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Galactose as inducer of the production of extracellular polymeric substances by *Acidithiobacillus ferrooxidans*

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Abstract. The presence of extracellular polymeric substances (EPS) is important for the formation of biofilms on mineral surfaces, increasing the bioleaching activity, as well as protecting the cells from adverse environmental conditions. The objective of this work was to study the effect of galactose for EPS production by *Acidithiobacillus ferrooxidans*. The work was 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 after oxidation to the broth. Cell growth stopped once ferric iron concentration increased up to 38 g/L. In order to determine the optimal conditions for EPS production, experiments were run in a chemostat of 0.5 L, operated at a constant dilution rate of 0.03 h^{-1}. Different steady states were obtained varying feeding concentrations of galactose (0.15%; 0.25% and 0.35%) and carbon dioxide (180 ppm and 360 ppm). Cells grown in the chemostat at optimum operational conditions were used as inoculum to determine oxidative capacity of the microorganisms overproducing EPS. The EPS was quantified using confocal laser scanning microscopy (CLSM), labelling the cells with propidium iodide and EPS carbohydrates with wheat germ agglutinin (WGA). The high volume production of EPS was observed in cells grown using 360 ppm of CO_2 and 0.35% of galactose. Also it was observed a size increase of cells, compared to cells grown in culture medium having 9 g/L of ferrous iron, where presence of EPS was not detected. The results revealed that EPS overproducing *At. ferrooxidans* showed a tolerance to ferric iron concentration almost 9.5 g/L higher than the natural tolerance of cells grown in absence of galactose. Presence of galactose in culture medium stimulated the EPS production.

Introduction

The extracellular polymeric substances (EPS) appear to play a role in the uptake of metals as in bioleaching[1] and the heavy metal tolerance of microorganisms, thanks to their capacity of interaction with different metal ions [2]. This feature has been most studied in bioleaching, biocorrosion, biomechanics and bioremediation [3,4,5,6]. *At. ferrooxidans* an iron(II) ion-oxidizing acidophilic microorganism , , is the most studied mesophilic microorganism for the extraction of metals from sulfidic ores by bioleaching [7]. EPS production in *At. ferrooxidans* has been studied by various researchers [8], although it is not yet clear how it is produced. Several exogenous substances have been described for the production of EPS in *At. ferrooxidans*. Besides, the presence of some carbohydrates can lead to an increased production of EPS [9]. It has been described that galactose at a concentration of 0.35% in culture medium 9K can stimulate production of EPS, because it possesses metabolic pathways for synthesis [10,11]. Moreover, the inhibition of ferrous iron bio oxidation by *At. ferrooxidans* at high ferric ion concentrations is well known [12]. A tolerance of 15 g/L ferric ion has been reported [13]. This research aims to study the production of EPS in *At. ferrooxidans* in the presence of a defined concentration of galactose, and how it can affect its tolerance to high concentrations of ferric ions.

Methodology

The tolerance of *At. ferrooxidans* to ferric ions was determined in a batch culture. Cells were cultured in 500 mL modified 9K medium in a reactor of 1000 mL, at 30 °C, initial pH of 1.8, 200 rpm and an inoculum of 10% v/v. The high concentrations of ferric ion were obtained by adding consecutively the equivalent of 9 g/L of ferrous ion to observe a total inhibition of cell growth and ferrous ion oxidation. The assays were done in triplicate. Precultures for chemostat cultivation were grown overnight at 30 °C and 200 r.p.m. in 500 mL shake flasks containing 100 mL mineral medium with 44 g/L of ferrous sulphate. The experiments were carried out in a chemostat of 1 L. Experimental conditions were a working volume of 0.5 L, pH controlled at 1.8 by automatic addition of concentrated H_2SO_4, and a constant temperature of 30 °C. Agitation was set at 200 rpm, and the air flow into the reactor was 0.5L/min. The chemostat operation was preceded by a batch cultivation. After the ferrous ions had been exhausted, the batch mode was switched to continuous mode at a dilution rate (D) of 0.03 h^{-1}. The culture was assumed to be in steady state, after at least three volume changes. Different steady states were obtained varying feeding concentrations of galactose (0.15%, 0.25% and 0.35%) and carbon dioxide (180 ppm and 360 ppm). The feed of carbon dioxide was controlled by a trap of 3M NaOH. The samples were performed by triplicate, and results are presented as the averages and mean deviations. 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 to labell 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

The ferric ion tolerance of *At. ferrooxidans* grown in absence of galactose was 37.9 g/L (Fig. 3A). After determining its tolerance, the amount of galactose necessary for greater production of EPS was determined.

In chemostat was observed that when galactose increased in the reactor, the cell concentration decreased (Fig. 1) probably because galactose exerted some inhibition on bacterial growth and ferrous ion oxidation [14].

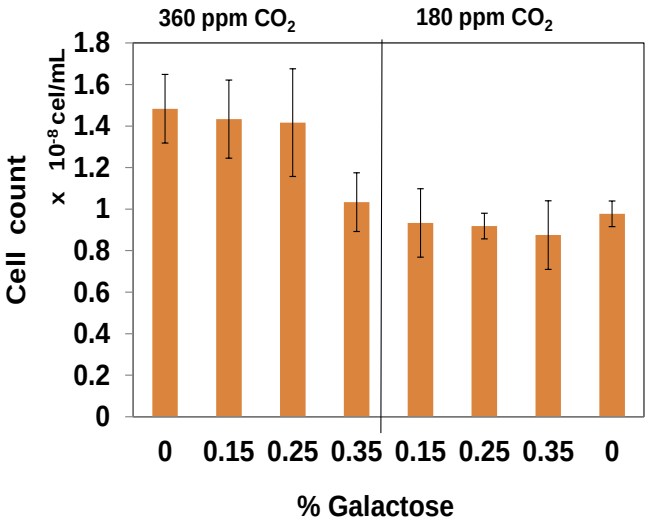

Figure 1. Cell counts at steady state condition in chemostat operated at a dilution rate of 0.03 h^{-1}.

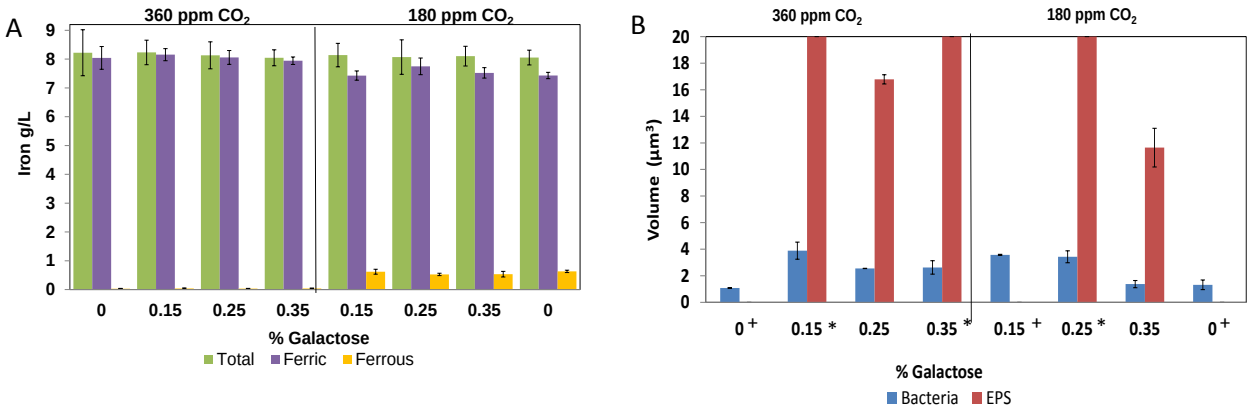

Figure 2. Chemostat culture operated at a dilution rate of 0.03 h^{-1} at different galactose and CO_2 concentrations at each steady state condition. (A) Iron, (B) Volume of cells and EPS. * EPS exceeded not measurable, $^{+}$ Not detected.

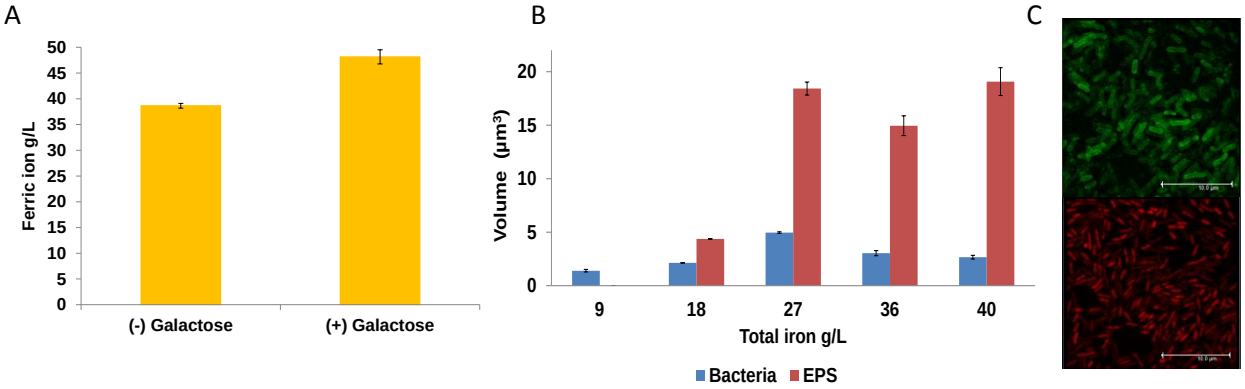

Figure 3. Ferric ion tolerance with and without addition of 0.35% galactose (A) Tolerance ferric iron, (B) Volume of cell and EPS and (C) CLSM microphotograph: EPS (green) and the bacteria (red).

The residual ferrous ion concentration in the reactor increased as CO_2 content in the gas feed was lowered to 180 ppm, indicating a decrease in the oxidation (Fig. 2). However, the major differences in the production of EPS were observed by the microscopic analysis.

The effect of galactose addition on the production of EPS was performed in the chemostat. An increased presence of EPS for cells when galactose concentrations were 0.25% and 0.35% at 360 ppm CO_2, and 0.25% at 180 ppm CO_2 (Fig. 2B, 3B, 4D and 4F) was measurable, showing also under these conditions an increase of the cell size. Cells grown in presence of 0.35% of galactose and 360 ppm of CO_2 showed higher tolerance to ferric ions than untreated cells (Fig. 3A), coincident with an observed increment in cell size and EPS volume (Fig. 3B). Fig. 3C shows very clearly the EPS (green) surrounding the cells (red) all over its surface, completely covering and forming a thick layer of 1 to 2 microns of EPS. In trials, where galactose was absent, EPS was not detected, although some authors report their presence amid no added carbohydrates to media culture [2,11].

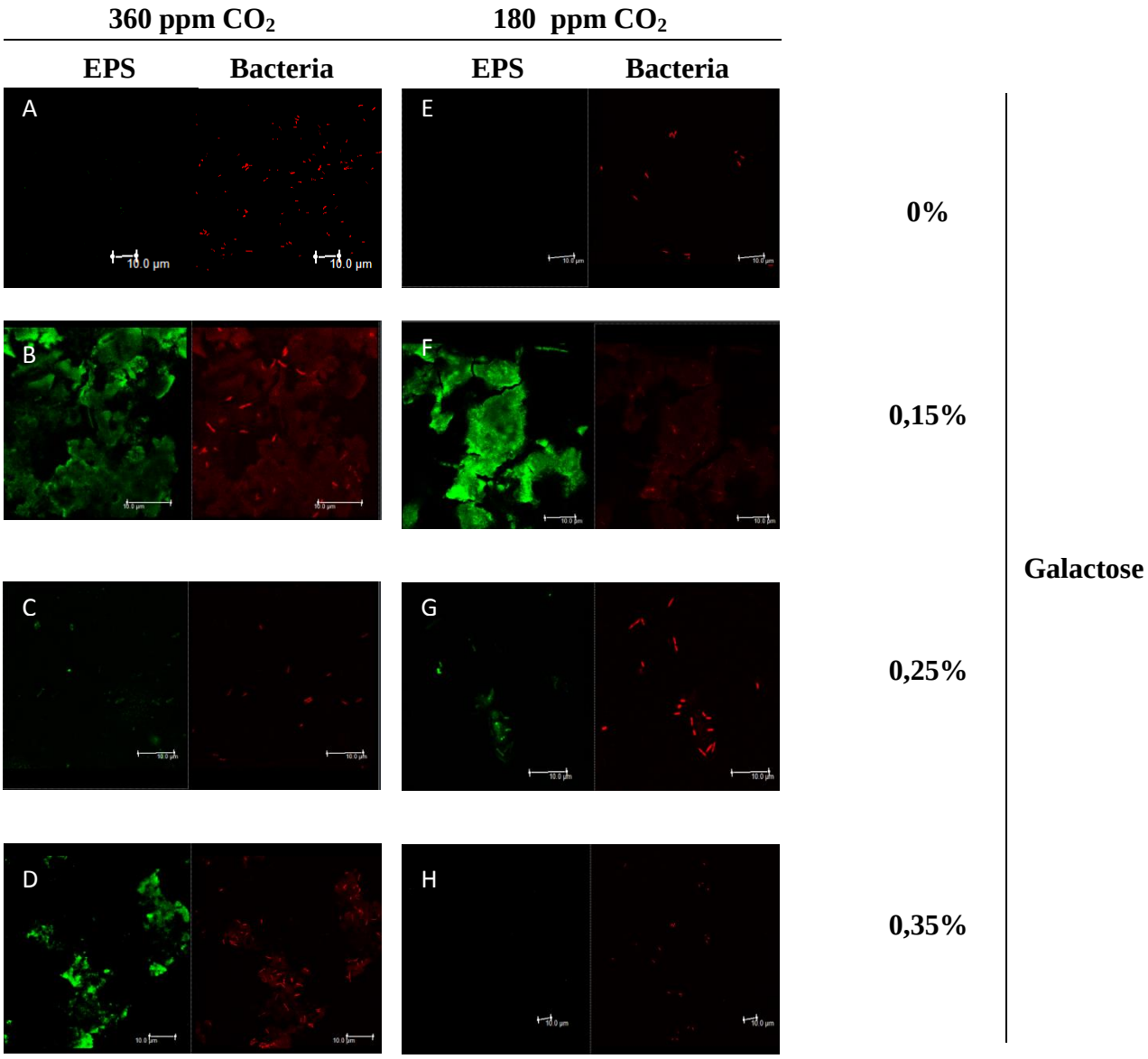

Figure 4. . CLSM microphotograph showing *At. ferrooxidans*. The right side shows the cells (red) labelling with propidium iodide and the left side the EPS carbohydrates (green) labelling with wheat germ agglutinin at different steady states in the chemostat. At 360 ppm of CO_2: galactose (A) 0%, (B) 0.15%, (C) 0.25%, (D) 0.35%. At 180 ppm CO_2: galactose (E) 0.15%, (F) 0.25%, (G) 0.35%, (H) 0% (Scale bars: 10 µm).

Conclusions

The presence of galactose in the medium restricted the cell growth moderately, however, it enhanced the production of EPS. The highest EPS production was obtained using 0.15 and 0.25% galactose and 360 ppm of CO_2. The tolerance to ferric ion concentrations with galactose was higher than in its absence. The cells showed an increased size in the presence of galactose.

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