Advanced Materials Research Vol. 825 (2013) pp 115-119
Online available since 2013/Oct/07 at www.scientific.net

doi:10.4028/www.scientific.net/AMR.825.115

EPS production during adaptation of *Acidithiobacillus ferrooxidans* to high ferric ion concentration

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Key words: *At. ferrooxidans*, extracellular polymeric substances, ferric ion.

Abstract. The ability of *Acidithiobacillus ferrooxidans* to derive its energy from the oxidation of ferrous iron and the inhibitory effect of high ferric iron concentrations on its growth behaviour has been extensively studied. Furthermore, it is known that *At. ferrooxidans* excretes organic substances called extracellular polymeric substances (EPS), which could play a role in its protection against adverse environmental conditions. In this context, the aim of this work was to study the production of EPS during adaptation of *At. ferrooxidans* to high ferric ion concentrations. The experiments were performed in shake flasks of 250 mL at 30 °C, 200 rpm and at an initial pH of 1.8. In order to establish the natural tolerance of the strain, its growth behaviour was evaluated at high ferric iron concentrations by adding consecutively the equivalent of 9 g/L of ferrous iron each time it was depleted in the broth. Cell growth stopped once ferric iron concentration increased up to 38 g/L.

The adaptation consisted in eight sub-cultures run in parallel at initial concentrations of ferrous iron of 18, 27 and 36 g/L. The EPS was quantified as micro volumes using confocal laser scanning microscopy (CLSM), labelling the cells with propidium iodide and EPS carbohydrates with wheat germ agglutinin (WGA). During the adaptation procedure an increase in the ferric ion volumetric productivity of subcultures run with 27 and 36 g/L was observed, as a result of cell adaptation. The amount of EPS excreted by cells was increased along with those experimental conditions having increased ferric iron concentrations. EPS on cells grown with 9 g/L of ferrous iron were not detected. This study found that the adapted strain showed higher production of EPS at high ferric ion concentrations and increased ferric ion tolerance than non-adapted ones.

Introduction

Acidithiobacillus ferrooxidans is an acidophilic bacterium, iron-oxidizing [1], important in different processes such as bioleaching, production of ferric sulfate, microbial fuel cells etc [2,3]. One of the problems latent in the various processes using this type of microorganism is the inhibition caused by ferric ion accumulation, leading to a low productivity of the process [4]. It is known that bacteria in adverse conditions often change their metabolism to adapt to new environmental conditions. Presumably, extracellular polymeric substances (EPS) are the result of these modifications [5]. *Acidithiobacillus ferrooxidans* capacity to oxidize ferrous ion has been extremely exploited, however it has been shown that oxidation and growth are subject to product inhibition. It has been reported that *At. ferrooxidans* presents competitive product inhibition to ferric ion, and a tolerance to this ion of up to 15 g/L [4].

In an adaptive experience *L. ferrooxidans* with ferric ion to ferrous ion by adding pulses growth was observed at concentrations close to 70 g/L of ferric ion (unpublished data), and for *At. ferrooxidans* it was reported the tolerance of up to 53 g/L of ferric ion in the presence of EPS [6]. The values are higher than those reported for inhibition of *At. ferrooxidans* in planktonic state. An increased sensitivity of *At. ferrooxidans* to copper ions by inhibiting the formation of EPS has been reported [8]. The aim of this work was to study the production of EPS during adaptation of *At. ferrooxidans* to high ferric ion concentrations.

Methodology

Tolerance of *At. ferrooxidans* to ferric ion was determined in a 1000 mL batch reactor containing 500 mL modified 9K medium at 30 °C, initial pH of 1.8, 200 rpm, and inoculum of 10% v/v. The increasing concentrations of ferric ion in the broth were obtained adding consecutively the equivalent of 9 g/L of ferrous ion until total inhibition of cell growth and ferrous ion oxidation was observed. The assay was carried out in duplicate.

Adaptation experiments were performed in 250 mL shake flasks having 100 mL of culture medium described by [9], at 30 °C, 200 rpm and initial pH of 1.8. Initially the ability of ferrous ion oxidation at high ferric ion concentrations in culture with increasing stages of ferrous ion was investigated. The adaptation consisted in consecutive sub-cultures at initial ferrous ion concentrations of 18, 27 and 36 g/L. During the experiments Fe^{2+}, Fe^{3+}, Fe_T (phenanthroline method), cell counts (Petroff Hausser chamber), Eh and pH were quantified. The analyses were performed in triplicate and results are presented as the average plus mean deviation.

EPS were visualized using confocal laser scanning microscopy (CLSM). 10 µL of samples were fixed in glass slide during 10 min and then immersed in methanol for 10 min. Two fluorophores were used: labelling the cells with propidium iodide and EPS carbohydrates with wheat germ agglutinin (WGA). Each fluorophore was incubated with the sample for 2 h and visualised under appropriate laser excitation wavelengths and optical filters for specific detection of fluorophore signals (Argon laser: 488 nm, 505–550 nm band pass filter; helium neon laser: 543 nm, >560 nm long pass filter). The EPS quantity was measured using an image processing software, allowing to quantify micro volumes (μm^3) of cells and EPS.

Results

Overproduction of EPS by *At. ferrooxidans* could be stimulated by different techniques, even though it is not clear what their specific functions are. One of the functions studied in this research is the tolerance to high concentrations of ferric ion, after being subjected to an adaptation by consecutive sub-cultures.

It was noticed that the bacteria after 8 sub-cultures had a higher tolerance than non-adapted cells. The ground state filed a ferric ion tolerance of 38.2 g/L and after adaptation presented a tolerance of 48.9 g/L, increasing tolerance in 23,6% (Fig. 1 and 4).

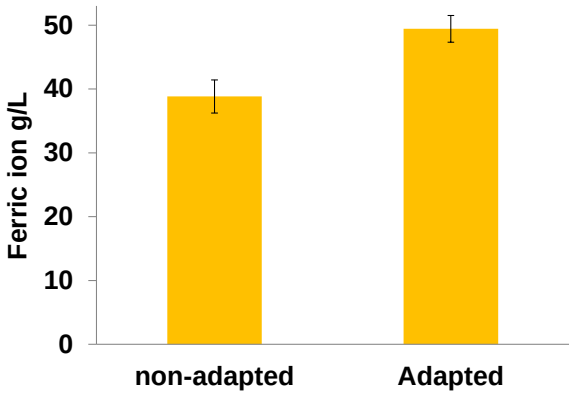

Figure 1. Tolerance to ferric iron by adapted and non-adapted bacteria.

EPS production by bacteria undergpoing adverse conditions has been reported by several investigators [7]. It was observed that adapted bacteria had a higher volume of EPS on their surface, possibly for conferring the ability to tolerate high concentrations of iron. While gradually the concentration of ferric iron in the test increased also the volume of EPS measured increased, suggesting that it may play a specific role in the adaptation of *At. ferrooxidans*.

During the sub-cultures it was observed that the volumetric productivity of ferric ion (Q_p) (Fig. 2A and 2B) increased considerably for the cases of 27 and 36 g/L of initial ferrous iron concentrations, but not for the case of 18 g/L. This suggests that the cells in the latter condition can grow without being inhibited by the accumulated ferric ion

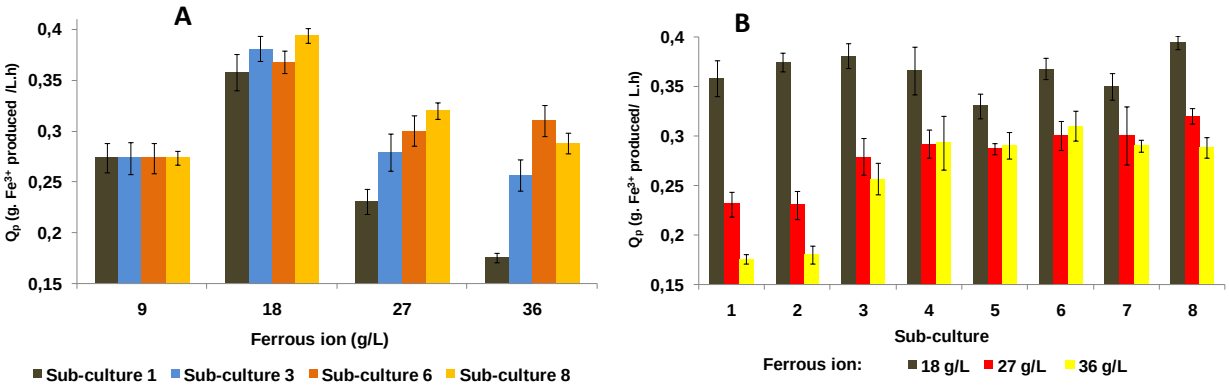

Figure 2. (A y B) Volumetric productivity of ferric ion in different adaptation sub-cultures at initial concentrations 18, 27 and 36 g/L of ferrous iron.

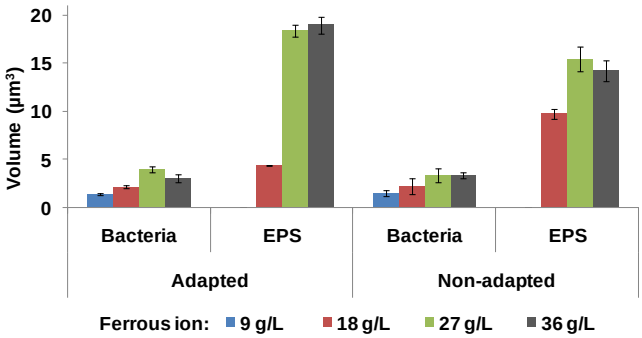

Figure 3. Volume of cell and EPS of cell adapted and non-adapted at different concentrations initial of ferrous iron.

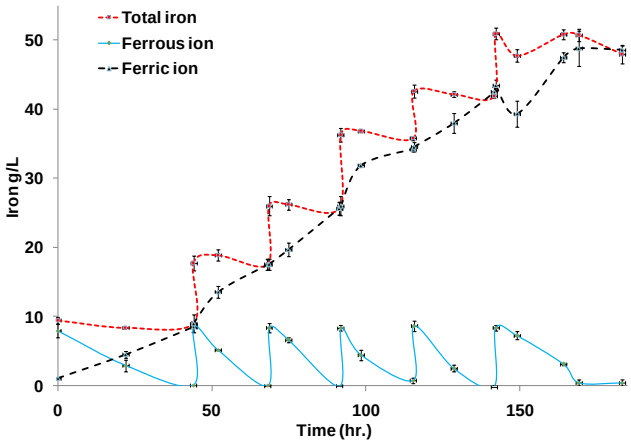

Figure 4. Fe^{2+}, Fe^{3+} and total iron concentration behaviour during tolerance test for bacteria previously adapted to high ferric iron concentrations.

It has been reported that iron can be absorbed by the EPS of acidophilic bacteria [10], providing a potential participating system for increased tolerance to heavy metals.

Figure 3 shows that at different levels of ferrous iron the volume of EPS gradually increased and also in the case of adapted and non- adapted bacteria, as well as bacterial volume, however, at 27 and 36 g/L volumes were similar. Fig. 4 shows the ferric, ferrous and total iron profiles of the tolerance test to adapted cells. Total iron increases stepwise as a consequence of each Fe^{2+} addition while Fe^{3+} increases steadily. During the last Fe^{2+} addition a decrease of Fe^{2+} biooxidation is observed, indicating that the accumulated ferric iron is hardly tolerated by the cells.

In Figure 5, it can be observed the EPS surrounding the surface of the bacteria, whose volume increases with increasing iron presence. This structure and micro cell volumes is consistent with those reported by [8]. The ability to produce EPS in adverse conditions can be taken as a physiological strategy for *At. ferrooxidans* against foreign agents, in this case to high concentrations of iron.

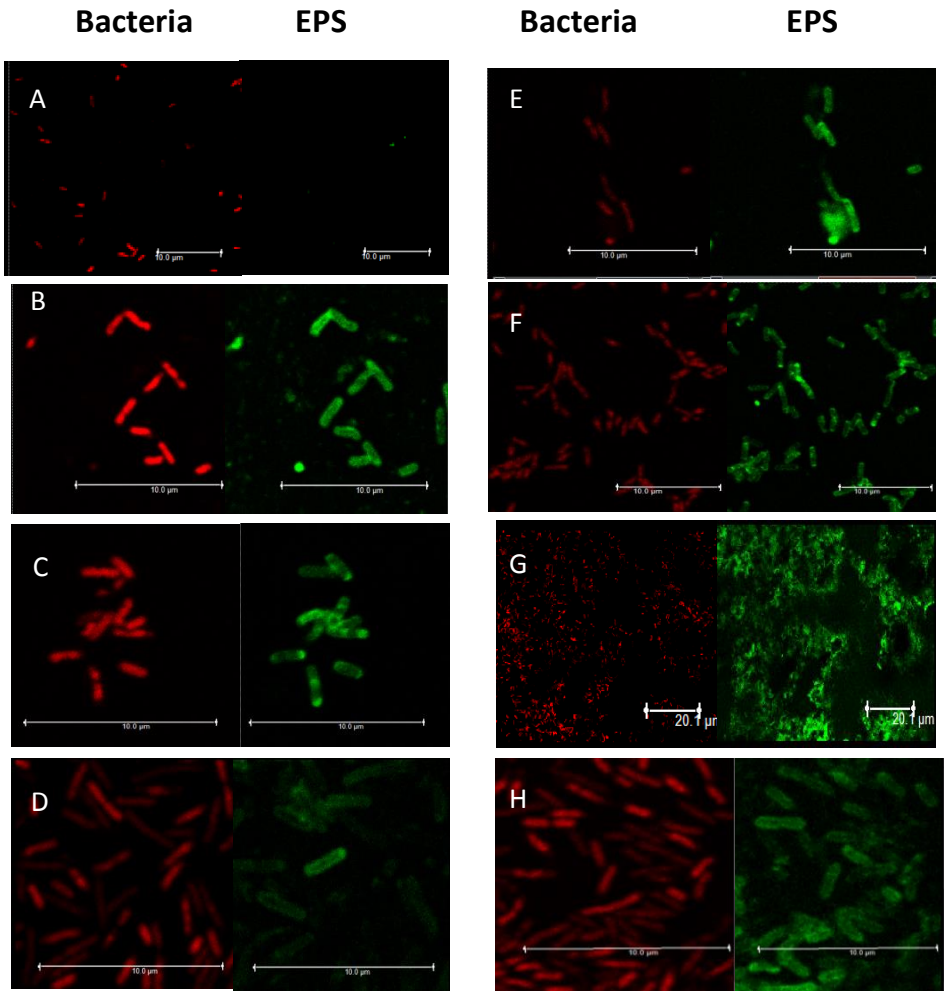

Figure 5. . CLSM microphotograph of *At. ferrooxidans*. Cells (red) are labelled with propidium iodide and EPS carbohydrates (green) with wheat germ agglutinin. Non-adapted: (A) 9 g/L, (B) 18 g/L, (C) 27 g/L, (D) 36 g/L. Adapted: (E) 18 g/L, (F) 27 g/L, (G) 36 g/L, (H) 40 g/L.

Conclusions

EPS production in *At. ferrooxidans* is increased as the bacterium is adapted to high concentrations of ferric ion. Also, it is possible to increase its capacity of ferrous ion oxidation at high concentrations of ferric iron.

Acknowledgments

School of Biochemical Engineering, P. Catholic University of Valparaiso, Chile; for their support in this investigation.

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10.4028/www.scientific.net/AMR.825

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10.4028/www.scientific.net/AMR.825.115