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Chloride ion tolerance and pyrite bioleaching capabilities of pure and mixed halotolerant, acidophilic iron- and sulfur-oxidizing cultures

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ABSTRACT

The search for halotolerant acidophilic microorganisms to increase the efficiency of bioleaching processes in regions of fresh water scarcity has been ongoing for the past two decades. In this study, three pure cultures (V6, V8 and M8) and four enrichment cultures (14C, L2-21, L4-9 and L6-11) from low pH, high saline environments were characterized for their ability to oxidize soluble iron and inorganic sulfur in the presence of increasing concentrations of chloride ion. The mixed cultures 14C and L2-21 contained predominantly *Acidihalobacter* and *Acidithiobacillus* spp., respectively, while L4-9 and L6-11 predominantly contained *Ferroplasma* spp. Cultures V6, V8, 14C and L2-21 were assessed for their ability to oxidize 1% pyrite at 9, 15 and 30 g/L chloride ion. Results showed that pure cultures V6 and V8 and mixed culture 14C were able to oxidize pyrite at chloride ion concentrations of 30 g/L, which is higher than the chloride concentration found in seawater (19 g/L). L2-21 was unable to oxidise pyrite, possibly due to the predominant presence of sulfur oxidizing microorganisms in the mixed culture. This illustrates the potential applicability of the cultures for saline water bioleaching and biooxidation of sulfide ores.

1. Introduction

Microorganisms capable of oxidizing metal sulfides are predominantly acidophilic in nature, having an optimum pH range below 3 (Baker-Austin and Dopson, 2007). These microorganisms are useful for bioleaching applications, in which microorganisms are used to catalyze the extraction of metals from ore. Bioleaching allows the economic extraction of metals from low-grade and complex ores, the processing of which would not be feasible using traditional mining methods (Jerez, 2009; Rawlings, 2002). It also provides the benefits of having relatively low energy demand and atmospheric emissions, making it a more environmentally benign alternative compared to more traditional extraction methods such as roasting and smelting or leaching with strong inorganic acids (Rohwerder et al., 2003).

The use of acidophilic microorganisms has already been successfully applied to a number of sulfide ores for the extraction of base metals such as copper, nickel, cobalt and zinc (Watling, 2016). These microorganisms have also been used for the biooxidation of refractory gold minerals (Viera et al., 2007; Watling, 2006). It has been estimated that the utilizable copper, zinc and nickel ore reserves in the world may only last another twenty to forty years (Norgate and Jahanshahi, 2010). As higher grade ores become less available, the mineral industry is faced with the challenge of finding low carbon footprint technologies, such as bioleaching, that may improve the economic viability of the mining sector (Johnson, 2013). However, the applicability of typical bioleaching microorganisms is restricted in areas like parts of Western Australia and Chile where chloride content of soils and source waters is extremely high (> 100 g/L) and access to fresh water is scarce, often leading to the use of seawater or brackish or brine ground and surface waters at some mines (Rea et al., 2015; Shiers et al., 2005; Watling, 2016; Zammit et al., 2012). This has led to a strong interest in the search for bacterial cultures that are able to actively bioleach in seawater media (Watling, 2016).

The ability of bioleaching microorganisms to tolerate chloride ion varies between domain, genus and species, but most of these microorganisms cannot tolerate the levels of chloride ion present in seawater and can be inhibited by concentrations as low as 6.6 g/L (Shiers et al., 2005; Suzuki et al., 1999; Zammit et al., 2012; Zammit et al., 2009). For example, *Acidithiobacillus ferrooxidans* is inhibited by 4.2 g/L chloride and *Leptospirillum* (*L.*) *ferriphilum* by 12.3 g/L chloride (Gahan et al., 2010; Rea et al., 2015; Zammit et al., 2012). *Sulfobacillus thermosulfidooxidans* has been found to grow at up to 12 g/L chloride ion (Zammit et al., 2012). Some archaea, such as *Sulfolobus* spp., are inhibited by 18 g/L chloride (Grogan, 1989; Salo-Zieman et al., 2006).

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Chloride ion stress is thought to affect the viability of acidophiles used in bioleaching by affecting the mechanisms they use to tolerate low pH. Cell membranes are permeable to chloride and when chloride enters the cell, the negative charge of the ion leads to the collapse of the inside positive membrane potential that is normally maintained by the acidophilic cell (Slonczewski et al., 2009; Suzuki et al., 1999). This causes protons to simultaneously enter the cell disrupting to the internal pH (Matin, 2007). The cytoplasm becomes acidified, reducing metabolic activity and ultimately causes cell death (Alexander et al., 1987). Another effect of chloride ions may be on the osmotic potential of the cells. Ojumu et al. (2008) showed that energy stress is created by the osmotic gradient formed between the interior and external environment due to an increased ionic strength, decreasing the growth and bioleaching efficiency of *L. ferriphilum*.

While low pH environments and those high in osmotic stress can be found in a diverse range of locations, environments where both stresses co-exist are rare. There are a few places where the geological conditions provide both high salinity and low pH, such as acidic lakes and drains and volcanoes near seawater, hence only a limited number of microorganisms capable of simultaneously tolerating both stresses have been isolated to date. In this study, iron oxidation and the bioleaching capabilities of a number of halotolerant acidophiles, both pure isolates and mixed cultures, were investigated. The ability of these acidophilic environmental cultures for pyrite leaching under high chloride ion stress was evaluated to explore the potential for using these microorganisms in the bioleaching and biooxidation of sulfide ores in arid regions where access to freshwater is limited.

2. Materials and methods

2.1. Environmental cultures and growth conditions

The cultures used in this study (Table 1) were obtained from culture collections at CSIRO Land and Water (L2-21, L4-9, L6-11) and the University of Exeter (V6, V8, M8, 14C). The mixed cultures from CSIRO Land and Water were enriched from acidic, saline soils and surface waters from the Western Australian Wheat belt and Southwest region. The pure and mixed cultures from the University of Exeter were obtained from shallow acidic pools at the Aeolian Islands, Vulcano, Italy (V6, V8 and 14C) and Milos, Greece (M8). The growth conditions for the maintenance of the cultures are shown in Table 1.

2.2. Chloride tolerance and bioleaching tests

The tolerance of the environmental cultures to chloride ion was determined in triplicate in 50 mL of the following media: V6, V8, M8 and 14C in basal salts (in g/L Milli-Q™ H_2O: 0.4, $(NH_4)_2SO_4$, 0.5, $MgSO_4{\cdot}7H_2O$ and 0.2, K_2HPO_4 acidified to pH 2.0 with concentrated H_2SO_4) supplemented with 13.9 g/L $FeSO_4{\cdot}7H_2O$, 1.51 g/L $K_2S_4O_6$ and 1 mL/L of filter sterilized (0.2 μm cellulose acetate filter, Millipore) trace element solution (mg/L Milli-Q™ H_2O; $MnCl_2{\cdot}2H_2O$, 62; $ZnSO_4{\cdot}7H_2O$, 68; $CoCl_2{\cdot}6H_2O$, 64; H_3BO_3, 30; Na_2MoO_4, 10; $CuCl_2{\cdot}2H_2O$, 66; $NaVO_3$); L2-21, L4-9 and L6-11 in basal salt media consisting of 1.5 g/L $(NH_4)_2SO_4$ plus 0.01% yeast extract adjusted to pH 1.8 with concentrated H_2SO_4 and supplemented with 10 g/L $FeSO_4{\cdot}7H_2O$, 5 g/L tyndallized elemental sulfur and 1 mL/L filter sterilized trace element solution (as above). All cultures were incubated on a rotary incubator at 100 rpm at 30 °C except L4-9 and L6-11 which were at incubated at 45 °C. Tolerance to chloride was tested at 2, 9, 15, 30 and 45 g/L chloride ion, where chloride was provided as sodium chloride (V8, V6, M8 and 14C) or synthetic Sea salts (Sigma Aldrich) (L2-21, L4-9 and L6-11).

Pyrite (FeS_2) concentrates (milled to < 0.75 μm) were sterilized by gamma irradiation (50 kGray). The elemental composition, as determined by inductively coupled plasma—atom emission spectroscopy (ICP-AES) after borax flux and re-dissolution in 5% (vol/vol) HNO_3 was (wt/wt) 36.6% Fe, 0.24% Cu, 0.04% Ni, and 39.8%S. Isolates V6, and V8 and mixed cultures 14C and L2-21 were incubated in 100 mL of the media as described (minus the yeast extract) with 9, 15 and 30 g/L chloride ion and 1% pyrite (the sole source of iron and sulfur) and incubated on a rotary incubator at 100 rpm at 30 °C.

Table 1
Growth conditions of the pure and mixed environmental cultures used in the study.

	Type of culture	Maintenance chloride ion (g/L)	Source of chloride ion	Growth temperature (°C)
V6 *Acidihalobacter prosperus* DSM 14174	Pure	12	Sodium chloride	30
V8 *Acidihalobacter ferrooxidans* DSM 14175	Pure	12	Sodium chloride	30
M8 *Acidihalobacter ferrooxidans*	Pure	12	Sodium chloride	30
14C	Mixed	30	Sodium chloride	30
L2-21	Mixed	21	Sea salts	30
L4-9	Mixed	9	Sea salts	45
L6-11	Mixed	11	Sea salts	45

2.3. Sampling and sample processing

Samples for pH, redox potential, iron and sulfur assays for chloride ion tolerance and bioleaching tests were taken at T_0 and then every 24 h for cultures V6, V8 and 14C, and 48 h for cultures L2-21, L4-9 and L6-11. The chloride ion tolerance tests were run for 96 h for the pure cultures and 144 h for the mixed cultures. For the bioleaching tests, samples were taken every 7 days for a total duration of 28 days for all cultures tested.

The samples (2 mL) were filtered (0.2 μm cellulose acetate filter, Millipore) to remove precipitates and cells and the filtrate used for the assays. Cultures containing elemental sulfur (L2-21, L4-9 and L6-11), were centrifuged at 700*g* for 3 min at room temperature prior to filtering the media, in order to remove excess sulfur particles. Iron oxidation was determined based on ferric and total dissolved iron concentrations using the ferric chloride assay as described by Govender et al. (2012) against a standard curve for ferric chloride. Samples for sulfur oxidation were prepared by adding 500 μL of filtrate to 4.5 mL of 0.07 M nitric acid (in Milli-Q™ H_2O). Soluble sulfur release (only for bioleaching tests) was determined using inductively coupled plasma atom emission spectrometry (ICP-AES). Solution pH of the samples was measured using Ionode IJ44A pH electrode. Redox potential (ORP) was measured using Ionode IJ64 oxidation reduction potential electrode, with results recorded in millivolts against a double junction Ag/AgCl primary reference. To account for losses due to evaporation in the experimental flasks at 45 °C, flasks were weighted before each sampling and sterile Milli-Q™ H_2O water of pH 1.8 was added.

2.4. Microbial community profiling

Genomic DNA was extracted from cultures as described by Zammit et al. (2011). Diversity profiling of the total genomic DNA was performed at the Australian Genome Research Facility (Perth, Western Australia). Amplicon sequencing at a read length of paired-end 300 bp was performed using the primer pair 341F (5′-CCTAYGGGRBGCASAG-3′) – 806R (5′-GGACTACNNGGGTATCTAAT-3′) (Muyzer et al., 1993) specific for the 16S rRNA gene of bacteria and archaea. PEAR version 0.9.5 was used to assemble paired-ends reads by aligning the forward and reverse reads (Zhang et al., 2014). Primers were trimmed