Inhibition of the bioleaching microorganism *Acidithiobacillus thiooxidans* by SDS Addition

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The inhibition of bioleaching by sodium dodecyl sulphate (SDS) known for large scale percolators in Romania (Schippers *et al.* 2001) was shown for pure cultures of sulphur-oxidizing *Acidithiobacillus thiooxidans* DSM 622 and German sand samples.

A decrease of 25 to 75 % in planctonic cell number counted for 10^{10} *At. thiooxidans* cells with a Thoma-chamber 30 minutes after exposure to SDS concentrations from 0.5 to 10 g/L suggested a cell lysis. Additionally a release of nucleic acids was found.

To apply these results in a more natural habitat columns filled with aquifer material from an East German lignite mining area containing 1 % pyrite were treated. Columns were washed once with 2 g/L SDS and afterwards with rainwater. Most-probable-number determinations of flow-through or sand revealed no growth of iron- and sulphur-oxidizing microorganisms within 25 weeks while up to 10^6 cells per millilitre were determined in the control. Elution of sulphate dropped to 25 %.

Keywords: Acid rock drainage, Bioleaching, Bacteria, Environmental

INTRODUCTION

The oxidation of sulphide containing minerals e.g. pyrite and marcasite leads with water to sulphuric acid containing products. The dissolved metals contaminate ground and surface water known as acid mine drainage (AMD). Microorganisms mediate and accelerate this process. Occurrence of microorganisms and mechanism of bacterial sulphide oxidation in AMD areas was reviewed by Rohwerder *et al.* (2003). In a study of an East German lignite mining area carried out in 2006 the acidophilic iron- and sulphur-oxidizing bacteria *Acidithiobacillus ferrooxidans*, *At. thiooxidans* and *L. ferrooxidans* were identified. In overburden *At. ferrooxidans* and *At. thiooxidans* were equally distributed, in heap samples *At. ferrooxidans* was the dominating acidophile (Siebert *et al.* 2009).

Inhibition of bacterial and chemical leaching is necessary to protect the environment from AMD. The application of sulphonated tensides like sodium dodecyl sulphate, resulting in inhibition or enhancement, was previously described (Onysoko *et al*. 1984; Sand 1984; Dugan 1986). Schippers *et al.* (2001) showed the efficient inhibition of leaching bacteria by SDS application and alkaline layers in large percolators. Effects of other substances e.g. quaternary ammonium compounds or hydrocarbons to planctonic microorganisms were described by Sikkema *et al.* (1995), Kourai *et al.* (2006), and Sumitomo *et al.* (2006). The influence of surface active compounds on the adhesion of microorganisms to surfaces was reviewed by Neu (1996).

Cell lysis in Gram-positive and -negative bacteria and various ways of its induction has been studied for more than 100 years (reviewed by Rice and Bayles 2008). Tsuchido *et al.* (1976) showed that lysis in *Bacillus subtilis* was triggered by long

chain fatty acids. An induced release of genomic DNA plus the development of competence in bacterial populations in the lytic stage was also reported (Steinmoen *et al.* 2002; Kreth *et al.* 2009).

The objective of this study was a first approach to collect arguments for a lysis mechanism explaining the sensitivity of bioleaching for an inhibition by SDS.

MATERIAL AND METHODS

Growth experiments

The sulphur-oxidizing bacterium *At. thiooxidans* DSM 622 was pre-cultivated in medium according to Hutchinson *et al.* (1965) containing 10 g/L sulphur. For lysis experiments cultures were transferred in modified medium according to Mackintosh (1978) containing 1 mM ammonium chloride instead of ammonium sulphate and 5 g/L sulphate. Cells were cultured until late logarithmic phase and harvested by centrifugation. Afterwards a bacterial suspension was adjusted to 10^{10} cells per millilitre with fresh medium. As inhibitory substance SDS was dissolved in double-distilled water to a 10 % stock solution, diluted to appropriate concentrations and filter-sterilized. Equal volumes of SDS dilutions were added to 1 ml of suspension for final concentrations of 0.5, 1, 2.5, 5, or 10 g/L. Solutions were mixed and incubated for 30 minutes at room temperature. Cell number determination was performed in a Thoma-chamber with appropriate dilutions, before and after SDS treatment. All given data are mean values of duplicate measurements. The percentages given as results were calculated in reference to the appropriate negative control.

For analysis of nucleic acid release, 500 µl of bacterial suspensions after SDS treatment were centrifuged at 13,000 x g for 10 minutes. Nucleic acids in the

supernatants were precipitated by isopropanol, washed with 70 % ethanol and suspended in 20 µl water. Cell pellets were suspended in 20 µl water. Equal amounts of pellet or supernatant samples were analysed by electrophoresis on 0.8 % agarose gels and stained with ethidium bromide.

Column experiments

For leaching experiments 20 ml columns (Bio-Rad, USA) were used. Up to 16 g aquifer material, containing 0.4 to 1 % pyrite, from an East German lignite mining area were filled into the columns. The experiments were performed at 21 ℃.

For inhibition experiments SDS was solubilised in rainwater to a final concentration of 2 g/L. Columns were washed with 85 ml SDS rainwater mixture or rainwater for the control. Afterwards the columns were washed with 5 ml rainwater. The eluent was collected for one week, adjusted to 5 ml with fresh rainwater and again applied to the column. As a positive control a further column was loaded every week with 5 ml fresh rainwater.

Sulphate measurement of the leaching solution was performed once per month with Spectroquant test (Merck, Germany) following manufacture instructions. Additionally pH value was measured every two to four weeks.

Cell counts of acidophilic iron- and sulphur oxidizing microorganisms were determined by most-probable-number technique in liquid media according to Mackintosh (1978) and Hutchinson *et al.* (1965). Cultures were incubated at 28 ℃ for three to four weeks. Soil samples were analysed before the experiment and 26 weeks after SDS addition to the column, leaching solution additional after 13 weeks.

RESULTS AND DISCUSSION

SDS caused cell number decrease and nucleic acid release

Planctonic cells of *At. thiooxidans* DSM 622 counted 30 minutes after exposition to 0.5 to 10 g/L SDS showed a decrease in cell number between 25 and 75 % compared to the initial cell count (Figure 1). High molecular weight nucleic acids above 10 kb were released in the pellet fraction after addition of 5 or 10 g/L SDS, whereas lower SDS concentrations resulted only in a release of nucleic acids up to 2 kb (Figure 2). Additionally, in comparison to the control sample more nucleic acids below 500 bp were visible. In the supernatant nucleic acids were not detectable (data not shown).

Decrease in cell number and survival of a subpopulation of bacteria after autolysis was described by Qin *et al.* (2007) for *Staphylococcus epidermis*. The *Staphylococcus* wild-type showed a 30 % decrease of the initial OD_{580} after treatment with Triton X-100, which is similar to our observation at 0.5 g/L SDS.

It was shown that release of extracellular nucleic acids is important in biofilm formation and cell-cell adhesion (Allesen-Holm *et al.* 2006; Qin *et al.* 2006; Rice *et al.* 2007). Since *At. thiooxidans* is known as biofilm producer, this microorganism should possess a mechanism for secretion of nucleic acids into the biofilm. The release of low as well as high molecular weight nucleic acids triggered by SDS, shown for *At. thiooxidans* in this study for the first time, fits to that biofilm model. Release of nucleic acids by external influence was described by Kreth *et al.* (2009), showing that hydrogen peroxide induced DNA release in *Streptococcus sanguinis* and *S. gordonii*. The induction of a competent state initiated release of DNA from a *S. pneumoniae* subpopulation was shown by Steinmoen *et al.* (2002). This DNA can be taken up by

competent cells. This would explain the 25 % not-lysed subpopulation of *At. thiooxidans* even after treatment with 10 g/L SDS.

SDS treatment in small scale inhibited bioleaching

To apply these results in a more natural habitat experiments in 20 ml columns were performed. These columns were filled with material containing iron- and sulphur-oxidizing microorganisms in concentrations between $1x10^4$ and $5x10^4$ cells per gram sand. After 26 weeks in control columns with rainwater around two orders of magnitude more iron-oxidizing microorganisms were measured per gram soil. Sulphur-oxidizing microorganisms were found in the range from $5x10^1$ to $1x10^5$ cells per gram sand for fresh and recycled rainwater. In the SDS treated sample iron- and sulphur-oxidizing microorganisms were not detectable. In leaching solution of control columns 13 weeks after start of the experiments iron- and sulphur oxidizing microorganisms were found in concentrations between $1x10^4$ and $5x10^5$ cells per millilitre (Figure 3). After additional 13 weeks both controls showed a slight decrease of iron- oxidizing microorganisms. Sulphur-oxidizing microbes were found in concentrations of $1x10^5$ or $5x10^1$ cells per millilitre for fresh or recycled rainwater, respectively. In the leaching solution of SDS treated sample iron- and sulphur-oxidizing microorganisms were not detectable over the complete run. The decrease below the detection limit of leaching microorganisms after SDS treatment correlates with examinations in column experiments with addition of 1 mM SDS (Schippers *et al.* 1998). Repeated SDS treatment performed by Schippers *et al.* (2001) resulted in long term inhibition of leaching microorganisms in large percolators. The rapid decrease of leaching microorganisms after single SDS treatment in our experiments is explainable by higher surfactant concentration and smaller scale of our columns.

The control washed with fresh rainwater showed four weeks pH values around 4.0, than dropped to pH 2.0. In the sample with recycled rainwater pH values dropped within 12 weeks from 3.0 to 1.5. The SDS treated sample showed over the complete run pH values between 4.5 and 4.0. In the SDS treated column a decrease of sulphate release of more than 75 % was measured, compared to the controls (Figure 4). This supports the results shown above. A stable pH around 4 and a reduction in sulphate release after SDS treatment over 26 weeks indicate long term inhibition of leaching microorganisms in column scale experiments.

CONCLUSIONS

Release of DNA and significant but incomplete disappearance of *At. thiooxidans* cells after SDS treatment suggest a lysis mechanism for the planctonic high cell density system. In the more complex sand samples not only sulphur- but also iron- oxidizing microorganisms were not detectable and sulphate release reduced by SDS treatment indicating the sensitivity of bioleaching.

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FIGURES

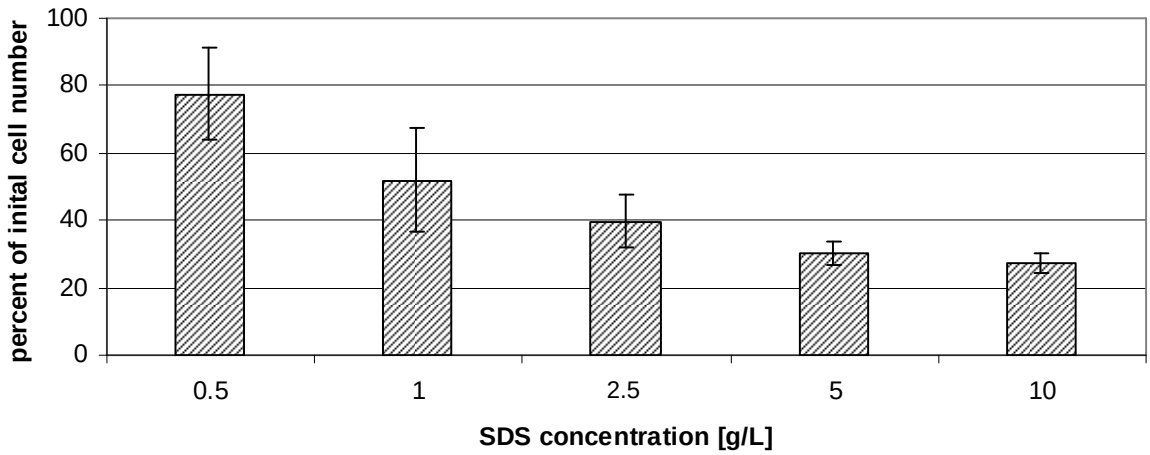

Figure 1. Lysis of planctonic *At. thiooxidans* DSM 622. Cell number counted 30 minutes after exposition to different concentrations of SDS in relation to the control.

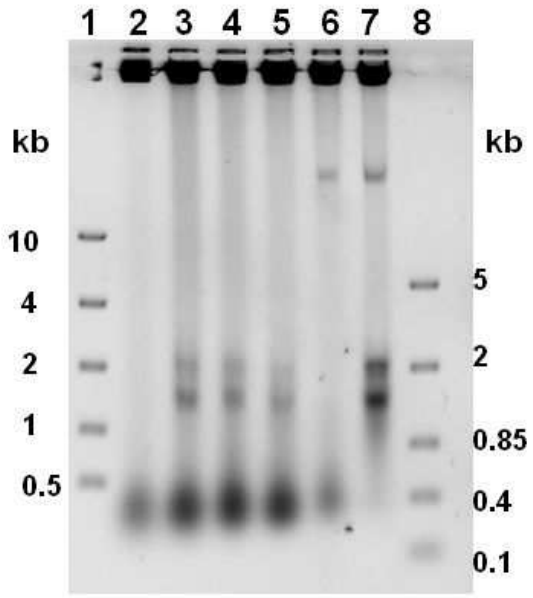

Figure 2. Agarose gel of nucleic acids released from *At. thiooxidans* pellet fraction after SDS treatment. Lanes: 1, high range marker (Fermentas); 2, control without SDS; 3, 0.5 g/L SDS; 4, 1 g/L SDS; 5, 2.5 g/L SDS; 6, 5 g/L SDS; 7, 10 g/L; 8, middle range marker (Fermentas)

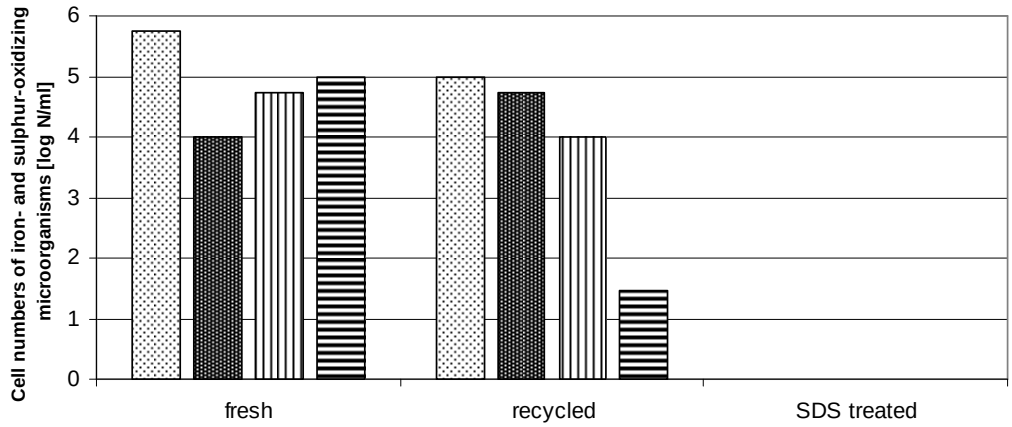

Figure 3. Cell number determined by MPN- technique from leaching solution of three columns. Cultivation at 28°C in medium according to Mackintosh (1978) for iron-oxidizing microorganisms (▨ 13 weeks, ▩ 26 weeks) or Hutchinson *et al.* (1965) for sulphur-oxidizing microorganisms (▥ 13 weeks, ▤26 weeks) for three to four weeks.

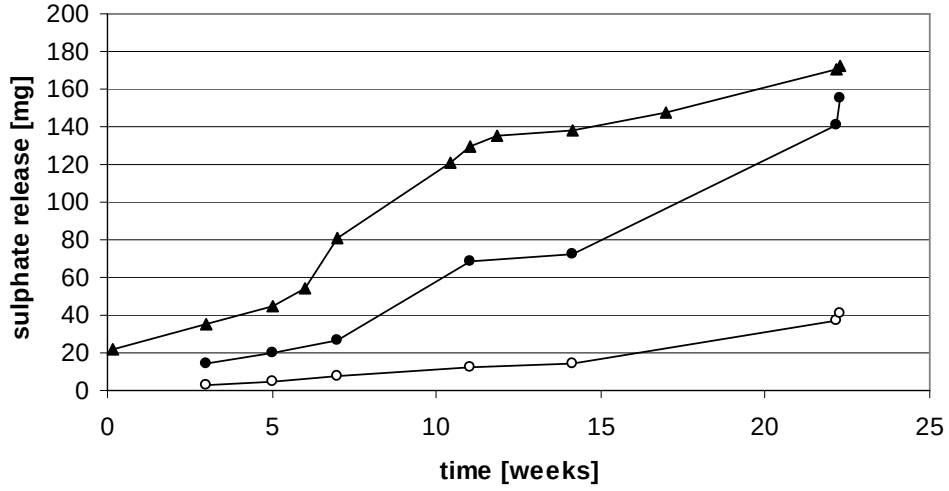

Figure 4. Time course of sulphate released from 20 ml sand columns run with fresh (—▲—), recycled (●) or 2g/L SDS containing (○) rainwater.

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