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## **Bioleaching of pyrite by iron - oxidizing acidophiles under the influence of reactive oxygen species**

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### **Abstract**

After 24h of exposure to acidic media, pyrite generates reactive oxygen species (ROS). Freshly-crushed pyrite with grain sizes between 50-100  $\mu\text{m}$  at 5 % (w/v) pulp density generated  $0.17 \pm 0.01$  mM  $\text{H}_2\text{O}_2$ , while 10% pyrite generated  $0.29 \pm 0.01$  mM and 30 % pyrite generated  $0.83 \pm 0.06$  mM. These levels of  $\text{H}_2\text{O}_2$  probably inhibited iron oxidation in iron-grown cells of *Acidithiobacillus ferrooxidans*<sup>T</sup> but not in pyrite-grown cells. ROS originating from pyrite, which was incubated for 24 h in acidic medium, likely prohibited pyrite dissolution by iron-grown cells, while pyrite-grown cells were probably adapted to these concentrations of ROS. Periodical addition of 100  $\mu\text{M}$   $\text{H}_2\text{O}_2$  to pyrite cultures inoculated with pyrite-grown cells did not decrease iron dissolution. By high throughput proteomics analysis, an increased expression of proteins related to oxidative stress management, iron- and sulfur oxidation systems, carbon fixation and biofilm formation was observed in biofilm cells grown on pyrite compared to iron-grown cells.

### **Introduction**

In acidic conditions, sulfide minerals, especially pyrite, generate toxic ROS, resulting in oxidative stress of acidophilic metal oxidizing microorganisms [1, 2]. Pyrite spontaneously generated  $\text{H}_2\text{O}_2$ ,  $(\text{O}_2\cdot^-)$  and  $\text{OH}\cdot$  radicals in aqueous solution either in the presence or in the absence of oxygen [3, 4]. As a result, bioleaching bacteria are likely to be confronted with high levels of oxidative stress. It is indicated that in comparison with iron-grown cells, pyrite- grown cells of *A. ferrooxidans*<sup>T</sup> are better adapted to  $\text{H}_2\text{O}_2$  [5]. Studies on the oxidative stress response of acidophiles have been carried

out [6, 7], though the influence of ROS on bioleaching and strategies to withstand ROS of *A. ferrooxidans*<sup>T</sup> are still inadequately understood. The aim of this study was to characterize the effect of H<sub>2</sub>O on *A. ferrooxidans*<sup>T</sup> grown with iron or pyrite as growth substrate and subsequently on pyrite bioleaching efficiency. High throughput proteomics analysis was used to get insights into the mechanisms for ROS tolerance in *A. ferrooxidans*<sup>T</sup>.

## Materials and Methods

**Bacteria and cultivation conditions.** *A. ferrooxidans*<sup>T</sup> was grown in Mackintosh medium [8] adjusted to pH 1.8, supplied either with ferrous iron 3 gL<sup>-1</sup> or pyrite at 5% w/v (50-100 µm grain size); cultures were incubated at 28<sup>o</sup>C and shaking at 120 rpm.

**Pyrite grains.** Pyrite was prepared as described [9] and sterilized by autoclaving at 120<sup>o</sup>C for 4h under N<sub>2</sub> atmosphere.

**Quantification of H<sub>2</sub>O<sub>2</sub>:** H<sub>2</sub>O<sub>2</sub> was quantified using the spectrophotometric determination method of Baga [10].

**Iron determination.** Pyrite dissolution was assessed by quantification of ferrous and total iron concentration using the 1,10-phenanthroline method according to German standards [5].

**Proteomics analysis.** Proteins from biofilm cells grown on pyrite surfaces and cells grown on ferrous iron after 5 days of incubation were extracted, purified, and analyzed as described [11].

## Results and Discussion

**Generation of H<sub>2</sub>O<sub>2</sub> in pyrite-containing acidic MAC media.** Table 1 summarizes the concentrations of H<sub>2</sub>O<sub>2</sub> produced by pyrite after 24 h incubation in MAC media. Obviously, pyrite generated H<sub>2</sub>O<sub>2</sub> in dependence of pyrite grain size and pulp density. Pyrite with the grain size of 50-100 µm generated higher concentrations of H<sub>2</sub>O<sub>2</sub> than the fraction with 100-200 µm grain size. Also, pulp densities of 30% (w/v) generated H<sub>2</sub>O<sub>2</sub> concentrations 5 times higher than that of 5% (w/v).

**Tab. 1.** H<sub>2</sub>O<sub>2</sub> generation (mM) by pyrite of different grain size and pulp density after 24h in MAC medium.

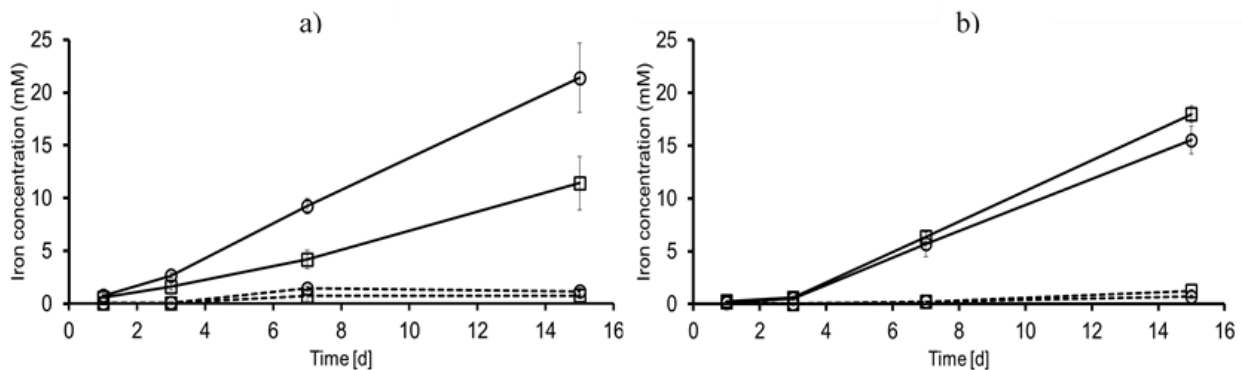
| Grain sizes (µm) | Pulp density % (w/v) |             |             |
|------------------|----------------------|-------------|-------------|
|                  | 5                    | 10          | 30          |
| 50-100           | 0.17 ± 0.01          | 0.29 ± 0.01 | 0.83 ± 0.06 |
| 100-200          | 0.05 ± 0.00          | 0.09 ± 0.01 | 0.25 ± 0.01 |

About 0.9 mM H<sub>2</sub>O<sub>2</sub> is formed by a pyrite load of 10% (pyrite sizes <106 µm) at pH 4.5 and more H<sub>2</sub>O<sub>2</sub> is formed at lower pH [12]. The H<sub>2</sub>O<sub>2</sub> generated in this study was lower than in the study by Nooshabadi et al [12]. However, H<sub>2</sub>O<sub>2</sub> concentrations of ≥ 50µM have significant toxic effects on *A.ferrooxidans* [13, 14]. Therefore, 5% (w/v) pyrite loading, a size of 50-100, generates toxic levels of H<sub>2</sub>O<sub>2</sub> for *A.ferrooxidans*.

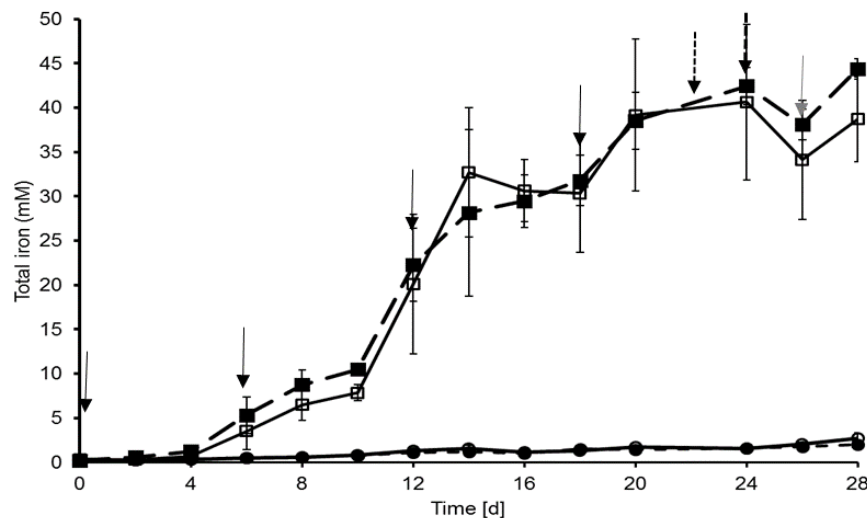
**Pyrite dissolution.** Figure 1 shows the dissolution of pyrite by *A.ferrooxidans*<sup>T</sup>. Total concentration of iron in solution without the preincubation of pyrite was nearly two times the total iron concentration in solution with pyrite-generated H<sub>2</sub>O<sub>2</sub> in the case of iron-grown cells (**a**). In contrast, there was no significant reduction in pyrite dissolution when pyrite-grown cells were used (**b**).

Also, in case of pyrite-grown cells no significant differences in pyrite dissolution assays with the periodical additions of external H<sub>2</sub>O<sub>2</sub> concentrations occurred (Figure 2). In abiotic assays, with and without addition of H<sub>2</sub>O<sub>2</sub>, the total iron ions remained relatively constant at low concentration.

In biotic assays, regardless of the addition of external H<sub>2</sub>O<sub>2</sub>, total iron concentrations increased steadily and reached approximately 45 mM after 28 days of incubation.



**Fig. 1.** Pyrite dissolution with a) iron-grown cells and b) pyrite-grown cells of *A.ferrooxidans*<sup>T</sup>. Control without preincubation of pyrite (*circles*) and assay with 24h preincubation of pyrite in MAC media (*squares*) were compared. Iron ion concentrations include ferrous iron (*dashed line*) and total iron (*solid line*). The initial cell inoculation was  $5 \cdot 10^7$  cells/mL. All assays in experiment were performed in triplicate; error bars show standard deviation of the mean as indicated.



**Fig. 2.** Effect of external addition of H<sub>2</sub>O<sub>2</sub> on pyrite dissolution using pyrite grown-cells of *A.ferrooxidans*<sup>T</sup>. The additions of H<sub>2</sub>O<sub>2</sub> were 100 μM at day 0, 6, 12 and 18 (*black arrows*), 500 μM at day 22, 24 (*dotted black arrows*) and 1 mM at day 26 (*grey arrows*). Total iron concentrations were measured in sterile (*circles*) and inoculated (*squares*) pyrite dissolution assays, with (*empty symbols*) and without (*filled symbols*) periodic addition. The initial cell inoculum was  $5 \cdot 10^7$  cells/mL. All assays in experiment were performed in triplicate; error bars show standard deviation of the mean as indicated.

Those results are similar to previous findings, indicating that compared to iron-grown cells, pyrite -grown cells of *A.ferrooxidans*<sup>T</sup> were probably adapted to the presence of elevated levels of ROS, which are generated on metal sulfide surfaces [5]. Additionally, although H<sub>2</sub>O<sub>2</sub> is commonly known as a toxic agent, at low levels it may also function as signaling agent [15]. Also, pre-exposure to low concentrations of H<sub>2</sub>O<sub>2</sub> allows microorganisms to resist efficiently detrimental effects of oxidative stress [16]. Genetic response is of importance among strategies to protect enzymes and DNA against oxidative stress damage [16]. Bioleaching acidophiles possess several proteins responsible for coping with oxidative stress, involving several oxygen detoxification and repair

systems [6]. Proteomic data showed that during the growth with pyrite as energy source, cells of *A.ferrooxidans*<sup>T</sup> utilized ROS degradation, redox balance, macromolecule repair mechanisms, metal and oxygen homeostasis as fundamental adaptation strategies to the elevated presence of ROS (data not shown). Also, our shot-gun proteomics analysis indicated that there was a remarkable increase in the expression of proteins related to oxidative stress response in biofilm cells grown on pyrite surfaces compared to iron-grown cells of *A.ferrooxidans*<sup>T</sup>.

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