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Preservation of salt-tolerant acidophiles used for chalcopyrite bioleaching: Assessment of cryopreservation, liquid-drying and cold storage

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ABSTRACT

Microorganisms with importance to mineral biotechnology are commonly maintained through live culture maintenance, which can be labour intensive and expensive. Live culture maintenance can also result in contamination, genetic drift and loss of traits, or whole strains, that are crucial to some biotechnological applications. This study aimed to investigate alternative preservation methods, and to determine the best method required for the preservation of mixed microbial cultures capable of bioleaching chalcopyrite under salt stress. Live culture maintenance followed by cold storage at 4 °C, liquid drying and temperature-controlled cryopreservation were used to preserve mesophilic (30 °C), moderately thermophilic (45 °C) and thermophilic (60 °C) bioleaching cultures. Recovery of cells and bioleaching activity were determined following revival. Results were compared to cultures routinely maintained by subculturing. Across all temperatures, cryopreservation with 10% (v/v) glycerol as a cryoprotectant resulted in the highest post-revival cell recovery. Further research is required to determine if the microbial diversity in each culture is impacted by the preservation method employed.

1. Introduction

Cultures used for application and development of mineral biotechnologies are commonly maintained through continual live culturing. Live culture maintenance ensures continued culture growth through the serial transfer of strains from nutrient depleted growth medium to fresh medium (Spring, 2006). However, repeated culturing can be inappropriate for culture collections or long-term maintenance as it is costly, highly labour intensive, can negatively affect strain genetic integrity and has increased contamination risks (Tedeschi and De Paoli, 2011; Malik, 1998).

Alternative long-term storage techniques use freezing or drying to suspend cell metabolism, thus arresting cell functions and aging (Benson, 2008). However, due to differences in cell structure and the sensitivity of some microbial species to low temperatures and desiccation, there are currently no published preservation methods that can be applied for all microorganisms or whole mixed microbial communities (Morgan et al., 2006). In addition, research organisations range in levels of funding, staff availability and expertise, and hence it is important to select methods to maintain cultures that are cost and time effective, and that do not require specialised skills (Belt, 1996).

Comprehensive microbial preservation protocols exist for well-developed industries, such as pharmaceutical production and wine making (Lopez et al., 2011). These industries primarily use microorganisms isolated from environments with moderate temperatures and pH, and often with little or no contaminants present (Lopez et al., 2011). Microorganisms adapted to moderate environments are easier to preserve due to lower sensitivities to dry or cold conditions required for standard long-term preservation methods (Cleland et al., 2004). Extremophilic microorganisms commonly implemented for bioleaching of low-grade and polymetallic ores have a variety of specialised physical and biochemical adaptations that allow their survival in the harsh conditions required for metal extraction. These can include novel membrane structures and lipids and tolerance to pH, temperature and contaminant concentration extremes (Morozkina et al., 2010). Such adaptations can result in increased vulnerability during long-term preservation (Koga, 2012; Morozkina et al., 2010). Overall, very few preservation guides exist for bioleaching microorganisms (Prakash et al., 2013), and as a result, research institutions and

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industry are required to maintain live cultures for their research and/or industrial purposes.

Evaluation of alternatives to live culture maintenance is necessary to improve preservation of bioleaching microorganisms that have importance in minerals biotechnology. In addition, establishment of a long-term preservation method for these specialised microorganisms is required to ensure genetic integrity and that industrially relevant functions are not lost over time. Development of reliable alternatives preservation methods for these industrially relevant microorganisms would reduce costs associated with long-term culture maintenance and allow establishment of comprehensive culture collections, which ultimately would improve the efficiency of industrial research and development (Janssens et al., 2010).

The objective of this study was to determine the effect of various preservation methods on mixed cultures of salt-tolerant acidophilic microorganisms that were capable of bioleaching copper from chalcopyrite at high sulfate concentrations in order to provide guidance for best-practice preservation of industrially relevant cultures. The development of preservation methodology can be a difficult process, as there are a plethora of variables that can affect preservation success including (but not limited to) ensuring that all species are equally preserved and represented in a revived culture and that there is no selective preservation of species due to the preservation method or the medium used for revival of cells (Benson, 2008; Hubálek, 2003). In this study, three existing methods were investigated for their applicability for preserving mixed salt-tolerant bioleaching microorganisms. These included cryopreservation at −80 °C (Pegg, 2007; Fuller, 2004), liquid-drying (Malik, 1998, 1990; Sakane et al., 1992), and cold storage at 4 °C. The viability of revived cultures was determined by total cell counts and bioleaching activity tests, and the results were compared to the viability of maintained live cultures as the standard method of culture maintenance currently used in many laboratories.

2. Materials and methods

2.1. Cultures and growth conditions

Three mixed cultures of salt-tolerant, acidophilic bioleaching microorganisms of unknown species composition and different optimum growth temperatures were selected from the CSIRO Biotechnology Culture Collection (Perth, Australia) for use in this study (Table 1). These cultures were prepared previously through salt tolerance adaptation experiments by Rea et al. (2015a) and incorporated a number of CSIRO Biotechnology Culture Collection strains and enrichment cultures sourced from samples collected at locations throughout the Wheatbelt and Collie mine areas in Western Australia.

All cultures were grown on 3% w/v sterile chalcopyrite concentrate in modified basal salts medium (MBSM, g L^{-1}: $(NH_4)_2SO_4$, 1.50; KH_2PO_4, 0.25; yeast extract, 0.10 and $MgSO_4{\cdot}7H_2O$ concentrations specified in Table 1) and acidified to pH 1.8 with concentrated H_2SO_4. Rotary incubators at 150 rpm were used for incubation at the growth temperature for each culture (Table 1).

2.2. Ore concentrate

Chalcopyrite ore concentrate sourced from Mount Isa Mines (MIM; Queensland, Australia) was used for this study. Mineralogy of this ore concentrate was previously determined by quantitative X-ray diffraction analysis (Rea et al., 2015b) and comprised of 64 weight% (wt.%) chalcopyrite ($CuFeS_2$), 5 wt.% pyrite (FeS_2), 0.6 wt.% pyrrohotite ($Fe_{(1-x)}S$ x = 0–0.17), 5 wt.% talc ($Mg_3Si_4O_{10}(OH)_2$), 9 wt.% quartz (SiO_2), 3 wt.% jarosite ($KFe_3(OH)_6(SO_4)_2$), 3 wt.% melanterite ($FeSO_4{\cdot}7H_2O$) and 10.4 wt.% acid consuming and other gangue minerals. To remove organic solvents that may be present from flotation processing, the concentrate was washed 3 times in ethanol, rinsed 3 times in deionised water and dried at 50 °C. The dry concentrate was sterilised by tyndallisation by heating to 105 °C for 1 h each day for 3 successive days.

2.3. Cell density determination

Cell concentration was determined in triplicate using a Thoma ruled counting chamber (ProSciTech) and phase contrast microscopy (Leica) using 100× objective. Cell survival was determined by calculating the percentage of cells remaining at a certain time point as a total of those present at a starting time point. Cell survival of preserved cultures was determined after preserved cells were stored for 1 or 5 weeks.

2.4. Live culture maintenance

As a control, cultures (100 mL) were maintained by weekly subculturing. Flasks containing 90 mL of fresh growth medium (MBSM) were inoculated with 10 mL of the previous culture (10% v/v) and incubated at temperatures specified in Table 1 for 5 weeks, with the subculturing process repeated every 7 days. Bioleaching tests were carried out to determine leaching activity of the live cultures in duplicate after a total of 5 weeks of weekly subculturing. Cell survival was determined after 1 week and 5 weeks of weekly subculturing as described previously.

2.5. Culture preservation

2.5.1. Preparation of cells for preservation

For all preservation methods, cells were harvested from cultures in the exponential phase of growth. The exponential phase of growth was determined through preliminary bi-weekly cell density measurements taken from unpreserved cultures for 3 weeks after inoculation and found to occur after 3–5 days depending on the culture (results not shown) (Santivarangkna et al., 2007). Culture samples (1 mL) with a cell density of 1×10^8 cells mL^{-1} were used for each preservation treatment. Preservation of cells was undertaken in triplicate for each method tested.

2.5.2. Cryopreservation

Culture samples were diluted in an equal volume of 20% v/v glycerol solution in 2 mL Nunc cryovials (Sigma Aldrich) and incubated at room temperature for 15 min to allow equilibration of intercellular solutes before freezing (Spring, 2006). Glycerol was

Table 1
List of mixed, aerobic chalcopyrite bioleaching cultures from the CSIRO Biotechnology Culture Collection used in this study.

Culture identification number	Organism thermal classification	Growth temperature (°C)	$MgSO_4{\cdot}7H_2O$ concentration (g L^{-1})	pH
L1-100	Mesophile	30	257	1.8
L3-40	Moderate thermophile	45	100	1.8
L5-80	Thermophile	60	200	1.8