

**Western Australian School of Mines
Faculty of Science and Engineering**

Biomining of Rare Earth Elements from Phosphate Ores and Minerals

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Author's Declaration

To the best of my knowledge and belief, this thesis contains no material previously published by any other person except where due acknowledgment has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

Signature: Slavica

Date: 30/10/2018

Dedication

To my beloved parents, my amazing sister, and hope.

زندگی صحنه یکتای، سزمنندی ماست، هر کسی نغمه خود خواند و از صحنه رود،

صحنه پیوسته بجاست، ترم آن نغمه که مردم بسیارند به یاد

ژاله اصفهانی

"You sing your song and depart.

The scene is eternal.

Greet the song that stays

in the people's memory to infinity."

Jaleh Esfahani

"Be happy for this moment. This moment is your life"

Omar Khayyam

Abstract

Rare earth elements (REEs) are known as strategic and critical elements and, in recent years, have gained great interest as key materials that play an integral role in smart technologies following development of sustainable energy technologies. Extraction of REEs is of great environmental and economic importance. Currently, multistep hydrometallurgical extraction of REEs requires significant processing with high-energy consumption, where the conventional REEs production relies on either an alkaline process that uses concentrated sodium hydroxide or an acidic process that uses concentrated sulfuric acid, both at high temperatures. This has led to the development of processes which are more economically feasible and environmentally friendly.

The exploration of extracting REEs with phosphate solubilizing bacteria (PSB) has started recently. The use of PSM for bioleaching of REEs provides a biotechnical approach for the extraction of REEs from primary and secondary sources. However, understanding the microbial-mineral interaction and the mechanisms of phosphate mineral bio-solubilisation in order to develop a successful method for bioleaching of REEs still remains a major challenge.

As REEs are typically complexed with chemical groups including phosphate (monazite and xenotime), this study focused on the biomining of REEs by PSM from monazite bearing minerals. Bacterial communities were enriched from the highest grade of known REEs deposit in the world, Mt. Weld, in arid Western Australia. The indigenous PSB enriched from Mt. Weld deposit was dominated by *Actinobacteria*, *Proteobacteria*, and *Firmicutes*.

Furthermore, a series of monazite dissolution experiments have provided a detailed understanding of the mineral dissolution process, with the investigation of the leaching behaviour of individual REEs and the mechanism attributed to their mobilisation.

Bioleaching experiments with *Acidithiobacillus ferrooxidans*, *Enterobacter aerogenes*, and indigenous PSB enriched from Mt. Weld mine site were conducted in P-free media: basal salt media (BSM), modified National Botanical Research Institute Phosphate (NBRIP) medium, and modified NBRIP, respectively. A systemic study of the mechanisms of bioleaching REEs from monazite with *E. aerogenes* provided the first evidence of the microbial bio-mobilization mechanisms involved in REE dissolution in terms of the importance of microbial colonization of mineral surfaces. A conceptual model describing the main phenomena affecting REE leaching, namely contact, non-contact, and cooperative leaching was proposed. The bioleaching results along with comparative genomic study of the potential mechanisms of phosphate metabolism during the interaction of *E. aerogenes* and *A. ferrooxidans* showed that *A. ferrooxidans* compared to *E. aerogenes* achieved a more efficient stress response which potentially increased the overall REEs dissolution from monazite. Monazite dissolution (1% pulp density) as a phosphate source during bioleaching with PSB was observed to decrease in the following order: Co-culture of *E. aerogenes* and *A. ferrooxidans* with FeSO_4 and $\text{K}_2\text{S}_4\text{O}_6$ > *A. ferrooxidans* with FeSO_4 and $\text{K}_2\text{S}_4\text{O}_6$ > *A. ferrooxidans* with pyrite > *E. aerogenes* in the presence of glucose >> Abiotic controls with FeSO_4 and $\text{K}_2\text{S}_4\text{O}_6$ > > Abiotic controls with pyrite \approx Abiotic controls with glucose at 30 °C with the total concentration of REEs (Ce, La, Pr, Nd,

and Y) after 12 days of 40, 23.6, 10.6, 5.84, 2.73, 0, 0, and 0 mg L⁻¹, respectively. Also, higher REEs dissolution was observed when inoculation of *A. ferrooxidans* in BSM sterile monazite started at a lower starting pH (initial pH 2.00±0.15 and final pH= 1.50 ± 0.15, respectively) compared to a higher starting pH (initial pH= 2.50 ± 0.15 and final pH= 2.50 ± 0.15, respectively) with a final concentration of REEs (Ce, La, Pr, Nd, and Y) after 12 days of 106 mg L⁻¹ and 23.6 mg L⁻¹, respectively. However, in the leaching solution supplemented with glycine (1 g L⁻¹) (initial pH 2.16±0.01 and final pH= 1.70 ± 0.01, respectively), the final total REEs concentration with *A. ferrooxidans* decreased (87 mg L⁻¹). Genomic analysis of the potential mechanisms of glycine metabolism of *A. ferrooxidans* demonstrated that in the presence of glycine some REEs in the leachate are lost due to oxalate-REEs precipitate formation.

The characterization of changes in REEs and Th fractionation during bioleaching with *E. aerogenes* and *A. ferrooxidans* demonstrated that bacterial dissolution was effective in stimulating REEs mobility as indicated by the increase of REEs in easily extractable and acid soluble fractions followed by the increase of Th in residual fraction. This suggested that bioleaching favour solubilisation of REEs over actinides, potentially decreasing environmental hazards associated with these minerals during chemical leaching.

The combination of biogeochemical processes and genomic analysis of metabolic characteristics of specific elements provided a better understanding of various patterns that controlled the bioavailability and mobility of phosphate and REEs in monazite bioleaching. This study examined an environmentally benign processes for the extraction of REEs from various phosphate ores and highlighted the potential applicability of the use of members of PSB for the extraction of REEs.

Acknowledgment

"Behind every great man is a woman rolling her eyes". Such a hilarious quote. Still makes me laugh! Well, some may say I am not a great man, like Cyrus the Great, or there is no woman to roll her eyes. Nevertheless, enduring and completing a daring adventure, a PhD, is a great achievement. Of course there were moments and it was not easy but "no pain no gain", right? More or less everyone is obsessed with leaving a mark upon the world. I wanted to leave a mark, too. To be remembered, precisely! "Not the marks that leave scars". Something to connect us all to each other regardless of our ethnic background or language barriers. It appeared to happen through science. The journey started exactly 14 years ago when I got admission into University of Tehran to study soil science. And considering that I was born on "Iran's Student day" meant that I was destined to be a student, searching for answers and do research. But it is over now.

There are individuals that during this journey put up with me and were ever patient. To my wonderful parents, *Simin* and *Heidar*. You two are the reason I'm here. I would not be here, sitting in my room in Perth, chasing liberty, knowledge and integrity far away from Tehran. Thank you both for guiding me on the right path to being a better person. I have learned from you two that "It is nice to be important, but it is more important to be nice". To my amazing sister, *Negar*, *goori joonam! Je vous aime!* You have inspired me from the beginning, with your precious heart and curious mind. There are not enough words to express how grateful I am to have such a loving, caring, and supportive family. *Negari*, you still amaze me, gooriiiiii! November 17th 2017, when I was in the lab, you extended the border of your love with *David* and with a message attached with a cute picture of *Berenice*, made me a proud uncle!

مامان، بابا، مکار دوستتان دارم بی انتها. مامان و بابا ممنون بابت تک تک زحماتان. ممنون از محبت بی دریغتان. مکاری خواهرم و یاورم که وجودت شادی بخش همه ی ماست. کلمات بسی عاجز از بیان احساساتم برای هر سه تان. نمی توانم واژه ای بالاتر از تقدیر بر زبانم جاری سازم، که هر چه گویم، کم گفته ام. دوستتان دارم بی نهایت.

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This journey is over now. The end of this means the start of another. I sang my heart, soul, and brain out. See you soon!

List of publications

This thesis is assembled by publications (either published, accepted, submitted or prepared for submission) which form the individual chapters of the thesis. The published papers are in Appendix 1.

The publications are listed as follows:

Chapter 2

Fathollahzadeh, H., Eksteen, J. J., Kaksonen, A. H., & Watkin, E. L. (2018). Role of microorganisms in bioleaching of rare earth elements from primary and secondary resources: A review. *Applied Microbiology and Biotechnology* (under review)

Chapter 3

Fathollahzadeh, H., Eksteen, J. J., Kaksonen, A. H., & Watkin, E. L. (2018). Diversity and function of phosphate solubilizing bacteria enriched from Mount Weld rare-earth mine, Australia. *Bioresource Technology Reports* (prepared for submission)

Chapter 4

Fathollahzadeh, H., Becker, T., Eksteen, J.J., Kaksonen, A.H., Watkin, E.L. 2018. Microbial contact enhances bioleaching of rare earth elements. *Bioresource Technology Reports*, 3, 102-108.

Chapter 5

Fathollahzadeh, H., Hackett, M. J., Khaleque, H. N., Eksteen, J. J., Kaksonen, A. H., & Watkin, E. L. (2018). Better together: Potential of co-culture microorganisms to enhance bioleaching of rare earth elements from monazite. *Bioresource Technology Reports*, 3, 109-118.

Chapter 6

Fathollahzadeh, H., Khaleque, H. N., Eksteen, J. J., Kaksonen, A. H., & Watkin, E. L. (2018). Effect of glycine on bioleaching of rare earth elements by phosphate solubilising microorganisms from Western Australian monazite. *Hydrometallurgy* (prepared for submission)

Statement of Contribution by Others

I hereby declare that the work presented in this thesis was primarily designed, experimentally executed, interpreted, and written by the first author of the individual manuscripts (Homayoun Fathollahzadeh). Contributions by colleagues are described in the following. The signed statement by co-authors are in Appendix 2.

Chapter 2

HF drafted the outline, generated the figure, and wrote the manuscript. HF conceived of the study and JJE, AHK, and ELJW critically reviewed, revised, and approved the final manuscript.

Chapter 3

HF drafted the outline, generated the figure, and wrote the manuscript. HF conceived of the study and JJE, AHK, and ELJW critically reviewed, revised, and approved the final manuscript.

Chapter 4

HF drafted the outline, generated the figure, and wrote the manuscript. TB performed the Co-localised atomic force microscopy (AFM) and confocal Raman microscopy (CRM) measurements. HF conceived of the study and TB, JJE, AHK, and ELJW critically reviewed, revised, and approved the final manuscript.

Chapter 5

HF drafted the outline, generated the figure, and wrote the manuscript. MJH performed the Ce L_{III} -Edge X-ray absorption spectroscopy (XAS). HNK performed bioinformatics analyses and generation of images. HF conceived of the study and MJH, HNK, JJE, AHK, and ELJW critically reviewed, revised, and approved the final manuscript.

Chapter 6

HF drafted the outline, generated the figure, and wrote the manuscript. HNK performed bioinformatics analyses and generation of images. HF conceived of the study and HNK, JJE, AHK, and ELJW critically reviewed, revised, and approved the final manuscript.

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Chapter 1: Introduction

1.1 Background to this study

Rare earths elements (REEs) are critical components to many technologies that drive the modern world. Though REEs are relatively abundant in the Earth's crust, they are not evenly distributed around the world, and they are produced and processed mostly in China (Ganguli & Cook, 2018; Zepf, 2013). Considering Chinese export quota, limited economical high grade resources, susceptible REEs global market, insufficient recovery of REEs from wastes, growing demand for electric vehicles, wind turbines, and smart technologies, will likely leave customers no alternative but to pay more (Ganguli & Cook, 2018).

The decrease in global supply and an ever increasing demand for REEs offer pre-eminent opportunities for Australia to become a major player in the REEs industry as Australian deposits are known as the highest grade deposit of REEs in the world (Haque et al., 2014). These deposits are normally richest in one of three major REE bearing minerals: bastnasite, monazite and xenotime, of which WA has large resources in monazite (Mt. Weld mine, operated by Lynas Corporation) and xenotime (Browns Range, operated by Northern Minerals); both of which are REE phosphate minerals. In addition, monazite is also associated with mineral sands and iron oxide, copper, gold, such as Olympic dam.

Currently, the opportunities for major improvement in the mining, extraction and recovery of REEs are increasingly limited by three factors: (1) extraction of REEs is achieved by using harsh acidic and/or basic conditions at high temperature, which release a large amounts of toxic and radioactive waste, (2) REEs are found mixed in the ore, and thus chemical separation of each type leads to an inefficient recovery overall, and (3) the extraction efficiency is dependent on ores containing high concentrations of REEs, which limits potential sources of recovery (Zhuang et al., 2015).

Beneficiation, recovery and separation of the REEs from the ore matrix is material and energy intensive processes. They are complex and involve various unit processes including but not limited to: grinding, screening, gravity concentration, low intensity magnetic separation, and flotation, acidic and/or alkaline roasting, under high temperatures followed by solvent extraction and calcination (Falconer, 2003; Fuerstenau, 2013; Jha et al., 2016; Zhang et al., 2016). As these methods are relatively non-selective, there is excessive reagent consumption and waste generation, as well as co-dissolution of radionuclides. Also, as REEs-bearing ores may contain thorium and uranium up to 10% of the total ore matrix (Ragheb, 2011), emission of radioactive waste associated with REEs mining and extraction result in complicated disposal protocols or contamination of the final REEs concentrate (Ault et al., 2015).

It has been demonstrated that the environmental life cycle impacts of REEs production during chemical leaching are far greater than those for other metals (Vahidi & Zhao, 2016). Consequently, due to environmental restrictions, sustainable mining and production are now encouraged.

The use of microorganisms to extract metals from ore matrix is called biomining. Biomining occurs at relatively low temperature and atmospheric pressure through the natural ability of certain microorganisms to catalyse reactions, leading to the solubilisation of metals from the minerals without relying on expensive and aggressive reagents common in hydrometallurgical processing (Bryan et al., 2015). Biomining has clear advantages over conventional processes including, simplicity of operation, lower capital/operation costs and lower impact on the environment. *Acidithiobacillus (A.) ferrooxidans*, the most studied obligate chemolithoautotrophic bioleaching bacterium, requires small amounts of inorganic nutrients, such as ferrous iron and reduced sulfur compounds for iron bio-oxidation and generation of sulphuric acid (Watling, 2016; Zhuang et al., 2015). Biomining is successfully used in industrial operations to extract several metals such as copper, nickel, zinc and cobalt with 20% of the world's copper production originating from heap or dump/stockpile bioleaching (Jerez, 2017).

This research project proposes a biomining approach for REE phosphate minerals using phosphate solubilising microorganisms (PSMs) to liberate REEs. Biomining of REE-phosphate ore has the potential to more selectively solubilise REEs over radionuclides, offering an additional benefit that may solve one of the largest challenges in REE processing. PSMs have been used to mobilize insoluble phosphate from organic and inorganic (mineral) phosphate resources derived over hundreds year of agronomic studies (Goldstein & Krishnaraj, 2007).

Phosphate mineral bio-solubilisation mechanisms are not well understood, however, in microcosm studies inverse correlations between pH and phosphorus release are frequently observed (Farhat et al., 2009; Feng et al., 2011). Gluconic, acetic, citric and other organic acids are produced during bio-solubilisation of phosphate. Organic acids may have both acidifying and ligand (complexation) actions which may act individually or simultaneously to enhance the solubilisation of phosphorus (Banfield et al., 1999; Welch et al., 2002). Recently, evidence for microbial solubilisation of phosphate from REE-phosphate minerals has been reported (Brisson et al., 2016; Corbett et al., 2017; Hassanien et al., 2013a; Shin et al., 2015). Although very little research has been done on the application of PSMs on monazite, no detailed studies have been done on the leaching behaviour of individual REEs and the mechanism attributed to their mobilisation.

1.2 Aim, objectives and significance of this study

The overall aim of this research project was to explore the use of PSMs for extracting REEs from monazite ore, and elucidate possible bioleaching mechanisms and the leaching behaviour of individual REEs in the process. The specific objectives are:

- To isolate and identify indigenous phosphate solubilising bacteria obtained from REEs ore and evaluate their potential application in bioleaching REEs from various phosphate ores
- To investigate the role of the phosphate solubilizing bacterium, *Enterobacter (E.) aerogenes*, in REEs leaching from monazite

- To elucidate the mechanisms of phosphate and REEs solubilisation
- To propose bioleaching of monazite by combining heterotrophic and autotrophic acidophilic microorganisms
- To study the effects of glycine on the dissolution of REEs from various phosphate ores in the absence and presence of PSMs

The achievement of above objectives can potentially beneficially impact the REEs industries through:

- The introduction of an environmentally benign processes for the extraction of REEs from various phosphate ores
- A greater understanding on the limitations, risks and potential of PSMs
- Potential reduction in waste generation and in the consumption of reagents

1.3 Thesis overview

This thesis is divided into the seven chapters. The key aspects discussed in each chapter are described below:

- Chapter 2** is dedicated to a detailed review of literature on the major aspect of the thesis including but not restricted to: i) importance of biomining of REEs, ii) microbial processes related to the mobilisation of REEs and phosphates, iii) mechanisms of monazite bioleaching, iv) phosphate stabilisation-related genes, v) current status of bioleaching of REEs from primary and secondary resources, and vi) mining of REEs and sustainability.
- Chapter 3** introduces isolated indigenous phosphate solubilising bacterial strains from Mt. Weld deposit and evaluates their capabilities for the bioleaching of REEs from three different grades of monazite bearing minerals present in WA.
- Chapter 4** presents a systemic study of mechanisms of bioleaching REE from monazite with *E. aerogenes* and proposes a new conceptual model of the possible mechanisms of monazite bioleaching.
- Chapter 5** explores the bioleaching of REEs from monazite by a co-culture of autotrophic, acidophilic *A. ferrooxidans* and heterotrophic *E. aerogenes* and compares the efficiency to those of individual pure cultures.

- v. **Chapter 6** describes the capabilities of glycine-bioleaching system for the bioleaching of REEs from three different grades of monazite bearing minerals obtained from WA.
- vi. **Chapter 7** summarizes the major results found in this study and highlights recommendations for further work.

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Chapter 2: Role of microorganisms in bioleaching of rare earth elements from primary and secondary resources: A review.

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Role of microorganisms in bioleaching of rare earth elements from primary and secondary resources: A review

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Abstract

In an era of environmental degradation, and water, and mineral scarcity, enhancing microbial function in sustainable mining has become a prerequisite for the future of the green economy. In recent years, the extensive use of rare earth elements (REEs) in green and smart technologies has led to an increase in the focus on recovery and separation of REEs from ore matrices. However, the recovery of REEs using traditional methods is complex and energy intensive, leading to the requirement to develop processes which are more economically feasible and environmentally friendly. The use of phosphate solubilizing microorganisms for bioleaching of REEs provides a biotechnical approach for the recovery of REEs from primary and secondary sources. However, managing and understanding the microbial-mineral interactions in order to develop a successful method for bioleaching of REEs still remains a major challenge. This review focuses on the use of microbes for the bioleaching of REEs and highlights the importance of genomic studies in order to narrow down potential microorganisms for the optimal extraction of REEs.

Keywords: Phosphate solubilising microorganisms; Monazite bioleaching; Rare earth elements; Sustainable mining

2.1 Introduction

In view of escalating environmental degradation, water scarcity, and the depletion of high grade mineral deposits, the need for a new integrated approach towards sustainable mining has become more urgent. In addition, market demands for green and digital high-technology including but not limited to wind energy, electric cars, and smart phones are growing at an accelerated rate (Goodenough et al., 2018b). Rare earth elements (REEs) are known to be critical for green economy, modern life and society (Jowitt et al., 2018). However, annual global demand of REEs has not yet exceeded the annual supply, which ranged from 120,000 t to 140,000 t (Klinger, 2018).

Recent advances in biomining reflect the exciting potential of developing microbial miners to mobilise a range of metals (Watling, 2016). The diversity and capabilities of bioleaching microorganisms for mineral dissolution are ample, providing adaptability of these microorganisms to extreme and challenging environments (Watling et al., 2010).

This review summarises the fundamental understanding of the interactions of REEs with the microorganisms as well as microbial processes related to the mobilisation of REEs to provide insight into the potential application of microorganisms in the extraction of REEs from REEs-bearing minerals. Subsequently, the studies on the use of phosphate solubilising bacteria in the bioleaching of REEs from primary and secondary resources will be reviewed, and the current state and future outlook will be discussed.

2.2 Global situation of rare earth elements: Turning crisis into opportunity

REEs are an essential component in most modern technologies. The current development of electric vehicles and wind turbines relies extensively on dysprosium and neodymium in REEs magnets (Deady et al., 2016). It is anticipated that further development of these technologies will result in a disproportionate increase in the demand for REEs (Alonso et al., 2012). On the one hand, there is limited recycling technologies available for REEs and less than 1% of the REEs in end-of-life consumer products are recycled globally (Tkaczyk et al., 2018). On the other hand, global primary resources of REEs are limited due to geopolitical controls (i.e. limiting of export quotas for REEs by China in 2010), and they have been identified as critical metals (DOE, 2011; Hoatson et al., 2011; Jaireth et al., 2014b). It has been found that REEs are relatively abundant in the Earth's crust (Zepf, 2013). However, the concentrated forms suitable for viable extraction are less common and separation procedures for REEs are more difficult compared to most other exploited metals (Hoatson et al., 2011).

REEs are typically complexed with chemical groups including oxides [Anatase: $(\text{Ti,REEs})\text{O}_2$], carbonates [Bastnäsite: $(\text{Ce,La})(\text{CO}_3)\text{F}$] or phosphates [Monazite: $(\text{Ce,La,Nd,Th})\text{PO}_4$; Xenotime: YPO_4] (Jaireth et al., 2014b). Of the 250 known REEs minerals only three major REEs bearing minerals (bastnäsite, monazite, and xenotime) are currently exploited commercially (Jordens et al., 2013). The main operating mines are Bayan Obo in China, Mountain Pass in the US and Mount Weld in Australia with China producing 70 - 90% of the global REEs supply (Binnemans et al., 2018). Since the REEs crisis in 2010 and the current

control by China's of REEs supply, countries with a high dependency on REEs products have pursued multiple measures to transform the industry and secure their REEs supply (Klinger, 2018). The decrease in global supply and an ever increasing demand for REEs, offer potential opportunities for Australia to become a major player in the REEs industry as Australian deposits are known as the richest deposit of REEs in the world (Haque et al., 2014).

Currently, industrial extraction of REEs requires significant processing, and the conventional REEs production relies on either an alkaline process that uses concentrated sodium hydroxide or an acidic process that uses concentrated sulfuric acid and high temperatures. These generate large amounts of toxic waste containing thorium, uranium, hydrogen fluoride, and acidic waste water (Abreu & Morais, 2010; Hurst, 2010). Chemical leaching efficiencies of 85% for La, Nd and Sm have been reported (Kim et al., 2009). In Australia, until 1995 REEs were largely produced from mineral sands containing monazite associated with high thorium content (Hoatson et al., 2011). The Chinese REEs production has resulted in environmental pollution that was attributed to the imposition of an export quota by the Chinese government in 2010 (Vahidi et al., 2016).

Indeed, the environmental life cycle impact of REE production through chemical leaching is significantly higher compared to other metals (Haque et al., 2014; Thompson et al., 2017a; Vahidi & Zhao, 2016). Therefore, alternative approaches offering environmental benefits have received increased attention. Regrettably, in contrast to the biomining of sulfide minerals, much less effort has been devoted to studying the interaction of REEs with microorganisms in bioleaching systems and the application of biohydrometallurgy to REE-bearing resources. To date, there are no available reports on the bioleaching of bastnasite and xenotime, and only a few studies on monazite bioleaching. Nevertheless, there is considerable incentive to establish green technologies for the recovery of REEs which may contribute to a more sustainable process (Barmettler et al., 2016a).

2.3 Rare earth elements: Connecting chemistry to biology

While the first REE in an oxide form (Y_2O_3) was discovered in 1794, non-radioactive REEs were not characterized/separated until 113 years later, as these elements are highly insoluble and scarce in pure form (Bünzli, 2014). In 1891, Karl Auer von Welsbach, used a mix of various lanthanide oxides, thorium and other metals for the first gas mantles (Bünzli, 2014).

According to recommendations by the International Union of Pure and Applied Chemistry (IUPAC), REEs include 17 elements with special chemical and physical properties, namely: yttrium (Y) and scandium (Sc) as well as lanthanides such as lanthanum (La), cerium (Ce), praseodymium (Pr), neodymium (Nd), promethium (Pm), samarium (Sm), europium (Eu), gadolinium (Gd), terbium (Tb), dysprosium (Dy), holmium (Ho), erbium (Er), thulium (Tm), ytterbium (Yb) and lutetium (Lu). The REEs are commonly divided into light (LREEs) and heavy (HREEs). However, there is no absolute agreement on which elements are included in each category, the LREEs being commonly referred to as the first sixth elements of REEs series, namely La, Ce, Pr, Nd, Pm and Sm and the HREEs being Eu, Gd, Tb, Dy, Ho, Er, Tm, Yb, Lu, and Y (Ramos et al., 2016; Šmuc et al., 2012). Y is grouped with the HREEs as its ionic radius is

nearly identical to that of Ho (Chakmouradian & Wall, 2012). The LREEs have lower atomic numbers and masses but larger ionic radius, higher solubility and alkalinity, whereas the group of HREEs have higher atomic numbers and masses but smaller ionic radius and lower alkalinity (Li et al., 2017; Ramos et al., 2016).

REEs have very similar chemical and physical properties, and this uniqueness stems from their electronic configuration (mainly f orbitals), generally forming a particularly stable oxidation state (3+) (e.g., Ce³⁺ with electron configuration [Xe] 4f¹), and a very small but regular decrease in the ionic radius, with an increase in atomic number (from La to Lu), known as “lanthanide contraction” (Aide & Aide, 2012). However, Ce and Eu usually present in the oxidation state (3+), may also occur as Ce⁴⁺ and Eu²⁺ under oxidizing and reducing conditions, respectively. The significance of the lanthanide contraction phenomena is observed in the greater stability of hydrolysis and complex constants with increasing atomic number (from the LREEs to HREEs). The higher stability behaviour of HREEs, can also be explained by Pearson's Rule (Pearson, 1963) which suggested that hard cations (e.g., REE³⁺) will preferentially bond with hard anions (i.e., ligands such F⁻, OH⁻, and PO₄³⁻), through ionic bonds, whereas soft or polarizable cations will bond with soft anions through covalent bonds.

Due to similarities between Ce³⁺/La³⁺ and Ca²⁺ in terms of ionic radius, coordination environment and ligand preferences, REEs have been used as analogues for multiple applications (Lim & Franklin, 2006; Pagano et al., 2015; Pang et al., 2002). Although, displacement of Ca²⁺ by Ce³⁺ and Ga³⁺ ions for bone tissue engineering has long been applied (Deliormanlı, 2015), only a few recent studies suggest a biological role for REEs. Until recently, the biologists and enzymologists believed REEs to be inert because of the low solubility of these elements in the environment. However, in 2011 it has been discovered that Ce³⁺ and La³⁺ are required for the activity of the enzyme methanol dehydrogenase (MDH) in some bacteria to oxidize methanol for carbon and energy (Hibi et al., 2011; Pol et al., 2014; Skovran et al., 2011)).

Furthermore, it has been postulated that REEs may play pivotal roles not only in biogeochemical processes of single carbon compounds but may also be involved in the metabolism of a broader range of compounds, in a wide range of microorganisms (Chistoserdova, 2016). Thus, recent studies on the biological roles of REEs have emerged as a new field which may contribute to the isolation of potential strains for biomining of REEs (Shiller et al., 2017; Skovran & Martinez-Gomez, 2015).

2.4 Why do we need biomining?

Due to the growth in the numbers of hybrid electric vehicles (HEVs) and full electric vehicles (EVs), the use of REEs by the automotive industry is forecasted to increase (Goodenough et al., 2018b). The production of HEVs and EVs is estimated to increase from 2.3 million units in 2016 to over 10.1 million units in 2026, which is likely to drive an increased demand for neodymium-iron-boron (NdFeB) magnets (Roskill, 2017).

Currently, the opportunities for major improvement in the mining, extraction, and recovery of REEs are increasingly limited by three factors: (1) the extraction of REEs is achieved by using

harsh acidic and/or alkaline conditions with either concentrated sulfuric acid and/or sodium hydroxide extraction at high temperature, which release toxic and radioactive waste, (2) REEs are found mixed in the ore, and thus chemical separation of each type leads to an inefficient overall recovery, and (3) the extraction efficiency is dependent on ores containing high concentrations of REEs, which limits potential sources of recovery (Zhuang et al., 2015).

The environmental life cycle impacts of REEs production during chemical leaching are far greater than those for other metals currently measured with life cycle assessment tools (Vahidi & Zhao, 2016). Consequently, due to environmental restrictions, sustainable mining and production are now encouraged on environmental grounds in many countries. Biomining is successfully used in industrial operations, to extract several metals such as copper, nickel, cobalt and zinc, with 20% of the world's copper production originating from heap or dump/stockpile bioleaching (Jerez, 2017). Bioleaching methods offer environmentally friendly alternatives to classical approaches. A variety of microbial groups have the potential to be applied for bioleaching of REEs from solid matrices (Barmettler et al., 2016a). In most of these studies, phosphate solubilising microorganisms (PSMs) were used to solubilise REEs from REEs containing materials (Hopfe et al., 2018).

2.5 Phosphate solubilising microorganisms

Most studies to date on the bioleaching of REEs have been carried out with PSMs. However, the majority of the previous research on PSMs has been conducted in agricultural systems. The reason may be the simplicity of using PSMs to liberate insoluble phosphate from organic and inorganic (mineral) phosphate resources present in soil for enhanced crop production derived after hundreds year of agronomic studies (Goldstein & Krishnaraj, 2007). A large number of studies have been conducted over the last hundred years on the isolation and characterization of PSMs, mainly using tricalcium phosphate (TCP) as model phosphate mineral. However, it has recently been reported that TCP, is unreliable as a universal selection factor for isolating and testing the direct contribution of biological P solubilisation (Bashan et al., 2013). Perhaps, this is the main reason why amongst potential isolated PSMs, a low number of isolates proved to be successful in the direct mobilisation of phosphate (Banik & Dey, 1983). These discrepancies suggest that other processes and variables of considerable importance, as will be discussed in the following sections, also control the rates of P solubilisation.

Gram-negative bacteria have been found to be more efficient at dissolving mineral phosphates than Gram-positive bacteria (Sashidhar & Podile, 2010). A large range of Gram-negative bacterial species including *Klebsiella*, *Enterobacter*, *Pseudomonas*, *Bacillus*, *Rhizobium*, *Erwinia*, *Agrobacterium*, *Flavobacterium*, *Enterobacter*, *Micrococcus*, *Thiobacillus*, *Acetobacter*, *Burkholderia* spp., *Clavibacter*, *Serratia* and *Streptomyces* are capable of P solubilisation as well as some fungi such as *Penicillium*, *Aspergillus*, *Rhizopus*, and *Fusarium* (Illmer & Schinner, 1992; Rodríguez & Fraga, 1999; Zhao & Lin, 2001).

In this review, the term "PSMs" is used in recognition of the fact that microorganisms are capable of transforming insoluble phosphate into more soluble form which directly and/or

indirectly contributes to the metabolism of microorganism via increasing phosphate availability in the cell-mineral interface and the solution. Phosphate minerals are dissolved by acidification (Goldstein & Krishnaraj, 2007). Therefore, any microorganism that acidifies the media can potentially release some level of phosphate and hence REEs which are in a phosphate mineral matrix.

2.6 Mechanisms of phosphate and REEs solubilisation

It is well established that the solubilisation of phosphate from poorly soluble mineral phosphates is mainly related to the production of organic acids (Babu-Khan et al., 1995; Goldstein & Krishnaraj, 2007; Goldstein, 2007), however the precise mechanism of P solubilisation by different PSMs still remains poorly understood (Park et al., 2009b). The following sections will address a number of proposed theories to explain the mechanism of phosphate solubilisation.

2.6.1 Organic acid release

As heterotrophic microorganisms in bioleaching systems rely mainly on organic carbon sources (e.g., glucose), heterotrophic metabolism (biologic oxidation of organic compounds to yield ATP) lowers the pH of the leachate either by H⁺ extrusion (Illmer & Schinner, 1995) or by the secretion of organic acids such as gluconic, citric, acetic, lactic, malic, succinic, tartaric, 2-ketogluconic, and oxalic acids (Bolan et al., 1994). The proton release from the cytoplasm to the outer surface occurs in exchange for a cation (Sashidhar & Podile, 2010). The addition of a various carbon sources has been shown to affect the type of secreted organic acids by *Penicillium rugulosum*. With sucrose, most of the glucose molecules were converted to gluconic acid while fructose was strongly associated with the production of citric acid through the tricarboxylic acid cycle (Reyes et al., 1999). While some of the organic acids are responsible for energy production as intermediates in the tricarboxylic (TCA) cycle (e.g., citrate, malate), others are primarily present in cells for cation charge balancing or for maintaining osmotic potential (e.g., malate, malonate, oxalate) (Jones, 1998).

In most heterotrophic bacteria and fungi, the release of low molecular weight organic acids has been demonstrated to be the most common reason for phosphate solubilisation (Rodríguez & Fraga, 1999). The heterotrophic leaching involves several mechanisms but organic acids play a dominant and central role in the overall process, supplying both protons and a REEs-complexing organic acid anions. Therefore, the ability of organic acid production by PSMs could be considered a phenotype, defined as “a specific characteristic of the microorganism” (Goldstein & Krishnaraj, 2007). Beyond organic acids, Illmer and Schinner. (Illmer & Schinner, 1995) also reported that the proton-excretion associated with ammonium ion assimilation could be another mechanism of P solubilisation without organic acid production.

Although, organic acids have been suggested as the principal mechanism of P solubilisation, it has been demonstrated that the higher amount of free organic acids was not directly correlated with higher phosphate solubilisation (Banik & Dey, 1983; de Oliveira Mendes et al.,

2014). Amongst 57 fungal strains (with a clear predominance of *Penicillium* and *Aspergillus* species) it has been found that phosphate solubilisation mechanisms not only differ between strains but are also dependent on the applied P sources where solubilisation rate increased as follows: TCP > AlPO₄ >> FePO₄ >> Rock phosphate (de Oliveira Mendes et al., 2014).

The rate of P mobilisation during phosphate minerals bioleaching varied based on i) the microbial strains, ii) the concentration and types of organic acid produced, iii) and the physicochemical properties of the mineral which is governed by the mineral composition, phosphate uptake rate, and growth rate of the biomass (Brisson et al., 2016; Corbett et al., 2017). It may be seen that any member of PSMs community either from a same or different family, may release different metabolites including organic acids depending on the solubility of the phosphate mineral and the growth media condition (Table 1). Buch et al. (Buch et al., 2008) have described the metabolic flexibility of two *Pseudomonads* strains related to gluconic acid secretion and P solubilisation. Under P-deficient conditions, both *Pseudomonas* strains secreted only gluconic acid. However, under P-sufficient conditions the secretion of pyruvic and acetic acids in addition to gluconic acid by both strains indicated increased carbon flow through the phosphorylative pathway of glucose oxidation (Buch et al., 2008). Under normal metabolic conditions, three different strains of a well-known PSMs family, *Enterobacter (E.) aerogenes* have been reported to produce similar range of gluconic acid up to 1 mM (Stella & Halimi, 2015). However, in another recent study, the type and levels of secreted organic acids by the same strain (*E. aerogenes* ATCC 13048) on the same monazite have been found to be different, even though the concentration of the principle metabolite, glucose (3% w/v) was similar (Corbett et al., 2017; Fathollahzadeh et al., 2018a) (Table 1).

2.6.2 Phosphatase activity

Recently, it has been reported that besides organic acids, other biological and chelating factors play a significant role in monazite solubilisation (Corbett et al., 2018). Production of phosphatases by the microorganisms may have aided in releasing phosphate from the monazite with microbial incorporation of phosphate shifting the solution equilibrium, further promoting leaching of the REE matrix (Corbett et al., 2018). The authors concluded that the combined action of acid phosphatase activity and organic acids undoubtedly increased REEs solubilisation (Corbett et al., 2018).

2.6.3 The importance of bacterial attachment

Successful and efficient interaction of microbial strains and mineral surfaces are another key component that control mineral dissolution (Sand & Gehrke, 2006). Adhesion and then colonization of microbes on the mineral surfaces are a survival mechanism, and nutrients in aqueous environments are more accessible at surfaces (Busscher & van der Mei, 2012). However, the extent to which measured REEs concentration over bio-mobilization and bio-mineralization relate to the bacterial attachment on a phosphate mineral has not been studied till recently. Although, a few studies have been conducted to investigate the role of attachment of bacteria on the dissolution of phosphate and silicate based minerals such as

apatite, fluorapatite, and feldspar (Feng et al., 2011; Hutchens, 2009; Hutchens et al., 2006; Hutchens et al., 2003), no report of the role of bacterial attachment in the monazite dissolution was available till recently.

To improve bioleaching performance, it is essential to have a detailed understanding of the bioleaching mechanism. In an attempt to understand the fundamental mechanisms of monazite bioleaching by *E. aerogenes*, it has been demonstrated that the contact of bacteria with minerals can have a significant effect on their capacity to enhance mineral dissolution, even though the same types of organic acids with similar concentration were present during non-contact leaching (Fathollahzadeh et al., 2018a). It has been suggested by Fathollahzadeh et al. (Fathollahzadeh et al., 2018a) that the ability of PSMs to solubilise REEs-phosphate minerals is by contact mechanisms, non-contact mechanism, or a cooperative mechanism (a combination of both) (Fig. 1).

In contact leaching (Fig 1.a), attached cells mobilize phosphate within a matrix of extracellular polymeric substances (EPS) and release REE^{3+} into the solution. Organic acid anions (OA) generated by the cells from organic substrates complex with REE^{3+} . Protons released from organic acids also attack the ore surface resulting in further phosphate dissolution. Incorporation of phosphate into the biomass increases REE^{3+} solubility. In the non-contact mechanism (Fig 1.b) suspended cells generate REE^{3+} complexing organic acids and biologically incorporate phosphate increasing REE^{3+} solubility. The protons released from organic acids attack the ore resulting in further REE^{3+} and PO_4^{3-} dissolution. In the cooperative mechanism (Fig 1.c) attached cells mobilise phosphate from the monazite and incorporate it into cells releasing REE^{3+} while suspended cells generate organic acids for REE^{3+} complexation and protons released from organic acids attack the ore. Alternatively, attached cells may play a role in organic acid generation while suspended cells take up PO_4^{3-} from solution increasing REE^{3+} solubility.

Table 1 – Biological phosphate mobilisation and secreted organic acids by phosphate solubilising microorganisms from various phosphate resources.

Microbial strain	Final concentration of organic acids (mM)	P released (mM)	Organic substrate, phosphate source, final pH and leaching time	Reference
<i>Pseudomonas aeruginosa</i> P4	gluconic acid: 46	0.44	100 mM glucose, rock phosphate, final pH = 4.80, 96 h	(Buch et al., 2008)
<i>Burkholderia cepacia</i> CC-A174	gluconic acid: 16, 2-keto gluconic acid: 3.8	2.10	29 mM sucrose, tri-calcium phosphate, final pH = 3, 199 h	(Lin et al., 2006)
<i>Burkholderia caribensis</i> FeGI03	gluconic acid: 31, acetic acid: 0.02	8.17	55 mM glucose, hydroxy apatite, final pH = 4.04, 168 h	(Delvasto et al., 2009)
<i>Burkholderia ferrariae</i> FeGI01	gluconic acid: 0.03, acetic acid: 0.04	5.07	55 mM glucose, hydroxy apatite, final pH = 4.82, 168 h	(Delvasto et al., 2009)
<i>Enterobacter asburiae</i> PS13	acetic acid: 55	0.89	75 mM glucose, rock phosphate, final pH = 4, 60 h	(Sharma et al., 2005)
<i>Serratia marcescens</i> CTM 50650	acetic acid: 229	19	55 mM glucose, hydroxy apatite, final pH = NR, 48 h	(Farhat et al., 2009)
<i>Serratia marcescens</i> CTM 50650	Citric acid: 56	3.42	55 mM fructose, hydroxy apatite, final pH = NR, 48 h	(Farhat et al., 2009)
<i>Enterobacter aerogenes</i> ATCC 13048	gluconic acid < 0.01, formic acid < 0.01, citric acid: 0.03, malic acid: 0.10	< 0.02	166 mM glucose, monazite, final pH = 3.8, 192 h	(Corbett et al., 2017)
<i>Pseudomonas aeruginosa</i> DSMZ 50071	gluconic acid < 0.01, formic acid < 0.01, citric acid: 0.03, malic acid: 0.16	< 0.02	166 mM glucose, monazite, final pH = 4, 192 h	(Corbett et al., 2017)
<i>Aspergillus niger</i> DSMZ 821	gluconic acid: 0.27, acetic acid: 1.4, formic acid < 0.01, malic acid: 0.04	< 0.02	166 mM glucose, monazite, final pH = 2, 192 h	(Corbett et al., 2017)
<i>Aspergillus terreus</i> ML3-1	itaconic acid > 20, succinic acid:4	0.48	55 mM glucose, monazite, final pH = 2-2.8, 168 h	(Brisson et al., 2016)
<i>Gluconobacter oxydans</i> NRRL B58	gluconic acid: 233	Not reported	222 mM glucose, waste materials, final pH = 2.14, 40 h	(Thompson et al., 2017a)
<i>Enterobacter aerogenes</i> ATCC 13048	gluconic acid: 1, acetic acid: 1, malic acid: 11	< 0.02	166 mM glucose, monazite, final pH = 3.4, 72 h	(Fathollahzadeh et al., 2018a)

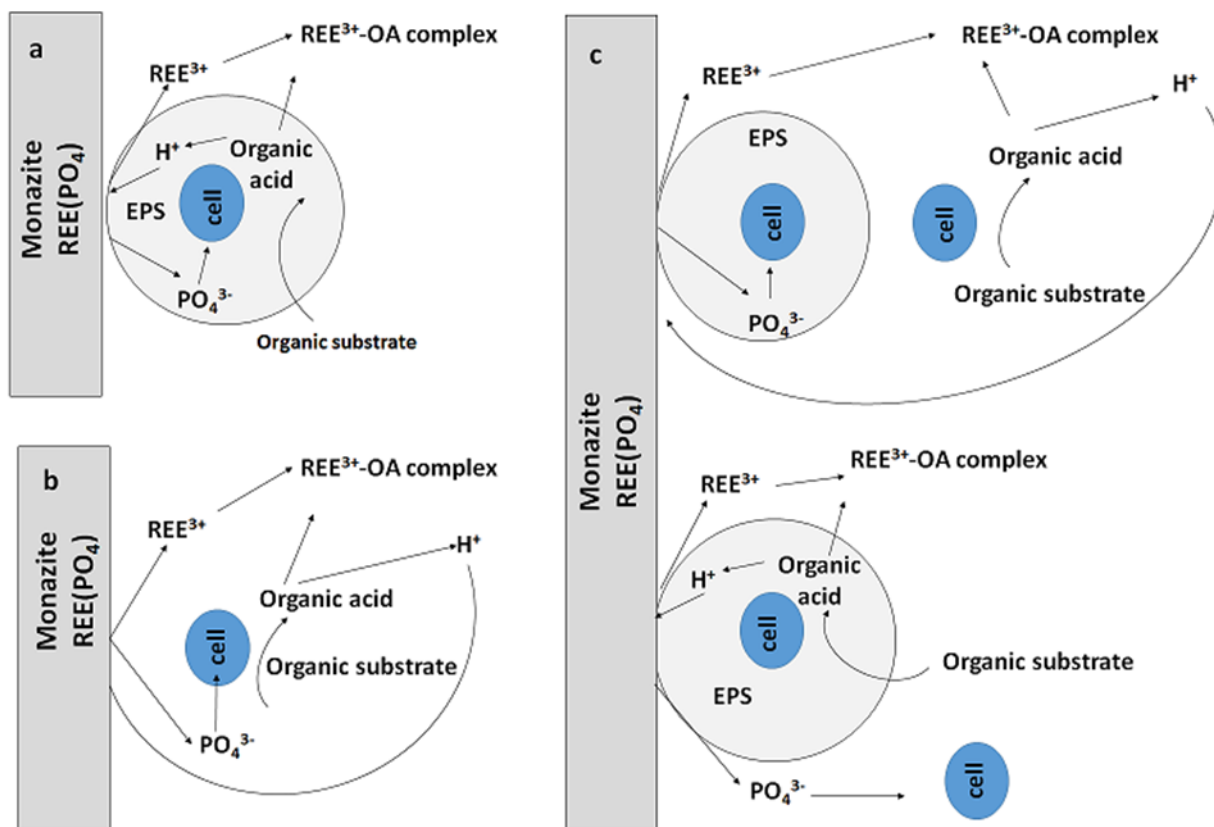


Fig.1 A conceptual model showing the proposed mechanisms of monazite bioleaching. In contact leaching attached microbial cells mobilize phosphate (PO_4^{3-}) within a matrix of extracellular polymeric substances (EPS) releasing REE^{3+} into solution. Organic acids (OA) generated by the cells from organic substrates complex REE^{3+} . Protons released from organic acids attack the ore resulting in further PO_4^{3-} dissolution. Incorporation of PO_4^{3-} into the cells increases REE^{3+} solubility. (b) In non-contact mechanism suspended cells generate REE^{3+} complexing organic acids and incorporate PO_4^{3-} into cells increasing REE^{3+} solubility. The protons released from organic acids attack the ore resulting in further REE^{3+} and PO_4^{3-} dissolution. (c) In cooperative mechanisms attached cells mobilise PO_4^{3-} from monazite and incorporate it into cells releasing REE^{3+} while suspended cells generate organic acids for REE^{3+} complexation and protons released from organic acids attack the ore. Alternatively, attached cells may play a role in organic acid generation while suspended cells take up PO_4^{3-} from solution increasing REE^{3+} solubility. Reprinted from (Fathollahzadeh et al., 2018a) with permission from Elsevier.

2.6.4 Spent medium and abiotic leaching

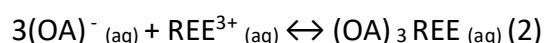
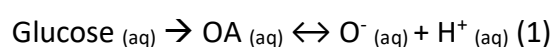
Apart from direct leaching, where bioleaching is achieved in the presence of the organism, efforts have been made to investigate the effects of metabolites present in spent media as well as abiotic leaching with synthesized organic acids (Brisson et al., 2016; Fathollahzadeh et al., 2018a; Hassanien et al., 2013b).

When exploring the possible leaching mechanisms of REEs from monazite, it has been demonstrated that monazite spent medium leaching resulted in lower REEs leaching compared to biotic contact and non-contact leaching (Fathollahzadeh et al., 2018a). The authors suggested that as no phosphate consumption occur during spent media leaching, the precipitation and formation of secondary phosphate minerals increased, resulting in a decrease in overall REEs solubilisation. Furthermore, as microorganism are not present in the system, organic acids are not continually produced in the spent media. These findings suggested that the lower REEs leaching in spent media and abiotic than in the inoculated system was related to the presence and attachment of bacteria (Fathollahzadeh et al., 2018a).

It has also been demonstrated that abiotic leaching of REEs from Egyptian monazite (0.6% pulp density) with synthesized organic acids (chemical leaching using 74 mM citric and 14 mM oxalic acids) resulted in lower recovery, of 58.8% compared to 75.4% with bioleaching directly by *Aspergillus ficuum* (Hassanien et al., 2013b). In another study, the solubilised REEs concentrations from monazite sand by all detected organic acids and with cell-free spent medium were substantially lower than those observed for the active cultures (*Aspergillus terreus* strain ML3-1 and a *Paecilomyces* spp. strain WE3-F.), confirming applied strains secreted as yet unidentified metabolites into solution that are more effective than the identified organic acids at solubilizing phosphate and REEs from monazite (Brisson et al., 2016). This also suggests that to some extent the synthesized organic acids may substitute conventional lixiviants in REEs mineral leaching, whereas in the presence of the microbes and biogenic organic acids, the overall leaching efficiency for REEs can be increased (Corbett et al., 2017; Corbett et al., 2018; Fathollahzadeh et al., 2018a). These results emphasize the importance of implementing contact leaching in the bioleaching of REEs from monazite.

2.7 Processes related to the mobilisation of REEs

As mentioned previously, PSMs can acidify the medium naturally and therefore are able to release phosphate and REEs present in a phosphate mineral matrix. In most heterotrophic bioleaching systems, proton substitution reactions rely on the production of organic acids (OA) as follows:

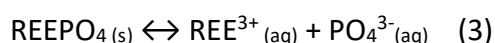


The proton activity is a key parameter affecting the dissolution of REEs-phosphate minerals. It has been reported that the normalized dissolution rate of La and Ce (by increasing acidity from 0.1 M to 1 M HNO₃) increased by one and two order of magnitude, respectively (Gausse et al., 2018).

A complex formation between OA anions and metal cations depends on the number and position of carboxylic (COOH) and hydroxy (OH) functional groups in the organic acids (Bolan et al., 1994). Depending on the dissociation properties and the number of these functional groups, organic acids can carry different negative charges, thereby allowing the complexation of REEs cations in solution and the displacement of anions from the bioleaching matrix (reaction 1 and 2). Therefore, the higher degree of protonation of the organic molecules is associated with i) provision of a lower pH condition which helps to maintain the stability of dissolved REEs³⁺ in solution; ii) affecting the degree of proton and ligand attack on the mineral surface, and iii) influencing the kinetics and strength of the complex formed with the relevant ligands, either in solution or at the bacterial-mineral surface interfaces.

Overall, during the bio solubilisation process of monazite, the organic acids secreted by PSMs with differing numbers of donating protons governs the leaching behaviour of REEs and phosphate. Naturally occurring organic ligands secreted by microorganisms, such as acetate, gluconate, citrate, malate, formate, oxalate, and succinate, play a crucial role in the mobility of REEs (Goynes et al., 2010).

From chemistry point of view, the solubility of the trivalent REEs-phosphates is very low (10⁻¹³ M in pure water) compared to calcium phosphate minerals (Firsching & Brune, 1991). The equilibrium solubility products (K_{sp}) expression for the equilibrium reaction of monazite dissolution is as follow:



$$K_{sp} = [\text{REE}^{3+}] \times [\text{PO}_4^{3-}] \quad (4)$$

Apart from the complexity of REEs-phosphate minerals, PSMs may stimulate or suppress mineral dissolution by the transport of solubilised inorganic phosphate for intercellular metabolism. As mentioned previously, according to the reaction (3), the incorporation of phosphate into the biomass shifts the reaction to the right and increases REE³⁺ solubility. Also, the precipitation (formation of secondary phosphate minerals), adsorption and desorption (formation of aqueous REE³⁺/PO₄³⁻) control the concentration of phosphate ions in the solution. It has been well established that the level of released P should not be relied upon as an indicator of ore solubilisation as microbial incorporation of inorganic phosphorus from the surrounding medium for immediate use in metabolic processes lowers the soluble P concentration (Brisson et al., 2016; Corbett et al., 2017). The diffusion of released phosphate into microorganisms influences phosphate concentrations in solution and promotes the dissolution and desorption reactions. Thus, phosphate regulation between the cell-mineral interface and the solution through bio-sorption, precipitation and co-precipitation may influence REEs dissolution rate during bioleaching. Additionally, a number of low molecular

weight organic acids can be rapidly degraded by microorganisms (Corbett et al., 2017). The retention mechanisms of citric and malic acids have demonstrated that higher Fe concentration under acidic conditions decreased the biodegradation of citric acid and malic acid (Yang et al., 2016). Therefore, the presence of Fe either originating from the mineral or regenerated during bio-oxidation in the bioleaching system can modulate the stability of organic acids by complexolysis, and hence prolong their complexing capacity with REEs (Fathollahzadeh et al., 2018c). Moreover, iron supplementation can be used for phosphate removal (Bunce et al., 2018)

Apart from the above mentioned factors associated with REEs mobilisation, perhaps the more urgent challenge is a better understanding of the molecular genetics of mineral phosphate solubilisation mechanisms and the associated metabolic pathways to phosphate regulation in response to P stress to develop efficient and environmentally friendly processing pathways, for bioleaching of REEs-phosphate minerals. Therefore, the following section focuses on the phosphate (Pho) regulon, the unique mechanism responsible for the regulation of phosphate and phosphate solubilisation in the bacteria.

2.8 Utilizing omics for enhanced bioleaching

Inorganic phosphate is an essential nutrient for energy, nucleic acid and phospholipid biosynthesis in microorganisms. Microbial communities have evolved appropriate regulatory mechanisms to survive and adapt rapidly to changes in phosphate availability through the presence of a “Pho regulon”. The characterisation of the Pho regulon was first reported for *Escherichia coli*, and later for many other bacterial species (Wanner & Chang, 1987). The Pho regulon in Gram-negative bacteria is controlled by a two-component regulatory system (phoR-phoB) which involves an inner-membrane histidine kinase sensor protein (phoR) and a cytoplasmic transcriptional response regulator (phoB) (Santos-Beneit, 2015). The *phoB* gene encodes the soluble DNA-binding response regulator and the *phoR* encodes the cytoplasmic membrane-associated sensor kinase (White & Metcalf, 2007). The P signalling pathway requires seven proteins (phoB, phoR, pstS, pstC, pstA, pstB, phoU), all of which probably interact in a membrane-associated signalling complex (Hsieh & Wanner, 2010). Inorganic extracellular phosphate depletion in the medium has been highlighted to be essential for the activation of the pho regulon and P signalling pathway in bacteria (Hsieh & Wanner, 2010). Figure 2 represents a model for Pi signal transduction of the *E. coli* pho regulon, showing how it is activated in response to P levels (Hsieh & Wanner, 2010).

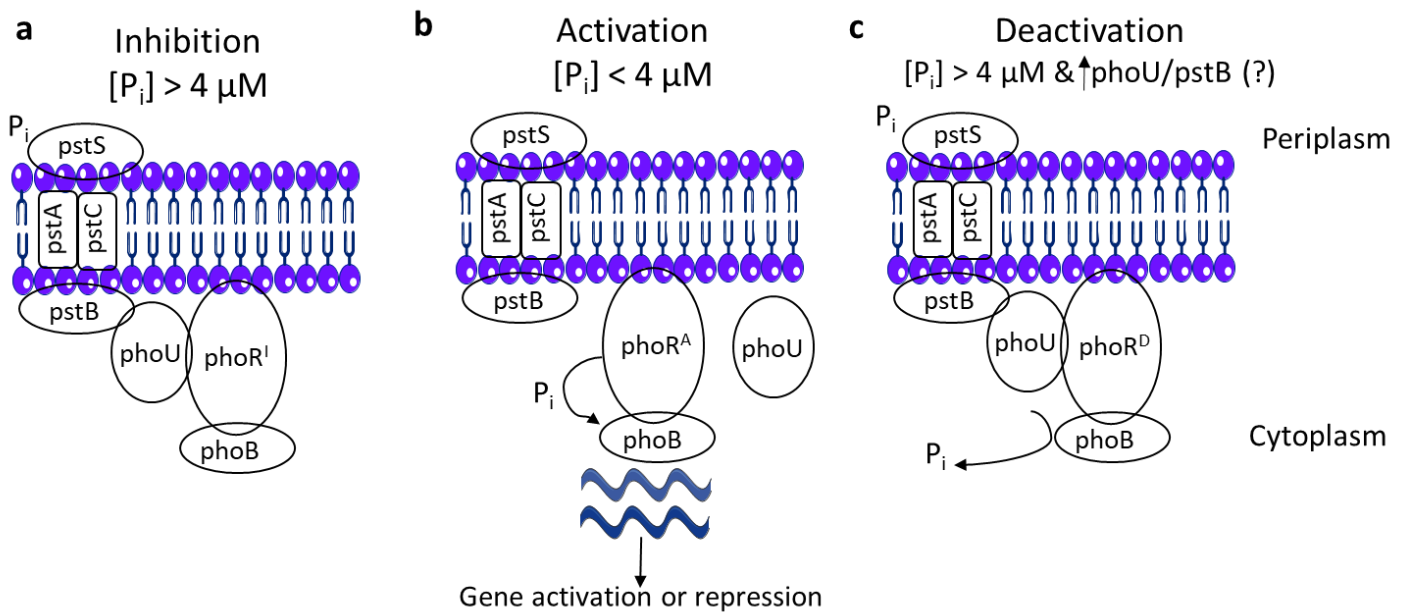


Fig.2 Biogeochemical model for sensing extracellular inorganic phosphate (P_i) and transduction of the signal to control gene expression in *Escherichia coli* (adapted from (Hsieh & Wanner, 2010)). The signalling processes of inhibition, activation, and deactivation are proposed to correspond to different states of phoR: an inhibition state (phoR^I), an activation state (phoR^A), and a deactivation state (phoR^D). With excess extracellular P (> 4 μM for *E. coli*) (Figure 2.a), the pstSCAB complex pumps P across the cell membrane into the cytoplasm, where phoU proteins forms a complex with pstSCAB transporter system and the phoR histidine kinase, which prevents auto phosphorylation of the kinase caused by the interaction with phoU, a chaperon like phoR-phoB inhibitory protein. When external P is low (< 4 μM for *E. coli*) (Figure 2.b) and the pstSCAB complex is inactive, phoU dissociates and auto phosphorylation of phoR occurs. The phosphorylated phoR activates the transcription factor phoB, which then activates the transcription of at least 31 genes, where one of activated genes, phoA, encodes the phoA protein (bacterial alkaline phosphatase). This protein is transported across the membrane into the periplasm to degrade organic polyphosphates to release P which is then taken up into the cell to overcome the P stress (Blätke et al., 2012). When the cell's P requirement is met, this system is switched off again and phoBP is dephosphorylated (Figure 2.c). Reprinted from (Hsieh & Wanner, 2010; Wanner, 1996) with permission from Elsevier.

With excess extracellular P ($> 4 \mu\text{M}$ for *E. coli*) (Fig 2.a), the pstSCAB complex pumps P across the cell membrane into the cytoplasm, where PhoU proteins form a complex with the pstSCAB transporter system and the PhoR histidine kinase, which prevents auto-phosphorylation of the kinase caused by the interaction with PhoU, a chaperon-like PhoR-PhoB inhibitory protein. When external P is low ($< 4 \mu\text{M}$ for *E. coli*) (Fig 2.b) and the pstSCAB complex is inactive, PhoU dissociates and auto-phosphorylation of PhoR occurs. The phosphorylated PhoR activates the transcription factor PhoB, which then activates the transcription of at least 31 genes, where one of the activated genes, *phoA*, encodes the PhoA protein (bacterial alkaline phosphatase). This protein is transported across the membrane into the periplasm to degrade organic polyphosphates to release P which is then taken up into the cell to overcome the P stress (Blätke et al., 2012). When the cell's P requirement is met, this system is switched off again and PhoB is dephosphorylated (Fig 2.c) (Hsieh & Wanner, 2010; Wanner, 1996).

A recent study has demonstrated that the distinct organization of the master regulator, *phoBR*, in *Acidithiobacillus ferrooxidans* compared to *E. aerogenes* led to more efficient stress response which potentially increased the overall REEs dissolution from monazite (Fathollahzadeh et al., 2018c). Furthermore, previous research on several PSMs as well as *E. coli* have identified a variety of different genes/operons with homology to those which play a role in phosphate uptake, regulation and solubilisation mechanisms of mineral phosphate, including *pqq*, *phoR*, *phoB*, *pstC*, *pstA*, *pstB*, *pstS*, *phoU*, *phoR*, *pKKY*, *pK1M10*, and *gabY* (Chhabra et al., 2013; Rodríguez et al., 2006; Tsurumaru et al., 2015). These may have pivotal roles in the solubilisation of phosphates and, therefore, in the recovery of REEs through phosphate bioleaching.

In addition to the *pho* regulon, gene cloning studies have also revealed that genes directly or indirectly involved in organic acid synthesis as well as expression and regulation of direct oxidation pathways participate in phosphate mobilisation (Babu-Khan et al., 1995; Rodríguez et al., 2006). Amongst organic acids, gluconic acid was highlighted as the metabolic basis of inorganic phosphate solubilisation, through the oxidation of glucose by a periplasmic glucose dehydrogenase (*gdh*) enzyme (encoded by *gcd* gene) that requires pyrrolo quinoline quinone (PQQ) as a redox cofactor (An & Moe, 2016). The second periplasmic oxidation, catalyzed by gluconate dehydrogenase (*gad*) results in the production of 2-ketogluconic acid ($\text{pK}_a=2.6$) while minimizing the respiratory chain component PQQ and bypassing the NADPH-generating glucose-6-phosphate dehydrogenase (G6PD) (Ebert et al., 2011). In addition to providing carbon for intracellular metabolism, the direct oxidation of glucose to gluconic acid produces a transmembrane proton motive force (PMF) that may be used for bioenergetics and/or membrane transport functions which results in the uptake of exogenous amino acids and other compounds (Goldstein, 2007). Moreover, several acid phosphatase genes (*acp*) from Gram-negative bacteria have been characterized for improving organic phosphate solubilisation from organic compounds in soil (Rossolini et al., 1998). It has been suggested that acid phosphatase generated by isolated PSMs does not act directly on inorganic P solubilisation. However, phosphatase activity may participate in lowering the pH of the culture medium by the dephosphorylating action and the production of organic acids (Achal et al., 2007; Park et al., 2011) corresponding to no significant correlation between the concentration

of phosphate, phosphate solubilisation and the phosphatase activity (Braz & Nahas, 2012; Mihalache et al., 2015).

Extending beyond genomic studies, transcriptome profiling also provides potential in identifying molecular mechanisms that affect bioleaching. For example, transcriptome profiling of PSM strain *Burkholderia multivorans* at three levels of exogenous soluble phosphate (0, 0.5, and 20 mM) identified 446 differentially expressed genes, among which 44 genes were continuously up-regulated when soluble phosphate concentration was increased and 81 genes were continuously down-regulated (Zeng et al., 2017). Furthermore, genes involved in glucose metabolism were continuously down-regulated, which indicated that metabolic channelling of glucose towards the phosphorylative pathway was negatively regulated by soluble phosphate, which may in turn might affect the organic acid secretion and subsequently the phosphate-solubilizing activity (Zeng et al., 2017).

Altogether, a better understanding of complex interactions among PSM genotypes, phenotypes, environmental conditions and microbiome structure provides indispensable information in their metabolic properties and helps to identify novel strains and genes for optimum biological REEs/phosphate mobilisation. Alongside REEs/phosphate solubilisation tests with conventional strains, novel omics techniques are enabling the discovery of the core genetic elements that increasingly drive the regulation of PSMs adaptation strategies/physiological responses based on the growth media and the types of mineral. This genetic material has the potential to be transferred to other microorganisms in order to enable them to solubilise phosphate and REEs. Despite the great potential of applying omics to construct microbial communities with modified phosphate starvation–responsive genes for bioprocessing of REE-phosphate minerals, the exploration of opportunities in which core microbiomes can be integrated into “smart bioleaching” is only emerging.

2.9 Current status of bioleaching of REEs from primary and secondary resources

Microorganisms are effective in the mobilisation of elements mainly through three principles including acidolysis (formation of organic/inorganic acids), complexolysis (excretion of complexing agents), and redoxolysis (oxidation/reduction reactions) (Brandl, 2008). Biotechnological mineral processing approaches where microorganisms generate bioleachants have been developed as a sustainable alternative to chemical leaching of primary and secondary ores and waste streams (Watling, 2016). Bioleaching processes are generally operated at relatively low temperature and atmospheric pressure, without relying on expensive and aggressive reagents common in hydrometallurgical processing, or high temperature, energy cost and gas emissions related to pyrometallurgical processing (Bryan et al., 2015). There have been several studies and patents published recently addressing the interactions of microorganisms and REEs including both REEs mobilisation from solids and immobilization from liquids (Barmettler et al., 2016a). However, the role of microorganisms in REEs immobilisation is not included in the scope of this review as most of available and economical resources of REEs are present as solid matrices and the current bioleaching efforts are directed to releasing REEs from minerals. In the following sections, the latest studies on bioprocessing of REEs from primary and secondary REEs resources will be discussed.

2.9.1 Primary resources

Prominent currently operating mines with primary REEs minerals (i.e., monazite, bastnasite, and xenotime) are Mount Weld in Australia, Bayan Obo in China, and recently reopened Mountain Pass in the US (Haque et al., 2014). The Mount Weld deposit, Laverton, in Western Australia has been identified as the richest and highest grade known deposit of REEs in the world dominated with secondary REEs phosphate (monazite) encapsulated in iron oxide minerals (Haque et al., 2014).

In 2017, the laboratory bioleaching studies of Mount Weld Monazite (MWM) (500 mL shake flasks containing 0.5% ore (w/v) with 3% glucose and modified PVK media, initial pH = 7, for 14 days at 130 rpm, at either 30 or 37 °C depending on the inoculum species) demonstrated that a number of traditional PSMs could solubilize REEs from a lateritic monazite concentrate into the leachate (Corbett et al., 2017). After 8 d, *Penicillium* sp. released a total concentration of 12.32 mg L⁻¹ of Ce, La, Nd, and Pr with little release of Th and Fe into solution (Corbett et al., 2017). Furthermore, bioleaching experiments conducted on non-sterile MWM with a known PSM (*Penicillium* sp.CF1) (8 d and pulp density of 0.5% w/v) resulted in greater mobilisation of REEs into solution (23.7 mg L⁻¹) in comparison to experiments conducted on sterile monazite (Corbett et al., 2018). The authors suggested that the presence of indigenous microbes (*Firmicutes*) on the non-sterile monazite increased the bioleaching of MWM by PSMs at rates greater than what was recorded with either the indigenous organisms or the PSMs separately (Corbett et al., 2018). This syntrophic effect of indigenous and inoculated microorganisms was in good agreement with enhanced REEs bioleaching from monazite with a co-culture system (up to a final concentration of 40 mg L⁻¹ REEs) (Fathollahzadeh et al., 2018c).

The two mine deposits outside of Australia (Bayan Obo in China and Mountain Pass in California) principally contain bastnasite where in Bayan Obo bastnasite and monazite co-occur in ore minerals (Haque et al., 2014). Recently, bioleaching of REEs from bastnasite-bearing rock by Gram-positive *Actinobacteria* has been investigated (Zhang et al., 2018). These authors have reported that in a nutrient-rich growth medium, the total concentration of bioleached REEs ranged from 56 to 342 µg L⁻¹, whereas in an oligotrophic medium, only one strain (*Streptomyces* sp.) grew in the presence of the bastnasite (0.5% w/v), and leached up to 548 µg L⁻¹ of total REEs (Zhang et al., 2018). Coincidentally, a combination of low solubility of bastnasite, the lack of nutrients from the mineral, the precipitation of REEs minerals, and the re-sorption of leached REEs to cell and residual mineral surfaces may have contributed to the observed low leaching efficiency (0.008–0.08%) (Zhang et al., 2018).

Despite many known deposits available for the evaluation of the bioleaching of REEs from primary minerals, the other available published studies have reported REEs bioleaching efficiencies for monazite sand, or synthesized and poorly characterized REEs matrices (Brisson et al., 2016; Hassanien et al., 2013b). The use of synthesized REEs matrices for studying the solubilisation mechanisms of REEs/phosphate has disadvantages because of a variety of factors such chemical composition and surface chemistry.

An enrichment culture of heterotrophic REEs-phosphate solubilizing fungi was established with 10 g L⁻¹ glucose and NdPO₄ (Brisson et al., 2016). A 1 mL aliquot of a spore suspension of the three isolated fungal strains (*A. niger*, *A. terreus*, and *Paecilomyces* spp.) were incubated for 6 days in 250 mL Erlenmeyer flasks at room temperature, 250 rpm, with 1% pulp density of synthesized monazite sand. Bioleaching efficiencies were tested in comparison to abiotic leaching (HCl, organic acid, and spent medium). Cell-free spent medium and active cultures leached REEs to concentrations 1.7–3.8 and 5 times higher than those of HCl solutions of comparable pH (3% of leaching efficiency for the cell-free spent medium and 5% of leaching efficiency for active cultures), indicating that metabolites secreted by these organisms contribute substantially to REEs leaching.

In another study, the feasibility of 10 species of PSMs to develop a bioleaching process of REEs from a monazite bearing ore was determined by halo zone formation on agar media, where *Pseudomonas fluorescens*, *P. putida*, *P. rhizosphaerae*, *Mesorhizobium ciceri*, *Bacillus megaterium*, and *Acetobacter aceti* formed halo zones, with the zone of *A. aceti* being the widest (Shin et al., 2015). Furthermore, with respect to higher Ca and phosphate solubilisation of *Ac. aceti* from TCP comparing to the other strains, this strain was used for further REEs bioleaching experiments. The strain released up to 5.7 mg L⁻¹ of Ce on day 4 (0.13% of leaching efficiency).

2.9.2 Secondary resources

To tackle the REEs supply challenge, extraction of REEs from secondary resources and waste streams has been proposed (Binnemans et al., 2013b). However, up to 2011 less than 1% of the REEs were recovered (Tkaczyk et al., 2018). In contrast to recycling of REEs from the REEs wastes streams and/or the low grade ores such as mineral sands, more attention has been devoted to red mud (bauxite residue), waste electrical and electronic equipment (WEEE) shredding, waste phosphors/cracking catalysts, and fluorescent powder (Hopfe et al., 2017; Marra et al., 2018; Qu & Lian, 2013; Reed et al., 2016).

A filamentous, acid-producing fungi named RM-10, identified as *Penicillium tricolor*, was isolated from red mud and the fungal bioleaching efficiency of REEs from red mud was investigated under various bioleaching processes (one-step and two-step) and pulp densities (2, 5, and 10% w/v) (Qu & Lian, 2013). Higher production of citric and oxalic acids with the increased pulp density of red mud in both processes demonstrated that both acids played major roles in the bioleaching of REEs from red mud with leaching efficiencies varying from 36% to 78%.

Marra et al. (Marra et al., 2018) proposed a two-step bioleaching process for the extraction of REEs from WEEE dust with *Acidithiobacillus thiooxidans* and *P. putida*. The first leaching step with *At. thiooxidans* showed yields of > 99% for Ce and Eu, and > 80% for La and Y while pH of the leaching solution dropped from 3.5 to 1.0 (with 1% pulp density after 8 days). In the second step, the cyanide producing *P. putida* released 48% of gold within 3 h from the residue of the first step (Marra et al., 2018). As sulfur was not present in the WEEE dust, the production of biogenic sulfuric acid from elemental sulfur was responsible for the

solubilisation of the REEs contained in WEEE. The leaching efficiency of Y in this study was higher than in a study where a mixed culture of acidophiles leached up to 70% of Y from fluorescent powder (with 10% pulp density after 16 days) (Beolchini et al., 2012).

The potential of *Gluconobacter oxydans* in bioleaching of REEs from spent fluid catalytic cracking (FCC) catalyst and retorted phosphor powder (RPP) was demonstrated to be controlled by the production of gluconic acid. The leaching efficiency was up to 49% of the total REEs from the FCC material and up to 2% of total REEs from the RPP (Reed et al., 2016). The optimized batch process (agitation intensity, oxygen levels, glucose concentrations, and nutrient additions) increased leaching efficiency of REEs from FCC up to 56% (Thompson et al., 2017a). As a result, the authors suggested that microorganisms producing gluconic and other organic acids can effectively leach REEs from waste materials, and that increasing organic acid production will improve the overall recovery. Another readily accessible secondary resource of REEs, fluorescent phosphor (FP) which contains about 10% of REE-oxides has been bioleached by Kombucha metabolites (i.e., acetic and gluconic acid) with leaching efficiency of up to 8% (Hopfe et al., 2017). These studies revealed that the interaction of the different microorganisms and the sample material contribute to the leaching behaviour of REEs. Bioleaching could also potentially be applied to other waste products.

2.10 Mining of REEs and sustainability: The missing link

In a world of metal scarcity, the development of new sustainable technologies for REEs extraction from both primary and secondary resources would be extremely beneficial. It must be realized that in a market where the global consumption of a resource grows by more than 1% per annum, REEs recycling cannot replace primary mining of REE ores (Binnemans et al., 2013b), thereby recycling, investigating new potential REEs resources, and primary mining of rare earths are complementary activities for securing long term REEs supply. Among the available processes, bioleaching can be a sustainable technique for extracting these elements (Ilyas et al., 2017).

The major drawback of bioleaching compared to conventional REEs extraction is slower dissolution kinetics and need for a growth substrate for microbial growth. A techno-economic analysis showed glucose to be the single largest expense for the heterotrophic bioleaching process of REEs, constituting 44% of the total cost (Thompson et al., 2017a). Therefore, lower expenditure on carbon and energy in addition to an improved leaching efficiencies with core PSMs would increase the overall profit.

2.11 Challenges and future prospects

Until recently relatively little research has been conducted on the solubilisation of REEs from phosphate minerals with only a few papers focusing on the biomining of REEs having been published. In addition, knowledge of the underlying metabolic basis of phosphate solubilisation is limited with no information on the fate of phosphate and REEs within the bacteria-mineral-solution interface being available. One study has determined the concentration of P in the biomass of a Gram-positive PSM, *Staphylococcus aureus*, (grown on

K_2HPO_4) (Mechler et al., 2015), however the method does not reflect the complex multicomponent thermodynamics of monazite. Moreover, due to the low solubility of REEs, the biological functions of these elements have not been extensively studied. As a result, the understanding of the biochemistry of these elements is limited. The rate of biomass growth is controlled by nutrient availability including phosphate (Rodríguez & Fraga, 1999; Rodríguez et al., 2006) as well as environmental stressors such as low pH and REEs toxicity which may suppress further REEs bio-mobilization. Further studies using different pure and mixed cultures are required to better understand the mechanisms of phosphate diffusion to and from the mineral and the effects of toxic elements in bioleaching of REEs.

The mineralogy of monazite governs the dissolution and transport of REEs and phosphate due to its very low solubility compared to other mineral types, such as apatite and/or orthophosphates are more susceptible to leaching. The mobility of REEs, Th, and U is also controlled by their oxidation state. Nevertheless, limited information on the characteristics of the different oxidation states of REEs and their mobility and transition in biogeochemical processes is available in the literature.

REEs-phosphate minerals are composed of inorganic P that can be utilized by PSMs for metabolic purposes. It has been demonstrated that, the mineral composition together with the speciation of elements in the phosphate minerals is one key factor influencing the proliferation of microbial communities in the bioleaching systems (Fathollahzadeh et al., 2018c). Studying the effects of bioleaching on REEs mobility from monazite has demonstrated that during REEs mobilisation from different fractions of REEs, solubilised REEs does not necessarily remain in the solution but shifts to the other fractions in the residue. In this context, the mobility of REEs and phosphate in monazite depends on microbial activity, attachment of bacteria on the surface, phase association of the REEs, (distribution of labile and non-labile REE), and which physiochemical and biological processes (Fathollahzadeh et al., 2018c) these phases are subjected to.

Another aspect of the mineralogical challenge is the shift of REEs phases in the REEs-phosphate minerals. Sequential extraction procedures (SEP) provide evidence on the fractionation of elements of interest for evaluating their potential mobility and bioavailability (Fathollahzadeh et al., 2015; Fathollahzadeh et al., 2014). A method for REEs fractionation has only recently been proposed (Mittermüller et al., 2016). It has been demonstrated that bacteria are capable of modulating the fate and speciation of metals in contaminated sediments (Fonti et al., 2015), however, the effects of bioleaching on REEs mobility from phosphate minerals have only recently been reported (Fathollahzadeh et al., 2018c).

Future attempts to understand biofilm formation and the development of relevant industrial strains, and their interaction with mineral surfaces in mixed species cultures, as well as the development of biomarkers to analyse the microbial biodiversity within field operations are major challenges that need addressing to enhance our knowledge for future bioleaching processes development and monitoring.

2.12 Conclusions

Bioleaching of REEs is considered a greener and environmentally friendly technology than conventional techniques. Although a number of endeavours have been made to investigate the effects of PSMs on the bioleaching of phosphates and REEs from phosphate minerals, there are not enough studies of the mechanisms responsible for the mobilisation of REEs. As REEs exist as either primary or secondary resources, it is mandatory to study the viability of different strains for bioleaching. Organic acids, phosphatases, bacterial attachment, phosphate regulation, the fractionation of REEs and mineralogy determine the fate and mobility of REEs and phosphate, which is crucial for the optimal extraction of REEs. Metagenomic, transcriptomic, proteomic and metabolic studies are needed to unveil the biochemical and molecular mechanisms used by PSMs during bioleaching.

2.13 Notes

2.13.1 Funding

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2.14 Compliance with ethical standards

2.14.1 Conflict of interest

The authors declare that they have no conflict of interest.

2.14.2 Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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Chapter 3: Diversity and function of phosphate solubilizing bacteria enriched from Mount Weld rare-earth mine, Australia

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Diversity and function of phosphate solubilizing bacteria enriched from Mount Weld rare-earth mine, Australia

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Abstract

Currently the known high grade easily-acquirable reserves of rare earth element (REEs) containing phosphate minerals are depleting. The objective of this study was to enrich indigenous phosphate solubilising bacterial strains from various phosphate ores and evaluate their potential application in the bioleaching REEs from the ores. Bacterial communities were enriched from the highest grade known deposit of REEs in the world, in arid Western Australia. The dominant taxa enriched from the monazite concentrate were *Actinobacteria*, *Proteobacteria*, and *Firmicutes*. The consortium of indigenous bacteria solubilized REEs (Ce, La, Nd) up to a total final concentration of 0.836 mg L⁻¹.

Keywords: Phosphate solubilising bacteria; Indigenous bacteria; Monazite bioleaching; Rare earth elements; Sustainable mining

3.1 Introduction

Phosphorous (P) deficiency in agricultural soils is a major constraint to sustainable crop production. The availability of P to plants and soil microbiota is known to be governed by the forms of P, such as water-soluble, exchangeable, non-exchangeable, and minerals, the first two forms are easily available to biota (Werner et al., 2017). It has been demonstrated that in response to P deficiency, phosphate solubilizing bacteria (PSB) are able to solubilise P from poorly available sources (Goldstein & Krishnaraj, 2007).

As economies around the world decarbonise, demand for more sustainable agriculture systems and green technologies such as electric vehicles and wind turbines, using new energy materials (i.e., Rare Earth Elements [REEs]) is forecast to increase (Binnemans et al., 2013a). Economic ore deposits are also becoming more difficult to find, partly due to the increased costs of exploration in remote locations or at greater depths, as well as decreasing the average grade of the deposits (Dunbar, 2017). Biotechnology could provide innovative alternatives for mitigating the constraints on current methods for metal extraction, and could fundamentally change the mining industry (Dunbar, 2017).

The phosphate solubilising capacity of PSB has been demonstrated to provide an environmentally friendly strategy for biomining of REEs from phosphate waste materials (Thompson et al., 2017b). Therefore, bio-dephosphorization of iron phosphate ores by *Burkholderia caribensis*, *Leptospirillum ferrooxidans*, and a mixed culture of acidophilic bacteria have been explored (Chime, 2013; Delvasto et al., 2009; Priha et al., 2014). A large range of bacterial species including *Klebsiella*, *Enterobacter*, *Pseudomonas*, *Bacillus*, *Rhizobium*, *Erwinia*, *Agrobacterium*, *Flavobacterium*, *Enterobacter*, *Micrococcus*, *Thiobacillus*, *Acetobacter*, *Burkholderia spp.*, *Clavibacter*, *Serratia* and *Streptomyces* are capable of P solubilisation (Illmer & Schinner, 1992; Rodríguez & Fraga, 1999; Zhao & Lin, 2001). It has been indicated that the P solubility was directly correlated with the release of organic acids (Park et al., 2009a). Therefore, the main mechanism of P solubilisation by heterotrophic PSB has been associated with production of organic acids such as gluconic, citric, oxalic, acetic, lactic and itaconic acids (Chen et al., 2006; Khan et al., 2014; Zeng et al., 2017).

Two of the three primary REEs minerals (monazite [(Ce, La, Nd, Th)PO₄] and xenotime [YPO₄]) are associated with phosphate groups (Jaireth et al., 2014a). Early studies in the fields of biomining of REEs-phosphate minerals recognized the potential of microbial communities to release REEs from mineral solids (Barmettler et al., 2016b; Corbett et al., 2017). Most previous research has been focused on the application of exogenous and pure strains in the bioleaching of monazite (Brisson et al., 2016; Corbett et al., 2017). Metallomorphic and extreme environments provide a unique habitat for microbial life and Western Australia (WA) has many natural and man-made environments ideal for bioprospecting purposes (Kaksonen et al., 2018).

Moreover, it has been suggested that indigenous microorganisms are more competitive in terms of adaptation than exogenous microorganisms (Delvasto et al., 2009; Priha et al., 2014).

However, the bioprospecting of indigenous microorganisms from the natural environments where the REEs ore is present has still been largely unexplored.

The Mount (Mt.) Weld deposit, Laverton, in WA has been known as the richest and highest grade known deposit of REEs in the world dominated with secondary REEs phosphate (monazite) encapsulated in iron oxide minerals (Haque et al., 2014). The objective of this study was to evaluate the capability of indigenous PSB strains from Mt. Weld deposit in the bioleaching of REEs from three different grade of monazite bearing minerals present in WA regions.

3.2 Material and methods

3.2.1 Monazite ore

Three monazite samples from WA were used for the leaching experiments. A high grade weathered yellowish monazite concentrate was collected from the Mt. Weld Mine (Lynas Corporation), referred to as MWM (Mt. Weld Monazite), a medium grade brownish monazite ore from the Mt. Weld Mine (Lynas Corporation) referred to as MWO, and a monazite ore sourced from the Busselton Mineral Sands deposit, WA (Cable Sands Pty Ltd) referred to as CSM. Sample preparation and composition analysis for MWM and CSM were described elsewhere (Corbett et al., 2017). The monazite concentrate of CSM was diluted 1:10 with Silica flour to obtain a safe Th/U working concentration (Corbett et al., 2017). The mineralogical composition of MWM, MWO, and CSM was determined by X ray diffraction (XRD) at CSIRO Minerals, Waterford, Western Australia. The XRD analysis revealed that the MWM was mainly composed of monazite, florencite, and nontronite whereas CSM contained zircon and monazite. MWO sample was dominated by goethite followed by monazite, florencite, and nontronite. The ores were ground in a rod mill, pulverized in a ring mill and finally sieved to <38 μm in particle size. The elemental composition of the MWO was analysed by inductively coupled plasma optical emission spectrometry (ICP-OES, CSIRO Minerals, Waterford, WA). The MWO contained (%): 3.30 La, 4.81 Ce, 0.741 Pr, 2.27 Nd, 0.049 Y, 0.043 Th, 31.5 Fe, 2.95 P, 0.703 Ca, 0.524 Mg, 3.84 Si, 0.426 Ti, 0.012 Zr, and <0.003 U. Before bioleaching experiments, the mineral samples were sterilized by gamma irradiation at 50kGy for 11 h (ChemCentre, Bentley, Western Australia).

3.2.2 Isolation and enrichment of indigenous bacteria from the ore sample

The isolation and enrichment of indigenous microbes were conducted from the non-sterile MWO on two different inorganic P sources; $\text{Ca}_3(\text{PO}_4)_2$ (TCP) and $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ (CaP). For the isolation of heterotrophic PSB, MWO (2.5 g) was incubated in 50 mL of National Botanical Research Institute's phosphate growth medium (NBRIP) broth (Nautiyal, 1999) with either TCP or CaP as the phosphate source, at $\text{pH}=6.85\pm 0.25$, for 7 d at 30 °C, agitated at 140 rpm. To detect the alteration in the microbial community with subculturing, cultures enriched on TCP or CaP were continuously grown on TCP or CaP as shown in table 1.

To investigate the bioleaching efficiency of the mixed populations from the enrichment cultures, the culture originally enriched on TCP and the culture originally enriched on CaP were combined and incubated in NBRIP broth. This is referred to as TCP:CaP.

Table 1 – The order of subculturing of original cultures for studying changes in the diversity of microbial community.

	Original enrichment	Subculture
TCP-TCP	TCP	TCP
TCP-CaP	TCP	CaP
CaP-CaP	CaP	CaP
CaP-TCP	CaP	TCP

Bacterial cells were also detached from one gram of MWO using 9 ml of Ringer's solution and shaken (140 rpm) (Merck, Darmstadt, Germany) for 24 h, cultured on NBRIP plates as a dilution series (10 fold), and incubated at 30 °C for 24 h. PSB isolated from Ringer's solution were identified based on the formation of visible halo/zone on agar plates (NBRIP either with TCP or CaP as the phosphate source + 15 g L⁻¹ agar) indicating P solubilisation of TCP or CaP by bacterial isolates.

3.2.3 Diversity profiling of enrichment cultures

DNA was extracted from one mL of enrichment cultures (TCP or CaP) using the FastDNA™ SPIN KIT (MP Biomedicals). DNA quality and concentration required for diversity profiling (10 ng in 10 µL) was confirmed using a Nanodrop (ThermoFisher) and by PCR.

Diversity profiling of the total genomic DNA was carried out by the Australian Genomics Research Facility using universal primers 341F (5'-CCTAYGGGRBGCASAG-3') and 806R (5'-GGACTACNNGGTATCTAAT-3') specific for the 16S rRNA gene of bacteria and archaea (Muyzer et al., 1993). Sequence analysis was conducted as described elsewhere (Khaleque et al., 2018). The operating Taxonomic Units (OTUs) representing less than 1% of the communities were excluded. Bacterial diversity was evaluated by calculating the Shannon diversity index (H) according to the equation (Shannon, 2001):

$$H = - \sum p_i \ln (p_i)$$

where, p_i is corresponding to OUT's values. The flowchart of the bioprospecting is presented in Fig. 1.

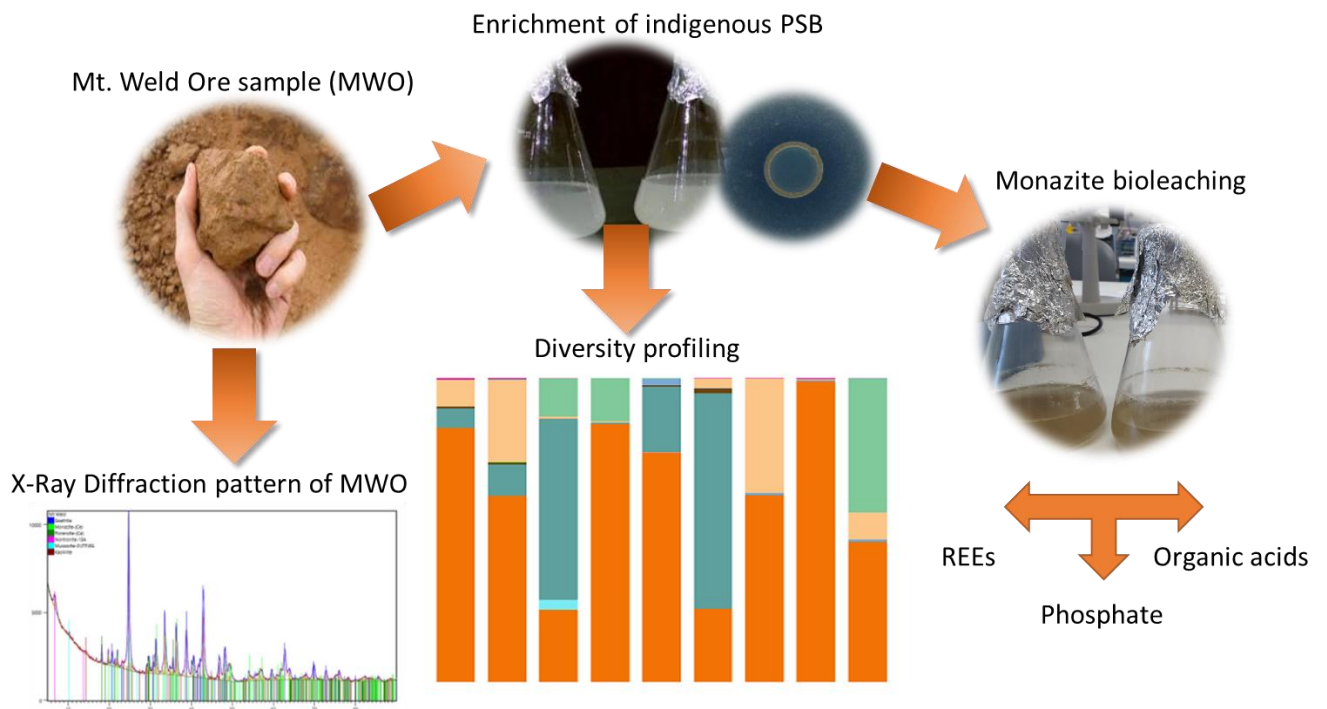


Fig.1 The flowchart of bioprospecting phosphate solubilising bacteria (PSB) for monazite bioleaching from Mount weld ore (MWO).

3.2.4 Bioleaching of phosphate minerals by enrichment cultures

The ability of indigenous bacteria (cultures enriched on TCP and the mixed culture, TCP:CaP) to utilise MWO, MWM, and CSM as a phosphate source, was evaluated in 500 mL Erlenmeyer flasks. Cultures enriched on TCP and CaP were grown at 30 °C in NBRIP medium with available P sources either TCP or CaP and the mixed culture with both TCP and CaP (Nautiyal, 1999), with shaking at 140 rpm, and harvested by centrifugation (3,600 g, 10 min). Cells were resuspended in sterile Tris-HCl buffer (100 mM, pH 7.2), centrifuged (3,600 g, 5 min) and washed twice more to remove any trace of phosphate.

Bioleaching was carried out over 7 days at 30 °C in triplicate, at 140 rpm in an orbital shaking incubator (RATEK, Model No: OM11) in 200 mL of NBRIP media (pH 7.00±0.25), with 1% v/v bacterial inoculum (initial density 1×10^7 cells mL⁻¹) and 1% pulp density of sterilized ore sample. For bioleaching with a mixed culture of these bacteria, a mixed bacterial inoculum of each culture (0.5% v/v TCP enrichment + 0.5% of CaP enrichment, initial density 1×10^7 cells mL⁻¹) was prepared and used for the bioleaching experiment. Cell-free abiotic controls were carried out under the same conditions. Bioleaching was monitored by measuring the soluble concentration of Ce, La, Pr, Nd, Fe, P, Th, U, and Y in the leachate.

3.2.5 Analytical Methods

Samples were taken at 0, 2, 3, 5, 7 d and pH measured using a pH meter (Ionode IJ series pH probe). Thereafter, samples were filtered (0.20 μm , cellulose acetate/surfactant-free, Sartorius) and assayed for REEs, Y, Th, U, and Fe concentrations by ICP-MS on day 2 and 7 (Agilent Technologies 7700 series, Bureau Veritas Australia Pty Ltd, Canning Vale, Western Australia) and the average values were reported. Soluble phosphate concentration was determined by colorimetric method (Murphy & Riley, 1962).

Organic acids were analysed at day 7 by high performance liquid chromatography (HPLC) (Agilent 1200, Curtin Water Quality Research Centre, Bentley, Western Australia) coupled with a diode array detector (DAD, Agilent). The injection volume was set as 50 μL for the samples. Compound separation was achieved with a C18 reverse phase column (Agilent, 5 μm , 4.6 \times 250 mm). The isocratic elution flow rate was 1.0 mL min^{-1} . The mobile phase consisted of 70% methanol and 30% phosphate buffer (pH=2.0). A detection wavelength of 220 nm was used. The identity and concentration of organic acid was determined by comparing the retention times and peak areas of chromatograms of the samples with standards. Organic acid identity was confirmed by liquid chromatography tandem-mass spectrometry (LC-MS/MS). The experimental setup for LC-MS/MS including some exemplary mass chromatograms of organic acids were described elsewhere (Busetti et al., 2014). Organic acids standards included gluconic, malic, formic, butyric, citric, acetic, lactic, oxalic, and pyruvic acids.

3.3 Results and Discussion

3.3.1 Bio-solubilisation of REEs from monazite by PSB

Previous studies on the microbial solubilisation of REEs-phosphate minerals and release of REEs have focused mainly on the impact of a single species of bacteria or fungi (Keekan et al., 2017; Shin et al., 2015). However, little is known of the indigenous microbial consortia present on the ores. In order to assess the bioleaching potential of native microbial consortia in the leaching of REEs from monazite, a series of leaching experiments was conducted. Regardless of the source of the enrichment cultures the same decline in pH was observed (Fig. 2). The pH when grown in the presence of CSM decreased to 2.6, MWO and MWM to 3.9-4.1. The pH in the abiotic controls did not decrease. This decrease in the pH can be attributed to the production of the organic acids resulting from glucose oxidation, bacterial respiration and nitrification (Corbett et al., 2018). Moreover, the concentrations of soluble P in the leachate of all samples were below than detection limit ($< 2 \text{ mg L}^{-1}$). This confirms that phosphate release into solution is not a reliable indicator of the breakdown of the phosphate matrix and the release of REEs. This is most likely due to the bacteria utilising any phosphate that has been released for their metabolic requirements (Corbett et al., 2017). This is in good agreement with phosphate starved environment for PSB present in this study.

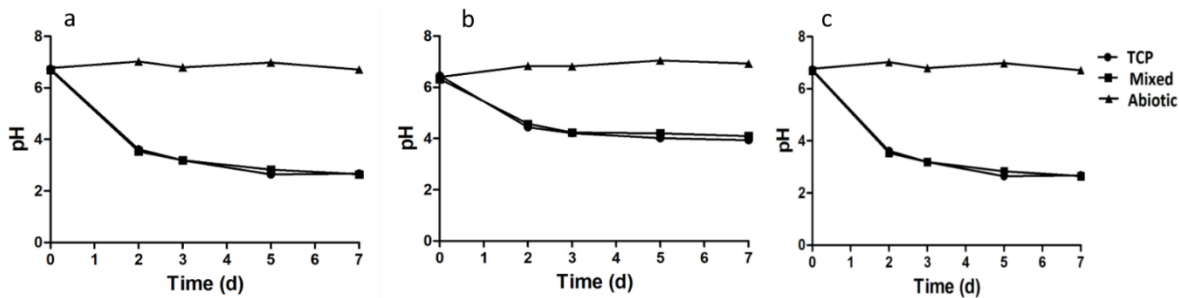


Fig.2 Observed pH change over seven days after inoculation of monazite minerals (a) Cable sand monazite, (b) Mt. Weld mine ore, and (c) Mt. Weld monazite with heterotrophic indigenous phosphate solubilising bacteria enriched on $\text{Ca}_3(\text{PO}_4)_2$ (TCP) or $\text{Ca}_3(\text{PO}_4)_2 + \text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ (Mixed). Error bars represent standard error between three replicate flasks. Error bars not visible are smaller than symbols.

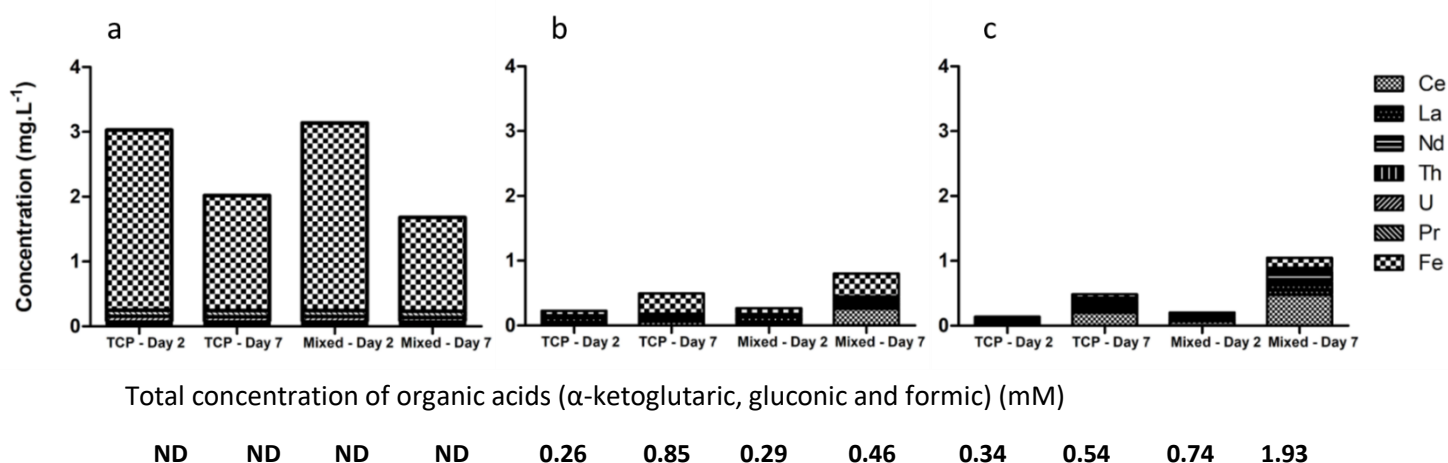


Fig.3 Total concentration of Ce, La, Nd, Th, U, Pr, and Fe released into the leachate from (a) Cable sand monazite, (b) Mt. Weld mine ore, and (c) Mt. Weld monazite 2 and 7 days after inoculation with heterotrophic indigenous phosphate solubilising bacteria enriched on $\text{Ca}_3(\text{PO}_4)_2$ (TCP) or $\text{Ca}_3(\text{PO}_4)_2 + \text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ (Mixed). Data are averages \pm SD of triplicate biological replicates. Error bars not visible are smaller than symbols.

All enrichment cultures used in this study produced organic acids at varying concentrations (Fig. 3). Organic acids production of heterotrophic metabolism by enrichment PSB on MWO and MWM where glucose was provided as the carbon source were α -ketoglutaric, gluconic and formic acids (Fig. 3). However, no organic acids were detected in the leachate of CSM bioleaching flasks (Fig. 3). The total concentration of organic acids were higher in the presence of MWM (1.93 mM) compared to MWO (0.45 mM). The final concentration of REEs leached from the CSM, MWO, and MWM is shown in Fig. 3. The mixed culture of enrichment cultures

on MWM was the most efficient at leaching REEs with a total of 0.836 mg L⁻¹ REE (Ce, La, and Nd) by day 7. When MWO and CSM was provided as a phosphate source, bacterial species released lower levels of total REEs: MWO (0.351 mg L⁻¹) and CSM (< 0.1 mg L⁻¹) (Fig. 3).

Bioleaching of MWO and MWM with TCP enrichment cultures resulted in the lower release of REEs (up to 0.135 and 0.415 mg L⁻¹, respectively) by day 7 compared to the mixed culture (Fig. 3). Enhanced organic acid production and REEs release in this study was observed by the mixed culture (TCP:CaP). It has been also demonstrated that synergistic action between strains of a mixed cultures enhanced phosphate solubilisation and organic acid production than either less diverse microbial consortium or species alone (Braz & Nahas, 2012; Corbett et al., 2018).

Lower release of Ce, La, and Nd from CSM in this study may be explained with the 10:1 dilution with silica flour that was necessary to work within radiation safety levels or the heterogeneous particle size of CSM, as well as P availability for microorganisms (Corbett et al., 2017). However, the total concentration of contaminants such as Fe in the leachate of CSM was higher than REEs (up to a final total concentration of 1.78 and 1.45 mg L⁻¹ after inoculation with PSB enriched on TCP and mixed, respectively). The presence of accessible iron on the surface CSM has been suggested to govern higher Fe concentration (Corbett et al., 2017).

The competition between bacteria in the mixed cultures of native PSB may have affected the quantity and the type of secreted organic acids as well (Corbett et al., 2017). It has also been demonstrated that dicarboxylic (malic and oxalic) and tricarboxylic (citric) acids rather than monocarboxylic acids (acetic, formic, and gluconic) dissolve REEs more effectively due to having a high affinity and stability to trivalent metals such as REEs (Jones, 1998). No dicarboxylic or tricarboxylic acid were detected in this study, possibly resulting in a lower REEs release in this study compared to a previous research where demonstrated PSB were used in the bioleaching of REEs from the same matrix ore (Corbett et al., 2017). Shin et al. (Shin et al., 2015) investigated the bioleaching of REEs from monazite-bearing ore (with 3.5% Ce) by *Acetobacter aceti* and detected citric, malic, tartaric, and acetic acids and a total concentration of 5.8 mg L⁻¹ of Ce in the leachate on day 4. In this study the Ce concentration reached 0.47 mg L⁻¹ by day 7 with the mixed culture. Moreover, Corbett et al. (Corbett et al., 2017) reported that *Enterobacter aerogenes* produced gluconic, citric, formic, and malic acids with a total of 1 mg L⁻¹ Ce leached from MWM after 8 days.

Traditional REEs extraction methods allow high levels of REE recovery from various ore concentrates (>90%); however, greener strategies of REE extraction and recycling are in high demand with a focus on bioleaching and ion exchange (Corbett et al., 2017; Park et al., 2017; Rozelle et al., 2016). The application of phosphate solubilising microorganisms (PSMs) to the recovery of REEs is an emerging trend in biomining with a number of studies demonstrating varying leaching efficiencies between 0.1-25% (Brisson et al., 2016; Corbett et al., 2017; Corbett et al., 2018; Qu & Lian, 2013; Shin et al., 2015). This study successfully demonstrated

the release of REEs by enrichment cultures derived from MWO. Nevertheless, levels of REEs (Ce, La, and Nd) leached from the CSM, MWO, MWM in this study were not comparable to conventional methods as the maximum recovery from MWM by PSB in the mixed culture was less than 0.1%.

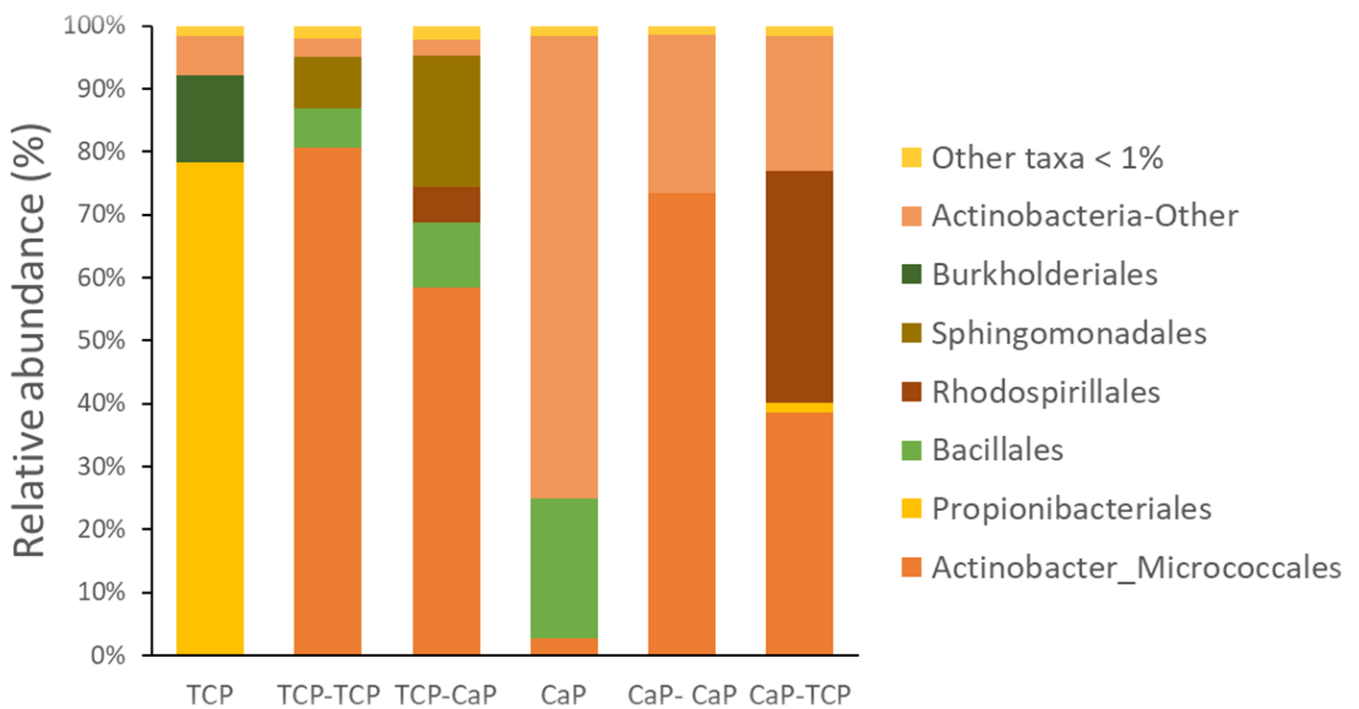
Considering the results presented in this study (Fig. 3), it is apparent the contribution of the enrichment cultures in the mobilisation of REEs were of minor importance. Therefore, inoculation with effective strains with a higher solubilisation potential is necessary. Previous research on the occurrence and diversity of PSB in soils indicated that although there were many PSB in the tested soils, only a few (5%) of the total isolates were effective in terms of phosphate solubilisation (Ndung'u-Magiroyi et al., 2012). The reason for this difference is unknown, but may be due to differences in the respective thermodynamic and chemical composition of the materials as the solubility of the trivalent REEs-phosphates is very low (10^{-13} M in pure water) compared to calcium phosphate minerals (Firsching & Brune, 1991).

Further investigation is also required to investigate the interactions between PSM and mineral phases where potentially toxic elements present in monazite such as Th and U may suppress microbial activity. Therefore, to determine the performance of the efficient strains identified from the ores, further tests and studies in terms of their direct contribution to P release and REEs are required.

3.3.2 Microbial community profile

In this study two approaches were used to isolate and characterise PSB from MWO: direct isolation using Ringer solution and the enrichment of bacteria with TCP or CaP as a phosphate source. No microbial growth were observed from the Ringer's solution. This is most likely due to the fact that the chemical composition of the solution did not provide appropriate growth condition for heterotrophic microorganisms.

Diversity profiling of the MWO enrichment cultures using the 16S rRNA genes showed that the microbial composition varied depending on the type of inorganic phosphate source (TCP and CaP) (Fig. 4). *Actinobacteria* was identified in all samples and *Micrococcales* was the most abundant orders in the subcultured enrichments (TCP-TCP, TCP-CaP, CaP-CaP, and CaP-TCP) (Fig. 4). *Propionibacteriales* was the most abundant order in the TCP enrichment and *Actinobacteria* the most abundant in the CaP enrichment. *Actinobacteria* are common rock-dwelling bacteria present in metal rich acidic ecosystems and have a range of mechanisms to counteract heavy metal toxicity (Baker & Banfield, 2003; Haferburg & Kothe, 2007). Other orders identified were *Bacillales*, *Rhodospirillales* and *Sphingomonadales* and *Burkholderiales* (Fig. 4). This is consistent with bacterial communities in mine tailings dump in arid Western Australia reported to be dominated by *Proteobacteria*, *Actinobacteria* and *Firmicutes* (Wakelin et al., 2012).



Shannon diversity index values

0.73 0.76 1.25 0.83 0.65 1.20

Fig.4 Relative abundance of bacterial orders detected in enrichment cultures from Mt. Weld ore when provided with $\text{Ca}_3(\text{PO}_4)_2$ (TCP) and $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ (CaP). (TCP: original culture enriched on TCP, TCP-TCP: original culture enriched on TCP and continued cultivation on TCP, TCP-CaP: original culture enriched on TCP and continued cultivation on CaP, CaP: original culture on CaP, CaP-CaP: original culture enriched on CaP and continued cultivation on CaP, CaP-TCP: original culture enriched on CaP and continued cultivation on TCP). Numeric values correspond to the Shannon diversity index.

The proportions of microbial order changed after they were subcultured either on the original (TCP or CaP) or alternative P source (Fig. 4). In the original cultures enriched either on TCP or CaP with further cultivation on the same P source, *Actinobacteria* were 83-84% and 75-98% of the total community, respectively. The proportion of *Actinobacteria* dropped to 60% when the source of P was changed. It can be concluded that the bacterial community structure was related to changes in the nature of available P. The Shannon diversity index ranged from 0.65 to 1.25, which implies the nature of inorganic phosphate source has affected the diversity profile of the PSB cultures enriched from MWO. The Shannon diversity index for cultures enriched on CaP (0.83) was higher than those enriched on TCP (0.73) which is consistent with higher solubility of CaP compared to TCP. A higher *H* where the source of P were changed (TCP-CaP or CaP-TCP) suggests that inclusion and substitution of inorganic phosphate sources with different solubility, increased the diversity of the potential PSB (Fig. 4), consequently REEs mobilisation in the mixed culture was enhanced (Fig. 3).

From the bioleaching perspective, there is little known about role of *Actinobacteria* in the bioleaching of REE minerals. In a recent study, *Actinobacteria* strains isolated from bastnasite (another primary REE mineral), released a total REEs concentration of 0.342 to 0.548 mg L⁻¹ from bastnasite (<75 µm, 0.5 % pulp density) in a nutrient-rich and oligotrophic medium, respectively (Zhang et al., 2018).

Species belonging to *Proteobacteria* are often shown to be dominant taxa in mine tailings and survive in oligotrophic environments (Wakelin et al., 2012; Yu-Qing et al., 2008). Moreover, another study of the diversity profile of MWM enrichment cultures showed that *Proteobacteria* (*Burkholderia* and *Sphingomonas*) contributed to monazite bioleaching (Corbett et al., 2018). The presence of heterotrophic, acid tolerant species, *Burkholderiaceae*, common in P limited environments, explains the production of organic acid which also produce extracellular acid phosphatases when they require P (Corbett et al., 2018). *Sphingomonas* species are also capable of chemotaxis towards inorganic phosphate sources hydrolysing organophosphate substrates (Dennis et al., 2013). The presence of the mentioned species within the enrichment cultures may explain the phosphate solubilisation capacity of the consortium. However, the microbial diversity of the cultures from the bioleaching experiments on the various ores was not determined, but it is reasonable to assume that to some extent the enhanced dissolution of REEs from MWO and MWM by enriched native cultures occurred as result of microbial action not limited to organic acids.

As previously described the presence of *Actinobacteria* in all samples were evident. The *Actinobacteria* are a phylum of Gram-positive bacteria. It has been also demonstrated that the presence of the carboxyl groups in the bacterial polysaccharide structure of Gram-positive bacteria (*Actinobacteria* and *Firmicutes*) induce significant REEs accumulation (Emmanuel et al., 2012; Emmanuel et al., 2011). This may explain the lowered concentration of REEs in the solution in the presence of native bacteria in this study compared to exogenous bacteria (Corbett et al., 2017). Examination of the microbial diversity of enrichment cultures established on MWM have also shown that *Firmicutes* and *Proteobacteria* were dominated phyla (Corbett et al., 2018) which is consistent with diversity profiling of MWO enrichment

cultures in this study. Although it is evident that native consortia are capable of catalysing REEs mobilisation, their activities and bioleaching performance appear to be varied by mineralogy of the ore and how they respond to environmental stress (i.e., phosphate-nutrient limited environment). For example, in the presence of CSM, organic acid production and REEs were negligible, whereas in the presence of MWO and MWM, the enrichment cultures increased the organic acid production and REEs dissolution. Further examination is required to identify and understand the effects of environmental stress on microbial produced organic acids, and release of P and REEs.

In order to confirm the significance of the metabolites produced by identified phylum in the dissolution of REEs from monazite, further tests are needed. It is in fact possible that what was observed in this study is not only due to organic acids generated by indigenous strains, but the combined result of unknown metabolites, synergistic interactions between indigenous strains, and the complexing effects of organic acids. Previous research have shown that the production of phosphatases have contributed to phosphate solubilisation and further leaching of the REE matrix (Corbett et al., 2018). The finding of this study suggested that the synergistic action of acid phosphatase activity and organic acids increased REEs solubilisation (Corbett et al., 2018).

There is a high degree of functional and genetic diversity among the PSB. Due to their innate potential of producing an array of metabolites such as organic acids, siderophores, and enzymes, studying molecular genetic of these phosphate solubilizing strains is considered to play a vital role in understanding the mobilisation mechanisms of phosphate, REEs and the enhancement of bioleaching efficiencies. Further examination is required to identify key genes for phosphate solubilisation and glucose metabolism to understand the effects of microbial metabolites on various REEs bearing ores and to optimize bioleaching parameters with the purpose of a higher REEs extraction yield. Nevertheless, further bioprospecting from extreme environment for the characterisation of novel indigenous microorganisms could provide tools for the isolation and selection of highly stress-resistant biomining microbes, which would ultimately increase bioleaching efficiency.

3.4 Conclusion

The present study investigated the application of native phosphate solubilising bacteria (PSB) present in the Mt. Weld deposit for bioleaching REEs. The data obtained from diversity profiling, organic acids, and leaching experiments suggested that bioleaching of REEs-containing phosphate minerals with *Actinobacteria*, *Proteobacteria*, and *Firmicutes* dominated cultures solubilized REEs (Ce, La, Nd) up to a final concentration of 0.836 mg L⁻¹. Further research is required to optimize the bioleaching to develop an economically viable alternative to conventional REEs extraction processes.

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Chapter 4: Microbial contact enhances bioleaching of rare earth elements

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Microbial contact enhances bioleaching of rare earth elements

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Abstract

The mobility of rare earth elements (REEs) in monazite depends on microbial activity, attachment of bacteria on the mineral surface, phase association of the REEs, and which physiochemical and biological processes these phases are subjected to. To better understand the role of the phosphate solubilizing bacterium, *Enterobacter aerogenes*, in REEs leaching, a series of monazite dissolution experiments was performed. The contact of bacteria with monazite was demonstrated to be advantageous for REEs bioleaching even though the same types of organic acids with similar concentrations were present during non-contact leaching. Monazite dissolution was observed to decrease in the following order: Biotic contact >> Biotic non-contact >> Spent media \approx Abiotic at 30 °C. The attachment of bacteria on monazite surface by a co-localised atomic force microscopy (AFM) and confocal Raman microscopy (CRM) indicated no preferential attachment of bacteria to specific site on the monazite surface.

Keywords: Monazite bioleaching; Rare earth elements; Phosphate solubilising bacteria; AFM

4.1 Introduction

In the last decade, rare earth elements (REEs), have been considered as “critical and strategic metals”, due to China’s monopoly position and increased global demand in green technologies. Although REEs are relatively abundant in the Earth’s crust, they are not evenly distributed around the world, and they are mainly produced and processed in China (Ganguli & Cook, 2018; Zepf, 2013). Consequently, the prediction of exhaustible resources such as REEs is of profound significance, in that it not only aids governments to establish long-term resource plans but also contribute to maintain sustainable social and economic development (Wang et al., 2015). Considering the constant development of REEs industries, the Generalized Weng model, a widely used quantitative model in exhaustible resource forecast has been adopted to predict the production of the three major REEs in China (i.e., mixed rare earth, bastnasite and ion-absorbed rare earth) (Wang et al., 2015). The results suggested that countries with REEs resources should commence or continue their production to gradually decline dependency on China’s supply (Wang et al., 2015).

Apart from the geopolitical challenges in REEs production, environmental issues can be a major concern where the extraction of REEs requires significant processing (Goodenough et al., 2018a). The current conventional REE production, relies on high temperatures and harsh chemical treatments has high energy consumption, and generates large volumes of toxic waste containing thorium, uranium, hydrogen fluoride, and acidic waste water (Hurst, 2010). Furthermore, as REEs-bearing ores may contain thorium and uranium up to 10% of the total ore matrix (Ragheb, 2011), emission of radioactive waste associated with REEs mining and extraction resulted to complicated disposal protocols or contamination of the final REEs concentrate (Ault et al., 2015). It has been reported that the environmental life cycle impacts of REEs production during chemical leaching are far greater than those for other metals (Vahidi & Zhao, 2016). Consequently, due to environmental restrictions, sustainable mining and production are now encouraged. The use of microorganisms to recover metals from ore matrix is called biohydrometallurgy. Biotechnological mineral processing approaches have been developed as a sustainable alternative to chemical leaching of ores and waste streams. Biohydrometallurgy utilises microorganisms to generate bio-lixiviants which accelerate the dissolution of elements from their ores or other materials (Watling, 2016). Bioleaching processes are generally operated at relatively low temperature and atmospheric pressure, which reduces energy cost and gas emissions, and without relying on expensive and aggressive reagents (Bryan et al., 2015).

Despite the great contribution of bioleaching to the extraction of base metals from sulfide minerals, very few studies have explored the application of microbes, in particular phosphate solubilizing microorganisms (PSMs), to monazite and other phosphate minerals hosting REEs. Brisson et al. (Brisson et al., 2016) demonstrated bioleaching of REEs (3-5% recovery) from monazite sand as the sole phosphate source by three phosphate solubilizing fungi. In another study, Shin et al. (Shin et al., 2015) examined the feasibility of using phosphate solubilizing bacteria (PSB) for the bioleaching of REEs from monazite-bearing ore with maximum leaching yield for cerium (up to 0.13%).

The previous studies of REE bioleaching have focused on efficiency (Brisson et al., 2016; Hassanien et al., 2013a; Shin et al., 2015) whereas very little is known of the mechanisms involved and benefits of REEs dissolution to the microbes. The novelty of this study is designing experiments to allow the bacteria to be either in contact or non-contact with the monazite surface. Adhesion and then colonization of the mineral surface are survival mechanism for bacteria and nutrients in aqueous environments are more accessible at surfaces (Busscher & van der Mei, 2012). Many studies on sulfide minerals demonstrate that microbial attachment and biofilm formation can stimulate pyrite bioleaching (Sand & Gehrke, 2006). Corbett et al. (Corbett et al., 2017) demonstrated that *Enterobacter aerogenes* leached 43% of the phosphate from tricalcium phosphate ($\text{Ca}_3(\text{PO}_4)_2$ or TCP) after 192 h, and of 12 known PSB released the greatest amount of REE from a monazite. Therefore, in this study, the mechanisms of bioleaching REE from monazite were systematically investigated with *E. aerogenes*. A series of monazite dissolution experiments were performed to i) obtain initial knowledge on microbial bio-mobilization mechanisms involved in REE dissolution in terms of the importance of microbial colonization on mineral surface, and ii) evaluate the change in organic acids profile during bioleaching. Experimental data from monazite dissolution was used to develop a conceptual model to integrate the main phenomena affecting REE leaching. The results from this study will facilitate the development of sustainable bio-mining approaches REE extraction.

4.2 Material and methods

4.2.1 Monazite ore

The high grade weathered yellowish monazite ore was collected from the Mount Weld Mine (Lynas Corporation), and is hereafter referred to as MWM (Mt. Weld Monazite). Sample preparation and composition analysis were described elsewhere (Corbett et al., 2017). The total surface area of the MWM was $24000 \text{ cm}^2 \text{ g}^{-1}$ as determined by Brunauer–Emmett–Teller (BET) analysis at CSIRO Minerals, Waterford, Western Australia. The BET surface area ($\text{cm}^2 \text{ g}^{-1}$) was analysed by the N_2 adsorption method at the temperature of liquid nitrogen ($-196 \text{ }^\circ\text{C}$) in a Micromeritics Gemini III 2375 (USA). Prior to the nitrogen adsorption measurements, each sample (approximately 0.6 g in weight) was degassed at $150 \text{ }^\circ\text{C}$ for 3 h in vacuum. The BET surface area was determined by using the N_2 adsorption data at 5 different standard pressures (0.05, 0.15, 0.2, 0.25 and 0.3) at $-196 \text{ }^\circ\text{C}$. Any results were rejected and the samples re-tested if the correlation coefficient of a plot of the 'BET Function' through the 5 points was lower than 0.9997. Before bioleaching experiments, the mineral samples were sterilized by gamma irradiation at 50kGy for 11 h (ChemCentre, Bentley, Western Australia).

4.2.2 Bioleaching experiment

Enterobacter aerogenes (ATCC® 13048™) was grown to exponential phase at $30 \text{ }^\circ\text{C}$ in National Botanical Research Institute Phosphate (NBRIP) medium (Nautiyal, 1999), with shaking at 140 rpm, and harvested by centrifugation (3600 g, 10 min). Cells were resuspended in sterile Tris-

HCl buffer (100 mM, pH 7.2), centrifuged (3600 g, 5 min) and washed twice more to remove any trace of phosphate. The ability of *E. aerogenes* to bioleach MWM as a phosphate source, was evaluated in 500 mL Erlenmeyer flasks. Bioleaching was carried out over 18 days at 30 °C in triplicate, at 120 rpm in an orbital shaking incubator (RATEK, Model No: OM11) in 200 mL of modified NBRIP media (3% w/v glucose and pH 7.00±0.25), with 0.5% v/v bacterial inoculum (initial density 1×10^7 cells mL⁻¹) and 1% pulp density of sterilized monazite. Cell-free abiotic controls were carried out under the same conditions. Bioleaching was monitored by measuring the soluble concentration of Ce, La, Pr, Nd, Fe, P, Th, U, and Y in the leachate.

Non-contact experiments were conducted in similar conditions to those described above. Snakeskin® dialysis tubing (10K MWCO, 35 mm, ThermoFisher SCIENTIFIC, catalogue number 88245) with Snakeskin™ Dialysis Clips (ThermoFisher SCIENTIFIC, catalogue number 68011) were used to study the possible mechanisms for leaching REE from monazite as follows:

1. For biotic contact leaching of monazite, dialysis bag, 200 mL media, and 1 mL of bacterial suspension were placed in 500 mL Erlenmeyer flasks. The monazite in this experiment was not sealed in the dialysis bag, so that bacteria were free to colonize monazite surfaces.
2. For abiotic contact leaching monazite and media were placed in 500 mL Erlenmeyer flasks.
3. For biotic non-contact leaching the monazite was sealed in the dialysis bag. This sealed dialysis bag, media, and 1 mL of bacterial culture were placed in Erlenmeyer flasks.
4. For abiotic non-contact leaching monazite was sealed in dialysis bag. This sealed dialysis bag and media were placed in Erlenmeyer flasks.

The pore size of the dialysis bag is sufficiently small to prevent bacterial migration through the bag, but large enough to allow the homogenous transfer of nutrients for bacterial growth.

Molar dissolution rates (r) per surface area of the ore and time ($\text{mol cm}^{-2} \text{s}^{-1}$) were calculated as using Equation 1:

$$r = \frac{r_{\text{volumetric}}}{c_{\text{solids}} \times M \times A} \quad (1)$$

where $r_{\text{volumetric}}$ refers to the volumetric leaching rate ($\text{g L}^{-1} \text{s}^{-1}$) obtained from the slope of the soluble element concentration versus time plot, c_{solids} represents the initial solid concentration in the flasks (10 g L^{-1}), M is molar mass of the element (140.1, 138.9, and 88.9 g mol^{-1} for Ce, La, and Y, respectively), and A is the total mineral surface area ($\text{cm}^2 \text{ g}^{-1}$) obtained with BET.

4.2.3 Leaching of MWM with spent media

Pregnant solutions were prepared as described in section 2.2. After 24 h incubation and pH decrease (pH = 3.4), the media was aseptically filtered (0.20 μm , Satorius). One gram of MWM was added to 50 mL of the filtered spent medium in 200 mL flask and incubated at 30 °C with shaking at 120 rpm for six days. Leaching was monitored by measuring the soluble concentration of Ce, La, Pr, Nd, Fe, P, Th, U, and Y in the leachate.

4.2.4 Analytical Methods

Samples were taken at 0, 2, 3, 6, 9, 12, 18 d and pH measured using a pH meter (Ionode II series pH probe). Thereafter, samples were filtered (0.20 µm, Satorius) and assayed for REEs, Y, Th and U concentrations by ICP-MS (Agilent Technologies 7700 series, Bureau Veritas Australia Pty Ltd, Canning Vale, Western Australia) and the average values were reported. Organic acids were identified by high performance liquid chromatography (HPLC) (Agilent 1200, Curtin Water Quality Research Centre, Bentley, Western Australia) coupled with a diode array detector (DAD, Agilent). Injection volume was set as 50 µL for the samples. Compound separation was achieved with a C18 reverse phase column (Agilent, 5 µm, 4.6 × 250 mm). The isocratic elution flow rate was 1.0 mL min⁻¹. The mobile phase consisted of 70% methanol and 30% phosphate buffer (pH=2.0). A detection wavelength of 220 nm was used. The identity and concentration of organic acid was determined by comparing the retention times and peak areas of chromatograms of the samples with standards. Organic acid identity was confirmed by liquid chromatography tandem-mass spectrometry (LC-MS/MS), and the experimental setup for LC-MS/MS including some exemplary mass chromatograms of organic acids were described elsewhere (Buseti et al., 2014). Organic acids standards included gluconic, malic, formic, butyric, citric, acetic, lactic, oxalic, and pyruvic acids. Microbial cells were counted using a Helber bacteria counting chamber (Thoma rule, Hawksley UK) at 400 X magnification.

Scanning electron microscopy (SEM) of the bioleaching residue was performed on a Zeiss Evo 40XVP SEM (John de Laeter Centre, Curtin University, Western Australia).

Co-localised atomic force microscopy (AFM) and confocal Raman microscopy (CRM) measurements were performed on a WITec Alpha 300SAR (WITec GmbH, Ulm, Germany). The samples were mounted on a purpose built re-location stage, allowing returning to the same sample area. AFM data were acquired in intermittent contact mode in air utilizing standard probes with a resonant frequency of 300 kHz and a spring constant of 40 N m⁻¹ (type NCH-VA, Bruker, Santa Barbara, USA).

For the confocal Raman measurements a frequency double NdYAG laser ($\lambda=532\text{nm}$) was used for excitation and the Raman spectra were collected through a 100x objective with a numerical aperture of 0.9 (Zeiss, Germany) and fed via a 50 µm optical fibre into the spectrometer. For AFM and CRM measurements, a pure monazite crystal (Lynas, Australia) was embedded in epoxy resin, cut and polished to obtain a suitable flat sample surface (with a thickness of 1 up to 2 mm and size of 1 cm³). MWM fine grains were also embedded in epoxy resin. Both samples were cleaned and sterilized in ethanol, nitrogen gas and an UV/ozone cleaner prior to exposure to *E. aerogenes*. The sample was exposed to 1% v/v bacterial inoculum (initial density 1 × 10⁷ cells mL⁻¹) and 18 mL of modified NBRIP with shaking at 120 rpm in 100 mL Erlenmeyer flask for 24 h at 30 °C.

4.3 Results and Discussion

4.3.1 Organic acid profile

Glucose was the carbon source available to *E. aerogenes* in both the contact and non-contact bioleaching experiments, where the bacteria produced malic, acetic and gluconic acid (Figure. 1). Corbet et al. (Corbett et al., 2017) have reported the release of citric and formic acids in addition to gluconic and malic acids, by *E. aerogenes*. It has been suggested that dicarboxylic (malic and oxalic) and tricarboxylic (citric) acids rather than monocarboxylic acids (acetic, formic, and gluconic) govern REE dissolution due to having a high affinity and stability to trivalent metals such as REEs (Jones, 1998). Previous studies with *E. aerogenes* and insoluble phosphate complexes have reported gluconic acid concentration up to 1 mM at day 4 (Stella & Halimi, 2015) which is in good agreement with this study. Johnston (Johnston, 1952) reported that the phosphate solubilisation potential of organic acids is related to the structural characteristics of the acid, thereby the concentration of organic acids as well as their structure and stability of ligands should be taken into account. Of those organic acids detected, only malic acid is dicarboxylic which make it a stronger acid ($pK_a = 3.40$) comparing to acetic acid ($pK_a = 4.75$), however, gluconic acid has a pK_a of approximately 3.60. Citric and formic acids were not detected in the present study, and hence may not be attributed to REE dissolution by *E. aerogenes* when grown on NBRIP. The short half-life of organic acids (e.g., citrate 2-6 h) (Van Hees et al., 2003), unidentified acids with no standards solution (Brisson, 2015), and the overlapping of the peaks of different acids in HPLC may have hindered the detection of some other organic acids in this study which may have been effective at solubilising REE from monazite.

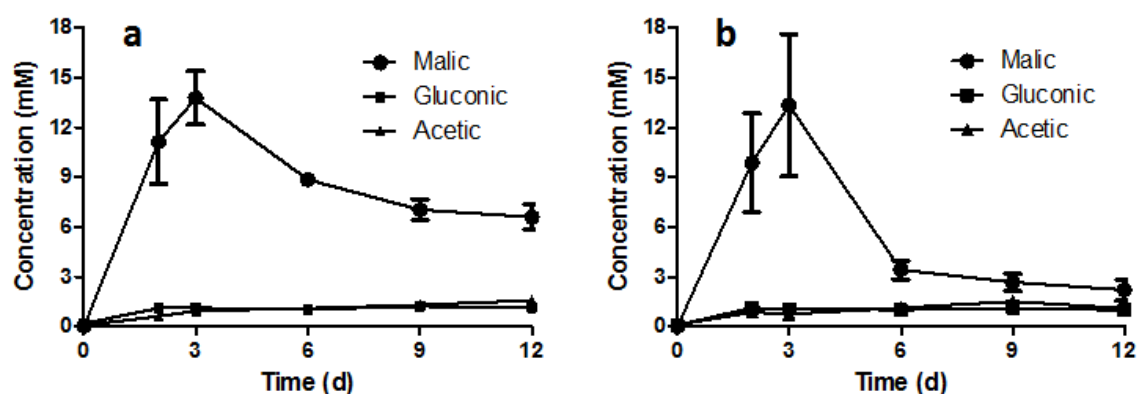


Fig.1 Organic acid concentration (mM) by *Enterobacter aerogenes* after 12 days of incubation in the presence of Mount Weld monazite (MWM) under (a) biotic contact and (b) biotic non-contact conditions. Error bars (SE) represent standard error between three replicate flasks. Error bars not visible are smaller than symbols.

Although, the organic acid profile of both contact and non-contact bioleaching were similar (Figure 1), contact bioleaching resulted in higher REE dissolution compared to non-contact leaching (Figure 2). Therefore, monazite dissolution may not be solely achieved by organic acids.

4.3.2 Monazite dissolution during contact, non-contact, and spent medium bioleaching

In order to assess the necessity of contact between microbial cells and mineral in the leaching of REE from monazite, a series of monazite dissolution experiments was conducted. During the contact bioleaching experiments with monazite the pH of the leachate decreased to 3.39 ± 0.08 by day 2, whereas with non-contact bioleaching it decreased to 3.47 ± 0.04 by day 2. The concentration of soluble Ce, La, and Nd in contact bioleaching (2.55, 0.57, and 0.36 mg L^{-1} on day 18, respectively) was higher than other elements as expected due to higher content in the ore. On the other hand, much lower soluble Ce, La, and Nd concentration were detected in non-contact leachate (0.66, 0.16, and 0.12 mg L^{-1} , respectively on day 18). After 48 h, soluble Ce concentrations were 2.61 times higher for contact bioleaching than for non-contact bioleaching and reached to 3.82 times higher concentration by day 18 (Figure 2). When exploring the possible leaching mechanisms in the present study, monazite dissolution was observed to decrease in the following order: Biotic contact \gg Biotic non-contact \gg Spent media \approx Abiotic. Exposure of MWM to spent media resulted in lower REEs leaching compared to biotic contact and non-contact (data not shown). On the one hand, as there is no phosphate consumption in spent media leaching precipitation and formation of secondary phosphate minerals increased, where overall REEs leaching decreased.

Furthermore, as metabolic activity was not occurring, continuous organic acid production was minimized in spent media as well which is consistent with increased pH up to 4.50 ± 0.01 by day 6. At higher pH the precipitates would remain in insoluble forms. The soluble concentrations of elements in the spent media and abiotic controls were near or below detection limits (Ce, La, Pr, Nd, Th, U, and Y $< 1 \mu\text{g L}^{-1}$; Fe $< 0.5 \text{ mg L}^{-1}$, and P $< 2 \text{ mg L}^{-1}$). As noted above, lower REEs leaching in spent media and abiotic suggesting that the presence and attachment of bacteria contributed directly to higher REEs leaching.

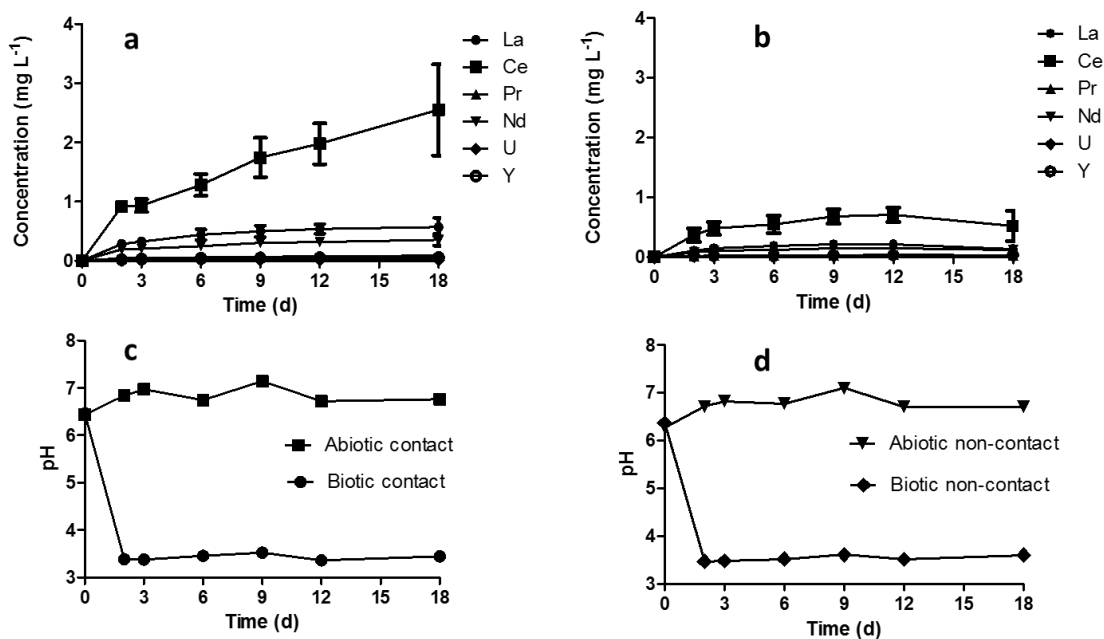
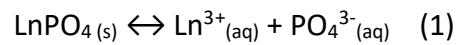


Fig.2 Leaching of MWM by *Enterobacter aerogenes*. Dissolved La, Ce, Pr, Nd, U, and Y under (a) biotic contact and (b) biotic non-contact, observed pH change under (c) contact and (d) non-contact conditions. Error bars represent standard error between three replicate flasks. Error bars not visible are smaller than symbols. The concentration of REEs and U in abiotic flasks remained below detection levels throughout the experiment (data not shown).

In comparison to conventional monazite processing, where most of Th is leached to solution (Peelman et al., 2014), in the present study no Th was observed in leachate of either the contact or non-contact bioleaching. On the other hand, soluble Y concentration reached an average of 0.0563 ± 0.010 and 0.0262 ± 0.005 mg L^{-1} , during contact and non-contact bioleaching, respectively, suggesting preferential release of Y over actinides considering the similar contents of Th and U in the MWM.

Non-steady Ce, La, and Y dissolution rates during contact bioleaching of MWM with *E. aerogenes* at 30 °C on day 3 were 1.14×10^{-17} , 3.93×10^{-18} , and 5.7×10^{-19} $\text{mol cm}^2 \text{ s}^{-1}$, respectively. By day 18 the rates slowed down to 4.37×10^{-18} , 9.30×10^{-19} , and 1.52×10^{-19} $\text{mol cm}^2 \text{ s}^{-1}$, respectively. On the other hand, non-steady Ce, La, and Y dissolution rates during non-contact bioleaching from MWM at 30 °C on day 3 were 5.60×10^{-18} , 1.73×10^{-18} , and $2 \times 60 \times 10^{-19}$ $\text{mol cm}^2 \text{ s}^{-1}$, respectively, and by day 18 the rates slowed down to 1.07×10^{-18} , 2.64×10^{-19} , and 8.14×10^{-20} $\text{mol cm}^2 \text{ s}^{-1}$, respectively. This confirmed higher REE dissolution (2 to 4 times) attributed to bacterial attachment. Moreover, when considering the equilibrium between monazite, the dissolved ions (Ln^{3+} represents REEs) and very low solubility of monazite (10^{-13} M) (Firsching & Brune, 1991), pico-molar concentrations of REEs and

phosphate can produce saturation and supersaturation, according to reaction (1). Thus, phosphate abundance through REE precipitation and co-precipitation may influence REE distribution in solution, exhibiting distinctive REE dissolution rate (Goyne et al., 2010).



The SEM photomicrographs (Figure 3) also demonstrated breakdown of the monazite surface (due to biofilm formation) with contact leaching while the mineral surface remained intact after non-contact leaching. Thus, the data suggested a microbially mediated REE dissolution on mineral surfaces through contact mechanism.

The atomic force microscopy scans of the surface of the monazite crystal and MWM after 24 h of exposure to *E. aerogenes* clearly show the attachment of bacteria to the crystal and MWM surface (Figure 4). The images show a range of clusters as well as solitary bacteria on the monazite surface. The confocal Raman microscopy image indicates that the surface chemistry of the investigated sample is composed of REEs (i.e., Ce^{3+}) and PO_4^{3-} and no other major heterogeneous mineral phases were observed. Changes in the intensity in the presented Raman map are due to variations in the sample topography. Comparing the Raman image with the distribution and arrangement of bacteria clusters seen in the AFM images for both monazite crystal and MWM, it appears that there is no preferential attachment to specific area relevant to chemical composition (either REE^{3+} or PO_4^{3-}).

4.3.3 A conceptual modelling for microbial-mineral interactions during monazite bioleaching

Monazite particles are composed of inorganic P that can be used by PSB for metabolic purposes. Thus, mineral composition is a key factor influencing bacterial communities and their activities, especially in bioleaching. Bacteria can either exist in bulk solution (suspended), or attached to surfaces or within EPS (Donlan, 2002). Cell counting in the present study showed that the number of *E. aerogenes* cells in the leachate dropped by 48 h from 10^7 to 10^3 cells mL^{-1} most likely as a result of attachment to monazite (data not shown). However, it has been unclear whether microbial attachment and biofilm formation is a prerequisite for monazite dissolution.

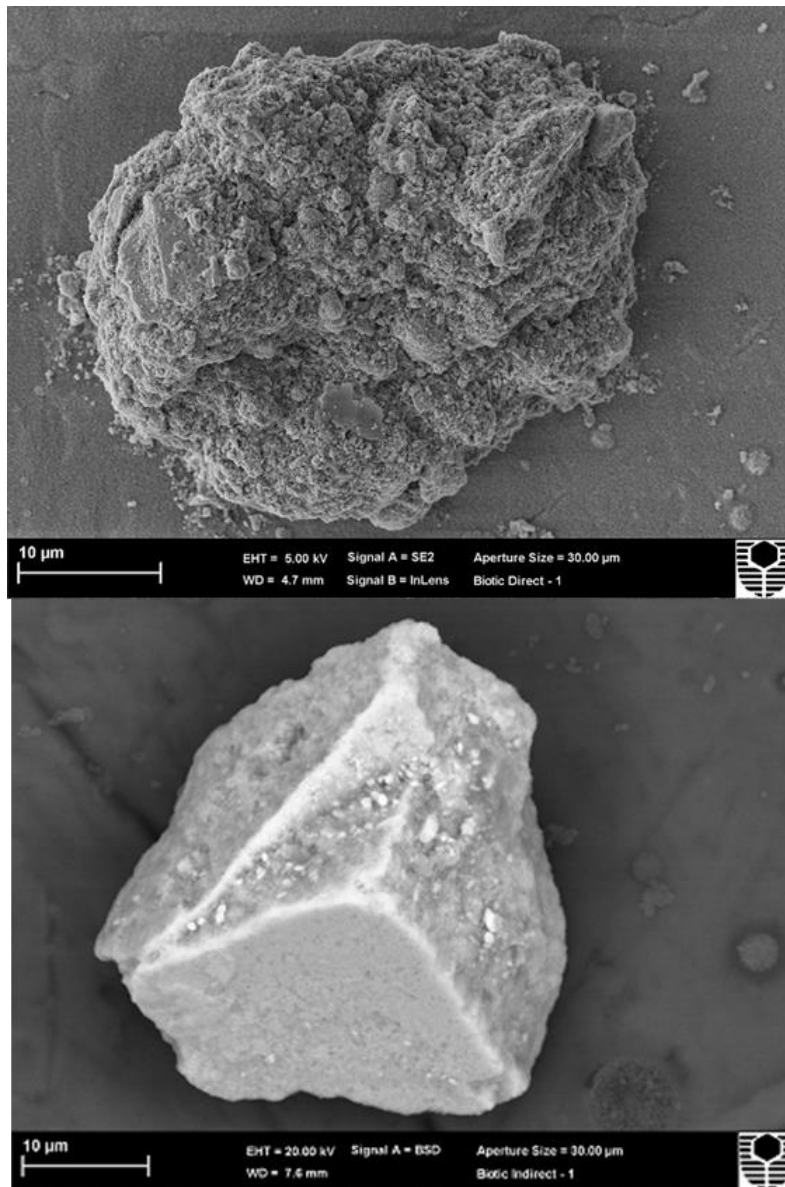


Fig.3 Scanning electron microscopy images of the MWM after 18 days of contact bioleaching (top) and non-contact bioleaching (bottom) with *Enterobacter aerogenes*.

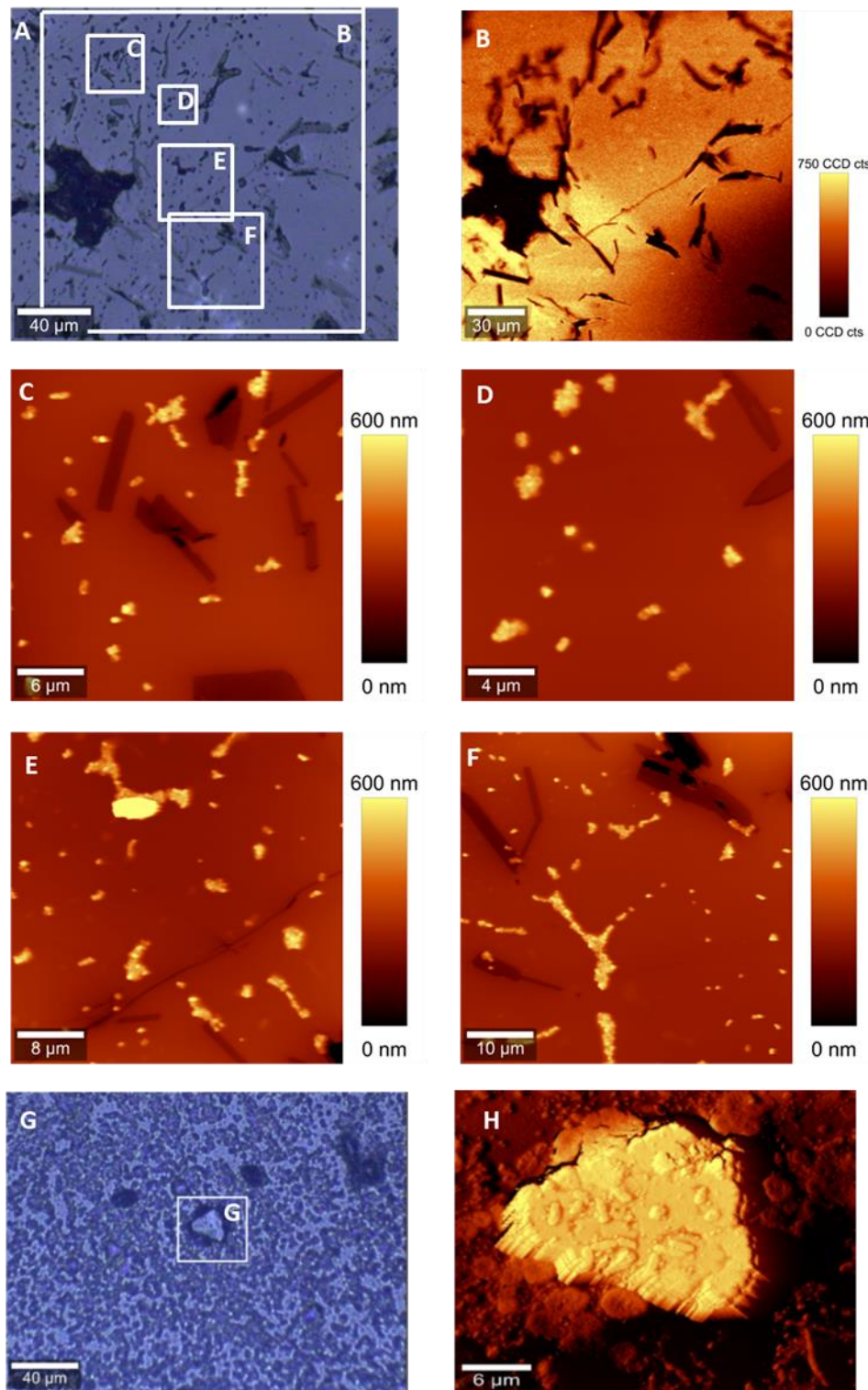


Fig.4 (A) Optical microscopy image of monazite crystal and (G) grain after 24 h exposure to *Enterobacter aerogenes* with squares indicating where Confocal Raman microscopy (CRM) (B) and atomic force microscopy (AFM) measurements (C-F, H) were recorded. Confocal Raman microscopy image generated using a sum filter over the peak at 970 rel. 1/cm. (C-F, H) atomic force microscopy topography images showing clusters of bacteria attached to the monazite surfaces.

As previously mentioned, within bacteria-mineral interfaces, the rate of REE release during monazite bioleaching depends on i) the concentration of organic acid ligand in solution, ii) nature of the mineral surface (distribution of labile and non-labile REE), and iii) concentration of phosphate in solution which is governed by mineral composition, phosphate uptake rate, and growth rate of the biomass. The rate of biomass growth is controlled by nutrient availability including phosphate as well as environmental stressors such as low pH and REE toxicity which may inhibit REE bio-mobilization. However, the extent to which measured REE concentration during bio-mobilization and bio-mineralization relate to the bacterial attachment on monazite has not been previously studied.

It has been suggested by Rawlings et al. (Rawlings et al., 1999) that the ability of microorganisms to oxidize sulfide minerals is possibly due to contact and non-contact mechanisms, or a combination of both (cooperative mechanism). However, bioleaching pathways for phosphate minerals (i.e., monazite) are very different than for sulfide minerals, where ferric iron lixiviant is regenerated by iron oxidizing microorganisms either in the bulk solution or in EPS (Crundwell, 2003). Here, we propose a new conceptual model of the possible mechanisms of monazite bioleaching including contact (Figure 5.a), non-contact (Figure 5.b), and cooperative (Figure 5.c) leaching as shown in Figure 5. In contact leaching (Figure 5.a) attached microbial cells mobilize phosphate (PO_4^{3-}) within a matrix of EPS and release REE cations (REE^{3+}) into solution. Organic acids (OA) generated by the cells from organic substrates complex REE^{3+} . Protons released from organic acids attack the ore resulting in further PO_4^{3-} dissolution. Incorporation of PO_4^{3-} into the cells increases REE^{3+} solubility, according to reaction (1). In the non-contact mechanism (Figure 5.b) suspended cells generate REE^{3+} complexing organic acids and incorporate PO_4^{3-} into cells increasing REE^{3+} solubility. The protons released from organic acids attack the ore resulting in further REE^{3+} and PO_4^{3-} dissolution. In cooperative mechanism (Figure 5.c) attached cells mobilise PO_4^{3-} from monazite and incorporate it into cells releasing REE^{3+} while suspended cells generate organic acids for REE^{3+} complexation and protons released from organic acids attack the ore. Alternatively, attached cells may play a role in organic acid generation while suspended cells take up PO_4^{3-} from solution increasing REE^{3+} solubility.

Previous studies (Brisson et al., 2016; Corbett et al., 2017) demonstrated that microbial solubilisation of monazite is promising, however to be competitive with conventional processes, the recovery rates via bioleaching need to be increased. To enable scale up of the approach, a solid understanding of which factors are most important for controlling REEs mobilisation is required. This study provided preliminary data on significance of microbial colonisation for nutrient acquisition by PSM, particularly phosphate via monazite dissolution. Overall our findings suggest that attachment of bacteria on mineral surface enhance REEs bioleaching. Further evaluation of potential PSMs for bioleaching REEs from monazite in large scale experiments will be considered.

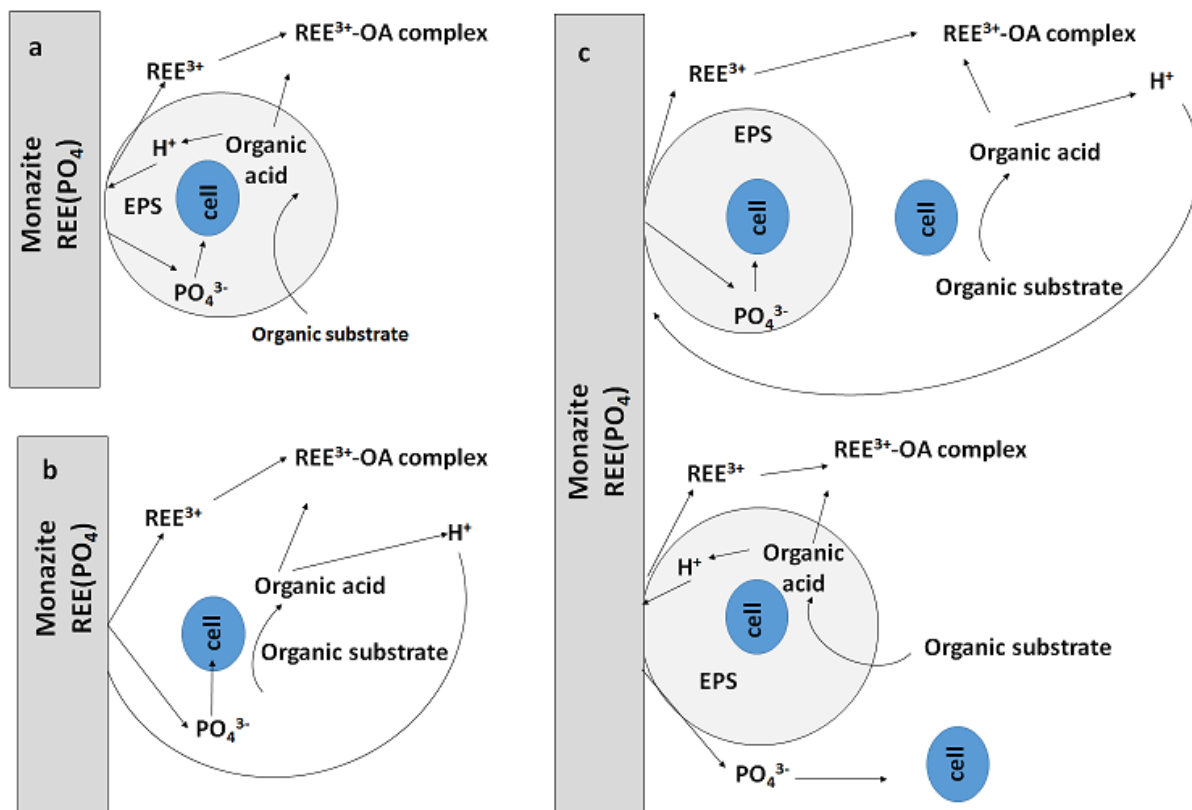


Fig.5 A conceptual model showing the proposed mechanisms of monazite bioleaching.

4.4 Conclusion

The present study explored the possible mechanisms for bioleaching REEs from monazite. While a similar range and concentration of organic acids were secreted regardless of the ability of the bacteria to have contact with the mineral surface it was demonstrated that monazite dissolution was enhanced with bacterial contact by *E. aerogenes* with the monazite surface. No preferential attachment of bacteria to the monazite surface was observed by a co-localised AFM and CRM observed for either crystal monazite of the Mt. Weld Monazite. The data obtained from the organic acids profile and the contact and non-contact leaching experiments show promising scope for further research in the bioleaching of REEs-containing phosphate minerals.

4.5 Acknowledgments

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Chapter 5: Better together: Potential of co-culture microorganisms to enhance bioleaching of rare earth elements from monazite.

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Better together: Potential of co-culture microorganisms to enhance bioleaching of rare earth elements from monazite

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Abstract

The aim of this study was to develop continuous bioleaching of monazite by combining heterotrophic and autotrophic acidophilic microorganisms. The results showed that a co-culture of autotrophic, acidophilic *Acidithiobacillus ferrooxidans* and heterotrophic *Enterobacter aerogenes* was more effective in bioleaching rare earth elements (REEs) from monazite than either species alone. This was likely due to a synergic interaction through the biogenic generation of both organic acids and sulfuric acid. In conclusion, the consortium of *E. aerogenes* and *A. ferrooxidans* solubilized REEs (Ce, La, Nd, Pr, and Y) up to a final concentration of 40 mg L⁻¹.

Keywords: bioleaching; monazite; *Acidithiobacillus ferrooxidans*; *Enterobacter aerogenes*; rare earth elements; co-culture

5.1 Introduction

The shift to a low carbon future is expected to accelerate the deployment of rare earth elements (REEs) in the wind and solar energy sectors. Therefore, countries rich in REEs resources (i.e., Australia) can establish long-term benefits through sustainable REE mining. Besides the primary REEs bearing minerals (i.e., monazite), large rare-earth bearing ores hosting iron-rich minerals (Fe-oxide phosphate) including goethite and hematite (Hoatson et al., 2011), may contribute to global REEs supply (Faris et al., 2017). Currently, industrial extraction of REEs from monazite involves either a basic process that uses concentrated sodium hydroxide or an acidic process that uses concentrated sulfuric acid. These generate large amounts of hazardous waste containing thorium and uranium (Abreu & Morais, 2010). Biohydrometallurgy has been studied as a more environmentally sustainable alternative to extract REEs from phosphate minerals including monazite (Keekan et al., 2017). Previously reported REEs bioleaching efficiencies from monazite with both bacteria and fungi have been very low compared to chemical leaching (Brisson et al., 2016; Shin et al., 2015). Recently, bioleaching of REEs from bastnasite-bearing rock by Gram-positive bacteria, Actinobacteria has been investigated (Zhang et al., 2018). These authors have reported that in a nutrient-rich growth medium, the total concentration of bioleached REEs ranged from 56 to 342 $\mu\text{g L}^{-1}$, whereas in an oligotrophic medium, only one strain (*Streptomyces sp.*) grew in the presence of the bastnasite (0.5% w/v), and leached up to 548 $\mu\text{g L}^{-1}$ of total REEs (Zhang et al., 2018). Coincidentally, a combination of low solubility of bastnasite, a lack of nutrients from the mineral, the precipitation of REEs minerals, and re-sorption of leached REEs to cell and residual mineral surfaces may have contributed to the observed low leaching efficiency (0.008–0.08%) (Zhang et al., 2018). However, comparing to the conventional extraction of REEs, bioleaching can be considered as an “eco-friendly technology” to minimize the high cost and negative environmental impact.

In phosphate-based environments “phosphate solubilizing microorganisms” (PSMs) can be introduced to enhance the solubilisation of insoluble inorganic phosphate via acidification, chelation, and exchange reactions (Son et al., 2006). As a consequence, heterotrophic PSMs such as *Enterobacter aerogenes* can be used for the solubilisation of REEs from a phosphate mineral such as monazite via secretion of organic acids (Corbett et al., 2017). Earlier studies demonstrated that the recovery of REEs using heterotrophic microorganisms is possible, although, the bioleaching mechanisms are not yet clearly and explicitly understood (Brisson et al., 2016).

It has been demonstrated that optimizing microbial community structure in co-culture systems are an effective way of improving microbial community function (Ma et al., 2017). The continuous requirement of heterotrophic bioleaching microorganisms for a carbon and energy source to maintain microbial activity is problematic at industrial level, however the addition of acidophilic autotrophic bioleaching microorganisms (e.g., *Acidithiobacillus ferrooxidans*) to these systems can potentially improve their performance. Autotrophic acidophiles requires small amounts of inorganic nutrients, such as ferrous iron and reduced sulfur compounds for bio-oxidation (Zhuang et al., 2015). In addition, the ability of acidophilic

bacteria to tolerate toxic heavy metal ions, enhances their capacity for the bioleaching of metals. *A. ferrooxidans* is the most studied obligate chemolithoautotrophic bioleaching bacterium. It gains energy from the aerobic oxidation of ferrous iron and/or reduced sulfur compounds to ferric iron and sulfuric acid, respectively (Watling, 2016). Although *A. ferrooxidans* has been used to leach phosphorous from different types of rock phosphates (Bhatti & Yawar, 2010), to the best of our knowledge, despite the commercial application of acidophilic bioleaching for a diverse range of elements from sulfide minerals, the acidophilic bioleaching of REEs-bearing minerals has not been previously studied. It has been demonstrated that the microbial consortia have greater bioleaching rates than pure cultures (Johnson, 2001), we therefore propose a two-step bioleaching system where the metabolites generated by *E. aerogenes* result in pH reduction negating the need for manual pH adjustment required for *A. ferrooxidans*.

In this context, the aim of this work was to investigate the bioleaching of REEs from monazite by a co-culture of autotrophic, acidophilic *A. ferrooxidans* and heterotrophic *E. aerogenes* and compare the efficiency to those of individual pure cultures.

5.2. Material and methods

5.2.1 Phosphate and sulfide minerals

The high grade weathered yellowish monazite ore was collected from the Mount Weld Mine (Lynas Corporation), and is hereafter referred to as MWM (Mt. Weld Monazite). The ore was ground by a rod mill, pulverized in a ring mill and finally sieved to <38 µm in particle size. The elemental composition of the MWM was analysed by inductively coupled plasma optical emission spectrometry (ICP-OES, CSIRO Minerals, Waterford, Western Australia). The ore contained (%): 10.1 La, 12.6 Ce, 2.10 Pr, 6.25 Nd, 0.165 Y, 0.162 Th, 1.23 Fe, 9.93 P, 1.75 Ca, 0.199 Mg, 1.96 Si, 0.554 Ti, 0.031 Zr, and <0.003 U. Pyrite concentrate (p80 passing 120 µm) used as a source of Fe and S was obtained from Kalgoorlie Consolidated Gold Mines Pty Ltd. (KCGM), Australia (Bryan et al., 2015). The mineralogical composition of MWM was determined by X ray diffraction (XRD) at CSIRO Minerals, Waterford, Western Australia. The XRD Analysis of MWM revealed that samples were mainly constituted by 51 % monazite, 41 % florencite, and 8 % nontronite. Pyrite concentrate contained 60 % pyrite, 12.5 % quartz, 9 % albite and 7.5 % dolomite. Before bioleaching experiments, the mineral samples were sterilized by gamma irradiation at 50 kGy for 11 h (ChemCentre, Bentley, Western Australia).

5.2.2 Bioleaching experiment

Entrobacter aerogenes (ATCC 13048, obtained from ATCC) was grown to exponential phase at 30 °C in National Botanical Research Institute Phosphate (NBRIP) medium (Nautiyal, 1999), with shaking at 120 rpm, and harvested by centrifugation (3,600 × g, 10 min). Cells were resuspended in sterile Tris-HCl buffer (100 mM, pH 7.2), centrifuged (3,600 × g, 5 min) and

washed twice more to remove any trace of phosphate. *Acidithiobacillus ferrooxidans* (ATCC 23270, obtained from DSMZ) was grown to exponential phase at 30 °C, with shaking at 120 rpm, in the basal salt media (BSM) at pH 2.0 which is described elsewhere (Zammit et al., 2011). Cells were resuspended in sterile Tris buffer (20 mM, pH 2.0), centrifuged (3,600 × g, 5 min) and washed twice more to remove any trace of phosphate.

All bioleaching experiments were conducted in 250 mL Erlenmeyer flasks containing 100 mL of the relevant media, in triplicate at 30 °C with shaking at 120 rpm in an orbital shaking incubator (RATEK, Model No: OM11) over 12 days.

The ability of *A. ferrooxidans* to bioleach MWM as a phosphate source, was evaluated in BSM (pH 2.50±0.15), supplied with either FeSO₄ (13.9 g L⁻¹) and K₂S₄O₆ (1.51 g L⁻¹) (filter sterilized 0.20 µm, Sartorius) or sterilized pyrite (1% pulp density), with 1% v/v bacterial inoculum (initial density 1 × 10⁶ cells mL⁻¹) and 1% pulp density of sterilized monazite.

In the co-culture experiment, *E. aerogenes* was first cultivated in modified NBRIP media (3% w/v glucose and pH 7.00±0.25) with 1% v/v bacterial inoculum (initial density 1 × 10⁷ cells mL⁻¹) and 1% pulp density of sterilized monazite. Three days later, when the pH dropped to < 3.5, a 10 mL aliquot of *A. ferrooxidans* (initial density 1 × 10⁶ cells mL⁻¹ before inoculation) in BSM was added to the leachate, and the combined culture supplied with FeSO₄ (13.9 g L⁻¹) and K₂S₄O₆ (1.51 g L⁻¹) (filter sterilized 0.20 µm, Sartorius).

Cell-free abiotic controls were carried out under the same conditions. Samples were taken at 0, 2, 3, 6, 9, 12 d and pH measured using a pH meter (Ionode IJ series pH probe). Samples were then filtered with disposable syringe filters (0.20 µm, Sartorius) and assayed for REEs, Y, Th, U, Fe, and P concentrations by inductively coupled plasma-mass spectrometry (ICP-MS) Agilent Technologies 7700 series, Bureau Veritas Australia Pty Ltd, Canning Vale, Western Australia) and the average values were reported.

5.2.3 Comparison of the phosphate and iron regulation of *E. aerogenes* and *A. ferrooxidans*

In order to investigate the potential metabolic pathways involved in inorganic phosphate solubilisation by both strains in the co-culture system, a genome-based comparison of phosphate pathways was carried out.

The genomes of *E. aerogenes* (ATCC 13048 – KCTC 2190) and *A. ferrooxidans* (ATCC 23270) were downloaded from the NCBI ftp site (<ftp://ftp.ncbi.nlm.nih.gov/>). For the purpose of this comparison, the genomes were annotated using the Rapid Annotation using Subsystem Technology (RAST) server (<http://rast.nmpdr.org/>) using the ClassicRAST annotation scheme (Overbeek et al., 2013). Comparisons were performed using the SEED and RAST servers and Geneious v.10.2.3 bioinformatic software (Kearse et al., 2012).

5.2.4 Synchrotron analysis

Synchrotron radiation is a powerful technique that can be used to determine elemental oxidation state of REEs for a wide range of environmental samples.

5.2.4.1 Sample Preparation

Ce L_{III}-Edge X-ray absorption spectroscopy (XAS) data were collected on two solutions of monazite leachate from co-culture with ferrous sulfate and potassium tetrathionate at day 3 and 6 after *A. ferrooxidans* addition, as well as the MWM residue at end of bioleaching experiment. The leachates were prepared with 30% glycerol, and flash frozen with liquid nitrogen cooled iso-pentane, into 1 mm × 3 mm × 23 mm acrylic sample cuvettes. The cuvettes were covered and with closed with metal free kapton adhesive tape, which served as an X-ray transparent window. The powder sample was ground with mortar and pestle to a fine, homogenous powder, and then adhered as a thin film to metal free Kapton adhesive tape.

5.2.4.2 XAS Data Collection

Ce L_{III}-Edge XAS data were collected at beamline 7-3, at the Stanford Synchrotron Radiation Lightsource (SSRL). The beamline utilised a Si (220) double-crystal monochromator with harmonic rejection obtained by setting the 7 collimating mirror cut-off to 9 keV. The incident and transmitted X-ray intensities were recorded using N₂-filled gas ionization chambers (sweeping voltage of 1.8 kV). The X-ray absorption near edge spectrum (XANES) was measured as the Ce L_{III}-Edge fluorescence excitation spectrum, with X-ray fluorescence collected with an array of 30 germanium detectors (Canberra) equipped with a vanadium filter and a Soller slit assembly. Spectra were collected with the sample temperature maintained at approximately 10 K, using an Oxford instruments liquid helium flow cryostat. Each Ce L_{III}-Edge was obtained from the co-addition of 10 replicate spectra. X-ray energy was calibrated by reference to the K_α-edge absorption of a metallic Cr foil (first inflection point calibrated to 5989 eV).

5.2.4.3 Data Processing

Ce L_{III}-Edge XAS spectra were processed using the EXAFSPAK suite of programs (George). Individual spectra were combined, a linear background subtracted, and the edge jump normalized to a value of 1 absorbance unit. Spectral comparison was performed on data without any smoothing filters applied, as well as on data with a 0.5 gaussian smoothing function applied, to reduce noise levels, which were the result of low Ce concentration present in the leachates. Due to the low concentration of Ce in the samples, and relatively larger noise levels in the raw data, data analysis was limited to visual inspection of the edge position and shape, and fitting of the spectra to model Ce³⁺ and Ce⁴⁺ compounds was not performed.

5.2.5. Sequential extraction procedure (SEP)

In order to evaluate the mobilization behaviour of REEs, sequential extraction of elements from the feed ore and bioleaching residue of pure cultures was carried out according to the modified the Community Bureau of Reference (CBR) five-step procedure (Mittermüller et al., 2016) with an additional determination of the residual fraction using sodium peroxide fusion

(Fig. 1). The method determines five well defined fractions (speciation) in samples: easily soluble and ion-exchangeable fraction (F1), carbonate bound and mobilized by complexation fraction (F2), reducible fraction (F3), acid soluble fraction (F4), and residual fraction (R). The total content of the elements of interest in the mineral was determined by conducting a peroxide fusion analysis for the original minerals (same as the residue of modified SEP). All reagents used to perform the extraction were of analytical grade. Prior to the extraction, all tubes and glassware were soaked in diluted nitric acid (10 %v/v) for 8 h and rinsed with ultra-pure Milli-Q™ water (Millipore, 18 MΩ/cm resistivity). All extractions were carried out in triplicate.

The recovery percentage of the stepwise extraction was determined by comparing the sum of the five individual fractions (F1, F2, F3, F4 and R) to the total content determined by peroxide fusion of the original ore, according to following equation (Equation 1):

$$Recovery (\%) = \left[\frac{F1+F2+F3+F4+R}{Total\ content} \right] \times 100 \quad (1)$$

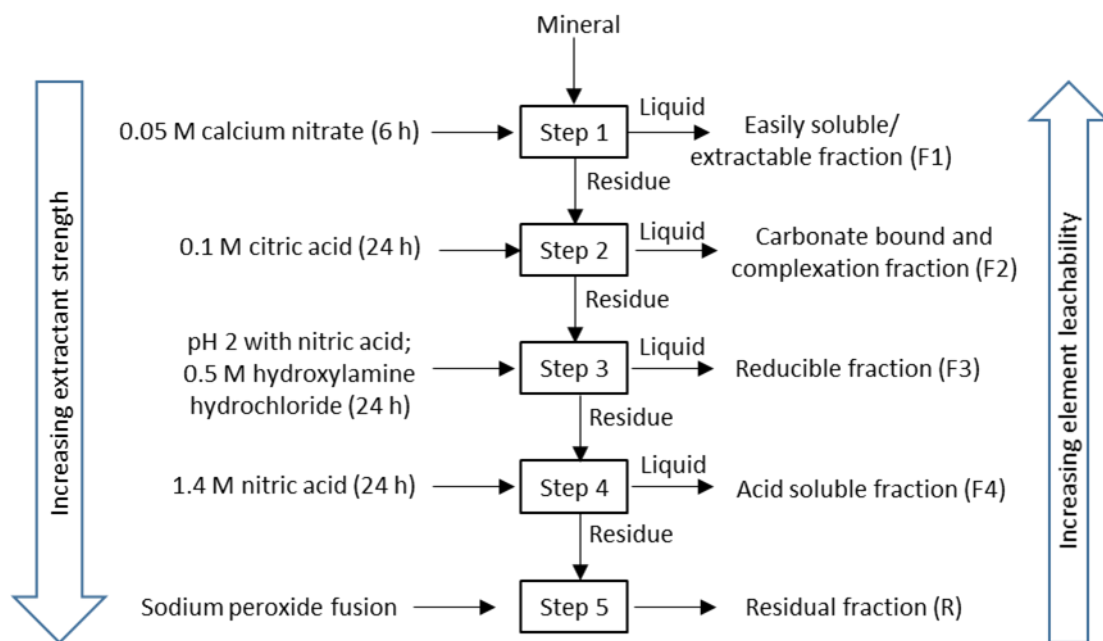


Fig. 1 Modified sequential extraction procedure (SEP) for REEs partitioning in Mt. Weld Monazite.

5.3 Results and Discussion

5.3.1 Mineral characterisation

The total concentration of REEs (mg kg⁻¹ dry material) for MWM (through the peroxide fusion) and partitioning of REEs (with modified SEP) are presented in Table 1. The geochemical fraction in which REEs occur is critical for understanding their mobility, therefore within SEPs, the sample is progressively dissolved in extraction solution of increasing strength.

Table 1 – Total content and fractionation of elements in feed Mt. Weld Monazite ore as determined by sequential extraction procedure. Values <0.0001% are not shown. Data are averages of triplicate biological replicates.

Element	Total content (mg.kg ⁻¹)	Easily soluble F1 (%)	Carbonate F2 (%)	Reducible F3 (%)	Acid soluble F4 (%)	Residual R (%)
La	99075		0.37	0.69	12.0	86.9
Ce	182250	0.00067	0.35	1.88	12.5	85.2
Pr	20675		0.58	0.64	13.7	85.0
Nd	73000	0.00048	0.65	0.64	14.4	84.3
Th	1838		0.10	0.0	6.2	93.7
Y	2220		0.98	0.87	12.7	85.4

Ce had the highest content of all REEs in MWM followed by La and Nd. Based on the comparison of the total content and the sum of all fractions (F1+F2+F3+F4+R), satisfactory recovery was achieved for the most of REEs in MWM feed ore, ranging between 105% and 126%, suggesting the method to be consistent and reproducible. Recoveries greater than 100% indicated that the stepwise procedure was more efficient in extracting REEs than peroxide fusion. Comparison of the SEP results with previous studies was not possible as to best of our knowledge, no reports of fractionation of monazite by SEP are available.

The sequential extraction procedure revealed that Nd was the most mobile REE, with 15.7% of the total content in the non-residual (labile) fractions, and the residual fraction accounted for 84.3%. The residue contained 85.0-86.9% of other REEs and 93.7% of Th. The distribution behaviour of La, Ce, Pr, Nd, and Y in each labile fraction were in a similar range where the dominant scavenging phase in the acid soluble fraction was most likely represented by phosphate groups naturally present in monazite and florencite. Therefore, the SEP results confirm that amongst labile fractions, REEs associated with phosphate fractions can be released through biochemical pathways in which the cleavage of REEs-phosphate either directly or indirectly may be influenced by PSMs (*Azospirillum brasilense*, *Bacillus megaterium*, *Burkholderia glathea*, *Pseudomonas aeruginosa*, *Pseudomonas putida*,

Aspergillus niger, *Aspergillus tubigensis*, and *Penicillium sp*) and/or other microbial species (*Firmicutes*) (Corbett et al., 2017; Corbett et al., 2018).

5.3.2 Bio-solubilisation of REEs from monazite

Bio-solubilisation of REEs from the monazite was explored with individual cultures of *E. aerogenes* and *A. ferrooxidans* as well as a combination of the two species.

5.3.2.1 Phosphate solubilizing bacteria

Following the inoculation of *E. aerogenes* into sterile media plus MWM, the total concentration of REEs in the leachate increased from 2.90 at day 2 to 5.84 mg L⁻¹ at day 12 (Fig. 2). No solubilisation of REEs, Fe or P occurred in abiotic flasks with the soluble concentration of all elements being below detection limits (Ce, La, Pr, Nd, Th, U, and Y < 1 µg L⁻¹; Fe < 0.5 mg L⁻¹, and P < 2 mg L⁻¹), indicating that metabolites secreted by microbial cells contributed to REEs mobilization. With microbial growth, the pH of the media decreased from 6.50±0.02 to 3.38±0.05 (Fig. 2). This decrease in the pH can be attributed to the production of organic acids resulting from glucose oxidation, bacterial respiration and NH₄ assimilation (Corbett et al., 2017). *E. aerogenes* was reported to be efficient in solubilizing tricalcium phosphate [Ca₃PO₄(TCP)] (Corbett et al., 2017; Prasanna et al., 2011). However, only a few studies have reported microbial solubilisation of natural monazite by PSMs (Corbett et al., 2017; Shin et al., 2015). Shin et al. (Shin et al., 2015) examined bioleaching of REEs from monazite-bearing ore and reported a total concentration of Ce in the leachate of 5.8 mg L⁻¹ on day 4 (0.13% of leaching efficiency) with *Acetobacter aceti*. For comparison in this study, the Ce concentration was 2.6 mg L⁻¹ at day 3 (0.20% of leaching efficiency) for monazite bioleaching with *E. aerogenes* and by day 9, the Ce and La concentrations had increased to 4 mg L⁻¹ (0.31% of leaching efficiency) (Fig. 2). In the study by Shin et al. (Shin et al., 2015), the Ce concentration dropped to less than 2 mg L⁻¹ (0.02% of leaching efficiency). Given the difference in experimental conditions (supplement of soluble of phosphate source in Shin et al. 2015) and the ore complexity, it is not surprising that the leaching behaviour of REEs was found to differ between studies.

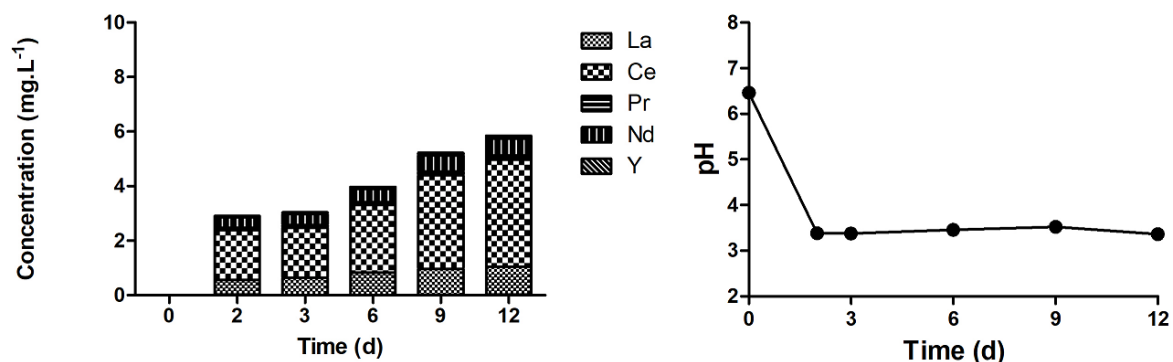


Fig. 2 Concentrations of dissolved La, Ce, Pr, Nd, and Y (left) and pH of leachate (right) during the bioleaching of Mt. Weld Monazite with *Enterobacter aerogenes* in the presence of glucose. The concentration of REEs, Th, and U in abiotic flasks remained below detection level throughout the experiment. Data are averages \pm SD of triplicate biological replicates. Error bars not visible are smaller than symbols.

Corbett et al. (Corbett et al., 2017) reported that *E. aerogenes* released a total of 1.93 mg L⁻¹ REEs (Ce, La, Nd, and Pr) from a similar ore sample (MWM) after 8 days. In comparison, the maximum total REEs concentration observed in this study was 3.97 mg L⁻¹ after 6 days (Fig. 2). Differences in experimental conditions (media composition and growth temperature), and the type and concentration of secreted organic acids may have contributed to the differing results. In contrast, the concentration of P, Fe, Th and U solubilised from monazite were much lower than observed for REEs and most were lower than detection limits (< 2 mg L⁻¹ for phosphate, < 0.5 mg L⁻¹ for Fe, < 5 μ g L⁻¹ for Th, and < 0.02 mg L⁻¹ for U), which is consistent with previous studies (Brisson et al., 2016; Corbett et al., 2017). As cells are present in the bioleaching system, incorporation and surface attachment of phosphate groups within microbial biomass and structures could be expected (Corbett et al., 2017). Also XRD analysis of the bioleached residue in this study confirmed the formation of secondary minerals such as cheralite [(Ce_{0.4}Ca_{0.3}Th_{0.3}) (PO₄) (SiO₄)] and woodhouseite [CaAl₃ (PO₄) (SO₄) OH₆] which may explain the very low concentration of elements, especially phosphate in solution (Supplementary Fig. S1a). According to these considerations, the preferential release of REEs over Th and U favours the selective recovery of radionuclides for further downstream processing.

These data indicated that *E. aerogenes* is a promising organism for microbial dissolution of phosphate REEs with almost no Th and U mobilization. However, in order to make it competitive with conventional extraction, further examination of factors influencing the release rate of REEs in conjunction with other microorganisms is required.

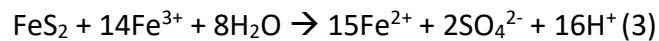
5.3.2.2 Acidophilic bacteria

The capacity of *A. ferrooxidans* to tolerate exceptionally high level of metals has been demonstrated (Dopson et al., 2014). Tsaplina et al. (Tsaplina et al., 2015) reported leaching yields of 15-30% for REEs with acidophilic chemolithotrophic microorganisms from a 30% pulp density of ash slag waste at pH 0.76. It has also been demonstrated that the addition of pyrite can enhance metal bioleaching from electronic wastes (Bryan et al., 2015). However, no studies on the solubilisation of REE phosphates by acidophilic bacteria in the presence of various growth substrates have been reported. It was therefore decided to adopt an experimental setup with *A. ferrooxidans* as a model microorganism to evaluate the bioleaching of REEs from MWM under acidic conditions.

The presence of *A. ferrooxidans* and availability of ferrous iron and reduced sulfur compounds impacted the pH change and mobilization behaviour of REEs (Fig. 3). The pH of the media with both $\text{FeSO}_4/\text{K}_2\text{S}_4\text{O}_6$ and pyrite initially increased as bio-oxidation of Fe^{2+} to Fe^{3+} is acid consuming (reaction 1).



Pyrite can be solubilised only by a combination of proton and oxidative attack, according to “thiosulfate pathway”, where the main product is sulfate (reactions 2 and 3) (Fonti et al., 2016).



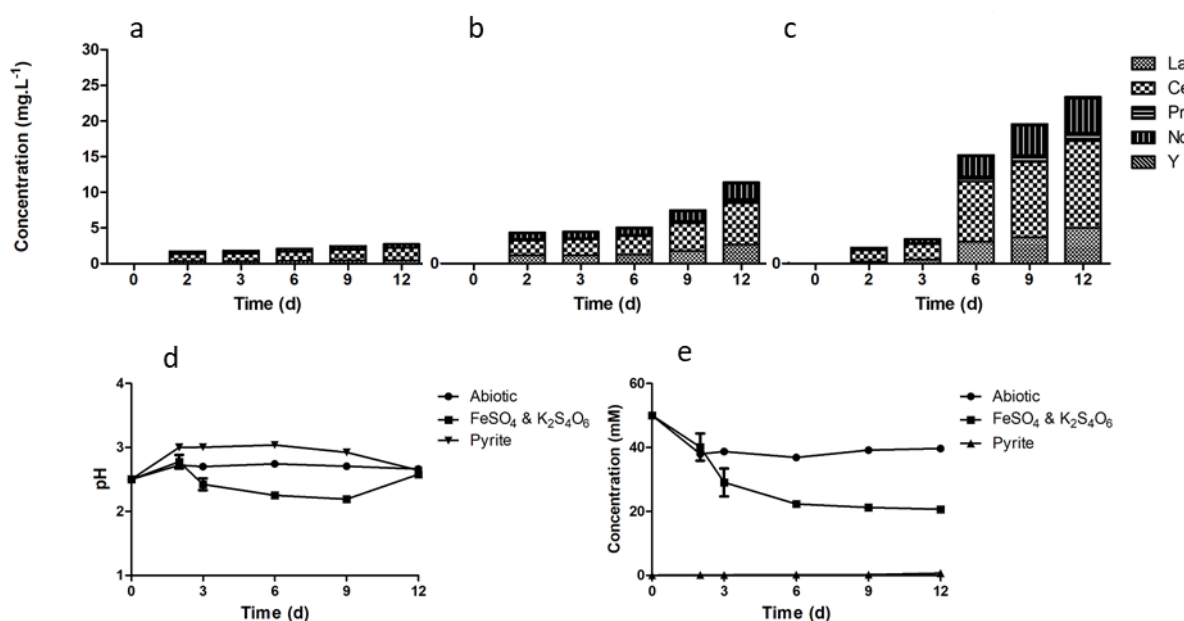
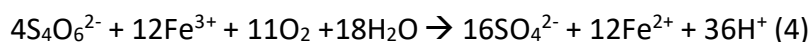
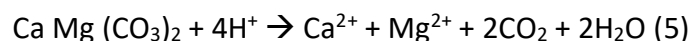


Fig. 3 Concentrations of dissolved La, Ce, Pr, Nd, and Y during the bioleaching of Mt. Weld Monazite *Acidithiobacillus ferrooxidans*. (a) Abiotic controls with FeSO₄ and K₂S₄O₆, (b) *Acidithiobacillus ferrooxidans* with pyrite, (c) *Acidithiobacillus ferrooxidans* with FeSO₄ and K₂S₄O₆, (d) solution pH, and (e) concentration of soluble Fe under all conditions. Abiotic controls with pyrite; concentration of REEs and Fe remained below detection level throughout the experiment. Data are averages ± SD of triplicate biological replicates. Error bars not visible are smaller than symbols.

Subsequently, after 9 days, the pH of the media in the presence of FeSO₄ and K₂S₄O₆ had decreased to 2.19±0.02, whereas the pH of the media inoculated with pyrite was 2.92±0.07. Bacterial oxidation of K₂S₄O₆ by *A. ferrooxidans* with molecular oxygen to sulphate is an acid-producing process according to reaction 4.

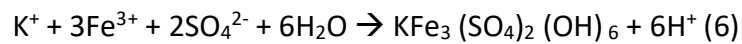


The final pH at day 12 in both conditions were in a similar range to the initial pH (2.5). As dolomite was present in the pyrite concentrate, a higher H⁺ consumption due to the buffering capacity of the dolomite (reaction 5) and the heterogeneous nature of the concentrate (presence of quartz and albite) can reasonably be assumed to be responsible for the differences in pH and lower REEs solubilisation in the presence of pyrite.



Moreover, the initial concentration of Fe and S within FeSO₄ and K₂S₄O₆ were 50 and 5 mM, respectively; which was adequate for bacterial growth.

The total concentration of Fe in solution by day 12 in the presence of FeSO₄ and K₂S₄O₆ gradually decreased from 50 to 21 mM whereas in solution in the presence of pyrite it increased slowly (from 0.10 to 0.66 mM by day 12). The formation of Fe co-precipitate, jarosite (reaction 6) in MWM residue after incubation with FeSO₄ and K₂S₄O₆ was confirmed by XRD (Supplementary Fig. S1b), whereas with pyrite, jarosite formation did not occur.



The presence of different substrates influenced the REEs solubilisation by *A. ferrooxidans* (Fig. 3). REEs solubilisation was greatest in the presence of FeSO₄ and K₂S₄O₆ (average final total REEs concentration during 12 days of incubation: 23.6 mg L⁻¹, 1% percentage recovery of Ce). On the other hand, the growth of *A. ferrooxidans* on pyrite as a source of Fe and S resulted in lower REEs solubilisation (average final total REEs concentration: 10.6 mg L⁻¹) which was still double of that achieved with *E. aerogenes*.

5.3.2.2.1 Phosphorous solubilisation

In the presence of *A. ferrooxidans*, the combined low pH with active Fe/S oxidising bacteria which produce biogenic sulfuric acid, favoured the mobilization of P up to 33 mg L⁻¹. Phosphorous solubilisation from REE phosphates relies on protons to proceed (Chi et al., 2006), however, as previously mentioned (3.2.2) the pH of the inoculated media in the presence of pyrite did not decrease, potentially limiting phosphate release into the leachate. Increasing the concentration of pyrite to 30 g L⁻¹, has been proposed to improve the fraction of phosphorous leached from rock phosphate (P content 12%)(Chi et al., 2006).

5.3.2.2.2 Thorium and uranium solubilisation

The tolerance of *A. ferrooxidans* to Th²⁺ and UO₂²⁺ has previously been demonstrated to be in a range of 900-1880 mg L⁻¹, where Th was more toxic to Fe oxidation than UO₂²⁺ (Leduc et al., 1997). This range is much higher than could be solubilised from the MWM based on its total content of Th and U, and therefore the application of *A. ferrooxidans* for MWM can be justified. As MWM also contains trace amount of radioactive elements with higher Th content (0.162%) relative to U (0.003%), early saturation of Th in the solution was expected. Nevertheless, Th concentrations in all scenarios were lower than U (0.057±0.006 vs 0.118±0.021). The amount of Th and U solubilised by *A. ferrooxidans* increased gradually during 12 days of incubation (0.30% and 32% of leaching efficiency, respectively). However, Singh et al. (Singh et al., 2009b) reported 98% uranium dissolution from silicate-apatite ore in 40 days by *A. ferrooxidans* under the optimum conditions (pH 1.7, temperatures 35° C). This is much higher than the overall solubilisation of Th and U from MWM in this study. Providing phosphate sources for bacterial growth could be one controlling factor in Th and U solubilisation as in this study bacteria obtained their phosphate from MWM which is not as easily accessible as common phosphate sources in 9K media (Singh et al., 2009b).

The major controlling factors in the release of U in this study was likely to be pH (reaction 7), and the increased concentration of Fe³⁺ (reaction 8) which can oxidise tetravalent uranium to water-soluble hexavalent uranium. While phosphate solubilising bacteria (section 3.2.1) only

solubilised U in the range of $0.013 \pm 0.001 \text{ mg L}^{-1}$ at day 2 up to $0.020 \pm 0.000 \text{ mg L}^{-1}$, by day 12, [comparable to concentration of U in abiotic controls ($0.023 \pm 0.002 \text{ mg L}^{-1}$)], *A. ferrooxidans* at day 12 solubilised U up to $0.118 \pm 0.021 \text{ mg L}^{-1}$ in the presence of $\text{FeSO}_4/ \text{K}_2\text{S}_4\text{O}_6$ and $0.105 \pm 0.000 \text{ mg L}^{-1}$ in the presence of pyrite.



The generation of ferric ions through the bio-oxidation of Fe^{2+} by *A. ferrooxidans* led to increasing solubility of Th. In the presence of $\text{FeSO}_4/ \text{K}_2\text{S}_4\text{O}_6$, the Th concentration in the leachate increased up to $0.057 \pm 0.006 \text{ mg L}^{-1}$ by day 12 compared to 0.007 mg L^{-1} in abiotic leaching. Solubilisation of Th can be attributed to the presence of an oxidising agent (Fe^{3+}). The formation of secondary minerals such as cheralite in the MWM residue also may explain the lower concentration of soluble Th in solution. These findings are in agreement with the results of thermodynamic speciation of monazite where the solubility of Th was restricted in the presence of phosphate due to the passivation layer of REE phosphates (Lapidus & Doyle, 2015).

5.3.2.3 Synergistic effect of co-culture

Of the three experimental conditions (*E. aerogenes* on glucose, *A. ferrooxidans* on $\text{FeSO}_4/ \text{K}_2\text{S}_4\text{O}_6$, and *A. ferrooxidans* on pyrite), *A. ferrooxidans* supplied with $\text{FeSO}_4/ \text{K}_2\text{S}_4\text{O}_6$ resulted in the greatest solubilisation of REEs, Y, as well as Th and U. This can be explained in terms of mechanisms by which elements are released into the leachate (biogenic acid and ferric iron generation) and the minimization of co-precipitation of soluble complexes. Phosphate solubilizing bacterium, *E. aerogenes* can lower pH naturally by the production of organic acids, as shown in section 5.3.2.1, producing conditions suitable for growths of *A. ferrooxidans*. Therefore, in this study an attempt was made to assess the potential of microbial co-culture system for REEs extraction by using *A. ferrooxidans* and *E. aerogenes*.

Glucose fermentation and the secretion of organic acids led to the natural acidification of the media by *E. aerogenes* with the first sharp decrease in pH (2.84 ± 0.03) observed by day 2. The addition of *A. ferrooxidans* plus $\text{FeSO}_4/ \text{K}_2\text{S}_4\text{O}_6$ at day 3 resulted in a further pH decrease (to 2.48 ± 0.01). A stable pH (2.46 ± 0.04) was reached at the end of the experiment (Fig. 4 b). Due to the inhibition of Fe^{2+} oxidation activity by *A. ferrooxidans* reported at pH values above 3.0 (Meruane & Vargas, 2003), the *A. ferrooxidans* inoculum was added when pH was 2.84 ± 0.03 which is close to the optimum pH of the species (2.5) (Schippers, 2007).

MWM was more efficiently solubilised in media co-inoculated with *E. aerogenes* and *A. ferrooxidans* (Fig 4.a) than with individual cultures (Fig. 2 and 3). An increase in REEs solubilisation from MWM with the co-culture was evident from day 6 (3 days after the addition of *A. ferrooxidans*) (Fig. 4 a) with a total REEs concentration of 40 mg L^{-1} on day 9. The major increase was observed for Ce as the concentration of released Ce in co-culture was on average 2.4 times higher than when inoculated with *A. ferrooxidans* and 7.4 times higher than with *E. aerogenes* (Fig. 4 a). However, the concentrations of solubilised La, Pr, Nd, U, and

Y detected in the co-culture solution at day 12 were in similar range as *A. ferrooxidans* in the presence of $\text{FeSO}_4/\text{K}_2\text{S}_4\text{O}_6$. This provided an opportunity to selectively extract REEs while minimising Th and U solubilisation.

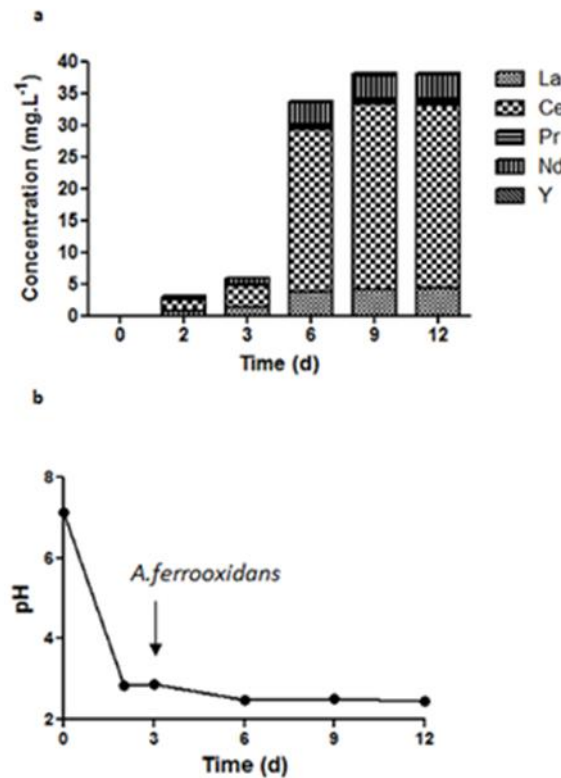


Fig. 4 Concentrations of dissolved La, Ce, Pr, Nd, and Y during the bioleaching Mt. Weld Monazite with a co-culture of *Enterobacter aerogenes* and *Acidithiobacillus ferrooxidans* (a) in the presence of FeSO_4 and $\text{K}_2\text{S}_4\text{O}_6$ and (b) pH of leachate. The concentration of REEs, Th, and U in abiotic controls remained below detection level throughout the experiment (data not shown). Data are averages \pm SD of triplicate biological replicates. Error bars not visible are smaller than symbols.

The concentration of P in the co-culture leachate was $3.33 \pm 0.94 \text{ mg L}^{-1}$. This is higher than in the presence of *E. aerogenes* ($< 2 \text{ mg L}^{-1}$) and lower than released by *A. ferrooxidans* (33 mg L^{-1}). Previous results have demonstrated the enhanced ability of an *Aspergillus niger* and *Burkholderia cepaciathan* co-culture (both known as PSM) to solubilize phosphates compared to their individual performance (Braz & Nahas, 2012). *Enterobacter* species have been characterized as one of the most efficient PSM (Corbett et al., 2017). However, in this study no detectable P was observed with *E. aerogenes*. This indicates that P solubilisation from MWM by *E. aerogenes* can be inhibited by environmental stresses such as a very low pH, phosphate deficiency, and toxic elements. The heterotrophic dissolution of REEs and P from monazite is mostly governed by acido-complexolysis of organic acids. An alternative

possibility is that the bacteria, in particular *E. aerogenes* have utilised the soluble P for cellular requirements. After co-inoculation with *A. ferrooxidans*, overall P solubilisation improved.

A number of low molecular weight organic acids can be rapidly degraded by microorganisms (Corbett et al., 2017). Heterotrophic culture of *E. aerogenes* generated a range of organic acids including gluconic, malic and acetic acids (data not published). The retention mechanisms of citric and malic acids have been investigated in some detail, and it has been demonstrated that higher Fe concentration under acidic conditions decreased the biodegradation of citric acid and malic acid (Yang et al., 2016). The enhanced dissolution of REEs from monazite as a result of the excretion of organic acids by heterotrophic bacteria and sulphuric acid from *A. ferrooxidans*, occurred by complexolysis of complexing agents and REEs. Therefore, the higher REEs concentration in the leachate of co-culture system can also be explained with higher and stable release of Fe in the co-culture (60 mM) compared to *A. ferrooxidans* (21 mM) which potentially contributed to the stability of organic acids, thereby prolonging their complexing capacity with REEs.

As a result, it could be summarized that the synergistic function of co-culture system can generate more lixiviant (i.e., acid), which promotes the bioleaching process of monazite. In this context, the synergistic action of *E. aerogenes* and *A. ferrooxidans* in co-culture, in comparison to pure culture, offers potential alternatives to conventional techniques.

5.3.3 Phosphate utilization

As mentioned above, different P solubilisation was seen in pure cultures of *E. aerogenes* and *A. ferrooxidans* as well as the co-culture. A comparative genomic study of the potential mechanisms of phosphate metabolism during the interaction of *E. aerogenes* and *A. ferrooxidans* was performed (Fig. 5). It has previously been demonstrated that polyphosphate kinase (*ppk*) is responsible for the accumulation of long polymers of inorganic phosphates (known as polyphosphate) and that polyphosphate can be hydrolysed to liberate inorganic P by the enzyme exopolyphosphatase (*ppx*) (Rao et al., 2009). A genomic comparison revealed the *ppx* gene was directly downstream of the complete *pho* regulon (*phoB-phoR-ptsSCAB-phoU*) in *A. ferrooxidans*, as has previously been shown (Vera et al., 2003). However, the genome of *E. aerogenes* showed major differences in the organization in the *pho* regulon, as shown by the absence of the *pstA* gene and the lack of co-localization of the *phoB-phoR* operon with *pstSCB* (Fig. 5). Furthermore, *ppk* and *ppx* in *E. aerogenes* are located in the same operon, and not as part of the *pho* regulon, as has previously also been described for *Escherichia coli* (Kornberg et al., 1999). As previously speculated by Vera et al. (Vera et al., 2003) the presence of *ppk* and *ppx* on the same operon suggests that the genes may be co-transcribed and therefore indicates limited accumulation of polyphosphate. Therefore, in *E. aerogenes* it is likely that inorganic P is directly used to meet the cell's phosphate requirements, resulting in less P available in the solution and the requirement for a constant source of P in order to avoid phosphate starvation. However, in *A. ferrooxidans* *ppk* is not found as part of the operon with *ppx*, suggesting the genes are transcribed separately,

allowing the accumulation of polyphosphate in this strain. *A. ferrooxidans* would be able to store P and also to liberate it depending on the cell's phosphate requirement. As *E. aerogenes* is likely to only transiently accumulate polyphosphate, it would require uptake of any phosphates released by *A. ferrooxidans* in order to overcome phosphate starvation. This could then explain the reduced concentration of phosphate in the media when *A. ferrooxidans* and *E. aerogenes* are in co-culture, as compared to the concentration of phosphate present when *A. ferrooxidans* is used as a pure culture.

Bacterial adaptation to phosphate and energy deficient environments represent key factors that can compromise the feasibility of bioleaching of REEs from MWM. However, there is limited understanding concerning the potential components involved in phosphate and iron dynamics within bacterial-mineral surfaces. Further study of the accumulation and regulation of the inorganic phosphate during the solubilisation process is essential in order to enhance the functioning of these microorganisms for the efficient leaching of REEs-phosphate minerals.

E. aerogenes



A. ferrooxidans

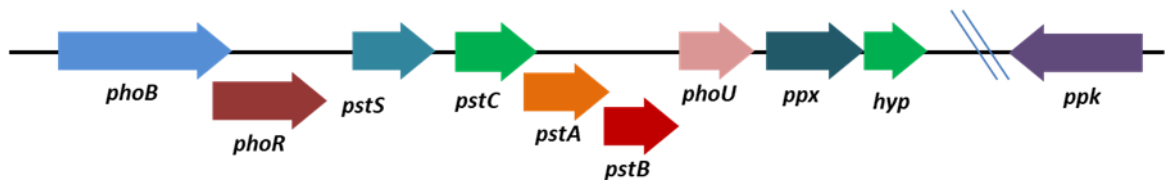


Fig. 5 Genetic organization of the *pho* Operon in *Enterobacter aerogenes* and *Acidithiobacillus ferrooxidans* (*phoB* – phosphate regulon transcriptional regulatory protein; *phoR* – phosphate regulon sensor protein; *pstS* – phosphate ABC transporter, periplasmic phosphate-binding protein; *pstC* – phosphate transport system permease protein; *pstA* – phosphate transport system permease protein; *pstB* – phosphate transport ATP-binding protein; *phoU* – phosphate transport system regulatory protein; *ppx* – exopolyphosphatase; *hyp* – hypothetical protein; *ppk* – polyphosphate kinase).

5.3.4 Oxidation state of Ce and Nd

The X-ray absorption near-edge spectrum (XANES) provides valuable insight into the chemical form of a specific element, including oxidation state, molecular geometry, and ligand type. In

many cases, simple visual inspection of the position of the edge energy and the general shape of the edge (features such as height, width, and splitting) is sufficient to draw conclusions about oxidation state (Hackett et al., 2012). This holds true for Ce, and differentiating Ce^{3+} from Ce^{4+} based on visual inspection of the L_{III} -Edge is relatively easy. The published literature highlights that the Ce^{3+} edge has a distinctive white line feature at 5726 eV (López-Moreno et al., 2010). As would be expected, at higher oxidation state, a shift of the edge to higher energy occurs, and the edge of Ce^{4+} is shifted to ~ 5730 eV. Further, Ce^{4+} contains two stable ground state electronic configurations, $4f^0$ and $4f^1$, and consequently the Ce^{4+} L_{III} -Edge contains a distinctive “double-peak” white line feature (Shahin et al., 2005).

The results from this study revealed that in the bioleached MWM residue and resultant leachate, the Ce L_{III} -Edge occurs at 5726 eV, and contains only a single edge feature (Fig. 6). Therefore, it was concluded that the major oxidation state of Ce found in the MWM and leachates are Ce^{3+} . These results suggest that oxidation of REEs (Ce^{3+} to Ce^{4+}) most likely did not occur during the bioleaching of MWM, possibly as the dissolution mechanism of Ce was controlled by chelation with anions from organic acids.

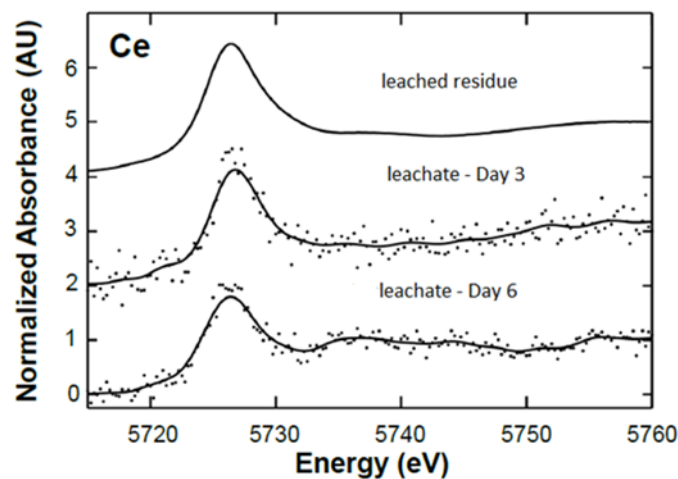


Fig. 6 Ce L_{III} -Edge XAS spectra collected from leachate at day 3 and day 6 after *Acidithiobacillus ferrooxidans* addition, and Mt. Weld Monazite residue during co-culture experiment. Dotted lines show individual data points, and solid lines show resulting spectra produced from a 0.5 Gaussian smoothing filter. A single white line feature, centred at 5726 eV was observed for all spectra, indicating that Ce was mainly present as Ce^{3+} .

5.3.5 Changes in the partitioning of REEs and Th during bioleaching

Changes in REEs and Th fractionation due to bioleaching with *E. aerogenes* and *A. ferrooxidans* were evident (Table 1 and 2). The major changes were observed for the non-residual fractionation of La, Ce, Pr, Nd, and Y, while Th showed smaller variations, in congruence with

their high association to the residual fraction (i.e., over 93.7 %). Bioleaching was effective in stimulating REEs mobility as indicated by the increase of REEs in easily extractable and acid soluble fractions F1 and 4 (Table 2). The increase of REEs mobility in F1 and F4 was associated with a concomitant decrease of these elements in fractions F2, F3, and R (Table 2).

Table 2 – Fractionation of elements in bioleached residue of Mt. Weld Monazite ore as determined by sequential extraction procedure after bioleaching with either *Enterobacter aerogenes* or *Acidithiobacillus ferrooxidans*. The results <0.0001% are not shown. Data are averages of triplicate biological replicates.

Fraction Content of MWM (mg kg ⁻¹ and %) after bioleaching with <i>E. aerogenes</i>					
	Easily soluble F1 (%)	Carbonate F2 (%)	Reducible F3 (%)	Acid soluble F4 (%)	Residual R (%)
La	90 (0.07)	238 (0.19)	444 (0.35)	27800 (22.0)	97433 (77.3)
Ce	244 (0.11)	680 (0.32)	1456 (0.69)	44800 (21.1)	164667 (77.7)
Pr	15 (0.06)	69 (0.29)	77 (0.33)	5456 (23.0)	18033 (76.2)
Nd	59 (0.07)	286 (0.34)	280 (0.34)	19790 (23.8)	62700 (75.4)
Th				89 (4.34)	1950 (95.6)
Y	3.7 (0.15)	9.6 (0.38)	10 (0.41)	558 (22.4)	1907 (76.6)
Fraction Content of MWM (mg kg ⁻¹ and %) after bioleaching with <i>A. ferrooxidans</i>					
La	120 (0.12)	79 (0.08)	259 (0.26)	17680 (17.8)	80937 (81.7)
Ce	247 (0.13)	223 (0.12)	829 (0.45)	35800 (19.6)	145152 (79.6)
Pr	27 (0.13)	23 (0.11)	49 (0.24)	3813 (18.4)	16763 (81.0)
Nd	100 (0.14)	92 (0.12)	166 (0.23)	12987 (17.8)	59655 (81.7)
Th		5.7 (0.31)		48 (2.63)	1784 (97.0)
Y	3 (0.13)	2 (0.09)	4.3 (0.19)	334 (15.0)	1877 (84.5)

Although previous research has shown the impact of bioleaching on fractionation of metals in the contaminated sediments (Fonti et al., 2015), to the best of our knowledge, there has no previous reports on the effects of bioleaching on REEs mobility from phosphate minerals.

The first step of sequential extraction obtains the easily soluble/exchangeable fraction (F1) of elements that are weakly associated with organic and inorganic sites (Beckett, 1989) which can be released by the action of protonation (pH change) and ion exchange of cations such as Ca²⁺, K⁺, and Mg²⁺ which have a comparable ion radius (Coordination number = 6) to the trivalent state of REEs (e.g., Na⁺: 1.02 Å, Ca²⁺: 1.00 Å, K⁺: 1.38 Å, La³⁺: 1.03 Å, Ce³⁺: 1.01 Å, Pr³⁺: 0.99, Nd³⁺: 0.98 Å, and Y³⁺: 0.90) (Jia, 1991; Shannon, 1976). The results of this fraction have shown that without bacteria only Ce (1.4 mg kg⁻¹) and Nd (0.4 mg kg⁻¹) may be released through weak electrostatic interactions or ion-exchange reaction. However, after bioleaching with *E. aerogenes* and *A. ferrooxidans*, an increase of Ce, La, Nd, Pr, and Y association with the easily soluble/exchangeable fraction of the leach residue was observed (0.06-0.15 and

0.12-0.14 % of their total concentration in *E. aerogenes* and *A. ferrooxidans*, respectively). Higher association of REEs in the F1 for *A. ferrooxidans* were in good agreement with higher concentration of REEs in *A. ferrooxidans* culture as illustrated in Fig. 3.

The second fraction (F2) extracted with citric acid ($pK_a = 3.13$) accounts for the carbonate and complexation fraction from which REEs can be solubilised with low molecular weight organic acids. Among the REEs in bioleaching residue of *E. aerogenes* the highest content in F2 was recorded for Y followed by Nd, and Ce. As citric acid was not detected in this study (data not shown), smaller extractable concentration of REEs in bioleach residue can be attributed to the microbial generation of weaker acids such as gluconic, acetic, and malic acids and consequently complexation of REE with organic acids. These results agree with previous study by Mittermüller et al. (Mittermüller et al., 2016) that reported extraction yields of the organic acids for both soil and tailings material generally increasing with increasing complexation capacity in the order: acetic acid < malic acid < citric acid. As the nature of acids generated by *E. aerogenes* and *A. ferrooxidans* are different, comparison of REEs complexation with biogenic sulfuric and organic acids is difficult.

The elements bound to Fe-Mn oxy/hydroxides, the reducible fraction (F3), are normally mobilized with reductive conditions (Zimmerman & Weindorf, 2010) by changing either the oxidation state of the element or the host mineral elements (i.e., iron ore manganese hydroxides) (Mittermüller et al., 2016). A couple of recent studies have reported that MnO_2 can oxidize Ce^{3+} to Ce^{4+} (Yu et al., 2016). Fe-Mn oxy/hydroxides can also scavenge cations such as REEs by recrystallization products such as hematite, lepidocrocite, goethite, and maghemite (Lottermoser, 1990). However, based on results described in section 3.4 changes to the oxidation state of Ce^{4+} were not detected, and hence Eh may not contribute to REEs mobilisation.

The fourth fraction of extracted elements, the acid soluble fraction (F4), includes the REEs that are usually associated with barely soluble phosphates. The widely used CBR extraction method (Rao et al., 2010) includes REE phosphates within the residual fraction. Thus, a separate step was required to evaluate the extent to which REE are available in a phosphate form for further bioleaching by microorganisms. The bioleaching induced REEs association into the acid soluble fraction suggests that phosphate chemistry in solution was most likely the limiting step to REEs mobilisation. Due to differences in the final pH of media for *E. aerogenes* and *A. ferrooxidans* cultures, the phosphate metal complexes would be less solubilized at higher pH (*E. aerogenes*), resulting in higher precipitated P, whereas the soluble P would be more prevalent in the experiments with *A. ferrooxidans* due to lower pH and solubilisation of P-metal complexes (higher insoluble P content for *E. aerogenes*). At lower pH, these REEs phosphate complexes would solubilize, allowing for the release of higher soluble levels of P, but at higher pH the precipitates would remain insoluble. Therefore, providing acidic conditions that maintain phosphate complexes dissolved in solution potentially contribute to increase in overall REEs dissolution.

Considering the sum of so-called non-residual (labile) fractions (F1+F2+F3+F4), the observed increase in partitioning in mobile fraction was not associated with REEs solubilisation during

bioleaching processes, as the total concentration of all REEs in the non-residual fractions of the residue for *E. aerogenes* (22-24%) were higher than *A. ferrooxidans* (15-20%), while *A. ferrooxidans* displayed higher REEs solubilisation into solution phase during bioleaching (Fig. 2 and 3; Table 1 and 2). In addition, REEs released from the residual fraction did not remain in the solution phase but shifted to the other fraction of the monazite, particularly the acid soluble fraction, implying that bacterial metabolisms (phosphate acquisition, storage, and complexation) are likely to play a key role in controlling overall solubilisation processes. These findings suggest that an increase in REEs mobility does not necessarily govern a specific element solubilisation into the solution.

Based on the results from bioleaching, SEP and XANES, it can be concluded that a combination of biogeochemical processes and physio-chemical characteristic of specific elements generated complex patterns that controlled the bioavailability and mobility of REEs in monazite.

5.4 Conclusion

This study provides the first direct evidence of a synergistic effect of a heterotrophic-autotrophic co-culture on the bioleaching of REEs from monazite. The combination of *A. ferrooxidans* and *E. aerogenes* increased REEs bioleaching from monazite (up to a final concentration of 40 mg L⁻¹ REEs including: Ce, La, Nd, Pr, and Y) as compared to the pure cultures of *A. ferrooxidans* (23.6 mg L⁻¹) or *E. aerogenes* (5.84 mg L⁻¹) owing to a synergic interaction through the biogenic generation of both organic acids and sulfuric acid.

5.5 Acknowledgments

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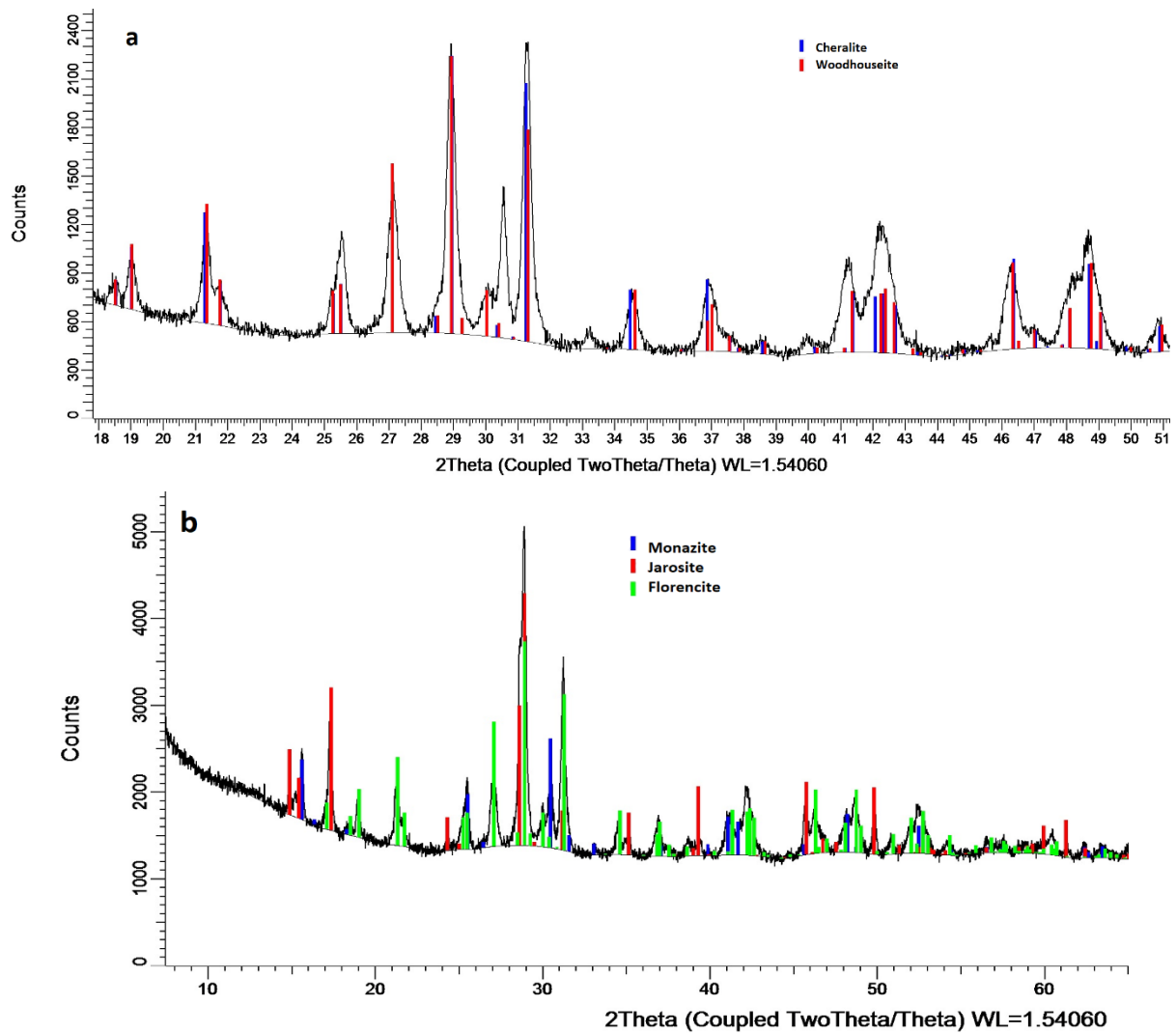
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5.7 Supplementary Files



Supplementary Figure S1. XRD patterns of secondary minerals formation of Mt. Weld Monazite after bioleaching with *Enterobacter aerogenes*: (a) cheralite/ woodhouseite; *Acidithiobacillus ferrooxidans* (b) Jarosite

Chapter 6: Effect of glycine on bioleaching of rare earth elements by phosphate solubilising microorganisms from Western Australian monazite.

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Effect of glycine on bioleaching of rare earth elements by phosphate solubilising microorganisms from Western Australian monazite.

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6.1. Introduction

The use of concentrated sodium hydroxide or sulfuric acid in basic and acidic hydrometallurgical processes for leaching of rare earth elements (REEs) bearing phosphate minerals containing thorium and uranium (i.e. monazite) result in the production of large amounts of toxic wastes that contain thorium, uranium, hydrogen fluoride, and acidic waste water (Abreu & Morais, 2010; Hurst, 2010). Unless treated properly, the waste streams have a significant damaging effect on the environment. Techno-economic and life cycle analysis of REEs bioleaching from waste materials have demonstrated that bioleaching offered significant environmental benefits in most of the impact categories compared to conventional hydrometallurgical processes (Thompson et al., 2017b).

Earlier studies have demonstrated the possibility of bioleaching REEs using heterotrophic and acidophilic autotrophic bioleaching microorganisms (Corbett et al., 2017; Fathollahzadeh et al., 2018b; Fathollahzadeh et al., 2018d). It has been demonstrated that phosphate solubilising microorganisms (PSMs) can acidify the leaching media naturally by the use of microbial metabolites (i.e., organic acids and acid phosphatase) (Corbett et al., 2018; Fathollahzadeh et al., 2018b) and therefore are able to release the phosphate and REEs present in a phosphate mineral matrix.

Considering the input chemicals utilized in the REEs extraction procedures show significant contributions to most impact categories in the environmental life cycle assessment studies (Arshi et al., 2018), there is urgent need to find lixivants that cause less pollution and are more sustainable for ore processing. Recently, it has been suggested that glycine ($\text{NH}_2\text{-CH}_2\text{-COOH}$) can be used as a benign biodegradable lixiviant for the extraction of precious elements, including gold from polymetallic ores (Eksteen & Oraby, 2015; Oraby & Eksteen, 2014).

Glycine is one of the simplest and cheapest amino acids (the bulk cost of glycine is approximately USD 1000–1800 per tonne). It is also non-flammable, environmentally safe and stable, enzymatically degradable and is easily metabolised in most living organisms (Oraby & Eksteen, 2014). Depending on the solution pH, glycine can exist in three different forms as either a cation ($^+\text{H}_3\text{NCH}_2\text{COOH}$), a zwitterion ($^+\text{H}_3\text{NCH}_2\text{COO}^-$), or an anion ($\text{H}_2\text{NCH}_2\text{COO}^-$) (Aksu & Doyle, 2001). In acidic solutions ($\text{pH} < 2.35$) the cationic form predominates (Aksu & Doyle, 2001). Due to its ability to form complexes, glycine can form 1:1 and 1:2 complexes with REEs via the carboxylic or the amino group (Kiss et al., 1991).

In order to dissolve insoluble materials effectively and biologically, it has been suggested that the leaching reagents must satisfy the following criteria: i) occur naturally within the cell or in its environment, ii) act under physiological pH and temperature, and iii) be a part of the cell metabolism, and constantly regenerated (Mandl & Neuberg, 1956). Recent studies have shown that the addition of microbial metabolites such as amino acids enhanced the bioleaching of sulfide and phosphate minerals (Li et al., 2013; Rojas-Chapana & Tributsch, 2000). Consequently, glycine can be hypothetically considered as a potential lixiviant as it appears to meet the criteria listed above.

Although heterotrophic and acidophilic autotrophic bioleaching microorganisms have been used to leach REEs from monazite (Corbett et al., 2017; Corbett et al., 2018; Fathollahzadeh et al., 2018b; Fathollahzadeh et al., 2018d), to the best of our knowledge, the effect of amino acids such as glycine on the bioleaching of REEs has not been previously studied.

In order to explore such a possibility, several experiments were performed with two strains of known PSMs: *Enterobacter (E.) aerogenes* and *Acidithiobacillus (A.) ferrooxidans* as model organisms for heterotrophs and chemolithoautotrophs, respectively. First, the performance of *E. aerogenes* for bioleaching of REEs from high grade monazite in the presence and absence of glycine was evaluated. Thereafter, as *A. ferrooxidans* has been previously demonstrated to leach more REEs in comparison to *E. aerogenes* (Fathollahzadeh et al., 2018d), the bioleaching of REEs from high grade monazite (MWM) and another two monazite concentrates (MWO and CSM) with *A. ferrooxidans* in the presence and absence of glycine was investigated.

In this context, the aim of this work was to evaluate the performance of an integrated glycine-bioleaching system for the extraction of REEs from various grades of monazite bearing minerals obtained from Western Australia (WA).

6.2 Materials and methods

6.2.1 Monazite ore

Three monazite samples from WA were used for the leaching experiments (Table 1). Sample preparation and composition analysis of MWM and CSM has been previously reported in Corbett et al. (Corbett et al., 2017). The elemental composition of MWO, MWM, and CSM was analysed by inductively coupled plasma optical emission spectrometry (ICP-OES, CSIRO Mineral Resources, Waterford, WA) (Table 1). The MWO sample (<38 µm) was dominated by goethite followed by monazite, florencite, nontronite, muscovite, and kaolinite as determined by X-ray diffraction at CSIRO Mineral Resources, Waterford, Western Australia. The monazite concentrate of CSM was diluted 1:10 with ground silica to obtain a safe Th/U working concentration (Corbett et al., 2017). Before bioleaching experiments, the mineral samples were sterilized by gamma irradiation at 50kGy for 11 h (ChemCentre, Bentley, Western Australia).

6.2.2 Bioleaching experiment

Enterobacter aerogenes (ATCC 13048, obtained from ATCC) and *Acidithiobacillus ferrooxidans* (ATCC 23270, obtained from DSMZ) were grown to exponential phase in National Botanical Research Institute Phosphate (NBRIP) medium (Nautiyal, 1999) and in basal salt media (BSM) (Zammit et al., 2011), respectively, at 30 °C with shaking at 120 rpm, and harvested by centrifugation (3,600 × g, 10 min), as reported in detail in Fathollahzadeh et al. (Fathollahzadeh et al., 2018d).

All bioleaching experiments were conducted in 250 mL Erlenmeyer flasks containing 100 mL of the relevant media, in triplicate at 30 °C with shaking at 120 rpm in an orbital shaking incubator (RATEK, Model No: OM11) over 12 days.

Table 1 – ICP-EOS analysis for elemental composition of Monazite samples used in this study

Sample ID	Content (%)		
	Medium grade Mt Weld monazite (MWO)	High grade Mt Weld monazite (MWM)	Cable Sand monazite (CSM)
Lanthanum (La)	3.30	10.1	9.41
Cerium (Ce)	4.81	12.6	14.0
Praseodymium (Pr)	0.741	2.10	0.184
Neodymium (Nd)	2.27	6.25	5.46
Yttrium (Y)	0.049	0.165	0.851
Thorium (Th)	0.043	0.162	4.98
Uranium (U)	<0.003	<0.003	0.194
Iron (Fe)	31.5	1.23	0.458
Phosphorus (P)	2.95	9.93	9.45
Calcium (Ca)	0.703	1.75	0.693
Magnesium (Mg)	0.524	0.199	0.079
Silicon (Si)	3.84	1.96	1.72
Titanium (Ti)	0.426	0.554	0.325
Zirconium (Zr)	0.012	0.031	1.80

The ability of *E. aerogenes* to bioleach sterilized MWM (1% pulp density) was explored in the modified NBRIP media (3% w/v glucose and initial pH 7.00±0.25) with 1% v/v bacterial inoculum (initial density 1×10^7 cells mL⁻¹).

The ability of *A. ferrooxidans* to bioleach sterilized ore samples (MWO, MWM, CSM) as a phosphate source, was evaluated in BSM (initial pH 2.00±0.15), supplied with FeSO₄ (13.9 g L⁻¹) and K₂S₄O₆ (1.51 g L⁻¹) (filter sterilized through 0.20 µm cellulose acetate/surfactant-free membrane, Sartorius), with 1% v/v bacterial inoculum (initial density 1×10^6 cells mL⁻¹) and 1% pulp density of sterilized monazite ore.

Moreover, bioleaching of sterilized ore samples with *E. aerogenes* (1% v/v, initial density 1×10^7 cells mL⁻¹) in modified NBRIP media (3% w/v glucose and initial pH 7.00±0.25) and *A. ferrooxidans* (1% v/v, initial density 1×10^6 cells mL⁻¹) in BSM (initial pH 2.00±0.15) supplied with FeSO₄ (13.9 g L⁻¹) and K₂S₄O₆ (1.51 g L⁻¹) (filter sterilized through 0.20 µm cellulose acetate/surfactant-free membrane, Sartorius) in the presence of sterilized glycine (1 g L⁻¹, Sigma) was carried out over 12 days at 30 °C in triplicate, at 120 rpm in an orbital shaking incubator.

Cell-free abiotic controls were carried out under the same conditions. Samples were taken at 0, 2, 3, 6, 9, 12 d and pH measured using a pH meter (Ionode IJ series pH probe). Samples were then filtered with disposable syringe filters (0.20 μm , Sartorius) and assayed for REEs (La, Ce, Pr, Nd, and Y), Th, U, Fe, and P concentrations by inductively coupled plasma-mass spectrometry (ICP-MS) Agilent Technologies 7700 series (Bureau Veritas Australia Pty Ltd, Canning Vale, Western Australia) and the average values were reported.

The effect of glycine on the growth of *E. aerogenes* (20% inoculum) was evaluated in 10 mL of modified NBRIP medium (5 g L⁻¹ K₂HPO₄ instead of monazite source) and determined by cell growth in the absence and presence of glycine (1 g L⁻¹, Sigma). The growth was assessed by taking 200 μL aliquots of the bacterial suspension and placing the samples in a 96-well plate for measuring optical density at 650 nm for cell growth using a microplate reader (EnSpire Multimode plate reader, PerkinElmer). Moreover, the effect of glycine on the activity of *A. ferrooxidans* was evaluated in 100 mL BSM (supplied with 5 g L⁻¹ K₂HPO₄ instead of monazite source) and determined by oxidation of ferrous to ferric in the absence and presence of glycine (1 g L⁻¹, Sigma). Iron oxidation was determined based on ferric and total dissolved iron concentrations using the modified ferric chloride assay as described by Khaleque et al. (Khaleque et al., 2018).

6.2.3 Glycine metabolism of *E. aerogenes* and *A. ferrooxidans*

Glycine is the simplest amino acid and can be catabolized by way of several metabolic pathways (Kikuchi et al., 2008). In order to investigate the potential metabolic pathways involved in glycine metabolism by *E. aerogenes* and *A. ferrooxidans*, a genome-based analysis of glycine pathways was performed. The genome of *E. aerogenes* (ATCC 13048 – KCTC 2190; NCBI accession: CP002824.1) and *A. ferrooxidans* (ATCC 23270; NCBI accession: CP001219.1) were downloaded from the NCBI ftp site (<ftp://ftp.ncbi.nlm.nih.gov/>).

For the purpose of this study, the genome of *E. aerogenes* and *A. ferrooxidans* was annotated using the Rapid Annotation Subsystem Technology (RAST) server (<http://rast.nmpdr.org/>) and the ClassicRAST annotation scheme (Overbeek et al., 2013). The Kyoto Encyclopaedia of Genes and Genomes (KEGG) was used for verification of metabolic pathways (Kanehisa et al., 2015) (<http://www.genome.jp/kegg/>).

6.3 Results and Discussion

The bio-solubilisation of REEs from the monazite samples was explored with cultures of *E. aerogenes* and *A. ferrooxidans* as well as a combination of each strain with glycine.

6.3.1 Bio-solubilisation of REEs from monazite with heterotrophic PSM

Following the inoculation of heterotrophic PSM into sterile media containing MWM, *E. aerogenes* solubilized REEs (La, Ce, Nd, Pr, and Y) up to a final concentration of 6 mg L⁻¹ (Fig.

1) consistent with the leaching results of a previous study (Fathollahzadeh et al., 2018b). The preferential release of REEs over Th and U corresponded to the formation of cheralite and woodhouseite confirmed by XRD (Fathollahzadeh et al., 2018d). Incorporation of phosphate into microbial biomass and formation of secondary phosphate minerals may also explain the lower concentration of phosphate in the leachate compared to REEs ($P \approx 2 \text{ mg L}^{-1}$) consistent with the previous studies (Corbett et al., 2017; Fathollahzadeh et al., 2018d). With microbial activity, the pH of the media decreased from 6.50 ± 0.02 to 3.36 ± 0.05 (Fig. 1). This decrease in the pH can be attributed to the production of organic acids and phosphatases resulting from glucose oxidation (Corbett et al., 2017; Corbett et al., 2018). No solubilisation of REEs, Fe or P occurred in abiotic flasks with the soluble concentration of all elements being below detection limits ($\text{Ce, La, Pr, Nd, Th, U, and Y} < 1 \mu\text{g L}^{-1}$; $\text{Fe} < 0.5 \text{ mg L}^{-1}$, and $\text{P} < 2 \text{ mg L}^{-1}$), indicating that metabolites secreted by microbial cells enhanced REEs mobilization.

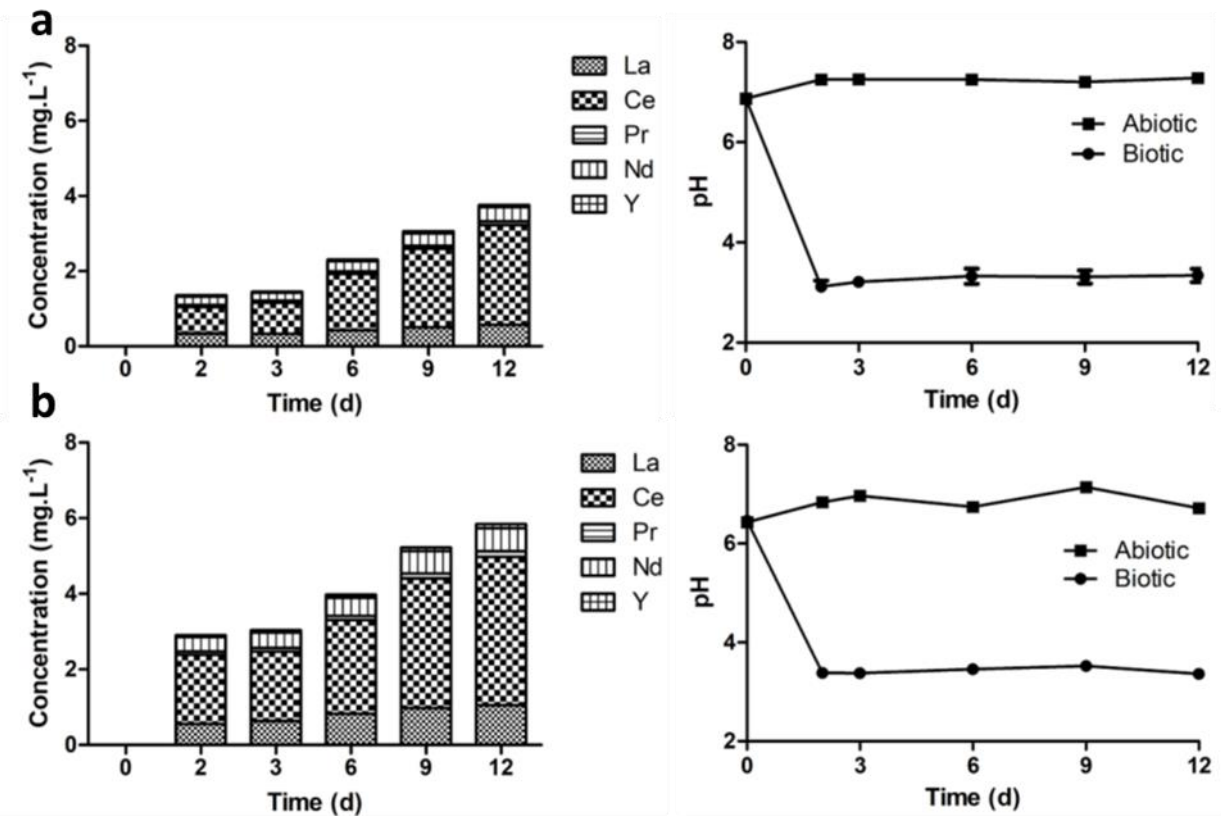


Fig. 1. The concentrations of dissolved La, Ce, Pr, Nd, and Y and pH of leachate during the bioleaching of Mt. Weld Monazite (MWM) in the presence of *Enterobacter aerogenes* (a) with and (b) without glycine and pH during abiotic leaching. The concentration of REEs, Th, and U in abiotic flasks remained below detection level throughout the experiment (data not shown). The pH data for both abiotic and biotic flask are averages \pm standard deviations (SD) of triplicate replicates. Error bars not visible are smaller than symbols.

The final pH of the media in the presence of glycine was in similar range (3.34 ± 0.05) to the flasks with no glycine (3.36 ± 0.05) (Fig. 1). The cell yield was higher in the presence than in the absence of glycine (Supplementary Fig. S1). However, REEs solubilisation with *E. aerogenes* decreased in the presence of glycine (average final total REEs concentration after the 12 days of incubation: 4 mg L^{-1}) (Fig. 1). These data indicated that *E. aerogenes* produce greater biomass in the presence of glycine, however, overall REEs dissolution from monazite in the presence of glycine was reduced. Moreover, *E. aerogenes* used in this study was not provided with soluble phosphate source during bioleaching and therefore had to acquire phosphate from monazite. This was confirmed by the low concentration of P in the leachate ($< 2 \text{ mg L}^{-1}$), and phosphate deficiency can also reasonably be assumed to be responsible for slowing down the overall microbial growth and affecting further REEs solubilisation.

6.3.2 Bio-solubilisation of REEs from monazite with autotrophic PSM

Any microorganism that acidifies the media can potentially release some level of phosphate and hence REEs. Although bioleaching of sulfide minerals by *A. ferrooxidans* has been extensively studied (Dopson et al., 2014; Watling et al., 2010), autotrophic bioleaching of REEs-bearing phosphate minerals has only been reported recently (Fathollahzadeh et al., 2018d). The previous research (Fathollahzadeh et al., 2018d) demonstrated that *A. ferrooxidans* (initial pH= 2.50 ± 0.15) could solubilize MWM up to a final total REEs concentration of 23.6 mg L^{-1} in the presence of FeSO_4 and $\text{K}_2\text{S}_4\text{O}_6$. In this study, in order to elucidate the effect of amino acids on the bioleaching of REEs, several experiments were performed with *A. ferrooxidans* (initial 2.00 ± 0.15) with the addition of glycine to evaluate the effect of glycine on the bioleaching of REEs from MWM, MWO, and CSM under acidic conditions.

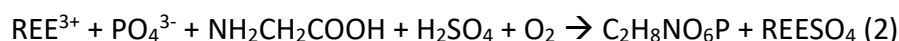
6.3.2.1 Solution pH, iron, and sulfate concentration

The changes in pH, and iron concentration during the leaching experiments are shown in Figure 2. Solution pH and total soluble iron concentration decreased in all biotic flasks over time, whereas in abiotic flasks pH and iron concentration remained relatively stable (Fig. 2). Total soluble sulfate concentration increased in both biotic and abiotic flasks (Fig. 2). However, the total soluble sulfate concentration after day 2 remained steady in abiotic flasks and decreased gradually in biotic flasks (Fig. 2).

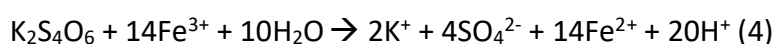
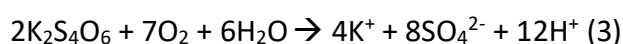
The presence of *A. ferrooxidans* impacted the pH change as well as the total concentration of soluble iron and sulfate (Fig.2). The pH of the media in the presence of $\text{FeSO}_4/\text{K}_2\text{S}_4\text{O}_6$ initially increased as bio-oxidation of Fe^{2+} to Fe^{3+} is an acid consuming (reaction 1).



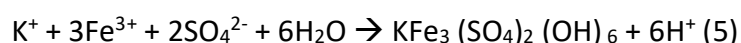
Furthermore, in the presence of glycine, the pH of the media initially increased as the reaction is acid consuming as follows (reaction 2):



Subsequently, after 12 days incubation with *A. ferrooxidans*, the pH of the media in the presence of FeSO₄ and K₂S₄O₆ decreased to 1.48, whereas the pH of the media inoculated with glycine was 1.70 (Fig.2). The final pH in the abiotic flasks at day 12 in both conditions were in similar range to the initial pH (2.20-2.34). Bacterial oxidation of K₂S₄O₆ by *A. ferrooxidans* with molecular oxygen and ferric iron to sulphate is an acid-producing process according to reaction 3 and 4. This reaction also explains the enhanced sulfate concentration in biotic flasks (Fig.2). Initial increase of total soluble sulfate in abiotic flasks was observed which cannot be easily explained as no pH adjustment with sulphuric acid was carried out in this study. Also, in abiotic conditions the kinetics of tetrathionate oxidation to generate sulfate cannot be as rapid as in the biotic conditions.



After the incubation of *A. ferrooxidans* in the presence of FeSO₄ and K₂S₄O₆ with and without glycine, the concentration of total soluble iron and sulfate decreased as the Fe³⁺ precipitated as jarosite (reaction 5):



The total concentration of Fe in the leachate by day 12 in the presence of *A. ferrooxidans* and *A. ferrooxidans* with glycine gradually decreased from 50 to 15.71±0.07 and 18.98±0.15 mM, respectively whereas in the leachate of the abiotic flasks iron concentrations remained relatively stable (48-53 mM).

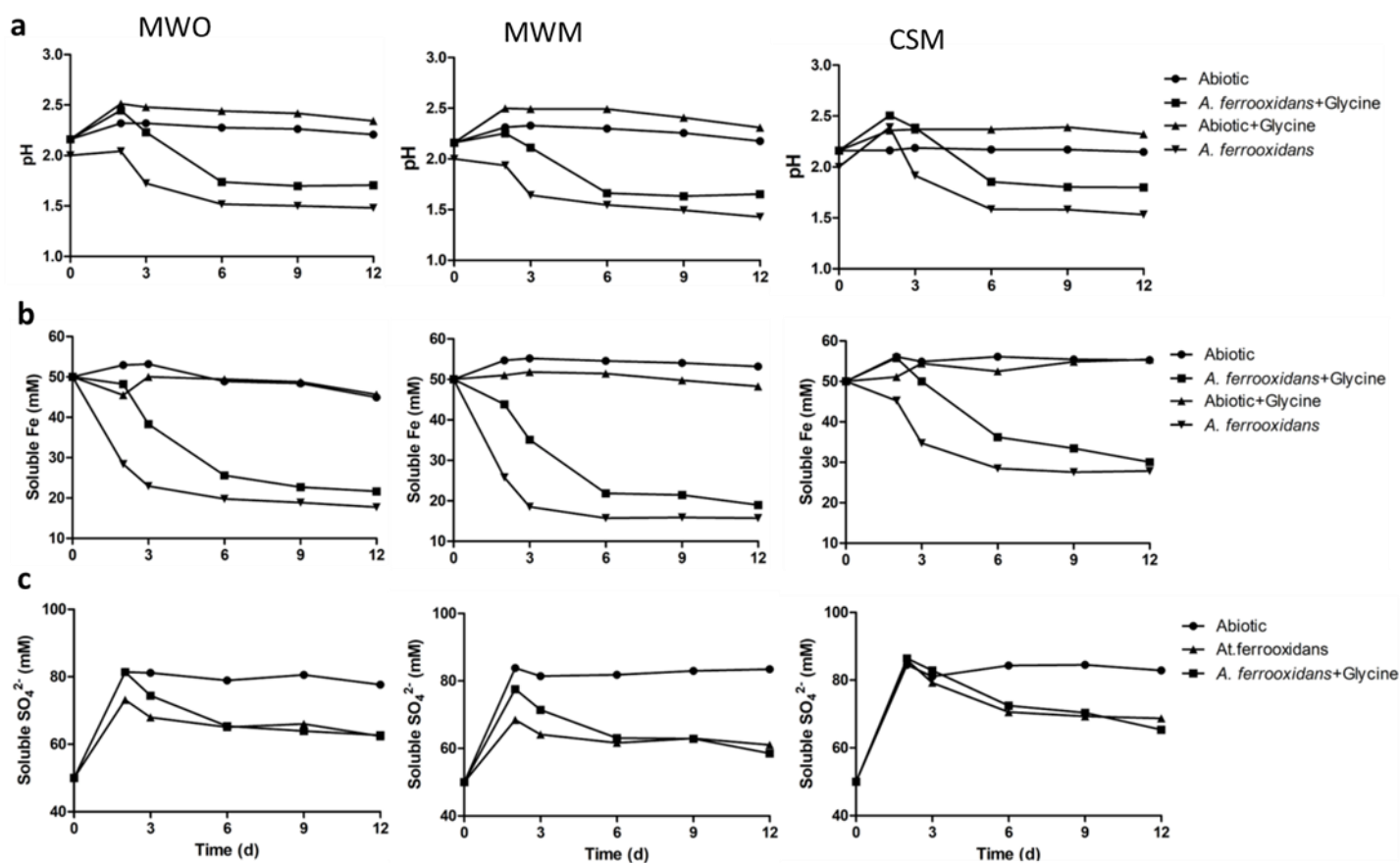
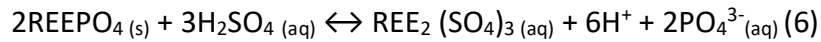


Fig. 2 (a) Solution pH change, (b) concentration of total soluble Fe, and (c) concentration of total soluble SO_4^{2-} during the leaching of Mt. Weld ore (MWO), Mt. Weld monazite (MWM), and Cable Sand monazite (CSM) with and without *Acidithiobacillus ferrooxidans* and/or glycine. Data for both abiotic and biotic flask are averages \pm standard deviations (SD) of triplicate replicates. Error bars not visible are smaller than symbols.

6.3.2.2 REEs leaching

The overall dissolution reaction of monazite in acidic media can be written as (reaction 6):



In addition to phosphate utilisation by bacteria, the precipitation (formation of secondary phosphate minerals) and adsorption and desorption (formation of aqueous REE^{3+} and PO_4^{3-}) equilibrium may control the concentration of phosphate and REEs ions in the solution (Fathollahzadeh et al., 2018d).

The concentrations of P in the leachate of *A. ferrooxidans* on sterile MWM, MWO, and CSM at day 12 were 4, 2.7, and < 2 mg L⁻¹, respectively (data not shown). However, no detectable concentration of P (< 2 mg L⁻¹) was observed where glycine was present in the medium. The lower concentration of P in the presence of glycine can be attributed to the formation of glycine phosphate complex according to reaction 2. As MWM and MWO also contain trace amounts of radioactive elements with higher Th content (MWM: 0.162%; MWO: 0.043%) comparing to U (0.003%), higher concentration of Th over U in the solution was expected. The amount of Th and U solubilised by *A. ferrooxidans* increased gradually during the 12 days of incubation in all conditions (MWO: Th = 0.08 ± 0.00 mg L⁻¹, U = 0.09 ± 0.00 mg L⁻¹; MWM: Th = 0.18 ± 0.00 mg L⁻¹, U = 0.06 ± 0.00 mg L⁻¹). These results are in good agreement with previous studies (Corbett et al., 2017; Fathollahzadeh et al., 2018d) showing that the solubilisation of Th and U can be attributed to the presence of an oxidising agent (Fe^{3+}) and acidic pH. It has also been demonstrated that in the presence of bacteria and phosphate released by bacteria, Th mobilisation is restricted. This can be attributed to the formation of secondary Th precipitate, resulting in preferential release of REEs over Th and U (Corbett et al., 2018; Fathollahzadeh et al., 2018d).

It has been suggested that the activity and bioleaching of *A. ferrooxidans* is enhanced at near optimum pH (1.5-2.5) (Bosecker, 1997; Schippers, 2007). Since the initial and final pH of leachate in this study (2.00 ± 0.15 and pH= 1.50 ± 0.15, respectively) was lower than found in the previous study (pH= 2.50 ± 0.15 and pH= 2.50 ± 0.15, respectively) (Fathollahzadeh et al., 2018d), higher REEs dissolution was expected in this study for MWM. The previous study conducted on sterile MWM in BSM (initial pH 2.50±0.15) demonstrated that average final total REEs concentration during 12 days of incubation with *A. ferrooxidans* was 23.6 mg L⁻¹ (Fathollahzadeh et al., 2018d), whereas in this study after inoculation with *A. ferrooxidans* in BSM sterile MWM at a lower starting pH (initial pH 2.00±0.15), average final total REEs concentration increased up to 106 mg L⁻¹ (Fig. 3), 2.1 times higher than abiotic leaching with glycine (50 mg L⁻¹) and 1.6 times higher than abiotic leaching without glycine (66 mg L⁻¹).

As shown in Fig. 3, the change of REEs leached from MWM over time was determined in the leaching solution supplemented with glycine. It can be seen that glycine could markedly diminish the bioleaching of monazite, for which the final total REEs concentration was 1.2 times less (87 mg L⁻¹) than the bioleaching in the absence of glycine (106 mg L⁻¹). Ferric iron generation in *A. ferrooxidans* seemed to be delayed by the presence of glycine, however, the final concentrations of Fe^{3+} in the presence and absence of glycine was in similar range (4.31±0.14 mM and 5.87±0.19 mM, respectively) (Supplementary Table S1). Previous studies

have shown that there is an optimal concentration of glycine (0.5 g L^{-1}) for bioleaching of collophanite (a phosphate mineral) by *A. ferrooxidans* that could facilitate bacterial growth, decrease the leaching pH, and ultimately resulted in the enhancement of bioleaching (Li et al., 2013). However, the bioleaching supplemented with 1 or 2 g L^{-1} glycine resulted in lower acidity and fraction of phosphate leached (Li et al., 2013).

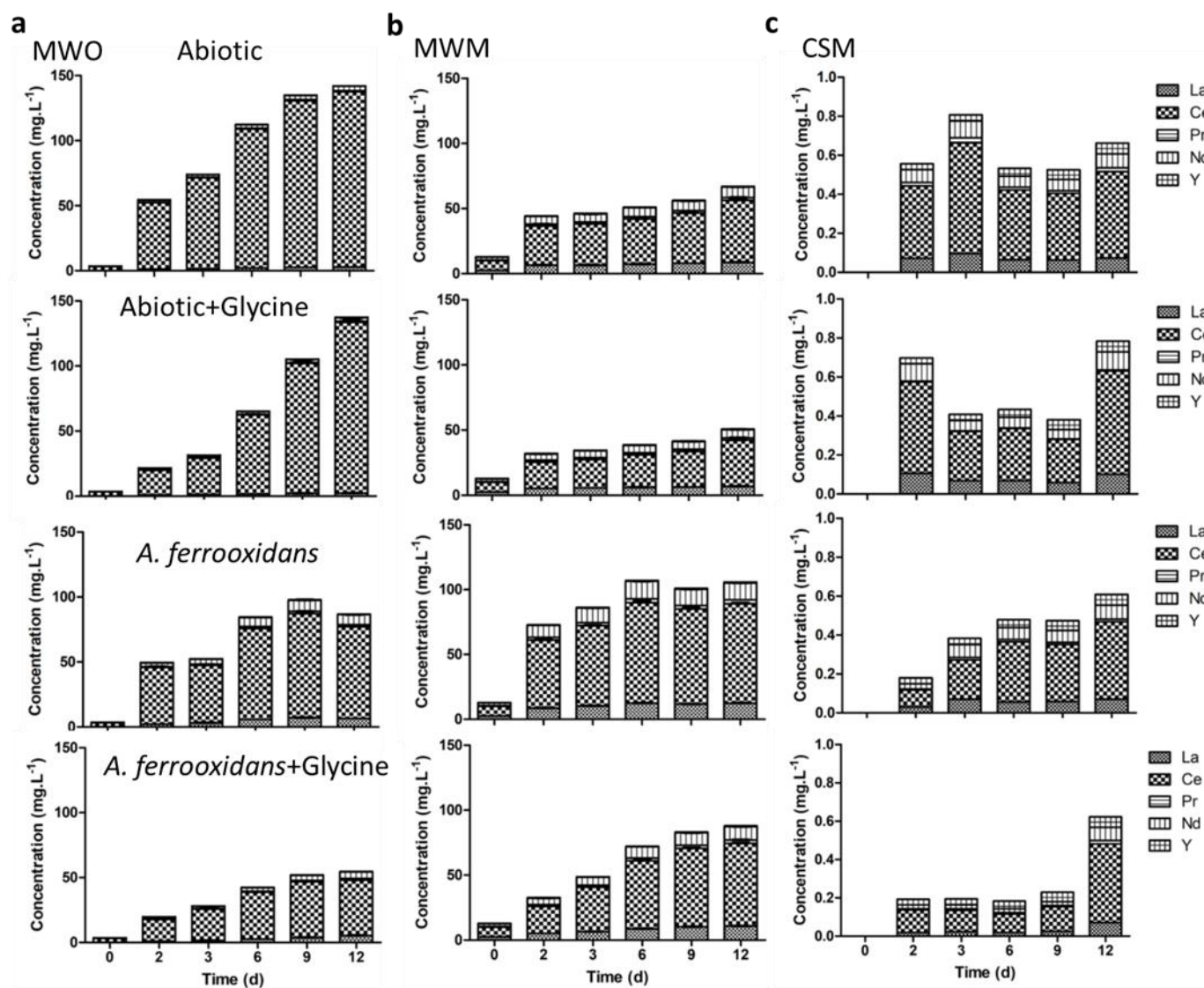


Fig. 3 The concentrations of dissolved La, Ce, Pr, Nd, and Y during the leaching of (a) Mt. Weld ore (MWO), (b) Mt. Weld Monazite (MWM), and (c) Cable Sand monazite (CSM) in the abiotic controls in the presence of FeSO_4 and $\text{K}_2\text{S}_4\text{O}_6$, and in the presence of FeSO_4 and $\text{K}_2\text{S}_4\text{O}_6$ and glycine, and in the presence of *Acidithiobacillus ferrooxidans* with FeSO_4 and $\text{K}_2\text{S}_4\text{O}_6$, and *A. ferrooxidans* with FeSO_4 and $\text{K}_2\text{S}_4\text{O}_6$ and glycine. Data for both abiotic and biotic flasks are averages of triplicate replicates.

The leaching of REEs from MWO was more efficient in the absence of bacteria or glycine than with *A. ferrooxidans* cultures alone or with glycine (Fig. 3). An increase in REEs solubilisation from MWO with BSM was evident from day 2 with a total REEs concentration of 54 to 142 mg L⁻¹ on day 12. The major increase was observed for Ce (Fig.3). The presence of goethite as the dominant mineral in MWO, did not seem to slow down the dissolution of REEs, although the solubilisation of undesirable elements associated with goethite may cause problems in downstream processing. As Mt. Weld deposit's mineralogy has been described as a secondary REEs phosphate, encapsulated in iron oxide minerals (Haque et al., 2014), it is reasonable to assume the REEs bound to surface of Fe-Mn oxy/hydroxides, were remobilized with direct proton attack (Tao & Dongwei, 2014).

The bioleaching of REEs, Th, and U from CSM by *A. ferrooxidans* in all the conditions tested was not comparable to leaching of MWO and MWM (Fig. 3). As highlighted in *section 6.2.1*, in order to work within radiation safety levels, original samples were diluted (10:1 dilution) with silica flour which affected the overall leaching performance (Corbett et al., 2017).

According to the monazite dissolution reaction (reaction 6), the ability of REEs ions to remain in solution upon monazite solubilisation and REEs immobilisation relies on protons and the rate of dissolution will increase as the pH of the leaching solution decreases. Furthermore, glycine leaching tests showed that the presence of glycine to some extent, inhibited both abiotic and biotic leaching. In terms of abiotic leaching processes, the formation of glycine complexes such as glycine sulfate (C₆H₁₇N₃O₁₀S) and glycine phosphate (C₂H₈NO₆P) could suppress overall monazite dissolution according to reaction 2. Obviously further studies on the characterization of glycine complex compounds can contribute to a better understanding of possible role of glycine in monazite dissolution mechanisms. Nevertheless, it has previously been shown that glycine–sulfate buffer has no stimulatory or inhibitory effect on *A. ferrooxidans* (Schnaitman et al., 1969).

6.3.3 Glycine metabolism

Genomic analysis of the potential mechanisms of glycine metabolism of *A. ferrooxidans* demonstrated the presence of glycine oxidase (EC 1.4.3.19) which catalyzes the following chemical reaction (7):



The transformation of glyoxylate to oxalate has been reported in certain bacteria (Singh et al., 2009a), therefore, it is possible that in the presence of glycine some REEs in the leachate are lost due to oxalate-REEs precipitate formation (Goynes et al., 2010; Lazo et al., 2017).

A. ferrooxidans lacks genes for the SoxRS two-component regulator as well as for OxyR, which are regulated in response to superoxide and peroxides respectively in Gram negative bacteria (Valdés et al., 2008). Genomic analysis of *A. ferrooxidans* also showed absence of genes such as catalases for the breakdown of peroxides but *A. ferrooxidans* has previously been found to contain a Fur family regulator similar to PerR which may play a role in the control of its

inducible stress response through regulation of a AhpC family peroxidase (Valdés et al., 2008). However, currently *A. ferrooxidans* response to hydrogen peroxide has not been investigated therefore the direct effects of H_2O_2 accumulation are unknown.

From a molecular perspective, decreased REEs bioleaching by *A. ferrooxidans* in the presence of glycine may also be attributed to changes in membrane potential (Fig. 4).

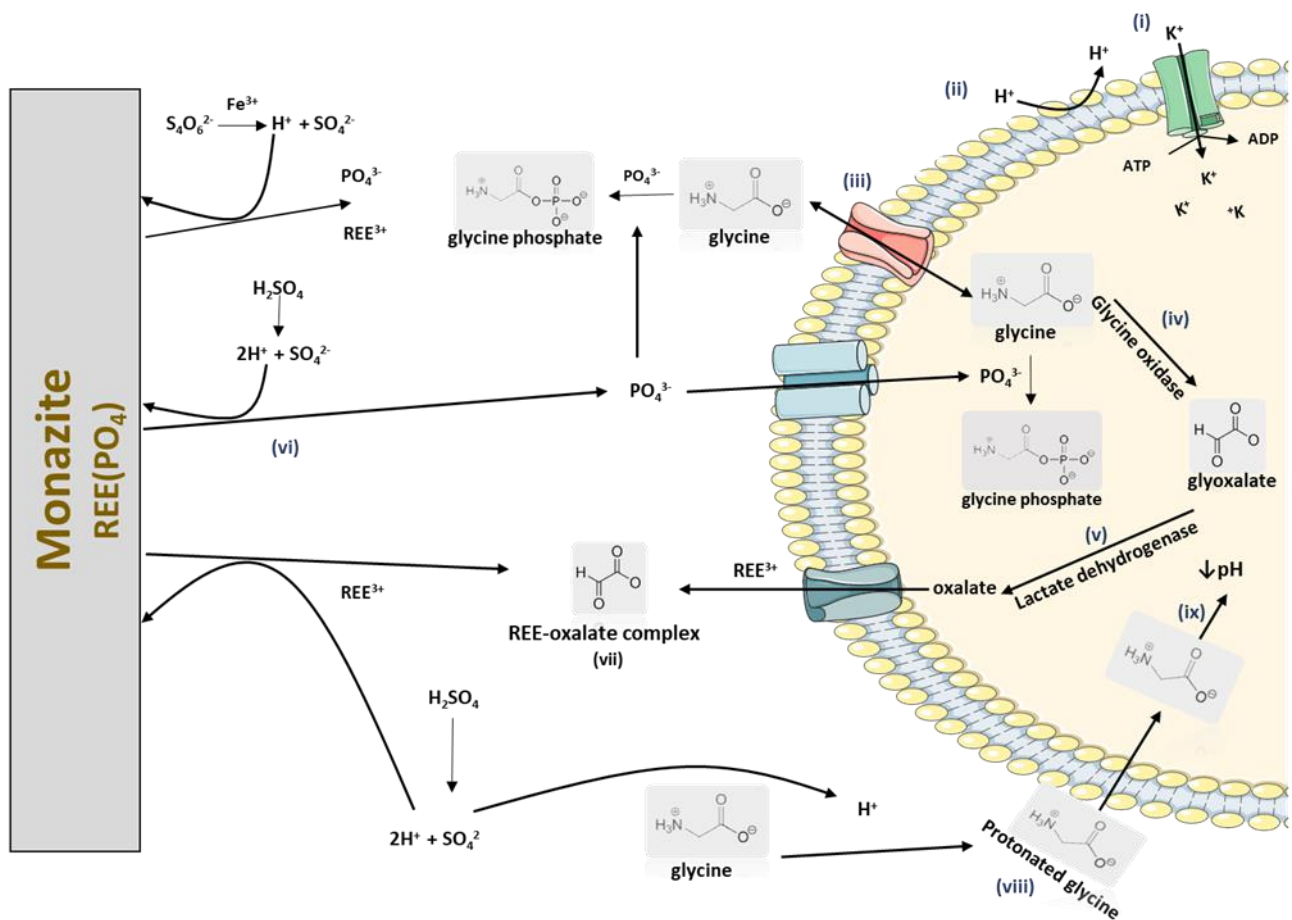


Fig. 4. Schematic representation of monazite dissolution with *Acidithiobacillus ferrooxidans* in the presence of glycine. (i) Active uptake of K⁺ for maintenance of a positive transmembrane potential, (ii) Exclusion of H⁺ as a result of K⁺ accumulation (electrostatic repulsion) (iii) Uptake of glycine through amino acid transporters and permeases, (iv) Conversion of glycine to glyoxylate by glycine oxidase, (v) Conversion of glyoxylate to oxalate by lactate dehydrogenase and subsequent export out of the cell (vi) Attack of monazite by H⁺, resulting in release of PO₄³⁻, which is transported into the cell and forms precipitates with glycine (vii) Attack of monazite by H⁺ to release REEs, which is complexed with oxalate and forms REE-oxalate precipitate, (viii) Protonation of glycine due to excess protons, and (ix) Leakage of protonated glycine into the cell, resulting in decrease in intracellular pH.

The integrity of the bacterial membrane is critical in maintaining their viability and metabolic functions, particularly under stress conditions (Mykytczuk et al., 2007). *A. ferrooxidans* is able to survive an extremely acidic environment due to its ability to maintain a positive membrane potential which reduces the inward flow of protons by electrostatic repulsion (Baker-Austin & Dopson, 2007). In an acidic environment, glycine is protonated and exists as a cation ($^+H_3NCH_2COOH$). While the presence of protonated glycine should be repelled by the cell due to its more positive internal charge, the excess protons outside in combination with protonated glycine may result in H^+ flux that drives the protonated glycine into the cell. Therefore, when protonated glycine enters the cell, it adds to the proton charge, thereby disrupting the transmembrane potential (ÜNLÜ et al., 2002; Vanhauteghem et al., 2012; Vanhauteghem et al., 2013). This increased internal charge in *A. ferrooxidans* and acidification of the cytoplasm, would disrupt membrane integrity and pH homeostasis, ultimately inhibiting growth/oxidation by *A. ferrooxidans* (Baker-Austin & Dopson, 2007).

Genomic analysis of the potential mechanisms of glycine metabolism of *E. aerogenes* demonstrated the absence of glycine dehydrogenase (glyoxylate forming) (EC 1.4.1.10), glycine dehydrogenase (cyanide forming) (EC 1.4.99.5), and glycine oxidase (glyoxylate forming) (EC 1.4.3.19). However, numerous strains of *Pseudomonas aeruginosa* are known to solubilise phosphate and REEs (Hassanien et al., 2013a; Nautiyal, 1999). In a recent study (Kao et al., 2017) reported that the presence of various amino acids slowed down the biofilm formation by *Pseudomonas aeruginosa* but it did not completely inhibit it. Therefore, in this study it may be hypothesized that the addition of amino acids to a PSM strain such as *E. aerogenes* may result in a similar response and thereby inhibit the ability of the microorganisms to solubilise REEs from monazite.

6.4 Conclusions

This study provides the first direct evidence on effect of glycine (1 g L^{-1}) on the bioleaching of REEs by *E. aerogenes* and *A. ferrooxidans* from various grades of monazite obtained from WA. The combination of *E. aerogenes* and glycine decreased REEs bioleaching from monazite (from 6 down to a final concentration of 4 mg L^{-1} REEs including: Ce, La, Nd, Pr, and Y) as compared to abiotic leaching. Also, the combination of *A. ferrooxidans* and glycine decreased REEs bioleaching from monazite (from 106 down to a final concentration of 87 mg L^{-1} REEs including: Ce, La, Nd, Pr, and Y) as compared to abiotic leaching. This was likely attributed to possible toxicity of glycine to the microorganisms at acidic pH. The maximum leaching (142 mg L^{-1} on day 12) was obtained during the abiotic leaching of MWO with BSM (initial pH 2.00 ± 0.15) most likely due to direct attack by protons derived from sulphuric acid.

6.5 Acknowledgments

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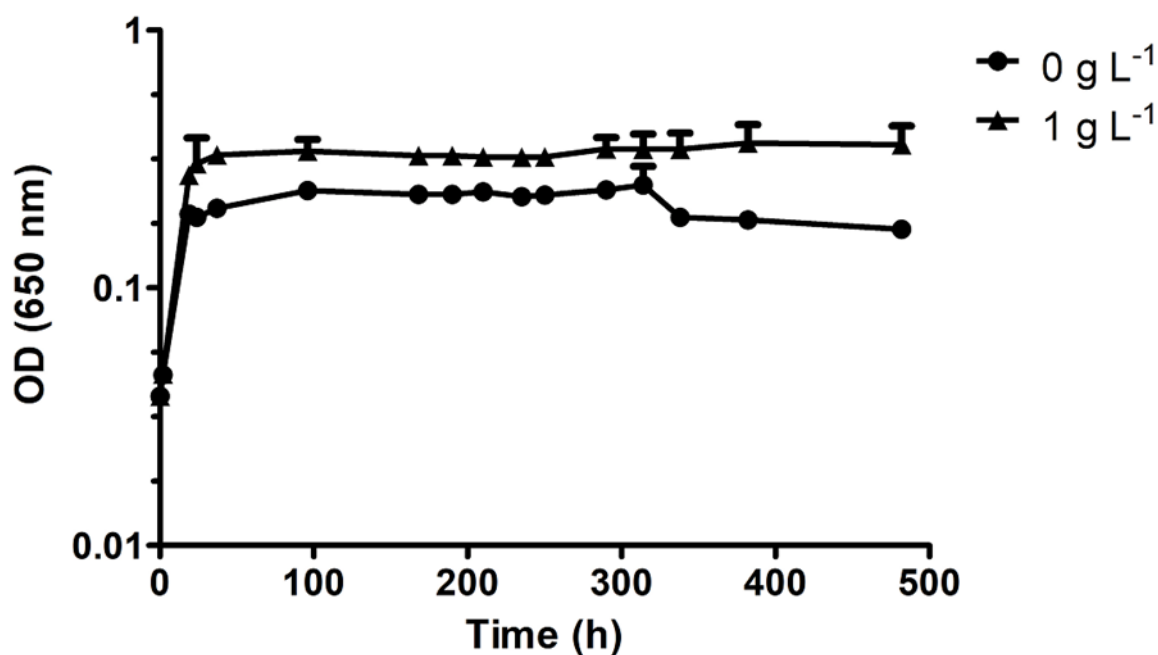
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6.7 Supplementary Files



Supplementary Figure S1. Growth of *Enterobacter aerogenes* without and with glycine (1 g L⁻¹) based on optical density (OD).

Supplementary Table S1 – The total soluble concentration of ferrous and ferric with *Acidithiobacillus ferrooxidans* in the presence of FeSO₄ and K₂S₄O₆, and *A. ferrooxidans* in the presence of FeSO₄ and K₂S₄O₆ and glycine.

	<i>A. ferrooxidans</i>		<i>A. ferrooxidans</i> + glycine	
	Total Fe (mM)	Fe ³⁺ (mM)	Total Fe(mM)	Fe ³⁺ (mM)
0	41±0.79	< 1	41±0.80	< 1
4	44±0.51	< 1	50±2.34	< 1
12	55±1.48	1.85±0.32	55±0.76	< 1
24	55±0.72	7.01±0.15	52±3.47	< 1
32	44±0.18	5.87±0.19	51±0.98	4.31±0.14

Chapter 7: Final discussion and recommendation for future research

Chapter 7: Discussion

7.1 General overview of the research

The ultimate objective of this study was to investigate the use of phosphate solubilising microorganisms (PSMs) in the extraction of rare earths elements (REEs) from phosphate ores, elucidate possible bioleaching mechanisms and the leaching behaviour of individual REEs in the process. In order to achieve the above-mentioned objectives the research plan was divided into three main phases (Fig 7.1), namely (i) bioprospecting, (ii) mechanisms, and (iii) leaching.

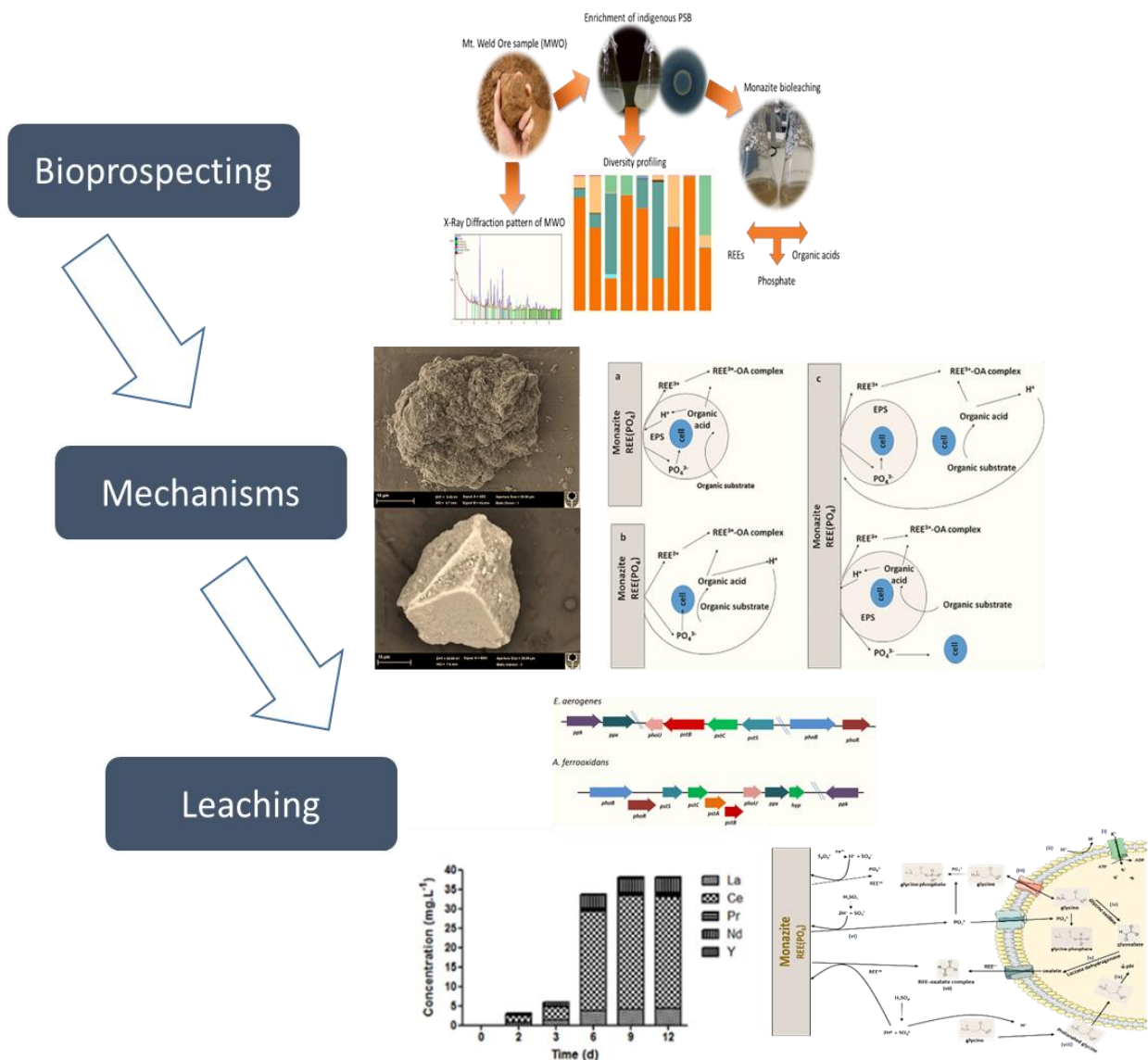


Figure 7.1. Overview of PhD research components.

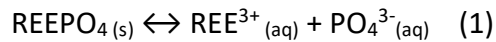
7.2 Final discussion

The initial objective of the study was to assess the function and diversity of indigenous phosphate solubilising bacteria (PSB) enriched from Mt. Weld deposit and evaluate their capabilities for the bioleaching of REEs from three different grades of monazite bearing ores. The detailed experimental setups were described in *chapter 3*. When grown in National Botanical Research Institute's phosphate growth medium (NBRIP) broth on two different inorganic P sources ($[\text{Ca}_3(\text{PO}_4)_2]$ referred to as TCP and $[\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}]$ referred to as CaP), it was found out that the nature of inorganic phosphate source affected the microbial composition of the PSB. The analysis of the community diversity profiles revealed that *Actinobacteria* was identified in all samples and *Micrococcales* was the most abundant order in the subcultured enrichments. The Shannon diversity index for cultures enriched on CaP (0.83) was higher than those enriched on TCP (0.73) which is consistent with higher solubility of CaP compared to TCP.

After bioprospecting of native PSB, bioleaching capabilities of the TCP enrichment cultures and the mixed culture (TCP:CaP) was carried out on 1% pulp density of sterilized monazite sample. The results of the monazite bioleaching showed that by day 7, the mixed cultures were able to mobilize REEs (Ce, La, and Nd) more efficiently than TCP enrichment cultures (up to a final concentration of 0.836 mg L^{-1} vs. 0.375 mg L^{-1}). It is also consistent with higher organic acid production by the mixed cultures. The total concentration of organic acids was higher in the mixed culture (1.93 mM) compared to TCP enrichment cultures (0.54 mM). Higher bioleaching by the mixed cultures was possibly due to established synergism between strains of the mixed cultures which enhanced phosphate solubilisation and organic acid production when compared to either of the less diverse microbial consortia.

The extent to which REEs solubilise from monazite depends on microbial activity, attachment of bacteria on the mineral surface, phase association of the REEs, and which physiochemical and biological processes these phases are subjected to. *Enterobacter aerogenes* was used as a model of demonstrated PSB to increase the understanding of the biological controls of the mineral dissolution process. The detailed experimental setups were described in *chapter 4*. A systemic study of the mechanisms of bioleaching REEs from monazite with *E. aerogenes* provided the first evidence of microbial bio-mobilization mechanisms involved in REE dissolution in terms of the importance of the microbial colonization of mineral surfaces. This was achieved by allowing the bacteria to either be in contact with the monazite surface or to prevent contact between the bacteria and the mineral. Contact of the bacteria with the monazite surface was found to result in greater leaching of REEs compared to the presence of the same types of organic acids with similar concentrations during non-contact leaching. Monazite dissolution (1% pulp density) was observed to decrease in the following order: Biotic contact \gg Biotic non-contact \gg Spent media \approx Abiotic at 30 °C. The lower REEs leaching in spent media and abiotic conditions suggested that the presence and attachment of bacteria contributed directly to the higher REEs mobilisation. No preferential attachment of bacteria to the monazite surface was demonstrated by a co-localised atomic force microscopy and confocal Raman microscopy. Data from the monazite dissolution was used to propose a

conceptual model to combine the main phenomena affecting REE leaching, namely contact, non-contact, and cooperative leaching. On the one hand, in the contact leaching, attached microbial cells mobilize phosphate within a matrix of EPS and release REE cations into solution. Organic acids released by the cells from organic substrates complex with REE cations. Protons released from organic acids attack the ore resulting in further phosphate dissolution. Incorporation of phosphate into the cells increases REE³⁺ solubility according to following reaction (1):



On the other hand, in the non-contact mechanism suspended cells generate REE³⁺ complexing organic acids and incorporate phosphate into cells increasing REE³⁺ solubility. The protons released from organic acids attack the ore resulting in further REE cations and phosphate dissolution.

It is likely that in addition to both contact and non-contact mechanisms, cooperative mechanism may also contribute to the leaching. In the cooperative mechanism, attached cells mobilize phosphate from monazite and incorporate it into cells releasing REE³⁺ while suspended cells generate organic acids for REE³⁺ complexation and protons released from organic acids attack the ore. Alternatively, attached cells may play a role in organic acid generation while suspended cells take up phosphate from solution increasing REE³⁺ solubility. These mechanisms ensure that the bacterial phosphate requirements are met, even when the external environment is phosphate depleted, via monazite dissolution.

The continuous carbon and energy requirement of heterotrophic bioleaching microorganisms (e.g., *E. aerogenes*) to maintain microbial activity can be problematic at large scale bioleaching operations, however the addition of acidophilic autotrophic bioleaching microorganisms (e.g., *Acidithiobacillus ferrooxidans*) to these systems can potentially advance bioleaching performance. Bioleaching capabilities of *E. aerogenes* and *A. ferrooxidans* were explored using 1% pulp density of sterilized monazite sample. Also, a two-step bioleaching system was proposed where the metabolites generated by *E. aerogenes* resulted in pH reduction negating the need for manual pH adjustment for *A. ferrooxidans*. The detailed experimental setups were described in *chapter 5*. Following the dissolution of monazite by *E. aerogenes*, the total concentration of REEs (Ce, La, Pr, Nd, and Y) in the leachate increased from 2.90 at day 2 to 5.84 mg L⁻¹ at day 12. Heterotrophic culture of *E. aerogenes* generated a range of organic acids including gluconic, malic and acetic acids. Higher REEs bioleaching with *E. aerogenes* comparing to the indigenous mixed culture described in *chapter 3* can be attributed to the production of dicarboxylic acids (i.e., malic acid) which dissolve REEs more effectively. On the other hand, the presence of *A. ferrooxidans* either with FeSO₄ and K₂S₄O₆ or sterilized pyrite as a source of Fe and S enhanced REEs solubilisation compared to that achieved with *E. aerogenes* (average final total REEs concentration during 12 days of incubation: 23.6 mg L⁻¹ and 10.6 mg L⁻¹, respectively). This can be explained in terms of mechanisms by which

elements are released into the leachate (biogenic acid and ferric iron generation) and the co-precipitation of soluble complexes is minimized.

Monazite was more efficiently solubilised in media co-inoculated with *E. aerogenes* and *A. ferrooxidans* than with individual cultures. The co-culture system demonstrated the enhanced REEs solubilisation with a total soluble REEs concentration of 40 mg L⁻¹ (Ce, La, Pr, Nd, and Y) on day 9. The heterotrophic dissolution of REEs and P from monazite is mostly governed by acido-complexolysis of organic acids. An alternative possibility was that the bacteria, in particular *E. aerogenes* have utilised the soluble P for cellular requirements. After co-inoculation with *A. ferrooxidans*, overall P solubilisation improved. The higher REEs concentration in the leachate of co-culture system also appeared to be in good agreement with higher and stable release of Fe in the co-culture (60 mM) compared to *A. ferrooxidans* (21 mM) which potentially contributed to the stability of organic acids, thereby prolonging their complexing capacity with REEs. Moreover, comparative genomic study of the potential mechanisms of phosphate metabolism during the interaction of *E. aerogenes* and *A. ferrooxidans* suggested the organization of pho operon was responsible for differences in monazite bioleaching. This confirmed that *E. aerogenes* was likely to only transiently accumulate polyphosphate and is required to uptake any phosphates released by *A. ferrooxidans* in order to overcome phosphate starvation. This also explained the reduced concentration of phosphate in the media of the co-culture system (3.33 mg L⁻¹), as compared to the concentration of phosphate present when *A. ferrooxidans* was used as a pure culture (33 mg L⁻¹).

The X-ray absorption near-edge spectrum (XANES) studies revealed that oxidation of REEs (Ce³⁺ to Ce⁴⁺) did not occur during the bioleaching of monazite. This confirmed that the dissolution mechanism of Ce as a model element for REEs was controlled by chelation with anions from organic acids. Moreover, characterization of changes in REEs and Th fractionation during bioleaching with *E. aerogenes* and *A. ferrooxidans* demonstrated that bacterial dissolution was effective in stimulating REEs mobility as indicated by the increase of REEs in easily extractable and acid soluble fractions. The bioleaching induced REEs association into the acid soluble fraction suggests that phosphate chemistry in solution was most likely the limiting step to REEs mobilisation. Also, increased association of Th with residual fraction after bioleaching, suggested that bioleaching favour solubilisation of REEs over actinides, potentially decreasing environmental hazards associated with these minerals. Bacterial metabolism (phosphate acquisition, storage, and complexation) appear to play a key role in controlling overall solubilisation processes. Therefore, providing acidic conditions that maintain REEs-phosphate complexes dissolved in solution, potentially contribute to increase in overall REEs dissolution.

Adding 1 g L⁻¹ of glycine into the leaching solution either with *E. aerogenes* or *A. ferrooxidans* was found to result in lower leaching of REEs compared to absence of glycine. The detailed experimental setups were described in *chapter 6*. The combination of *E. aerogenes* and glycine decreased REEs bioleaching from monazite (1% pulp density) (from 6 down to a final concentration of 4 mg L⁻¹ REEs including: Ce, La, Nd, Pr, and Y) as compared to abiotic leaching. Also, the combination of *A. ferrooxidans* and glycine decreased REEs bioleaching from

monazite (1% pulp density) (from 106 down to a final concentration of 87 mg L⁻¹ REEs including: Ce, La, Nd, Pr, and Y) as compared to abiotic leaching. This was likely attributed to increased internal charge and acidification of the cytoplasm and oxalate-REEs precipitate formation in *A. ferrooxidans*.

Overall, in this study, exploring REEs solubilisation by PSB has allowed for a deeper understanding of the mechanisms and highlighted the potential applicability of the use of members of bacteria as an alternative to current conventional processes used for REEs extraction that cannot selectively solubilise REEs over radionuclides. The laboratory based study strongly support the concept of sustainable mining for REEs extraction, especially if conditions for biogenic generation of organic acids and sulfuric acid for enhanced protonation and complexation is established.

7.3 Future Considerations

It has been shown that key differences exist between the members of PSMs in term of secreted metabolites, including organic acids and biogenic sulfuric acid. Further work is required to understand the underlying metabolic basis of phosphate and REEs solubilisation. This would also enable to highlight the fate of phosphate and REEs within the bacteria-mineral-solution interface. Furthermore, metagenomic, transcriptomic, proteomic as well as metabolic studies are needed to unveil the biochemical and molecular mechanisms used by PSMs during phosphate and REEs solubilisation.

8 Appendices

8.1 Appendix 1

The following pages contain the original reprints of publications.



Microbial contact enhances bioleaching of rare earth elements

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ABSTRACT

The mobility of rare earth elements (REEs) in monazite depends on microbial activity, attachment of bacteria on the mineral surface, phase association of the REEs, and which physiochemical and biological processes these phases are subjected to. To better understand the role of the phosphate solubilising bacterium, *Enterobacter aerogenes*, in REEs leaching, a series of monazite dissolution experiments was performed. The contact of bacteria with monazite was demonstrated to be advantageous for REEs bioleaching even though the same types of organic acids with similar concentrations were present during non-contact leaching. Monazite dissolution was observed to decrease in the following order: Biotic contact >> Biotic non-contact >> Spent media ≈ Abiotic at 30 °C. The attachment of bacteria on monazite surface by a co-localised atomic force microscopy (AFM) and confocal Raman microscopy (CRM) indicated no preferential attachment of bacteria to specific site on the monazite surface.

1. Introduction

In the last decade, rare earth elements (REEs), have been considered as “critical and strategic metals”, due to China's monopoly position and increased global demand in green technologies. Although REEs are relatively abundant in the Earth's crust, they are not evenly distributed around the world, and are mainly produced and processed in China (Ganguli and Cook, 2018; Zepf, 2013). Consequently, the prediction of exhaustible resources such as REEs is of profound significance, in that it not only aids governments to establish long-term resource plans but also contributes to maintain sustainable social and economic development (Wang et al., 2015). Considering the constant development of REEs industries, the Generalized Weng model, a widely used quantitative model in exhaustible resource forecast has been adopted to predict the production of the three major REEs in China (i.e., mixed rare earth, bastnasite and ion-absorbed rare earth) (Wang et al., 2015). The results suggested that countries with REEs resources should commence or continue their production to gradually decline dependency on China's supply (Wang et al., 2015).

Apart from the geopolitical challenges in REEs production, environmental issues can be a major concern as the extraction of REEs

from their ores requires significant processing (Goodenough et al., 2018). The current conventional REE production, relies on high temperatures and harsh chemical treatments, has high energy consumption, and generates large volumes of toxic waste containing thorium, uranium, hydrogen fluoride, and acidic waste water (Hurst, 2010). Furthermore, as REEs-bearing ores may contain up to 10% thorium and uranium (Ragheb, 2011), emission of radioactive waste associated with REEs mining and extraction results in either contamination of the final REEs concentrate or the requirement for complicated disposal protocols (Ault et al., 2015). It has been reported that the environmental life cycle impacts of REEs production during chemical leaching are far greater than those for other metals (Vahidi and Zhao, 2016). Consequently, due to environmental restrictions, sustainable mining and production are now encouraged. Biotechnological mineral processing approaches have been developed as a sustainable alternative to chemical leaching of ores and waste streams. Biohydrometallurgy utilises microorganisms to generate bio-lixivants which accelerate the dissolution of elements from their ores or other materials (Watling, 2016). Bioleaching processes are generally operated at relatively low temperature and atmospheric pressure, which reduces energy cost and gas emissions, and without relying on expensive and aggressive reagents (Bryan et al.,

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2015).

Despite the significant contribution of bioleaching to the extraction of base metals from sulfide minerals, very few studies have explored the application of microbes, in particular phosphate solubilising microorganisms (PSMs), to monazite and other phosphate minerals hosting REEs. [Brisson et al. \(2016\)](#) demonstrated the bioleaching of REEs (3–5% recovery) from monazite sand as the sole phosphate source by three phosphate solubilising fungi. In another study, [Shin et al. \(2015\)](#) examined the feasibility of using phosphate solubilising bacteria (PSB) for the bioleaching of REEs from monazite-bearing ore with maximum leaching yield for cerium (up to 0.13%).

The previous studies on REE bioleaching have focused on efficiency ([Brisson et al., 2016](#); [Hassanien et al., 2013](#); [Shin et al., 2015](#)) whereas little is known of the mechanisms involved and benefits of REEs dissolution to the microbes. Adhesion and colonization of the mineral surface are survival mechanisms for bacteria with nutrients in aqueous environments more accessible at surfaces ([Busscher and van der Mei, 2012](#)). Many studies on sulfide minerals demonstrate that microbial attachment and biofilm formation can stimulate pyrite bioleaching ([Sand and Gehrke, 2006](#)). [Corbett et al. \(2017\)](#) demonstrated that *Enterobacter aerogenes* leached 43% of the phosphate from tricalcium phosphate ($\text{Ca}_3(\text{PO}_4)_2$ or TCP) after 192 h, and of 12 known PSB released the greatest amount of REE from a monazite. Therefore, in this study, the mechanisms of bioleaching REE from monazite were systematically investigated with *E. aerogenes*. The study was designed to allow the bacteria to either be in contact with the monazite surface or to prevent contact between the bacteria and the mineral enabling us to investigate microbial bio-mobilization mechanisms involved in REE dissolution in terms of the importance of microbial colonization of mineral surfaces. Data from the monazite dissolution was used to develop a conceptual model to integrate the main phenomena affecting REE leaching. The results from this study will facilitate the development of sustainable bio-mining approaches REE extraction.

2. Material and methods

2.1. Monazite ore

The high grade weathered yellowish monazite ore was collected from the Mount Weld Mine (Lynas Corporation), and is hereafter referred to as MWM (Mt. Weld Monazite). Sample preparation and composition analysis were described elsewhere ([Corbett et al., 2017](#)). The total surface area of the MWM was $24,000 \text{ cm}^2 \text{ g}^{-1}$ as determined by Brunauer–Emmett–Teller (BET) analysis at CSIRO Minerals, Waterford, Western Australia. The BET surface area ($\text{cm}^2 \text{ g}^{-1}$) was analysed by the N_2 adsorption method at the temperature of liquid nitrogen ($-196 \text{ }^\circ\text{C}$) in a Micromeritics Gemini III 2375 (USA). Prior to the nitrogen adsorption measurements, each sample (approximately 0.6 g in weight) was degassed at $150 \text{ }^\circ\text{C}$ for 3 h in vacuum. The BET surface area was determined by using the N_2 adsorption data at 5 different standard pressures (0.05, 0.15, 0.2, 0.25 and 0.3) at $-196 \text{ }^\circ\text{C}$. Any results were rejected and the samples re-tested if the correlation coefficient of a plot of the ‘BET Function’ through the 5 points was lower than 0.9997. Before bioleaching experiments, the mineral samples were sterilized by gamma irradiation at 50 kGy for 11 h (ChemCentre, Bentley, Western Australia).

2.2. Bioleaching experiment

Enterobacter aerogenes (ATCC® 13,048™) was grown to exponential phase at $30 \text{ }^\circ\text{C}$ in National Botanical Research Institute Phosphate (NBRIP) medium ([Nautiyal, 1999](#)), with shaking at 140 rpm, and harvested by centrifugation (3600g, 10 min). Cells were resuspended in sterile Tris-HCl buffer (100 mM, pH 7.2), centrifuged (3600g, 5 min) and washed twice more to remove any trace of phosphate. The ability of *E. aerogenes* to bioleach MWM as a phosphate source, was evaluated in

500 mL Erlenmeyer flasks. Bioleaching was carried out over 18 days at $30 \text{ }^\circ\text{C}$ in triplicate, at 120 rpm in an orbital shaking incubator (RATEK, Model No: OM11) in 200 mL of modified NBRIP media (3% w/v glucose and $\text{pH } 7.00 \pm 0.25$), with 0.5% v/v bacterial inoculum (initial density $1 \times 10^7 \text{ cells mL}^{-1}$) and 1% pulp density of sterilized monazite. Cell-free abiotic controls were carried out under the same conditions. Bioleaching was monitored by measuring the soluble concentration of Ce, La, Pr, Nd, Fe, P, Th, U, and Y in the leachate.

Non-contact experiments were conducted in similar conditions to those described above. Snakeskin® dialysis tubing (10 K MWCO, 35 mm, ThermoFisher SCIENTIFIC, catalogue number 88245) with Snakeskin™ Dialysis Clips (ThermoFisher SCIENTIFIC, catalogue number 68011) were used to study the possible mechanisms for leaching REE from monazite as follows:

1. For biotic contact leaching of monazite, dialysis bag, 200 mL media, and 1 mL of bacterial suspension were placed in 500 mL Erlenmeyer flasks. The monazite in this experiment was not sealed in the dialysis bag, so that bacteria were free to colonize monazite surfaces.
2. For abiotic contact leaching monazite and media were placed in 500 mL Erlenmeyer flasks.
3. For biotic non-contact leaching the monazite was sealed in the dialysis bag. This sealed dialysis bag, media, and 1 mL of bacterial culture were placed in Erlenmeyer flasks.
4. For abiotic non-contact leaching monazite was sealed in dialysis bag. This sealed dialysis bag and media were placed in Erlenmeyer flasks.

The pore size of the dialysis bag is sufficiently small to prevent bacterial migration through the bag, but large enough to allow the homogenous transfer of nutrients for bacterial growth.

Molar dissolution rates (r) per surface area of the ore and time ($\text{mol cm}^{-2} \text{ s}^{-1}$) were calculated as using Eq. (1):

$$r = \frac{r_{\text{volumetric}}}{c_{\text{solids}} \times M \times A} \quad (1)$$

where $r_{\text{volumetric}}$ refers to the volumetric leaching rate ($\text{g L}^{-1} \text{ s}^{-1}$) obtained from the slope of the soluble element concentration versus time plot, c_{solids} represents the initial solid concentration in the flasks (10 g L^{-1}), M is molar mass of the element (140.1, 138.9, and 88.9 g mol^{-1} for Ce, La, and Y, respectively), and A is the total mineral surface area ($\text{cm}^2 \text{ g}^{-1}$) obtained with BET.

2.3. Leaching of MWM with spent media

Pregnant solutions were prepared as described in [Section 2.2](#). After 24 h incubation and pH decrease ($\text{pH} = 3.4$), the media was aseptically filtered ($0.20 \text{ } \mu\text{m}$, Satorius). One gram of MWM was added to 50 mL of the filtered spent medium in 200 mL flask and incubated at $30 \text{ }^\circ\text{C}$ with shaking at 120 rpm for six days. Leaching was monitored by measuring the soluble concentration of Ce, La, Pr, Nd, Fe, P, Th, U, and Y in the leachate.

2.4. Analytical methods

Samples were taken at 0, 2, 3, 6, 9, 12, 18 d and pH measured using a pH meter (Ionode IJ series pH probe). Thereafter, samples were filtered ($0.20 \text{ } \mu\text{m}$, Satorius) and assayed for REEs, Y, Th and U concentrations by ICP-MS (Agilent Technologies 7700 series, Bureau Veritas Australia Pty Ltd., Canning Vale, Western Australia) and the average values were reported. Organic acids were identified by high performance liquid chromatography (HPLC) (Agilent 1200, Curtin Water Quality Research Centre, Bentley, Western Australia) coupled with a diode array detector (DAD, Agilent). Injection volume was set as $50 \text{ } \mu\text{L}$ for the samples. Compound separation was achieved with a C18 reverse phase column (Agilent, $5 \text{ } \mu\text{m}$, $4.6 \times 250 \text{ mm}$). The isocratic

elution flow rate was 1.0 mL min^{-1} . The mobile phase consisted of 70% methanol and 30% phosphate buffer ($\text{pH} = 2.0$). A detection wavelength of 220 nm was used. The identity and concentration of organic acid was determined by comparing the retention times and peak areas of chromatograms of the samples with standards. Organic acid identity was confirmed by liquid chromatography tandem-mass spectrometry (LC-MS/MS), and the experimental setup for LC-MS/MS including some exemplary mass chromatograms of organic acids were described elsewhere (Busetti et al., 2014). Organic acids standards included gluconic, malic, formic, butyric, citric, acetic, lactic, oxalic, and pyruvic acids. Microbial cells were counted using a Helber bacteria counting chamber (Thoma rule, Hawksley UK) at $400\times$ magnification.

Scanning electron microscopy (SEM) of the bioleaching residue was performed on a Zeiss Evo 40XVP SEM (John de Laeter Centre, Curtin University, Western Australia).

Co-localised atomic force microscopy (AFM) and confocal Raman microscopy (CRM) measurements were performed on a WITec Alpha 300SAR (WITec GmbH, Ulm, Germany). The samples were mounted on a purpose built re-location stage, allowing returning to the same sample area. AFM data were acquired in intermittent contact mode in air utilizing standard probes with a resonant frequency of 300 kHz and a spring constant of 40 N m^{-1} (type NCH-VA, Bruker, Santa Barbara, USA).

For the confocal Raman measurements a frequency double NdYAG laser ($\lambda = 532 \text{ nm}$) was used for excitation and the Raman spectra were collected through a $100\times$ objective with a numerical aperture of 0.9 (Zeiss, Germany) and fed via a $50 \mu\text{m}$ optical fibre into the spectrometer. For AFM and CRM measurements, a pure monazite crystal (Lynas, Australia) was embedded in epoxy resin, cut and polished to obtain a suitable flat sample surface (with a thickness of 1 up to 2 mm and size of 1 cm^3). MWM fine grains were also embedded in epoxy resin. Both samples were cleaned and sterilized in ethanol, nitrogen gas and an UV/ozone cleaner prior to exposure to *E. aerogenes*. The sample was exposed to 1% v/v bacterial inoculum (initial density $1 \times 10^7 \text{ cells mL}^{-1}$) and 18 mL of modified NBRIP with shaking at 120 rpm in 100 mL Erlenmeyer flask for 24 h at 30°C .

3. Results and discussion

3.1. Organic acid profile

Glucose was the carbon source available to *E. aerogenes* in both the contact and non-contact bioleaching experiments, where the bacteria produced malic, acetic and gluconic acid (Fig. 1). Corbett et al. (2017) have reported the release of citric and formic acids in addition to gluconic and malic acids, by *E. aerogenes*. It has been suggested that dicarboxylic (malic and oxalic) and tricarboxylic (citric) acids rather than monocarboxylic acids (acetic, formic, and gluconic) govern REE dissolution due to having a high affinity and stability to trivalent metals such as REEs (Jones, 1998). Previous studies with *E. aerogenes* and insoluble phosphate complexes have reported gluconic acid concentration

up to 1 mM at day 4 (Stella and Halimi, 2015) which is in good agreement with this study. Johnston (1952) reported that the phosphate solubilisation potential of organic acids is related to the structural characteristics of the acid, thereby the concentration of organic acids as well as their structure and stability of ligands should be taken into account. Of those organic acids detected, only malic acid is dicarboxylic which make it a stronger acid ($\text{pK}_a = 3.40$) comparing to acetic acid ($\text{pK}_a = 4.75$), however, gluconic acid has a pK_a of approximately 3.60. Citric and formic acids were not detected in the present study, and hence may not be attributed to REE dissolution by *E. aerogenes* when grown on NBRIP. The short half-life of organic acids (e.g., citrate 2–6 h) (Van Hees et al., 2003), unidentified acids with no standards solution (Brisson, 2015), and the overlapping of the peaks of different acids in HPLC may have hindered the detection of some other organic acids in this study which may have been effective at solubilising REE from monazite.

Although, the organic acid profile of both contact and non-contact bioleaching were similar (Fig. 1), contact bioleaching resulted in higher REE dissolution compared to non-contact leaching (Fig. 2). Therefore, monazite dissolution may not be solely achieved by organic acids.

3.2. Monazite dissolution during contact, non-contact, and spent medium bioleaching

In order to assess the necessity of contact between microbial cells and mineral in the leaching of REE from monazite, a series of monazite dissolution experiments was conducted. During the contact bioleaching experiments with monazite the pH of the leachate decreased to 3.39 ± 0.08 by day 2, whereas with non-contact bioleaching it decreased to 3.47 ± 0.04 by day 2. The concentration of soluble Ce, La, and Nd in contact bioleaching (2.55 , 0.57 , and 0.36 mg L^{-1} on day 18, respectively) was higher than other elements as expected due to higher content in the ore. On the other hand, much lower soluble Ce, La, and Nd concentration were detected in non-contact leachate (0.66 , 0.16 , and 0.12 mg L^{-1} , respectively on day 18). After 48 h, soluble Ce concentrations were 2.61 times higher for contact bioleaching than for non-contact bioleaching and reached to 3.82 times higher concentration by day 18 (Fig. 2). When exploring the possible leaching mechanisms in the present study, monazite dissolution was observed to decrease in the following order: Biotic contact \gg Biotic non-contact \gg Spent media \approx Abiotic. Exposure of MWM to spent media resulted in lower REEs leaching compared to biotic contact and non-contact (data not shown). On the one hand, as there is no phosphate consumption in spent media leaching precipitation and formation of secondary phosphate minerals increased, where overall REEs leaching decreased. Furthermore, as metabolic activity was not occurring, continuous organic acid production was minimized in spent media as well which is consistent with increased pH up to 4.50 ± 0.01 by day 6. At higher pH the precipitates would remain in insoluble forms. The soluble concentrations of elements in the spent media and abiotic controls were near or below detection limits (Ce, La, Pr, Nd, Th, U, and Y $< 1 \mu\text{g L}^{-1}$;

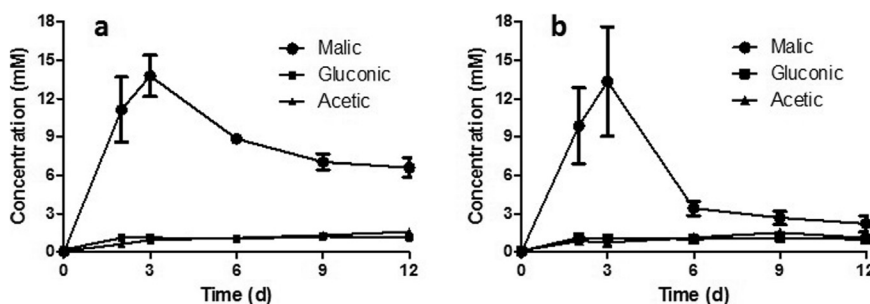


Fig. 1. Organic acid concentration (mM) by *Enterobacter aerogenes* after 12 days of incubation in the presence of Mount Weld monazite (MWM) under (a) biotic contact and (b) biotic non-contact conditions. Error bars (SE) represent standard error between three replicate flasks. Error bars not visible are smaller than symbols.

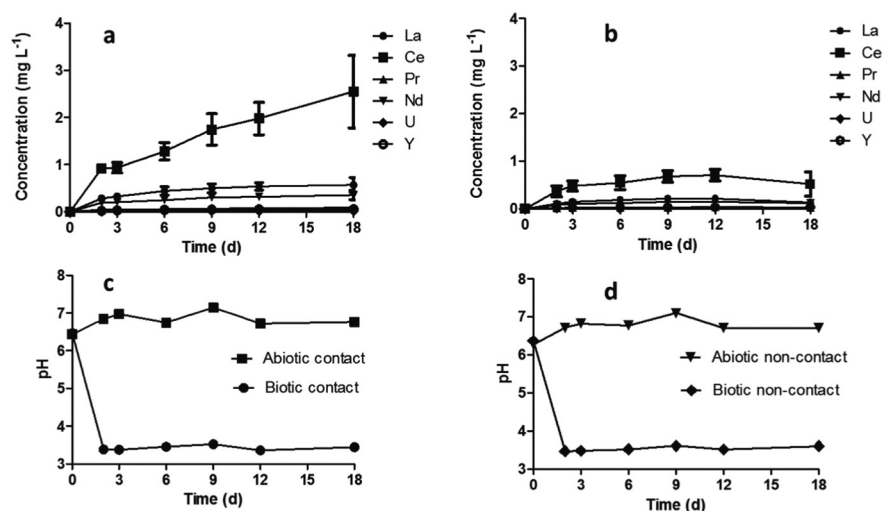


Fig. 2. Leaching of MWM by *Enterobacter aerogenes*. Dissolved La, Ce, Pr, Nd, U, and Y under (a) biotic contact and (b) biotic non-contact, observed pH change under (c) contact and (d) non-contact conditions. Error bars represent standard error between three replicate flasks. Error bars not visible are smaller than symbols. The concentration of REEs and U in abiotic flasks remained below detection levels throughout the experiment (data not shown).

$Fe < 0.5 \text{ mg L}^{-1}$, and $P < 2 \text{ mg L}^{-1}$). As noted above, lower REEs leaching in spent media and abiotic suggesting that the presence and attachment of bacteria contributed directly to higher REEs leaching.

In comparison to conventional monazite processing, where most of Th is leached to solution (Peelman et al., 2014), in the present study no Th was observed in leachate of either the contact or non-contact bioleaching. On the other hand, soluble Y concentration reached an average of 0.0563 ± 0.010 and $0.0262 \pm 0.005 \text{ mg L}^{-1}$, during contact and non-contact bioleaching, respectively, suggesting preferential release of Y over actinides considering the similar contents of Th and U in the MWM.

Non-steady Ce, La, and Y dissolution rates during contact bioleaching of MWM with *E. aerogenes* at 30°C on day 3 were 1.14×10^{-17} , 3.93×10^{-18} , and $5.7 \times 10^{-19} \text{ mol cm}^2 \text{ s}^{-1}$, respectively. By day 18 the rates slowed down to 4.37×10^{-18} , 9.30×10^{-19} , and $1.52 \times 10^{-19} \text{ mol cm}^2 \text{ s}^{-1}$, respectively. On the other hand, non-steady Ce, La, and Y dissolution rates during non-contact bioleaching from MWM at 30°C on day 3 were 5.60×10^{-18} , 1.73×10^{-18} , and $2 \times 60 \times 10^{-19} \text{ mol cm}^2 \text{ s}^{-1}$, respectively, and by day 18 the rates slowed down to 1.07×10^{-18} , 2.64×10^{-19} , and $8.14 \times 10^{-20} \text{ mol cm}^2 \text{ s}^{-1}$, respectively. This confirmed higher REE dissolution (2 to 4 times) attributed to bacterial attachment. Moreover, when considering the equilibrium between monazite, the dissolved ions (Ln^{3+} represents REEs) and very low solubility of monazite (10^{-13} M) (Firsching and Brune, 1991), pico-molar concentrations of REEs and phosphate can produce saturation and supersaturation, according to reaction (2). Thus, phosphate abundance through REE precipitation and co-precipitation may influence REE distribution in solution, exhibiting distinctive REE dissolution rate (Goyne et al., 2010).



The SEM photomicrographs (Fig. 3) also demonstrated breakdown of the monazite surface (due to biofilm formation) with contact leaching while the mineral surface remained intact after non-contact leaching. Thus, the data suggested a microbially mediated REE dissolution on mineral surfaces through contact mechanism.

The atomic force microscopy scans of the surface of the monazite crystal and MWM after 24 h of exposure to *E. aerogenes* clearly show the attachment of bacteria to the crystal and MWM surface (Fig. 4). The images show a range of clusters as well as solitary bacteria on the monazite surface. The confocal Raman microscopy image indicates that the surface chemistry of the investigated sample is composed of REEs (i.e., Ce^{3+}) and PO_4^{3-} and no other major heterogeneous mineral phases were observed. Changes in the intensity in the presented Raman map are due to variations in the sample topography. Comparing the

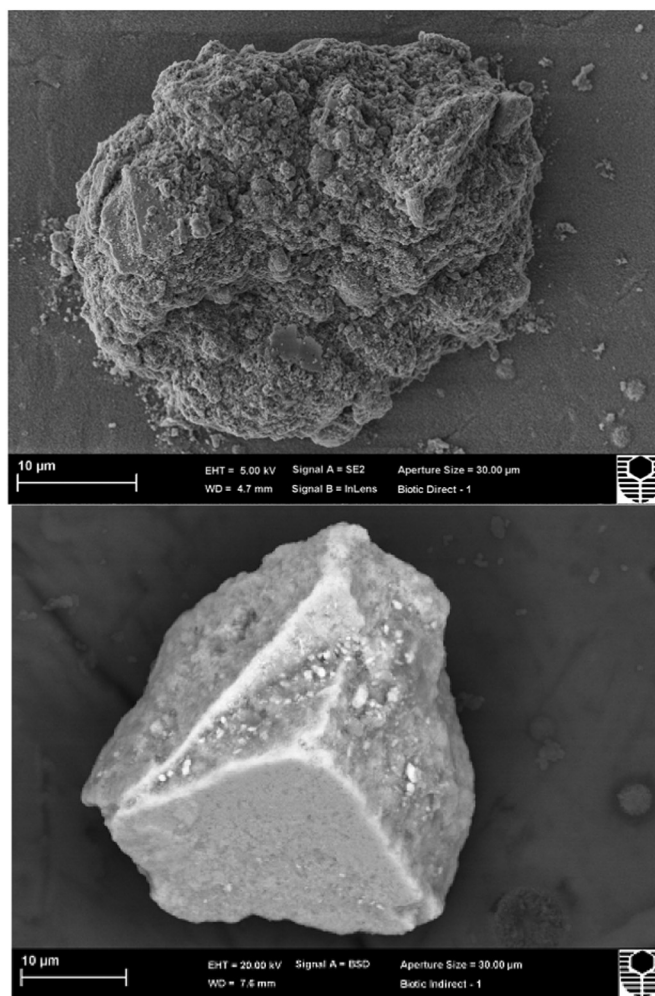


Fig. 3. Scanning electron microscopy images of the MWM after 18 days of contact bioleaching (top) and non-contact bioleaching (bottom) with *Enterobacter aerogenes*.

Raman image with the distribution and arrangement of bacteria clusters seen in the AFM images for both monazite crystal and MWM, it appears that there is no preferential attachment to specific area relevant to chemical composition (either REE^{3+} or PO_4^{3-}).

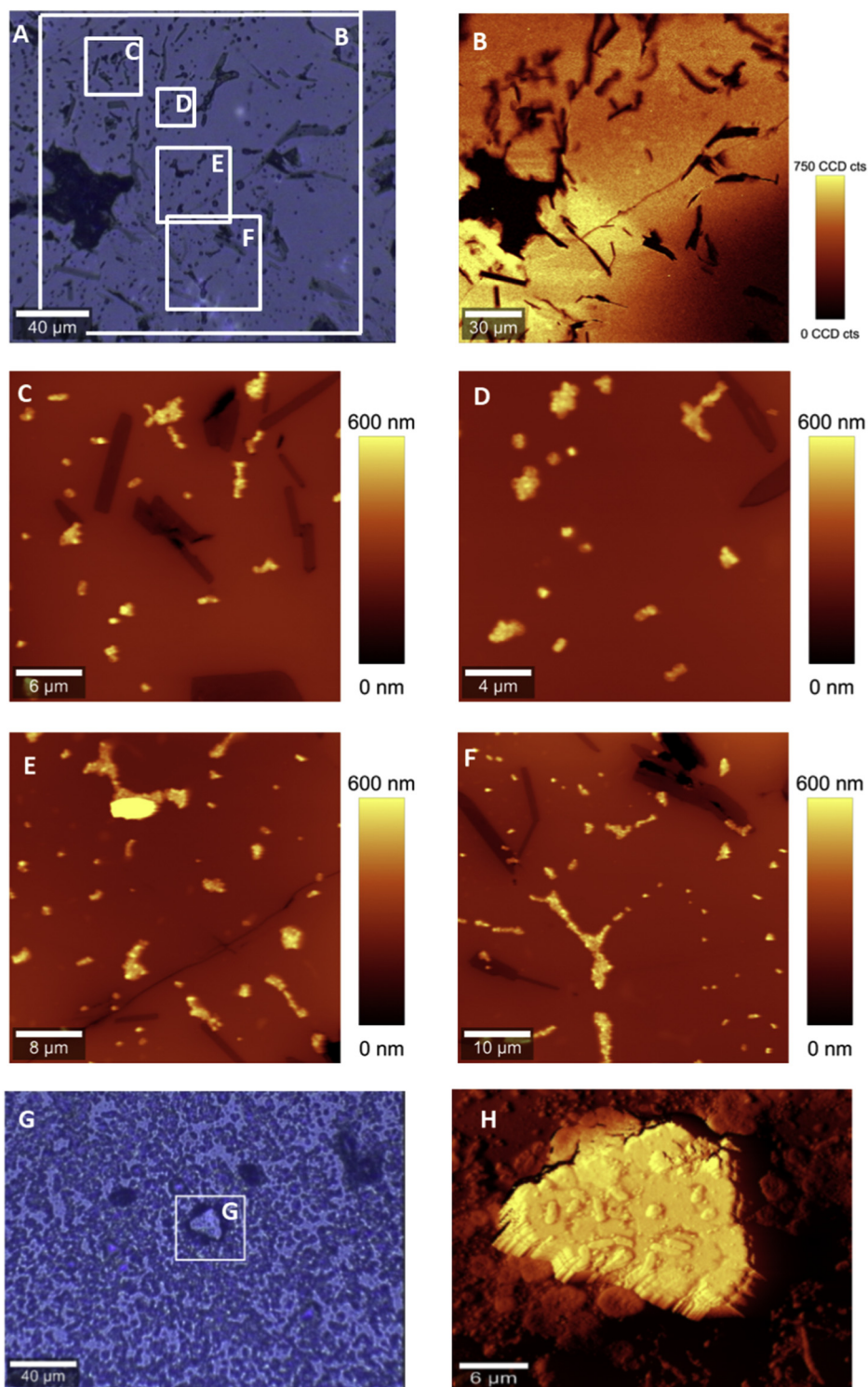


Fig. 4. (A) Optical microscopy image of monazite crystal and (G) grain after 24 h exposure to *Enterobacter aerogenes* with squares indicating where Confocal Raman microscopy (CRM) (B) and atomic force microscopy (AFM) measurements (C–F, H) were recorded. Confocal Raman microscopy image generated using a sum filter over the peak at 970 rel. 1/cm. (C–F, H) atomic force microscopy topography images showing clusters of bacteria attached to the monazite surfaces.

3.3. A conceptual modelling for microbial-mineral interactions during monazite bioleaching

Monazite particles are composed of inorganic P that can be used by PSB for metabolic purposes. Thus, mineral composition is a key factor influencing bacterial communities and their activities, especially in bioleaching. Bacteria can either exist in bulk solution (suspended), or

attached to surfaces or within EPS (Donlan, 2002). Cell counting in the present study showed that the number of *E. aerogenes* cells in the leachate dropped by 48 h from 10^7 to 10^3 cells mL⁻¹ most likely as a result of attachment to monazite (data not shown). However, it has been unclear whether microbial attachment and biofilm formation is a prerequisite for monazite dissolution.

As previously mentioned, within bacteria-mineral interfaces, the

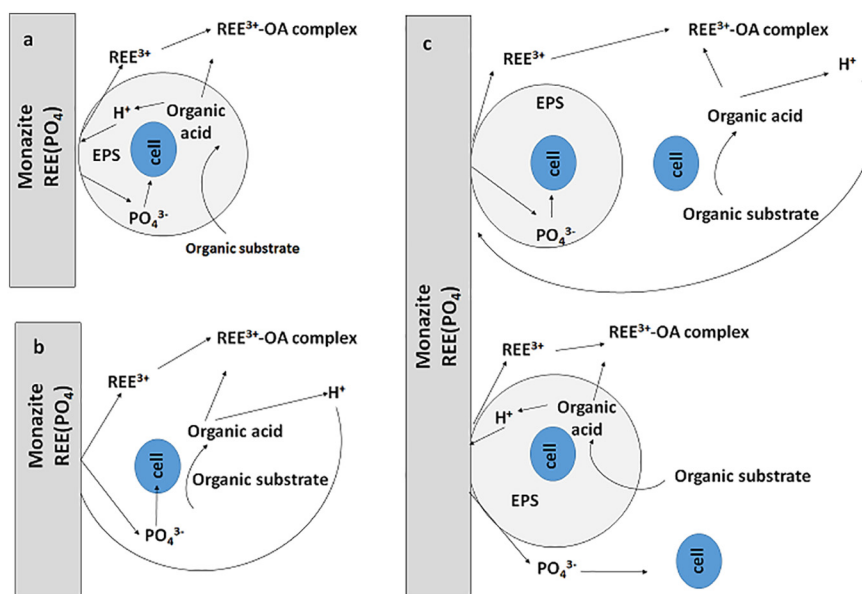


Fig. 5. A conceptual model showing the proposed mechanisms of monazite bioleaching.

rate of REE release during monazite bioleaching depends on i) the concentration of organic acid ligand in solution, ii) nature of the mineral surface (distribution of labile and non-labile REE), and iii) concentration of phosphate in solution which is governed by mineral composition, phosphate uptake rate, and growth rate of the biomass. The rate of biomass growth is controlled by nutrient availability including phosphate as well as environmental stressors such as low pH and REE toxicity which may inhibit REE bio-mobilization. However, the extent to which measured REE concentration during bio-mobilization and bio-mineralization relate to the bacterial attachment on monazite has not been previously studied.

It has been suggested by Rawlings et al. (1999) that the ability of microorganisms to oxidize sulfide minerals is possibly due to contact and non-contact mechanisms, or a combination of both (cooperative mechanism). However, bioleaching pathways for phosphate minerals (i.e., monazite) are very different than for sulfide minerals, where ferric iron lixiviant is regenerated by iron oxidizing microorganisms either in the bulk solution or in EPS (Crundwell, 2003). Here, we propose a new conceptual model of the possible mechanisms of monazite bioleaching including contact (Fig. 5.a), non-contact (Fig. 5.b), and cooperative (Fig. 5.c) leaching as shown in Fig. 5. In contact leaching (Fig. 5.a) attached microbial cells mobilize phosphate (PO_4^{3-}) within a matrix of EPS and release REE cations (REE^{3+}) into solution. Organic acids (OA) generated by the cells from organic substrates complex REE^{3+} . Protons released from organic acids attack the ore resulting in further PO_4^{3-} dissolution. Incorporation of PO_4^{3-} into the cells increases REE^{3+} solubility, according to reaction (2). In the non-contact mechanism (Fig. 5.b) suspended cells generate REE^{3+} complexing organic acids and incorporate PO_4^{3-} into cells increasing REE^{3+} solubility. The protons released from organic acids attack the ore resulting in further REE^{3+} and PO_4^{3-} dissolution. In cooperative mechanism (Fig. 5.c) attached cells mobilize PO_4^{3-} from monazite and incorporate it into cells releasing REE^{3+} while suspended cells generate organic acids for REE^{3+} complexation and protons released from organic acids attack the ore. Alternatively, attached cells may play a role in organic acid generation while suspended cells take up PO_4^{3-} from solution increasing REE^{3+} solubility.

Previous studies (Brisson et al., 2016; Corbett et al., 2017) demonstrated that microbial solubilisation of monazite is promising, however to be competitive with conventional processes, the recovery rates via bioleaching need to be increased. To enable scale up of the approach, a solid understanding of which factors are most important for

controlling REEs mobilization is required. This study provided preliminary data on significance of microbial colonization for nutrient acquisition by PSM, particularly phosphate via monazite dissolution. Overall our findings suggest that attachment of bacteria on mineral surface enhance REEs bioleaching. Further evaluation of potential PSMs for bioleaching REEs from monazite in large scale experiments will be considered.

4. Conclusion

The present study explored the possible mechanisms for bioleaching REEs from monazite. While a similar range and concentration of organic acids were secreted regardless of the ability of the bacteria to have contact with the mineral surface it was demonstrated that monazite dissolution was enhanced with bacterial contact by *E. aerogenes* with the monazite surface. No preferential attachment of bacteria to the monazite surface was observed by a co-localised AFM and CRM observed for either crystal monazite of the Mt. Weld Monazite. The data obtained from the organic acids profile and the contact and non-contact leaching experiments show promising scope for further research in the bioleaching of REEs-containing phosphate minerals.

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Better together: Potential of co-culture microorganisms to enhance bioleaching of rare earth elements from monazite



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ABSTRACT

The aim of this study was to develop continuous bioleaching of monazite by combining heterotrophic and autotrophic acidophilic microorganisms. The results showed that a co-culture of autotrophic, acidophilic *Acidithiobacillus ferrooxidans* and heterotrophic *Enterobacter aerogenes* was more effective in bioleaching rare earth elements (REEs) from monazite than either species alone. This was likely due to a synergistic interaction through the biogenic generation of both organic acids and sulfuric acid. In conclusion, the consortium of *E. aerogenes* and *A. ferrooxidans* solubilised REEs (Ce, La, Nd, Pr, and Y) up to a final concentration of 40 mg L⁻¹.

1. Introduction

The shift to a low carbon future is expected to accelerate the deployment of rare earth elements (REEs) in the wind and solar energy sectors. Therefore, countries rich in REEs resources (i.e., Australia) can establish long-term benefits through sustainable REE mining. Besides the primary REEs bearing minerals (i.e., monazite), large rare-earth bearing ores hosting iron-rich minerals (Fe-oxide phosphate) including goethite and hematite (Hoatson et al., 2011), may contribute to global REEs supply (Faris et al., 2017). Currently, industrial extraction of REEs from monazite involves either a basic process that uses concentrated sodium hydroxide or an acidic process that uses concentrated sulfuric acid. These generate large amounts of hazardous waste containing thorium and uranium (Abreu and Morais, 2010). Biohydrometallurgy has been studied as a more environmentally sustainable alternative to extract REEs from phosphate minerals including monazite (Keekan et al., 2017). Previously reported REEs bioleaching efficiencies from monazite with both bacteria and fungi have been very low compared to chemical leaching (Brisson et al., 2016; Shin et al., 2015). Recently, bioleaching of REEs from bastnasite-bearing rock by Actinobacteria has been investigated (Zhang et al., 2018). These authors have reported that in a nutrient-rich growth medium, the total concentration of

bioleached REEs ranged from 56 to 342 μg L⁻¹, whereas in an oligotrophic medium, only one strain (*Streptomyces* sp.) grew in the presence of the bastnasite (0.5% w/v), and leached up to 548 μg L⁻¹ of total REEs (Zhang et al., 2018). Coincidentally, a combination of the low solubility of bastnasite, a lack of nutrients from the mineral, the precipitation of REEs minerals, and re-sorption of leached REEs to cell and residual mineral surfaces may have contributed to the observed low leaching efficiency (0.008–0.08%) (Zhang et al., 2018). However, compared to the conventional extraction of REEs, bioleaching can be considered as an “eco-friendly technology” which minimizes the high cost and negative environmental impact.

In phosphate-based environments “phosphate solubilising microorganisms” (PSMs) can be introduced to enhance the solubilisation of insoluble inorganic phosphate via acidification, chelation, and exchange reactions (Son et al., 2006). As a consequence, heterotrophic PSMs such as *Enterobacter aerogenes* can be used for the solubilisation of REEs from a phosphate mineral such as monazite via secretion of organic acids (Corbett et al., 2017). Earlier studies demonstrated that the recovery of REEs using heterotrophic microorganisms is possible, although, the bioleaching mechanisms are not yet clearly and explicitly understood (Brisson et al., 2016).

It has been demonstrated that optimizing microbial community

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structure in co-culture systems are an effective way of improving microbial community function (Ma et al., 2017). The continuous requirement of heterotrophic bioleaching microorganisms for a carbon and energy source to maintain microbial activity is problematic at the industrial level, however the addition of acidophilic autotrophic bioleaching microorganisms (e.g. *Acidithiobacillus ferrooxidans*) to these systems can potentially improve their performance. Autotrophic acidophiles require small amounts of inorganic nutrients, such as ferrous iron and reduced sulfur compounds for bio-oxidation (Zhuang et al., 2015). In addition, the ability of acidophilic bacteria to tolerate toxic heavy metal ions, enhances their capacity for the bioleaching of metals. *A. ferrooxidans* is the most studied obligate chemolithoautotrophic bioleaching bacterium. It gains energy from the aerobic oxidation of ferrous iron and/or reduced sulfur compounds to ferric iron and sulfuric acid, respectively (Watling, 2016). Although *A. ferrooxidans* has been used to leach phosphorous from different types of rock phosphates (Bhatti and Yawar, 2010), to the best of our knowledge, despite the commercial application of acidophilic bioleaching for a diverse range of elements from sulfide minerals, the acidophilic bioleaching of REE-bearing minerals has not been previously studied. It has been demonstrated that microbial consortia have greater bioleaching rates than pure cultures (Johnson, 2001), we therefore propose a two-step bioleaching system where the metabolites generated by *E. aerogenes* result in pH reduction negating the need for manual pH adjustment required for *A. ferrooxidans*.

In this context, the aim of this work was to investigate the bioleaching of REEs from monazite by a co-culture of autotrophic, acidophilic *A. ferrooxidans* and heterotrophic *E. aerogenes* and compare the efficiency to those of individual pure cultures.

2. Material and methods

2.1. Phosphate and sulfide minerals

The high grade weathered monazite ore was collected from the Mount Weld Mine (Lynas Corporation), and is hereafter referred to as MWM (Mt. Weld Monazite). The ore was ground by a rod mill, pulverized in a ring mill and finally sieved to < 38 μm in particle size. The elemental composition of the MWM was analysed by inductively coupled plasma optical emission spectrometry (ICP-OES, CSIRO Minerals, Waterford, Western Australia). The ore contained (%): 10.1 La, 12.6 Ce, 2.10 Pr, 6.25 Nd, 0.165 Y, 0.162 Th, 1.23 Fe, 9.93 P, 1.75 Ca, 0.199 Mg, 1.96 Si, 0.554 Ti, 0.031 Zr, and < 0.003 U. Pyrite concentrate (p80 passing 120 μm) used as a source of Fe and S was obtained from Kalgoorlie Consolidated Gold Mines Pty Ltd. (KCGM), Australia (Bryan et al., 2015). The mineralogical composition of MWM was determined by X ray diffraction (XRD) at CSIRO Minerals, Waterford, Western Australia. The XRD analysis of MWM revealed that samples were mainly constituted by 51% monazite, 41% florencite, and 8% nontronite. Pyrite concentrate contained 60% pyrite, 12.5% quartz, 9% albite and 7.5% dolomite. Before bioleaching experiments, the mineral samples were sterilized by gamma irradiation at 50 kGy for 11 h (ChemCentre, Bentley, Western Australia).

2.2. Bioleaching experiment

Enterobacter aerogenes (ATCC 13048, obtained from ATCC) was grown to exponential phase at 30 °C in National Botanical Research Institute Phosphate (NBRIP) medium (Nautiyal, 1999), with shaking at 120 rpm, and harvested by centrifugation (3600 \times g, 10 min). Cells were resuspended in sterile Tris-HCl buffer (100 mM, pH 7.2), centrifuged (3600 \times g, 5 min) and washed twice more to remove any trace of phosphate. *Acidithiobacillus ferrooxidans* (ATCC 23270, obtained from DSMZ) was grown to exponential phase at 30 °C, with shaking at 120 rpm, in the basal salt media (BSM) at pH 2.0 which is described elsewhere (Zammit et al., 2011). Cells were resuspended in sterile Tris

buffer (20 mM, pH 2.0), centrifuged (3600 \times g, 5 min) and washed twice more to remove any trace of phosphate.

All bioleaching experiments were conducted in 250 mL Erlenmeyer flasks containing 100 mL of the relevant media, in triplicate at 30 °C with shaking at 120 rpm in an orbital shaking incubator (RATEK, Model No: OM11) over 12 days.

The ability of *A. ferrooxidans* to bioleach MWM as a phosphate source, was evaluated in BSM (pH 2.50 \pm 0.15), supplied with either FeSO₄ (13.9 g L⁻¹) and K₂S₄O₆ (1.51 g L⁻¹) (filter sterilized 0.20 μm , Satorius) or sterilized pyrite (1% pulp density), with 1% v/v bacterial inoculum (initial density 1 \times 10⁶ cells mL⁻¹) and 1% pulp density of sterilized monazite.

In the co-culture experiment, *E. aerogenes* was first cultivated in modified NBRIP media (3% w/v glucose and pH 7.00 \pm 0.25) with 1% v/v bacterial inoculum (initial density 1 \times 10⁷ cells mL⁻¹) and 1% pulp density of sterilized monazite. Three days later, when the pH dropped to < 3.5, a 10 mL aliquot of *A. ferrooxidans* (initial density 1 \times 10⁶ cells mL⁻¹ before inoculation) in BSM was added to the leachate, and the combined culture supplied with FeSO₄ (13.9 g L⁻¹) and K₂S₄O₆ (1.51 g L⁻¹) (filter sterilized 0.20 μm , Satorius).

Cell-free abiotic controls were carried out under the same conditions. Samples were taken at 0, 2, 3, 6, 9, 12 d and pH measured using a pH meter (Ionode IJ series pH probe). Samples were then filtered with disposable syringe filters (0.20 μm , Satorius) and assayed for REEs, Y, Th, U, Fe, and P concentrations by inductively coupled plasma-mass spectrometry (ICP-MS) Agilent Technologies 7700 series, Bureau Veritas Australia Pty Ltd., Canning Vale, Western Australia) and the average values were reported.

2.3. Comparison of the phosphate and iron regulation of *E. aerogenes* and *A. ferrooxidans*

In order to investigate the potential metabolic pathways involved in inorganic phosphate solubilisation by both strains in the co-culture system, a genome-based comparison of phosphate pathways was carried out. The genomes of *E. aerogenes* (ATCC 13048 – KCTC 2190) and *A. ferrooxidans* (ATCC 23270) were downloaded from the NCBI ftp site (<ftp://ftp.ncbi.nlm.nih.gov/>). For the purpose of this comparison, the genomes were annotated using the Rapid Annotation using Subsystem Technology (RAST) server (<http://rast.nmpdr.org/>) using the ClassicRAST annotation scheme (Overbeek et al., 2013). Comparisons were performed using the SEED and RAST servers and Geneious v.10.2.3 bioinformatic software (Kearse et al., 2012).

2.4. Synchrotron analysis

Synchrotron radiation is a powerful technique that can be used to determine elemental oxidation state of REEs for a wide range of environmental samples.

2.4.1. Sample preparation

Ce L_{III}-Edge X-ray absorption spectroscopy (XAS) data were collected on two solutions of monazite leachate from the co-culture supplied with FeSO₄ and K₂S₄O₆ at day 3 and 6 after *A. ferrooxidans* addition, as well as the MWM residue at end of bioleaching experiment. The leachates were prepared with 30% glycerol, and flash frozen with liquid nitrogen cooled iso-pentane, into 1 mm \times 3 mm \times 23 mm acrylic sample cuvettes. The cuvettes were covered and closed with metal free Kapton adhesive tape, which served as an X-ray transparent window. The powder sample was ground with mortar and pestle to a fine homogenous powder, and then adhered as a thin film to metal free Kapton adhesive tape.

2.4.2. XAS data collection

Ce L_{III}-Edge XAS data were collected at beamline 7-3, at the Stanford Synchrotron Radiation Lightsources (SSRL). The beamline

utilised a Si (220) double-crystal monochromator with harmonic rejection obtained by setting the 7 collimating mirror cut-off to 9 keV. The incident and transmitted X-ray intensities were recorded using N₂-filled gas ionization chambers (sweeping voltage of 1.8 kV). The X-ray absorption near edge spectrum (XANES) was measured as the Ce L_{III}-Edge fluorescence excitation spectrum, with X-ray fluorescence collected with an array of 30 germanium detectors (Canberra) equipped with a vanadium filter and a Soller slit assembly. Spectra were collected with the sample temperature maintained at approximately 10 K, using an Oxford instruments liquid helium flow cryostat. Each Ce L_{III}-Edge was obtained from the co-addition of 10 replicate spectra. X-ray energy was calibrated by reference to the K_α-edge absorption of a metallic Cr foil (first inflection point calibrated to 5989 eV).

2.4.3. Data processing

Ce L_{III}-Edge XAS spectra were processed using the EXAFSPAK suite of programs (George). Individual spectra were combined, a linear background subtracted, and the edge jump normalized to a value of 1 absorbance unit. Spectral comparison was performed on data without any smoothing filters applied, as well as on data with a 0.5 Gaussian smoothing function applied, to reduce noise levels, which were the result of low Ce concentration present in the leachates. Due to the low concentration of Ce in the samples, and relatively larger noise levels in the raw data, data analysis was limited to visual inspection of the edge position and shape, and fitting of the spectra to model Ce³⁺ and Ce⁴⁺ compounds was not performed.

2.5. Sequential extraction procedure (SEP)

In order to evaluate the mobilization behaviour of REEs, sequential extraction of elements from the feed ore and bioleaching residue of pure cultures was carried out according to the modified Community Bureau of Reference (CBR) five-step procedure (Mittermüller et al., 2016) with an additional determination of the residual fraction using sodium peroxide fusion (Fig. 1). The method determines five well defined fractions (speciation) in samples: easily soluble and ion-exchangeable fraction (F1), carbonate bound and mobilized by complexation fraction (F2), reducible fraction (F3), acid soluble fraction (F4), and residual fraction (R). The total content of the elements of interest in the mineral was determined by conducting a peroxide fusion analysis for the original minerals (same as the residue of modified SEP). All reagents used to perform the extraction were of analytical grade. Prior to the extraction, all tubes and glassware were soaked in diluted nitric acid (10%v/v) for

Table 1

Total content and fractionation of elements in feed Mt. Weld Monazite ore as determined by sequential extraction procedure. Values < 0.0001% are not shown. Data are averages of triplicate biological replicates.

Element	Total content (mg·kg ⁻¹)	Easily soluble F1 (%)	Carbonate F2 (%)	Reducible F3 (%)	Acid soluble F4 (%)	Residual R (%)
La	99,075		0.37	0.69	12.0	86.9
Ce	182,250	0.00067	0.35	1.88	12.5	85.2
Pr	20,675		0.58	0.64	13.7	85.0
Nd	73,000	0.00048	0.65	0.64	14.4	84.3
Th	1838		0.10	0.0	6.2	93.7
Y	2220		0.98	0.87	12.7	85.4

8 h and rinsed with ultra-pure Milli-Q™ water (Millipore, 18 MΩ/cm resistivity). All extractions were carried out in triplicate.

The recovery percentage of the stepwise extraction was determined by comparing the sum of the five individual fractions (F1, F2, F3, F4 and R) to the total content determined by peroxide fusion of the original ore, according to following equation (Eq. (1)):

$$\text{Recovery (\%)} = \left[\frac{F1 + F2 + F3 + F4 + R}{\text{Total content}} \right] \times 100 \quad (1)$$

3. Results and discussion

3.1. Mineral characterisation

The total concentration of REEs (mg kg⁻¹ dry material) for MWM (determined by peroxide fusion) and partitioning of REEs (determined by modified SEP) are presented in Table 1. The geochemical fraction in which REEs occur is critical for understanding their mobility, therefore within SEPs, the sample is progressively dissolved in extraction solution of increasing strength.

Ce had the highest content of all REEs in MWM followed by La and Nd. Based on the comparison of the total content and the sum of all fractions (F1 + F2 + F3 + F4 + R), satisfactory recovery was achieved for the REEs in the MWM feed ore, ranging between 105% and 126%, suggesting the method to be consistent and reproducible. Recoveries > 100% indicated that the stepwise procedure was more efficient in extracting REEs than peroxide fusion. Comparison of the SEP results with previous studies was not possible, as to best of our

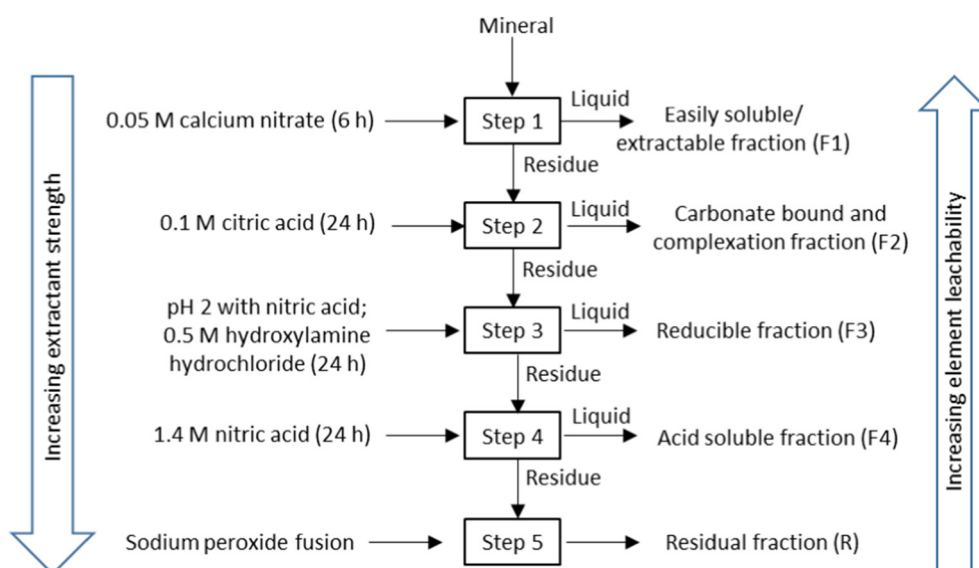


Fig. 1. Modified sequential extraction procedure (SEP) for REEs partitioning in Mt. Weld Monazite.

knowledge, no reports of fractionation of monazite by SEP are available.

The sequential extraction procedure revealed that Nd was the most mobile REE, with 15.7% of the total content in the non-residual (labile) fractions, and 84.3% of the residual fraction. The residue contained 85.0–86.9% of other REEs and 93.7% of the Th. The distribution behaviour of La, Ce, Pr, Nd, and Y in each labile fraction were in a similar range where the dominant scavenging phase in the acid soluble fraction was most likely represented by phosphate groups naturally present in monazite and florencite. Therefore, the SEP results confirm that amongst labile fractions, REEs associated with phosphate fractions can be released through biochemical pathways in which the cleavage of REEs-phosphate either directly or indirectly may be influenced by PSMs (*Azospirillum brasilense*, *Bacillus megaterium*, *Burkholderia glathei*, *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Aspergillus niger*, *Aspergillus tubigenis*, and *Penicillium* sp.) and/or other microbial species (*Firmicutes*) (Corbett et al., 2017; Corbett et al., 2018).

3.2. Bio-solubilisation of REEs from monazite

Bio-solubilisation of REEs from the monazite was investigated with individual cultures of *E. aerogenes* and *A. ferrooxidans* as well as a combination of the two species.

3.2.1. Phosphate solubilising bacteria

Following the inoculation of *E. aerogenes* into sterile media plus MWM, the total concentration of REEs in the leachate increased from 2.90 at day 2 to 5.84 mg L⁻¹ at day 12 (Fig. 2). No solubilisation of REEs, Fe or P occurred in abiotic flasks with the soluble concentration of all elements being below detection limits (Ce, La, Pr, Nd, Th, U, and Y < 1 µg L⁻¹; Fe < 0.5 mg L⁻¹, and P < 2 mg L⁻¹), indicating that metabolites secreted by microbial cells contributed to REEs mobilization. With microbial growth, the pH of the media decreased from 6.50 ± 0.02 to 3.38 ± 0.05 (Fig. 2). This decrease in the pH can be attributed to the production of organic acids resulting from glucose oxidation, bacterial respiration and NH₄ assimilation (Corbett et al., 2017). *E. aerogenes* was reported to be efficient in solubilising tricalcium phosphate [Ca₃PO₄(TCP)] (Corbett et al., 2017; Prasanna et al., 2011). However, only a few studies have reported microbial solubilisation of natural monazite by PSMs (Corbett et al., 2017; Shin et al., 2015). Shin et al. (2015) examined bioleaching of REEs from monazite-bearing ore with *Acetobacter aceti* and reported a total concentration of Ce in the leachate of 5.8 mg L⁻¹ on day 4 (0.13% of leaching efficiency). In this study, the Ce concentration was 2.6 mg L⁻¹ at day 3 (0.20% of leaching efficiency) for monazite bioleaching with *E. aerogenes* and by day 9, the Ce and La concentrations had increased to 4 mg L⁻¹ (0.31% of leaching efficiency) (Fig. 2). In the study by Shin et al. (2015), the Ce concentration dropped to < 2 mg L⁻¹ (0.02% of leaching efficiency). Given the difference in experimental conditions

(supplement of soluble of phosphate source in Shin et al. (2015)) and the ore complexity, it is not surprising that the leaching behaviour of REEs was found to differ between studies.

Corbett et al. (2017) reported that *E. aerogenes* released a total of 1.93 mg L⁻¹ REEs (Ce, La, Nd, and Pr) from a similar ore sample (MWM) after 8 days. In comparison, the maximum total REEs concentration observed in this study was 3.97 mg L⁻¹ after 6 days (Fig. 2). Differences in experimental conditions (media composition and growth temperature), and the type and concentration of secreted organic acids may have contributed to the differing results. In contrast, the concentration of P, Fe, Th and U solubilised from monazite were much lower than observed for REEs and most were lower than detection limits (< 2 mg L⁻¹ for phosphate, < 0.5 mg L⁻¹ for Fe, < 5 µg L⁻¹ for Th, and < 0.02 mg L⁻¹ for U), which is consistent with previous studies (Brisson et al., 2016; Corbett et al., 2017). As cells are present in the bioleaching system, incorporation and surface attachment of phosphate groups within microbial biomass and structures could be expected (Corbett et al., 2017). Also XRD analysis of the bioleached residue in this study confirmed the formation of secondary minerals such as cheralite [(Ce_{0.4}Ca_{0.3}Th_{0.3}) (PO₄) (SiO₄)] and woodhouseite [CaAl₃ (PO₄) (SO₄) OH₆] which may explain the very low concentration of elements, especially phosphate in solution (Supplementary Fig. S1a). According to these considerations, the preferential release of REEs over Th and U favours the selective recovery of radionuclides for further downstream processing.

These data indicated that *E. aerogenes* is a promising organism for microbial dissolution of phosphate REEs with almost no Th and U mobilization. However, in order to make it competitive with conventional extraction, further examination of factors influencing the release rate of REEs in conjunction with other microorganisms is required.

3.2.2. Acidophilic bacteria

The capacity of *A. ferrooxidans* to tolerate exceptionally high level of metals has been demonstrated (Dopson et al., 2014). Tsaplina et al. (2015) reported leaching yields of 15–30% for REEs with acidophilic chemolithotrophic microorganisms from a 30% pulp density of ash slag waste at pH 0.76. It has also been demonstrated that the addition pyrite can enhance metal bioleaching from electronic wastes (Bryan et al., 2015). However, no studies on the solubilisation of REE phosphates by acidophilic bacteria in the presence of various growth substrates have been reported. It was therefore decided to adopt an experimental setup with *A. ferrooxidans* as a model microorganism to evaluate the bioleaching of REEs from MWM under acidic conditions.

The presence of *A. ferrooxidans* and availability of ferrous iron and reduced sulfur compounds impacted the pH change and mobilization behaviour of REEs (Fig. 3). The pH of the media with both FeSO₄/K₂S₄O₆ and pyrite initially increased as bio-oxidation of Fe²⁺ to Fe³⁺ is acid consuming (reaction (1)).

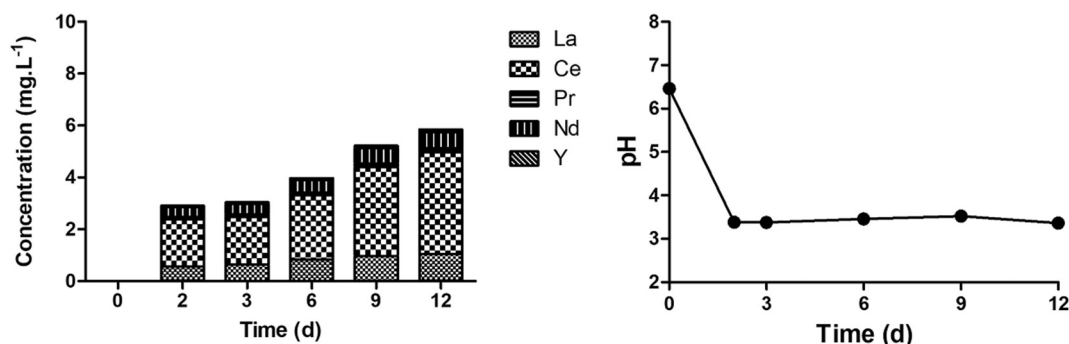


Fig. 2. Concentrations of dissolved La, Ce, Pr, Nd, and Y (left) and pH of leachate (right) during the bioleaching of Mt. Weld Monazite with *Enterobacter aerogenes* in the presence of glucose. The concentration of REEs, Th, and U in abiotic flasks remained below detection level throughout the experiment. Data are averages ± SD of triplicate biological replicates. Error bars not visible are smaller than symbols.

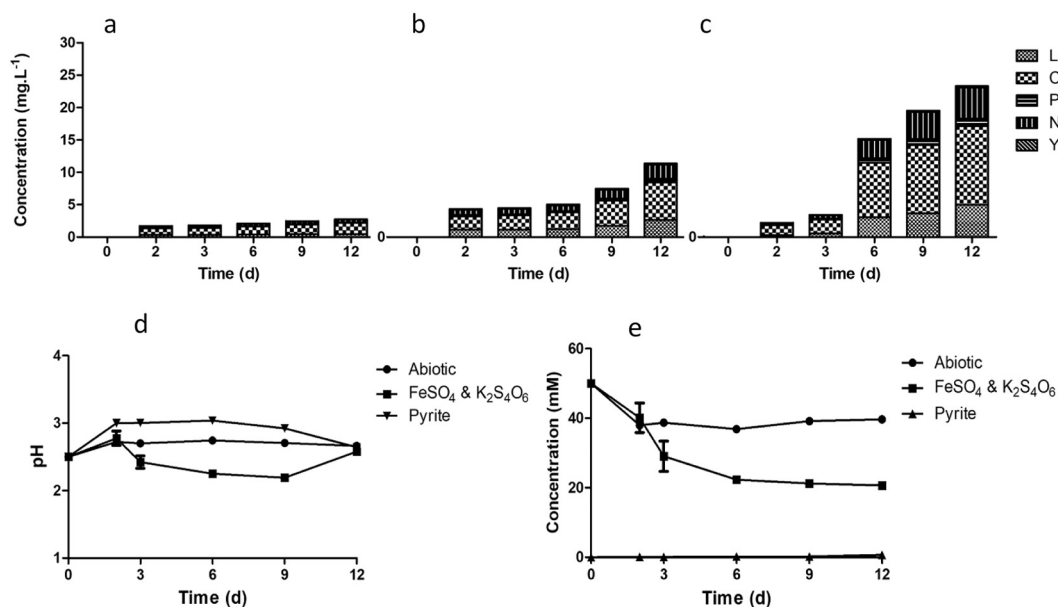
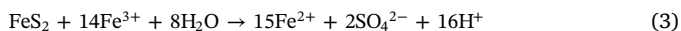


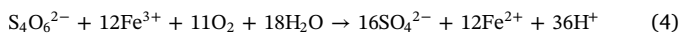
Fig. 3. Concentrations of dissolved La, Ce, Pr, Nd, and Y during the bioleaching of Mt. Weld Monazite with *Acidithiobacillus ferrooxidans*. (a) Abiotic controls with FeSO₄ and K₂S₄O₆, (b) *Acidithiobacillus ferrooxidans* with pyrite, (c) *Acidithiobacillus ferrooxidans* with FeSO₄ and K₂S₄O₆, (d) solution pH, and (e) concentration of soluble Fe under all conditions. Abiotic controls with pyrite; concentration of REEs and Fe remained below detection level throughout the experiment. Data are averages \pm SD of triplicate biological replicates. Error bars not visible are smaller than symbols.



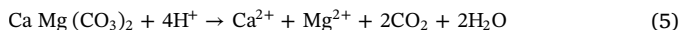
Pyrite can be solubilised only by a combination of proton and oxidative attack, according to “thiosulfate pathway”, where the main product is sulfate (reactions (2) and (3)) (Fonti et al., 2016).



Subsequently, after 9 days, the pH of the media in the presence of FeSO₄ and K₂S₄O₆ had decreased to 2.19 ± 0.02 , whereas the pH of the media inoculated with pyrite was 2.92 ± 0.07 . Bacterial oxidation of K₂S₄O₆ by *A. ferrooxidans* with molecular oxygen to sulfate is an acid-producing process according to reaction (4).

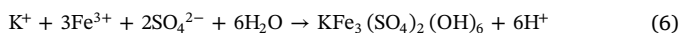


The final pH at day 12 in both conditions was in a similar range to the initial pH (Section 2.5). As dolomite was present in the pyrite concentrate, a higher H⁺ consumption due to the buffering capacity of the dolomite (reaction (5)) and the heterogeneous nature of the concentrate (presence of quartz and albite) can reasonably be assumed to be responsible for the differences in pH and lower REEs solubilisation in the presence of pyrite.



Moreover, the initial concentration of Fe and S within FeSO₄ and K₂S₄O₆ were 50 and 5 mM, respectively; which was adequate for bacterial growth.

The total concentration of Fe in solution by day 12 in the presence of FeSO₄ and K₂S₄O₆ gradually decreased from 50 to 21 mM whereas in the presence of pyrite it increased slowly (from 0.10 to 0.66 mM by day 12). The formation of Fe co-precipitate, jarosite (reaction (6)) in MWM residue after incubation with FeSO₄ and K₂S₄O₆ was confirmed by XRD (Supplementary Fig. S1b), whereas with pyrite, jarosite formation did not occur.



The presence of different substrates influenced the REEs solubilisation by *A. ferrooxidans* (Fig. 3). REEs solubilisation was greatest in the presence of FeSO₄ and K₂S₄O₆ (average final total REEs concentration

during 12 days of incubation: 23.6 mg L^{-1} , 1% percentage recovery of Ce). On the other hand, the growth of *A. ferrooxidans* on pyrite as a source of Fe and S resulted in lower REEs solubilisation (average final total REEs concentration: 10.6 mg L^{-1}) which was still double of that achieved with *E. aerogenes*.

3.2.2.1. Phosphorous solubilisation. In the presence of *A. ferrooxidans*, the combined low pH with active Fe/S oxidising bacteria which produce biogenic sulfuric acid, favoured the mobilization of P up to 33 mg L^{-1} . Phosphorous solubilisation from REE phosphates relies on protons to proceed (Chi et al., 2006), however, as previously mentioned (Section 3.2.2) the pH of the inoculated media in the presence of pyrite did not decrease, potentially limiting phosphate release into the leachate. Increasing the concentration of pyrite to 30 g L^{-1} , has been proposed to improve the fraction of phosphorous leached from rock phosphate (P content 12%) (Chi et al., 2006).

3.2.2.2. Thorium and uranium solubilisation. The tolerance of *A. ferrooxidans* to Th²⁺ and UO₂²⁺ has previously been demonstrated to be in a range of $900\text{--}1880 \text{ mg L}^{-1}$, where Th was more toxic to Fe oxidation than UO₂²⁺ (Leduc et al., 1997). This range is much higher than could be solubilised from the MWM based on its total content of Th and U, and therefore the application of *A. ferrooxidans* for MWM can be justified. As MWM also contains trace amount of radioactive elements with a higher Th content (0.162%) relative to U (0.003%), early saturation of Th in the solution was expected. Nevertheless, Th concentrations in all scenarios were lower than U (0.057 ± 0.006 vs 0.118 ± 0.021). The amount of Th and U solubilised by *A. ferrooxidans* increased gradually during 12 days of incubation (0.30% and 32% of leaching efficiency, respectively). However, (Singh et al., 2009) reported 98% uranium dissolution from silicate-apatite ore in 40 days by *A. ferrooxidans* under the optimum conditions (pH 1.7, temperatures 35 °C). This is much higher than the overall solubilisation of Th and U from MWM in this study. Providing a phosphate source for bacterial growth could be one controlling factor in Th and U solubilisation as in this study bacteria obtained their phosphate from MWM which is not as easily accessible as common phosphate sources in 9K media (Singh et al., 2009).

The major controlling factors in the release of U in this study was likely to be pH (reaction (7)), and the increased concentration of Fe^{3+} (reaction (8)) which can oxidize tetravalent uranium to water-soluble hexavalent uranium. While phosphate solubilising bacteria (Section 3.2.1) only solubilised U in the range of $0.013 \pm 0.001 \text{ mg L}^{-1}$ at day 2 to $0.020 \pm 0.000 \text{ mg L}^{-1}$, by day 12, [comparable to concentration of U in abiotic controls ($0.023 \pm 0.002 \text{ mg L}^{-1}$)], *A. ferrooxidans* at day 12 solubilised U up to $0.118 \pm 0.021 \text{ mg L}^{-1}$ in the presence of $\text{FeSO}_4/\text{K}_2\text{S}_4\text{O}_6$ and $0.105 \pm 0.000 \text{ mg L}^{-1}$ in the presence of pyrite.



The generation of ferric ions through the bio-oxidation of Fe^{2+} by *A. ferrooxidans* led to increasing solubility of Th. In the presence of $\text{FeSO}_4/\text{K}_2\text{S}_4\text{O}_6$, the Th concentration in the leachate increased to $0.057 \pm 0.006 \text{ mg L}^{-1}$ by day 12 compared to 0.007 mg L^{-1} in abiotic controls. Solubilisation of Th can be attributed to the presence of an oxidising agent (Fe^{3+}). The formation of secondary minerals such as cheralite in the MWM residue also may explain the lower concentration of soluble Th in solution. These findings are in agreement with the results of thermodynamic speciation of monazite where the solubility of Th was restricted in the presence of phosphate due to the passivation layer of REE phosphates (Lapidus and Doyle, 2015).

3.2.3. Synergistic effect of co-culture

Of the three experimental conditions (*E. aerogenes* on glucose, *A. ferrooxidans* on $\text{FeSO}_4/\text{K}_2\text{S}_4\text{O}_6$, and *A. ferrooxidans* on pyrite), *A. ferrooxidans* supplied with $\text{FeSO}_4/\text{K}_2\text{S}_4\text{O}_6$ resulted in the greatest solubilisation of REEs, Y, as well as Th and U. This can be explained in terms of mechanisms by which elements are released into the leachate (biogenic acid and ferric iron generation) and the minimization of co-precipitation of soluble complexes. Phosphate solubilising bacterium, *E. aerogenes* can lower pH naturally by the production of organic acids, as shown in Section 3.2.1 producing conditions suitable for the growth of *A. ferrooxidans*. Therefore, in this study an attempt was made to assess the potential of microbial co-culture system for REEs extraction by using *A. ferrooxidans* and *E. aerogenes*.

Glucose fermentation and the secretion of organic acids led to the natural acidification of the media by *E. aerogenes* with the first sharp decrease in pH (2.84 ± 0.03) observed by day 2. The addition of *A. ferrooxidans* plus $\text{FeSO}_4/\text{K}_2\text{S}_4\text{O}_6$ at day 3 resulted in a further pH decrease (to 2.48 ± 0.01). A stable pH (2.46 ± 0.04) was reached at the end of the experiment (Fig. 4b). Due to the inhibition of Fe^{2+} oxidation activity by *A. ferrooxidans* reported at pH values above 3.0 (Meruane and Vargas, 2003), the *A. ferrooxidans* inoculum was added when pH was 2.84 ± 0.03 which is close to the optimum pH of the species (Section 2.5) (Schippers, 2007).

MWM was more efficiently solubilised in media co-inoculated with *E. aerogenes* and *A. ferrooxidans* (Fig. 4a) than with individual cultures (Figs. 2 and 3). An increase in REEs solubilisation from MWM with the co-culture was evident from day 6 (3 days after the addition of *A. ferrooxidans*) (Fig. 4a) with a total REEs concentration of 40 mg L^{-1} on day 9. The major increase was observed for Ce as the concentration of released Ce in co-culture was on average 2.4 times higher than when inoculated with *A. ferrooxidans* and 7.4 times higher than with *E. aerogenes* (Fig. 4a). However, the concentrations of solubilised La, Pr, Nd, U, and Y detected in the co-culture solution at day 12 were in similar range as *A. ferrooxidans* in the presence of $\text{FeSO}_4/\text{K}_2\text{S}_4\text{O}_6$. This provided an opportunity to selectively extract REEs while minimising Th and U solubilisation.

The concentration of P in the co-culture leachate was $3.33 \pm 0.94 \text{ mg L}^{-1}$. This is higher than in the presence of *E. aerogenes* ($< 2 \text{ mg L}^{-1}$) and lower than released by *A. ferrooxidans* (33 mg L^{-1}). Previous results have demonstrated the enhanced ability of an *Aspergillus niger* and *Burkholderia cepaciathan* co-culture (both known as

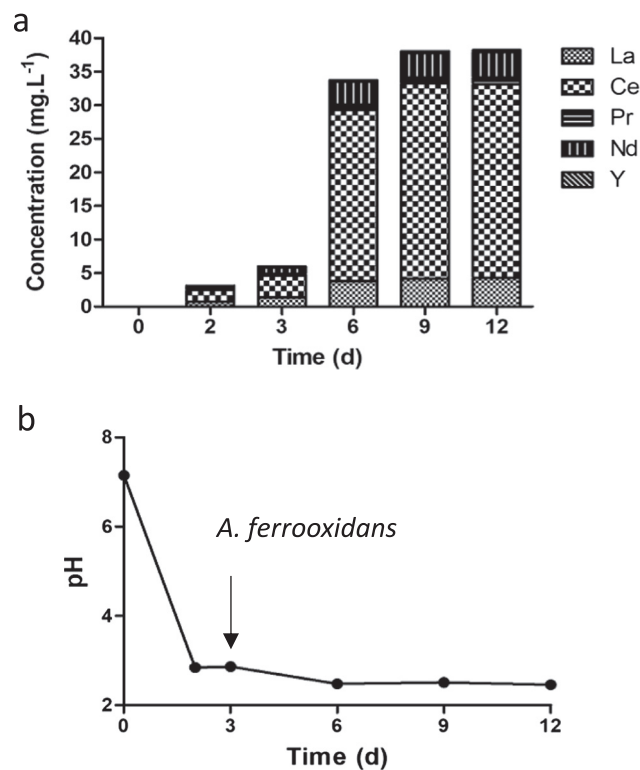


Fig. 4. Concentrations of dissolved La, Ce, Pr, Nd, and Y during the bioleaching Mt. Weld Monazite with a co-culture of *Enterobacter aerogenes* and *Acidithiobacillus ferrooxidans* (a) in presence of FeSO_4 and $\text{K}_2\text{S}_4\text{O}_6$ and (b) pH of leachate. The concentration of REEs, Th, and U in abiotic controls remained below detection level throughout the experiment (data not shown). Data are averages \pm SD of triplicate biological replicates. Error bars are smaller than symbols.

PSM) to solubilise phosphates compared to their individual performance (Braz and Nahas, 2012). *Enterobacter* species have been characterized as one of the most efficient PSM (Corbett et al., 2017). However, in this study no detectable P was observed with *E. aerogenes*. This indicates that P solubilisation from MWM by *E. aerogenes* can be inhibited by environmental stresses such as a very low pH, phosphate deficiency, and toxic elements. The heterotrophic dissolution of REEs and P from monazite is mostly governed by acido-complexolysis of organic acids. An alternative possibility is that the bacteria, in particular *E. aerogenes* have utilised the soluble P for cellular requirements. After co-inoculation with *A. ferrooxidans*, overall P solubilisation improved.

A number of low molecular weight organic acids can be rapidly degraded by microorganisms (Corbett et al., 2017). Heterotrophic cultures of *E. aerogenes* generated a range of organic acids including gluconic, malic and acetic acids (data not published). The retention mechanisms of citric and malic acids have been investigated in some detail, and it has been demonstrated that higher Fe concentration under acidic conditions decreased the biodegradation of citric acid and malic acid (Yang et al., 2016). The enhanced dissolution of REEs from monazite as a result of the excretion of organic acids by heterotrophic bacteria and sulfuric acid from *A. ferrooxidans*, occurred by complexolysis of complexing agents generated by the bacteria and REEs. Therefore, the higher REEs concentration in the leachate of the co-culture system can also be explained by the higher concentration of total Fe in co-culture (60 mM) compared to *A. ferrooxidans* (21 mM) which potentially contributed to the stability of organic acids, thereby prolonging their complexing capacity with REEs.

As a result, it could be summarized that the synergistic function of co-culture system can generate more lixiviant (i.e., acid), which

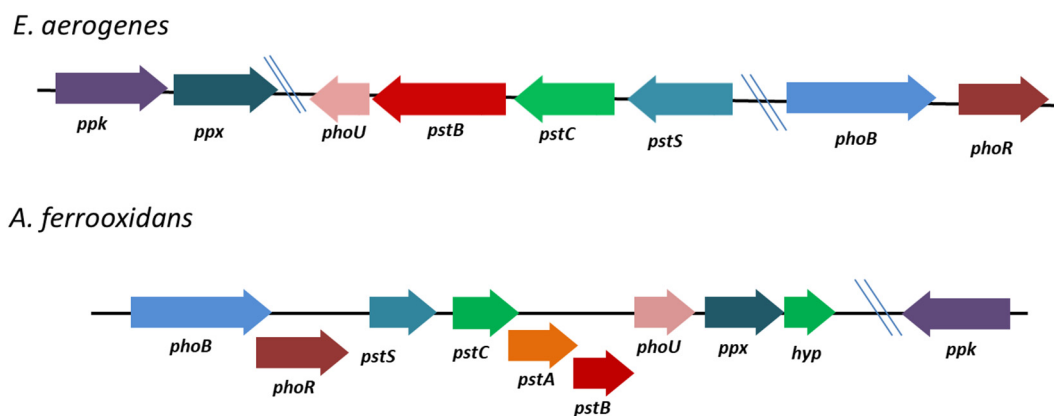


Fig. 5. Genetic organization of the *pho* Operon in *Enterobacter aerogenes* and *Acidithiobacillus ferrooxidans* (*phoB* – phosphate regulon transcriptional regulatory protein; *phoR* – phosphate regulon sensor protein; *pstS* – phosphate ABC transporter, periplasmic phosphate-binding protein; *pstC* – phosphate transport system permease protein; *pstA* – phosphate transport system permease protein; *pstB* – phosphate transport ATP-binding protein; *phoU* – phosphate transport system regulatory protein; *ppx* – exopolyphosphatase; *hyp* – hypothetical protein; *ppk* – polyphosphate kinase).

promotes the bioleaching process of monazite. In this context, the synergistic action of *E. aerogenes* and *A. ferrooxidans* in co-culture, in comparison to pure culture, offers potential alternatives to conventional techniques.

3.3. Phosphate utilization

As mentioned above, different P solubilisation was seen in pure cultures of *E. aerogenes* and *A. ferrooxidans* as well as the co-culture. A comparative genomic study of the potential mechanisms of phosphate metabolism during the interaction of *E. aerogenes* and *A. ferrooxidans* was performed (Fig. 5). It has previously been demonstrated that polyphosphate kinase (*ppk*) is responsible for the accumulation of long polymers of inorganic phosphates (known as polyphosphate) and that polyphosphate can be hydrolysed to liberate inorganic P by the enzyme exopolyphosphatase (*ppx*) (Rao et al., 2009). A genomic comparison revealed the *ppx* gene was directly downstream of the complete *pho* regulon (*phoB-phoR-ptsSCAB-phoU*) in *A. ferrooxidans*, as has previously been shown (Vera et al., 2003). However, the genome of *E. aerogenes* showed major differences in the organization in the *pho* regulon, as shown by the absence of the *pstA* gene and the lack of co-localization of the *phoB-phoR* operon with *pstSCB* (Fig. 5). Furthermore, *ppk* and *ppx* in *E. aerogenes* are located in the same operon, and not as part of the *pho* regulon, as has previously also been described for *Escherichia coli* (Kornberg et al., 1999). As previously speculated by Vera et al. (Vera et al., 2003) the presence of *ppk* and *ppx* on the same operon suggests that the genes may be co-transcribed and therefore indicates limited accumulation of polyphosphate. Therefore, in *E. aerogenes* it is likely that inorganic P is directly used to meet the cell's phosphate requirements, resulting in less P available in the solution and the requirement for a constant source of P in order to avoid phosphate starvation. However, in *A. ferrooxidans* *ppk* is not found as part of the operon with *ppx*, suggesting the genes are transcribed separately, allowing the accumulation of polyphosphate in this strain. *A. ferrooxidans* would be able to store P and also to liberate it depending on the cell's phosphate requirement. As *E. aerogenes* is likely to only transiently accumulate polyphosphate, it would require uptake of any phosphates released by *A. ferrooxidans* in order to overcome phosphate starvation. This could then explain the reduced concentration of phosphate in the media when *A. ferrooxidans* and *E. aerogenes* are in co-culture, as compared to the concentration of phosphate present when *A. ferrooxidans* is used as a pure culture.

Bacterial adaptation to phosphate and energy deficient environments represent key factors that can compromise the feasibility of bioleaching of REEs from MWM. However, there is limited

understanding concerning the potential components involved in phosphate and iron dynamics within bacterial-mineral surfaces. Further study of the accumulation and regulation of the inorganic phosphate during the solubilisation process is essential in order to enhance the functioning of these microorganisms for the efficient leaching of REEs-phosphate minerals.

3.4. Oxidation state of Ce and Nd

The X-ray absorption near-edge spectrum (XANES) provides valuable insight into the chemical form of a specific element, including oxidation state, molecular geometry, and ligand type. In many cases, simple visual inspection of the position of the edge energy and the general shape of the edge (features such as height, width, and splitting) is sufficient to draw conclusions about oxidation state (Hackett et al., 2012). This holds true for Ce, and differentiating Ce^{3+} from Ce^{4+} based on visual inspection of the L_{III} -Edge is relatively easy. The published literature highlights that the Ce^{3+} edge has a distinctive white line feature at 5726 eV (López-Moreno et al., 2010). As would be expected, at higher oxidation state, a shift of the edge to higher energy occurs, and the edge of Ce^{4+} is shifted to ~5730 eV. Further, Ce^{4+} contains two stable ground state electronic configurations, $4f^0$ and $4f^1$, and consequently the Ce^{4+} L_{III} -Edge contains a distinctive “double-peak” white line feature (Shahin et al., 2005).

The results from this study revealed that in the bioleached MWM residue and resultant leachate, the Ce L_{III} -Edge occurs at 5726 eV, and contains only a single edge feature (Fig. 6). Therefore, it was concluded that the major oxidation state of Ce found in the MWM and leachates are Ce^{3+} . These results suggest that oxidation of REEs (Ce^{3+} to Ce^{4+}) most likely did not occur during the bioleaching of MWM, possibly as the dissolution mechanism of Ce was controlled by chelation with anions from organic acids.

3.5. Changes in the partitioning of REEs and Th during bioleaching

Changes in REEs and Th fractionation due to bioleaching with *E. aerogenes* and *A. ferrooxidans* were evident (Tables 1 and 2). The major changes were observed for the non-residual fractionation of La, Ce, Pr, Nd, and Y, while Th showed smaller variations, in congruence with their high association to the residual fraction (i.e., over 93.7%). Bioleaching was effective in stimulating REEs mobility as indicated by the increase of REEs in easily extractable and acid soluble fractions F1 and 4 (Table 2). The increase of REEs mobility in F1 and F4 was associated with a concomitant decrease of these elements in fractions F2, F3, and R (Table 2).

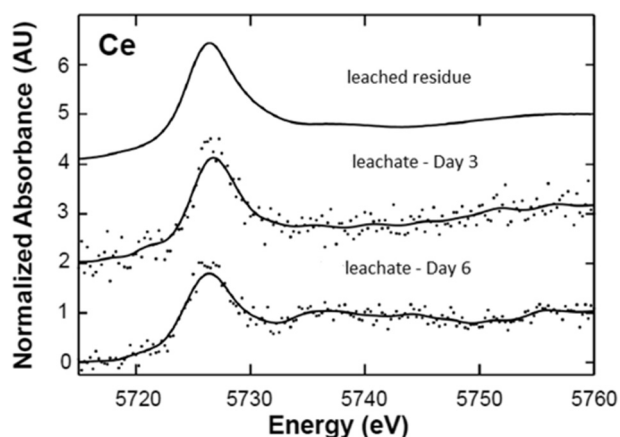


Fig. 6. Ce L_{III} -Edge XAS spectra collected from leachate solution at day 3 and day 6 after *Acidithiobacillus ferrooxidans* addition, and Mt. Weld Monazite residue during co-culture experiment. Dotted lines show individual data points, and solid lines show resulting spectra produced from a 0.5 Gaussian smoothing filter. A single white line feature, centred at 5726 eV was observed for all spectra, indicating that Ce was mainly present as Ce^{3+} .

Table 2

Fractionation of elements in bioleached residue of Mt. Weld Monazite ore as determined by sequential extraction procedure after bioleaching with either *Enterobacter aerogenes* or *Acidithiobacillus ferrooxidans*. The results < 0.0001% are not shown. Data are averages of triplicate biological replicates.

	Easily soluble F1 (%)	Carbonate F2 (%)	Reducible F3 (%)	Acid soluble F4 (%)	Residual R (%)
Fraction content of MWM (mg kg ⁻¹ and %) after bioleaching with <i>E. aerogenes</i>					
La	90 (0.07)	238 (0.19)	444 (0.35)	27,800 (22.0)	97,433 (77.3)
Ce	244 (0.11)	680 (0.32)	1456 (0.69)	44,800 (21.1)	164,667 (77.7)
Pr	15 (0.06)	69 (0.29)	77 (0.33)	5456 (23.0)	18,033 (76.2)
Nd	59 (0.07)	286 (0.34)	280 (0.34)	19,790 (23.8)	62,700 (75.4)
Th				89 (4.34)	1950 (95.6)
Y	3.7 (0.15)	9.6 (0.38)	10 (0.41)	558 (22.4)	1907 (76.6)
Fraction content of MWM (mg kg ⁻¹ and %) after bioleaching with <i>A. ferrooxidans</i>					
La	120 (0.12)	79 (0.08)	259 (0.26)	17,680 (17.8)	80,937 (81.7)
Ce	247 (0.13)	223 (0.12)	829 (0.45)	35,800 (19.6)	145,152 (79.6)
Pr	27 (0.13)	23 (0.11)	49 (0.24)	3813 (18.4)	16,763 (81.0)
Nd	100 (0.14)	92 (0.12)	166 (0.23)	12,987 (17.8)	59,655 (81.7)
Th		5.7 (0.31)		48 (2.63)	1784 (97.0)
Y	3 (0.13)	2 (0.09)	4.3 (0.19)	334 (15.0)	1877 (84.5)

Although previous research has shown the impact of bioleaching on fractionation of metals in the contaminated sediments (Fonti et al., 2015), to the best of our knowledge, there has no previous reports on the effects of bioleaching on REEs mobility from phosphate minerals.

The first step of sequential extraction obtains the easily soluble/exchangeable fraction (F1) of elements that are weakly associated with organic and inorganic sites (Beckett, 1989) which can be released by the action of protonation (pH change) and ion exchange of cations such as Ca^{2+} , K^+ , and Mg^{2+} which have a comparable ion radius (Coordination number = 6) to the trivalent state of REEs (e.g., Na^+ : 1.02 Å, Ca^{2+} : 1.00 Å, K^+ : 1.38 Å, La^{3+} : 1.03 Å, Ce^{3+} : 1.01 Å, Pr^{3+} : 0.99, Nd^{3+} : 0.98 Å, and Y^{3+} : 0.90) (Jia, 1991; Shannon, 1976). The results of this fraction have shown that without bacteria only Ce (1.4 mg kg⁻¹) and Nd (0.4 mg kg⁻¹) may be released through weak electrostatic interactions or ion-exchange reaction. However, after bioleaching with *E. aerogenes* and *A. ferrooxidans*, an increase of Ce, La,

Nd, Pr, and Y association with the easily soluble/exchangeable fraction of the leach residue was observed (0.06–0.15 and 0.12–0.14% of their total concentration in *E. aerogenes* and *A. ferrooxidans*, respectively). Higher association of REEs in the F1 fraction for *A. ferrooxidans* were in good agreement with higher concentration of REEs in *A. ferrooxidans* culture as illustrated in Fig. 3.

The second fraction (F2) extracted with citric acid ($pK_a = 3.13$) accounts for the carbonate and complexation fraction from which REEs can be solubilised with low molecular weight organic acids. Amongst the REEs in bioleaching residue of *E. aerogenes* the highest content in F2 was recorded for Y followed by Nd, and Ce. As citric acid was not detected in this study (data not shown), the smaller extractable concentration of REEs in the bioleach residue can be attributed to the microbial generation of weaker acids such as gluconic, acetic, and malic acids and consequently the complexation of REE with organic acids. These results agree with a previous study by (Mittermüller et al., 2016) that reported extraction yields of the organic acids for both soil and tailings material generally increasing with increasing complexation capacity in the order: acetic acid < malic acid < citric acid. As the nature of acids generated by *E. aerogenes* and *A. ferrooxidans* are different, comparison of REEs complexation with biogenic sulfuric and organic acids is difficult.

The elements bound to Fe-Mn oxy/hydroxides, the reducible fraction (F3), are normally mobilized with reductive conditions (Zimmerman and Weindorf, 2010) by changing either the oxidation state of the element or the host mineral elements (i.e., iron ore manganese hydroxides) (Mittermüller et al., 2016). A couple of recent studies have reported that MnO_2 can oxidize Ce^{3+} to Ce^{4+} (Yu et al., 2016). Fe-Mn oxy/hydroxides can also scavenge cations such as REEs by recrystallization products such as hematite, lepidocrocite, goethite, and maghemite (Lottermoser, 1990). However, based on results described in Section 3.4 changes to the oxidation state of Ce^{4+} were not detected, and hence Eh may not contribute to REEs mobilization.

The fourth fraction of extracted elements, the acid soluble fraction (F4), includes the REEs that are usually associated with barely soluble phosphates. The widely used CBR extraction method (Rao et al., 2010) includes REE phosphates within the residual fraction. Thus, a separate step was required to evaluate the extent to which REE are available in a phosphate form for further bioleaching by microorganisms. The bioleaching induced REEs association into the acid soluble fraction suggests that phosphate chemistry in solution was most likely the limiting step to REEs mobilization. Due to differences in the final pH of the media for *E. aerogenes* and *A. ferrooxidans* cultures, the phosphate metal complexes would be less solubilised at higher pH (*E. aerogenes*), resulting in higher precipitated P, whereas the soluble P would be more prevalent in the experiments with *A. ferrooxidans* due to lower pH and solubilisation of P-metal complexes (higher insoluble P content for *E. aerogenes*). At lower pH, these REEs phosphate complexes would solubilise, allowing for the release of higher soluble levels of P, but at higher pH the precipitates would remain insoluble. Therefore, providing acidic conditions that maintain phosphate complexes dissolved in solution potentially contribute to increase in overall REEs dissolution.

Considering the sum of the non-residual (labile) fractions (F1 + F2 + F3 + F4), the observed increase in partitioning in mobile fraction was not associated with REEs solubilisation during bioleaching processes, as the total concentration of all REEs in the non-residual fractions of the residue for *E. aerogenes* (22–24%) were higher than *A. ferrooxidans* (15–20%), while *A. ferrooxidans* displayed higher REEs solubilisation into solution phase during bioleaching (Figs. 2 and 3; Tables 1 and 2). In addition, REEs released from the residual fraction did not remain in the solution phase but shifted to the other fraction of the monazite, particularly the acid soluble fraction, implying that bacterial metabolism (phosphate acquisition, storage, and complexation) is likely to play a key role in controlling overall solubilisation processes. These finding suggest that an increase in REEs mobility does not necessarily govern a specific element solubilisation into the

solution.

Based on the results from bioleaching, SEP and XANES, it can be concluded that a combination of biogeochemical processes and physico-chemical characteristic of specific elements generated complex patterns that controlled the bioavailability and mobility of REEs in monazite.

4. Conclusion

This study provides the first direct evidence of a synergistic effect of a heterotrophic-autotrophic co-culture on the bioleaching of REEs from monazite. The combination of *A. ferrooxidans* and *E. aerogenes* increased REEs bioleaching from monazite (up to a final concentration of 40 mg L⁻¹ REEs including: Ce, La, Nd, Pr, and Y) as compared to the pure cultures of *A. ferrooxidans* (23.6 mg L⁻¹) or *E. aerogenes* (5.84 mg L⁻¹) owing to a synergic interaction through the biogenic generation of both organic acids and sulfuric acid.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.biteb.2018.07.003>.

Acknowledgments

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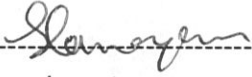
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8.2 Appendix 2

The following pages contain the written statements of the co-authors of published, submitted or under preparation manuscripts forming the chapters of this thesis.

To Whom It May Concern,

I, Homayoun Fathollahzadeh, as the first author of the publication entitled "**Role of microorganisms in bioleaching of rare earth elements from primary and secondary resources: A review**", declare that this work was primarily designed, experimentally executed, interpreted, and written by the first author of this manuscript.



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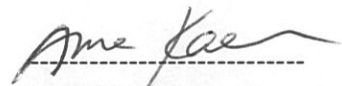
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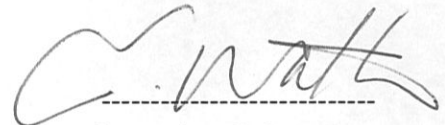
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I, Homayoun Fathollahzadeh, as the first author of the publication entitled "**Diversity and function of phosphate solubilizing bacteria enriched from Mount Weld rare-earth mine, Australia**", declare that this work was primarily designed, experimentally executed, interpreted, and written by the first author of this manuscript.

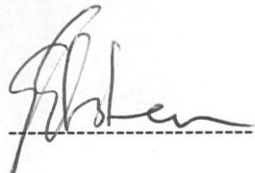


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Anna H. Kaksonen


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Elizabeth L.J. Watkin

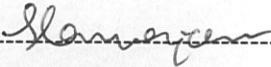
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Thomas Becker

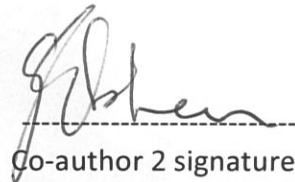
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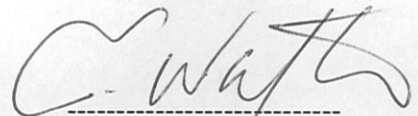
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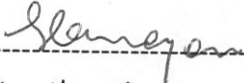
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I, Homayoun Fathollahzadeh, as the first author of the publication entitled "**Better together: Potential of co-culture microorganisms to enhance bioleaching of rare earth elements from monazite**", declare that this work was primarily designed, experimentally executed, interpreted, and written by the first author of this manuscript.



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Mark J. Hackett

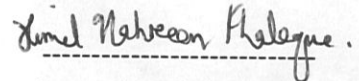
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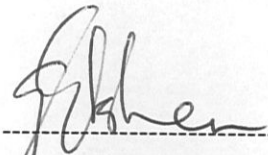
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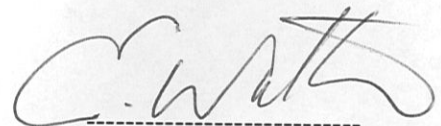
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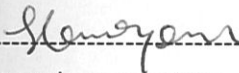
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I, Homayoun Fathollahzadeh, as the first author of the publication entitled "**Effect of glycine on bioleaching of rare earth elements by phosphate solubilising microorganisms from Western Australian monazite**", declare that this work was primarily designed, experimentally executed, interpreted, and written by the first author of this manuscript.



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Himel N. Khaleque

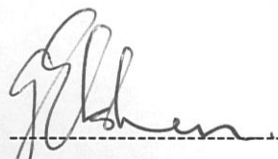
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Jacques J. Eksteen

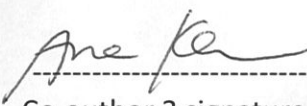
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8.3 Appendix 3

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1. H Fathollahzadeh, MJ Hackett, HN Khaleque, JJ Eksteen, AH Kaksonen, ELJ Watkin (2018) Better together: Potential of co-culture microorganisms to enhance bioleaching of rare earth elements from monazite. Bioresource Technology Reports 3 (2018)109–118
2. Homayoun Fathollahzadeh, Jacques J. Eksteen, Anna H. Kaksonen, Xi-Zhi Niu, Elizabeth L. J. Watkin (2018). Microbial Contact Enhances Bioleaching of Rare Earth Elements. Bioresource Technology Reports 3 (2018) 102–108

Many thanks

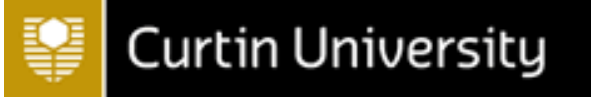
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