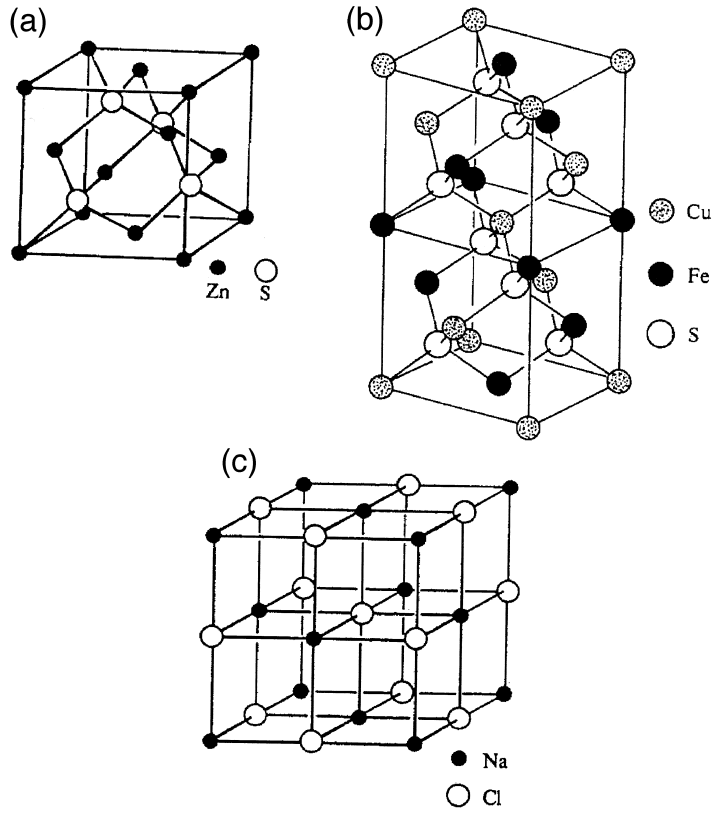

Fig. 6. Crystal structure of sphalerite a, chalcopyrite b, and galena (NaCl structure) c (reprinted from Vaughan and Craig [13]).

The formation of sulfur compounds in the course of iron(III) ion mediated chemical oxidation of these metal sulfides was analyzed. The results are shown in Table 1.

Whereas the oxidation products in the case of pyrite and molybdenite consisted of up to 90% of sulfate and to about 1% to 2% of polythionates, the other metal sulfides yielded elemental sulfur in amounts of more than 90% as the main intermediate. This result is caused by a mechanism, in which the metal sulfides are degraded via polysulfides as key intermediate. Due to their principal solubility in acid, the first reaction is assumed to be:

$$MS + 2H^+ \rightarrow M^{2+} + H_2S. \quad (6)$$

In contrast to pyrite oxidation, in these metal sulfides the M–S bonding is cleaved, before the sulfidic sulfur is oxidized. The kinetics of this reaction are dependent on the solubility product of the respective metal sulfide. Here only the general concept will be discussed [11]. The ensuing oxidation mechanism of aqueous sulfide has been described in detail by Steudel [12]. According to his work, the H_2S is subjected to a one electron oxidation by an iron(III) ion:

$$H_2S + Fe^{3+} \rightarrow H_2S^{*+} + Fe^{2+}. \quad (7)$$

The cation radical H_2S^{*+} may also directly be formed by an attack of iron(III) ions on a metal sulfide:

$$MS + Fe^{3+} + 2H^+ \rightarrow M^{2+} + H_2S^{*+} + Fe^{2+}. \quad (8)$$

By dissociation of the strong acid H_2S^{*+}, the radical HS^* occurs:

$$H_2S^{*+} + H_2O \rightarrow H_3O^+ + HS^*. \quad (9)$$

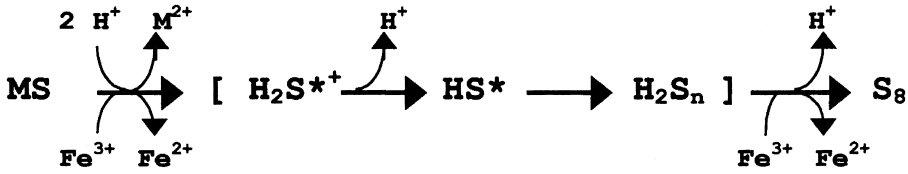

Fig. 7. Simplified scheme of the polysulfide mechanism (Schippers and Sand [1]).

Two of these radicals may react to a disulfide ion:

$$2HS^{*} \rightarrow HS_2^{-} + H^{+}. \quad (10)$$

The disulfide ion can further be oxidized by an iron(III) ion (Eq. (7)) or a HS^{*} radical:

$$HS_2^{-} + HS^{*} \rightarrow HS_2^{*} + HS^{-}. \quad (11)$$

Tetrasulfide can occur by dimerization of two HS_2^{*} or trisulfide by reaction of HS_2^{*} with HS^{*} radicals. Chain elongation of the polysulfides may proceed by analogous reactions. In acidic solutions, polysulfides decompose, liberating rings of elemental sulfur, mainly S_8 (> 99%):

$$HS_9^{-} \rightarrow HS^{-} + S_8. \quad (12)$$

This mechanism does not necessarily function only in the presence of iron(III) ions. An electron transfer from a semiconductor metal sulfide to an O_2 molecule is also possible. The O_2 molecule is reduced via a superoxide radical and a peroxide molecule to water [56]. However, iron(III) ions are generally much more efficient in extracting electrons from a metal sulfide lattice than O_2 [9,10].

The reactions 7–12 inherently explain the formation of elemental sulfur as the main sulfur compound. Minor amounts of sulfate and polythionates are products of thiosulfate reactions [12,28,57,58]. Thiosulfate may arise by a side reaction [12]:

$$HS_n^{-} + 3/2O_2 \rightarrow HS_2O_3^{-} + [(n-2)/8]S_8, \quad (13)$$

or be formed in the following one:

$$1/8S_8 + HSO_3^{-} \rightarrow HS_2O_3^{-}. \quad (14)$$

Also under anaerobic conditions only minor amounts of sulfate and polythionates were formed,

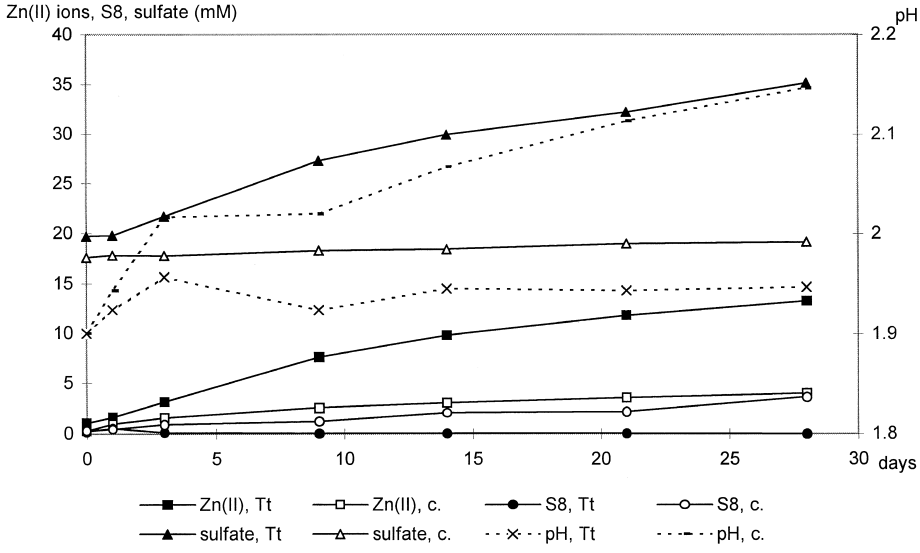

Fig. 8. Leaching of ZnS by *T. thiooxidans* strain R20 [60]. The organism was pregrown on ZnS, before the experiment was started by addition of 10^9 cells to 1 g ZnS (fine grained) in 50 ml salt solution in shake flasks at 28°C in the dark. Concentrations of Zn(II) ions, S_8, sulfate, and pH were measured. Tt, assays with *T. thiooxidans*; c., sterile control assays. Sulfuric acid for ZnS dissolution originates from biological oxidation of chemically formed elemental sulfur (S8). In sterile control assays elemental sulfur accumulates, simultaneously the pH increases, both lowering the dissolution rate of ZnS. Reprinted from Schippers and Sand [1].

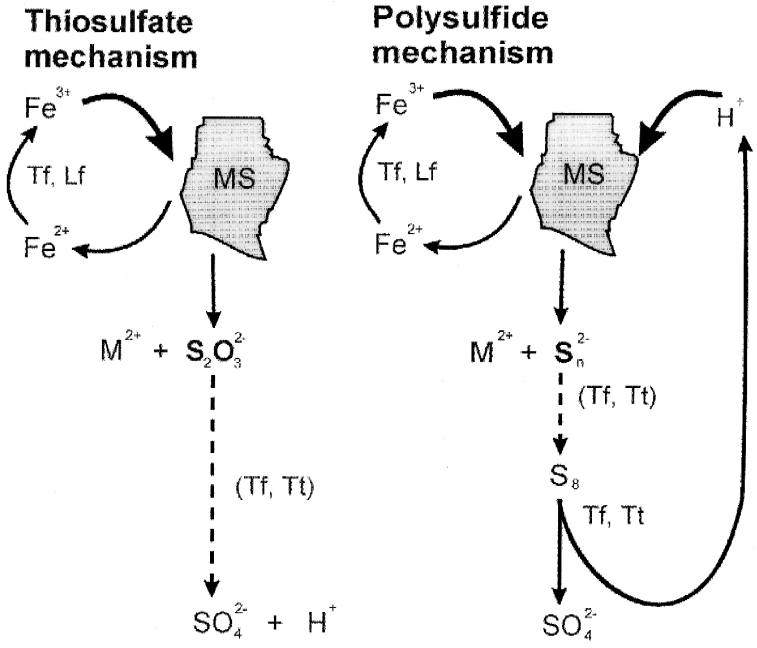

Fig. 9. Scheme of thiosulfate and polysulfide mechanism in (bio)leaching of metal sulfides. MS = metal sulfide; M^{2+} = metal ion; $S_2O_3^{2-}$ = thiosulfate; S_n^{2-} = polysulfide with chain length (n); S_8 = elemental sulfur; Tf, Lf, Tt = enzymatic reaction by *T. ferrooxidans*, *L. ferrooxidans*, and/or *T. thiooxidans*; (Tf, Tt) = enzymatic reaction possible (reprinted from Schippers and Sand [1]).

e.g. in the reaction of sphalerite or chalcopyrite with iron(III) ions (results not shown). To study whether thiosulfate (polythionates) may also be generated in the course of an anaerobic oxidation of polysulfides by iron(III) ions, polysulfides (synthesized according to Steudel et al. [59]) were added to an iron(III) ion containing, acidic solution under anaerobic conditions (glove-box). Formation of polythionates was detected, in contrast to control assays without iron(III) ions (results not shown). A reaction analogous to Eq. (13) with iron(III) ions instead of O_2 as oxidizing agent may explain this result:

$$HS_n^- + 6Fe^{3+} + 3H_2O \rightarrow HS_2O_3^- + [(n-2)/8]S_8 + 6Fe^{2+} + 6H^+. \quad (15)$$

Summarizing, thiosulfate and polythionates play a key role in the thiosulfate mechanism; however, these compounds play only a side role in the polysulfide mechanism. The complex mechanism is simplified in the following scheme (Fig. 7).

In any case, the main end-product is elemental sulfur. The latter is biologically oxidized to sulfuric acid. This explains the ability of *T. thiooxidans* to leach some metal sulfides, e.g. sphalerite (Fig. 8). The strain, with which the data in Fig. 8 have been produced, was not optimized by numerous precultures to grow on sphalerite. Thus, kinetics may not be derived from the graphs. The data only demonstrate the general mechanism of oxidation/dissolution.

As a consequence, two indirect oxidation mechanisms for metal sulfides exist, which are summarized by the following equations [1]: (1) Thiosulfate mechanism (FeS_2, MoS_2, and WS_2)

$$FeS_2 + 6Fe^{3+} + 3H_2O \rightarrow S_2O_3^{2-} + 7Fe^{2+} + 6H^+, \quad (16)$$

$$S_2O_3^{2-} + 8Fe^{3+} + 5H_2O \rightarrow 2SO_4^{2-} + 8Fe^{2+} + 10H^+; \quad (17)$$

(2) Polysulfide mechanism (e.g. ZnS, $CuFeS_2$, or PbS)

$$MS + Fe^{3+} + H^+ \rightarrow M^{2+} + 0.5H_2S_n + Fe^{2+} \quad (n \geq 2), \tag{18}$$

$$0.5H_2S_n + Fe^{3+} + \rightarrow 0.125S_8 + Fe^{2+} + H^+, \tag{19}$$

$$0.125S_8 + 1.5O_2 + H_2O \rightarrow SO_4^{2-} + 2H^+. \tag{20}$$

The scheme in Fig. 9 gives an overview, including some knowledge about biologically and/or chemically catalyzed parts.

5. Involvement of extracellular polymeric substances in (bio)leaching

Although much work has been done to elucidate the interfacial degradation process of metal sulfides [7,9,10] from the point of chemistry and physics [55] the importance and involvement of extracellular polymeric substances (EPS), excreted by leach bacteria, has almost been overlooked up to now. However, since electrochemical, biochemical, and surface specific mechanisms are jointly interacting, the function of the EPS needs to be taken into account. For the bacterial attack on the metal sulfide surface, the presence of EPS in the contact area between the bacterial cell and the sulfidic energy source seems to be a prerequisite. There is sufficient evidence for a critical role of organic film formation in bacterium–substratum interaction [61]. Such films have been observed with cells of *T. ferrooxidans* growing on pyrite [62]. In order to understand their functional sense for the bioleaching process, the chemical composition of these films (EPS) was analyzed for *T. ferrooxidans* grown on pyrite [16,63]. To achieve the dissolution of pyrite, cells of *T. ferrooxidans* attach to the mineral surface by means of excreted exopolymeric substances (lipopolysaccharides) and oxidize the mineral to sulfuric acid plus iron(III) ions. The primary attachment to pyrite (at pH 2) is mediated by exopolymer-complexed iron(III) ions as an electrochemical interaction with the negatively charged surface of the substrate/substratum. Cells, devoid of exopolymeric substances, neither attach to nor oxidize pyrite. The iron species are presumably bound by glucuronic acid subunits of the carbohydrate moiety. The molar ratio of both constituents amounted to 2 moles glucuronic acid to 1 mole iron(III) ions suggesting the formation of stable complexes [64]. These complexes render the cells with a net positive charge (three times positive, two times negative charge) and, thus, allow them to attach to the negatively charged pyrite surface in the course of an electrostatic interaction.

Furthermore, cells grown on sulfur exhibit a different composition of the exopolymers (leading to strongly hydrophobic surface properties) and do not attach to pyrite anymore. Glucuronic acids and iron species were, consequently, not detectable. However, a slight, but significant, increase of the phosphate content of the EPS was noted. Thus, the substrate/substratum influences the chemical structure of the exopolymers. The mechanism of regulation still needs to be clarified. Possibly, chaperones [65] are involved, because the change of substrate means physiological stress for the cells.

Considering the bacterial surface properties, attachment to sulfur is presumably dominated by hydrophobic (van der Waals) attraction forces, while sorption to pyrite is due to electrostatic interactions, probably combined with some involvement of hydrophobic forces. The involvement of charge effects is corroborated by earlier studies on the molecular structure of pyrite [25], indicating that those cations or molecules, which act as Lewis acids (willing to accept the unshared pair of electrons of pyritic sulfur), e.g. (EPS bound) iron species, will be preferentially attracted.

Obviously, iron(III) ions are of pivotal importance for cell attachment. In addition, these ions also mediate the primary steps in the degradation of metal sulfides. For the start of bioleaching, a sufficient amount of iron(III) ions in the medium is necessary. It could be demonstrated that the rate of pyrite oxidation remained negligible, until the iron(III) ion concentration had increased (by chemical oxidation of solubilized iron(II) ions or by supplementation) to a threshold value of ≥ 0.2 g/l (data not shown). In Fig. 10, the effect of an addition of iron(III) ions to the medium (0.5 g/l) is demonstrated. If a sufficient concentration of these ions was present, leaching of pyrite (by cells of *T. ferrooxidans*) started without lag phase.

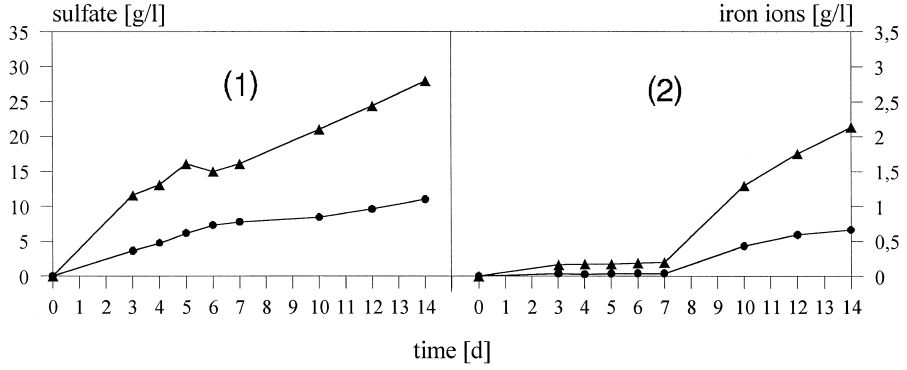

Fig. 10. Importance of iron(III) ions for pyrite dissolution by cells of *T. ferrooxidans*. Pyrite dissolution was measured as an increase of iron ion and sulfate concentration. Iron(III) ions (0.5 g/l) were added at the beginning of the experiment (1) or after 7 days (2). • = total iron ion concentration, ▲ = sulfate concentration, start cell concentration 10^9/ml (from Gehrke et al. [63]).

Consequently, the exopolymeric layer containing complexed iron(III) ions comprises a reaction space, in which the dissolution process takes place. It may be interpreted as a compartment, where special, up to now unknown conditions prevail, e.g. pH, redox potential, ion concentration, etc. The amount of the iron species in this layer was estimated to be approximately 53 g/l. This concentration can only be maintained by the formation of complexes, to avoid a precipitation of iron compounds. Again, only the indirect leaching mechanism, i.e. the catalytic effect of iron(III) ions, can unequivocally explain the findings.

Another aspect of bacterial leaching, which has not been extensively studied, is the attachment characteristics. Since attachment of cells of *T. ferrooxidans* was observed to be specific to (nutrient enriched) sulfide phase regions (e.g. FeS_2) on waste rock surfaces [66], there have been no further attempts to determine the location of attachment. Atomic force microscopy (AFM) images of colonized pyrite cubes illustrate that the mineral is only partially colonized by bacteria (Fig. 11).

In addition, visual inspection of attachment sites indicated that the majority of the cells adhered to locations with visible imperfections ("faults", "rivers", etc.). These findings suggest the occurrence of preferential attachment sites. Similar evidence was presented by Dziurla et al. [67]. Crystal defects, such as (corroding) emergences of dislocations and cracks, are probably the respective sites. In Fig. 12 the AFM image clearly illustrates that adhesion was specifically associated with a distinct indentation of a dislocation area (fault).

Corroding surface regions (exhibiting anodes and cathodes), thus, seem to be the preferential attachment sites for net positively charged bacteria like *T. ferrooxidans*, because an electrostatic interaction with the negatively charged cathode becomes possible. Furthermore, at the anode the substrate iron(II) ions are available. This hypothesis is in agreement with Berry and Murr [66], who reported that the crystal structure of a (sulfide) mineral is an important factor influencing the bioleaching process.

An attempt to determine localized anodic and cathodic sites by using the scanning vibrating electrode technique failed, because the anodes and cathodes seem to be separated by less than 10 μm (below the lateral resolution of the scan motor). Analysis of the current maps obtained showed neither distinguishable anodic nor cathodic activities (data not shown), thus suggesting the predominance of general, flat-spread corrosion phenomena.

Since electrically active sites of corrosion were not detectable, additional surface potential measurements have been performed using a Kelvin probe. These experiments demonstrated that the biologically driven process of pyrite degradation is electrochemical in nature. The experiments were repeated several times under different conditions, to evaluate the importance of EPS and the complexed iron(III) ions. The results are summarized in Table 2.

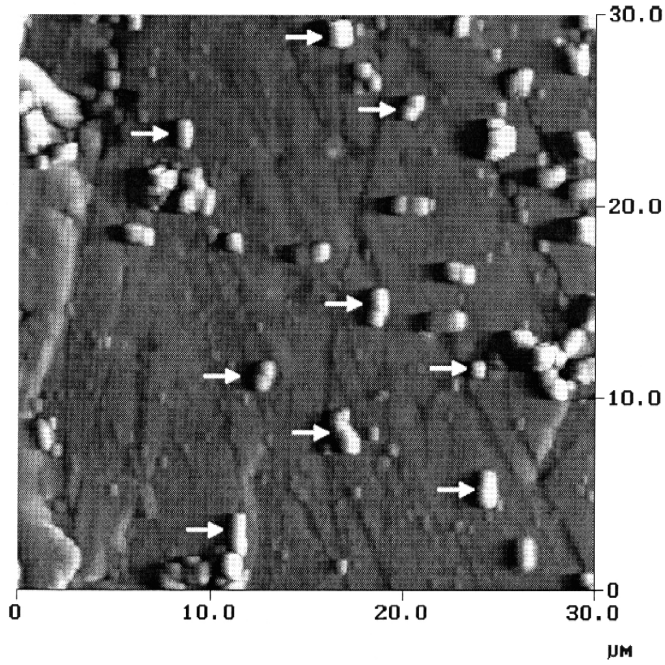

Fig. 11. Atomic force microscopy image of a pyrite surface with attached cells of *T. ferrooxidans*. The cells (some are indicated by arrows) are sparsely distributed over the surface.

The surface potential strongly increased over time in the presence of living, EPS-and iron(III) ion-containing bacteria, whereas EPS-deficient cells, although in the presence of iron(III) ions, caused a significantly reduced potential increase. Obviously, the latter cells had to produce their capsular material (EPS) prior to the onset of bio-oxidation [16,63]. Dead cells, containing EPS and iron(III) ions, did only negligibly influence the surface potential. The same was valid for living cells, which had been stripped of their EPS and been kept without iron(III) ions. Since the increased surface potentials can only be explained by the rapid bacterial (re)oxidation of the iron(II) ions originating from the anode (pyrite dissolution) and/or from iron(III) ion reduction at the cathode, the results clearly demonstrate the function of living, metabolically active bacteria in keeping the iron ions in an oxidized state. Moreover, the results, although obtained by a totally different method, allowed to draw the same conclusion as before, namely that exopolymers are a prerequisite for attachment and solubilization of a sulfide mineral.

Another important organism for bioleaching is *L. ferrooxidans*. Although thriving in the same habitat, *T. ferrooxidans* and *L. ferrooxidans* are genetically not related. Whereas *T. ferrooxidans* belongs to the beta-or gamma-subclass of the proteobacteria [68–70], *L. ferrooxidans* together with *Nitrospira moscoviensis* forms another class [71]. Consequently, the enzymes for iron(II) ion oxidation, causing metal sulfide dissolution under strongly acidic conditions, are completely different [72]. However, the attachment to a metal sulfide surface combined with EPS formation, prior to the onset of leaching, is achieved by a similar mechanism [16,63]. Again, glucuronic acids and iron(III) ions are key components of the EPS.

Furthermore, these findings allow to explain, why *L. ferrooxidans* exhibited increased leaching results (enhanced dissolution), when growing in mixed culture with *Acidiphilium* sp. [73], a chemoorganotrophic bacterium, on pyrite. *Acidiphilium* seems on one hand to further, by an up to now unknown mechanism (possibly quorum sensing), the EPS-production of *L. ferrooxidans*. This clearly would result

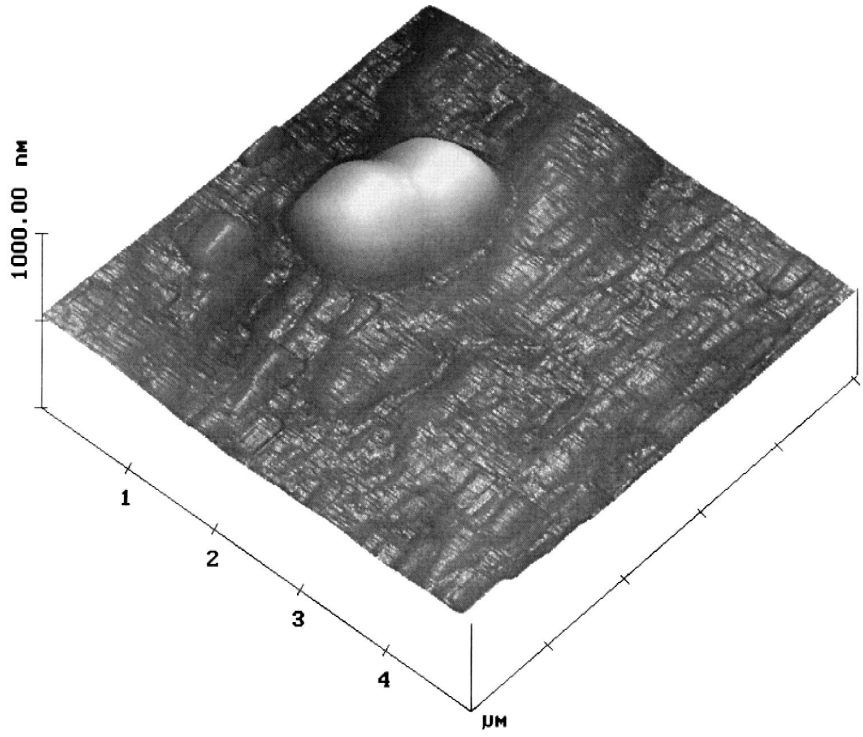

Fig. 12. Atomic force microscopy image of a cell of *T. ferrooxidans* being specifically attached to a dislocation area (surface fault).

in enhanced attachment. On the other hand *Acidiphilium* possesses and excretes exoenzymes, which are able to degrade the exopolymers of *L. ferrooxidans* (mainly lipopolysaccharides). The most frequently occurring neutral sugar in the EPS of *L. ferrooxidans* is glucose, the preferred carbon and energy source of *Acidiphilium* [74]. This is of special importance, since *L. ferrooxidans* seems to produce in general considerably more EPS than *T. ferrooxidans*. By digesting some of the exopolymers, parts of the metal sulfide surface may become available for other cells of *L. ferrooxidans* for attachment again (and degradation). The EPS, which *Acidiphilium* degrades, may either result from living, active cells of *L. ferrooxidans* [75] and/or simply be remaining footprints of predecessors. Because *T. ferrooxidans* produced only minor amounts of EPS, this finding may also explain, why enhancement of leaching was only noted for *L. ferrooxidans* in mixed culture, but not for mixed ones with *T. ferrooxidans* [73].

Table 2
Importance of EPS and metabolism for the onset of pyrite degradation (bioleaching) by cells of *T. ferrooxidans*

Pyrite surface covered with	Increase of surface potential [mV]	
	After 4 h	After 18 h
Dead cells + EPS, + iron(III) ions	48	59
Living cells + EPS, + iron(III) ions	245	344
Living cells − EPS, + iron(III) ions	150	212
Living cells − EPS, − iron(III) ions	5	18

Pyrite degradation was measured 4 and 18 h after inoculation with EPS-containing or EPS-deficient, living or dead iron(II) sulfate grown cells as an increase of the surface potential on a pyrite crystal (adapted from Gehrke et al. [16]).

In the light of these data, the discussion about metabolic inhibition of acidophilic lithotrophs by excreted organic acids like pyruvate becomes questionable too. An overflow of carbon compounds in these environments, which could result in an excretion of such compounds, seems highly unlikely.

However, the coating of the nutrient source by exopolymers seems to be much more likely. As a consequence, the planktonic leach bacteria would not be able anymore to attach to the metal sulfide and to degrade it, consequently, simply because they are facing an exopolymeric surface, not a mineral one. The above-described attachment mechanisms do not work under these circumstances.

It may also be assumed that the EPS constitute nucleation sites for the precipitation of minerals, as described by Douglas and Beveridge [76]. Consequently, precipitates like jarosites are formed, because of an interaction between the iron(III) ions and iron sulfates and/or hydroxides, etc., and, thus, would be of biogenic nature.

Summarizing, the iron(III) ion binding compounds (glucuronic acid) of the EPS of *T. ferrooxidans* and *L. ferrooxidans* are decisive for the interactions between cells and substrate/substratum. Geesey and Jang [64] reported too that the polysaccharides of bacterial EPS are commonly responsible for binding of metal ions through glucuronic acid subunits. The latter exhibit high complexation capacities. Similar evidence has been presented [77], especially for iron(III) ions. Oxygen atoms of hydroxyl groups of neighboring neutral sugars (e.g. glucose) shall also contribute to the coordinative binding of metal ions. Thus, the formation of stable complexes is promoted. The glucuronic acid content of the exopolymers provides, obviously, some selective ecological advantage, allowing the acidophilic iron oxidizers to attach to and to grow on metal sulfides. It may even be speculated that in other cases, like microbially influenced corrosion processes (MIC), the glucuronic acid components of the EPS of the relevant microorganisms have a comparable function in the adhesion and, finally, in the biocorrosion process [83].

6. Resume and outlook

The two different indirect oxidation mechanisms together with the role of the EPS have a fundamental importance for the debate about the "direct" or "indirect" mechanism of bacterial leaching. It becomes evident that a "direct", i.e. enzymatic attack, mechanism does not exist. The possibility of *T. ferrooxidans* to oxidize synthetic metal sulfides in the absence of iron ions [9,10,78,79] and the attachment of the bacterium to the mineral sulfide [66] were used up to now to prove the existence of a direct mechanism. However, the data presented here clearly demonstrate that without iron ions *T. ferrooxidans* does not oxidize FeS_2, MoS_2 and WS_2, whereas the leaching of sulfides, like ZnS, CdS, NiS, CoS, CuS, or Cu_2S, is correlated with their solubility products [9,10,16,79]. The addition of iron(III) ions to the cultures generally enhanced the leaching rates. Furthermore, it becomes obvious, why *T. thiooxidans*, a bacterium closely related to *T. ferrooxidans*, but without iron(II) ion oxidizing capacity, cannot leach FeS_2 (see above). In contrast, acid leaching of ZnS by *T. thiooxidans* has been confirmed here and in previous studies [80,81].

In the absence of iron ions *T. ferrooxidans* acts in the same manner as *T. thiooxidans* (by oxidation of sulfur). Consequently, the often cited "direct" mechanism of metal sulfide leaching is nothing else than the biological oxidation of the chemically formed elemental sulfur to sulfate. This conclusion is also supported by the recent finding that the solubilization of Cu^{2+} from a copper ore is determined by the sulfur oxidizing activity of *T. ferrooxidans* [82].

Summarizing, the findings discussed here end in a leaching model consisting only of the indirect thiosulfate or the indirect polysulfide mechanism. In both cases, the EPS with their iron(III) ions, probably complexed by glucuronic acid residues, play a pivotal role in the cell attachment to a metal sulfide surface and the ensuing degradation. The composition of the EPS is adapted to the respective substrate/substratum. Consequently, the EPS constitute an enlargement of the cells radius of action, and may be considered as a special reaction compartment.

Future research, in order to enhance (bio)leaching for precious metal winning, or to inhibit (bio)leaching for reducing the environmental impact, like acid rock drainage, must focus on the biochemical reactions in the course of metal sulfide degradation. Further research is needed to address the interfacial processes occurring between EPS, complexed iron(III) ions, and the metal sulfide. The latter clearly needs input from sources like electrochemistry, solid state physics, etc. From the present point of view, this future work clearly has the potential to allow for considerable achievements in bioleaching.

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