Bioleaching Potential of Filamentous Fungi to Mobilize Lithium and Cobalt from Spent Rechargeable Li-Ion Batteries

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Bioleaching Potential of Filamentous Fungi to Mobilize Lithium and Cobalt from Spent Rechargeable Li-Ion Batteries

by

Aldo Lobos

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Integrative Biology with a concentration in Ecology and Evolution Department of Integrative Biology College of Arts and Sciences University of South Florida

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Keywords: Aspergillus niger, Penicillium chrysogenum, Penicillium simplicissimum, Organic Acids, Cathode

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DEDICATION

I dedicate my thesis work to my family and friends. I am thankful for their constant support and love throughout this journey. I am also grateful to my family for being the world’s best cheerleaders at my side in this tough process. To my father, thank you for always offering words of encouragement and believing in me every step of the way.

I also dedicate this work to my fiancé Rosemary Mendez for being by my side and pushing me to accomplish every goal that I set.
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ABSTRACT

Demand for lithium (Li) and cobalt (Co) is on the rise, due in part to their increased use in rechargeable Li-ion batteries (RLIB). Current recycling processes that utilize chemical leaching efficiently recover Li and Co from the cathode material in spent batteries; however, these processes are costly and emit hazardous waste into the environment. Therefore, a more sustainable process for recycling Li and Co is needed, and bioleaching may provide a solution. Fungal bioleaching has been shown in previous studies to effectively mobilize metals (Pb, Al, Mn, Cu, and Zn) from mine tailings, electronic scrap, and spent batteries with organic acids. However, little is known regarding fungal tolerance to Li and Co, and if the concentrations of organic acids excreted by fungi can effectively leach Li and Co from the cathode material.

In order to address these questions, experiments were first conducted to test the Li and Co leaching efficiency with organic acids at concentrations similar to what has been previously reported in fungal cultures. The remaining experiments were performed with three fungal species: Aspergillus niger, Penicillium chrysogenum, and Penicillium simplicissimum. First, fungal biomass production, pH and organic acid excretion were examined when the fungi were grown in Czapek dox broth (CDB) or Sabouraud dextrose broth (SDB). Second, fungal biomass production and pH were examined when the fungi were grown in the presence of Li or Co. This determines tolerance of the fungi to the metals, and if fungal processes were inhibited by the metals. Third, bioleaching was performed with cathode material from RLIB in batch cultures to test the ability of organic acids excreted by A. niger to mobilize Li and Co. Three bioleaching
strategies, one-step, two-step, and spent-medium leaching techniques were used to mobilize Li and Co from the cathode in RLIB.

Low concentrations of organic acids similar to what is excreted by fungi have not been tested to leach Li and Co from the cathode in RLIB. Results from chemical leaching with low concentrations of organic acids in this study indicate that organic acid leaching efficiency can be increased by utilizing higher concentrations (above 50 mM) of citric or oxalic acid to mobilize Li or Co from the cathode in RLIB. Furthermore, 100 mM of citric acid or 100 mM of oxalic acid mobilized more Co or Li than mixtures of organic acids. Notably the addition of hydrogen peroxide to mixed concentrations of organic acids significantly improved mobilization of Li and Co under abiotic conditions.

Different growth media may alter biomass production and potentially organic acid excretion by the three fungal species. Analysis of biomass production by \textit{A. niger} and \textit{P. simplicissimum} showed that differences in media composition between CDB and SDB did not affect collected biomass for each species. However, CDB cultures with \textit{P. chrysogenum} had significantly less biomass than SDB cultures after 10 days of growth. Differences in growth by \textit{P. chrysogenum} between CDB and SDB may be attributed to preferred nutrients and/or low pH present in SDB cultures. Biomass production by the three fungi increased up to day 10 in CDB or SDB. This result indicated that nutrients in CDB or SDB were not limiting toward fungal growth. Cultures with \textit{A. niger} had the highest concentrations of organic acids (50 mM of oxalic acid), followed by cultures with \textit{P. simplicissimum} (30 mM oxalic acid), and \textit{P. chrysogenum} (less than 5 mM oxalic acid). Organic acids excreted by all three fungal species were detected in cultures in CDB, while only \textit{A. niger} and \textit{P. chrysogenum} excreted organic acids in SDB cultures.
Metals such as Li or Co present in the cathode of RLIB may be toxic to fungal processes when exposed to high metal concentrations. Metal tolerance experiments indicate that biomass production by the three fungi was significantly inhibited by 100 mg/L Co compared to controls, which contained no metal. Li at a concentration of 1000 mg/L inhibited biomass production by *A. niger* and *P. simplicissimum*. However, biomass production by *P. chrysogenum* was not significantly inhibited by 1000 mg/L Li. I found that *P. simplicissimum* was the most susceptible to toxic effects of Li and Co among the three fungi. In *A. niger* cultures amended with 100 mg/L Li or Co, pH at day 5 was similar to control cultures of *A. niger* without metals (pH 3.0 – 3.4), whereas pH was significantly higher in cultures with 1000 mg/L of Li or Co (pH 7.1 – 7.3).

Cultures of *A. niger* were exposed to the cathode material from RLIB to test the leaching efficiency of excreted organic acids after mobilizing Li and Co. In bioleaching experiments with *A. niger*, organic acids excreted in the presence of cathode material from RLIB were quantified at concentrations under 50 mM. At the end of bioleaching experiments with *A. niger*, 40 mM tartaric acid was detected and was the highest produced organic acid in bioleaching cultures. However, with conditions set in this study, organic acids excreted by *A. niger* mobilized only 7% of Co and 20% of Li when using spent medium with cathode material from RLIB. According to findings in chemical leaching experiments, concentrations of organic acids higher than 50 mM will be required in fungal cultures to increase mobilization of Li or Co from the cathode material in RLIB. Modifying growth media to include higher concentrations of sucrose will potentially increase organic acid excretion as demonstrated in previous publications. Future studies should focus on how to maximize organic acid excretion by fungi when exposed to metals found in the cathode of RLIB.
CHAPTER 1. INTRODUCTION AND OBJECTIVES

1.1. Li-ion Batteries and Fungal Bioleaching

Lithium and cobalt are metals that are used to form the lithium cobalt oxide cathode material in rechargeable lithium-ion batteries (Fergus, 2010). These metals have increased in demand as rechargeable lithium-ion batteries (RLIB) now power many electronic devices such as laptops, cell phones, power tools, and electric/hybrid cars (Wanger, 2011; Goonan, 2012). The future demand for lithium will partially depend on the quantity of vehicles using RLIB and the number of consumers that switch from gasoline to electric cars (Goonan, 2012). A recent estimate showed that the current US demand of RLIB is approximately 54,000 tons per year, and by the year 2020, the demand will be close to 70,000 tons per year to supply the mass production of electronic devices and electric vehicles (Martin et al., 2017).

Estimates for demand of RLIB will have to account for the fact that consumers will need to replace RLIB once they become spent batteries. Spent batteries are RLIB that have exceeded a lifetime of 10 – 15 years and can no longer maintain a charge (Gaines and Nelson, 2009). These batteries are typically discarded as solid waste when they have reached the end of life expectancy. The increase in production of RLIB, coupled with a finite lifetime, will result in a rise of battery waste into the solid waste stream. One estimate reported that the U.S will yearly dispose approximately 30,000 metric tons of RLIB by the year 2030 (Gaines and Nelson, 2009). That amount of battery waste disposed into landfills will be an environmental concern as toxic
metals enter the environment at an accelerated rate. Therefore, research has increased and focused on efficient methods to recycle spent batteries (Cui and Zhang, 2008). In order to mitigate the environmental impact of spent battery waste, battery recycling processes should include methods to efficiently recover lithium and cobalt.

Hydrometallurgical and pyrometallurgical methods are currently used for the recovery of valuable metals from battery waste; however, these processes involve extreme and costly conditions that include high temperature, high pressure, extreme chemical environment, and emission of toxic gases into the atmosphere (Cui and Zhang, 2008; Joulié et al., 2014; Li et al., 2009). Therefore, sustainable bioleaching processes have been reported as an alternative method to recover important metals from the solid waste stream (Brandl et al., 2001; Ilyas and Lee, 2013b; Horeh et al., 2016). At an industrial scale, this alternative method might be able to mitigate environmental impacts of battery waste and reduce industrial requirements for the recovery of lithium and cobalt from spent RLIB.

The term “bioleaching” is used in this study to describe the process of dissolving metals from the solid phase in batteries using a biological approach. Bacteria and fungi can be utilized in bioleaching processes to leach valuable metals from solid waste (Mishra and Rhee, 2014; Brandl et al., 2001). Bacteria have been studied for their bioleaching potential to recover valuable metals from spent batteries (Mousavi et al., 2014). However, these processes require a high level of control to maintain necessary temperature, dissolved oxygen concentrations and pH. These conditions may be costly to operate at an industrial scale. The bacteria used in bioleaching studies are cultured in controlled environments to produce a strong acid such as sulfuric acid (Mousavi et al., 2014). This strong acid can have an exothermic reaction with the cathode material (LiCoO₂) in spent batteries that releases toxic waste in the form of SO₃, Cl₂ and
NOx into the atmosphere (Santana et al., 2017). Another complication of using bacteria for bioleaching is that most bacteria are unable to tolerate the low pH levels (below 3) that are necessary for an effective bioleaching process (Rousk et al., 2009).

Bioleaching techniques with filamentous fungi have been utilized in multiple studies to recover valuable metals such as Pb, Cu, Zn, Mn, Cd, and Ni due to the high leaching efficiency of organic acids excreted by fungi at pH levels of 2-3 (Rousk et al., 2009). Filamentous fungi reportedly produce oxalic acid, citric acid, L-malic acid, tartaric acid and gluconic acid, which can dissolve metal ions from the solid phase (Burgstaller and Schinner, 1991; Bosshard et al., 1996). Aerobic filamentous fungi can produce some of these organic acids as intermediates in the tricarboxylic acid cycle (TCA), and others through the glyoxylate cycle (Magnuson and Lasure, 2004; Papagianni, 2007). Excretion of citric acid or oxalic acid is affected by multiple factors including pH, carbon source, trace metals, dissolved oxygen, phosphate and nitrogen limitation (Papagianni, 2007; Aghaie et al., 2009).

Researchers have reported effective leaching processes with filamentous fungi to recover various metals from solid waste including spent batteries (Table 1.1). Aspergillus niger, Penicillium chrysogenum and Penicillium simplicissimum were selected for this study based on the production of organic acids reported in previous bioleaching studies (Table 1.1). The effectiveness of the bioleaching process is highly dependent upon various chemical, physical and biological variables, including carbon and nitrogen source, concentration of inoculant, temperature, pH, oxygen supply, pulp density of waste material, and time (Rasoulnia and Mousavi, 2016).

In the natural environment fungi are typically growing under low nutrient conditions and excrete very low amounts of organic acids that are beneficial to surrounding plants. A
bioleaching process with fungi is designed to tweak the natural metabolism and growth of fungi in order to maximize excretion of organic acids. These organic acids can then be utilized to mobilize metals from solid waste such as spent batteries without creating hazardous waste (Table 1.1).

Table 1.1: Summary of previously published studies on fungal bioleaching.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Fungi</th>
<th>Identified Organic Acids</th>
<th>Solid Waste</th>
<th>Metal Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bosshard et al., 1996</td>
<td><em>Aspergillus niger</em></td>
<td>citric and gluconic</td>
<td>fly ash</td>
<td>Cd 81%; Zn 66%; Cu 57%; Pb 52%</td>
</tr>
<tr>
<td>Brandl et al., 2001</td>
<td><em>Aspergillus niger,</em></td>
<td>not reported</td>
<td>electronic scrap</td>
<td>Al 95%; Zn 95%; Cu 65%</td>
</tr>
<tr>
<td></td>
<td><em>Penicillium simplicissimum</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Santhiya and Ting, 2005</td>
<td><em>Aspergillus niger</em></td>
<td>Oxalic</td>
<td>spent refinery catalyst</td>
<td>Ni 58-63%; Al 54-58%; Mo 79-82%</td>
</tr>
<tr>
<td>Wu and Ting, 2006</td>
<td><em>Aspergillus niger</em></td>
<td>gluconic</td>
<td>fly ash</td>
<td>Zn 80-100%; Pb 60-70%</td>
</tr>
<tr>
<td>Amiri et al., 2011</td>
<td><em>Penicillium simplicissimum</em></td>
<td>gluconic</td>
<td>spent refinery catalysts</td>
<td>Mo 91-99%; Fe 78 – 100%</td>
</tr>
<tr>
<td>Ilyas and Lee, 2013</td>
<td><em>Penicillium chrysogenum</em></td>
<td>tartaric, oxalic, citric, gluconic</td>
<td>electronic scrap</td>
<td>Al 85-96%; Zn 81-98%; Ni 63-73%</td>
</tr>
<tr>
<td>Horeh et al., 2016</td>
<td><em>Aspergillus niger</em></td>
<td>malic, gluconic, oxalic, citric</td>
<td>spent Li-ion mobile phone battery</td>
<td>Cu 100%; Li 95%; Mn 70%; Al 65%; Co 45%; Ni 38%</td>
</tr>
</tbody>
</table>

The potential to recover lithium and cobalt from spent rechargeable Li-ion battery using a fungal bioleaching process mediated by *A. niger* has been demonstrated in one study (Horeh et al., 2016). That study reported a lithium recovery of 95% and a cobalt recovery of 45% from
spent battery cathode material (Horeh et al., 2016). A previous study demonstrated improved cobalt recovery in a chemical leaching procedure (i.e., not bioleaching) that modified the reactions with addition of 2% hydrogen peroxide by mass (Zhu et al., 2012). Experiments with hydrogen peroxide indicate that this chemical acts as a reducing agent under specific conditions (Zhu et al., 2012). These findings provide evidence for the application of hydrogen peroxide and its potential benefits in fungal bioleaching processes. Published literature is not available for the potential of *P. chrysogenum* and *P. simplicissimum* to improve recovery of Li or Co from spent RLIB. Also, there is limited published information on the toxicity of lithium and cobalt, which may negatively affect fungal growth or other important processes.

1.2. Metal Toxicity and its Effect on Fungal Processes

High metal concentrations generally inhibit microbial growth and can affect their survival (Burgstaller and Schinner, 1993). Previous studies on fungal tolerance to metals have included Ni, Mo, Fe, Zn, Cd, Cu, Pb, Mg and Mn. These studies used either a tolerance index or biomass production to determine metal tolerance (Anahid et al., 2011; Valix and Loon, 2003). The tolerance index is defined as the fungal growth rate in the presence of metals, divided by the fungal growth rate in a control absent of metals (Anahid et al., 2011). This index can be used to assess the fungal tolerance to each metal and whether, at certain concentrations, metal toxicity negatively affects fungal growth or survival.

Exposure to metals can impact organic acid excretion according to previous studies in *A. niger* cultures. Metals such as Mn, Fe and Zn have been previously reported as important limiting agents towards organic acid excretion by fungi. These studies show that high Mn concentrations in fungal cultures can decrease citric acid excretion by 20% (Gadd, 1999). The
effects of other surrounding metals on fungal processes are not completely clear; therefore, mechanisms behind organic acid excretion and how metal toxicity impacts these systems should be studied further.

1.3. Organic Acid Excretion by Filamentous Fungi

Oxalic acid (pKa$_1$ = 1.23) and citric acid (pKa$_1$ = 3.13) are reported as the strongest acids produced by filamentous fungi (Horeh et al., 2016). Commercial processes currently utilize the metabolic processes of filamentous fungi to produce and excrete large quantities of citric or oxalic acid. These acids are valuable for manufacture of various products, including food preservatives and household cleaners (Tkacz and Lange, 2004). These two organic acids have been the primary focus in previous bioleaching studies (Santhiya and Ting, 2005; Ilyas and Lee, 2013; Horeh et al., 2016). *A. niger* is the primary organism used in citric acid production due to its reported efficiency (about 90%) for converting polysaccharides to citric acid (Alkseev et al., 2015). This high efficiency of organic acid production and excretion is mediated by the activity of several enzymes and is dependent on specific environmental conditions which include but are not limited to: temperature, pH, carbon source and concentration, nitrogen and phosphorus limitation, concentration of trace metals, dissolved oxygen concentration, fungal morphology and inoculum concentration (Papagianni, 2007; Vandenberghe et al., 1999).

Researchers have examined the biochemical pathways that lead to the production and excretion of oxalic acid by *A. niger* (Ruijter et al., 1999). Previous studies demonstrated control over citric acid or oxalic acid production and excretion through pH modification of the culture medium (Auta et al., 2014; Andersen, et al., 2009; Ruijter et al., 1999). To further understand the effect of pH on citrate and oxalate production and excretion, scientists examined the
locations where oxalic acid and citric acid are produced in filamentous fungi biochemical pathways by NMR spectroscopy (Kubicek et al., 1988). Several studies with A. niger have shown that oxalic acid production occurs mainly in the glycolytic pathway located in the cytosol, and that citric acid is primarily produced via the TCA cycle in the mitochondria (Ruijter et al., 2000; Kubicek et al., 1988).

Oxaloacetate hydrolase catalyzes the conversion of oxaloacetate to oxalate (Ruijter et al., 1999), while citrate synthase catalyzes the formation of citric acid (Kubicek, 1988). Multiple factors are reported to affect the regulation of the activity of these two enzymes in filamentous fungi; however, current findings do not provide evidence that regulation of enzymes in fungi impacts organic acid production and excretion (Ruijter et al., 2000). Mechanisms behind organic acid production and excretion in fungi are complex and require a deeper understanding to improve process requirements for effective bioleaching of spent batteries.

Additional studies on A. niger and citrate synthase show that this enzyme is responsible for catalyzing the beginning step of the TCA cycle and is critical for cellular respiration (Kubicek, 1988). During the TCA cycle, citrate synthase catalyzes the condensation of acetylCoA and oxaloacetate to form citrate (Ruijter et al., 2000, Tkacz and Lange, 2004). The regulation of citrate synthase mainly varies depending on the availability of oxaloacetate and acetyl-CoA (Papagianni, 2007). The regulation of citrate synthase and the factors which mediate organic acid excretion have become a popular topic among microbial physiologists interested in the benefits of metabolic processes in filamentous fungi.

Researchers hypothesized that the regulation of citrate synthase played a key role in the excretion of citric acid (Ruijter et al., 2000). However, this hypothesis for effects of citrate synthesis regulations was disproved through a series of experiments with an A. niger mutant
which overexpressed citrate synthase (Ruijter et al., 2000). It was found that the mutant strain had a significantly higher citrate synthase activity than the control non-mutant strains of A. niger, but the difference in citrate synthase activity was not found to affect the excretion of citric acid (Ruijter et al., 2000). Therefore, the mechanism for citric acid excretion is dependent on the activity of other processes or enzymes in the TCA cycle of A. niger (Alkseev et al., 2015; Tkacz and Lange, 2004).

Studies of A. niger indicate that citric acid excretion is primarily mediated by other factors in their metabolism and/or environment (Andersen et al., 2009, Ilyas and Lee, 2013a). Maximum levels of citric acid were reported in previous studies at pH levels of 2-4 in the presence of various metals (Horeh et al., 2016; Ilyas and Lee, 2013a). Filamentous fungi such as A. niger are capable of acidifying their surrounding environment (pH 2-4) through active H+-ATPases for proton efflux, which increases in order to maintain balance of their proton gradient during growth (Gadd, 2004). Organic acids excreted by fungi can contribute to acidification of environments by the release of protons when organic acids come in contact with water. The role of low pH on increased citric acid excretion has been demonstrated in lab scale batch experiments, but the mechanism behind this finding is not clearly understood (Horeh et al., 2016; Ilyas and Lee, 2013a).

Previous research on A. niger provides evidence for a potential excretion mechanism that involves the affinity of a tricarboxylic acid carrier that is hypothesized to play a key role in the transport and excretion of citric acid (Andersen et al., 2009; Osiewacz, 2002). If the expression or activity of this tricarboxylic acid carrier increases it can directly compete with isocitrate dehydrogenase for citrate in the TCA cycle (Andersen et al., 2009). Current literature on citric acid excretion does not provide information for factors that mediate the affinity of this
tricarboxylic acid carrier in *A. niger*. Collected information indicates that the mechanism behind the excretion of citric acid involves multiple factors that may all have a role in developing optimal conditions.

Oxaloacetate hydrolase catalyzes the reaction in *A. niger* to produce oxalic acid (oxalate) and acetate: oxaloacetate + H₂O → oxalate + acetate (Figure 1.1 Ruijter *et al.*, 1999). Oxaloacetate hydrolase activity localized in the cytoplasm of *A. niger* is important for production of metabolic intermediates in biosynthesis such as lipid synthesis or other reactions (Figure 1.1 Kubicek *et al.*, 1988). The activity of this enzyme may also be a strategy by filamentous fungi, where oxalate production can be used to acidify their cytoplasm and excrete excess acid to the extracellular environment to reach optimal conditions for growth and survival (Garcia and Torres, 2011). Understanding the regulation of oxaloacetate hydrolase and the factors which mediate its activity (such as pH) is important when optimizing processes for oxalic acid production with filamentous fungi.

Figure 1.1: Schematic for excretion of oxalate in the cytoplasm of *A. niger*. (Information used to create schematic originated from Kubicek *et al.*, 1988; Ruijter *et al.*, 1999).
The optimal extracellular pH for maximum activity of oxaloacetate hydrolase is reported to be around 6 in cultures with fungi (Ruijter et al., 1999; Osiewacz, 2002). An extracellular pH of 6 is the same level of acidification at which several researchers have observed maximum production and excretion of oxalic acid, which was approximately 5 g/L (Kubicek et al., 1988). However, maintaining optimal extracellular pH for oxalic acid excretion will not be favorable in a bioleaching process due to the low pH requirements (2-3) necessary for effective leaching of metals (Rousk et al., 2009). Therefore, bioleaching studies with filamentous fungi should probably focus on excretion of citric acid rather than oxalic acid for effective metal mobilization.

One previous study examined the bioleaching potential of organic acids excreted by *A. niger* using growth media with high concentrations (100 g/L) of sucrose in bioleaching processes to mobilize Li and Co from the cathode material in RLIB (Horeh et al., 2016). The work in this thesis aims to test the bioleaching potential of organic acids excreted by *A. niger* when utilizing growth media with low concentrations of sucrose (30 g/L). This study is focused on mitigating costs of providing an abundance of sugar in bioleaching processes.

1.4. Objectives of Study

The following objectives were used in this thesis to develop experiments (more detailed analytical methods can be found in chapter 2):

1) Determine the amount of Li and Co that can be removed from spent Li-ion batteries through HCl digestion.

2) Analyze effectiveness of weak organic acids to leach Li and Co from spent RLIB.
3) Quantify biomass production and organic acid excretion by the three fungal species *A. niger*, *P. chrysogenum* and *P. simplicissimum* with two different growth media.

4) Assess the growth of *A. niger*, *P. chrysogenum* and *P. simplicissimum* in the presence of Li and Co at concentrations similar to levels found in bioleaching conditions.

5) Ascertaining the bioleaching potential of *A. niger*, *P. chrysogenum* and *P. simplicissimum* to determine if this process is effective for recovering Li and Co from spent rechargeable Li-ion batteries.

### 1.5. Significance and Expected Benefits of This Research

The purpose of this work is to determine the ability of the filamentous fungi *A. niger*, *P. chrysogenum* and *P. simplicissimum* to leach Li and Co from spent LIB via excreted organic acids. The work will expand knowledge on biochemistry and the organic acids produced by the three fungal species. This study is also expected to provide information on Li and Co toxicity to fungal growth and the effect of these metals on organic acid production. The expected benefits of this study are:

- Results from this work will help develop an environmentally friendly fungal bioleaching process which is cost effective for recycling Li and Co from spent rechargeable Li-ion batteries.
- An effective recycling process via fungal bioleaching can potentially mitigate mining of virgin Li or Co.
- The work done will advance knowledge on organic acid production and leaching potential of acids which can be used in commercial and/or industrial processes for economic gain.
• The knowledge gained may potentially apply to many other bioleaching applications, and contribute to recovery of valuable metals.

The completed work will also contribute to improve our understanding of organic acid excretion by filamentous fungi and how toxic metals affect their growth and production of organic acids. If successful, this method can be used as an alternative recycling process to mitigate waste from current recycling processes which are harmful to the environment. With technology increasing and demand rising for RLIB, it is imperative that environmentally sustainable and cost effective biological methods are developed to recycle electronic waste.
CHAPTER 2. EXPERIMENTAL AND ANALYTICAL METHODS

2.1. Preparation of Media, Reagents, and Standards

Lithium and cobalt standard solutions (1 mg/mL) for calibration of atomic absorption spectroscopy (AAS) were purchased from ACROS Organics. Standards for AAS calibration were prepared by diluting standard solutions of Li or Co with sterile deionized water and 0.7% HNO₃. Stock solutions of lithium and cobalt were prepared for metal tolerance tests with fungi. These stock solutions were prepared using lithium chloride (98.5%, Fisher Chemical) or cobalt chloride anhydrous (97%, ACROS Organics) with sterile deionized water to the concentration of 1,000 mg/L and 10,000 mg/L. Final concentrations of metal stock solutions were verified with AAS. Lithium and cobalt stock solutions were used to supplement media with various concentrations of each metal.

Organic acid solutions of the following organic acids were prepared in sterile deionized water at 300 mM concentration: oxalic acid (98%, SIGMA-ALDRICH), citric acid monohydrate (99%, SIGMA-ALDRICH), gluconic acid (98%, sodium salt, ACROS Organics), L-malic acid (99%, ACROS Organics), and tartaric acid (99%, Fisher Chemical). Organic acid stock solutions (100 mL total volume) were placed on a magnetic stirrer for 10 minutes to dissolve solutes. Stock solutions were diluted with sterile deionized water to appropriate volumes. Organic acid stock solutions with sterile deionized water were used in chemical leaching experiments with RLIB cathode or pure LiCoO₂ (97%, Alfa Aesar). Organic acid standards for
calibration of high performance liquid chromatography (HPLC) were prepared using HPLC-grade water (Fisher Chemical).

Sabouraud dextrose agar (BD-Difco, composition: 1 L Nanopure water, 5 g/L peptic digest of animal tissue, 5 g/L pancreatic digest of casein and 40 g/L of dextrose, 15 g/L of Agar) plates, Sabouraud dextrose broth (BD-Difco, composition: 1 L Nanopure water, 5 g/L peptic digest of animal tissue, 5 g/L pancreatic digest of casein and 20 g/L of dextrose) and Czapek Dox broth (BD-Difco, composition: 1L Nanopure water, 3 g/L NaNO₃, 1 g/L K₂HPO₄, 0.5 g/L MgSO₄, 0.5 g/L KCl, 0.01 g/L FeSO₄, 30 g/L Saccharose) were prepared per the manufacturer’s instructions. Agar and broth were freshly prepared 2-3 d before every experiment. Media were sterilized with an autoclave at 121 °C for 15 min, and then allowed to cool; cooled media were stored at 4 °C until day of experiment. Agar and broth were allowed to reach room temperature for 1 h before inoculation.

2.2. Analysis of Spent Batteries

Spent rechargeable mobile phone batteries were collected and stored at room temperature once separated by manufacture to determine the quantity collected. Maxwell rechargeable lithium-ion batteries were selected for all experiments based on quantity collected and the amount of cathode material required. Batteries were dismantled following previously published research (Li et al., 2013; Horeh et al., 2016). Pliers and cutting tools were utilized to open the steel casing and remove the cathode and anode strips, which were carefully separated. Wet weight of the cathode and anode was determined using an analytical gram scale. The cathode and anode was dried at 60 °C in a drying oven for 24 hours. The dried cathode material, which
contained Li and Co in the form LiCoO₂, was ground with a mortar and pestle in preparation for chemical digestion.

To determine maximum Li and Co recovery from 3 batteries with spent cathode material, chemical digestion was accomplished in a fume hood by placing 10 mL of 37% HCl in a 100 mL beaker with 0.2 g (2% pulp density, wt/vol %) of the grounded cathode material or 97% LiCoO₂ (following procedure by Mousavi et al., 2014). The solution was gently stirred with a magnetic stir bar while heating at 35 °C for 2 h. After chemical digestion, the solution was filtered through a 0.2 µm cellulose acetate syringe filter to remove particulates, and the filtrate was then analyzed for Li and Co concentrations via AAS. Percent mobilization of metals from cathode material was determined by assuming that 100% of the cathode material was LiCoO₂. Therefore, we expected that the supplemented cathode material consisted of approximately 7% Li and 59% Co in all experiments.

2.3. Chemical Leaching with Organic Acids of Cathode Material from RLIB

In order to simulate the fungal processes and determine leaching efficiency of organic acids, stock solutions of reagent grade organic acids (section 2.1) were diluted in 250 mL Erlenmeyer flasks with sterile deionized water to 100 mL of 20 mM, 50mM and 100 mM concentrations of each acid following previously published procedures (Aung and Ting, 2004). Duplicate control flasks were prepared with sterile deionized water without organic acid. Experimental and control flasks were supplemented with 2 g (2% pulp density, wt/vol) of cathode material from dismantled spent batteries, ground as described above. All flasks were incubated at 25 °C and shaken at 120 rpm. Chemical leaching of Li and Co was monitored by AAS every 48 h for 10 d in duplicate samples. Samples were filtered through a 0.2 µm cellulose
acetate syringe filter into sterile 10 mL plastic centrifuge tubes for AAS metal analysis. Metal concentrations in samples were measured in duplicate via AAS.

The chemical leaching experiment was repeated to test mixed organic acids over a 10 day period. Chemical leaching with mixed organic acids was conducted with and without the addition of a 2% hydrogen peroxide (added 6.042 mL of 30% H$_2$O$_2$) in experimental and control flasks (procedure by Zhu et al., 2012). Results of chemical leaching experiments were compared to determine effectiveness of weak organic acids as lithium or cobalt leaching agents. Different mixtures of organic acids were also investigated to simulate low concentrations of organic acid mixtures reported in fungal processes (Table 2.1).

<table>
<thead>
<tr>
<th>Organic Acid Mix</th>
<th>Oxalic Acid</th>
<th>Citric Acid</th>
<th>L-malic Acid</th>
<th>Tartaric Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50 mM</td>
<td>20 mM</td>
<td>10 mM</td>
<td>10 mM</td>
</tr>
<tr>
<td>2</td>
<td>100 mM</td>
<td>20 mM</td>
<td>10 mM</td>
<td>10 mM</td>
</tr>
<tr>
<td>3</td>
<td>20 mM</td>
<td>50 mM</td>
<td>10 mM</td>
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<td>4</td>
<td>50 mM</td>
<td>100 mM</td>
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<td>10 mM</td>
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<td>5</td>
<td>100 mM</td>
<td>20 mM</td>
<td>0 mM</td>
<td>0 mM</td>
</tr>
<tr>
<td>6</td>
<td>20 mM</td>
<td>100 mM</td>
<td>0 mM</td>
<td>0 mM</td>
</tr>
</tbody>
</table>

### 2.4. Culturing Fungi

The following filamentous fungal strains were examined in this thesis: *P. simplicissimum* (ATCC 48705), *A. niger* (ATCC 6275) and *P. simplicissimum* (ATCC 10108). Fungi were transferred every 2 weeks to fresh Sabouraud dextrose agar (SDA) plates to promote growth and spore production. Preparation of standard spore suspensions were accomplished following previously published procedures (Sabra et al., 2011). Agar plates with fungi were incubated at 30 °C for 5-7 d. After about 7 d, conidia were collected by placing 10 mL of sterile Nanopure water with 0.5% Tween 80 on fungal growth. The fungal surface containing spores was scraped
with a sterile microscope slide to suspend spores in the Tween 80 solution. Tween 80 helped prevent spores from clumping. Separation of spores was necessary in order to count and standardize spore suspensions. The agar plate with spores suspended in the Tween 80 solution was then placed over a sterile beaker with four layers of sterile cheesecloth. Solution was slowly poured through the four layers of cheesecloth to filter out fungal mass and hyphal branches so that only spores remained. Agar plate and cheesecloth were washed further with 30 mL of the Tween 80 solution to recover residual spores. After collection of spore suspensions, spores were counted using a Hauser Bright-LinePhase Hemacytometer counting chamber viewed through a phase contrast microscope (Nikon Eclipse E600) to standardize 1 mL of spore suspension by diluting the spore suspension with sterile deionized water to approximately 1 \times 10^7 spores. Collected spores were stored in 50 mL plastic centrifuge tubes at 4 °C until they were needed for an experiment.

The standard spore suspensions (approximately 1 \times 10^7 spores/mL) collected for each species were separately inoculated into 125 mL Erlenmeyer flasks containing 50 mL of either Sabouraud dextrose broth (SDB) or Czapek Dox broth (CDB). Broth cultures were incubated at 30 °C and shaken at 120 rpm in a shaker incubator in order to create aerobic conditions favorable for optimal growth. All flasks contained sterile cheesecloth plugs to allow air flow while preventing contamination from the air.

2.5. Biomass Production and Organic Acid Excretion Analysis

Fungal cultures in broth (CDB or SDB) were investigated for biomass production and organic acid excretion over a 10 day period. Wet biomass was collected every 24 hours using vacuum filtration with a Buchner funnel and a Whatman filter paper to separate the fungal mass
from the liquid nutrient broth. Wet biomass was dried on a preweighed aluminum tray at 60 °C for 24 hours. After drying for 24 hours dry biomass was measured on an analytical gram scale to determine the biomass production in g/L. Filtered broth without fungi was tested for media acidification by measuring pH (Fisher Scientific accumet AB15 pH meter). Furthermore, the filtered broth was centrifuged and filtered through a 0.2 µm cellulose acetate filter to prepare sample for organic acid analysis by HPLC. Analysis of filtered broth via HPLC was required to separate and quantify mixtures of organic acids produced by *A. niger*, *P. chrysogenum*, and *P. simplicissimum*. Samples were analyzed every 48 hours for 10 days to examine differences of organic acid production among the studied fungi and the two different growth media.

### 2.6. Fungal Tolerance to Li and Co

Aliquots of Li or Co from stock solutions were placed in flasks with prepared sterile Czapek dox broth (CDB) to cultivate filamentous fungi in various concentrations of Li or Co. Fungal cultures were exposed to 0.1 mg/L, 3 mg/L, 100 mg/L and 1000 mg/L of Li or Co to preliminarily assess toxicity and how biomass production and media acidification (a proxy for organic acid production) were affected over a 5 day period.

With a better understanding of fungal tolerance to Li or Co, experiments were then conducted to measure the growth (biomass) and pH in cultures every 5 days in the presence of 50 mg/L and 250 mg/L of Li or Co for 20 days to allow the fungi time to grow under toxic conditions. Tolerance index was used to divide biomass production in cultures with metals by biomass measured in control cultures without metals (Valix and Loon, 2003). Toxicity of Li or Co to fungi was positive if significant inhibition of biomass production and/or organic acid excretion was determined. Results indicating Li and/or Co toxicity were used to determine
which fungal species would not tolerate a bioleaching process with spent RLIB. Organic acid excretion by fungi was monitored as another factor in the determination of a fungal candidate for bioleaching Li-ion batteries.

2.7. Assessment of Fungal Bioleaching Potential

In bioleaching experiments, CDB was supplemented with a 2% pulp density of cathode material (containing LiCoO₂) from spent RLIB and incubated at 30°C while shaking at 120 rpm for 20 days. Bioleaching experiments were conducted in three separate experiments: one-step, two-step and spent medium bioleaching. One-step bioleaching was accomplished by inoculation of fungi and addition of cathode material containing LiCoO₂ on day zero. Two-step bioleaching experiments began by growing fungal cultures without metal for 48 h. Cathode material was supplemented into the broth at a 2% pulp density (2 g) 48 h post inoculation to provide time for fungal growth and organic acid excretion prior to exposure of RLIB cathode material.

The last experiment used spent-medium bioleaching, which was conducted by allowing the fungi to grow and produce organic acids (without metals throughout growth) up to 20 days. The nutrient broth was then filtered to remove fungal mass and spent medium was collected containing organic acids. Spent-medium was then supplemented with 2% pulp density of the cathode material (containing LiCoO₂) from spent RLIB. Results for each experiment were recorded every 5 days for up to 20 days using HPLC to separate and quantify organic acids. AAS was used to analyze concentrations of dissolved Li and Co in the nutrient broth solution.
2.8. Analytical Methods

HPLC analysis of organic acid concentrations was accomplished using a Perkin Elmer Series 200 Pump and a Perkin Elmer Series 200 UV/VIS Detector. Chromatographic separation was performed by isocratic elution of the 0.1 M phosphate buffer (KH₂PO₄, pH 2.5) mobile phase at a flow rate of 1.5 mL/min at room temperature. Twenty µL samples were passed through a Brownlee Choice Organic Acids column (300×4.6 mm) to separate organic acids produced by the three fungal species in two different nutrient broths (CDB and SDB). Once separated, organic acids were passed through the UV/VIS Detector set at 214 nm to detect organic acids. Organic acid concentrations were quantified using TotalChrom version 6.3.2 to measure peak areas of each organic acid. Peak areas of organic acid standards were used to construct a standard curve.

AAS analysis of dissolved Li and Co concentrations was accomplished by using VARIAN AA240FS to measure one metal at a time. Analysis of samples was repeated to determine concentrations of a second metal. Hollow cathode lamps for Li or Co were set to the appropriate wavelength per manufactures instruction. VARIAN SpectrAA version 5.01 was used to construct a standard curve using absorption of Li or Co standards. Metal analyses were conducted with an oxygen flow of 3.5 L/min and acetylene flow of 1.5 L/min.

2.9. Statistical Analysis

Experiments were conducted in duplicate or triplicate from batch experiments of fungi cultures and chemical leaching reactions. All statistical analyses were completed in R studio (R v. 3.3.2) utilizing the one-way or two-way analysis of variance test (ANOVA). R studio was also used to test for Pearson correlations. For all experiments, data from the last sampling event (Day
5, Day 10 or Day 20) were used to conduct ANOVA. An alpha level of 0.05 was set to determine significant differences or correlations.
CHAPTER 3. RESULTS

3.1. Chemical Digestion of Spent Batteries

The wet cathode material from dismantled rechargeable Li-ion mobile batteries was approximately 27% of the total battery wet weight (Figure 3.1). Chemical digestion of the cathode material with HCl and analysis via AAS showed that Li was 8.25% and Co was 47% of the total cathode weight (Table 3.1). Assuming the cathode material was 100% LiCoO₂, 37% HCl effectively mobilized more than 90% Li and close to 80% Co from spent RLIB cathode (Table 3.1). The HCl digestion method was verified by application to pure LiCoO₂, from which 94% Li and 96% Co were mobilized.

Figure 3.1: Percentages of components by weight in rechargeable Li-ion batteries.
Table 3.1: Metal mobilization after 2 hour HCl digestion of Maxwell Li-ion cathode material.

<table>
<thead>
<tr>
<th>HCl Digestion of Cathode Material (240 mg from each battery, n = 3)</th>
<th>Mean of Dissolved Metal</th>
<th>Percent of Total Cathode Weight</th>
<th>Mobilization Percent from Cathode (assuming cathode is LiCoO$_2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Li</td>
<td>19.8 mg ± 3.6 mg</td>
<td>8.25% ± 1.5%</td>
<td>115% ± 21%</td>
</tr>
<tr>
<td>Co</td>
<td>111.6 mg ± 1.8 mg</td>
<td>46.5% ± 0.75%</td>
<td>77% ± 1%</td>
</tr>
</tbody>
</table>

3.2. Chemical Leaching with Organic Acids and Cathode Material from Spent RLIB

Chemical leaching experiments were conducted to determine the capability of organic acids to solubilize Li and Co. Solutions containing organic acids (20 mM, 50 mM and 100 mM of oxalic, citric, L-malic, and tartaric acids) amended with 2% pulp density of cathode material (containing LiCoO$_2$) in Erlenmeyer flasks were shaken at 120 rpm and 25 °C for 10 days (procedure by Aung and Ting, 2004). With the assumption that 100% of the cathode material was LiCoO$_2$, oxalic acid at a concentration of 100 mM was the most effective for leaching Li (50% mobilized) and citric acid at a concentration of 100 mM was the most effective in leaching Co (30% mobilized) from the cathode material in spent Li-ion batteries (Figure 3.2). Citric acid at a concentration of 100 mM was the only organic acid to leach more than 20% of both Li and Co from the cathode material (Figure 3.2). Negative controls that contained the cathode material and water had less than 1% of Li and Co solubilized. Overall, weak organic acids were capable of leaching Li and Co from the cathode material when compared to negative controls (only cathode and water) after 10 days of leaching. However, the fraction leached was under 60% for all conditions tested.
Results from chemical leaching experiments utilizing mixed organic acids showed a maximum mobilization of Li and Co close to 40% and about 20%, respectively (Figure 3.3, Figure 3.4). Reactions reached equilibrium around day 8; metal dissolution did not increase between Day 8 and Day 10. As illustrated in Figure 3.3 and Figure 3.4, negative controls that contained cathode material and deionized water mobilized less than 1% of Li and Co in spent battery cathode material. Maximum Li mobilization was observed in reactions with organic acid mixtures that contained higher concentrations of oxalic acid (100 mM). Mobilization of Li with organic acid mixtures was not significantly different ($P = 0.117$) to maximum Li recovery with
individual organic acids. Peak removal of Co was observed in reactions with organic acid mixtures that contained higher concentrations of citric acid (100 mM). Mobilization of Co with mixed organic acids was significantly lower (P = 0.0404) than maximum Co recovery with only 100 mM citric acid. Minimal leaching (below 40%) of Li and Co was observed in this study with mixtures of organic acids at low concentrations similar to what is expected in fungal processes.

Figure 3.3: Chemical leaching of Li by mixtures of organic acids.

Figure 3.4: Chemical leaching Co by mixtures of organic acids.
In mixed organic acid reactions treated with hydrogen peroxide, the maximum mobilization of Li was significantly increased ($P = 0.0054$) close to 100%, and peak removal of Co was significantly increased ($P = 0.0078$) to approximately 70% in weak organic acid mixtures (Figure 3.5, Figure 3.6). Findings were consistent with previous results where organic acid mixtures that contained high concentrations of oxalic acid (100 mM) were the most effective in the mobilization of Li from the RLIB cathode material. Mixtures with 100 mM of citric acid were the most effective in the removal of Co from the RLIB cathode material. Negative controls with deionized water, 2% hydrogen peroxide, and cathode material were not effective. As illustrated in Figure 3.5 and 3.6, less than 1% Li or Co was mobilized from RLIB cathode material in the negative controls.

Figure 3.5: Chemical leaching Li by mixtures of organic acids and $\text{H}_2\text{O}_2$. 

![Graph showing percent recovery of Li over time with different acid mixtures and a control.](image)
3.3. Biomass Production and Organic Acid Excretion Analysis

Data was collected on biomass production, media acidification, and organic acid levels in the broth from *A. niger*, *P. chrysogenum*, and *P. simplicissimum* cultured in CDB or SDB for 10 d. The three fungal species produced 6-10 g/L of dry weight biomass after 10 d of growth. *P. simplicissimum* produced the greatest mean biomass (dry weight) of about 10 g/L (Figure 3.7). Around day 6 and day 8, an increase in the rate of biomass production for the three fungi was noted (Figure 3.7). On day 10, analysis of variance test indicated that production of fungal biomass in CDB and SDB was not significantly different for *P. simplicissimum* or *A. niger* cultures, but cultures with *P. chrysogenum* were significantly different between the two media. More biomass was recorded in SDB cultures of *P. chrysogenum* compared to CDB cultures. Biomass production among the three fungi continued to increase through Day 10; therefore, no nutrient had become limiting.

Figure 3.6: Chemical leaching Co by mixtures of organic acids and H$_2$O$_2$. 
Acidification of the culture medium was recorded as an indication of organic acid excretion by \textit{A. niger}, \textit{P. chrysogenum}, and \textit{P. simplicissimum}. Fungal cultures were monitored for changes in pH over a 10 day incubation period. Increased acidity was detected after 48 hours incubation for all three fungal species (Figure 3.8). Broth cultures with \textit{A. niger} had the lowest recorded pH (2.65 – 3.00) after 10 days in CDB or SDB (bottom panel Figure 3.9). Mean pH in CDB was not significantly different to SDB in \textit{A. niger} cultures after 10 days of growth. Results for \textit{P. simplicissimum} and \textit{P. chrysogenum} cultures indicate that pH values in CDB were significantly different than SDB after 10 days incubation. PH of cultures with \textit{P. simplicissimum} or \textit{P. chrysogenum} was significantly higher in CDB compared to SDB on Day 10 of growth (bottom panel Figure 3.9). \textit{A. niger}, \textit{P. simplicissimum} and \textit{P. chrysogenum} acidified cultures in both media, but pH remained unchanged in negative controls that contained either CDB or SDB, but no fungi (Figure 3.8). The $\Delta$ pH was significantly higher in CDB cultures compared to SDB cultures among the three fungal species (top panel Figure 3.9). PH and biomass were negatively correlated ($P < 0.01$) in SDB or CDB cultures, i.e., pH decreased as biomass increased over time.
Figure 3.8: Media acidification of fungal broth cultures in SDB and CDB. Error bars for standard deviation are shown on each marker.

Figure 3.9: Media acidification of fungal broth cultures after 10 days of growth. Top panel shows results for delta pH for the change in pH over time, bottom panel shows the final pH measured in cultures.
Organic acid levels excreted by fungi were measured in broth after 10 days of incubation. *A. niger* excreted significantly higher levels of oxalic acid (47 ± 14 mM) in SDB and citric acid (11 ± 1.5 mM) in CDB compared to *P. chrysogenum* and *P. simplicissimum* after 10 days of incubation (Figure 3.10). L-malic acid was only detected and quantified in *A. niger* SDB cultures. L-malic acid was not detected in CDB or SDB cultures with *P. chrysogenum* or *P. simplicissimum*. Oxalic acid and tartaric acid levels in *P. chrysogenum* cultures were measured at concentrations below 4 mM in CDB or SDB cultures (Figure 3.11). *P. simplicissimum* cultures contained oxalic, citric and tartaric acids at low concentrations (below 30 mM) only in CDB cultures, while organic acids were not detected in *P. simplicissimum* SDB cultures (Figure 3.12). Oxalic acid and tartaric acids levels in *A. niger* CDB vs SDB cultures were not significantly different. Organic acid levels in *P. chrysogenum* cultures were not tested for statistical differences between CDB and SDB cultures due to minimal concentrations of organic acids present after 10 days of growth. After 10 days of incubation, organic acids were not detected in SDB cultures with *P. simplicissimum*. Therefore, statistical difference between *P. simplicissimum* cultures in CDB or SDB was not tested.

![Figure 3.10](image_url)

**Figure 3.10:** Organic acid levels on day 10 in CDB or SDB cultures of *A. niger*. Acids not present were below detection limit < 0.5 mM.
Figure 3.11: Organic acid levels on day 10 in CDB or SDB cultures of *P. chrysogenum*. Acids not present were below detection limit < 0.5 mM.

Figure 3.12: Organic acid levels on day 10 in CDB or SDB cultures of *P. simplicissimum*. Acids not present were below detection limit < 0.5 mM.
3.4. Fungal Tolerance to Li and Co

Li or Co at various concentrations was added to fungal cultures to determine metal tolerance, which was measured by biomass production and media acidification over 5 days. CDB was amended with 0.1 mg/L, 3 mg/L, 100 mg/L or 1000 mg/L of Li or Co before inoculation. Biomass production by the three fungi was significantly inhibited compared to controls, which contained no metal, by 100 mg/L and 1000 mg/L Co (Figure 3.14, 3.18, and 3.23). *P. chrysogenum* was able to grow up to 1000 mg/L of Li with little inhibition (Figure 3.17) and *A. niger* was capable of growing up to 100 mg/L of Li with little inhibition (Figure 3.13). Biomass production by *P. simplicissimum* was significantly inhibited when growing in 100 mg/L of Li or Co (Figure 3.21 and Figure 3.23). Media pH was significantly higher for the three fungal species growing in 1000 mg/L of Co compared to controls without metal (Figure 3.16, 3.20, and 3.24). In a 5 day growth period, *A. niger*, *P. chrysogenum*, and *P. simplicissimum* showed low tolerance to concentrations above 100 mg/L Co. However, in this study, *P. chrysogenum* did show tolerance to Li at concentrations higher than expected peak exposure in a RLIB bioleaching process which is around 700 mg/L Li (Horeh *et al.*, 2016).

![Graph showing biomass production by A. niger in CDB after 5 days with Li concentrations](image)

Figure 3.13: Li toxicity to biomass production by *A. niger* in CDB after 5 days.
Figure 3.14: Effect of Li on media pH in CDB cultures with A. niger.

Figure 3.15: Co toxicity to biomass production by A. niger in CDB.

Figure 3.16: Effect of Co on media pH in CDB cultures with A. niger.
Figure 3.17: Li toxicity to biomass production by *P. chrysogenum* in CDB.

Figure 3.18: Effect of Li on media pH in CDB cultures with *P. chrysogenum*.

Figure 3.19: Co toxicity to biomass production by *P. chrysogenum* in CDB.
Figure 3.20: Effect of Co on media pH in CDB cultures with *P. chrysogenum*.

Figure 3.21: Li toxicity to biomass production by *P. simplicissimum* in CDB.

Figure 3.22: Effect of Li on media pH in CDB cultures with *P. simplicissimum*. 
The previous study on metal tolerance only monitored fungal cultures for 5 days and did not show if fungi can tolerate toxic metals over time. Therefore, in order to more thoroughly determine if fungi can tolerate Li and Co, biomass production and media acidification was examined for inhibition over a 20 day growth period after exposure to 50 or 250 mg/L Li or Co. Tolerance index was used to monitor toxicity of Li or Co to biomass production by fungal species. Cultures with *A. niger* showed inhibition of biomass production in Li or Co cultures after 10 days of incubation (Figure 3.25 and 3.26). However, growth in 50 mg/L Li was similar to control cultures without metal throughout the 20 days of growth (Figure 3.25). After 15 days
of growth, biomass production in cultures with 250 mg/L Li exceeded biomass (dry weight) in control cultures without metals (Figure 3.25). After 20 days, *A. niger* cultures with Co were no longer inhibited when compared to controls cultures without metals (Figure 3.26). Media acidification in *A. niger* cultures with 50 mg/L or 250 mg/L of Li or Co was similar to positive control cultures without metals after 20 days exposure (Figure 3.27 and 3.28).

Inhibition of biomass was detected in *P. chrysogenum* cultures with Li or Co on day 5 and 10 in cultures with Li. However, biomass in *P. chrysogenum* cultures with Li exceeded biomass (dry weight) produced by control cultures after 15 days incubation (Figure 3.29). Cultures with *P. chrysogenum* and 250 mg/L Co showed inhibition of biomass throughout the 20 days of growth, but inhibition was reduced over time (Figure 3.30). After 20 days of growth, *P. chrysogenum* in cultures containing 50 mg/L of Li or Co was able to produce as much biomass as control cultures without metals (Figure 3.29 and 3.30). Media acidification in cultures with *P. chrysogenum* and 50 mg/L Li were similar to positive fungal control cultures. However, at 250 mg/L Li, *P. chrysogenum* cultures were significantly higher in pH than control cultures absent metal (Figure 3.31). Notably, media acidification in *P. chrysogenum* cultures with 50 mg/L or 250 mg/L Co were significantly reduced after 20 days of exposure (Figure 3.32).

*P. simplicissimum* cultures were significantly inhibited by 250 mg/L Li after 5 days, and 50 mg/L Li after 10 days of growth (Figure 3.33). Biomass production in *P. simplicissimum* cultures containing 50 mg/L or 250 mg/L Co exceeded biomass dry weight produced by control cultures without metal after 5 days of incubation (Figure 3.34). However, 10 days of incubation show inhibition of biomass production but only at 50 mg/L Co and not in cultures containing 250 mg/L Co (Figure 3.34). These results do not agree with my previous 5 day experiment where 100 mg/L Co was found to inhibit biomass production. The pH in *P. simplicissimum* cultures
with 50 mg/L Li was similar to positive control cultures without metal, while pH in cultures with 250 mg/L Li were significantly higher than control cultures (Figure 3.35). The pH in *P. simplicissimum* cultures with 50 mg/L or 250 mg/L Co were similar to positive control cultures absent metal (Figure 3.36).

Figure 3.25: Inhibition of biomass production by Li in *A. niger* cultures.

Figure 3.26: Inhibition of biomass production by Co in *A. niger* cultures.
Figure 3.27: Effects of Li on media acidification in *A. niger* cultures.

Figure 3.28: Effects of Co on media acidification in *A. niger* cultures.

Figure 3.29: Inhibition of biomass production by Li in *P. chrysogenum* cultures.
Figure 3.30: Inhibition of biomass production by Co in *P. chrysogenum* cultures.

Figure 3.31: Effects of Li on media acidification in *P. chrysogenum* cultures.

Figure 3.32: Effects of Co on media acidification in *P. chrysogenum* cultures.
Figure 3.33: Inhibition of biomass production by Li in *P. simplicissimum* cultures.

Figure 3.34: Inhibition of biomass production by Co in *P. simplicissimum* cultures.

Figure 3.35: Effects of Li on media acidification in *P. simplicissimum* cultures.
3.5. Bioleaching of Li and Co by *A. niger*

Li and Co were leached from spent RLIB cathode material using organic acids produced by cultures of *A. niger*. Fungal cultures in one step leaching began growth with the cathode material present in the growth medium. Positive control cultures without cathode material contained close to 20 mM oxalic acid and approximately 20 mM of citric acid (Figure 3.40). Results from one-step leaching indicate that this process was poor for the recovery of Li or Co, where less than 5% of available Li or Co was mobilized (Figure 3.37 and 3.38). At the end of the 10-d bioleaching process, one-step cultures contained less than 5 mM oxalic acid, and close to 40 mM of tartaric acid (Figure 3.40). Two-step leaching was accomplished by adding the cathode material 48 hours post inoculation. Less than 7% of Li or Co was mobilized from the cathode when using a two-step leaching process with *A. niger* cultures (Figure 3.39).

However, mobilization of Li was increased to almost 20% when using spent medium to leach Li from RLIB cathode material (Figure 3.37). In two-step cultures, organic acid production was similar to one-step where less than 5 mM of oxalic acid and close to 40 mM tartaric acid was excreted by *A. niger*. In addition, spent medium cultures after 10 days leaching, contained less than 5 mM oxalic acid and close to 20 mM tartaric acid (Figure 3.40).
concentrations of tartaric acid was quantified in spent-medium after 10 days leaching and was not detected before exposure to RLIB cathode material. Citric acid and L-malic acid were not detected in one-step, two-step or spent-medium leaching cultures after 10 days incubation.

Spent medium contained excreted organic acids but not fungi while leaching Li or Co from the cathode. The final leaching method supplemented spent medium with 2% hydrogen peroxide as an attempt to improve mobilization of Li and Co from spent RLIB cathode material. After 10 days leaching, spent medium cultures with hydrogen peroxide mobilized less Li and Co from the cathode than spent medium without hydrogen peroxide (Figure 3.37 and 3.38). In spent medium with hydrogen peroxide, concentrations of oxalic acid and tartaric acid were slightly lower but similar to spent medium cultures without hydrogen peroxide (Figure 3.40).

Figure 3.37: Bioleaching Co from spent RLIB cathode with *A. niger*. Negative control contained CDB and cathode material without fungi or organic acids.
Figure 3.38: Bioleaching Li from spent RLIB cathode with *A. niger*. Negative control contained CDB and cathode material without fungi or organic acids.

Figure 3.39: Bioleaching Li and Co from spent RLIB cathode after 10 days with *A. niger*. 
Figure 3.40: Organic acids excreted by *A. niger* on day 10 of bioleaching RLIB cathode. Malic acid was not detected on day 10 in bioleaching cultures or positive controls.
CHAPTER 4. DISCUSSION

4.1. Analysis of Spent Batteries

The components of dismantled spent rechargeable Li-ion battery were similar to those observed in other studies (Li et al., 2013; Horeh et al., 2016). Variability in weight of components may be due to differences in composition between spent Maxwell batteries and RLIB dismantled in previously published work. In this thesis, the same model and manufacture of battery was used for every experiment to avoid any variation due to differences in composition. Chemical digestion of the cathode material with concentrated HCl was very effective (> 90% recovery of Li and Co). The observed reaction with HCl and the cathode during chemical digestion was vigorous and released visible fumes into the fume hood, which supports the literature on hazardous conditions present when dissolving cathode material with strong acids such as HCl. Recovery percentages reported were based on the assumption that 100% of the scraped cathode material was LiCoO₂.

4.2. Chemical Leaching with Organic Acids and Cathode Material from Spent RLIB

Chemical leaching experiments were undertaken to determine the ability of organic acids to leach Li and Co from cathode material. These findings informed the feasibility of the fungal bioleaching process in later experiments. Oxalic acid at 100 mM effectively mobilized 50% Li from 20 g/L of cathode material; however, 100 mM of oxalic acid mobilized less than 10% of Co from 20 g/L of cathode material. Other researchers obtained higher recoveries in chemical
leaching experiments when utilizing higher concentrations of organic acids, i.e. oxalic acid above 1 M was effective in leaching more than 80% of Li and Co from the cathode material in spent batteries (Sun and Qiu, 2012; Zeng et al., 2015). These studies indicate that a higher concentration of oxalic acid is necessary for effective mobilization (above 90%) of Li and Co from Li-ion cathode material (Zeng et al., 2015). I found citric acid to be the most effective leaching agent in the recovery of Co, as 100 mM was effective in the mobilization of 30% Co and 40% Li from the cathode material. Citric acid may be more effective as a chelator to mobilize Li and Co due to its chemical structure as a tricarboxylic acid which provides three protons to water (Horeh et al., 2016).

The leaching efficiency of L-malic acid and tartaric acid for the removal of Li and Co from Li-ion battery cathodes has not been previously studied. My results indicate that concentrations of 100 mM of tartaric acid or 100 mM of L-malic acid were able to mobilize 20% of Co and approximately 30% of Li from the RLIB cathode material. The leaching efficiencies of these organic acids are slightly lower than that of citric acid, but leach Co more effectively than oxalic acid. These results provide valuable knowledge on the leaching potential of fungal processes if similar concentrations of organic acids are excreted into the reaction with cathode material from spent rechargeable Li-ion batteries.

Filamentous fungi frequently excrete a combination of organic acids as observed in previous studies (Ilyas and Lee, 2013b; Horeh et al., 2016). Therefore, the efficiency of a mixture of organic acids for chemical leaching was explored. Mixtures of oxalic, citric, L-malic and tartaric acids at various concentrations mobilized up to 40% of Li and less than 20% of Co over 10 days. The removal of Li was less efficient but statistically similar compared to 100 mM oxalic acid alone, which mobilized almost 50% of the Li in the spent battery cathode.
Mobilization of Co from the cathode with mixed organic acids was significantly lower than that of 100 mM of citric acid alone. Results for low mobilization of Li and Co with low concentrations of individual and mixed organic acids were expected. These results were expected based on previous studies that demonstrate a higher removal of Li and Co from the cathode in spent RLIB when utilizing higher concentrations of organic acids (Sun and Qiu, 2012; Zeng et al., 2015).

Analysis of metal mobilization in modified chemical leaching reactions with 2% hydrogen peroxide and concentrations of mixed organic acids up to 100 mM indicated a strong reaction with hydrogen peroxide where removal of Li and Co increased above 90% and up to 70% respectively. This observation agrees with previous experiments that demonstrate the effect of hydrogen peroxide, which acts as a reducing agent to improve cobalt dissolution by accelerating the reaction to converting Co$^{3+}$ to Co$^{2+}$ (Sun and Qiu, 2012). Some researchers observed leaching efficiency above 90% for Li and Co when utilizing 2% hydrogen peroxide as a reducing agent in chemical leaching reactions with Li-ion cathode material (Li et al., 2013). However, previous studies with hydrogen peroxide only examined reactions with high concentrations (0.5 – 2 M) of organic acids and spent battery cathode material (Sun and Qiu, 2012; Li et al., 2013).

Based on performance of chemical leaching with concentrations of individual and mixed organic acids, fungal processes that excrete concentrations of organic acids below 50 mM have low potential to mobilize Li and Co from cathode material in spent RLIB. Chemical leaching experiments with hydrogen peroxide as a reducing agent indicate that the presence of 2% hydrogen peroxide might improve Li or Co leaching efficiency when utilizing weak organic acids produced by fungi.
4.3. Biomass Production and Organic Acid Excretion Analysis

In order to determine if sucrose would be more favorable toward growth and/or excretion of organic acids than glucose by fungi, two different growth media were used to culture fungi. Biomass production in CDB cultures by *A. niger* or *P. simplicissimum* was not significantly different than that in SDB. Biomass of *P. chrysogenum* was significantly greater in SDB compared to CDB. Biomass production by all three fungal species continued to increase through day 10, which was an indication that nutrients were not limiting in SDB or CDB and fungal cultures would continue to produce biomass if allowed more time for growth beyond 10 days. This is supported by a previously published growth curve for *A. niger* (Santhiya and Ting, 2005), where biomass increased up to 30 days then reached stationary phase of growth which lasted up to day 70.

Acidification of culture media was monitored in fungal cultures, and a decrease in media pH was used as an indicator for organic acid excretion. All three fungal species were able to significantly reduce the pH of SDB or CDB after 48 hours of growth. According to previous researchers, this observed acidification of media in fungal cultures may be caused by organic acid excretion and/or efflux of protons via H⁺- ATPases (Gadd, 2004). Results show that *A. niger* cultures in SDB was not significantly lower in pH compared to cultures in CDB after 10 days of growth. However, SDB cultures of *P. chrysogenum* or *P. simplicissimum* were significantly higher in pH compared to CDB cultures after 10 days of incubation. Furthermore, media was acidified by the three fungal species to pH levels below 5 in SDB and under 4 in CDB cultures. The change in pH over time as Δ pH showed that the three fungi had a significantly higher change in pH in CDB compared to SDB cultures. Due to the acidity observed in fungal
cultures, organic acid excretion was expected in CDB and SDB cultures with *A. niger*, *P. chrysogenum*, and *P. simplicissimum*.

Analysis of excreted organic acids was necessary to determine potential for fungi to efficiently leach Li and Co from spent RLIB cathode. After 10 days of growth, oxalic acid, citric acid, L-malic acid and tartaric acid was excreted by fungi in CDB or SDB cultures and detected via HPLC. The dominant organic acid excreted by *A. niger* was oxalic acid in CDB and SDB cultures. The pH levels in *A. niger* CDB or SDB cultures were lower than 3, and previous studies have reported inhibition of oxalic acid excretion in cultures below a pH of 3 (Ruijter *et al.*, 1999). Therefore, the growth media can be modified to buffer pH levels around 6 to increase excretion of oxalic acid by fungi (Ruijter *et al.*, 1999). However, maintaining a pH of 6 in a bioleaching process would decrease leaching efficiency of organic acids excreted by fungi to mobilize Li and Co from the cathode material.

Researchers found that high levels of citric acid were excreted by fungi in cultures below a pH of 3 (Horeh *et al.*, 2016; Ilyas and Lee, 2013a). Therefore, bioleaching processes with fungi should focus on production of citric acid. Samples collected from fungal cultures had low concentrations of citric acid (below 15 mM) after 10 days of incubation. Notably, citric acid was only excreted by *A. niger*, and *P. simplicissimum* in CDB cultures (Figure 3.10 and 3.12). A previous study demonstrated that decreased citric acid excretion by fungi can occur if concentrations of sugar are less than 14 – 22% in the total volume (Max *et al.*, 2010). Sugar concentration in cultures before inoculation was approximately 3% in CDB cultures compared to 2% cultures with SDB. These findings indicate that sugar concentrations in this study were too low when compared to previous studies, where researchers used sucrose-based cultures with 10% sugar (sucrose) and produced higher levels of citric acid (Horeh *et al.*, 2016). The effect of
sugar concentration on organic acid yield should be examined further in future research involving fungal bioleaching processes.

Tartaric acid and L-malic acid are weaker organic acids that were also present in fungal cultures. Concentrations of tartaric acid and L-malic acid were lower than 10 mM in CDB or SDB cultures. Regulation of tartaric acid and L-malic acid excretion has not been studied in published literature. Therefore, there is a lack of information on methods to improve and increase excretion of tartaric and L-malic acid. I found that fungal processes under set conditions in this study produced less organic acid than previously published work with the same species of fungi (Table 1.1). Results from one study indicate that 10 day incubation should be extended to 20 or 30 days to maximize excretion of oxalic acid and citric acid in A. niger cultures (Santhiya and Ting, 2005).

Cultures of P. chrysogenum in CDB or SDB did not produce more than 5 mM of oxalic acid, citric acid, tartaric acid, and L-malic acid, but results from media acidification analysis indicated increased acidity over time in cultures. Acidification observed could have been caused by the presence of other organic acids such as formic acid, acetic acid or succinic acid produced by P. chrysogenum (Gadd, 2004). Furthermore, P. chrysogenum likely acidified cultures through the efflux of protons via H⁺-ATPases or excretion of other organic acids that were not monitored (Gadd, 2004). Organic acid concentrations in fungal cultures varied over time and in some cases decreased below the limit of detection. These findings align with previous studies that demonstrated potential issues with fungi catabolizing excreted organic acids after utilizing remaining carbon provided by the media (Gadd, 1999). One study demonstrated that A. niger was capable of completely hydrolyzing 80 g/L of sucrose to glucose and fructose within 48 hours.
of incubation (Aung and Ting, 2005). Nevertheless, methods to supply enough carbon and energy to prevent organic acid catabolism should be explored further.

After comparing organic acid excretion by fungi cultured in CDB or SDB, I concluded that future experiments should involve culturing fungi only in CDB. This conclusion is based on the results found, where *A. niger*, *P. chrysogenum*, and *P. simplicissimum* excreted organic acids in CDB. While organic acids in SDB cultures with *P. simplicissimum* were not detected in this study. Furthermore, the presence of sucrose in CDB provides more carbon per molecule for organic acid excretion compared to dextrose in SDB (Papagianni, 2007). Growth media mainly comprised of sucrose has been explored in previous studies that demonstrate higher organic acid excretion by fungi (Ilyas *et al*., 2013a; Ilyas and Lee, 2013b; Ren *et al*., 2005; Santhiya and Ting, 2005). In order to support these findings, growth and organic acid excretion by fungi should be studied further by culturing fungi in various growth media to test the effects of varying levels of different carbon sources, N sources, P sources, and trace metals.

### 4.4. Fungal Tolerance to Li and Co

Growth, metabolism, and survival of fungi can be affected by surrounding metals that alter their chemical and physical state (Gadd, 2010). The effects of metals on fungal processes have been demonstrated in multiple studies, where various metals inhibited growth and organic acid excretion in broth cultures with *A. niger* (Burgstaller and Schinner, 1993; Gadd, 1999). My assessment of Li or Co toxicity in 5-day-old cultures indicated that concentrations at or above 100 mg/L Co inhibited biomass production in cultures with *A. niger*, *P. chrysogenum*, or *P. simplicissimum*. The pH in metal-contaminated cultures with *A. niger* or *P. chrysogenum* decreased similar to control cultures without metals. However, pH was significantly higher in
fungal cultures with 1,000 mg/L Co when compared to controls absent metals. Therefore, results indicate inhibition of biomass can occur without reduced media acidification by fungi.

Results from metal tolerance experiments showed potential issues with high levels of Co that could negatively affect bioleaching processes with the three fungi. These results agree with previous studies, where biomass production from 5-day-old cultures with fungi such as *P. simplicissimum* and *A. niger* were inhibited by 500 mg/L and 1000 mg/L of Co (Anahid *et al.*, 2011; Valix *et al.*, 2001). Notably, an increase in tolerance to Co was previously observed over time when culturing fungi up to 10 days (Anahid *et al.*, 2011; Valix *et al.*, 2001). These studies indicate that fungi would potentially improve their tolerance to Co if their growth period was extended to 10 or 20 days. An extended growth period does not necessarily provide evidence of fungi building metal tolerance but does show if fungi grow slowly due to their stress response from exposure to metals. It was expected that fungi could potentially be exposed up to 3,000 mg/L of Co, which was observed in chemical leaching experiments with 20 g/L RLIB cathode material and 100 mM citric acid. Potential exposure to Co at high levels in fungal bioleaching cultures will depend on the amount of organic acids excreted by fungi and their leaching efficiency to mobilize Co from RLIB cathode material.

Cultures of fungi containing 100 mg/L Li produced biomass with little inhibition, except for biomass production by *P. simplicissimum*, which was significantly inhibited after 5 days of growth. The relative toxicity of Li to fungal growth was more severe at higher concentrations, at which inhibition of biomass after 5 days of growth was detected at a significant level for *A. niger* and *P. simplicissimum* cultures with 1000 mg/L Li. While growing in cultures containing 100 mg/L Li, *A. niger* and *P. chrysogenum* acidified the media similar to controls without metals. However, in cultures with 1,000 mg/L Li, pH was significantly higher in 5 day old cultures of *A.
*niger* or *P. simplicissimum* compared to fungal control cultures without metals. Previous studies examined metal tolerance in fungi but did not include Li toxicity. However, these studies indicate that fungi such as *P. simplicissimum* and *A. niger* can increase tolerance of other metals in prolonged growth periods to 10 or 20 days (Anahid *et al.*, 2011; Valix *et al.*, 2001). A longer growth period may be necessary to allow fungi time to recover from the stress caused by high concentrations of Li.

Chemical leaching experiments with 20 g/L RLIB cathode indicate that fungi could be exposed up to 700 mg/L Li in a bioleaching process with 100 mM oxalic acid. Notably, biomass production and media acidification in *P. chrysogenum* cultures with 1,000 mg/L Li were similar to control cultures. These results show that *P. chrysogenum* would be capable of growing and acidifying surrounding media in the presence of Li at high concentrations up to 1,000 mg/L. Previous studies on metal toxicity to fungi did not supplement Li in submerged fungal cultures. Therefore, Li toxicity to biomass production in fungi was not supported by current literature and requires more research to understand fungal stress response on a macro and molecular level.

Li or Co toxicity to fungi is dependent on the concentration of surrounding metals and the ability of fungi to recover from environmental stress over time (Gadd, 1993). The growth period in the previous experiment did not allow time for fungi to recover from the initial stress response to Li or Co in submerged broth cultures. Therefore, *A. niger, P. chrysogenum,* and *P. simplicissimum* were cultured in the presence of Li or Co up to 20 days of growth. Maximum concentration of 250 mg/L for Li or Co was tested based on expected exposure when leaching RLIB cathode with low concentrations of organic acids below 50 mM (low organic acid excretion by fungi observed in section 3.3.).
Notably, an extended growth period revealed that 20 day old cultures with *A. niger* or *P. chrysogenum* in 250 mg/L of Li or Co were able to produce biomass similar to control cultures without metals. Tolerance index for these fungal species increased over time, which was an indication of improved tolerance to Li or Co in fungal cultures. These results may also indicate that *A. niger* or *P. chrysogenum* were just growing very slowly due to stress response from metal exposure. However, in 50 mg/L of Li, biomass production by *P. simplicissimum* cultures was inhibited throughout the 20 day growth period. Furthermore, *P. simplicissimum* showed a higher tolerance to Co than Li in submerged cultures. In addition, tolerance to cultures with 250 mg/L Co was higher than cultures with 50 mg/L Co in *P. simplicissimum* cultures. These results agree with one study, where fungal cultures were more tolerant to higher concentrations of metal than low concentrations in their surrounding environment (Valix *et al.*, 2001). However, my results (Figure 3.34) do not agree with findings in the initial metal tolerance study where *P. simplicissimum* was inhibited by 100 mg/L of Co in the first 5 days of growth (Figure 3.23). This disagreement may be attributed to experimental error in preparing metal solutions or cultures of fungi and will need to be repeated to support findings.

The mechanism behind tolerance and how fungi can tolerate higher but not lower concentrations of metals is not explained in previous studies. To improve understanding of the toxic effects of Li and Co, researchers should focus on genes associated with stress response over time and how altered gene expression can impact the physical and chemical processes in fungi. A complete analysis on metal tolerance should include studies on organic acid excretion in order to accurately determine the effect of metals on fungal suitability for bioleaching processes with RLIB cathode.
4.5. Bioleaching Li-ion cathode with *A. niger*

Chemical leaching with acids is expensive and can negatively impact the environment through the release of hazardous waste (Cui and Zhang, 2008; Joulié *et al.*, 2014; Li *et al.*, 2009; Santana *et al.*, 2017). Therefore, bioleaching with organic acids produced by fungi was investigated to determine leaching efficiency during mobilization of Li or Co from RLIB cathode material. *A. niger* was selected to test bioleaching of Li and Co due to previously collected results on organic acid excretion and toxicity to Li or Co. These results indicate that the *Penicillium* species were not tolerant of metals tested or capable of excreting the concentration of organic acids required to effectively mobilize metals from the cathode in RLIB. Bioleaching potential of *A. niger* was determined by testing methods from previous studies for one-step, two-step, and spent-medium leaching (Horeh *et al.*, 2016). These leaching methods altered conditions in bioleaching experiments to change the point of cathode exposure to a particular growth stage in fungi. Utilization of spent media prevented cathode exposure to fungi throughout the process to avoid issues with metal toxicity, while organic acid waste excreted by fungi was used to separately leach Li and Co from the cathode in RLIB absent fungi.

One-step leaching with *A. niger*, where cathode and spores were in the culture at the start, resulted in only 4% mobilization of Li or Co after 10 days of incubation. Furthermore, bioleaching with the two-step method only increased Li and Co mobilization by 2%, which was approximately 6% total recovery for each metal. In one-step and two-step fungal bioleaching, the presence of fungi can immobilize metals dissolved in solution through precipitation, fungal uptake and intracellular sequestration (Gadd, 2004). Immobilization of Li or Co may have contributed to lower metal recovery from culture supernatants. In addition, I found that exposure of fungi to metals in cathode material during one-step and two-step leaching inhibited processes
for excretion of oxalic and citric acid but improved production and excretion of tartaric acid. Organic acid levels in one-step and two-step leaching were below limit of detection for citric acid and below 5 mM for oxalic acid. Notably, in both one-step and two-step leaching, close to 40 mM of tartaric acid was detected on Day 10. This observation was not expected due to the lack of tartaric acid detected on 10 day old control cultures without cathode material. Similar findings have been reported in previous publications, where exposure of fungi to metals resulted in increased excretion of certain organic acids, depending on the fungal strain and conditions during exposure (Horeh et al., 2016; Santhiya and Ting, 2005).

Spent medium was collected from A. niger cultures on Day 10 and analyzed for excreted organic acids. HPLC analysis indicated that the filtered spent medium contained approximately 18 mM oxalic acid and roughly 20 mM citric acid before supplementation of the cathode material. Leaching cathode material with spent media resulted in a decrease of Co removal down to 5%, while Li mobilization was increased close to 20%. More oxalic acid and citric acid was present in spent medium compared to one step or two step leaching processes. Higher concentration of oxalic acid likely improved Li removal from RLIB cathode material. The effect of oxalic acid on Li mobilization was supported by previous chemical leaching results in section 3.2. In addition, levels of oxalic acid in the spent medium decreased from 18 mM to 2 mM, while citric acid decreased below the limit of detection after 10 days of leaching. These results indicate that oxalic acid and/or citric acid was potentially altered and/or complexed with Li or Co while leaching cathode material. Moreover, chelation and precipitation may have led to complexes that immobilize organic acids from a detectable state in the supernatant of cultures.

Hydrogen peroxide in fungal spent medium did not increase leaching efficiency of organic acids to mobilize Li and Co from cathode material in RLIB. However, in previous
chemical leaching experiments absent fungi (section 3.2), 2% hydrogen peroxide with organic acids was noted to significantly increase mobilization of Li and Co from the cathode (Zhu et al., 2012). I noted that chemical leaching experiments only contained water, pure organic acids, and RLIB cathode material. While bioleaching experiments with fungi contained various levels of excreted waste such as amino acids, organic acids, proteins, lipids, enzymes and other metabolites (Gadd, 2004). These products can potentially reduce or decompose hydrogen peroxide and inhibit the reduction of Co\(^{3+}\) in the cathode material. Negative controls that contained cathode in CDB with and without hydrogen peroxide mobilized less than 1% of Li or Co.

Results from bioleaching experiments indicate that concentrations of organic acids excreted by fungi in this study have a low potential to mobilize Li or Co from the cathode in RLIB. Mobilization of Li and Co in this study was significantly less than what was reported in a previous study with A. niger, where 95% Li and 45% Co was leached with organic acids (Horeh et al., 2016). However, that study included higher concentrations of sucrose (100 g/L) compared to 30 g/L of sucrose that was present in cultures in this study. These results indicate that a high concentration of sucrose may be necessary in order to increase organic acid excretion and improve Li and Co mobilization from the cathode in RLIB.

Citric acid was not detected on day 10 for each leaching experiment but was present in positive control cultures at 20 mM. Metals from the RLIB cathode such as manganese can inhibit excretion of citric acid in one-step or two-step leaching which is supported in previous studies (Gadd, 1999). This work requires more research to modify leaching conditions with fungi. Therefore, alteration of fungal metabolism will be necessary to increase excretion of organic acids. Fungal metabolism can be affected by available nutrients, concentration of sugar,
temperature, pH, dissolved oxygen, and many other variables which exist in a bioleaching environment (Papagianni, 2007; Aghaie et al., 2009). Results from chemical leaching experiments (section 3.2) indicate that higher concentrations of organic acids can increase mobilization of Li or Co from spent RLIB cathode. This is supported in previously published studies that demonstrated the Li and Co leaching efficiency of organic acids in a range of concentrations (Jha et al., 2013). Furthermore, bioleaching studies should include more fungal strains, particularly fungi that are able to tolerate high metal concentrations while excreting high levels of organic acids such as citric acid.
CHAPTER 5. SUMMARY AND CONCLUSIONS

Results from this study indicate that organic acid leaching efficiency can be increased when utilizing higher concentrations of citric or oxalic acid to mobilize Li or Co from RLIB cathode. Previous studies support these findings, where higher concentrations of citric or oxalic acid lead to increased mobilization of Li or Co from spent battery cathode (Jha et al., 2013; Li et al., 2013). Hydrogen peroxide was effective when included in chemical leaching experiments to significantly increase mobilization of Li and Co from RLIB cathode material. However, the effects of hydrogen peroxide decreased mobilization of both metals when supplemented into fungal bioleaching cultures with cathode.

Furthermore, analysis of biomass production by A. niger and P. simplicissimum showed that differences in media composition between CDB and SDB did not affect collected biomass for each species. However, CDB cultures with P. chrysogenum had significantly less biomass than SDB cultures after 10 days of growth. Differences in growth by P. chrysogenum between CDB and SDB may be attributed to preferred nutrients and/or low pH present in SDB cultures. In this study biomass production continued to increase among the three fungal species throughout the 10 days of growth. Therefore, nutrients provided in CDB or SDB were not limiting towards fungal growth during 10 days of incubation.

Notably, organic acids analyzed in this study were excreted below expected concentrations when compared to previous publications (Horeh et al., 2016; Ilyas et al., 2013a;
Ilyas and Lee, 2013b; Ren et al., 2005; Santhiya and Ting, 2005). These previous studies all had one component in common in bioleaching cultures, which was sugar concentration. Therefore, growth media for culturing fungi should be modified and include at least 100 g/L of carbon source to increase organic acid excretion. One study used a sucrose based medium with 100 g/L of sucrose and reported high concentrations of excreted organic acids (Ren et al., 2005).

Providing fungi with sucrose as a carbon source was determined to increase acid excretion in a previous publication (Papagianni, 2007). That study found that fungi such as A. niger have the enzyme invertase on its cell membrane which can cleave sucrose to 1 molecule of glucose and 1 molecule of fructose providing more carbon and energy for organic acid excretion.

Fungal biomass and media acidification in metal toxicity tests indicated that fungi have a low tolerance for Li and Co found in the cathode of RLIB. To improve simulation of bioleaching processes, future studies should examine the effects of supplementing fungal cultures with a combination of metals from RLIB cathode. However, I expect that the toxic effects of metals in combination would be more severe toward fungal processes. Furthermore, the effects of metals on organic acid excretion should be examined to improve analysis of metal toxicity. To improve fungal tolerance of metals in one step and two step leaching processes, several methods should be considered. Future research can involve subculturing fungi on gradually increasing concentrations of Li and/or Co to build fungal tolerance over time. In addition, researchers may benefit from isolating fungi which have metal resistant genes from a metal contaminated environment such as an electronic scrap yard or landfill.

To further improve understanding of metal toxicity researchers should focus on the stress response by fungi. Examining the expression of genes and their regulation with quantitative polymerase chain reactions (QPCR) while fungi are stressed by metals, will allow researchers to
identify which processes in fungi are affected and the how particular genes control fungal stress response. In addition, genetic modification may be utilized to express specific genes to improve the response of fungi, which will increase their performance in bioleaching processes. However, genetic modification would only be necessary if fungi isolated from contaminated environments did not have desired stress response genes.

Mobilization of Li and Co from the cathode in RLIB with various types of fungal bioleaching steps resulted in a very poor removal of Li or Co compared to one study (Horeh et al., 2016). This result may be attributed to the low amounts of sucrose in the CDB growth media and resulted in low concentrations of organic acids excreted by *A. niger* in leaching cultures. Therefore, results indicate that high concentrations of sugar may be a requirement for a successful bioleaching process with *A. niger*. In addition, the presence of fungi in one step and two step leaching processes increase potential for sorption or uptake by fungi removing the metal from the leaching culture (Gadd, 2004). Spent medium leaching was the most effective for the mobilization of Li from RLIB cathode. However, spent medium leaching did not improve removal of Co in cultures with and without hydrogen peroxide. Previously collected results indicate that higher concentrations of organic acids will be necessary to increase mobilization of Li and Co from cathode material.

The leached metals in this study were analyzed in cultures but analysis did not include chemical composition of products from chelation or precipitation. Previous studies have used NMR to examine organic metalloid complexes found in similar leaching reactions (Gadd, 1999). Recommendation for future research would include examining leaching cultures for organic metalloid complexes such as lithium oxalate to understand the next steps required to completely recover Li or Co from this fungal bioleaching process. In addition, future research should
include a before/after X-ray diffraction analysis of the cathode material in the bioleaching process to accurately determine Li and Co recovery.
REFERENCES


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