Functional genomic and metabolomics characterisation of bioleaching fungi from mine tailings

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DEDICATION

To my beloved children, Neo Ntsako and Amukelani Tsholofelo, who have filled my life with boundless joy and unwavering support. To my husband, Thulani Rich Nkuna, whose steadfast love and encouragement has been my guiding light throughout this journey. To my late granny, Rosina Makofane, whose wisdom, and strength continue to inspire me. To my beloved mother, Flora Maite Makofane, a beacon of unconditional love and unwavering belief. This work is dedicated to you, the pillars of my strength and the sources of my inspiration.

DECLARATION

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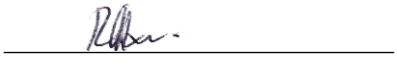

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RESEARCH OUTPUT

PhD research output

- **Nkuna, R**., Ijoma, G. N., & Matambo, T. S. (2022). Applying EDTA in Chelating Excess Metal Ions to Improve Downstream DNA Recovery from Mine Tailings for Long-Read Amplicon Sequencing of Acidophilic Fungi Communities. *Journal of Fungi*, *8*(5), 419**.**
- **Nkuna, R.,** & Matambo, T. S. (2024). Determining the Metabolic Processes of Metal-Tolerant Fungi Isolated from Mine Tailings for Bioleaching. Minerals, 14(3), 235.

SUMMARY

Bioleaching is a biological and an environmentally friendly alternative to traditional leaching methods for extracting metals from ores or wastes. However, its reliance on microorganism, which are sensitive to bioleaching extreme conditions, result in challenges of slow kinetics and low metal recovery that limit its potential. This study applied functional genomics to gain insights into the bioleaching process. The focus was on fungal communities that can survive in extreme conditions. Tailing samples, which represent an extreme environment, with high metal concentration and low pH, similar to bioleaching conditions, were used to profile, isolate and identify fungal communities. Genera such as *Trichoderma, Penicillium,* and *Talaromyces*, were obtained. Further screening of this organisms for metal tolerance, growth rates, carbon utilisation and organic acid production revealed *Trichoderma* as the most promising bioleaching fungus. *Trichoderma* exhibited high growth rates in the presence of metals, likely due to its high metal tolerance index of >1 to 300 mg/l Al, 100 mg/l Zn and Ni, with a minimum inhibitory concentration of 1600 mg/l for Al, 1000 mg/l for Zn and 400 mg/l for Ni. When grown in glucose media, the organism produced up to 300 g/l of citric acid. Thus, the fungus's bioleaching capacity to extract metals from two different metal-containing samples, namely tailings and ore samples, was assessed using one-step, two-step, and spent media approaches. The results showed that the fungus's bioleaching efficiency was unaffected by the sample type and led to the recovery of various metals (>62% of Ni in ore samples and >54% Zn in tailings samples). Other metals such as Mg, As, Mn, Cu and Co were leached in both tailing and ore samples. Metabolite analysis during bioleaching revealed that *Trichoderma* produced oxalic acid (up to 930.13 mg/l) as the main bioleaching agent, while citric acid was produced in low amounts. Transcriptomics analysis revealed that genes related to biological processes, cellular components, and molecular functions were differentially expressed. Further enrichment of the differentially expressed genes revealed that genes associated with the structure and function of the cell membrane were consistently upregulated, indicating how *Trichoderma* tolerates high metal exposure. Notably, the genes related to organic acid production were not differentially expressed, and we further concluded that *Trichoderma*'s metal toxicity defence mechanism does not disrupt the metabolic activities involved in the production of organic acid.

Keywords: Bioleaching, metal tolerance, transcriptomics, organic acids, metabolites.

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LIST OF ABBREVIATION

HNO_3: Nitric acid

EDTA: Ethylene diamine tetraacetic acid

GYEM: Glucose yeast extract media

GYEA: Glucose yeast extract agar

GYEB: Glucose yeast extract broth

YSM: Yeast sucrose media

DNA: Deoxyribonucleic acid

RNA: Ribonucleic acid

PCR: Polymerase chain reaction

OTU: Operational taxonomic units

KEGG: Kyoto Encyclopedia of Genes and Genomes

GC-MS: Gas chromatography-mass spectrometry

HPLC: High-performance liquid chromatography

ICP-AES: Inductively coupled plasma atomic emission spectroscopy.

TI: Tolerance index

RPM: revolutions per minute

MIC: Minimum inhibitory concentration

CHAPTER 1: INTRODUCTION

1.1 Background

It is widely acknowledged that microbial communities function in a way that enables them to survive in harsh conditions posed by biotic and abiotic stress. For instance, microbial communities in the environment can survive by expressing specific enzymes, synthesizing molecules that aid in repairing damage, relieving stress, breaking down certain compounds, and so forth (Bogati & Walczak, 2022; Walker & White, 2017). Moreover, advancements in molecular technologies over the years have demonstrated that microorganisms have the ability to switch on or off the activity of enzymes based on their surroundings, to best suit their need to survive regardless of the environmental conditions (Chi *et al.,* 2022). Consequently, these abilities have been harnessed and adapted by the scientific community for application in various industries for their advantages such as environmental friendliness and pollutant removal. Currently, the world is promoting the use of eco-friendly approaches in industries known to have a negative impact on the environment, and biological processes such as bioleaching are gaining significant attention (Nayak, 2022; Krishnan *et al.*, 2021; Arya & Kumar, 2020; Sajjad *et al.,* 2019). Bioleaching is a biological method of extracting metals from ores or waste (tailings and electronic waste) using microorganisms (Klaus, 1997).

Recently, there has been an increasing interest in optimizing bioleaching processes using bacteria, fungi, or yeast due to many potential benefits such as cost-effectiveness, eco-friendliness, and low energy requirements (Sodha *et al.,* 2020; Villares *et al.,* 2016; Kim *et al.,* 2010; Li & Ke, 2001; Remonsellez *et al.,* 2009). The type of ore being treated, and the microbial domain involved play a crucial role in the mechanism and efficiency of bioleaching. Bacteria are known for their ability to solubilize metal sulfides efficiently and are the most researched domain in the bioleaching space (Chen *et al.,* 2019; Borja *et al.,* 2016; Martinez, *et al.,* 2015; Martínez *et al.,* 2012; Ruiz *et al.,* 2012; Valdés *et al.,* 2008). Thus, the increase in commercialization of bioleaching processes is mostly by bacteria in the past two decades (Yin *et al.,* 2018). In contrast, bioleaching by filamentous fungi is considered efficient for oxides and carbonaceous minerals, with *Aspergillus Niger* serving as the model organism (Dusengemungu *et al.,* 2021; Keshavarz *et al.,* 2021). Despite the increase in bioleaching research by filamentous fungi in the last decade, there is limited research on its industrial application compared to bacteria.

Regardless of the potential benefits of bioleaching by heterotrophic fungi, there are still several challenges that need to be addressed in order to increase the commercialization of this process. One of the challenges is the cultivation time of heterotrophic fungi compared to

chemolithotrophic bacteria commonly used in commercial bioleaching (Roy *et al.,* 2021). However, it's important to remember that faster growth doesn't always equate to better bioleaching effectiveness. Recent studies have shown that under optimal conditions, fungi can actually solubilize metals faster than bacteria (Dusengemungu *et al.,* 2021). Nonetheless, slow process kinetics, metal toxicity, low solid to liquid ratio (pulp density), variability in the performance of different fungal strains and the need for optimal growth conditions remain key concerns that hinder the scalability of the process (Roy *et al.,* 2021).

To overcome these challenges, further research is necessary to optimize the use of heterotrophic fungi in bioleaching. This includes investigating the mechanisms that underlie metal solubilization by fungi and identifying the most efficient fungal strains. Furthermore, development of more cost-effective and efficient bioleaching processes that can be scaled up for large-scale operations is required. Efforts in this regard could involve exploring the potential of genetic engineering to enhance the bioleaching efficiency of fungi or the use of co-culture systems that combine different microbial strains to enhance the overall effectiveness of the process. Overall, with the right research and development, bioleaching by heterotrophic fungi has the potential to become a promising and sustainable alternative to traditional metal extraction methods.

1.2 Problem statement

Despite the recognized environmental benefits of bioleaching over chemical methods, industrial implementation, especially for fungi, faces significant hurdles, including slow kinetics and low metal leaching yields (Clark *et al.,* 2006). These challenges are exacerbated by the complex enzymatic reactions facilitated by microorganisms such as *Aspergillus niger* in often suboptimal conditions. Furthermore, the bioleaching environment itself becomes increasingly extreme, characterized by increasing concentrations of metals as they are bioleached, with potential to inhibit microbial activity (Zhu et al., 2018; Siezen and Wilson 2009). While heavy metals are essential for microbial metabolism, exceeding optimal concentrations can induce toxicity, leading to protein denaturation and impaired microbial function. Although certain microorganisms can adapt to such conditions, accelerating the bioleaching process necessitates the isolation of metal-tolerant fungal strains. Consequently, recent efforts have focused on bioprospecting for such fungi, employing 'omics' techniques to enhance process understanding and optimization.

1.3 Research aim and objectives.

1.3.1 Aim

The study aim was to provide a holistic molecular basis for understanding the bioleaching process by fungal isolate and the possibility of process improvement based on a concise understanding of transcriptional and functional genomics.

1.3.2 Objectives

- Profile fungal communities in various mine tailings collected from different depths using culture-independent method.
- Isolate and evaluate fungal isolates for their bioleaching potential, focusing on metal tolerance, growth rate, and carbon source utilization, particularly for organic acid production.
- Profile and identify targeted metabolites, particularly those associated with organic acids, based on carbon source utilization using LCMS.
- Assess the efficiency of different bioleaching processes, such as one-step, two-step, and spent media bioleaching, using the selected fungal isolate.
    - Quantify bioleached metals using ICP-AES and measure produced organic acids using HPLC.
    - Investigate the transcriptome of the selected fungus, *Trichoderma*, during the bioleaching process to understand its functional behaviour at the molecular level.

1.4 Thesis structure

Chapter One:

Introduction of the research and the significance of the study

Chapter Two:

This section discusses the use of filamentous fungi in bioleaching and focuses on the mechanisms involved, particularly the role of organic acids. The literature review delves into the optimization of conditions that promote high production of organic acids, metal tolerance as well as involved metabolic pathways.

Chapter Three

This chapter employed culture-independent techniques to profile the fungal communities in mine tailings and establish a connection between their presence and the physical and chemical

properties of the tailings as their habitat. The main goal of this analysis was to identify possible contenders for bioleaching. In addition, optimization of DNA isolation from metal rich samples was investigated.

Chapter Four

In this chapter, the fungal communities identified as dominant in Chapter 3 were isolated and assessed for their bioleaching abilities. The evaluation criteria included their growth rates, metal tolerance, carbon utilization, and production of organic acids. This was followed by metabolomic profiling of metabolites involved during the growth of four selected fungal isolates using glucose as a carbon source.

Chapter Five

In this chapter one fungal isolate, *Trichoderma asperellum*, selected from Chapter 4 was used for bioleaching experiments. The bioleaching experiments were conducted to compare the fungus's ability to extract metals from tailings, which represent its natural habitat, and ore, which is different from its habitat. The bioleaching experiments were carried out using one-step, two-step, and spent media and the resulting bioleached metals and organic acids were quantified using ICP-AES and HPLC, respectively. Furthermore, RNA was extracted from the fungi during the bioleaching process to study its transcriptome and better understand its function, related to organic acid production and metal tolerance during bioleaching.

Chapter Six

Overall conclusion and recommendation.

1.5 References

Arya, S., & Kumar, S. (2020). Bioleaching: urban mining option to curb the menace of E-waste challenge. *Bioengineered*, *11*(1), 640–660. https://doi.org/10.1080/21655979.2020.1775988

Bogati, K., & Walczak, M. (2022). The Impact of Drought Stress on Soil Microbial Community, Enzyme Activities and Plants. In *Agronomy* (Vol. 12, Issue 1). MDPI. https://doi.org/10.3390/agronomy12010189

Borja, D., Nguyen, K., Silva, R., Park, J., Gupta, V., Han, Y., Lee, Y., & Kim, H. (2016). Experiences and Future Challenges of Bioleaching Research in South Korea. *Minerals*, *6*(4), 128. https://doi.org/10.3390/min6040128

Chen, A., Hao, X., Xiao, Y., Zou, K., Liu, H., & Liu, X. (2019). *Responses of Acidithiobacillus thiooxidans A01 to Individual and Joint Nickel (Ni2+) and Ferric (Fe3+)*. 1–12. https://doi.org/10.3390/min9020082

Clark, M. E., Batty, J. D., van Buuren, C. B., Dew, D. W., & Eamon, M. A. (2006). Biotechnology in minerals processing: Technological breakthroughs creating value. *Hydrometallurgy*, *83*(1–4), 3–9. https://doi.org/10.1016/J.HYDROMET.2006.03.046

Chi, Z., Kong, C. C., Wang, Z. Z., Wang, Z., Liu, G. L., Hu, Z., & Chi, Z. M. (2022). The signaling pathways involved in metabolic regulation and stress responses of the yeast-like fungi Aureobasidium spp. In *Biotechnology Advances* (Vol. 55). Elsevier Inc. https://doi.org/10.1016/j.biotechadv.2021.107898

Dusengemungu, L., Kasali, G., Gwanama, C., & Mubemba, B. (2021). Overview of fungal bioleaching of metals. *Environmental Advances*, *5*, 100083. https://doi.org/10.1016/j.envadv.2021.100083

Keshavarz, S., Faraji, F., Rashchi, F., & Mokmeli, M. (2021). Bioleaching of manganese from a low-grade pyrolusite ore using Aspergillus niger: Process optimization and kinetic studies. *Journal of Environmental Management*, *285*. https://doi.org/10.1016/j.jenvman.2021.112153

Kim, D. J., Pradhan, D., Ahn, J. G., & Lee, S. W. (2010). Enhancement of metals dissolution from spent refinery catalysts using adapted bacteria culture - Effects of pH and Fe(II). *Hydrometallurgy*, *103*(1–4), 136–143. https://doi.org/10.1016/j.hydromet.2010.03.010

Klaus, B. (1997). Bioleaching: metal solubilization by microorganisms. *FEMS Microbiology Reviews*, *20*(1–2), 591–604. http://www.sciencedirect.com/science/article/pii/S0168644597000363

Krishnan, S., Zulkapli, N. S., Kamyab, H., Taib, S. M., Din, M. F. B. M., Majid, Z. A., Chaiprapat, S., Kenzo, I., Ichikawa, Y., Nasrullah, M., Chelliapan, S., & Othman, N. (2021). Current technologies for recovery of metals from industrial wastes: An overview. *Environmental Technology and Innovation*, *22*, 101525. https://doi.org/10.1016/j.eti.2021.101525

Li, H., & Ke, J. (2001). Technical note influence of Cu 2+ and Mg 2+ on the growth and activity of Ni 2+ adapted Thiobacillus ferrooxidans. *Science*, *14*(1), 113–116.

Martínez P, Gálvez S, Ohtsuka N, Budinich M, Cortés MP, Serpell C, Nakahigashi K, Hirayama A, Tomita M, Soga T, Martínez S, Maass A, P. P. (2012). Metabolomic study of Chilean biomining bacteria Acidithiobacillus ferrooxidans strain Wenelen and Acidithiobacillus thiooxidans strain Licanantay. *Metabolomics*, *9*(1), 247–257. https://doi.org/10.1007/s11306-012-0443-3

Martínez, P., Vera, M., Bobadilla-Fazzini, R. A., Haghshenas, D. F., Alamdari, E. K., Bonakdarpour, B., Darvishi, D., Nasernejad, B., Elissawy, A., Ebada, S. S., & Ashour, M. L. (2015). Omics on bioleaching: current and future impacts. *Applied Microbiology and Biotechnology*, *99*(20), 8337–8350. https://doi.org/10.1007/s00253-015-6903-8

Nayak, N. P. (2022). Microorganisms and their application in mining and allied industries. *Materials Today: Proceedings*, *2022*(xxxx). https://doi.org/10.1016/j.matpr.2022.07.392

Remonsellez, F., Galleguillos, F., Moreno-Paz, M., Parro, V., Acosta, M., & Demergasso, C. (2009). Dynamic of active microorganisms inhabiting a bioleaching industrial heap of low-grade copper sulfide ore monitored by real-time PCR and oligonucleotide prokaryotic acidophile microarray. *Microbial Biotechnology*, *2*(6), 613–624. https://doi.org/10.1111/j.1751-7915.2009.00112.x

Roy, J. J., Cao, B., & Madhavi, S. (2021). A review on the recycling of spent lithium-ion batteries (LIBs) by the bioleaching approach. In *Chemosphere* (Vol. 282). Elsevier Ltd. https://doi.org/10.1016/j.chemosphere.2021.130944

Ruiz, L. M., Castro, M., Barriga, A., Jerez, C. A., & Guiliani, N. (2012). The extremophile Acidithiobacillus ferrooxidans possesses a c-di-GMP signalling pathway that could play a

significant role during bioleaching of minerals. *Letters in Applied Microbiology*, *54*(2), 133–139. https://doi.org/10.1111/j.1472-765X.2011.03180.x

Sajjad, W., Zheng, G., Din, G., Ma, X., Rafiq, M., & Xu, W. (2019). Metals Extraction from Sulfide Ores with Microorganisms: The Bioleaching Technology and Recent Developments. *Transactions of the Indian Institute of Metals*, *72*(3), 559–579. https://doi.org/10.1007/s12666-018-1516-4

Sodha, A. B., Tipre, D. R., & Dave, S. R. (2020). Optimisation of biohydrometallurgical batch reactor process for copper extraction and recovery from non-pulverized waste printed circuit boards. *Hydrometallurgy*, *191*. https://doi.org/10.1016/j.hydromet.2019.105170

Siezen, R. J., & Wilson, G. (2009). Bioleaching genomics. *Microbial biotechnology*, *2*(3), 297. https://doi.org/10.1111/j.1751-7915.2009.00108.x

Valdés, J., Pedroso, I., Quatrini, R., Dodson, R. J., Tettelin, H., Blake, R., Eisen, J. A., & Holmes, D. S. (2008). Acidithiobacillus ferrooxidans metabolism: From genome sequence to industrial applications. *BMC Genomics*, *9*, 1–24. https://doi.org/10.1186/1471-2164-9-597

Villares, M., Işildar, A., Mendoza Beltran, A., & Guinee, J. (2016). Applying an ex-ante life cycle perspective to metal recovery from e-waste using bioleaching. *Journal of Cleaner Production*, *129*, 315–328. https://doi.org/10.1016/j.jclepro.2016.04.066

Walker, G. M., & White, N. A. (2017). Introduction to Fungal Physiology. In *Fungi* (pp. 1–35). John Wiley & Sons, Inc. https://doi.org/10.1002/9781119374312.ch1

Yin, S., Wang, L., Kabwe, E., Chen, X., Yan, R., An, K., Zhang, L., Wu, A., Dusengemungu, L., Kasali, G., Gwanama, C., Mubemba, B., Debaraj Mishra, Dong-Jin Kim, Jong-Gwan Ahn, & Young-Ha Rhee. (2018). Copper bioleaching in China: Review and prospect. *Minerals*, *11*(2), 100083. https://doi.org/10.3390/min8020032

Zhu, J., Zhou, S., Qiu, G., Liu, X., & Gan, M. (2018). Assessment of Bioleaching Microbial Community Structure and Function Based on Next-Generation Sequencing Technologies. *Minerals*, *8*(12), 596. https://doi.org/10.3390/min8120596

CHAPTER 2: LITERATURE REVIEW

2.1 Environmental impact of mining industry

The mining industry plays a critical role in society's prosperity and development (Liapun & Motola, 2023). However, mining industry has been identified as a significant contributor to environmental pollution (Priester *et al.,* 2019; Karaca *et al.,* 2018; Johnson, 2013; Bian *et al.,* 2012; Acheampong *et al.,* 2010; Dold, 2008). This is primarily due to the traditional methods of metal production, which include several steps ranging from excavation to waste disposal, which in the process destroys or disrupt ecosystem, emit greenhouse gasses, and pollutes soil and water bodies (Xu *et al.,* 2019; Moyé *et al.,* 2017; Phillips, 2016). Countries such as South Africa, for example, have been severely impacted by the continuous environmental pollution resulting from mining waste, which still occur many years after some mining processes have since concluded. This is because mining wastes are stockpiled in dumps referred to as tailings and overburden (spoils) material, which are heaps of waste stockpiled in open areas (Priester *et al.,* 2019; Karaca *et al.,* 2018).

Tailings are a significant environmental problem due to their high metal content, which may have been overlooked in the past due to ore grade, lack of commercial interest, or outdated technology used at the time (Guo *et al.,* 2022; Nkuna *et al.,* 2022). Because tailings are typically stored in open areas, they are susceptible to natural and microbial leaching of metals, resulting in acid mine drainage (AMD) in the soil and water. Mixed sulfide minerals, such as pyrite and pyrrhotite often found in tailings, are responsible for acid generation and the solubilization of metals. Despite this, stockpiling of tailings remains the primary method of storage and disposal (Gan *et al.,* 2022; Shengo, 2021). In addition to water and soil pollution, this stockpiling method also contaminate other areas due to wind-blown dust particles. The dust contains toxic metals, such as chromium (Cr), nickel (Ni), copper (Cu), cadmium (Cd), zinc (Zn), lead (Pb), cobalt (Co), arsenic (As), mercury (Hg), and tin (Sn), which can be disseminated over greater distances (Mileusnić *et al.,* 2014). Therefore, the accumulation of tailings in urban areas is highly detrimental to human health, with some containing significant quantities of radionuclides (Kolo *et al.,* 2017).

Various strategies have been employed to mitigate the environmental impact of mine tailings, such as covering sulfidic tailings with non-sulfidic topsoil to prevent water penetration (RoyChowdhury *et al.,* 2015; Nason *et al.,* 2013; Bell, 2001) and diverting surface and ground water from acid producing pyritic waste piles (Baniasadi *et al.,* 2019; RoyChowdhury *et al.,* 2015). Growing plants on tailings to prevent erosion and dust dispersion has been employed as

well (Gil-Loaiza *et al.*, 2016; Bell, 2001). However, despite these efforts, environmental issues related to tailings remain unresolved. One solution being considered is to view tailings as a source of metals, particularly as high-grade ores become depleted and demand for metals increases (Guo *et al.*, 2022; Xu *et al.*, 2019). Compared to other low-grade ores, re-extracting metals from tailings is less costly since the minerals are already finely ground and exposed on the surface (Andrews *et al.*, 2013). However, conventional extraction methods may cause additional environmental pollution (Kržanović *et al.*, 2019; Watling, 2014), highlighting the need for environmentally friendly alternatives such as bioleaching. Tailings contain multiple metals, making it challenging to extract them all at once using conventional methods (Kržanović *et al.*, 2019; Watling, 2014; Johnson, 2013). Bioleaching is an attractive option due to its cost-effectiveness and ability to extract several metals simultaneously.

Bioleaching is a biotechnological process that utilizes microbial communities to extract metals from ores or low-grade ores. Microbial communities involved include chemolithotrophic prokaryotes such as *Acidithiobacillus ferrooxidans, Acidithiobacillus ferrivorans, Acidithiobacillus caldus, Acidithiobacillus thiooxidans* and *Leptospirillum ferrooxidans* as well as heterotrophic eukaryotes such as *Aspergillus niger* and *Penicillium* (Qu *et al.*, 2022; Keshavarz *et al.*, 2021; Castro *et al.*, 2020; Clark *et al.*, 2019; Borja *et al.*, 2016; Martínez *et al.*, 2012; Valdés *et al.*, 2008). Although the commercialization of bioleaching processes has increased in the past two decades, most commercial processes still use chemolithotroph prokaryotes such as *Acidithiobacillus ferrooxidans* (Yin *et al*, 2018; Bosecker, 1997). Despite increasing research into the bioleaching ability of heterotroph eukaryotes, their commercial use it still limited.

2.2 Bioleaching by filamentous fungi

In general, bioleaching mechanisms can be classified as direct or indirect, where direct bioleaching involves the use of enzymes to breakdown mineral solid structure, while indirect bioleaching does not (Cho *et al.*, 2023; Yang *et al.*, 2019; Richter *et al.*, 2018). However, indirect bioleaching involves the use of corrosive metabolic products like organic acids and ligands secreted by microorganisms (Nasab *et al.*, 2020; Chaerun *et al.*, 2017). In bacteria, both bioleaching mechanisms are used, while filamentous fungi use organic acids, amino acids, and other secreted metabolites to dissolve metals. Most of these acids are monocarboxylic acids like lactic acid and propionic acid; dicarboxylic acids like oxalic acid, malic acid, and fumaric acid; tricarboxylic acids like citric acid, and sugar acids like gluconic acids, all these acids play

an important role in bioleaching (Mendes *et al.,* 2022; Karaffa *et al.,* 2021; Brisson *et al.,* 2020; Malekian *et al.,* 2019; Faraji *et al.,* 2018; Astuti *et al.,* 2016). Fungi like *Aspergillus, Penicillium, Trichoderma, Phanerochaete, Paecilomyces* and *Talaromyces* have been reported to be effective in bioleaching processes that involves organic acids (Liu *et al.,* 2022; Qu *et al.,* 2022; Brisson *et al.,* 2020; Faraji *et al.,* 2018; Farkas *et al.,* 2021; Tansengco *et al.,* 2018; Reed *et al.,* 2016; Babu *et al.,* 2014; Amiri *et al.,* 2011; Ren *et al.,* 2009). *Aspergillus* and *Penicillium* are the two common bioleaching fungi, with *Aspergillus niger* species representing the most studied species in the *Aspergillus* genus (Castro *et al.,* 2020; Din *et al.,* 2020; Keshavarz *et al.,* 2021; Muddanna & Baral, 2019a; Horeh *et al.,* 2016). The use and optimization of the other listed fungal genera for bioleaching applications is still relatively limited as compared to *Aspergillus* and *Penicillium* genera. However, as the field of bioleaching continues to evolve, it is expected that new research may provide further insights into their capabilities or uncover additional species with bioleaching potential.

2.2.1 Overview of carbon metabolic pathway in relation to bioleaching

Aspergillus niger serves as a model-organisms for bioleaching-related processes and most of the information available in literature is derived from bioleaching experiment using this species (Farkas *et al.,* 2021; Behera, 2020; Din *et al.,* 2020; Zheng *et al.,* 2019; Faraji *et al.,* 2018; Odoni *et al.,* 2017; Seh-Bardan *et al.,* 2012; Ren *et al.,* 2009; Santhiya & Ting, 2006; Kubicek *et al.,* 1988; Barratt *et al.,* 1965;). As mentioned, bioleaching by filamentous fungi is an indirect process where the fungus produces organic acids, which are responsible for bioleaching. Therefore, application of metabolomics on *A. niger* or any other fungi with bioleaching potential, is primarily focused on metabolic pathways of organic acid production, in this case, in the presence of metals. Organic acids are produced mainly from the breakdown of carbon sources and glucose and sucrose are the most reported carbon sources for bioleaching by fungi (Pathak *et al.,* 2021a; Shah *et al.,* 2020; Srichandan *et al.,* 2019). An overview of the glucose metabolic pathways in *A. niger* that could indirectly contribute to bioleaching process is represented in Figure 2.1. The sucrose metabolic pathway differs from glucose metabolic pathway by just a few steps, where sucrose is initially hydrolysed to glucose and fructose after entering the cell (Gupta & Gupta, 2021). The produced glucose will then be metabolised as indicted in the Figure 2.1, while fructose will be phosphorylated to produce glycolytic intermediate that will be converted to pyruvate (Li *et al.,* 2022; Fathollahzadeh *et al.,* 2019). According to previous studies, organic acids such as citric acid and oxalic acid, have been reported as the best leaching agents for the recovery of metals from various sources (Arshadi

et al., 2022; Pathak, *et al.*, 2021a; Liu *et al.*, 2020). The production of these organic acids is not only observed in the presence of metals (or during bioleaching), but even during growth of fungi under normal conditions. Organic acids are produced as part of fungal normal metabolic process for reasons such as nutrient acquisition, energy production and biosynthesis of important precursors (Andrino *et al.*, 2021; Wösten, 2019). Furthermore, organic acids can be used as defence mechanisms, where organic acids such as acetic and propionic acid are known to have antimicrobial properties, regulating pH as well as adaptation to different environments (Barnett *et al.*, 2020; Chen *et al.*, 2021; Schnürer & Magnusson, 2005). The two common organic acid are discussed below.

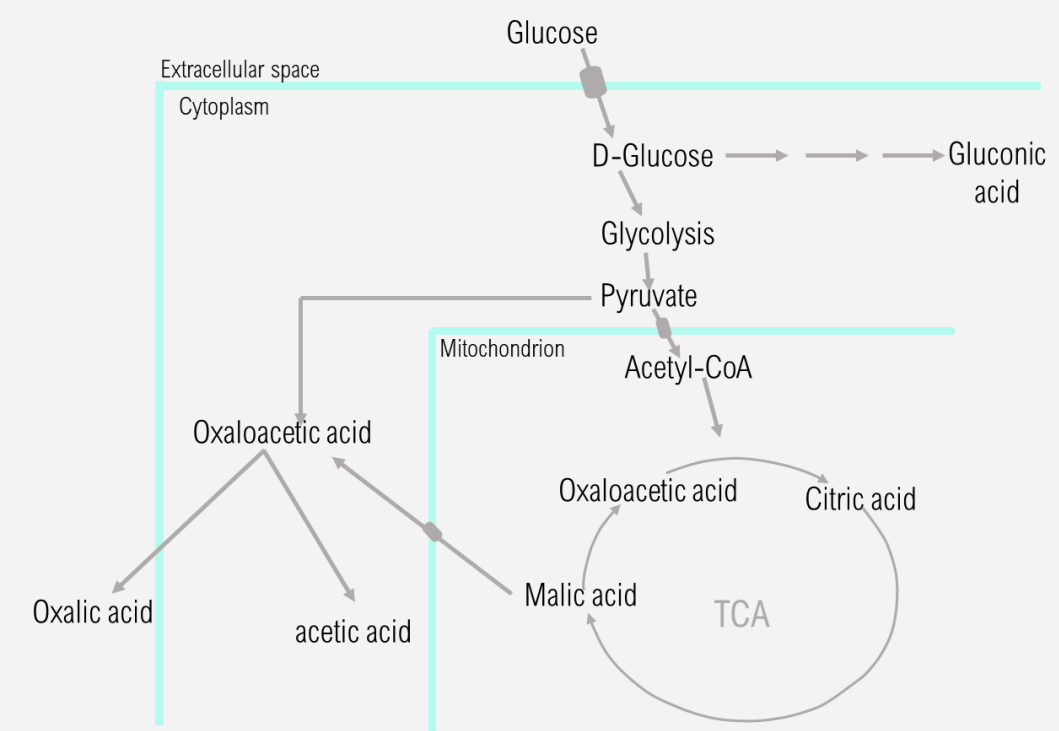

Figure 2.1: Metabolic pathway glucose by of *Aspergillus niger*. Adapted from Yoshioka *et al.* (2020); Kirimura *et al.* (2019); Odoni *et al.* (2017) and Kobayashi *et al.* (2014).

2.2.1.1 Citric acid

Citric acid producing fungi such as the well-studied *A. niger*, contains the necessary enzymes required to convert carbon such as glucose and sucrose to citric acid through tricarboxylic acid (TCA) cycle (also known as Krebs cycle or citrate cycle) (Figure 2.1) (Gupta & Gupta, 2021; Kirimura *et al.*, 2019). Citric acid production involves a number of enzymes and intermediates as reported by Gupta & Gupta (2021). Media containing high carbon sources, deficiency in manganese ions (Mn^{2+}) as well as limited nitrogen source is known to strongly influence the

accumulation of citric acid (Gupta & Gupta, 2021; Behera, 2020; Dutta *et al.*, 2019). Acidic pH also is a physiological condition that contribute to accumulation of citric acid (Behera, 2020). A series of metabolic reactions are triggered when citric acid producing fungi are grown in a medium that contains high carbon source (sugar). These metabolic reactions lead to rapid formation of an essential intermediate called fructose-2,6-bisphosphate (through glycolysis) (Behera, 2020). This intermediate serves as an allosteric activator of the enzyme phosphofructokinase I (PFK I), an essential enzyme for citric acid regulation (Zuo *et al.*, 2021). Additionally, high concentration of ammonium ions (NH_4^+), which means a limited nitrogen source, in the medium also serves as an activator for the enzyme PFK I (Gupta & Gupta, 2021). On the other hand, high concentration of NH_4^+ suppresses the biosynthesis of another important enzyme called α-ketoglutarate dehydrogenase (AGE) (Max *et al.*, 2010). PFK I in citric acid controls the rate of glycolysis, which is an essential pathway that provide precursors for citric acid production (Figure 2.1), therefore if expressed, it means tricarboxylic pathway will proceed, leading to production of citric acid. The repression of α-ketoglutarate dehydrogenase on the other hand, diverts metabolic flux away from TCA, leading to increased production of citric acid (Max *et al.*, 2010).

Under metal-rich conditions, such as those found in bioleaching processes, the interaction between metals and fungi can also stimulate the production of citric acid. However, the extent of this stimulation varies among different fungi and the specific metals present. The production of citric acid in response to metals is believed to be a survival mechanism against the potential toxic effects of metals on the organism (Shivakumar *et al.*, 2014). For example, a comparative study by Muddanna & Baral, (2019b) demonstrated that citric acid, generated through one-step bioleaching process of spent fluid catalytic cracking catalyst by *A niger*, acted as the dominant bioleaching agent. This citric acid demonstrated the ability to leach up to 50% of aluminium (Al), 32% of titanium (Ti), and 42% of vanadium (V) from the catalyst, implying that its production may have been influenced by the presence of Al, Ti and V. However, this is not always the case, the presence of metals such as manganese (Mn) has the potential to inhibit the production of citric acid in *A niger*. This was reported by Santhiya & Ting, (2006) and also observed in a study by Alavi *et al.* (2021) where the presence of Mn in battery powder inhibited citric acid production. As mentioned above, for maximum production of citric acid, the media should be deficient in Mn^{2+}. This is because deficiency in Mn^{2+} contribute to the increase in intracellular NH_4^+ through breakdown of cellular proteins, high NH_4^+ leads to activation of PFK 1 and repression of AGE (Gupta & Gupta, 2021). Therefore, if the media contains Mn^{2+},

the opposite will occur, and citric acid production will be inhibited. Consequently, in these studies, oxalic acid was reported as the main organic acid produced instead of citric acid. Thus, the main/dominant organic acid produced by a particular fungal species is also influenced by the environmental factors to which it is exposed.

2.2.1.2 Oxalic acid

Oxalic acid is another important extracellular organic acid reported for its bioleaching efficiency. Two main microbial metabolic pathways are available for the production of oxalic acid carried out by enzymes referred to as glyoxylate dehydrogenase (GDH) and oxaloacetate hydrolase (OAH: EC.3.7.1.1) (Kobayashi *et al.,* 2014; Kubicek *et al.,* 1988). The first enzyme, GDH, is responsible for hydrolysis of glyoxylic acid and the second enzyme (OAH) is responsible for hydrolysis oxaloacetate (Han *et al.,* 2007; Kobayashi *et al.,* 2014; Kubicek *et al.,* 2011). For fungal organisms with bioleaching potential, pathway by OAH is common, while the other pathway is mainly reported in non-bioleaching organisms (Naseri *et al.,* 2022; Munir *et al.,* 2001). The activity of OAH lead to the production of oxalate ions (important for metal solubilization) and acetate. According to Yoshioka *et al.* (2020), in *A niger*, oxalic acid is produced only by the metabolic pathway that involves the hydrolysis of oxaloacetate. Another important enzyme in the production of oxalic acid is pyruvate carboxylate, which is responsible for the production of oxaloacetate directly from pyruvate, that is, without the reaction of TCA cycle (Kubicek *et al.,* 1988). This cytoplasmic enzyme was reported in *A niger* (Kubicek *et al.,* 1988). As seen from Figure 2.1, some of the pyruvate molecules from glucose enter the TCA cycle for citric acid production while other pyruvate molecules are converted to oxaloacetate by pyruvate carboxylase.

When it comes to the leaching of Ni, V and molybdenum (Mo), oxalic acid has been reported to be the best leaching agent (Muddanna & Baral, 2019b; Santhiya & Ting, 2005). Just like citric acid, the overproduction of oxalic acid can be a consequence of the presence of nickel ions that activates oxalic acid biosynthesis pathway such as the expression of *oahA* gene, a segment of DNA that instructs the production of the enzyme OAH. A study by Santhiya & Ting (2005) reported that Ni and Mo may have contributed the highest oxalate production when *A. niger* was used to bioleach metals found in spent refinery catalyst. Higher oxalate production is linked to high oxalic acid production, since the enzymatic conversion of oxaloacetate by OAH yield oxalate, which is then hydrolysed to oxalic acid.

2.3 Mechanisms of bioleaching by secreted organic acids

Organic acids dissolve metals during bioleaching through a process known as ligand-assisted dissolution (Li *et al.*, 2021a). This process involves the formation of complexes between organic acids and metal ions, enhancing their solubility in solution (Li *et al.*, 2021a). Ligand-assisted dissolution can be further explained by acidolysis, complexation and redoxolysis mechanisms as described below (Figure 2.2). Also, it is important to note that active mechanism in bioleaching by different fungal species is determined by the matrix of the targeted mineral. All the different mechanisms, that is, acidolysis, complexolysis, and redoxolysis are effective in solubilizing metals during bioleaching (Dusengemungu *et al.*, 2021; Priyadarshini *et al.*, 2021). Acidolysis is effective for the solubilization of metal ions in sulfide minerals, whereas complexolysis is effective for metals that do not react to acids (Srichandan *et al.*, 2019). Redoxolysis on the other hand is effective for the solubilization of metal ions that can be oxidised or reduced by microorganisms (Srichandan *et al.*, 2019).

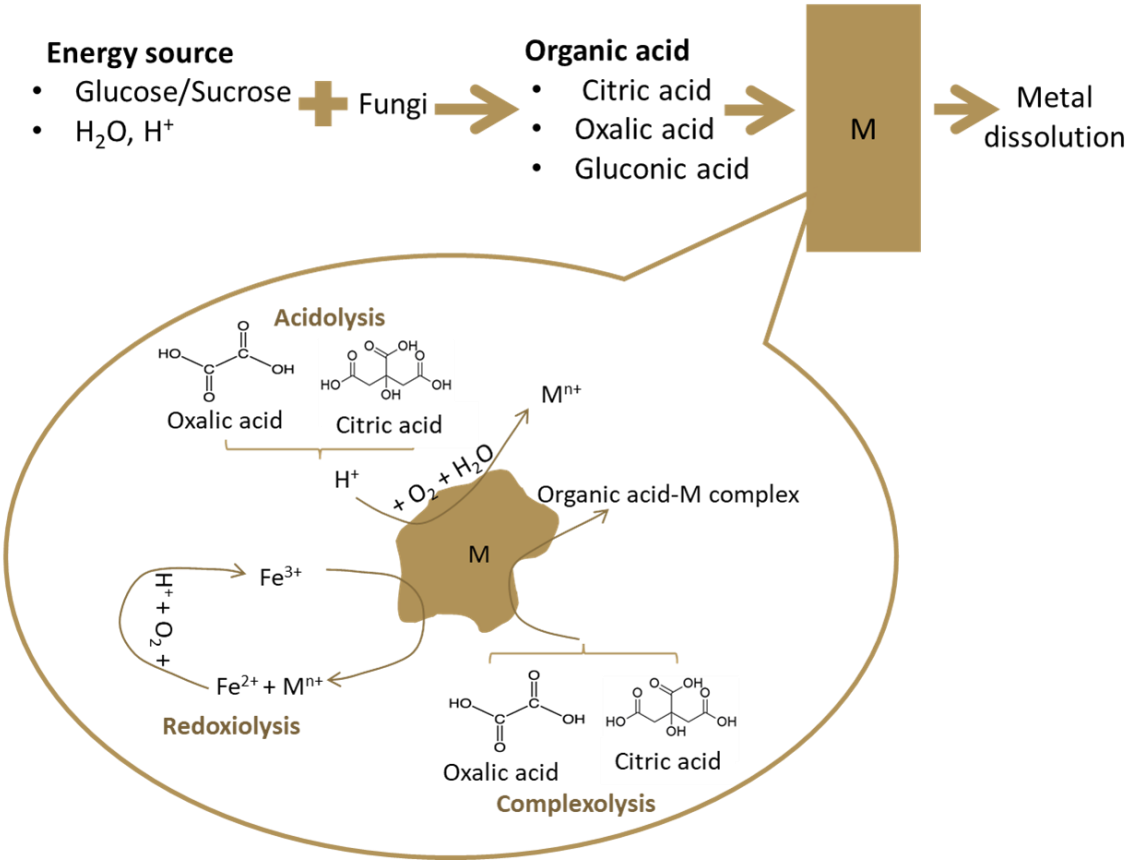

Figure 2.2: Overview of bioleaching mechanisms through acidolysis, complexolysis and redoxolysis. Adapted from Li *et al.* (2021b) and Tezyapar Kara *et al.* (2023).

2.3.1 Acidolysis

Acidolysis is an indirect mechanism by which organic acids assist in the dissolution of metal from minerals during bioleaching (Dusengemungu *et al.,* 2021). In this process, the organic acid, first attaches to the mineral surface through hydrogen bonding and then triggers the protonation of the metal oxide surface (Pathak, *et al.,* 2021a). Protonation occurs when the adsorbed organic acid supplies protons (H^+) to the oxygen on the mineral surface (Ocampo-López *et al.,* 2022). The protective layer of oxides or hydroxides is then removed due to the combination of protonated oxygen with water (equation 2.1). This reaction occurs at low pH which is reached due to the presence of organic acids (Dusengemungu *et al.,* 2021). Sources of protons such as microbially produced organic acids and proton – translocation ATPase located in the plasma membrane, are known to lower the availability of anions to cations in metal compounds (Ilyas *et al.*, 2014). This leads to the solubilization of metals ions.

$$MO + 2H^+ \leftrightarrow M^{2+} + H_2O \qquad (2.1)$$

Where MO is metal oxide such as $CaCO_3$ (Calcium carbonate) or NiO (Nickel oxide)

2.3.2 Complexolysis

The complexolysis process is a complementary mechanism to acidolysis where it is involved in stabilizing the metal ions created during acidolysis (Dusengemungu *et al.,* 2021). According to Brandl & Faramarzi (2006), this procedure entails organic compounds acting as chelators and forming complexes (ligand-induced metal solubilization) with the metal ions released during acidolysis. Metal-organic acid complexes form through chelation between the carboxyl and hydroxyl functional groups of the organic acid and the metal ions (Naseri *et al.,* 2022). The solubilization of metal ions depends on the ability of a molecule to complex with metal ions (Pathak, *et al.,* 2021a). Metals will be effectively leached out of the solid particles if the bonds between metal ions and the complexing molecule, ligands, are stronger than the lattice bonds between metal ions and solid particles (Ilyas *et al.,* 2014). These metal complexes have higher solubility than the original mineral. The stability of these metal complexes helps to minimise the toxic effect of metal ions on fungi (Li *et al.,* 2021a; Srichandan *et al.,* 2019). The solubilized complexes in the solution can support the microbial community's metabolism and can be harvested for industrial purposes. Complexolysis is slower than acidolysis, and equation 2.2 below provides an example of the complexolysis reaction involving citric acid as the chelating agent (Pathak, *et al.,* 2021a).

$$M^{2+} + C_6H_8O_7 \leftrightarrow M(C_6H_8O_7)^- + 3H^+ \quad (2.2)$$

2.3.3 Redoxolysis

Redoxolysis is a process by which microorganisms use electrons to solubilize metal ions from solid substrates (minerals) during bioleaching (Liapun & Motola, 2023). The attachment of microorganism to the sulfide mineral is the first step in redoxolysis (Vardanyan *et al.*, 2022). This attachment is made possible by microbially produced extracellular polymeric substances (EPS), which have glue like characteristics and allows the cell to attach to the mineral surface (Sethurajan & Gaydardzhiev, 2021; Vardanyan *et al.*, 2022). Once attachment stage is completed, organic acids are produced and secreted into the environment. The organic acid form metal-organic acids complexes on the surface of the mineral which facilitate metal solubilization (Dusengemungu *et al.*, 2021).

2.4 Steps in indirect fungal bioleaching

Since bioleaching by fungi primarily relies on organic acids, it allows for three possible ways in which bioleaching can be carried out (Figure 2.3). The first method referred to as one-step involves mixing the media, fresh fungi, and the metal-containing sample together at the beginning of the experiment. This differs from the second method, where in two-step, fresh fungus is inoculated to the media and allowed to grow and produce organic acid. The production of organic acids ultimately leads to decrease in pH and when a specific pH is reached, the metal containing samples are added (Yang *et al.*, 2008). Lastly, in spent media the inoculated fungus is allowed to completely use the carbon source and nutrients in the media to produce organic acids and other by products which will be used to leach metal containing samples (Pathak, *et al.*, 2021a).

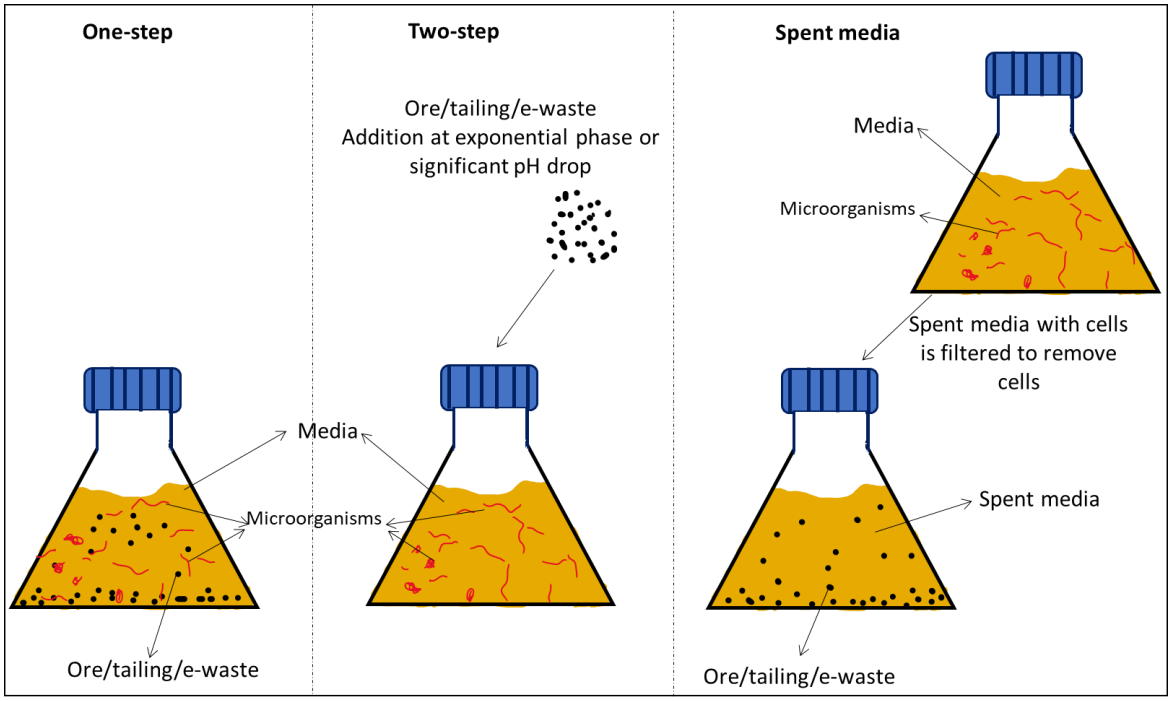

Figure 2.3: A schematic diagram depicting different fungal bioleaching steps: one-step, two-steps and spent media bioleaching.

Several studies comparing the above methods showed that the efficiency of each method is dependent on the type of fungi as well as metals to be leached. A comparative study by Netpae & Suckley (2020) also showed that the type of media used does contribute to the efficiency of each method. In this case, out of the three media compared, Richards's broth through two-step bioleaching leached over 80% of Ni and Cd. Two-step bioleaching process has also been reported by Qu & Lian (2013), Amiri *et al.* (2010), and Yang *et al.* (2008) as the best leaching process using organisms *Penicillium simplicissimum*, *Penicillium tricolor* and *A. niger* respectively. This may be attributed to one of the advantages of two-step methods, where the organism is allowed to grow and produce organic acid without the disturbances that may come with the presence of metal-containing samples that may delay the lag phase or affect metabolic activities. Studies on the efficiency of one-step over two-step bioleaching are mostly on some of the metals compared and not all just like in two-steps. And the best example of this is from a study by Qayyum *et al.* (2019), where the solubilization of Pb, Cd and Zn were compared using one-step and two-step. Only the solubilization of Pb was highest in one-step, while the rest were highest in two-step. This may be attributed to the disadvantages that come with the process, where metal toxicity may affect the growth and activity of the microorganisms. Spent media bioleaching is also effective especially because microbial cells are not used. This allows for bioleaching of high pulp densities (that is, more metal-containing sample), at very acidic

pH without the risk of toxicity to cells as observed in one-step (Adetunji *et al.*, 2023; Reed *et al.*, 2016). Thus, a study by Alavi *et al.* (2021) found spent media to be the best when compared to the other two, though their investigation was based on co-culture of *A. niger* and *A. tubingensis* comparing different carbohydrate sources as well. Similar results were reported by Horeh *et al.* (2016). In studies where spent media was the least effective it could be due to no continuous organic acid production since microorganisms are removed (Fathollahzadeh *et al.*, 2019). One more proposed reason is likely because spent media may not contain organic acid only, but also other by-products that may affect the metal solubilization. However, this may still need to be confirmed through research.

2.5 Challenges in fungal bioleaching

As seen above, organic acids play an important role in fungal bioleaching. Any challenges that lead to a disruption in organic acid production may negatively impact the efficiency of the bioleaching process. The production of organic acids is influenced by various operational parameters, such as temperature, pH, carbon source and many more others (Saim & Darteh, 2023). Furthermore, the characteristics of the ore or tailing must also be considered, especially in the case of tailings which may contain various chemicals from previous processing methods, such as flotations, which may pose toxic effects on the microorganisms. A brief explanation of some of the parameters and how their optimization contributes towards bioleaching efficiency is given below.

2.5.1 General growth conditions

Organic acids are extracellular metabolites that are part of the secondary metabolites produced during fungal growth (Din *et al.*, 2020). Bioleaching fungi are referred to as acidophiles because they can grow at low pH, ranging from 4 to 6, and even at extreme pH of about 2 due to their resilient ability (Gao *et al.*, 2021; Williams & Mayfield, 1971). The importance of pH in fungal growth can be explained in two stages. Once a mycelium comes to contact with the suitable substrate/media, the germination of conidiophores occurs. This germination usually occurs at pH above 5, and to facilitate this stage, most fungal general growth media, such as potato dextrose, are prepared at pH of 5-6. If pH fluctuations are observed at this stage, the result may be detrimental (Pathak *et al.*, 2021b). After germination, the fungi start to produce organic acids and other compounds such as ammonium, which causes a decrease in pH in the media (Pathak *et al.*, 2021b). Thus, in the laboratory, the simplest method of screening for organic acid production involves a colour change test during fungal growth using indicators

such as bromophenol blue/green (Din *et al.,* 2020). During bioleaching, a low pH is necessary to keep dissolved metal ions in solution after solubilization (Islam, 2008). Some organisms can produce various organic acids, such as citric acid, oxalic acids, and gluconic acids. However, the proportions of these acids may differ among different organisms, depending on the pH of the growth media and other environmental parameters (Mendes *et al.,* 2022; Keshavarz *et al.,* 2021; Din *et al.,* 2020b; Nasab *et al.,* 2020; Seh-Bardan *et al.,* 2012). The production of citric acid is reported to be favoured when the media pH reaches 3, whereas for gluconic acid and oxalic acid it is preferable to have a pH between 4 - 6 and above 6 respectively (Pathak, *et al.,* 2021a). It should be noted that organic acid production arises from the activities or expression of different enzymes. For example, oxaloacetate hydrolase, a cytoplasmic enzyme, produces oxalic acid and its reported optimal expression pH is within the range of 4.5-6.5 (Kristiansen *et al.,* 2002). Similarly, the enzyme glucose oxidase catalyzes the production of gluconic acids, and its reported optimal expression is above a pH of 6 (Kubicek *et al.,* 1988). When the pH values are no longer within the optimal range, their activity is inhibited. As suggested by Kristiansen *et al.* (2002) the activities of both glucose oxidase and glucose oxidase are inhibited at pH 3.

2.5.2 Carbon source utilisation

Carbon sources are essential for fungi involved in bioleaching because the production of organic acids is directly dependent on their presence. Moreover, these carbon sources provide nutrients and energy sources to the organisms. Glucose and sucrose are two important carbon sources often used in bioleaching and comparative bioleaching studies have shown that either glucose or sucrose as a carbon source can result in an efficient bioleaching process (Naseri *et al.,* 2022; Arshadi *et al.,* 2020; Qayyum *et al.,* 2019). For example, Qayyum *et al.* (2019) compared the efficiency of bioleaching by *Aspergillus flavus* when glucose or sucrose were used. Their results showed that sucrose resulted in more efficient bioleaching as compared to glucose. Malic acid was the main organic acid produced by *A. flavus*, and according to Qayyum *et al.* (2019), glucose as a carbon source limits the secretion of malic acid. However, if a different fungal species is used, the opposite is true. For instance, a study by Keshavarz *et al.* (2021) reported glucose to have more efficient bioleaching than sucrose. In this case, the main organic acid produced by *A. niger* was citric acid. The same study also reported on the order of organic acid production when the two carbon sources were used. For sucrose-containing media, the order is as follows: "citric > gluconic > malic > oxalic acids." For glucose-containing media, the order is as follows: "citric > gluconic > oxalic > malic acids." This demonstrates

why sucrose was the best carbon source for bioleaching by *A. flavus* and why glucose was the best carbon source for *A. niger*. This also goes on to show that the diversity of fungi can even be observed within the same genera. Thus, the importance or need for optimization studies on fungal organisms with bioleaching potential.

2.5.3 Metal toxicity

The process of bioleaching is considered extreme to the microbial community involved due to the presence of metals which results in metal toxicity that negatively impacts the efficiency of bioleaching by fungi on a cellular, biochemical and molecular levels (Priyadarshini *et al.*, 2021). Although filamentous fungi are known for their resilience and tolerance to various environmental stressors, the bioleaching process could still limit their activity. As metals are extracted from ore/tailings/electronic waste (e-waste) during bioleaching, their concentration in the leached solution rises. For instance, in a study by Zhang *et al.* (2023) biomass of *A. niger* showed no difference when grown in Pb of up to 500 mg/l, however as concentration was increased a decrease in biomass was observed. When the same organism was grown in the presence of copper, an increase from 0-50 mg/l inhibited cell growth. The death of a cell comes through a number of ways, but the production reactive oxygen species (ROS) is the prime reason for heavy metal induced cell death. In fungi, metals like Cu, iron (Fe), and Cd are known to trigger a defence mechanism that results in the production of an excessive amount of reactive oxygen species (ROS). Superoxide (O_2^-), hydrogen peroxides (H_2O_2) and hydroxyl radicals (OH^-) are examples of ROS (Wang & Jiao, 2000). Although these ROS are produced in fungi as part of normal cellular metabolism, the presence of metals creates stressful conditions and ROS production increases excessively. Cell death due to increased production of ROS results from ROS damaging fungal cell structures (through lipid peroxidation) and altering metabolism (Shahid *et al.*, 2014). Furthermore, excessive accumulation of metals in fungal cells damages nucleic acid and proteins, preventing cell division (Priyadarshini *et al.*, 2021). On a biochemical or metabolic level, in which bioleaching depends, enzymatic activities are inhibited. According to Burgstaller & Schinner (1993), high metal concentrations in bioleached solutions have been shown to inhibit the active sites of enzymes. Another method in which metal toxicity impacts enzymatic activity of fungi during bioleaching is through essential metals displacement (Burgstaller & Schinner, 1993). It is important to consider that the exact effects and degree of influence on growth and activity are determined by factors such as the type of metal, concentration as well as exposure time (Lobos *et al.*, 2021; Aung & Ting, 2005). Fungi have developed a variety of efficient resistance detoxification mechanisms for them to

survive such extreme conditions. This includes detoxification mechanisms induced by environmental conditions, such as metallothionein production and generation of antioxidant enzymes, as well as those induced genetically through mutation (Lobos *et al.,* 2021; Bazzicalupo *et al.,* 2020; Azevedo *et al.,* 2007) some which will be discussed in the next section.

2.6 Optimization of fungal bioleaching: adaptation studies

Metal toxicity can significantly inhibit fungal growth and activity, consequently disrupting bioleaching efficiency. Understanding the extent of this damage is crucial for identifying and utilizing metal-resistant/tolerant fungal isolates, which are essential for optimizing bioleaching processes. One way of archiving this is by isolating potential bioleaching fungi from extreme environments characterized by high metal content such as tailing, acid mine drainage or even polluted soils (Din *et al.,* 2020; Cecchi *et al.,* 2019; Chiacchiarini *et al.,* 2010). Even though the organisms are obtained from extreme environment, their metal tolerance still need to be verified and further subjected to a series of metal adaptation (pre-exposure to metals) where the organisms are grown in an increasing concentration of metal of interest to obtain the highest tolerance. For instance, it was determined by Yang *et al.* (2009) that Al and Fe significantly inhibited the growth of two un-adapted *A. niger* strains (AS 3.879 and AS 3.40). However, after six months of a series of metal exposure, the two strains could tolerate up to 3500 mg/l Al and 700 mg/l Fe. Another study by Amiri *et al.* (2011), highlighted another important feature of bioleaching. They used *Pnenicillium simplicissimum* (*P. simplicissimum*) which was able to tolerate up to 8000 mg/l, 1500 mg/l, 3000 mg/l and 8000 mg/l of Mo, Ni, Fe, and tungsten (W) respectively when the metals were tested separately. However, when the metals were combined, to test for multi metal tolerance, *P. simplicissimum* could tolerate up to 200 mg/l, 300 mg/l, 150 mg/l, and 2500 mg/l of Mo, Ni, Fe and W respectively. This highlights another important feature with regards to metal toxicity which according to Anahid *et al.* (2011) and Le *et al.* (2006) multi-metals impose a greater toxicity than single metals to fungal growth. Low grade ore such as tailings are known for their polymetallic characteristics and one of the advantages of bioleaching is its ability to leach more than one metal. Thus, multi metal tolerance adaptation studies are very crucial. Once adaptation to metals has been induced, it means that the organism has developed/enhanced metal toxicity survival mechanisms. These mechanisms can be obtained through intrinsic properties, where processes such as metal ion flux, metal sequestration and compartmentalisation take place or through modification of toxicity where metal chelation and detoxification take place (Siddiquee *et al.,* 2015; Zouboulis

et al., 2004). As metal triggers excessive production of ROS, fungi also developed a mechanism that protects the cell against ROS activity by producing metallothionein (MTs) (Priyadarshini *et al.,* 2021). These are cysteine rich proteins produced as a defence mechanism against metal ions (Gardea-Torresdey *et al.,* 1998). MTs have high affinity for metal ions, thus they play a role in metal ions detoxification and sequestration. They prevent cellular related damage by binding to metal ions and preventing the interaction with cellular components (Chiaverini & De Ley, 2010; Cai *et al.,* 2005). Therefore, if the metal ions are sequestered, the cell will not produce excessive ROS. In addition to the storage of metal ions, MTs ensures that these metal ions, which some of them are essential, are available for cellular function (Maret, 2011, 2021). Further defence mechanisms against metal induced ROS cause fungi to possess efficient antioxidant system along with enzymes responsible for scavenging ROS (Tang *et al.,* 2021). Antioxidant function by neutralising ROS and an example is Glutathione (GSH) System (Pradedova *et al.,* 2011).

To determine if the organism has developed or enhanced its survival to metal toxicity, tolerance index (TI) is used, which describes the growth rate in the presence of metals as compared to the control growth rate in the absence of metals. The growth is measured after a number of days (5-21 day) and then used to calculate the tolerance index, which is normally interpreted by saying organisms with a tolerance index of or above 1 are tolerant, while below 1 means susceptible to the metal tested (Priyadarshini *et al.,* 2021; Anahid *et al.,* 2011; Valix *et al.,* 2001). Overall, studies from Naseri *et al.* (2023), Shah *et al.* (2020), and Bahaloo-Horeh *et al.* (2018), have shown the benefit of this adaptation process where pre-adapted *A. niger*, *P. citrinum* and *P. simplicissium* enhanced bioleaching efficiency. According to Priyadarshini *et al.* (2021) using tolerance index as a measure for tolerance may provide limited information since fungal growth rate is measured based on the extension of hyphal over incubation period. And suggested that quantification of biomass should be used along with tolerance index, which provides more information about the density of mycelia rather than just measuring the extension of mycelia. However, the tolerance index method is still popular especially for studies involving the screening for fungal isolates for metal tolerance (Lobos *et al.,* 2021; Anahid *et al.,* 2011; Valix & Loon, 2003; Valix *et al.,* 2001; Le *et al.,* 2006). One drawback of adaptation is that the process can be time consuming, especially when it comes to fungi whom their growth peak is normally reached after a number of days ranging from 5 -21 days.

2.7 Metal tolerance mechanism in fungi

In the previous sections, it is explained that metal ions are detoxified or sequestered or chelated through binding etc. However, what was left out is what really happens to the metal ions during those highlighted processes and this section will elaborate while focusing only on three important mechanisms. The first one is metal chelation where organic acid are involved because there is a link between metal tolerance and organic acid production. As much as organic acids are the main leaching agents for bioleaching, their production is also a defence mechanism against the presence of metals (Šebesta *et al.,* 2022). Thus, the reason why the presence of metals as described in section 2.2.1 induce the production of organic acids. Organic acids such as citric, oxalic, gluconic, and malic acids are effective metal chelators that efficiently bind to metals. This metal chelation ability that eventually result in metal dissolution is described in section 2.3 through acidolysis, complexolysis and redoxolysis. When it comes to metal ion detoxification or chelation, molecules such as metallothioneins, peroxidases as well as homogeneous and heterogeneous proteins are produced in addition to organic acids (Šebesta *et al.,* 2022; Geetha *et al.,* 2021; Cánovas *et al.,* 2004). They function by forming nontoxic complex of metals that are sequestered into different cellular organelles. The second and third mechanisms occur after metal dissolution by the action of organic acid has occurred. They require metal ions that are already in a soluble state and are described below.

- *Biosorption*

Once metals are dissolved, the fungal biomass adsorbs the metal ions in the leaching solution through a mechanism called biosorption. Which simply imply the biosorption of metal ions to the surface of fungal cells or biomass to minimise their damage to the cell and cellular activities. This process is made possible through van der Waal forces, covalent bonding, electrostatic interaction, or a combination (Ahalya *et al.,* 2003; Priyadarshini *et al.,* 2021). This take place on microbial surface, which is characterized by a net negative charge arising from the presence of molecules such as proteins and polysaccharides which have different functional groups that allows this interaction (Geetha *et al.,* 2021; Gadd, 2009). For example, if polysaccharides contain the hydroxyl (-OH) functional group, it can form hydrogen bonds with metal ions and if contain carboxyl group (-COOH), can ionise and generate negatively charged carboxylate ions that can form coordination bonds with metal ions (Samaddar *et al.,* 2019). If we look at the characteristics of fungal cell walls, especially those with metal tolerance, it is made up of over 80% polysaccharides (Priyadarshini *et al.,* 2021; Latha *et al.,* 2012). Thus, the reason fungi such as *Aspergillus* and *Penicillium* are reported for their efficiency in biosorption

(Priyadarshini *et al.,* 2021). Once metal ions are bound to the cell surfaces or extracellular matrix, they form complexes that are revisable and prevent metal ion uptake into the cytoplasm (Priyadarshini *et al.,* 2021). Though this process is important for fungal survival and activity under high metal ion concentration, it can also be regarded as a drawback for bioleaching efficiency. Thus, according to Dusengemungu *et al.* (2021) additional leaching process of metal ions from biosorption system should be evaluated to increase bioleaching efficiency.

- *Bioaccumulation and compartmentalization*

This process normally occurs after mechanisms such as acidolysis, complexolysis and redoxolysis have solubilized metals into metals ions. This differs from biosorption because no active uptake and metabolism of adsorbed metal ion by the fungal cells (Priyadarshini *et al.,* 2021). While in bioaccumulation, metal ion uptake and their intracellular accumulation occurs (Dusengemungu *et al.,* 2021), the uptake of metal ions involves an active transport system present in fungal cell membranes and energy is required for this process (Ahalya *et al.,* 2003). Once transported into the cytoplasm, the metal ions precipitated in cellular compartments such as organelles or vacuoles (Priyadarshini *et al.,* 2021). Metal ion bioaccumulation process was reported by Sedlakova-Kadukova *et al.* (2020), where a comparison of different bioleaching systems (bacteria, fungi, and yeast) for recovery of lithium (Li) was conducted. What was eminent was the difference between the bacterial bioleaching mechanisms as compared to fungi and yeast. Lithium (Li) accumulation was observed in fungi and yeast (*A. niger* and *Rodotorula mucilaginosa*), while no accumulation was observed in bacterial (*Acidithiobacillus ferrooxidans* and *Acidithiobacillus thiooxidans*) bioleaching. They concluded that bioleaching by *A. niger* and *R. Mucilaginosa* is a combination of two processes, metal solubilization which is followed by bioaccumulation of metal ions. They further added that additional processes such as thermal, chemical, or microbiological can be used to recover or extract Li from microbial biomass. Overall, the occurrence of both mechanisms within a single organism is possible. It was reported by Ezzouhri *et al.* (2010) that biosorption of Pb was followed by bioaccumulation of Pb as a way of resisting Pb toxicity by *Penicillium.*

2.8 Functional mechanisms for bioleaching optimization

Functional molecular methods employ techniques that confirm the production and activity of enzymes. Unlike metagenomic studies, such as targeted sequencing, which primarily focus on profiling the microbial community, functional studies aim to provide insights into the specific capabilities of the microbial community. While bioinformatics software packages like

PICRUSt exist to predict potential function from marker genes in metagenomes (Douglas *et al.,* 2020), experimental testing is crucial to validate these predictions. Therefore, functional-related studies are necessary to gain a deeper understanding of bioleaching at the functional level, ultimately this will have a positive effect towards bioleaching optimization. Thus far, it has been clearly elaborated that key component of bioleaching is organic acids, which their production may be affected by factors such as pH, metal toxicity, carbon sources and other parameters. Thus, in understanding transcriptome of fungi during bioleaching experiments, optimization should be done under a combination of parameter reported to increase bioleaching efficiency.

- *Identification and overexpression of key genes*

The importance of monitoring transcriptomes during organic acid production aids in identification of up-regulated genes. These genes can be cloned, identified, and characterized for function. The next step from here will be to overexpress these genes in a suitable organism. This has been accomplished in a study by Kobayashi *et al.* (2014). Oxaloacetate hydrolase gene (*oahA*), a single gene encoding oxaloacetate hydrolase (OAH; EC 3.7.1.1), was overexpressed in citric acid producing *A. niger*. They isolated, cloned, and inserted the *oahA* gene in a constructed plasmid. The plasmid was then used to transform a citric acid producing *A. niger* WU-2223L. The over expression of oxalic acid was successful because when the transformed organism was grown in glucose containing media under favorable growth conditions, 28.9 g/l oxalic acid was produced as compared to 15.6 g/l oxalic acid produced in a non-transformed citric producing *A. niger*.

Another study applied the logic of growth morphology to influence citric acid production by *A. niger*. From literature there are various morphological characteristics observed in fungi, however, for submerged fermentation (such as lab scale bioleaching process), pellet morphology is common (Gibbs *et al.,* 2000; Cui *et al.,* 1998; Pirt, 1966). Fungal pellet morphology can be described as aggregation of fungal cells forming a compact spherical structure, which may limit mass transfer and oxygen penetration (Veiter *et al.,* 2018; Hille *et al.,* 2005). Zheng *et al.* (2022) disrupted and conditionally expressed protein kinase A (PKA), a signaling cascade that is involved in controlling cell division and growth in fungi, which is also responsible for carbon sensing (nutrient sensing). They did this by using a gene encoding the catalytic subunit of PKA called *pkaC.* When the *pkaC* gene was overexpressed an increase of intracellular and extracellular citric acid was observed, while most intracellular intermediate

of TCA decreased. Overall, an improvement of up to 1.87-fold citric acid titres was reached by modifying the hyphal growth at pellet surface to enable better mass transfer of nutrients, oxygen as well as secreted products.

In an attempt to provide a better understanding of physiological response to metal using morphological, physico-chemical and transcriptomics analysis, Zhang *et al.* (2023) subjected *A niger* to Pb, Cd and Cu stress. They established that this organism could tolerate more Pb than Cu and Cd. They linked the tolerance to Pb to the significant upregulation of the gene encoding for cell wall α-1.3-glucan synthase, while under Cu and Cd the gene was not differentially expressed. The function of cell wall α-1.3-glucan synthase is to reinforce cell wall (through polysaccharide production) to provide mechanical strength and protection against metal stress (Zhang *et al.* 2023). On metabolic function, production of oxalic acid was not affected by up to 500 mg/l of Pb, but a reduction was observed when Pb concentration was increased to 1000 mg/l Pb. They also observed that the presence of Cu or Cd significantly led to a decline in oxalic acid. Furthermore, when they investigated the stress response level, genes encoding AAA family ATPase and efflux pump antibiotic resistance protein were not affected in Pb stress, but down regulated in Cu and Cd. Pump efflux proteins are responsible for removal of toxic metals, while ATPases are crucial for energy production, important for cellular activities (Zhang et al. 2023). Overall, Pb stress did not inhibit metal detoxification processes while Cu and Cd did. The application of transcriptomics in this study was able to identify important genes for metal tolerance in *A. niger* and showed how the same organism differ in terms of metal toxicity exposure. This information can be used to optimize bioleaching by this organism. It is clear that this organism will be efficient in bioleaching of Pb than Cu and Cd.

2.9 Exploring metabolomics for insights into bioleaching processes

Metabolomics is the study of metabolites that are present in biological systems as a result of metabolic activity. Applying metabolomics methods in bioleaching may provide a better knowledge through the detection of variety of small molecules generated either as a defence mechanism or connected to bioleaching effectiveness (Brisson *et al.*, 2020). As mentioned in section 2.2.1, metabolic pathways of organic acid production depend on a number of factors which also differ based on variation observed within the same genera. Thus, identifying key organic acid production pathway in bioleaching fungi is important and such a study has been conducted by Deng *et al.* (2019). Their study characterized metabolites of *P. chrysogenum* during bioleaching of metals found in contaminated soil. Key metabolic pathways were observed. This includes the upregulation of glycolysis and the tricarboxylic acid as the main

catabolic pathways for glucose metabolism. At the end it was observed that the organic acids from TCA, mainly citric acid, was responsible for leaching of metals. However, when they analyzed metabolites related to Glutathione (GSH), they found that GSH was down regulated. GSH is responsible for removing ROS to ensure proper cell function. This makes sense, since up regulation of GSH in relation to its function and the reason that the cell is exposed to metals would mean GSH accumulation. However, this down regulation implies that biosynthesized GSH is consumed while combating the stress imposed by the presence of Pb ions (Xu *et al.,* 2014).

2.10 References

Acheampong, M. A., Meulepas, R. J. W., & Lens, P. N. L. (2010). Removal of heavymetals and cyanide from gold mine wastewater. *Journal of Chemical Technology and Biotechnology*, *85*(5), 590–613. https://doi.org/10.1002/jctb.2358

Adetunji, A. I., Oberholster, P. J., & Erasmus, M. (2023). Bioleaching of Metals from E-Waste Using Microorganisms: A Review. *Minerals*, *13*(6), 828. https://doi.org/10.3390/min13060828

Ahalya, N., Ramachandra, T. V, & Kanamadi2, R. D. (2003). Biosorption of Heavy Metals. In *Research Journal of Chemistry and Environment* (Vol. 7).

Alavi, N., Partovi, K., Majlessi, M., Rashidi, M., & Alimohammadi, M. (2021). Bioleaching of metals from cellphones batteries by a co-fungus medium in presence of carbon materials. *Bioresource Technology Reports*, *15*. https://doi.org/10.1016/j.biteb.2021.100768

Amiri, F., Mousavi, S. M., & Yaghmaei, S. (2011). Enhancement of bioleaching of a spent Ni/Mo hydroprocessing catalyst by Penicillium simplicissimum. *Separation and Purification Technology*, *80*(3), 566–576. https://doi.org/10.1016/j.seppur.2011.06.012

Amiri, F., Yaghmaei, S., & Mousavi, S. M. (2010). Comparison of different methods in bioleaching of tungsten- rich spent hydro-cracking catalyst using adapted Penecillum simplicissimum BBRC-20019. *Chemical Engineering Transactions*, *21*, 1483–1488. https://doi.org/10.3303/CET1021248

Anahid, S., Yaghmaei, S., & Ghobadinejad, Z. (2011). Heavy metal tolerance of fungi. *Scientia Iranica*, *18*(3 C), 502–508. https://doi.org/10.1016/j.scient.2011.05.015

Andrews, W. J., Moreno, C. J. G., & Nairn, R. W. (2013). *Potential recovery of aluminum , titanium , lead , and zinc from tailings in the abandoned Picher mining district of Oklahoma*. 61–69. https://doi.org/10.1007/s13563-013-0031-7

Andrino, A., Guggenberger, G., Kernchen, S., Mikutta, R., Sauheitl, L., & Boy, J. (2021). Production of Organic Acids by Arbuscular Mycorrhizal Fungi and Their Contribution in the Mobilization of Phosphorus Bound to Iron Oxides. *Frontiers in Plant Science*, *12*. https://doi.org/10.3389/fpls.2021.661842

Arshadi, M., Esmaeili, A., & Yaghmaei, S. (2020). Investigating critical parameters for bioremoval of heavy metals from computer printed circuit boards using the fungus Aspergillus niger. *Hydrometallurgy*, *197*. https://doi.org/10.1016/j.hydromet.2020.105464

Arshadi, M., Esmaeili, A., Yaghmaei, S., & Arab, B. (2022). Evaluation of Aspergillus niger and Penicillium simplicissimum for their ability to leach Zn–Ni–Cu from waste mobile phone printed circuit boards. *Journal of Material Cycles and Waste Management*, *24*(1), 83–96. https://doi.org/10.1007/s10163-021-01299-0

Astuti, W., Hirajima, T., Sasaki, K., & Okibe, N. (2016). Comparison of effectiveness of citric acid and other acids in leaching of low-grade Indonesian saprolitic ores. *Minerals Engineering*, *85*, 1–16. https://doi.org/10.1016/J.MINENG.2015.10.001

Aung, K. M. M., & Ting, Y. P. (2005). Bioleaching of spent fluid catalytic cracking catalyst using Aspergillus niger. *Journal of Biotechnology*, *116*(2), 159–170. https://doi.org/10.1016/j.jbiotec.2004.10.008

Azevedo, M. M., Carvalho, A., Pascoal, C., Rodrigues, F., & Cássio, F. (2007). Responses of antioxidant defenses to Cu and Zn stress in two aquatic fungi. *Science of the Total Environment*, *377*(2–3), 233–243. https://doi.org/10.1016/j.scitotenv.2007.02.027

Babu, A. G., Shim, J., Bang, K. S., Shea, P. J., & Oh, B. T. (2014). Trichoderma virens PDR-28: A heavy metal-tolerant and plant growth-promoting fungus for remediation and bioenergy crop production on mine tailing soil. *Journal of Environmental Management*, *132*, 129–134. https://doi.org/10.1016/j.jenvman.2013.10.009

Bahaloo-Horeh, N., Mousavi, S. M., & Baniasadi, M. (2018). Use of adapted metal tolerant Aspergillus niger to enhance bioleaching efficiency of valuable metals from spent lithium-ion mobile phone batteries. *Journal of Cleaner Production*, *197*, 1546–1557. https://doi.org/10.1016/j.jclepro.2018.06.299

Baniasadi, M., Vakilchap, F., Bahaloo-Horeh, N., Mousavi, S. M., & Farnaud, S. (2019). Advances in bioleaching as a sustainable method for metal recovery from e-waste: A review. In *Journal of Industrial and Engineering Chemistry* (Vol. 76, pp. 75–90). Korean Society of Industrial Engineering Chemistry. https://doi.org/10.1016/j.jiec.2019.03.047

Barnett, M. J., Palumbo-Roe, B., Deady, E. A., & Gregory, S. P. (2020). Comparison of three approaches for bioleaching of rare earth elements from bauxite. *Minerals*, *10*(8), 1–19. https://doi.org/10.3390/min10080649

Barratt, R. W., Johnson, G. B., & Ogata, W. N. (1965). Wild-type and mutant stocks of Aspergillus nidulans. *Genetics*, *52*(1), 233–246. https://doi.org/10.1093/genetics/52.1.233

Bazzicalupo, A. L., Ruytinx, J., Ke, Y. H., Coninx, L., Colpaert, J. V., Nguyen, N. H., Vilgalys, R., & Branco, S. (2020). Fungal heavy metal adaptation through single nucleotide polymorphisms and copy-number variation. *Molecular Ecology*, *29*(21), 4157–4169. https://doi.org/10.1111/mec.15618

Behera, B. C. (2020). Citric acid from Aspergillus niger: a comprehensive overview. In *Critical Reviews in Microbiology* (Vol. 46, Issue 6, pp. 727–749). Taylor and Francis Ltd. https://doi.org/10.1080/1040841X.2020.1828815

Bell, L. C. (2001). Establishment of native ecosystems after mining-Australian experience across diverse biogeographic zones. In *Ecological Engineering* (Vol. 17). www.elsevier.com/locate/ecoleng

Bian, Z., Miao, X., Lei, S., Chen, S. E., Wang, W., & Struthers, S. (2012). The challenges of reusing mining and mineral-processing wastes. *Science*, *337*(6095), 702–703. https://doi.org/10.1126/science.1224757

Borja, D., Nguyen, K., Silva, R., Park, J., Gupta, V., Han, Y., Lee, Y., & Kim, H. (2016). Experiences and Future Challenges of Bioleaching Research in South Korea. *Minerals*, *6*(4), 128. https://doi.org/10.3390/min6040128

Bosecker, K. (1997). Bioleaching: metal solubilization by microorganisms. *FEMS Microbiology Reviews*, *20*(3–4), 591–604. https://doi.org/10.1111/j.1574-6976.1997.tb00340.x

Brandl, H., & Faramarzi, M. A. (2006). Microbe-metal-interactions for the biotechnological treatment of metal-containing solid waste. in *china particuology* (Vol. 4, Issue 2).

Brisson, V. L., Zhuang, W. Q., & Alvarez-Cohen, L. (2020). Metabolomic Analysis Reveals Contributions of Citric and Citramalic Acids to Rare Earth Bioleaching by a Paecilomyces Fungus. *Frontiers in Microbiology*, *10*. https://doi.org/10.3389/fmicb.2019.03008

Burgstaller, W., & Schinner, F. (1993). Leaching of metals with fungi. In *Journal of Biotechnology* (Vol. 27).

Cai, L., Li, X.-K., Song, Y., & Cherian, M. G. (2005). Essentiality, Toxicology and Chelation Therapy of Zinc and Copper. In *Current Medicinal Chemistry* (Vol. 12).

Cánovas, D., Vooijs, R., Schat, H., & De Lorenzo, V. (2004). The role of thiol species in the hypertolerance of Aspergillus sp. P37 to arsenic. *Journal of Biological Chemistry*, *279*(49), 51234–51240. https://doi.org/10.1074/jbc.M408622200

Castro, L., Blázquez, M. L., González, F., & Muñoz, J. A. (2020). Bioleaching of phosphate minerals using aspergillus niger: Recovery of copper and rare earth elements. *Metals*, *10*(7), 1–13. https://doi.org/10.3390/met10070978

Cecchi, G., Ceci, A., Marescotti, P., Persiani, A. M., Di Piazza, S., & Zotti, M. (2019). Interactions among microfungi and pyrite-chalcopyrite mineralizations: tolerance, mineral bioleaching, and metal bioaccumulation. *Mycological Progress*, *18*(3), 415–423. https://doi.org/10.1007/s11557-018-01466-y

Chaerun, S. K., Sulistyo, R. S., Minwal, W. P., & Mubarok, M. Z. (2017). Indirect bioleaching of low-grade nickel limonite and saprolite ores using fungal metabolic organic acids generated by Aspergillus niger. *Hydrometallurgy*, *174*, 29–37. https://doi.org/10.1016/j.hydromet.2017.08.006

Chen, H., Yan, X., Du, G., Guo, Q., Shi, Y., Chang, J., Wang, X., Yuan, Y., & Yue, T. (2021). Recent developments in antifungal lactic acid bacteria: Application, screening methods, separation, purification of antifungal compounds and antifungal mechanisms. In *Critical Reviews in Food Science and Nutrition*. Taylor and Francis Ltd. https://doi.org/10.1080/10408398.2021.1977610

Chi, Z., Kong, C. C., Wang, Z. Z., Wang, Z., Liu, G. L., Hu, Z., & Chi, Z. M. (2022). The signaling pathways involved in metabolic regulation and stress responses of the yeast-like fungi Aureobasidium spp. In *Biotechnology Advances* (Vol. 55). Elsevier Inc. https://doi.org/10.1016/j.biotechadv.2021.107898

Chiacchiarini, P., Lavalle, L., Giaveno, A., & Donati, E. (2010). First assessment of acidophilic microorganisms from geothermal Copahue-Caviahue system. *Hydrometallurgy*, *104*(3–4), 334–341. https://doi.org/10.1016/j.hydromet.2010.02.020

Chiaverini, N., & De Ley, M. (2010). Protective effect of metallothionein on oxidative stress-induced DNA damage. In *Free Radical Research* (Vol. 44, Issue 6, pp. 605–613). https://doi.org/10.3109/10715761003692511

Cho, K. H., Kim, H. S., Lee, C. G., Park, S. J., & Choi, N. C. (2023). A Comparative Study on Bioleaching Properties of Various Sulfide Minerals Using Acidiphilium cryptum. *Applied Sciences (Switzerland)*, *13*(10). https://doi.org/10.3390/app13105997

Clark, M. E., Batty, J. D., van Buuren, C. B., Dew, D. W., Eamon, M. A., Marhual, N. P., Pradhan, N., Kar, R. N., Sukla, L. B., Mishra, B. K., Citation, S., Amiri, F., Mousavi, S. M., Yaghmaei, S., Barati, M., Ballester, A., González, F., Blázquez, M. L., Mier, J. L., … Revitzer, H. (2019). Bioinformatic prediction of gene functions regulated by quorum sensing in the bioleaching bacterium Acidithiobacillus ferrooxidans. *Hydrometallurgy*, *9*(1–4), 187–199. https://doi.org/10.1016/j.biortech.2008.03.003

Cui, Y. Q., Okkerse, W. J., Van Der Lans, R. G. J. M., & Luyben, K. C. A. M. (1998). Modeling and measurements of fungal growth and morphology in submerged fermentations. *Biotechnology and Bioengineering*, *60*(2), 216–229. https://doi.org/10.1002/(SICI)1097-0290(19981020)60:2<216::AID-BIT9>3.0.CO;2-Q

Deng, X., Yang, Z., & Chen, R. (2019). Study of characteristics on metabolism of penicillium chrysogenum f1 during bioleaching of heavy metals from contaminated soil. *Canadian Journal of Microbiology*, *65*(9), 629–641. https://doi.org/10.1139/cjm-2018-0624

Din, G., Hassan, A., Rafiq, M., Hasan, F., Badshah, M., Khan, S., Chen, G., Ripp, S., & Shah, A. A. (2020). Characterization of Organic Acid Producing Aspergillus tubingensis FMS1 and its Role in Metals Leaching from Soil. *Geomicrobiology Journal*, *37*(4), 336–344. https://doi.org/10.1080/01490451.2019.1701585

Dold, B. (2008). Sustainability in metal mining: From exploration, over processing to mine waste management. *Reviews in Environmental Science and Biotechnology*, *7*(4 SPEC. ISS.), 275–285. https://doi.org/10.1007/s11157-008-9142-y

Douglas, G. M., Maffei, V. J., Zaneveld, J. R., Yurgel, S. N., Brown, J. R., Taylor, C. M., Huttenhower, C., & Langille, M. G. I. (2020). PICRUSt2 for prediction of metagenome functions. In *Nature Biotechnology* (Vol. 38, Issue 6, pp. 685–688). Nature Research. https://doi.org/10.1038/s41587-020-0548-6

Dusengemungu, L., Kasali, G., Gwanama, C., & Mubemba, B. (2021). Overview of fungal bioleaching of metals. *Environmental Advances*, *5*, 100083. https://doi.org/10.1016/j.envadv.2021.100083

Dutta, A., Sahoo, S., Mishra, R. R, Pradhan, B., Das, A., & Bikash Chandra Behera, B. C. (2019). A comparative study of citric acid production from different agro-industrial wastes by Aspergillus niger isolated from mangrove forest soil. *Environmental and Experimental Biology*, *17*(3). https://doi.org/10.22364/eeb.17.12

Ezzouhri, L., Ruiz, E., Castro, E., Moya, M., Espínola, F., Cherrat, L., Er-Raioui, H., Lairini, K., & Essaadi, A. B. (2010). *Mechanisms of lead uptake by fungal biomass isolated from heavy metals habitats*.

Faraji, F., Golmohammadzadeh, R., Rashchi, F., & Alimardani, N. (2018). Fungal bioleaching of WPCBs using Aspergillus niger: Observation, optimization and kinetics. *Journal of Environmental Management*, *217*, 775–787. https://doi.org/10.1016/j.jenvman.2018.04.043

Farkas, B., Bujdoš, M., Polák, F., Matulová, M., Cesnek, M., Duborská, E., Zvěřina, O., Kim, H., Danko, M., Kisová, Z., Matúš, P., & Urík, M. (2021). Bioleaching of manganese oxides at different oxidation states by filamentous fungus aspergillus niger. *Journal of Fungi*, *7*(10). https://doi.org/10.3390/jof7100808

Fathollahzadeh, H., Eksteen, J. J., Kaksonen, A. H., & Watkin, E. L. J. (2019). Role of microorganisms in bioleaching of rare earth elements from primary and secondary resources. In *Applied Microbiology and Biotechnology* (Vol. 103, Issue 3, pp. 1043–1057). Springer Verlag. https://doi.org/10.1007/s00253-018-9526-z

Gadd, G. M. (2009). Biosorption: Critical review of scientific rationale, environmental importance and significance for pollution treatment. In *Journal of Chemical Technology and Biotechnology* (Vol. 84, Issue 1, pp. 13–28). https://doi.org/10.1002/jctb.1999

Gan, C. dan, Yang, J. yan, Liu, R., Li, X. yu, & Tang, Q. xuan. (2022). Contrasted speciation distribution of toxic metal(loid)s and microbial community structure in vanadium-titanium magnetite tailings under dry and wet disposal methods. *Journal of Hazardous Materials*, *439*. https://doi.org/10.1016/j.jhazmat.2022.129624

Gao, H., Wang, Y., Luo, Q., Yang, L., He, X., Wu, J., Kachanuban, K., Wilaipun, P., Zhu, W., & Wang, Y. (2021). Bioactive Metabolites From Acid-Tolerant Fungi in a Thai Mangrove Sediment. *Frontiers in Microbiology*, *11*. https://doi.org/10.3389/fmicb.2020.609952

Gardea-Torresdey, J. L., Arenas, J. L., Francisco, N. M. C., Tiemann, K. J., & Webb, R. (1998). Ability of Immobilized Cyanobacteria to Remove Metal Ions From Solution and Demonstration of the Presence of Metallothionein Genes in Various Strains. *Journal of Hazardous Substance Research*, *1*(1). https://doi.org/10.4148/1090-7025.1001

Geetha, N., Bhavya, G., Abhijith, P., Shekhar, R., Dayananda, K., & Jogaiah, S. (2021). Insights into nanomycoremediation: Secretomics and mycogenic biopolymer nanocomposites for heavy

metal detoxification. *Journal of Hazardous Materials*, *409*. https://doi.org/10.1016/j.jhazmat.2020.124541

Gibbs, P. A., Seviour, R. J., & Schmid, F. (2000). Growth of filamentous fungi in submerged culture: Problems and possible solutions. In *Critical Reviews in Biotechnology* (Vol. 20, Issue 1, pp. 17–48). CRC Press LLC. https://doi.org/10.1080/07388550091144177

Gil-Loaiza, J., White, S. A., Root, R. A., Solís-Dominguez, F. A., Hammond, C. M., Chorover, J., & Maier, R. M. (2016). Phytostabilization of mine tailings using compost-assisted direct planting: Translating greenhouse results to the field. *Science of the Total Environment*, *565*, 451–461. https://doi.org/10.1016/j.scitotenv.2016.04.168

Guo, D., Hou, H., Long, J., Guo, X., & Xu, H. (2022). Underestimated environmental benefits of tailings resource utilization: Evidence from a life cycle perspective. *Environmental Impact Assessment Review*, *96*. https://doi.org/10.1016/j.eiar.2022.106832

Gupta, R., & Gupta, N. (2021). *Tricarboxylic acid cycle. Fundamentals of Bacterial Physiology and Metabolism*. Springer. https://doi.org/10.1007/978-981-16-0723-3_12

Han, Y., Joosten, H. J., Niu, W., Zhao, Z., Mariano, P. S., McCalman, M., Van Kan, J., Schaap, P. J., & Dunaway-Mariano, D. (2007). Oxaloacetate hydrolase, the C-C bond lyase of oxalate secreting fungi. *Journal of Biological Chemistry*, *282*(13), 9581–9590. https://doi.org/10.1074/jbc.M608961200

Hille, A., Neu, T. R., Hempel, D. C., & Horn, H. (2005). Oxygen profiles and biomass distribution in biopellets of Aspergillus niger. *Biotechnology and Bioengineering*, *92*(5), 614–623. https://doi.org/10.1002/bit.20628

Horeh, N. B., Mousavi, S. M., & Shojaosadati, S. A. (2016). Bioleaching of valuable metals from spent lithium-ion mobile phone batteries using Aspergillus Niger. *Journal of Power Sources*, *320*, 257–266. https://doi.org/10.1016/j.jpowsour.2016.04.104

Ilyas, S., Lee, J. C., & Kim, B. S. (2014). Bioremoval of heavy metals from recycling industry electronic waste by a consortium of moderate thermophiles: Process development and optimization. *Journal of Cleaner Production*, *70*, 194–202. https://doi.org/10.1016/j.jclepro.2014.02.019

Islam, M. M. (2008). *Bioleaching of refinery hydroproccessing Ni-Mo catalyst by heterotrophic fungi*.

Johnson, D. B. (2013). Development and application of biotechnologies in the metal mining industry. *Environmental Science and Pollution Research*, *20*(11), 7768–7776. https://doi.org/10.1007/s11356-013-1482-7

Karaca, O., Cameselle, C., & Reddy, K. R. (2018). Mine tailing disposal sites: contamination problems, remedial options and phytocaps for sustainable remediation. *Reviews in Environmental Science and Biotechnology*, *17*(1), 205–228. https://doi.org/10.1007/s11157-017-9453-y

Karaffa, L., Fekete, E., & Kubicek, C. P. (2021). The role of metal ions in fungal organic acid accumulation. In *Microorganisms* (Vol. 9, Issue 6). MDPI AG. https://doi.org/10.3390/microorganisms9061267

Keshavarz, S., Faraji, F., Rashchi, F., & Mokmeli, M. (2021). Bioleaching of manganese from a low-grade pyrolusite ore using Aspergillus niger: Process optimization and kinetic studies. *Journal of Environmental Management*, *285*. https://doi.org/10.1016/j.jenvman.2021.112153

Kirimura, K., Kobayashi, K., & Yoshioka, I. (2019). Decrease of citric acid produced by Aspergillus Niger through disruption of the gene encoding a putative mitochondrial citrate-oxoglutarate shuttle protein. *Bioscience, Biotechnology and Biochemistry*, *83*(8), 1538–1546. https://doi.org/10.1080/09168451.2019.1574205

Kobayashi, K., Hattori, T., Honda, Y., & Kirimura, K. (2014). Oxalic acid production by citric acid-producing Aspergillus niger overexpressing the oxaloacetate hydrolase gene oahA. *Journal of Industrial Microbiology and Biotechnology*, *41*(5), 749–756. https://doi.org/10.1007/s10295-014-1419-2

Kolo, M. T., Amin, Y. M., Khandaker, M. U., & Abdullah, W. H. B. (2017). Radionuclide concentrations and excess lifetime cancer risk due to gamma radioactivity in tailing enriched soil around Maiganga coal mine, Northeast Nigeria. *International Journal of Radiation Research*, *15*(1), 71–80. https://doi.org/10.18869/acadpub.ijrr.15.1.71

Kristiansen, B., Linden, J. , & Mattey, M. ,. (2002). *Citric Acid Biotechnology*, *CRC Press*.

Kržanović, D., Conić, V., Bugarin, D., Jovanović, I., & Božić, D. (2019). Maximizing economic performance in the mining industry by applying bioleaching technology for extraction of polymetallic mineral deposits. *Minerals*, *9*(7). https://doi.org/10.3390/min9070400

Kubicek, C. P., Punt, P., & Visser, J. (2011). *Production of Organic Acids by Filamentous Fungi*.

Kubicek, C. P., Schreferl-Kunar,'t Wilfried Wohrer, G., & Rohr2, M. (1988). Evidence for a Cytoplasmic Pathway of Oxalate Biosynthesis in Aspergillus niger. In *APPLIED AND ENVIRONMENTAL MICROBIOLOGY* (Vol. 54, Issue 3). https://journals.asm.org/journal/aem

Latha J.L, P. Naga Babu, P. Rakesh1, K., Ashok Kumar, M. Anupama, & and L. Susheela. (2012). *Fungal cell walls as protective barriers for toxic metals* (Berhardt Leon V, Ed.; pp. 181–198). Nova Science.

Le, L., Tang, J., Ryan, D., & Valix, M. (2006). Bioleaching nickel laterite ores using multi-metal tolerant Aspergillus foetidus organism. *Minerals Engineering*, *19*(12), 1259–1265. https://doi.org/10.1016/j.mineng.2006.02.006

Li, J., Chroumpi, T., Garrigues, S., Kun, R. S., Meng, J., Salazar-Cerezo, S., Aguilar-Pontes, M. V., Zhang, Y., Tejomurthula, S., Lipzen, A., Ng, V., Clendinen, C. S., Tolić, N., Grigoriev, I. V., Tsang, A., Mäkelä, M. R., Snel, B., Peng, M., & de Vries, R. P. (2022). The Sugar Metabolic Model of Aspergillus niger Can Only Be Reliably Transferred to Fungi of Its Phylum. *Journal of Fungi*, *8*(12), 1315. https://doi.org/10.3390/jof8121315

Li, J., Xu, T., Liu, J., Wen, J., & Gong, S. (2021b). Bioleaching metals from waste electrical and electronic equipment (WEEE) by Aspergillus niger: a review. In *Environmental Science and Pollution Research* (Vol. 28, Issue 33, pp. 44622–44637). Springer Science and Business Media Deutschland GmbH. https://doi.org/10.1007/s11356-021-15074-z

Li, Q., Luo, J., Xu, R., Yang, Y., Xu, B., Jiang, T., & Yin, H. (2021a). Synergistic enhancement effect of Ag+ and organic ligands on the bioleaching of arsenic-bearing gold concentrate. *Hydrometallurgy*, *204*. https://doi.org/10.1016/j.hydromet.2021.105723

Liapun, V., & Motola, M. (2023). Current overview and future perspective in fungal biorecovery of metals from secondary sources. In *Journal of Environmental Management* (Vol. 332). Academic Press. https://doi.org/10.1016/j.jenvman.2023.117345

Liu, Q., Bai, J. feng, Gu, W. hua, Peng, S. juan, Wang, L. cai, Wang, J. wei, & Li, H. xin. (2020). Leaching of copper from waste printed circuit boards using Phanerochaete chrysosporium fungi. *Hydrometallurgy*, *196*. https://doi.org/10.1016/j.hydromet.2020.105427

Liu, Q., Bai, J., Li, R., Gu, W., Peng, S., Wang, J., Tang, Z., & Yu, C. (2022). Electrochemical oxidation of copper-clad laminate for manufacturing printed circuit boards via bioleaching by

the fungus Phanerochaete chrysosporium. *Bioelectrochemistry*, *144*. https://doi.org/10.1016/j.bioelechem.2021.108002

Lobos, A., Harwood, V. J., Scott, K. M., & Cunningham, J. A. (2021). Tolerance of three fungal species to lithium and cobalt: Implications for bioleaching of spent rechargeable Li-ion batteries. *Journal of Applied Microbiology*, *131*(2), 743–755. https://doi.org/10.1111/jam.14947

Malekian, H., Salehi, M., & Biria, D. (2019). Investigation of platinum recovery from a spent refinery catalyst with a hybrid of oxalic acid produced by Aspergillus niger and mineral acids. *Waste Management*, *85*, 264–271. https://doi.org/10.1016/j.wasman.2018.12.045

Maret, W. (2011). Metals on the move: Zinc ions in cellular regulation and in the coordination dynamics of zinc proteins. *BioMetals*, *24*(3), 411–418. https://doi.org/10.1007/s10534-010-9406-1

Maret, W. (2021). An appraisal of the field of metallomics and the roles of metal ions in biochemistry and cell signaling. In *Applied Sciences (Switzerland)* (Vol. 11, Issue 22). MDPI. https://doi.org/10.3390/app112210846

Martínez P, Gálvez S, Ohtsuka N, Budinich M, Cortés MP, Serpell C, Nakahigashi K, Hirayama A, Tomita M, Soga T, Martínez S, Maass A, P. P. (2012). Metabolomic study of Chilean biomining bacteria Acidithiobacillus ferrooxidans strain Wenelen and Acidithiobacillus thiooxidans strain Licanantay. *Metabolomics*, *9*(1), 247–257. https://doi.org/10.1007/s11306-012-0443-3

Max, B., Salgado, J. M., Rodríguez, N., Cortés, S., Converti, A., & Manuel Domínguez, J. (2010). BIOTECHNOLOGICAL PRODUCTION OF CITRIC ACID. *Brazilian Journal of Microbiology*, *41*, 862–875.

Mendes, G. de O., Dyer, T., Csetenyi, L., & Gadd, G. M. (2022). Rock phosphate solubilization by abiotic and fungal-produced oxalic acid: reaction parameters and bioleaching potential. *Microbial Biotechnology*, *15*(4), 1189–1202. https://doi.org/10.1111/1751-7915.13792

Mileusnić, M., Mapani, B. S., Kamona, A. F., Ružičić, S., Mapaure, I., & Chimwamurombe, P. M. (2014). Assessment of agricultural soil contamination by potentially toxic metals dispersed from improperly disposed tailings, Kombat mine, Namibia. *Journal of Geochemical Exploration*, *144*(PC), 409–420. https://doi.org/10.1016/j.gexplo.2014.01.009

Moyé, J., Picard-Lesteven, T., Zouhri, L., El Amari, K., Hibti, M., & Benkaddour, A. (2017). Groundwater assessment and environmental impact in the abandoned mine of Kettara (Morocco). *Environmental Pollution*, *231*, 899–907. https://doi.org/10.1016/j.envpol.2017.07.044

Muddanna, M. H., & Baral, S. S. (2019a). A comparative study of the extraction of metals from the spent fluid catalytic cracking catalyst using chemical leaching and bioleaching by Aspergillus niger. *Journal of Environmental Chemical Engineering*, *7*(5). https://doi.org/10.1016/j.jece.2019.103335

Muddanna, M. H., & Baral, S. S. (2019b). Leaching of nickel and vanadium from the spent fluid catalytic cracking catalyst by reconnoitering the potential of Aspergillus niger associating with chemical leaching. *Journal of Environmental Chemical Engineering*, *7*(2). https://doi.org/10.1016/j.jece.2019.103025

Munir, E., Yoon, J. J., Tokimatsu, T., Hattori, T., & Shimada, M. (2001). *A physiological role for oxalic acid biosynthesis in the wood-rotting basidiomycete Fomitopsis palustris*. www.pnas.orgcgidoi10.1073pnas.191389598

Naseri, T., Mousavi, S. M., & Kuchta, K. (2023). Environmentally sustainable and cost-effective recycling of Mn-rich Li-ion cells waste: Effect of carbon sources on the leaching efficiency of metals using fungal metabolites. *Waste Management*, *157*, 47–59. https://doi.org/10.1016/j.wasman.2022.11.043

Naseri, T., Pourhossein, F., Mousavi, S. M., Kaksonen, A. H., & Kuchta, K. (2022). Manganese bioleaching: an emerging approach for manganese recovery from spent batteries. In *Reviews in Environmental Science and Biotechnology* (Vol. 21, Issue 2, pp. 447–468). Springer Science and Business Media B.V. https://doi.org/10.1007/s11157-022-09620-5

Nasab, H. M., Noaparast, M., Abdollahi, H., & Amoozegar, M. A. (2020). Indirect bioleaching of Co and Ni from iron rich laterite ore, using metabolic carboxylic acids generated by P. putida, P. koreensis, P. bilaji and A. niger. *Hydrometallurgy*, *193*. https://doi.org/10.1016/j.hydromet.2020.105309

Nason, P., Alakangas, L., & Öhlander, B. (2013). Using sewage sludge as a sealing layer to remediate sulphidic mine tailings: A pilot-scale experiment, northern Sweden. *Environmental Earth Sciences*, *70*(7), 3093–3105. https://doi.org/10.1007/s12665-013-2369-0

Netpae, T., & Suckley, S. (2020). Comparison of three culture media for one-step and two-step bioleaching of nickel and cadmium from spent Ni-Cd batteries by Aspergillus niger. *Advances in Environmental Technology*, *6*(3), 167–172. https://doi.org/10.22104/AET.2021.4759.1286

Nkuna, R., Ijoma, G. N., Matambo, T. S., & Chimwani, N. (2022). Accessing Metals from Low-Grade Ores and the Environmental Impact Considerations: A Review of the Perspectives of Conventional versus Bioleaching Strategies. In *Minerals* (Vol. 12, Issue 5). MDPI. https://doi.org/10.3390/min12050506

Ocampo-López, C., Ospina-Sanjuan, Á., Ramírez-Carmona, M., & Rendón-Castrillón, L. (2022). Development of a Model to Estimate the Thermodynamic Stability of Organic Substances in Leaching Processes. *Metals*, *12*(9). https://doi.org/10.3390/met12091424

Odoni, D. I., van Gaal, M. P., Schonewille, T., Tamayo-Ramos, J. A., Martins dos Santos, V. A. P., Suarez-Diez, M., & Schaap, P. J. (2017). Aspergillus niger secretes citrate to increase iron bioavailability. *Frontiers in Microbiology*, *8*(AUG). https://doi.org/10.3389/fmicb.2017.01424

Pathak, A., Kothari, R., Vinoba, M., Habibi, N., & Tyagi, V. V. (2021b). Fungal bioleaching of metals from refinery spent catalysts: A critical review of current research, challenges, and future directions. In *Journal of Environmental Management* (Vol. 280). Academic Press. https://doi.org/10.1016/j.jenvman.2020.111789

Pathak, A., Vinoba, M., & Kothari, R. (2021a). Emerging role of organic acids in leaching of valuable metals from refinery-spent hydroprocessing catalysts, and potential techno-economic challenges: A review. *Critical Reviews in Environmental Science and Technology*, *51*(1), 1–43. https://doi.org/10.1080/10643389.2019.1709399

Phillips, J. (2016). Climate change and surface mining: A review of environment-human interactions & their spatial dynamics. In *Applied Geography* (Vol. 74, pp. 95–108). Elsevier Ltd. https://doi.org/10.1016/j.apgeog.2016.07.001

Pirt, S. J. (1966). *A theory of the mode of growth of fungi in the form of pellets in submerged culture*. https://royalsocietypublishing.org/

Pradedova, E. V., Isheeva, O. D., & Salyaev, R. K. (2011). Classification of the antioxidant defense system as the ground for reasonable organization of experimental studies of the oxidative stress in plants. In *Russian Journal of Plant Physiology* (Vol. 58, Issue 2, pp. 210–217). https://doi.org/10.1134/S1021443711020166

Priester, M., Ericsson, M., Dolega, P., & Löf, O. (2019). Mineral grades: an important indicator for environmental impact of mineral exploitation. *Mineral Economics*, *32*(1), 49–73. https://doi.org/10.1007/s13563-018-00168-x

Priyadarshini, E., Priyadarshini, S. S., Cousins, B. G., & Pradhan, N. (2021). Metal-Fungus interaction: Review on cellular processes underlying heavy metal detoxification and synthesis of metal nanoparticles. In *Chemosphere* (Vol. 274). Elsevier Ltd. https://doi.org/10.1016/j.chemosphere.2021.129976

Qayyum, S., Meng, K., Pervez, S., Nawaz, F., & Peng, C. (2019). Optimization of pH, temperature and carbon source for bioleaching of heavy metals by Aspergillus flavus isolated from contaminated soil. *Main Group Metal Chemistry*, *42*(1), 1–7. https://doi.org/10.1515/mgmc-2018-0038

Qu, Y., Li, H., Shi, B., Gu, H., Yan, G., Liu, Z., & Luo, R. (2022). Bioleaching Performance of Titanium from Bauxite Residue Under a Continuous Mode Using Penicillium Tricolor. *Bulletin of Environmental Contamination and Toxicology*, *109*(1), 61–67. https://doi.org/10.1007/s00128-022-03518-2

Qu, Y., & Lian, B. (2013). Bioleaching of rare earth and radioactive elements from red mud using Penicillium tricolor RM-10. *Bioresource Technology*, *136*, 16–23. https://doi.org/10.1016/j.biortech.2013.03.070

Reed, D. W., Fujita, Y., Daubaras, D. L., Jiao, Y., & Thompson, V. S. (2016). Bioleaching of rare earth elements from waste phosphors and cracking catalysts. *Hydrometallurgy*, *166*, 34–40. https://doi.org/10.1016/j.hydromet.2016.08.006

Ren, W. X., Li, P. J., Geng, Y., & Li, X. J. (2009). Biological leaching of heavy metals from a contaminated soil by Aspergillus niger. *Journal of Hazardous Materials*, *167*(1–3), 164–169. https://doi.org/10.1016/j.jhazmat.2008.12.104

Richter, C., Kalka, H., Myers, E., Nicolai, J., & Märten, H. (2018). Constraints of bioleaching in in-situ recovery applications. *Hydrometallurgy*, *178*, 209–214. https://doi.org/10.1016/j.hydromet.2018.04.008

Roy, J. J., Cao, B., & Madhavi, S. (2021). A review on the recycling of spent lithium-ion batteries (LIBs) by the bioleaching approach. In *Chemosphere* (Vol. 282). Elsevier Ltd. https://doi.org/10.1016/j.chemosphere.2021.130944

Saim, A. K., & Darteh, F. K. (2023). A Comprehensive Review on Cobalt Bioleaching from Primary and Tailings Sources. In *Mineral Processing and Extractive Metallurgy Review*. Taylor and Francis Ltd. https://doi.org/10.1080/08827508.2023.2181346

Samaddar, P., Kumar, S., & Kim, K. H. (2019). Polymer Hydrogels and Their Applications Toward Sorptive Removal of Potential Aqueous Pollutants. In *Polymer Reviews* (Vol. 59, Issue 3, pp. 418–464). Taylor and Francis Inc. https://doi.org/10.1080/15583724.2018.1548477

Santhiya, D., & Ting, Y. P. (2005). Bioleaching of spent refinery processing catalyst using Aspergillus niger with high-yield oxalic acid. *Journal of Biotechnology*, *116*(2), 171–184. https://doi.org/10.1016/j.jbiotec.2004.10.011

Santhiya, D., & Ting, Y. P. (2006). Use of adapted Aspergillus niger in the bioleaching of spent refinery processing catalyst. *Journal of Biotechnology*, *121*(1), 62–74. https://doi.org/10.1016/j.jbiotec.2005.07.002

Schnürer, J., & Magnusson, J. (2005). Antifungal lactic acid bacteria as biopreservatives. *Trends in Food Science and Technology*, *16*(1–3), 70–78. https://doi.org/10.1016/j.tifs.2004.02.014

Šebesta, M., Vojtková, H., Cyprichová, V., Ingle, A. P., Urík, M., & Kolenčík, M. (2022). Mycosynthesis of Metal-Containing Nanoparticles—Fungal Metal Resistance and Mechanisms of Synthesis. In *International Journal of Molecular Sciences* (Vol. 23, Issue 22). MDPI. https://doi.org/10.3390/ijms232214084

Sedlakova-Kadukova, J., Marcincakova, R., Luptakova, A., Vojtko, M., Fujda, M., & Pristas, P. (2020). Comparison of three different bioleaching systems for Li recovery from lepidolite. *Scientific Reports*, *10*(1). https://doi.org/10.1038/s41598-020-71596-5

Seh-Bardan, B. J., Othman, R., Wahid, S. A., Husin, A., & Sadegh-Zadeh, F. (2012). Bioleaching of heavy metals from mine tailings by aspergillus fumigatus. *Bioremediation Journal*, *16*(2), 57–65. https://doi.org/10.1080/10889868.2012.665958

Sethurajan, M., & Gaydardzhiev, S. (2021). Bioprocessing of spent lithium ion batteries for critical metals recovery – A review. *Resources, Conservation and Recycling*, *165*. https://doi.org/10.1016/j.resconrec.2020.105225

Shah, S., Cesar Palmieri, M., Regina Pombeiro Sponchiado, S., & Bevilaqua, D. (2020). Enhanced bio-recovery of aluminum from low-grade bauxite using adapted fungal strains. *Brazilian*

Journal of Microbiology, *51*, 1909–1918. https://doi.org/10.1007/s42770-020-00342-w/Published

Shahid, M., Pourrut, B., Dumat, C., Nadeem, M., Aslam, M., & Pinelli, E. (2014). Heavy-metal-induced reactive oxygen species: Phytotoxicity and physicochemical changes in plants. In *Reviews of Environmental Contamination and Toxicology* (Vol. 232, pp. 1–44). Springer New York LLC. https://doi.org/10.1007/978-3-319-06746-9_1

Shengo, L. M. (2021). Review of Practices in the Managements of Mineral Wastes: The Case of Waste Rocks and Mine Tailings. In *Water, Air, and Soil Pollution* (Vol. 232, Issue 7). Springer Science and Business Media Deutschland GmbH. https://doi.org/10.1007/s11270-021-05198-w

Shivakumar, C. K., Thippeswamy, B., & Krishnappa, M. (2014). Studies on Heavy Metals Detoxification Biomarkers in Fungal Consortia. *Carib.j.SciTech*, *2*, 496–502. http://caribjscitech.com/

Siddiquee, S., Rovina, K., & Azad, S. Al. (2015). Heavy Metal Contaminants Removal from Wastewater Using the Potential Filamentous Fungi Biomass: A Review. *Journal of Microbial & Biochemical Technology*, *07*(06). https://doi.org/10.4172/1948-5948.1000243

Srichandan, H., Mohapatra, R. K., Parhi, P. K., & Mishra, S. (2019). Bioleaching approach for extraction of metal values from secondary solid wastes: A critical review. *Hydrometallurgy*, *189*. https://doi.org/10.1016/j.hydromet.2019.105122

Tang, X., Huang, Y., Li, Y., Wang, L., Pei, X., Zhou, D., He, P., & Hughes, S. S. (2021). Study on detoxification and removal mechanisms of hexavalent chromium by microorganisms. In *Ecotoxicology and Environmental Safety* (Vol. 208). Academic Press. https://doi.org/10.1016/j.ecoenv.2020.111699

Tansengco, M., Tejano, J., Coronado, F., Gacho, C., & Barcelo, J. (2018). Heavy metal tolerance and removal capacity of trichoderma species isolated from mine tailings in Itogon, Benguet. *Environment and Natural Resources Journal*, *16*(1), 39–57. https://doi.org/10.14456/ennrj.2018.5

Tezyapar Kara, I., Kremser, K., Wagland, S. T., & Coulon, F. (2023). Bioleaching metal-bearing wastes and by-products for resource recovery: a review. In *Environmental Chemistry Letters*.

Springer Science and Business Media Deutschland GmbH. https://doi.org/10.1007/s10311-023-01611-4

Valdés, J., Pedroso, I., Quatrini, R., Dodson, R. J., Tettelin, H., Blake, R., Eisen, J. A., & Holmes, D. S. (2008). Acidithiobacillus ferrooxidans metabolism: From genome sequence to industrial applications. *BMC Genomics*, *9*, 1–24. https://doi.org/10.1186/1471-2164-9-597

Valix, M., & Loon, L. O. (2003). Adaptive tolerance behaviour of fungi in heavy metals. *Minerals Engineering*, *16*(3), 193–198. https://doi.org/10.1016/S0892-6875(03)00004-9

Valix, M., Usai, F., & Malik, R. (2001). Fungal bio-leaching of low grade laterite ores. *Minerals Engineering*, *14*(2), 197–203. https://doi.org/10.1016/S0892-6875(00)00175-8

Vardanyan, A., Vardanyan, N., Abrahamyan, N., Aatach, M., & Gaydardzhiev, S. (2022). Sequential biologically assisted extraction of Cu and Zn from printed circuit boards (PCB). *International Journal of Environmental Studies*. https://doi.org/10.1080/00207233.2022.2126122

Veiter, L., Rajamanickam, V., & Herwig, C. (2018). The filamentous fungal pellet—relationship between morphology and productivity. In *Applied Microbiology and Biotechnology* (Vol. 102, Issue 7, pp. 2997–3006). Springer Verlag. https://doi.org/10.1007/s00253-018-8818-7

Wang, S. Y., & Jiao, H. (2000). Scavenging capacity of berry crops on superoxide radicals, hydrogen peroxide, hydroxyl radical's, and singlet oxygen. *Journal of Agricultural and Food Chemistry*, *48*(11), 5677–5684. https://doi.org/10.1021/jf000766i

Watling, H. R. (2014). Review of biohydrometallurgical metals extraction from polymetallic mineral resources. *Minerals*, *5*(1), 1–60. https://doi.org/10.3390/min5010001

Williams, S. T., & Mayfield, C. I. (1971). Studies on the ecology of actinomycetes in soil iii. the behaviour of neutrophilic streptomycetes in acid soil. In *Soil Biol. Biochem* (Vol. 3). Pergamon Press.

Wösten, H. A. B. (2019). Filamentous fungi for the production of enzymes, chemicals and materials. In *Current Opinion in Biotechnology* (Vol. 59, pp. 65–70). Elsevier Ltd. https://doi.org/10.1016/j.copbio.2019.02.010

Xu, D. M., Zhan, C. L., Liu, H. X., & Lin, H. Z. (2019). A critical review on environmental implications, recycling strategies, and ecological remediation for mine tailings. In

Environmental Science and Pollution Research (Vol. 26, Issue 35, pp. 35657–35669). Springer. https://doi.org/10.1007/s11356-019-06555-3

Xu, P., Liu, L., Zeng, G., Huang, D., Lai, C., Zhao, M., Huang, C., Li, N., Wei, Z., Wu, H., Zhang, C., Lai, M., & He, Y. (2014). Heavy metal-induced glutathione accumulation and its role in heavy metal detoxification in Phanerochaete chrysosporium. *Applied Microbiology and Biotechnology*, *98*(14), 6409–6418. https://doi.org/10.1007/s00253-014-5667-x

Yang, J., Wang, Q., Wang, Q., & Wu, T. (2008). Comparisons of one-step and two-step bioleaching for heavy metals removed from municipal solid waste incineration fly ash. *Environmental Engineering Science*, *25*(5), 783–789. https://doi.org/10.1089/ees.2007.0211

Yang, J., Wang, Q., Wang, Q., & Wu, T. (2009). Heavy metals extraction from municipal solid waste incineration fly ash using adapted metal tolerant Aspergillus niger. *Bioresource Technology*, *100*(1), 254–260. https://doi.org/10.1016/j.biortech.2008.05.026

Yang, Y., Ferrier, J., Csetenyi, L., & Gadd, G. M. (2019). Direct and Indirect Bioleaching of Cobalt from Low Grade Laterite and Pyritic Ores by Aspergillus niger. *Geomicrobiology Journal*, *36*(10), 940–949. https://doi.org/10.1080/01490451.2019.1654045

Yin, S., Wang, L., Kabwe, E., Chen, X., Yan, R., An, K., Zhang, L., Wu, A., Dusengemungu, L., Kasali, G., Gwanama, C., Mubemba, B., Debaraj Mishra, Dong-Jin Kim, Jong-Gwan Ahn, & Young-Ha Rhee. (2018). Copper bioleaching in China: Review and prospect. *Minerals*, *11*(2), 100083. https://doi.org/10.3390/min8020032

Yoshioka, I., Kobayashi, K., & Kirimura, K. (2020). Overexpression of the gene encoding alternative oxidase for enhanced glucose consumption in oxalic acid producing Aspergillus niger expressing oxaloacetate hydrolase gene. *Journal of Bioscience and Bioengineering*, *129*(2), 172–176. https://doi.org/10.1016/j.jbiosc.2019.08.014

Zhang, L., Yang, X., Li, S., Tang, L., Chen, T., Gu, T., Chen, G., Gadd, G. M., & Li, Z. (2023). A contrast of Pb(II), Cd(II), and Cu(II) toxicities to Aspergillus niger through biochemical, morphological, and genetic investigations. *Journal of Hazardous Materials*, *446*. https://doi.org/10.1016/j.jhazmat.2022.130691

Zheng, X., Cairns, T. C., Ni, X., Zhang, L., Zhai, H., Meyer, V., Zheng, P., & Sun, J. (2022). Comprehensively dissecting the hub regulation of PkaC on high-productivity and pellet

macromorphology in citric acid producing Aspergillus niger. *Microbial Biotechnology*, *15*(6), 1867–1882. https://doi.org/10.1111/1751-7915.14020

Zheng, X., Yu, J., Cairns, T. C., Zhang, L., & Zhang, Z. (2019). *Comprehensive Improvement of Sample Preparation Methodologies Facilitates Dynamic Metabolomics of Aspergillus niger*. *1800315*, 1–9. https://doi.org/10.1002/biot.201800315

Zouboulis, A. I., Loukidou, M. X., & Matis, K. A. (2004). Biosorption of toxic metals from aqueous solutions by bacteria strains isolated from metal-polluted soils. *Process Biochemistry*, *39*(8), 909–916. https://doi.org/10.1016/S0032-9592(03)00200-0

Zuo, J., Tang, J., Lu, M., Zhou, Z., Li, Y., Tian, H., Liu, E., Gao, B., Liu, T., & Shao, P. (2021). Glycolysis Rate-Limiting Enzymes: Novel Potential Regulators of Rheumatoid Arthritis Pathogenesis. In *Frontiers in Immunology* (Vol. 12). Frontiers Media S.A. https://doi.org/10.3389/fimmu.2021.779787

CHAPTER 3: MICROBIAL PROFILING OF GOLD MINE TAILINGS

Chapter objective

The purpose of this chapter was to profile the fungal communities found in tailing samples with the ultimate aim of understanding their bioleaching capability. However, obtaining pure genomic DNA from enriched tailings proved challenging due to the presence of metal ions, which are abundant in tailings. Therefore, in addition to profiling fungal communities and understanding their potential application in bioleaching, this chapter also reports on optimization strategy DNA isolation/extraction from samples rich in metal ions which can lead to DNA degradation and inhibition of downstream processes such as polymerase chain reaction (PCR). This chapter was published as indicated below (see Appendix A3.1).

Publication

Nkuna, R., Ijoma, G. N., & Matambo, T. S. (2022). Applying EDTA in Chelating Excess Metal Ions to Improve Downstream DNA Recovery from Mine Tailings for Long-Read Amplicon Sequencing of Acidophilic Fungi Communities. Journal of Fungi, 8(5), 419.

Abstract

The hostile environment of gold mine tailings contains unique microbial life capable of bioleaching. The metagenomic analysis of such an environment provides an in-depth understanding of the microbial life and its potential, especially in biomining operations. However, DNA recovery from samples collected in those environments is challenging due to the presence of metal ions that interfere with the DNA analysis. A varied concentration of EDTA (4–13 µg/µl) was used to chelate the metal ions of enriched tailing samples prior to DNA extraction. The results show that 9 µg/µl of EDTA was effective in most samples. However, the increasing concentration of EDTA negatively affected the DNA recovery. The sequencing of the successfully extracted DNA revealed a diverse range of fungal genera, some of which have not been previously reported in tailing or bioleaching applications. The dominant genera include *Fodinomyces*, *Penicillium*, *Recurvomuces*, *Trichoderma*, and *Xenoacremonium*; their traits were determined using the FungalTraits database. This study demonstrates the need to include a preliminary metal-chelating step using EDTA before DNA extractions for samples collected from metal-rich environments. It further showed the need for optimization but provided a benchmark range, particularly for tailings. However, we caution that a further EDTA removal step from the extracted DNA should be included to avoid its interferences in downstream applications.

Keywords: bioleaching; DNA extraction; ethylene diamine tetraacetic acid (EDTA); fungi; mine tailing

3.1 Introduction

Mine tailings sites frequently contain distinct and phylogenetically diverse microbial communities that can be exploited for their specific metal-extraction capabilities (bioleaching) (Vázquez-Campos *et al.,* 2014). Despite the presence of both chemolithotrophic prokaryotes and heterotroph eukaryotes in these environments, the research is focused more on the chemolithotrophic members of the community in comparison to the eukaryotic members (Liang *et al.,* 2020; Jia *et al.,* 2018). However, in recent years, the characteristics of the eukaryotic members, particularly their acid-tolerance, have piqued the interest of scholars and scientists in studying and applying them in bioleaching processes (Huerta-Rosas *et al.,* 2020). Depending on the research question, bioleaching research employs either a culture-dependent method or a culture-independent method or a combination of the two (Selbmann *et al.,* 2021). However, profiling such microbial communities relies on metagenomics approaches as they have been shown to be very useful in rapidly elucidating large sample areas. Metagenomic DNA isolated from tailing environments is a potential genetic resource from which the uncultured microbial species' phylogenetic affiliation can be determined, and their genetic potential can be explored by identifying novel genes with potential applications in bioleaching processes (Desai & Madamwar, 2007: Zhang *et al.,* 2016). Metagenomic approaches are crucial for unravelling the complexities of these communities, especially for unculturable microorganisms. Recent studies in similar environments showcase the power of these tools. For instance, Dusengemungu *et al*. (2024) successfully employed the DADA2 ITS pipeline workflow for analysing fungal diversity in metal-rich mine tailings, demonstrating its effectiveness for such samples.

The foundation of various biological applications in microbial profiling depends on accurate and reliable DNA analysis. Separating pure DNA from cell matrix or biological samples has proven to be extremely complicated and challenging in a complex environment (Liu *et al.,* 2019). Tailings are one of the most challenging environmental samples to recover pure DNA from (Desai & Madamwar, 2007). They have high concentrations of heavy metals, which are impurities in the form of metal ions that have an impact on downstream DNA applications (Bernardino *et al.*, 2019; Colin *et al.*, 2019). According to published research, these metal ions

can interfere with the DNA analysis at various stages, from extraction to polymerase chain reaction (PCR) (Kuffel *et al.,* 2021; Moreno & McCord, 2017; Desai & Madamwar, 2007).

Metal ions cause interferences during DNA analysis; they act as cofactors required to increase the activity of various enzymes/nucleases such as Deoxyribonuclease (DNase) (Guéroult *et al.*, 2010). DNase is an endonuclease that hydrolyses double-stranded DNA, and divalent metal ions such as magnesium (Mg^{2+}) and calcium (Ca^{2+}) increase its activity (Schrader *et al.,* 2012; Guéroult *et al.,* 2010; Dominguez & Ward, 2009; Melgar & Goldthwait, 1968). Although DNA is packaged in a nucleus and protected from nucleases, it is exposed to nucleases during DNA extraction (El-Ashram *et al.,* 2016). Metal ions in extracted DNA can also prevent successful PCR (Kuffel *et al.,* 2021); Ca^{2+}, for instance, is an inorganic substance known to have inhibitory effects on PCR (Schrader *et al.*, 2012).

Commercial extraction kits/protocols are designed to extract DNA in its purest form, removing most impurities derived from the biological sample content or cell matrix (Heikrujam *et al.*, 2020). To address metal ion removal or stability during extraction, lysis buffer contains chelating agents that are capable of stabilizing metal ions such as iron, magnesium, manganese, cobalt, zinc, lead, copper, calcium, etc. (Kuffel *et al.,* 2021; Bonsu *et al.,* 2020; Feng & van Deventer, 2010). Chelating agents include ethylene diamine tetraacetic acid (EDTA) and ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA) (Kuffel *et al.,* 2021; Heikrujam *et al.,* 2020). EDTA and EGTA have similar properties and can both chelate similar metals, such as Mg^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+} ions (Lopata *et al.,* 2019). However, the affinity of EDTA and EGTA for magnesium varies, with EDTA having a high affinity and EGTA having a low affinity (Portzehl *et al.,* 1964). EDTA chelates metal ions at a 1:1 ratio regardless of cation charge and forms stable chelating complexes with cations (Zaitoun & Lin, 1997).

The metal content of environmental samples and the success of DNA extraction protocols differ depending on the sample's chemical composition. According to Kuffel *et al.* (2021), the impact of metal ion interference on the recovery of DNA is determined by the concentration of the various metals involved. As a result, the challenges of successful DNA extraction or PCR are expected with tailing samples with their associated high concentration of metal ions.

Nearly all of the previous investigations relating to metal interference in DNA extraction can only be found in forensic science, with studies conducted on methods of optimizing DNA extraction and PCR processes in relation to metal ion interferences on blood, bone, and other

samples collected from crime scenes (Kuffel *et al.,* 2021; Moreno & McCord, 2017; Khosravinia & Ramesha, 2007). Their findings thus far suggest the pre-treatment of samples containing significantly higher concentrations of metal ions with chelating agents such as EDTA, EGTA, and CHELEX (a chelating agent manufactured by Bio-Rad laboratories) before extraction (Hall & Axelrod, 1977). This pre-treatment is an accepted protocol addition despite the included presence of chelating agents in all DNA extraction buffers found in commercial kits (Fortin *et al.,* 2007). However, it appears that the studies carried out investigating this likely interference for environmental samples, especially tailings, are few to non-existent.

EDTA was chosen as the chelating agent in this study because it has a higher affinity for chelating Mg^{2+} ions (cofactor for DNase) compared to EGTA and does not interfere with most chemicals used in standard buffers. Moreover, previous studies demonstrated that the chelating ability of EDTA is effective between 4–13 µg/µl (Khosravinia & Ramesha, 2007). Beyond this concentration, EDTA may affect DNA recovery negatively. In this study, EDTA (4–13 µg/µl) was used as a metal ion chelating agent in tailing samples to optimize DNA recovery and PCR, with the goal of profiling the potential bioleaching fungal communities present in the tailing samples.

In this study, DNA isolation from metal-rich tailing samples was optimized using EDTA, a cheating agent, with the objective of profiling and identifying fungal genera in mine tailing samples with bioleaching potential. Subsequently, the FungiTraits database was employed to assign ecological and functional information to the dominant fungal taxa. This knowledge will be instrumental in guiding future studies aimed at isolating fungal communities with bioleaching potential.

3.2 Methodology

3.2.1 Site description and soil sampling

Gold mine tailing samples were collected at three different tailing storage sites in Krugersdorp, Gauteng province, South Africa (mixed tailing and tailing A -26°07'58.9"S 27°42'42.2"E, tailing B - 26°07'42.2"S 27°41'44.6"E, and tailing C - 26°08'28.6"S 27°43'02.8"E). A diagonal sampling method was used (Tang *et al.,* 2021), and the samples were collected at various depths: depth 1: 0-15 cm, depth 2: 15-30 cm, and depth 3: 30-45 cm, while mixed tailings (mixed tailings before re-processing) were collected on the surface. Each depth's samples were combined (for instance, all 0-15 cm depth samples were combined to form one sample per sampling site), and from each tailing storage site, a total of three samples were obtained

resulting in a total of 12 samples (3 depths + 3 mixed tailings sample). All samples were collected using sterile zipper bags and transported to the university laboratory on ice, and upon arrival, the samples were stored at 4 °C until further analysis.

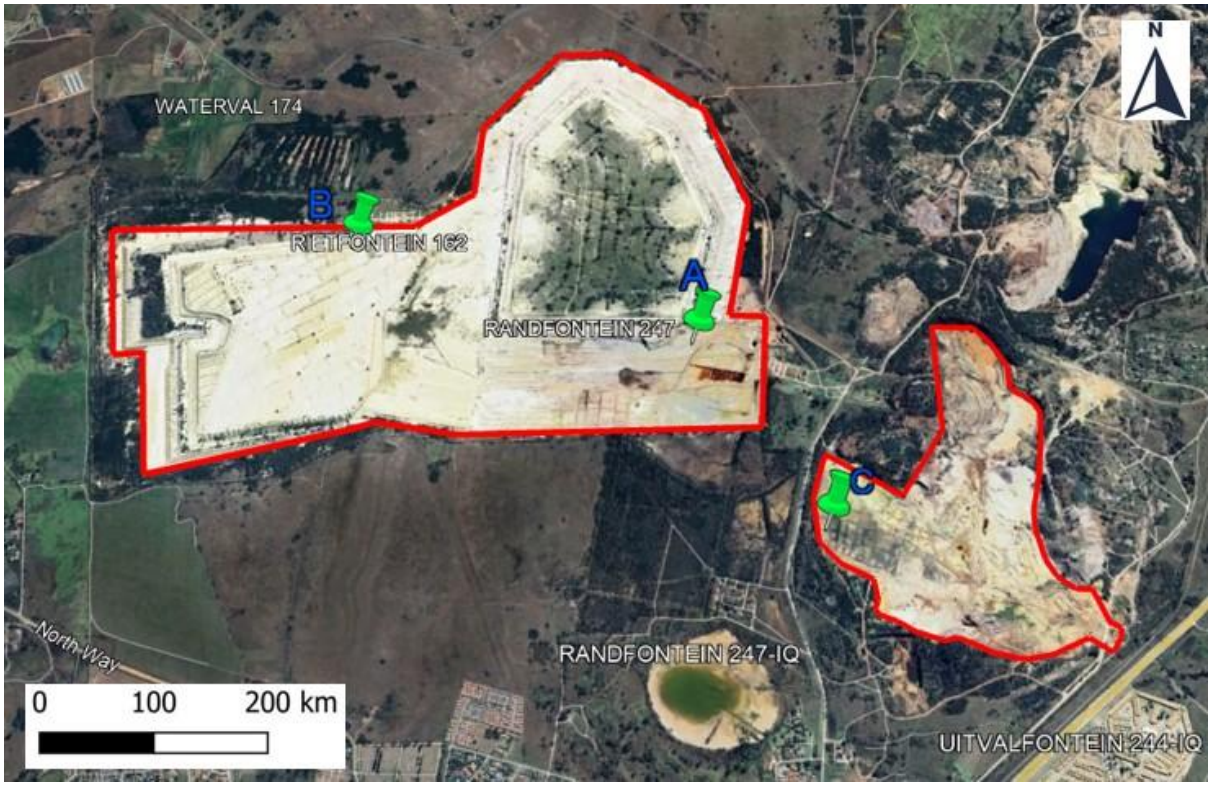

Figure 3.1: map showing the three sampling sites were tailing samples were collected in Krugersdorp, Gauteng province, South Africa.

3.2.2 Characterization of tailing samples

The pH of the tailings was determined by dissolving 1 g of tailings in 10 ml of dH_2O and allowing it to sit for an hour before taking pH measurements with an Eutech PH 1710 pH/mV bench meter (Inflitek). For heavy metal content analysis, 0.25 g samples were digested by mixing nitric acid (HNO_3) and hydrochloric acid (HCL) at a ratio of 3:1 in a microwave digester (CEM Mars-6using Xpress plus vessels, MAD TECHNOLOGY (PTY) LTD, Johannesburg, South Africa), heated to 180 °C, and held there for 10 min. The samples were then diluted up to 50 ml in falcon tubes. The metals in the solution were initially scanned, followed by quantification using inductively coupled plasma atomic emission spectroscopy (ICP-AES) (Shimadzu ICPE-9820, Gauteng, South Africa).

3.2.3 Microbial enrichment and DNA extraction

Two different selective media (Table 3.1) were used to enrich the fungal communities found in the tailing samples. The purpose of enrichment was to target the acidophilic/acid-tolerant communities with bioleaching potential and to increase their population for DNA isolation. The two media were prepared as follows:

Table 3.1. Media composition for enrichment of bioleaching microorganisms.

Solution	**Name**	**Composition**		
		General		
A	Basic bioleaching media composition	3.0 g/l $(NH_4)_2SO_4$, 0.5 g/l $MgSO_4 \cdot 7H_2O$, 0.5 g/l K_2HPO_4, 0.1 g/l KCl, and 1.5% Agar bacteriological		
B		44.2 g/l $FeSO_4 \cdot 7H_2O$ or 30 g/l sulfur		
	Compounds Different in Each Media		**pH**	**References**
A	Glucose yeast extract medium (GYEM)	5 g/l glucose, 0.05 g/l yeast extract	3	(Kishimoto & Tano, 1987)
A	Yeast sucrose media (YSM)	100 g/l sucrose, 1.5g/l $NaNO_3$, 1.6 g/l yeast extract	3	(Amiri *et al.*, 2011)

- Solution A contains all the compounds listed in Table 3.1 (for each media) except iron (II) sulfate or sulfur, which were sterilized by autoclaving at 112 °C for 30 min.
- Solution B contains either iron (II) sulfate (44.2 g/l in liquid media and 6 g/l on solid media, maintained at pH 2 to avoid oxidation) or sulfur (30 g/l in liquid media and 10 g/l) (Hassanshahian & Ghoebani, 2018; Martínez *et al.*, 2012). The solutions were sterilized by filtration using 0.2 µm sterile filters and added to solution A aseptically to prepare iron- or sulfur-containing media (Dong *et al.*, 2013).

For the initial enrichment, only solution A was prepared, in which 20 g of tailings was added to 250 ml of the two specific media in Duran Schott bottles. The contents of the tailings were used as an energy source. The samples were enriched for 12 days at 30 °C in a shaker incubator at 140 rpm (Labotec (PTY) LTD, Model 353, Gauteng, South Africa); this was referred to as S1. After 12 days, 20 ml was transferred to the fresh liquid media containing both solution A

and solution B (iron (II) sulfate heptahydrate served as the energy source) and incubated for a further 12 days; this was referred to as S2.

3.2.4 Optimization of DNA extraction

Several failed attempts at extracting DNA and running a successful PCR from the enriched tailing samples led to the assumption that metal ions may have been interfering with the DNA extraction process. To stabilize the metal ions in the samples, 10 ml of S1 and S2 samples was centrifuged at 14,000 rpm, and the supernatant was discarded. The pellet was resuspended with EDTA (4–13 µg/µl) and treated at room temperature for an hour on a rotating mixer at 60 rpm before extraction (Hall & Axelrod, 1977). The samples were further centrifuged to remove the EDTA and resuspended in 250 µl sterile dH_2O. The samples with no EDTA treatment were centrifuged and only resuspended in sterile dH_2O. DNA extractions were conducted using Zymo soil/faecal and BIOMICS DNA extraction kits (Zymo Research Corp, Irvine, California), according to the manufacturer's instructions. The ratio of absorbance at 260 and 280 nm was used to check the purity of the extracted DNA using a nanodrop (Mfg year 2016) and quantified using a QUBIT fluorometer (ThermoFisher, Edenvale, South Africa). To confirm the elimination of PCR inhibitors following EDTA treatment, the ITS region was amplified using ITS1 - 5′ CTTGGTCATTTAGAGGAAGTAA-3′ and ITS4 - 5′ CCTCCGCTTATTGATATGC-3′ primers (Adeleke *et al.,* 2010). The ITS gene was amplified in a 25 µl reaction containing DNA template, 5 µM of each primer, and 12.5 µl of Biolabs 2X Master mix (New England Biolabs). The PCR amplification was performed as follows: initial denaturation at 95 °C for 30 s, 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 1 min, extension at 68 °C for 1 min, and a final extension at 68 °C for 5 min. One percent agarose gel electrophoresis was used to view the amplified gene. The DNA samples treated with EDTA that resulted in a successful PCR amplification were submitted to Inqaba Biotechnical Industries (Pty) Ltd., Pretoria, South Africa for long amplicons sequencing with PacBio sequel II.

3.2.5 Bioinformatics and statistics

A total of 1,375,847 demultiplexed reads were obtained across 46 samples, with an average of 29,909 reads per sample. The DADA2 ITS pipeline workflow (Callahan *et al.,* 2020) with technology specific for PacBio (Callahan *et al.*, 2019) was used. The DADA2 R package v1.18.0 was installed on R studio. The sequences were quality filtered and denoised, and the chimeric sequences were removed (https://benjjneb.github.io/dada2/ITS_workflow.html). The

DADA2 default naïve Bayesian classifier (Wang *et al.,* 2007) was used to assign taxonomy to the amplicon sequence variants based on the UNITE database (Kõljalg *et al.*, 2013). The resulting OTU table with assigned taxonomy was exported to Excel for relative abundance plots at different levels. The alpha and beta diversities plots were generated using MicrobiomeAnalyst (a comprehensive statistical, visual, and meta-analysis of microbiome data) (Dhariwal *et al.,* 2017). The FungalTraits database was used to determine the dominant fungal taxa's ecological information and functional assignment (Tanunchai et al., 2022; Põlme et al., 2020). This database categorizes the functional assignment into various "traits", such as primary and secondary lifestyles, endophytic interaction capacity, aquatic habitat, and many others.

3.3 Results

3.3.1 Tailing characterization

Table 3.2 shows the pH measurements of the four different tailing samples, which ranged from 3.9 to 1.8 in all four tailing sites. The pH range in tailings A and B is the lowest. Figure 3.2 depicts the metal content analysis of the tailings at various depths using ICP-AES. The highest concentrations of iron and sulfur were observed in all the tailings, followed by aluminium in varying concentrations. The differences in the tailings revealed that tailings A and B had the highest concentrations of the three metals, with iron at 75.7 ppm and 87.3 ppm and sulfur at 88.1 ppm and 73.2 ppm, respectively. Rubidium and calcium were observed to be highest in tailing A only at 46.3 and 65.8 ppm, respectively. Rubidium (except in tailing A depth 1), Calcium (except in tailing A depth 1), potassium, and sodium were found in lower concentrations.

Table 3.2: pH measurements of the different tailing

Level	Mixed tailings	Tailing A	Tailing B	Tailing C
1-0-15 cm	3.27 ± 0.75	2.1 ± 0.017	3.0 ± 1.06	3.945 ± 0.02
2-15-30 cm	3.305 ± 0.46	1.8 ± 0.04	2.8 ± 1.12	3.7 ± 0.04
3-30-45 cm	3.25 ± 0.61	1.8 ± 0.005	2.8 ± 1.12	3.7 ± 0.01

*The total number of tailing samples is 12 (3 per sampling site + 3 for mixed tailing samples.
± - standard deviation

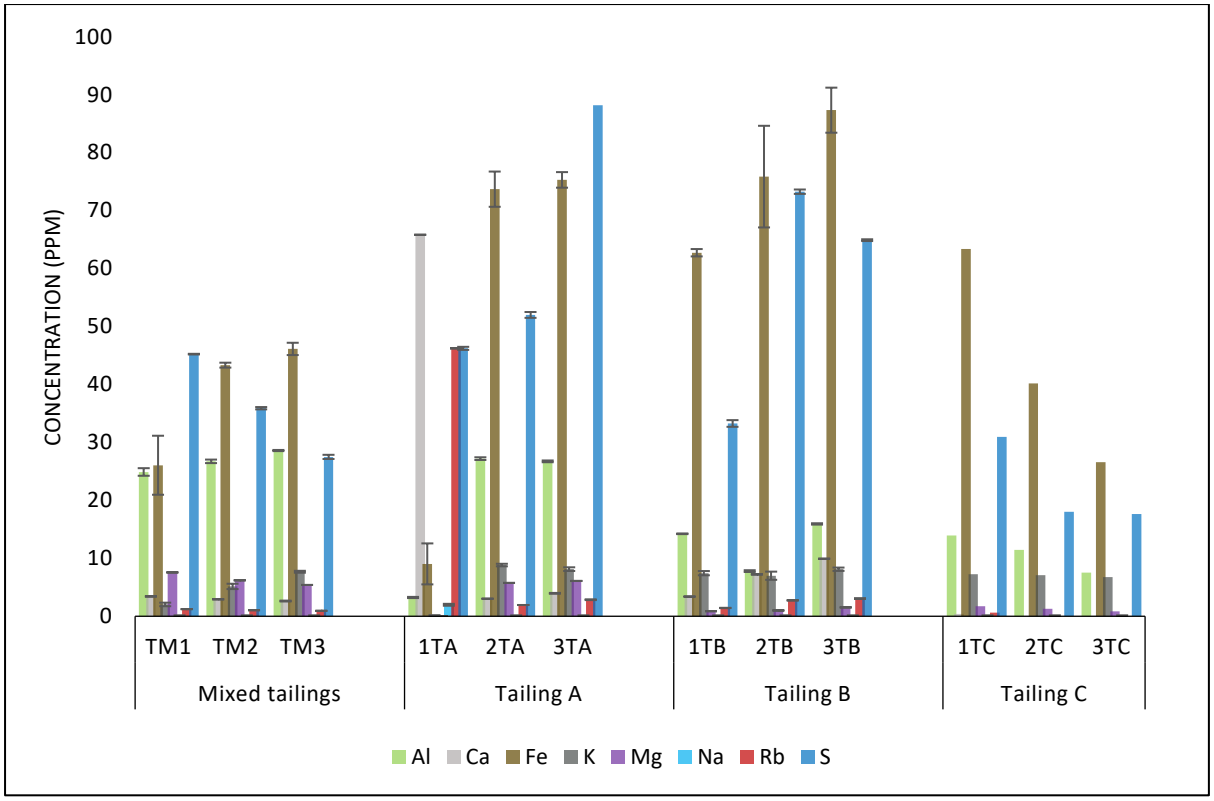

Figure 3.2: Heavy metal characterization of 4 different tailings at different levels. 1- 3 sampling depth, T- tailing, A-C - different tailings and M - mixed tailing

3.3.2 DNA optimization

An EDTA concentration of 9 µg/µl was found to be effective for metal ion chelation in S1 samples. This was confirmed by comparing the 260/280 ratio DNA concentration and the successful PCR amplification of the ITS1–ITS4 region in S1 samples from both enrichment media. The 260/280 ratio and the DNA concentration improved in the EDTA-treated samples (Table 3.3). The average ratio of the samples without EDTA treatment in GYEM and YSM was 1.68 and 1.18, respectively, and improved to 2.0 and 1.75, respectively, in the samples treated with EDTA. The DNA concentration increased from 1.31 ng/ml in GYEM and 0.35 ng/ml in YSM in the control samples to 74.4 ng/ml and 30.7 ng/ml, respectively, in the EDTA-treated samples. The PCR amplification of the ITS region was successful in all the EDTA-treated DNA samples (Appendix 3.2).

EDTA concentrations ranging from 9–13 µg/µl were used in the S2 samples. The average ratio of the samples without EDTA treatment in GYEM and YSM was 1.72 and 1.07, respectively, whereas the average ratio improvement in the samples treated with EDTA was 2.38 and 1.70, respectively. In GYEM, the average DNA concentration increased from 1.52 ng/ml in the

samples that were not treated with EDTA to 39.72 ng/ml in the samples that were treated with EDTA. In YSM S2, the samples without EDTA treatment yielded no DNA, whereas the samples with EDTA yielded 1.92 ng/ml on average. Amplification of the ITS region by PCR was mostly successful in the GYEM S2 samples treated with EDTA (Appendix A3.2).

Table 3.3: Effectiveness of EDTA as chelating agent based on DNA purity, concentration, and successful sequencing of ITS region. 1- 3 sampling depth, T- tailing, A-C – different tailings and M – mixed tailing.

Sample ID	**A260/A280**				**Fluorometer (ng/ml)**				**Reads count**	
	Glucose yeast exract media									
	S1		**S2**		**S1**		**S2**		**S1**	**S2**
	No EDTA	EDTA	No EDTA	EDTA	No EDTA	EDTA	No EDTA	EDTA	EDTA	EDTA
TM1	2.0	1.95	1.83	2.50	1.25	78.5	1.50	14.4	43557	25392
TM2	1.55	2.00	2.0	2.33	1.18	1.09	2.00	2.08	45882	43225
TM3	1.40	2.00	1.50	2.66	low	0.59	2.50	14.5	10319	76036
1TA	1.60	2.10	2.10	2.40	1.31	140	0.58	19.6	60803	3868
2TA	1.5	2.00	1.93	2.70	0.98	172	1.95	7.59	19980	6725
3TA	2.0	2.00	1.55	2.75	1.51	6.64	0.88	7.72	60633	6365
1TB	1.66	1.92	2.00	2.20	0.89	225	2.80	42.1	34189	125058
2TB	1.50	1.9	1.01	2.20	low	37.8	0.94	7.61	51553	44624
3TB	2.0	1.95	1.35	2.20	0.84	139	1.21	13.5	43365	22567
1TC	1.71	2.14	1.88	2.25	1.29	8.88	low	2.09	37915	45172
2TC	1.50	2.14	1.6	1.92	1.07	26.0	3.08	340	31218	100441
3TC	1.80	2.00	2.00	2.50	5.48	58	0.86	5.54	7045	75451
					Yeast sucrose media					

	S1		S2		S1		S2		S1	S2
	No EDTA	EDTA	No EDTA	EDTA	No EDTA	EDTA	No EDTA	EDTA	EDTA	EDTA
TM1	1.7	1.62	0.22	4.00	1.85	14.8	low	0.75	29426	9131
TM2	1.54	1.50	1.40	1.50	1.47	1.54	low	0.88	27541	681
TM3	1.62	1.88	1.35	1.50	0.98	5.74	low	1.32	38058	failed
1TA	1.70	1.7	0.18	--	low	9.31	low	0.50	22704	568
2TA	0.18	1.85	1.80	2.50	low	7.79	low	2.60	23805	33873
3TA	1.27	1.87	1.44	2.25	low	183	low	3.37	24542	20572
1TB	0.90	1.78	1.35	1.36	low	64.4	low	2.06	34033	1193
2TB	1.44	1.62	1.55	1.58	low	10.1	low	3.78	6816	1433
3TB	0.71	1.72	1.28	2.00	low	16.5	low	2.57	11643	1261
1TC	1.0	1.86	0.81	2.00	low	45.1	low	1.19	13849	3809
2TC	1.40	1.93	0.23	--	low	9.34	low	2.81	20693	1685
3TC	0.75	1.75	1.27	1.75	low	1.30	low	1.21	27148	failed

*-- refers to samples that had no reading (repeated 3 times)

3.3.3 Alpha and Beta Diversities

A total of 766,039 sequences were clustered into 564 OTUs, after filtering, using the following criteria - a minimum count of 4 OTUs per sample and the OTU number was 167 using MicrobiomeAnalyst (a comprehensive statistical, visual, and meta-analysis of microbiome data) (Dhariwal *et al.*, 2017). The alpha diversities were measured at the feature level using the Kruskal-Wallis statistical method (3.3); significant differences for alpha-diversity indices S1 and S2 were observed. The statistical p-values were as follows: observed and chao1 - $p < 0.002$, Shannon - $p < 0.003$ and Simpson - $p > 0.006$. The highest diversity index was observed in GYEM S2, and the lowest diversity index was observed in YSM S2. The beta diversity was also measured using the Bray–Curtis index distance method, and the PERMANOVA statistical method was used (F-value: 2.5639; R-squared: 0.15479; *p*-value < 0.001) (Figure 3.4). Beta

diversity compares the number of species shared between samples; from Figure 3.4, overlaps can be seen from all the four grouped samples (based on media (GYEM and YSM) and enrichment (S1 and S2).

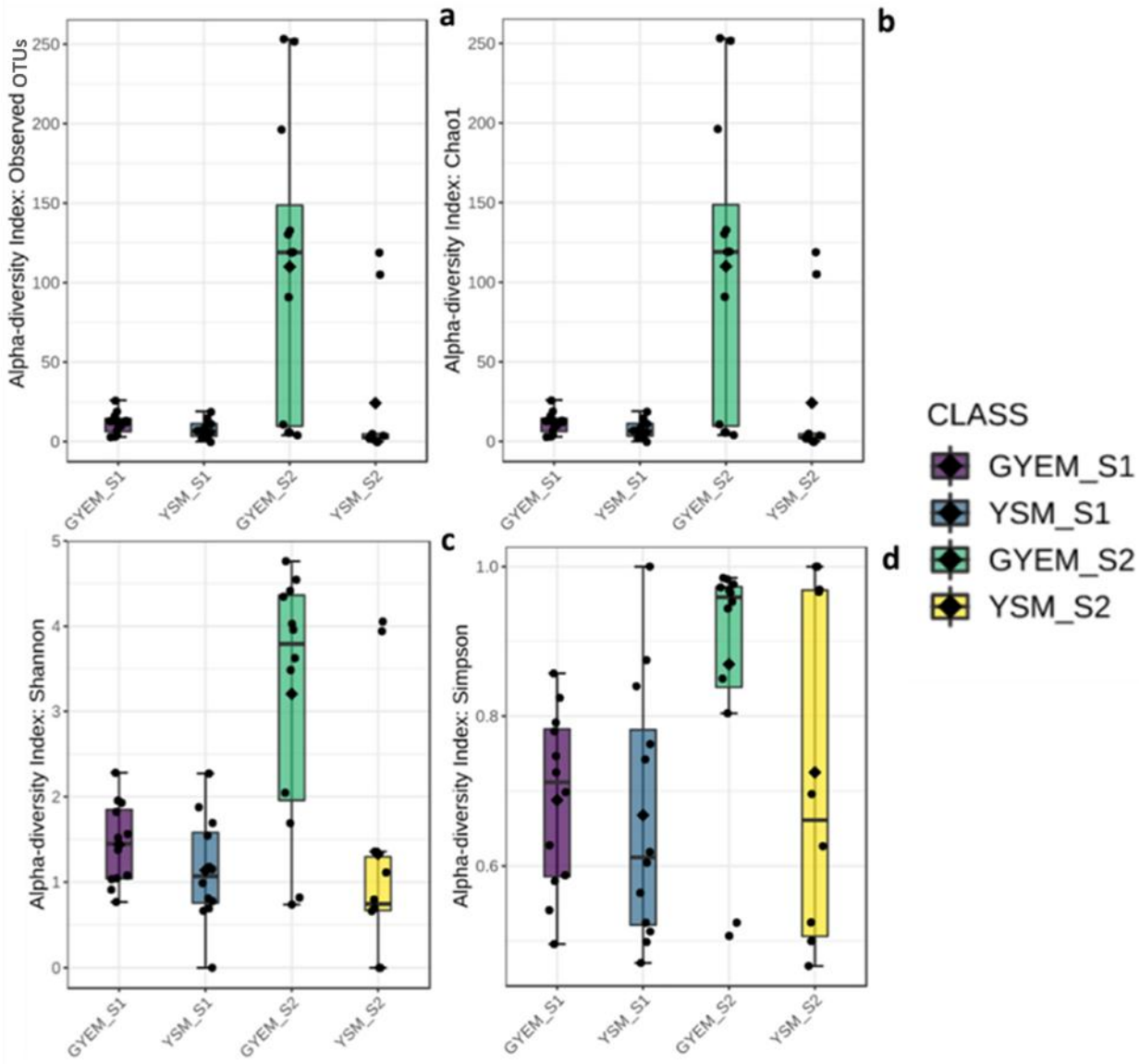

Figure 3.3. Alpha diversity indices of fungi: (**a**) - observed OTUs (richness index), (**b**) - Chao1 (estimation/estimator of the total richness), (**c**) - Shannon index (richness and evenness) and (**d**) - Simpson index (number of species present, as well as the relative abundance of each species).

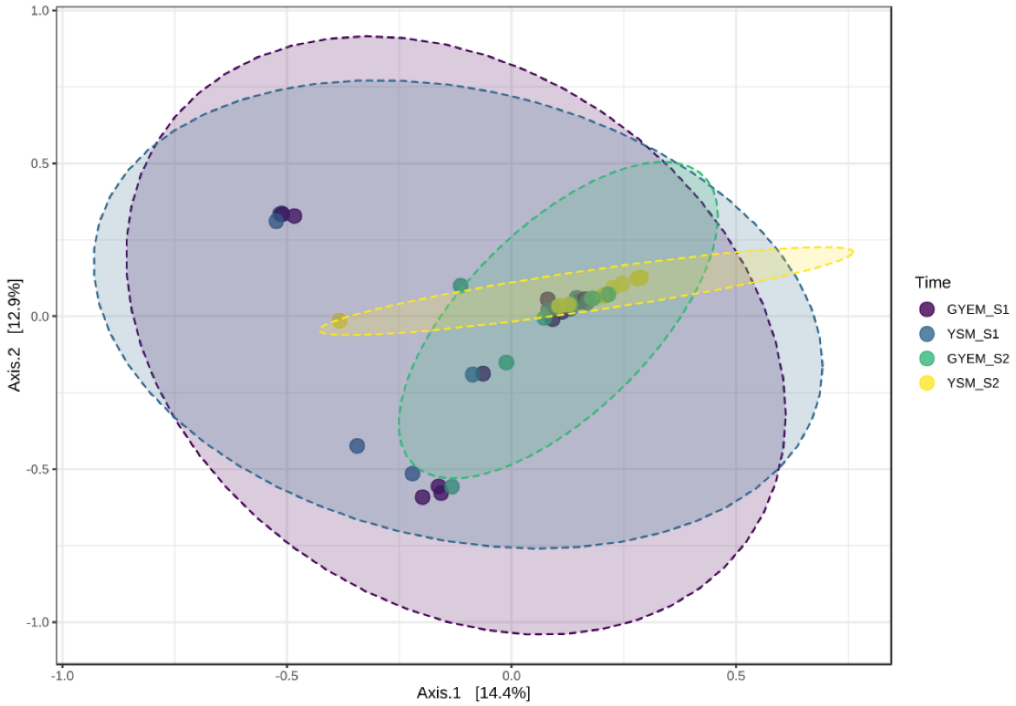

Figure 3.4. Bray-Curtis measures of beta-diversity visualized using principal coordinate analysis (PCoA) for the comparison of fungal diversity for enrichment (S1 and S2) of tailing samples using GYEM and YSM.

3.3.4 Taxonomic diversity of microbial community on GYEM and YSM enrichment

The relative abundance of the microbial communities enriched with glucose yeast extract and yeast sucrose media was determined at the phylum and genus depths comprising at least 1% in at least one sample (Figure 3.3 and Figure 3.4). There was an abundance of the Ascomycota, Basidiomycota, Chytridiomycota, and Rozellomycota phyla (Figure 3.5). Despite the apparent dominance of these phyla in the various tailing sites, each enrichment medium had its own distinct fungal community composition (Figure 3.6). The phylum Ascomycota was the most prevalent in both enrichment media, represented by the genera *Penicillium*, *Xenoacremonium*, *Talaromyces*, *Fodinomyces*, *Recurvomyces*, and *Trichoderma*. The phylum Basidiomycota was abundant in YSM-S2, with the genera *Rhodotorula* and *Malassezia* representing this phylum.

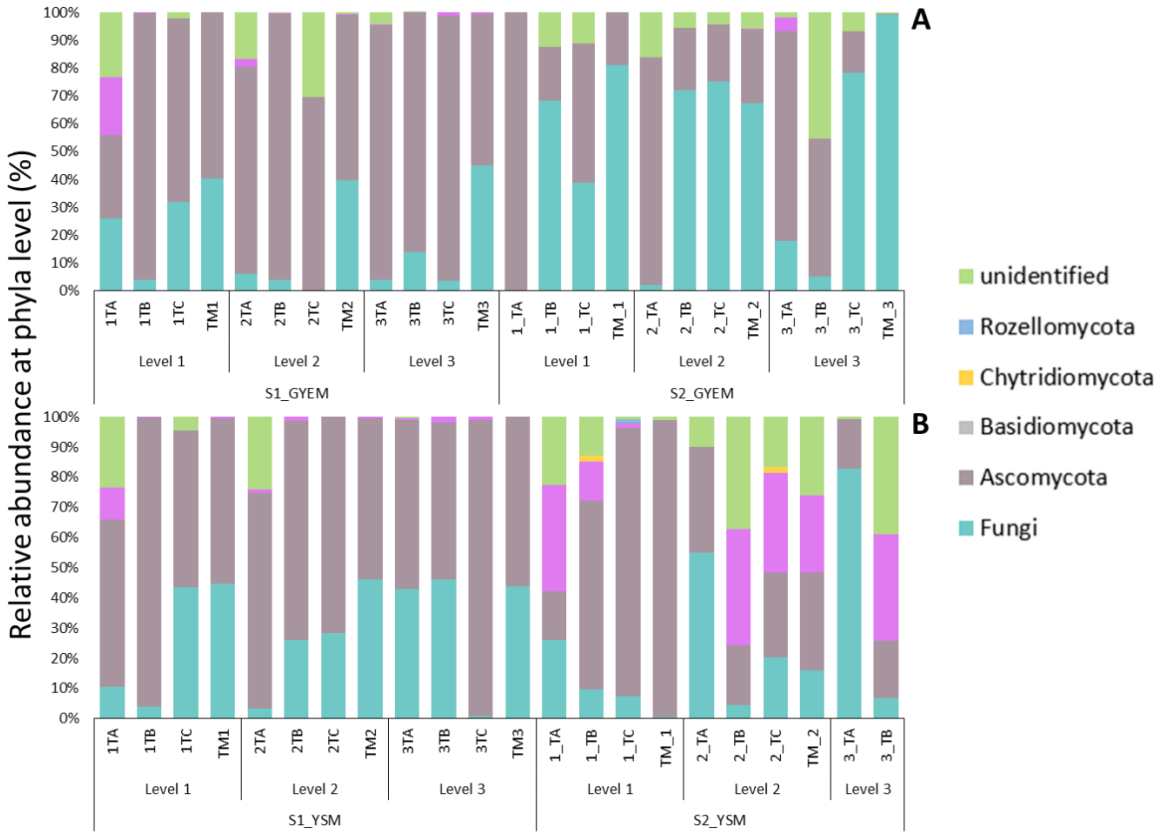

Figure 3.5: Relative abundance of fungi at phyla depth: **a** - glucose yeast extract media and **b** - yeast sucrose media.

The following paragraphs compare the diversity abundance of S1 and S2 on both enrichment media per tailing site at genus level (Figure 3.6). Furthermore, by using the PacBio platform, the taxonomic profile of some fungal populations in the tailing samples was resolved to the species level (Appendix A3.3)

Tailing A: The genera *Penicillium*, *Fodinimyces*, and *Recurvomyces* were observed in most depths of the tailing A samples for both media in the S1 and S2 enrichments, ranging from 4.2-45%. *Talaromyces* was observed only in GYES-S1 depth 1 and 2 (7.09% and 10.5%), while Trichoderma and *Xenoacremonium* were observed only in depth 1 and 2 of GYEM-S2 (12.6-50.6%). The genus *Rhodotorula* was only observed in depth 1 S1 of both media enrichment but was most dominant in GYEM-S1 at 20.6%. Unique genera to GYEM-S2 depth 1 and 3 were Trichoderma (50.6%), *Scoleobasidium* (6.6%), and *Acidothrix* (20.8%), whereas Paraphaesophaeria (11.3%) *Basidiomycota* (11.5%), and *Malassezia* (23.8%) were unique to YSM-S2 depth 1.

Tailing B: Similar to tailing A, *Penicillium*, *Fodinomyces*, and *Recurvomyces* were observed in most depths of the tailing B samples for both media in the S1 and S2 enrichments, ranging

from 15.7-52%. *Malassezia* was only observed in YSM-S2 depth 1 and 2 at 10.11% and 38.4%, respectively, while Acidothrix was observed in S2 depth 3 in both media. *Mollisia* (3.1%) was unique to GYEM-S2 depth 3, and *Spegazzinia* (5.3%) was unique to YSM-S2.

Tailing C: The genera *Penicillium*, *Fodinimyces*, *Acidomyces*, and *Recurvomyces* were observed in most depths of the tailing C samples for both media in S1 and S2 enrichments, ranging from 4.9-44%. *Penicillium* was observed in S1 of both media (GYEM-S1 depth 1 at 31.9% and 2 at 9.7%, YSM-S1 depth 1 at 4.1%). *Xenoacremonium* (8.9%) was observed in the GYEM-S1 depth, while in YSM-S2 it was observed at depth 1 and 2 (43.6% and 13.8%). *Talaromyces* (28.7%), *Coniochaeta* (6.2%), and *Acidothrix* (20.3%) were unique to GYEM-S1 depth 1 and 2, while *Candida* (41.8%) was unique to YSM-S2 depth 2 and *Fusarium* (9.5%) and Pleosporale (4.4%) were unique to YSM-S2 depth 2.

Mixed tailings: The genera *Penicillium* and *Xenoacremonium* were abundant in most depths of mixed tailing samples for both media in the S1 and S2 enrichments, ranging from 7.9-52.7%. Sorduriomycetes was observed in both media S1 but was most abundant in GYEM at 5.87%. *Scolecobasidium* (7.5%) was unique to GYEM-S1 depth 2, whereas *Scytalidium* (57.6%), *Malassezia* (25.6%), and *Spegazzinia* (5.3%) were unique to YSM-S2 depth 1 and 2.

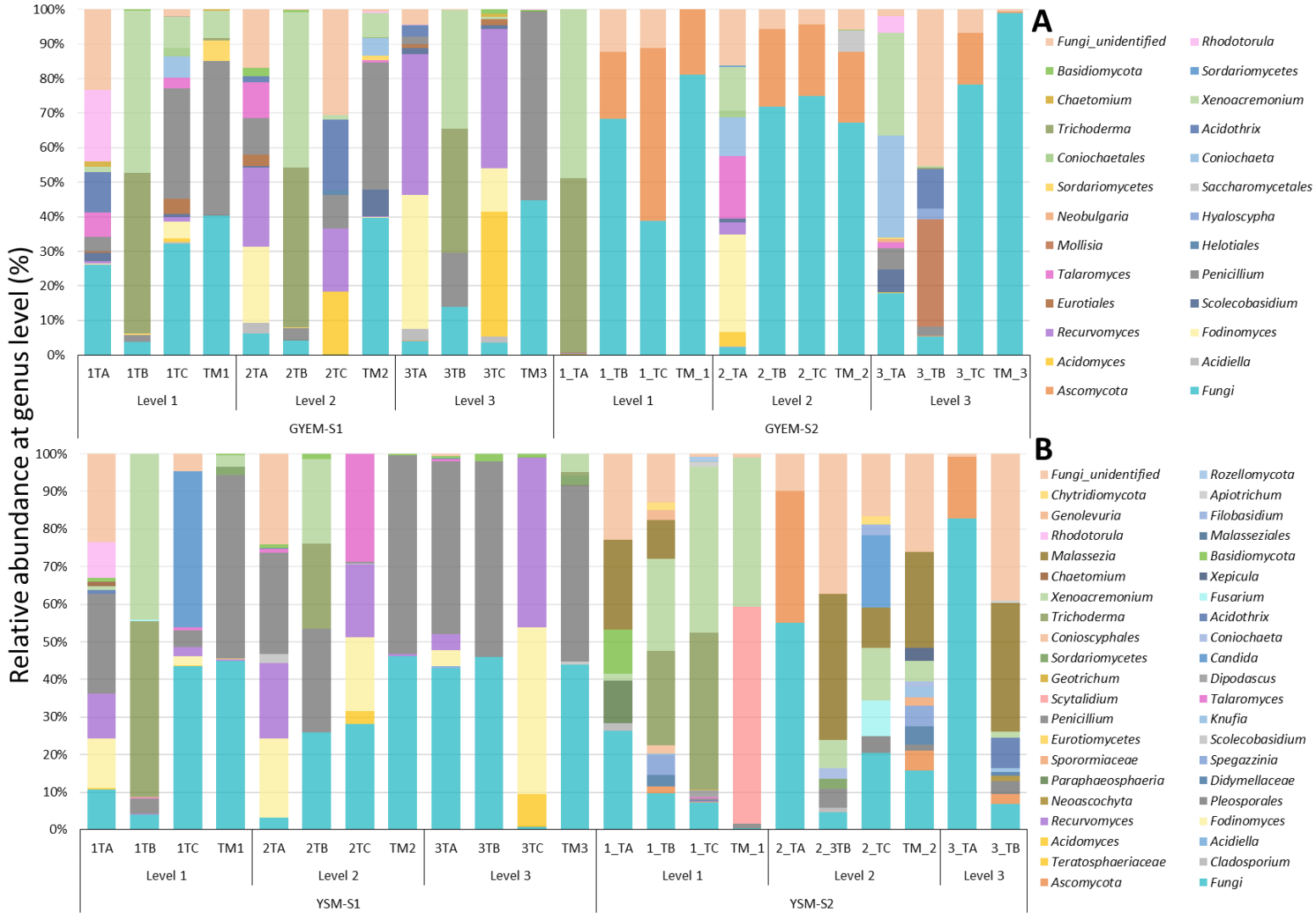

Figure 3.6: Relative abundance of fungi at genus depth: **a** - glucose yeast extract media and **b** - yeast sucrose media.

The FungalTraits database revealed ecological and functional information for eight of the 17 dominant genera. According to the findings of the ecological functional assignment, for primary lifestyle, the genera are mostly soil and wood saprotrophs. Their secondary lifestyle and endophytic interaction capacity are primarily foliar-endophytic, with a few genera assigned to root-associated and rock-inhabiting. Table 3.4 contains more detailed information on the ecological data and the functional assignment found for the fungal isolates present in this study.

Table 3.4: Ecological information and functional assignment of dominant fungal taxa using FungalTraits database.

Genera	primary lifestyle	Secondary lifestyle	Comment on lifestyle	Endophytic interaction capability	Plant pathogenic capacity	Decay substrate	Decay type	Aquatic habitat	Animal biotrophic capacity	Growth form	Fruitbody type
Penicillium	unspecified saprotroph	foliar endophyte	toxin-producing, animal parasite some species,mycoparasite some species	foliar endophyte	0	soil	mold	partly aquatic	opportunistic human parasite	filamentous mycelium	0
Recurvomyces	soil saprotroph	rock-inhabiting	0	0	0	soil	0	non-aquatic	0	filamentous mycelium	perithecium (hymenium hidden, narrow opening)
Talaromyces	unspecified saprotroph	0	hypervariable, thermophile, some species animal parasite some species various saprotrophs	0	0	leaf/fruit/seed, soil, animal material	mold	partly aquatic	opportunistic human parasite	filamentous mycelium	0
Xenoacremonium	wood saprotroph	0	0	0	0	wood	0	partly marine (partly non-aquatic)	opportunistic human parasite	filamentous mycelium	0
Rhodotorula	unspecified saprotroph	foliar endophyte	0	foliar endophyte	0	0	0	partly aquatic	opportunistic human parasite	yeast	none
Acidothrix	soil saprotroph	0	0	0	0	soil	0	non-aquatic	0	filamentous mycelium	none
Malassezia	soil saprotroph	root-associated	0	root-associated	root-associated	roots, soil	0	partly aquatic	animal-associated/opportunistic human parasite	yeast	none
Spegazzinia	wood saprotroph	0	0	no endophytic capacity	0	wood	0	non-aquatic	0	filamentous mycelium	perithecium (hymenium hidden, narrow opening)

Coniochaeta	unspecified saprotroph	foliar endophyte	hypervariable	foliar endophyte	leaf/fruit/seed pathogen	leaf/fruit/seed, soil, dung, animal material	0	non-aquatic	animal parasite	filamentous mycelium	cleistothecium (closed, spherical)

3.4 Discussion

Metal ion interference on DNA recovery is largely determined by metal concentration (Kuffel *et al.* 2021). As shown in S1 and S2 of both enrichment media, the effectiveness of EDTA in chelating metal ions varied with the metal content and concentration. In S1, the main energy source was the tailing contents (Figure 3.2), whereas in S2 the main energy source was the inclusion of iron (II) sulfate heptahydrate. As a result of the higher Fe^{2+} ion concentrations, the S2 samples may have required a higher EDTA concentration to complete chelation. Fe^{2+} may also be responsible for the low DNA recovery and the encountered difficulty in running PCR in S2 samples. These findings are similar to an observation made by Kuffel *et al.* (2021) when they investigated metal ion interferences in forensic DNA analysis from crime scenes, where DNA extractions were carried out from swabs of a variety of metal objects, including bullets, cartridge casings, gun surfaces, knives, metal wires, and surfaces, as well as calcium-rich bone samples. They caution that the metal ions' impacts on DNA recovery, extraction, and amplification are not entirely understood. However, their investigations demonstrate the effects of some metals on DNA amplification, showing that inhibitory effects were observed with zinc, tin, iron (II), and copper, with 50% of the inhibitory concentration (IC50) values much below 1 mM. Thus, their study finding supports the observation made in this present study regarding the inhibitory effects observed with Fe^{2+} ions.

The low recovery of DNA and failed PCR may also be attributed to the high concentration of EDTA used in S2 as compared to the S1 samples. According to a study conducted by Khosravinia & Ramesha, (2007), EDTA concentrations > 11 µg/µl are known to reduce DNA recovery. Beyond chelating, Lopata *et al.* (2019) also showed that EDTA affects enzyme activity by tightly binding to the active site of enzymes such as Taq DNA polymerase, dUTPase, and dNTPase, resulting in low or no PCR products. As an example, Taq polymerase requires Mg^{2+} for activation, while EDTA tends to compete with Mg^{2+} for binding, resulting in enzyme inhibition. This trend of the poor activity of enzymes in the presence of higher concentrations of EDTA and metal ions was also observed by Kuffel *et al.* (2021) and they highlighted that KOD polymerase was more metal-resistant than Taq polymerase in such instances.

A ratio of absorbance at 260 and 280 nm can be used to assess DNA purity, and a measured ratio of ≈1.8 and even up to 2.0 represents a pure DNA. However, a ratio above 2.0 shows the likelihood of RNA contamination of the DNA being assessed for purity. Moreover, a ratio of

>2 is related to DNA degradation and is showing, rather, a measurement of free nucleotides, as described in the protocol manual for the NanoDrop instrument (*Determination of DNA Concentration and Purity, NanoDrop*, n.d.). Moreover, a ratio of 1.6 suggests the presence of proteins, phenol, or other contaminants that absorb strongly at or near 280 nm (Lucena-Aguilar *et al.*, 2016). The S2 samples had an average ratio of 1.7 after treatment with EDTA, which is likely due to any one of the factors described.

Metamorphic environments such as tailings are characterized by high metal concentrations and extremely low pH ranges. These factors contribute to the diversity structure of the acidophilic or acid-tolerant communities found in that environment (Hu *et al.*, 2021; Xue *et al.*, 2018). As a result, the taxonomic abundance of the fungal community observed in the four tailing samples was influenced by their habitat. Furthermore, the nutrients provided by the enrichment media also contributed to the taxonomic abundance. Tailing A and B had the lowest pH ranges of <2.1, which decreased with each depth, whereas mixed tailing and tailing C had pH ranges of >3 for all depths. However, the four tailings had similar metal content with high concentrations of iron, sulfur, and aluminium, though the concentration differed (Figure 3.2). This explains why genera such *Penicillium*, *Fodinomyces*, and *Recurvomyces* were dominant in most depths of the different tailings. *Penicillium* and *Fodinomyces* are both acidophilic (growing only at pH below 5) and acid-tolerant (growing at pH ranging below or above 5) (Vázquez-Campos *et al.*, 2014). Furthermore, their abundance in both enrichment media demonstrates their ability to thrive in different carbon sources (Xia *et al.*, 2018; Vázquez-Campos *et al.*, 2014). *Penicillium* and *Fodinomyces* have been applied in bioleaching and have demonstrated metal tolerance and good bioleaching efficiencies (Begum *et al.*, 2021; Vázquez-Campos *et al.*, 2014). To the best of our knowledge, this is the first report of *Recurcomyces* in tailing samples. However, this genus has been described by Selbmann *et al.* (2008) as a rock-inhabiting yeast found in cold, dry environments, and this ecological feature is supported by analysis conducted using the FungalTraits database (Table 3.4). Furthermore, Coleine *et al.* (2021) suggested that nonsporulating fungi found in rock habitats may occasionally be dispersed between continents, for example via dust, and adapt to other ecologically similar habitats. As a result, the characteristics of *Recurvomyces*, such as growing in an extreme environment, as described by Coleine *et al.* (2021), Selbmann *et al.* (2021), Ametrano *et al.* (2019), and Selbmann *et al.* (2008) are evident enough to support the possibility of its existence in tailings. Further investigations of this genus' bioleaching efficiency are therefore required.

Another dominant genus that was observed in most of the tailings (except in tailing B), especially in depths 1 and 2, is *Xenoacremonium*. This genus, previously referred to as *Acremonium recifei* (now renamed *Xenoacremonium recifei* L. by Lombard and Crous) (Lombard *et al.*, 2015), is a plant pathogen involved in wood decay and has also been isolated from petroleum-contaminated sites (Patel *et al.*, 2021; Taha *et al.*, 2020). Ecological information from FungalTraits also shows that it is a wood saprotroph; thus, its characterization as a wood decaying pathogen is valid. To the best of our knowledge, this is the first report of this genus in tailing samples. No studies have yet linked this genus to bioleaching processes or applications. *Acidothrix* is also another genus observed to be dominant mostly in depth 3 of the tailing samples (except tailing C). *Acidothrix* exhibits both acid-tolerant and acidophilic characteristics and is mostly found in extreme habitats such as acidic soils (Hujslová *et al.*, 2014). This explains its detection only at depth 3, where the lowest pH ranges were observed.

Although similar genera were observed in most tailings, some genera were only found in specific tailing samples. For example, genera such as *Trichoderma, Rhodotorula*, and *Paraphaesophaeria* were only observed in tailing A, *Mollisia* in tailing B, whereas *Candida*, *Acidomyces*, *Coniochaeta*, *Fusarium*, and *Pleosporale* were observed in tailing C, and *Scytalidium* in mixed tailings. Though the following genera are unique to each tailing, *Trichoderma*, *Candida*, *Acidomyces*, *Rhodotorula*, *Fusarium*, *Coniochaeta*, and *Scytalidium* have been observed to share similar characteristics such as acid and metal tolerance. Their occurrence in tailing samples has been reported as well as their application to the bioleaching process (Begum *et al.*, 2021; Ou *et al.*, 2021; Tansengco *et al.*, 2018). The fungus *Paraphaesophaeria* was only found in tailing A and is known to be a plant-associated (primarily a desert plant) fungi with Cu, Cr, and Cd tolerance (An *et al.*, 2015). It has also been reported in manganese oxidation (Takano *et al.*, 2006). *Mollisia* is another genus found only in tailing B. It is an endophyte that is associated with plants and is also involved in plant decay (Tanney & Seifert, 2020; Clark *et al.*, 2019;). Its presence in tailings is likely due to the current efforts to revegetate tailings to prevent dust dispersion during windy days, as well as other environmental concerns linked to airborne pollution (Zappelini *et al.*, 2015).

A comparison of the observed genera in the two media revealed genera that were unique to each. For example, *Malassezia* and *Spegazzinia* were observed only in YSM S2 and *Scoleobasidium*, which was unique to GYEM. The genus *Malassezia* has been reported in flotation tailings of the Kevitsa mine in Finland (Bomberg *et al.*, 2020) and in zinc and copper tailings (Miettinen *et al.*, 2021). Because the genus is known to dominate parts of the human

microbiota (Sparber *et al.,* 2020), it was suggested by Miettinen *et al.* (2021) that its presence in tailing samples may have come as result of human contamination. However, ecological information from FungalTraits assigned *Malassezia* as a soil saprotroph that is associated with plant roots. This may be a demonstration of the genera's versatility and mobility. Thus, its ability to survive in low pH and high concentrations of iron (II) sulfate heptahydrate in the S2 media enrichment, as observed in this study, implies that members of this genus are potentially acid-tolerant and further investigations may be required to determine their bioleaching potential. According to Amend (2014), *Malassezia* are ecologically hyper-diverse and have been reported in diverse environmental sources, such as Antarctic soils (Garmendia *et al.,* 2021), hydrothermal vents, and deep-sea sediments (Amend, 2014). *Spegazzenia* is a wood saprotroph with no endophytic capability, as described in Table 3.4, obtained from FungalTraits. This genus of *Spegazzenia* was observed by Samarakoon *et al.* (2020) to be associated with *Musa* sp. (banana). No reports of *Spegazzenia* in the past have associated any member of this genus with tailing or bioleaching. *Scoleobasidium* is an endophyte that was previously isolated in tomato fruits, as reported by Mahmoud & Narisawa (2013). The genus showed the ability to increase plant biomass in the presence of an organic nitrogen source. The plant growth promoting ability was also reported by Hamayun *et al.* (2009) in soybean plants. Therefore, just like *Mollisia*, its introduction to tailings is likely due to the revegetation process at these tailing sites. These four genera, however, have not been previously reported in association with bioleaching processes and will require extensive investigations. This validates the need for routine bioprospecting of extreme environments for microorganisms that may potentially be useful for industrial applications and biomining operations.

3.5 Conclusion

In this work, the application of EDTA to stabilize divalent metal ions was explored as a result of the difficulties encountered in extracting DNA from the metal-rich environment of tailings. Our investigation showed that the use of EDTA improved the DNA recovery for S1 samples with no interference with the downstream application. However, in the S2 samples, the use of a high EDTA concentration led to low DNA recovery, which impacts the downstream application. It is therefore recommended that a necessary preliminary application of optimal concentrations of EDTA is required to extract DNA from environmental samples obtained from metal-rich environments such as those present in tailings. However, we caution that a further EDTA removal step must be included above the conventional wash step advised by the DNA

extraction kit manufacturers. The elimination of the additional EDTA is needed to ensure the integrity of the DNA extracts and to prevent enzyme inhibition during downstream analysis.

The fungal ITS1 region long-read amplicon sequencing revealed the presence and varied compositions of the acidophilic and acid-tolerant fungal diversity at different depths of the four tailing samples. Genera with applications in bioleaching research, such as Trichoderma, *Candida, Acidomyces, Rhodotorula, Fusarium, Coniochaeta,* and *Scytalidium,* were identified. The sequencing also elucidated novel fungal genera not previously found in tailing samples, likely unique to these mines in Krugersdorp, Gauteng province, South Africa. These include *Recurcomyces* and *Xenoacremonium,* which, to the best of our knowledge, have not been previously linked to metal-containing samples like tailings. The findings of this study clearly support the use of long-read amplicon sequencing to gain a more in-depth understanding of biodiversity. However, amplicon sequencing studies do not provide enough opportunities to visualize (morphological characteristics), to test functions (such as bioleaching ability), and to investigate the genetics and genomics of these acidophiles. This limitation is being solved by the creation of the FungalTraits database. Nonetheless, they provide great insights with their high throughput that prompt further investigation, particularly with regard to applications and further tests of microbial efficiencies and abilities. As such, they are excellent tools for bioprospecting vast sample areas.

3.6 References

Adeleke, Rasheed, Eugene Cloete, and D. K. (2010). Isolation and identification of iron ore - solubilising fungus. *South African Journal of Science*, *106*(9), 1–6. https://doi.org/10.4102/sajs.v106i9/10.254

Amend, A. (2014). From Dandruff to Deep-Sea Vents: Malassezia-like Fungi Are Ecologically Hyper-diverse. *PLoS Pathogens*, *10*(8), 8–11. https://doi.org/10.1371/journal.ppat.1004277

Ametrano, C. G., Muggia, L., & Grube, M. (2019). Fungi in Extreme Environments: Ecological Role and Biotechnological Significance. *Fungi in Extreme Environments: Ecological Role and Biotechnological Significance*, 119–143. https://doi.org/10.1007/978-3-030-19030-9

Amiri, F., Mousavi, S. M., & Yaghmaei, S. (2011). Enhancement of bioleaching of a spent Ni/Mo hydroprocessing catalyst by Penicillium simplicissimum. *Separation and Purification Technology*, *80*(3), 566–576. https://doi.org/10.1016/j.seppur.2011.06.012

An, H., Liu, Y., Zhao, X., Huang, Q., Yuan, S., Yang, X., & Dong, J. (2015). Characterization of cadmium-resistant endophytic fungi from Salix variegata Franch. in Three Gorges Reservoir Region, China. *Microbiological Research*, *176*, 29–37. https://doi.org/10.1016/j.micres.2015.03.013

Begum, S., Rath, S. K., & Rath, C. C. (2021). Applications of Microbial Communities for the Remediation of Industrial and Mining Toxic Metal Waste: A Review. *Geomicrobiology Journal*, *0*(0), 1–12. https://doi.org/10.1080/01490451.2021.1991054

Bernardino, A. F., Pais, F. S., Oliveira, L. S., Gabriel, F. A., Ferreira, T. O., Queiroz, H. M., & Mazzuco, A. C. A. (2019). Chronic trace metals effects of mine tailings on estuarine assemblages revealed by environmental DNA. *PeerJ*, *2019*(11), 1–18. https://doi.org/10.7717/peerj.8042

Bomberg, M., Miettinen, H., Musuku, B., & Kinnunen, P. (2020). First insights to the microbial communities in the plant process water of the multi-metal Kevitsa mine. *Research in Microbiology*, *171*(7), 230–242. https://doi.org/10.1016/j.resmic.2020.07.001

Bonsu, D. O. M., Higgins, D., & Austin, J. J. (2020). Forensic touch DNA recovery from metal surfaces – A review. *Science and Justice*, *60*(3), 206–215. https://doi.org/10.1016/j.scijus.2020.01.002

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2020). *DADA2 ITS pipeline workflow (1.8). Retreieved Novemeber 2021, from https://benjj neb.github.io/dada2/ ITS_ workf low.html*.

Callahan, B. J., Wong, J., Heiner, C., Oh, S., Theriot, C. M., Gulati, A. S., McGill, S. K., & Dougherty, M. K. (2019). High-throughput amplicon sequencing of the full-length 16S rRNA gene with single-nucleotide resolution. *Nucleic Acids Research*, *47*(18), e103. https://doi.org/10.1093/nar/gkz569

Clark, T. N., Ellsworth, K. T., Decken, A., Johnson, J. A., & Gray, C. A. (2019). The application of NMR and LC-HRMS based prioritization strategies for the discovery of natural products produced by endophytic fungi from medicinal plants. *University of New Brunswic*, *64*.

Coleine, C., Stajich, J. E., Ríos, A. D. L., Selbmann, L., Coleine, C., Stajich, J. E., Ríos, A. D. L., Selbmann, L., Coleine, C., & Stajich, J. E. (2021). Beyond the extremes: Rocks as ultimate refuge for fungi in drylands Beyond the extremes: Rocks as ultimate refuge for fungi in drylands. *Mycologia*, *113*(1), 108–133. https://doi.org/10.1080/00275514.2020.1816761

Colin, Y., Goberna, M., Verdú, M., & Navarro-Cano, J. A. (2019). Successional trajectories of soil bacterial communities in mine tailings: The role of plant functional traits. *Journal of Environmental Management*, *241*(October 2018), 284–292. https://doi.org/10.1016/j.jenvman.2019.04.023

Desai, C., & Madamwar, D. (2007). Extraction of inhibitor-free metagenomic DNA from polluted sediments, compatible with molecular diversity analysis using adsorption and ion-exchange treatments. *Bioresource Technology*, *98*(4), 761–768. https://doi.org/10.1016/j.biortech.2006.04.004

Determination of DNA Concentration and Purity, NanoDrop. (n.d.).

Dhariwal, A., Chong, J., Habib, S., King, I. L., Agellon, L. B., & Xia, J. (2017). MicrobiomeAnalyst: a web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. *Nucleic Acids Research*, 45(W1), W180-W188.

Dominguez, K., & Ward, W. S. (2009). A novel nuclease activity that is activated by Ca2+ chelated to EGTA. *Systems Biology in Reproductive Medicine*, *55*(5–6), 193–199. https://doi.org/10.3109/19396360903234052

Dong, Y., Lin, H., Xu, X., & Zhou, S. (2013). Hydrometallurgy Bioleaching of different copper sul fi des by Acidithiobacillus ferrooxidans and its adsorption on minerals. *Hydrometallurgy*, *140*, 42–47. https://doi.org/10.1016/j.hydromet.2013.05.009

Dusengemungu, L., Gwanama, C., & Mubemba, B. (2024). Data on fungal abundance and diversity in copper and cobalt contaminated tailing soils in Kitwe, Zambia. *Data in Brief*, 52, 109951.

El-Ashram, S., Al Nasr, I., & Suo, X. (2016). Nucleic acid protocols: Extraction and optimization. *Biotechnology Reports*, *12*, 33–39. https://doi.org/10.1016/j.btre.2016.10.001

Feng, D., & van Deventer, J. S. J. (2010). Thiosulphate leaching of gold in the presence of ethylenediaminetetraacetic acid (EDTA). *Minerals Engineering*, *23*(2), 143–150. https://doi.org/10.1016/j.mineng.2009.11.009

Fortin, N., Beaumier, D., Lee, K., & Greer, C. W. (2004). Soil washing improves the recovery of total community DNA from polluted and high organic content sediments. *Journal of microbiological methods*, *56*(2), 181-191.

Garmendia, G., Alvarez, A., Villarreal, R., Martínez-Silveira, A., Wisniewski, M., & Vero, S. (2021). Fungal diversity in the coastal waters of King George Island (maritime Antarctica). *World Journal of Microbiology and Biotechnology*, *37*(8), 1–12. https://doi.org/10.1007/s11274-021-03112-4

Guéroult, M., Picot, D., Abi-Ghanem, J., Hartmann, B., & Baaden, M. (2010). How cations can assist dnase i in DNA binding and hydrolysis. *PLoS Computational Biology*, *6*(11), 1–11. https://doi.org/10.1371/journal.pcbi.1001000

Hall, N. E. L., & Axelrod, D. E. (1977). Interference of cellular ferric ions with DNA extraction and the application to methods of DNA determination. *Analytical Biochemistry*, *79*(1–2), 425–430. https://doi.org/10.1016/0003-2697(77)90417-1

Hamayun, M., Khan, S.A., Kim, H.Y., Chaudhary, M.F., Hwang, Y.H., Shin, D.H., Kim, I.K., Lee, B.H. and Lee, I. J. (2009). *Gibberellin Production and Plant Growth Enhancement by Newly Isolated Strain of Scolecobasidium tshawytschae* (pp. 560–565). Journal of microbiology and biotechnology.

Hassanshahian, M., & Ghoebani, S. (2018). *Isolation and Characterization of Iron and Sulfur-Oxidizing Bacteria from Maiduk Copper Mine at Shahrbabk Province in Iran*. *0451*. https://doi.org/10.1080/01490451.2017.1348408

Heikrujam, J., Kishor, R., & Behari Mazumder, P. (2020). The Chemistry Behind Plant DNA Isolation Protocols. *Biochemical Analysis Tools - Methods for Bio-Molecules Studies*, 1–12. https://doi.org/10.5772/intechopen.92206

Hu, X., Wang, J., Lv, Y., Liu, X., & Zhong, J. (2021). *Effects of Heavy Metals / Metalloids and Soil Properties on Microbial Communities in Farmland in the Vicinity of a Metals Smelter*. *12*(August), 1–13. https://doi.org/10.3389/fmicb.2021.707786

Huerta-Rosas, B., Cano-Rodríguez, I., Gamiño-Arroyo, Z., Gómez-Castro, F. I., Carrillo-Pedroza, F. R., Romo-Rodríguez, P., & Gutiérrez-Corona, J. F. (2020). Aerobic processes for bioleaching manganese and silver using microorganisms indigenous to mine tailings. *World Journal of Microbiology and Biotechnology*, *36*(8), 1–16. https://doi.org/10.1007/s11274-020-02902-6

Hujslová, M., Kubátová, A., Kostovčík, M., Blanchette, R. A., de Beer, Z. W., Chudíčková, M., & Kolařík, M. (2014). Three new genera of fungi from extremely acidic soils. *Mycological Progress*, *13*(3), 819–831. https://doi.org/10.1007/s11557-014-0965-3

Jia, T., Wang, R., Fan, X., & Chai, B. (2018). A comparative study of fungal community structure, diversity and richness between the soil and the phyllosphere of native grass species in a copper tailings dam in Shanxi Province, China. *Applied Sciences (Switzerland)*, *8*(8). https://doi.org/10.3390/app8081297

Khosravinia, H., & Ramesha, K. P. (2007). Influence of EDTA and magnesium on DNA extraction from blood samples and specificity of polymerase chain reaction. *African Journal of Biotechnology*, *6*(3), 184–187. https://doi.org/10.4314/ajb.v6i3.56127

Kishimoto, N., & Tano, T. (1987). Acidophilic heterotrophic bacteria isolated from acidic mine drainage, sewage, and soils. *The Journal of General and Applied Microbiology*, *33*(1), 11–25. https://doi.org/10.2323/jgam.33.11

Kõljalg, U., Nilsson, R. H., Abarenkov, K., Tedersoo, L., Taylor, A. F. S., Bahram, M., Bates, S. T., Bruns, T. D., Bengtsson-Palme, J., Callaghan, T. M., Douglas, B., Drenkhan, T., Eberhardt, U., Dueñas, M., Grebenc, T., Griffith, G. W., Hartmann, M., Kirk, P. M., Kohout, P., … Larsson, K. H. (2013). Towards a unified paradigm for sequence-based identification of fungi. *Molecular Ecology*, *22*(21), 5271–5277. https://doi.org/10.1111/mec.12481

Kuffel, A., Gray, A., & Daeid, N. N. (2021). Impact of metal ions on PCR inhibition and RT-PCR efficiency. *International Journal of Legal Medicine*, *135*(1), 63–72. https://doi.org/10.1007/s00414-020-02363-4

Liang, J. liang, Liu, J., Yang, T. tao, Wang, P. deng, Zhang, S. chang, Jia, P., Liao, B., Shu, W. sheng, & Li, J. tian. (2020). Contrasting soil fungal communities at different habitats in a revegetated copper mine wasteland. *Soil Ecology Letters*, *2*(1), 8–19. https://doi.org/10.1007/s42832-020-0022-x

Liu, M., Ding, X., Wang, X., Li, J., Yang, H., & Yin, Y. (2019). Extraction of DNA from complex biological sample matrices using guanidinium ionic liquid modified magnetic nanocomposites. *RSC Advances*, *9*(40), 23119–23128. https://doi.org/10.1039/c9ra01505a

Lombard, L., van der Merwe, N. A., Groenewald, J. Z., & Crous, P. W. (2015). Generic concepts in Nectriaceae. *Studies in Mycology*, *80*, 189–245. https://doi.org/10.1016/j.simyco.2014.12.002

Lopata, A., Jójárt, B., Surányi, É. V., Takács, E., Bezúr, L., Leveles, I., Bendes, Á., Viskolcz, B., Vértessy, B. G., & Tóth, J. (2019). Beyond chelation: EDTA tightly binds taq DNA polymerase, MutT and dUTPase and directly inhibits dNTPase activity. *Biomolecules*, *9*(10), 1–19. https://doi.org/10.3390/biom9100621

Lucena-Aguilar, G., Sánchez-López, A. M., Barberán-Aceituno, C., Carrillo-Ávila, J. A., López-Guerrero, J. A., & Aguilar-Quesada, R. (2016). DNA Source Selection for Downstream Applications Based on DNA Quality Indicators Analysis. *Biopreservation and Biobanking*, *14*(4), 264–270. https://doi.org/10.1089/bio.2015.0064

Mahmoud, R. S., & Narisawa, K. (2013). A new fungal endophyte, Scolecobasidium humicola, promotes tomato growth under organic nitrogen. *PLoS ONE*, *8*(11), 1–8. https://doi.org/10.1371/journal.pone.0078746

Martínez P, Gálvez S, Ohtsuka N, Budinich M, Cortés MP, Serpell C, Nakahigashi K, Hirayama A, Tomita M, Soga T, Martínez S, Maass A, P. P. (2012). Metabolomic study of Chilean biomining bacteria Acidithiobacillus ferrooxidans strain Wenelen and Acidithiobacillus thiooxidans strain Licanantay. *Metabolomics*, *9*(1), 247–257. https://doi.org/10.1007/s11306-012-0443-3

Melgar, E., & Goldthwait, D. A. (1968). Deoxyribonucleic acid nucleases. II. The effects of metals on the mechanism of action of deoxyribonuclease I. *Journal of Biological Chemistry*, *243*(17), 4409–4416. https://doi.org/10.1016/S0021-9258(18)93208-4

Miettinen, H., Bomberg, M., Le, T. M. K., & Kinnunen, P. (2021). Identification and metabolism of naturally prevailing microorganisms in zinc and copper mineral processing. *Minerals*, *11*(2), 1–31. https://doi.org/10.3390/min11020156

Moreno, L. I., & McCord, B. R. (2017). Understanding metal inhibition: The effect of copper (Cu2+) on DNA containing samples. *Forensic Chemistry*, *4*(March), 89–95. https://doi.org/10.1016/j.forc.2017.03.005

Ou, S. N., Liang, J. L., Jiang, X. M., Liao, B., Jia, P., Shu, W. S., & Li, J. T. (2021). Physiological, Genomic and Transcriptomic Analyses Reveal the Adaptation Mechanisms of Acidiella bohemica to Extreme Acid Mine Drainage Environments. *Frontiers in Microbiology*, *12*(July), 1–14. https://doi.org/10.3389/fmicb.2021.705839

Patel JN, Parmar FA, U. V. (2021). *Isolation and Characterization of Pathogens Causing Disease in Pomegranate (Punica granatum L.), India*. *10*(5, may 2021). https://doi.org/10.15680/IJIRSET.2021.1005041

Põlme, S., Abarenkov, K., Henrik Nilsson, R., Lindahl, B. D., Clemmensen, K. E., Kauserud, H., Nguyen, N., Kjøller, R., Bates, S. T., Baldrian, P., Frøslev, T. G., Adojaan, K., Vizzini, A., Suija, A., Pfister, D., Baral, H. O., Järv, H., Madrid, H., Nordén, J., … Tedersoo, L. (2020). FungalTraits: a user-friendly traits database of fungi and fungus-like stramenopiles. *Fungal Diversity*, *105*(1). https://doi.org/10.1007/s13225-020-00466-2

Portzehl, H., Caldwell, P. C., & Rüegg, J. C. (1964). The dependence of contraction and relaxation of muscle fibres from the crab Maia squinado on the internal concentration of free calcium ions. *BBA - Specialised Section On Biophysical Subjects*, *79*(3), 581–591. https://doi.org/10.1016/0926-6577(64)90224-4

Samarakoon, B. C., Phookamsak, R., Wanasinghe, D. N., Chomnunti, P., Hyde, K. D., McKenzie, E. H. C., Promputtha, I., Xu, J. C., & Li, Y. J. (2020). Taxonomy and phylogenetic appraisal of Spegazzinia musae sp. nov. And S. deightonii (Didymosphaeriaceae, Pleosporales) on Musaceae from Thailand. *MycoKeys*, *70*, 19–37. https://doi.org/10.3897/MYCOKEYS.70.52043

Schrader, C., Schielke, A., Ellerbroek, L., & Johne, R. (2012). PCR inhibitors - occurrence, properties and removal. *Journal of Applied Microbiology*, *113*(5), 1014–1026. https://doi.org/10.1111/j.1365-2672.2012.05384.x

Selbmann, L., de Hoog, G. S., Zucconi, L., Isola, D., Ruisi, S., Gerrits van den Ende, A. H. G., Ruibal, C., De Leo, F., Urzì, C., & Onofri, S. (2008). Drought meets acid: three new genera in a dothidealean clade of extremotolerant fungi. *Studies in Mycology*, *61*(June 2014), 1–20. https://doi.org/10.3114/sim.2008.61.01

Selbmann, L., Stoppiello, G. A., Onofri, S., & Stajich, J. E. (2021). *Culture-Dependent and Amplicon Sequencing Approaches Reveal Diversity and Distribution of Black Fungi in Antarctic Cryptoendolithic Communities*.

Sparber, F., Ruchti, F., & LeibundGut-Landmann, S. (2020). Host Immunity to Malassezia in Health and Disease. *Frontiers in Cellular and Infection Microbiology*, *10*(May). https://doi.org/10.3389/fcimb.2020.00198

Taha, H., Shivanand, P., Khoo, D. H., Mohammad, Y. H., Matussin, N. B. A., & Metali, F. (2020). Identification of culturable petroleum-degrading bacteria and fungi from petroleum-contaminated sites in Brunei Darussalam. *Journal of Environmental Science and Health - Part A Toxic/Hazardous Substances and Environmental Engineering*, *55*(13), 1542–1547. https://doi.org/10.1080/10934529.2020.1826238

Takano, K., Itoh, Y., Ogino, T., Kurosawa, K., & Sasaki, K. (2006). Phylogenetic analysis of manganese-oxidizing fungi isolated from manganese-rich aquatic environments in Hokkaido, Japan. *Limnology*, *7*(3), 219–223. https://doi.org/10.1007/s10201-006-0177-x

Tang, C., Zhong, J., Lv, Y., Liu, X., Li, Y., Zhang, M., Yan, X., & Sun, W. (2021). Response and dynamic change of microbial community during bioremediation of uranium tailings by bacillus sp. *Minerals*, *11*(9), 1–14. https://doi.org/10.3390/min11090967

Tanney, J. B., & Seifert, K. A. (2020). Mollisiaceae: An overlooked lineage of diverse endophytes. *Studies in Mycology*, *95*, 293–380. https://doi.org/10.1016/j.simyco.2020.02.005

Tansengco, M., Tejano, J., Coronado, F., Gacho, C., & Barcelo, J. (2018). Heavy metal tolerance and removal capacity of trichoderma species isolated from mine tailings in Itogon, Benguet. *Environment and Natural Resources Journal*, *16*(1), 39–57. https://doi.org/10.14456/ennrj.2018.5

Tanunchai, B., Ji, L., Schroeter, S. A., Wahdan, S. F. M., Hossen, S., Delelegn, Y., Buscot, F., Lehnert, A. S., Alves, E. G., Hilke, I., Gleixner, G., Schulze, E. D., Noll, M., & Purahong, W. (2022). FungalTraits vs. FUNGuild: Comparison of Ecological Functional Assignments of Leaf- and Needle-Associated Fungi Across 12 Temperate Tree Species. *Microbial Ecology*, *0123456789*. https://doi.org/10.1007/s00248-022-01973-2

Vázquez-Campos, X., Kinsela, A. S., Waite, T. D., Collins, R. N., & Neilan, B. A. (2014). Fodinomyces uranophilus gen. nov. sp. nov. and Coniochaeta fodinicola sp. nov., two uranium mine-inhabiting Ascomycota fungi from northern Australia. *Mycologia*, *106*(6), 1073–1089. https://doi.org/10.3852/14-013

Wang, Q., Garrity, G. M., Tiedje, J. M., & Cole, J. R. (2007). Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology*, *73*(16), 5261–5267. https://doi.org/10.1128/AEM.00062-07

Xia, M., Bao, P., Liu, A., Wang, M., Shen, L., Yu, R., Liu, Y., Chen, M., Li, J., Wu, X., Qiu, G., & Zeng, W. (2018). Bioleaching of low-grade waste printed circuit boards by mixed fungal culture and its community structure analysis. *Resources, Conservation and Recycling*, *136*(January), 267–275. https://doi.org/10.1016/j.resconrec.2018.05.001

Xue, P., Carrillo, Y., Pino, V., Minasny, B., & Mcbratney, A. B. (2018). Soil Properties Drive Microbial Community Structure in a Large Scale Transect in South Eastern. *Scientific Reports*, *July*, 1–11. https://doi.org/10.1038/s41598-018-30005-8

Zaitoun, M. A., & Lin, C. T. (1997). Chelating behavior between metal ions and EDTA in sol - gel matrix. *Journal of Physical Chemistry B*, *101*(10), 1857–1860. https://doi.org/10.1021/jp963102d

Zappelini, C., Karimi, B., Foulon, J., Lacercat-Didier, L., Maillard, F., Valot, B., Blaudez, D., Cazaux, D., Gilbert, D., Yergeau, E., Greer, C., & Chalot, M. (2015). Diversity and complexity of microbial communities from a chlor-alkali tailings dump. *Soil Biology and Biochemistry*, *90*, 101–110. https://doi.org/10.1016/j.soilbio.2015.08.008

Zhang, X., Niu, J., Liang, Y., Liu, X., & Yin, H. (2016). Metagenome-scale analysis yields insights into the structure and function of microbial communities in a copper bioleaching heap. *BMC genetics*, *17*(1), 1-12.

CHAPTER 4: ISOLATION, IDENTIFICATION, AND CHARACTERIZATION OF FUNGAL ISOLATES

Objective

Bioleaching is a process in which metals are extracted from ore or tailing by the activity of microbial communities. For an organism to be used in bioleaching experiments, it should have abilities such as metal tolerance and production of organic acids. In this chapter, the dominant fungi identified in chapter 3 were isolated. Fifteen different isolated fungi were screened for their metal tolerance, growth rates in the presence and absence of metals, carbon utilization, and organic acid production Therefore, this chapter focused on screening 15 different fungal isolates, in which their isolation was guided by the results from chapter 3, for their metal tolerance, growth rates carbon utilization, and production of organic acids. The purpose was to ultimately select 1 isolated for use in bioleaching assay.

Publication

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Abstract

This study evaluated the bioleaching potential of fungal isolates from South African tailing samples. Following an initial screening of 15 isolates, four were selected for further analysis based on their potential for organic acid production and metal tolerance. In terms of tolerance to Al, Zn, Ni, and Cr, these four isolates - *Trichoderma*, *Talaromyces*, *Penicillium_3*, and *Penicillium_6* displayed varying degrees of resistance, with *Trichoderma* displaying better tolerance (TI>1). The isolates' growth rates (*kd*) were also assessed, and *Trichoderma* once more displayed greater growth rates under metal stress. High Performance Liquid Chromatography (HPLC) was used to measure organic acids and Gas Chromatography Mass Spectrometry (GC-MS) to identify metabolites. The four isolates were shown to mostly produce citric acid, with *Trichoderma* producing the most in the shortest amount of time. GC-MS results showed citric acid cycle is main pathways for organic acid production, though other pathways related to lipid biosynthesis and carbohydrate metabolism also play significant roles. Three compounds involved in furfural breakdown were abundant. Using KEGG, a link between these compounds and the citric acid cycle was established, where their breakdown generates an intermediate of citric acid cycle. This research offers perceptions into the metabolic procedures of metal-tolerant fungi, which may advance the development of bioleaching.

Keywords: Metal tolerance, Organic acid, Metabolites, Bioleaching

4.1 Introduction

The fungal domain is known for its remarkable metabolic versatility that enables these organisms to thrive in diverse environments such as metal-rich mine tailings (Shourie and Vijayalakshmi, 2022; Russo *et al.*, 2019). These microorganisms have gained attention for their ability to produce secondary metabolites, particularly organic acids, with wide-ranging industrial applications, from pharmaceuticals to bioleaching (Mishra *et al.*, 2023; Wösten, 2019). In bioleaching, an eco-friendly approach for metal extraction, organic acids are the main leaching agents (Pathak, *et al.*, 2021a, 2021b). Consequently, the efficiency of metal solubilization depends on the continuous production of organic acids (Din *et al.*, 2020; Narayanasamy *et al.*, 2018; Kolenčík *et al.*, 2013; Ren *et al.*, 2009; Wu and Ting, 2006). The production of organic acid is a result of microbial metabolic activities, and as a result, challenges such as slow kinetics and low yields are often expected during bioleaching processes (Moazzam *et al.*, 2021; Roy *et al.*, 2021; Pathak *et al.*, 2017).

Slow kinetics arise from various factors, including limited nutrient availability, metal toxicity, adaptability requirements, and environmental conditions (Sajjad *et al.*, 2019). For instance, before organic acids can be produced, cells must first utilize nutrients, grow, and multiply. Furthermore, the type of metal, carbon source and the fungal genus or species play pivotal roles in determining the type of organic acid produced. For example, when grown in glucose containing media, *Aspergillus niger* produces citric acid as the main organic acid whereas *Aspergillus flavus* produces malic acid (Keshavarz *et al.*, 2021; Qayyum *et al.*, 2019). Moreover, citric acid production varies when environmental conditions involve metals. For instance, in the presence of aluminium, *A. niger* produces citric acid as the main organic acid, but in the presence of manganese, citric acid production is inhibited while favouring the production of oxalic acid (Alavi *et al.*, 2021; Muddanna & Baral, 2019; Santhiya & Ting, 2005).

Organic acids are the main leaching agents in metal bioleaching, employing mechanisms like acidolysis (direct interaction of organic acid with metal-containing minerals) and complexolysis (formation of stable complexes between organic acids and metal ions) (Pathak *et al.*, 2021a, 2021b). Consequently, organic acids such as citric and oxalic acid are known to bioleach metals such as Aluminium (Al), Copper (Cu), Chromium (Cr), Iron (Fe), Nickel (Ni), Manganese (Mn), and Zinc (Zn), with citric acid being more efficient than oxalic acid (Brown *et al.*, 2023; Shekhar Samanta *et al.*, 2023; Sarkodie *et al.*, 2022; Dusengemungu *et al.*, 2021).

In fungi, the production of organic acids is intricately linked to their utilization of carbon sources. As a result, bioleaching studies determine and use optimal carbon sources for organic acid production to improve bioleaching process efficiency (Din *et al.,* 2020). While this is a crucial step in optimizing fungal bioleaching, gaining a deeper understanding of carbon utilization at the metabolic level is imperative. This can be achieved through approaches like metabolomics, offering insights into the upregulated metabolic pathways during fungal carbon utilization and subsequent conversion into organic acids (Brisson *et al.,* 2020). Such knowledge opens new avenues for process optimization.

In addition to organic acid production, fungal isolates with bioleaching potential should also demonstrate metal tolerance (Rose & Devi, 2018; Valix & Loon, 2003). The challenges encountered during bioleaching processes, particularly slow kinetics and low metal yields, are exacerbated by elevated metal concentrations, which trigger adaptation and stress response mechanisms in fungal organisms (Mani & Kumar, 2014). Metal toxicity can disrupt growth phases, such as prolonging the lag phase, hinder organic acid production, or even completely inhibit fungal growth (Anahid *et al.,* 2011). To address these challenges, harnessing indigenous microbial communities adapted to extreme environments with high metal concentrations and low pH becomes advantageous (Thavamani *et al.,* 2017). These native microorganisms likely possess stress response mechanisms that facilitate efficient growth and potential bioleaching activities in metal-rich environments such as tailings.

In this study, fungal isolates were obtained from mine tailings samples in South Africa with the objective of assessing their growth rates under various metal stresses to determine their metal tolerance and potential suitability for bioleaching. Furthermore, we investigated their organic acid production during growth stages at the metabolic level, using glucose as the carbon source to identify enriched metabolic pathways with significant importance towards organic acid production. This knowledge will contribute towards the optimization of the bioleaching process using fungal isolates with similar characteristics.

4.2 Material and methods

4.2.1 Fungal isolation and identification

Tailing samples were collected in Krugersdorp, a mining city in Gauteng province, South Africa, as described in Chapter 3, section 3.2.3 (Nkuna *et al.,* 2022). To isolate fungi, we initially enriched microorganisms in the tailing samples using the media listed in Table 4.1, following the media preparation as outlined in Chapter 3, section 3.2.3. GYEM and YSM were

also used as listed in Table 3.1 (Chapter 3). Fungal isolates were obtained from enriched tailing samples using the same media, slightly modified with the addition of 1.5% bacteriological agar to solidify the liquid media for isolation on petri dishes. The purified isolates were stored in slants. For identification, ITS1—5′ CTTGGTCATTTAGAGGAAGTAA-3′ and ITS4—5′ CCTCCGCTTATTGATATGC-3′ primers were used to amplify the ITS region of the pure isolates using extracted gDNA (BIOMICS extraction kit, Zymo Research Corp, Irvine, California) as a template for polymerase chain reaction (PCR) (Adeleke *et al.,* 2010). Amplified genes were visualized using 1% gel electrophoresis. The PCR product containing the amplified ITS genes were sequenced at Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa, using the ABI Big dye V3.1 kit following the manufacturer's instructions and sequenced using the ABI 3500XL genetic analyzer. The sequences were aligned using ClustalW multiple alignment on BioEdit and MAFFT- an online version of a multiple sequence alignment program (Katoh *et al.,* 2019). Operational taxonomic units (OTUs) were generated using MOTHUR (Reintroducing mothur: 10 Years Later – PMC (nih.gov)) to group similar species at a 97% similarity threshold. The representative OTU sequences were then compared to sequences in the GenBank database for identification.

Table 4.1: Media composition for enrichment and isolation of bioleaching microorganisms.

Name	**Composition**		
	General		
Basic composition of bioleaching media	3.0 g/l $(NH_4)_2SO_4$, 0.5 g/l K_2HPO_4, 0.5 g/l $MgSO_4 \cdot 7H_2O$, 0.1 g/l KCl, $FeSO_4 \cdot 7H2O$ or sulfur and 1.5% Agar bacteriological		
Compounds different in each media		**pH**	**References**
Silverman and Lundgren 9 K	0.01 g/l Ca $(NO_3)_2$	1.8	(Chen *et al.,* 2019)
KDM	0.145 g/l NaH_2PO_4, 0.021 g/l $CaCL_2$	1.8	(Martínez et.al., 2012)
Iron-tryptone soya broth (FeTSB)	0.05 g/l Ca $(NO_3)_2$, 0.25 g/l Tryptone soy broth	1.8	(Ilyas *et al.,* 2010)

4.2.2 Screening for organic acid production and metal tolerance.

Fifteen representative fungal isolates (based on OTUs) were screened for their organic acid production and metal tolerance using qualitative and quantitative assays.

4.2.2.1 Qualitative and quantitative assay

The isolates were grown in glucose yeast extract agar (GYEA) supplemented with 1% bromophenol blue indicator for organic acid screening. Five mm mycelial plugs of fresh pure isolates (5-7 days old) were inoculated and incubated at 30°C for 5-7 days. After incubation, the plates were observed for colour change, where a yellow coloration of the medium indicated the production of organic acids (Din *et al.,* 2020). The colony size as well as the yellow zone around the colony were measured using a digital calliper (mm) (RS PRO Pty (Ltd) ltd). Acid Unitage (AU) was calculated using formula 4.1 (Shaikh & Qureshi, 2013). The same 15 isolates were also screened for their tolerance to Al, Zn, Ni and Cr. Potato dextrose agar was used to grow the isolates and after 5 days of incubation at 30°C, the agar plate was covered with sterile dH_2O, and spores were collected. The collected spores were then serially diluted up to 10^7 (vortexing in between) (Krishnamoorthy et al., 2021; Faraji et al., 2018). Ten microliters of 10^7 fungal spore dilution were spread on GYEA media supplemented with 100 mg/l of each heavy metal (Rose & Devi, 2018). Hundred milligram per liter was selected based preliminary screening of few isolates. Metal tolerance was determined by visible growth after 72 hours of incubation at 30 °C.

$$Acid\ Unitage\ (AU) = \frac{Fungi\ zone\ with\ yellow\ halo\ (mm)}{Zone\ with\ Fungi\ (mm)} \qquad (4.1)$$

Fresh fungal isolates were grown in glucose yeast extract broth (GYEB) for 5 days at 30°C in a shaking incubator at 120 rpm. The pH of the medium was adjusted to 5.5. After incubation, the final pH was measured.

- **Metal tolerance and growth rate assays**

Based on the qualitative organic acid assay, metal tolerance and quantitative organic acid assay, isolates were selected for metal tolerance and growth rates assay. The isolates were grown in GYEA supplemented with increasing concentrations of metals as follows: Al from 100 – 1600 mg/l, Zn from 100 -1000 mg/l, Ni from 100-400 mg/l and Cr from 50-210 mg/l. To induce tolerance, each organism was allowed to grow for 5-7 days in one concentration of metal before being transferred to the next concentration. At each concentration, the measured growth diameter (colony zize) from the metal supplemented agar and the controls were used to calculate the tolerance index (TI) using formula 4.2 (Valix and Loon, 2003). TI values were interpreted as follows: a TI of ≥1 signified resistance or high metal tolerance, while a TI below 1 indicated low or no tolerance, implying susceptibility.

$$Tolerance\ Index\ (TI) = \frac{Fungal\ growth\ in\ the\ presence\ of\ metal\ (mm)}{Fungal\ growth\ without\ metal\ exposure\ (mm)} \quad (4.2)$$

Once minimum inhibitory concentration (MIC) of metals was reached, the lowest and highest metal concentration (or the ones that showed susceptibility) were used to study the metal tolerance behaviour, that is, the effect of metals on the growth phases of fungi and growth rates (kd) of each isolate. MIC refers to the minimum heavy metal concentration that entirely inhibits the noticeable growth of microorganisms. In the metal tolerance behaviour and growth rates experiment, fresh fungal mycelia plug (5 mm) was aseptically inoculated onto the centre of sterilized modified GYEA supplemented with each heavy metal. The plates were incubated at 30 °C for 5-7 days. The metal tolerance index was determined by measuring the diameter of the colonies with a digital calliper (RS PRO Pty (Ltd) ltd) daily and used to plot growth phase graph. The growth phase graph was interpreted using Figure 4.1 The *kd* of an individual fungus was determined by following equation 4.3 (Rose & Devi, 2018).

$$kd = D/T \quad (4.3)$$

Where:

D is the average diameter (mm) of the fungal colony (excludes diameter of inoculum, 5 mm), T is time (period) in hours (h).

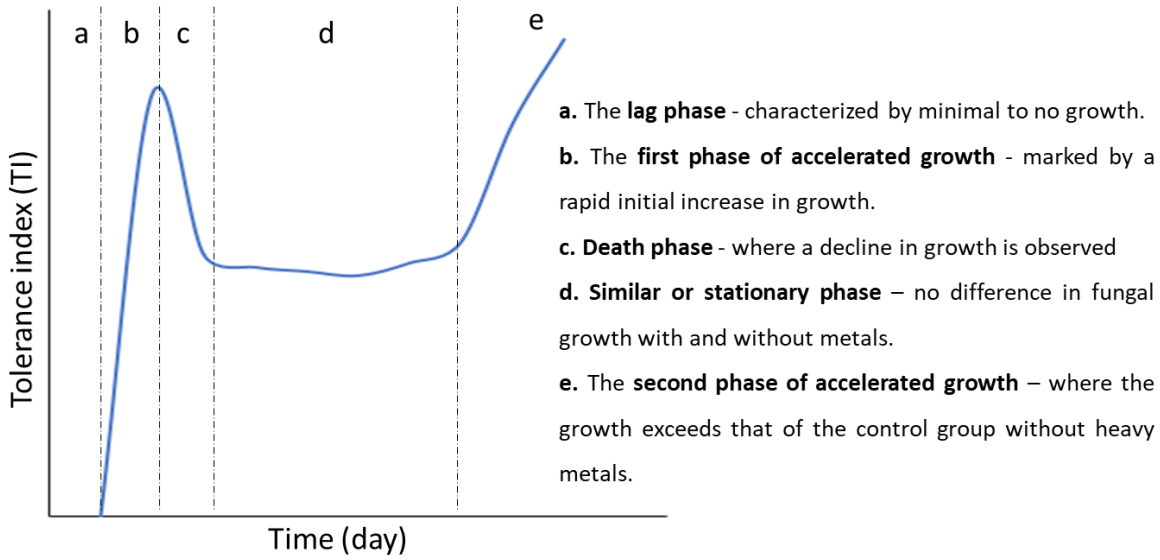

Figure 4.1: Five different growth phases of fungi in the presence of metals. This Figure was adapted and modified from those observed in Rose & Devi, (2018) and Valix *et al.* (2001).

4.2.3 Carbon source for organic acid production

- ***Screening for carbon source***

The ability of the four selected isolates to produce organic acids was further investigated using GYEB. The broth was supplemented with 30 g/l of either glucose, sucrose, or fructose, and the initial pH was adjusted to approximately 5.5 (Barrat *et al.,* 1965; Din *et al.*, 2020). Adjusting the initial pH to 5.5 for all isolates creates a standardized starting point. Five-millimetre mycelial plugs of freshly grown fungal isolates were inoculated into 100 ml broth containing either glucose, sucrose or fructose as the carbon source. The inoculated broth was incubated at 30°C in a shaking incubator at 120 rpm for 5 days. After incubation, a cell free extract was prepared by syringe filtering the fungal culture using 0.2 µm filters. The filtrate was collected in 5 ml tubes to analyze for a decrease in pH and to determine the molarity of produced organic acid. The filtrate was titrated against 0.1 M sodium hydroxide (NaOH) using phenolphthalein indicator (0.5 ml/100 ml). The molarity was determined by the formula below (Din *et al.*, 2020).

$$Ma = \frac{MbVb}{Va} \qquad (4.4)$$

Where: M_a is the unknown molarity of organic acid solution, V_a is the volume of organic acid sample, M_b is the known molarity of NaOH and V_b is the volume of NaOH.

- ***Organic acid and metabolites identification***

The carbon source best utilized by the four isolates was selected based on the results obtained above. The isolates were grown as indicated above, except that 100 g/l of glucose was used. The collected samples were sent to the University of the Witwatersrand, South Africa, for Gas Chromatography–Mass Spectrometry (GC-MS) and High-Performance Liquid Chromatography (HPLC) analysis. The HPLC was conducted as follows: The standard solutions were injected into the HPLC instrument (Agilent 1200 series) followed by the samples. The injection volume was 20 µl, with a flow rate of 1 ml/min, and 0.2 ml of sulfuric acid in 1L of deionized water was used as the mobile phase. The separation of components took place in a Bio-Rad fermentation column at a temperature of 65°C. The separated components were then detected by the Refractive Index column, and the profile was observed. Quantification and detection were performed using Agilent CDS Open Lab software Rev c.01.10 (287). The GC-MS analysis was performed as follows: A 1 µl sample was injected into a Shimadzu GC-MS-QP2010 Ultra instrument using the split injection method. The injector temperature was kept at 260 ˚C, while the oven temperature was held at 60˚C for 3 minutes

before being ramped up to 280˚C and held there for 19 minutes. This resulted in a total run time of 21 minutes. The ion source temperature was kept at 200˚ C.

4.3 Results

4.3.1 Microbial isolation

A total of 60 fungal isolates were obtained from the different enriched tailing samples. Three percent of the isolates were obtained from sulfur containing media, whereas the rest were isolated from iron (II) sulfate containing media. The breakdown percentages of isolates obtained from each media was as follows; GYEA– 67%, YSM – 23%, FeTSB – 5%, 9K – 3%, and KDM – 2%. The sanger sequences of the isolates were clustered into operational taxonomic units (OTUs) at similarity of 97% and 15 different OTUs were obtained, Appendix A4.2 shows morphological characteristics of each of the 15 OTUs. Comparison of the 15 representative sequences with that of NCBI showed 8 different genera with the *Penicillium* genus as the most abundant followed by the genus *Trichoderma* (Appendix A4.3). The representative OTUs, different genera that are grouped together and their frequency as well as accession numbers of each sequence are listed in Appendix A4.3. As mentioned above, *Penicillium* was the most dominant genera with 7 out of the 15 OTUs belonging to this genus. As a result, the OTUs belonging to *Penicillium* were differentiated by a number at the end (e.g., *Penicillium_3*)

4.3.2 Qualitative screening

Two critical qualities for potential bioleaching fungi are organic acid production and metal tolerance (Bahaloo-Horeh *et al.,* 2018; Le *et al.,* 2006). To assess these qualities, the 15 isolates underwent screening. Among these 15 isolates, 12 were found to be positive for organic acid production, as indicated by the formation of a yellow zone on GYEA plates supplemented with bromophenol blue. Notably, the highest AU, exceeding 2, was observed in *Penicillium_2* and *Penicillium_3* (Table 4.2). Further evaluation of organic acid production employed a quantitative assay, wherein the isolates were cultured in GYEB, and the resulting decrease in pH was measured. As depicted in Table 4.2, *Penicillium_6, Penicillium_3*, *Trichoderma, Fusarium,* and *Talaromyces* exhibited a decrease in pH to below 4, with *Talaromyces* recording the lowest at 3.46.

For qualitative metal tolerance screening, growth appearance was used to determine positive results. These results were categorized as follows: visible growth (+), good visible growth (++), and very good visible growth (+++) (Rose & Devi, 2018). Most fungal isolates displayed very

good visible growth when cultivated on GYEA supplemented with 100 mg/l of Al, Zn, and Ni. However, only *Penicillium_6, Penicillium_3*, and *Trichoderma* exhibited positive results for Cr tolerance. Consequently, based on the results of quantitative organic acid assays and qualitative metal tolerance screening, *Trichoderma*, *Talaromyces Penicillium_3* and *Penicillium_6,* were selected for further analysis due to their ability to achieve the lowest pH levels and their tolerance to all five metals. *Talaromyces*, while able to tolerate only four metals, was included due to having the lowest pH. *Fusarium* on the other hand was not included since its growth was inhibited by 100 mg/l of all the tested metals.

Table 4.2: Qualitative and quantitative screening of fungal isolates for organic acids production and metal tolerance at 100 mg/l for Aluminium (Al), Zinc (Zn), Nickel (Ni), and Chromium (Cr). ± represent standard deviation; n=3. Visible growth (+), good visible growth (++), and very good visible growth (+++)

Organism	**Organic acid**				**Metal tolerance screening**				
	Qualitative		**Quantitative**						
	colony zone (mm)	**yellow zone (mm)**	**acid unitage (AU)**	**pH**	Al	Zn	Fe	Ni	Cr
Penicillium_7	27.91±0.39	49.04±0.41	1.75±0.06	6.25±0.10	+	-	-	-	-
Coniochaeta	28.06±0.54	44.68±0.39	1.59±0.03	6.16±0.14	+++	++	-	+++	-
Acidiella	0.00	0.00	0.00	6.15±0.14	+	++	++	+++	-
Penicillium_4	23.91±0.21	44.74±0.11	1.87±0.06	6.01±0.02	+++	++	++	+++	-
Penicillium_1	23.55±0.70	34.42±0.31	1.46±0.03	6.04±0.03	+++	++	++	+++	-
Acidomyces	0.00	0.00	0.00	5.97±0.01	-	-	-	-	-
Penicillium_2	21.76±0.45	44.99±0.60	2.03±0.03	5.88±0.4	+++	++	++	+++	-
Talaromyces_2	25.34±0.56	36.67±0.45	1.45±0.14	5.76±0.02	+++	++	++	+++	-
Fodinomyces	0.00	0.00	0.00	5.53±0.06	++	++	-	++	-
Penicillium_5	26.21±0.09	46.55±0.07	1.77±0.35	5.07±0.0	+++	++	++	+++	-
Fusarium	32.50±0.03	37.75±0.03	1.16±0.05	4.14±0.0	-	-	-	-	-
Penicillium_6	21.50±0.09	28.81±0.08	1.33±0.17	4.16±0.03	+++	++	++	+++	+
Penicillium_3	17.86±0.05	37.97±0.09	2.13±0.11	4.08±0.01	++	++	++	++	++
Trichoderma	35.71±0.15	48.81±0.11	1.37±0.007	3.70±0.003	+++	++	+	+++	++
Talaromyces_1	8.56±0.3	15.62±0.10	1.82±0.02	3.46±0.01	+++	++	++	++	-

4.3.3 Quantitative screening

The four selected isolates underwent a quantitative screening for metal tolerance. The tolerance index (TI) served as the metric to assess the influence of different concentrations of metals, such as Al, Zn, Ni, and Cr, on the growth of *Trichoderma*, *Talaromyces*, *Penicillium_3*, and *Penicillium_6*. TI values were interpreted as follows: a TI of ≥1 signified resistance or high metal tolerance, while a TI below 1 indicated low or no tolerance, implying susceptibility. Comparison among the four isolates revealed that *Trichoderma* exhibited higher tolerance to 300 mg/l of Al and Zn, as well as 100 mg/l of Ni (Figure 4.2). In contrast, the other three isolates demonstrated a TI<1 at 100 mg/l of Al, Zn, and Ni, as well as 50 mg/l of Cr, with *Talaromyces* exhibiting the lowest TI of below 0.6. The cell growth of the isolates was only inhibited at concentrations of 1600 mg/l for Al, 1000 mg/l for Zn, and 400 mg/l for Ni across all four isolates.

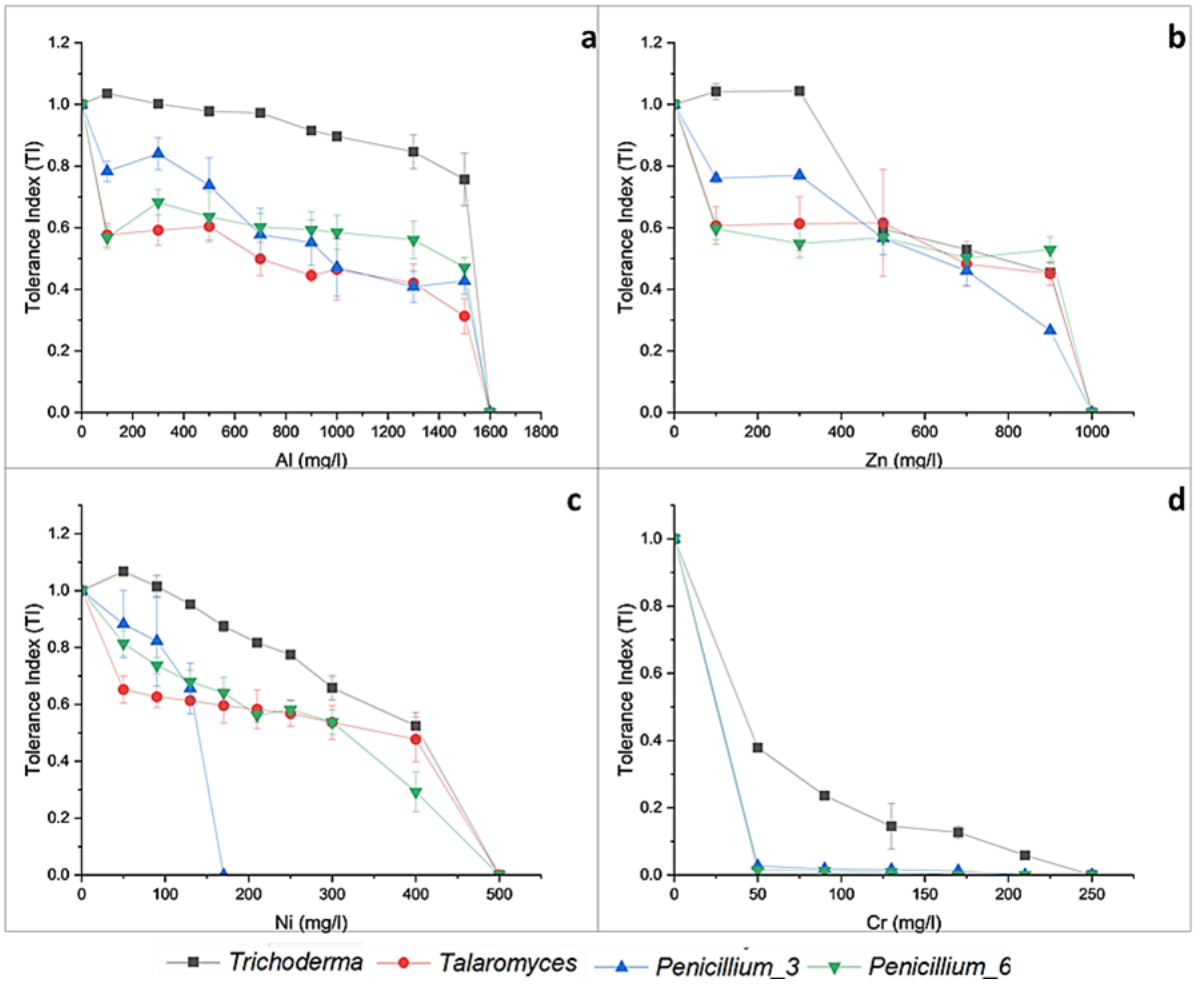

Figure 4.2: Tolerance index of the four selected isolates to increasing concentration of four different metals: **a-** Aluminium, **b-** Zinc, **c-** Nickel and **d –** Chromium.

The growth rates (kd) of the four isolates were assessed as shown in Figure 4.3. It was observed that at 100 mg/l of Al, Zn, and Ni, *Trichoderma* exhibited higher growth rates compared to the control without added metal, while at the highest metal toxicity affected the growth rates of *Trichoderma*. The other three isolates demonstrate lower kd when compared to their controls.

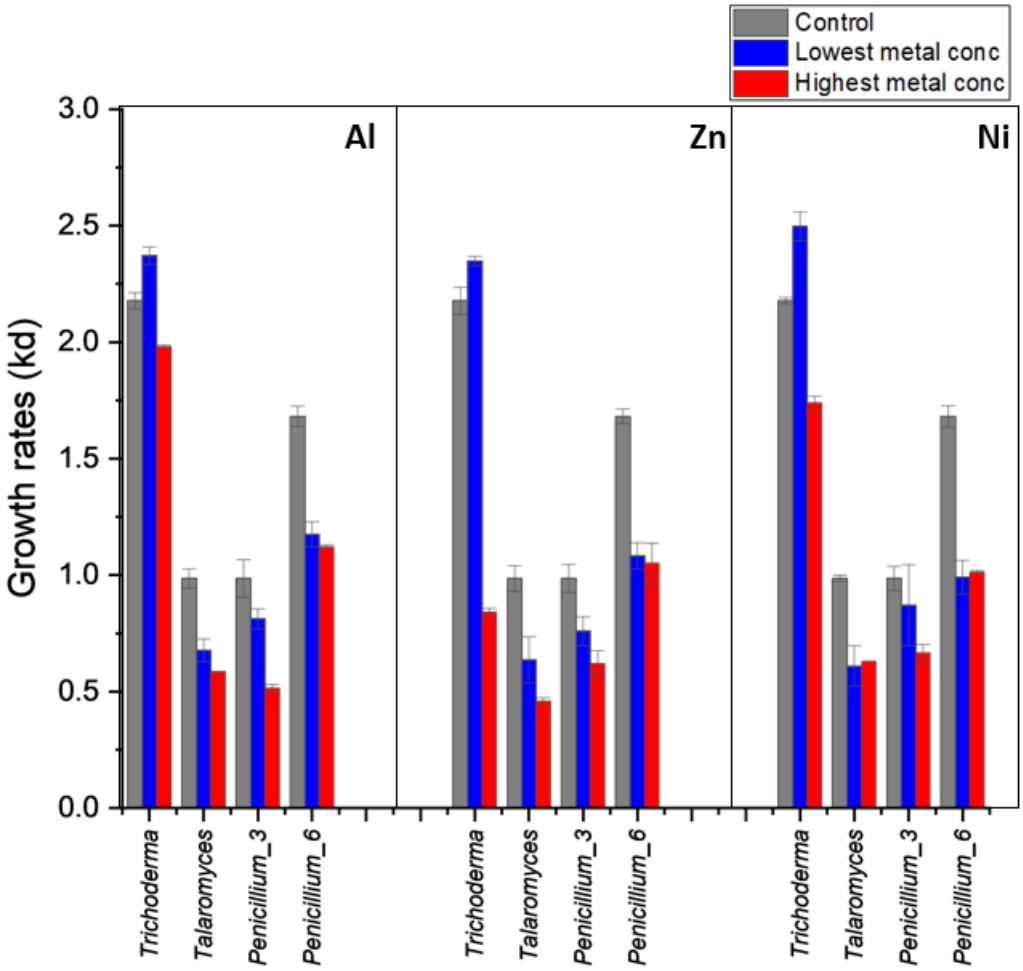

Figure 4.3: Illustrates a comparison of the growth rates of *Trichoderma, Talaromyces*, *Penicillium_3,* and *Penicillium_6* under varying metal concentrations. The growth rate of the cultures was determined by dividing the average diameter measured after 5-7 days by the corresponding time period in hours. Lowest metal concentration (100 mg/l) and the highest metal concentrations (1500 mg/l for Al, 600 mg/l for Zn, and 200 mg/l for Ni). This was in contrast to the control group (no added metal).

4.3.3.1 Effect of heavy metal concentration on fungal growth phases

To efficiently monitor the effect of metals on the growth of fungi, a five-growth phase behavior of fungi under metal stress was used. This growth phase was initially proposed by Valix *et al.*

(2001) and Valix & Loon, (2003) and was also reported by Rose & Devi, (2018). The five phases are indicated in Figure 4.1 and each phase is explained.

To assess the growth behaviour of the four isolates in the presence of metals, two different concentrations (Highest and lowest – which differed per metal) of Al, Zn, Ni, and Cr were used as indicated in Figure 4.2. Ideally, the lowest concentration should be the one at which the organism demonstrates a TI greater than 1. However, this criterion is only applicable to *Trichoderma*. Nevertheless, we still evaluated the effect of metals on the growth rates of the other three organisms, using the initial metal concentration as the lowest. When examining the growth behaviour of *Trichoderma* (Figure 4.4a), it is evident that it does not follow the five-growth phase behaviour for Ni and Zn, both at the lowest and highest concentrations. For Al and Cr, we did observe a semblance of the five-growth phase for *Trichoderma*, where phases b, c, and d were noticeable. In the case of *Talaromyces*, *Penicillium_3*, and *Penicillium_6*, a five-growth phase was observed for most metals at the lowest metal concentration. However, a noteworthy observation is the demonstration of susceptibility through an extended lag phase, especially in *Talaromyces* (Figure 4.4b) and *Penicillium_3* (Figure 4.4c)

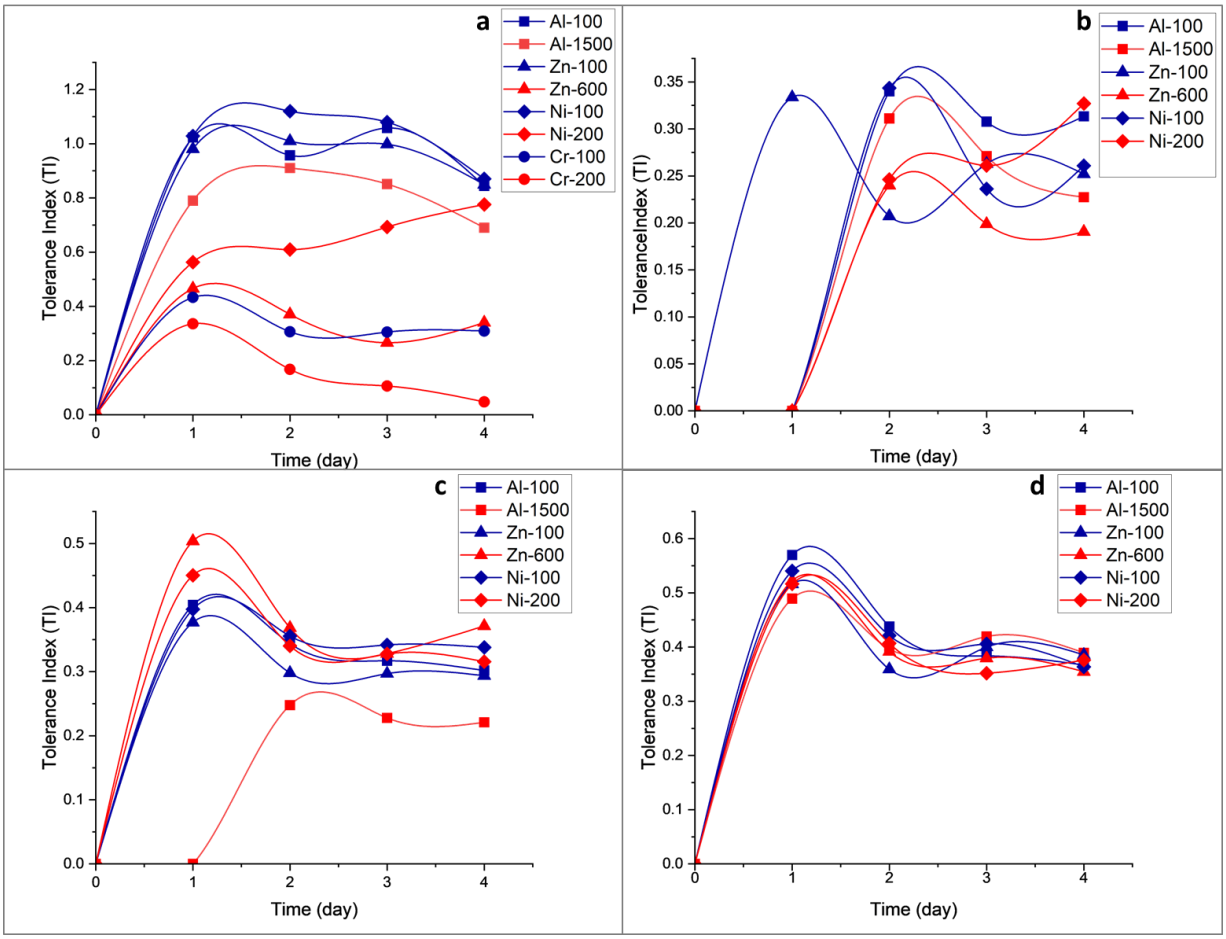

Figure 4.4: Growth phases of selected isolates in the presence of metals. **a**- *Trichoderma*, **b**- *Talaromyces*, **c** - *Penicillium*_**3** and d- *Penicillium_6*.

4.3.3.2 Carbon utilization

To determine the optimal carbon source for organic acid production, the four isolates were cultivated in glucose, sucrose, and fructose. As illustrated in Figure 4.5a, utilization of glucose as a carbon source resulted in the lowest pH for all four isolates, indicating that glucose is the most effectively utilized carbon source by these isolates. When comparing pH to molarity, as shown in Figure 4.5a and b, a significant difference is observed between sucrose and glucose pH levels, especially in *Talaromyces*.

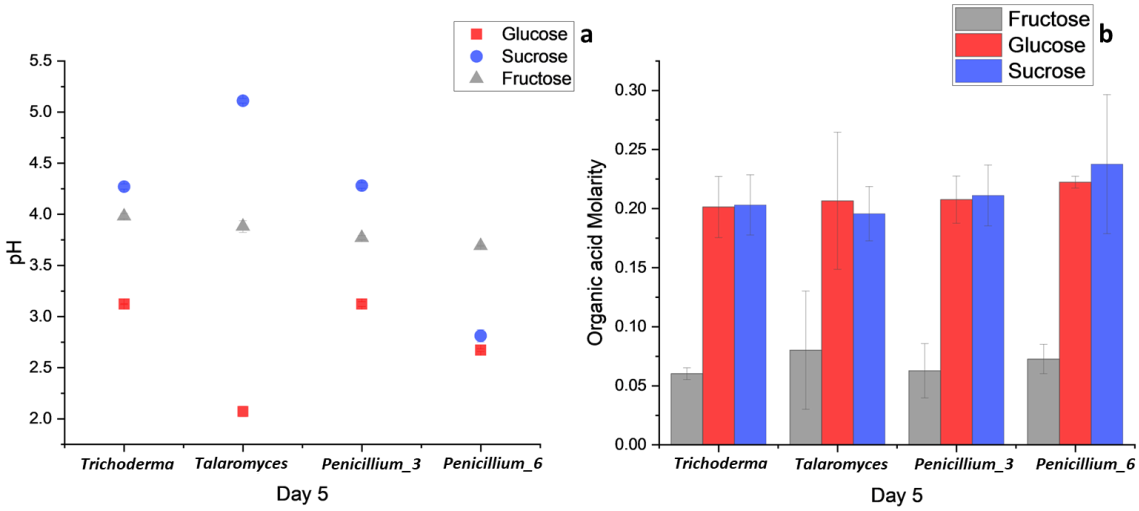

Figure 4.5: Glucose, sucrose and fructose utilization by the four selected isolates. **a**- represents pH decrease after 5 days of incubation, and **b**- represents the calculated molarity of organic acids. Carbon source utilisation is directly linked to production of organic acids which causes a decrease in pH. The use of glucose resulted the lowest pH for *Trichoderma*, *Talaromyces*, *Penicillium_3* and *Penicillium_6*

The isolates were subsequently grown in glucose containing media and samples were collected every other day for 15 days for organic acid and metabolite analysis using HPLC and GC-MS respectively. In Figure 4.6, the pH profiles during the growth of these isolates revealed a difference in the metabolic activities of *Trichoderma* compared to the other isolates. *Trichoderma* exhibited the lowest pH reached within just 5 days of growth, in contrast to day 9 for *Penicillium_6* and day 15 for *Talaromyces* and *Penicillium_3*. The organic acids produced during the growth of these four isolates were identified and quantified using HPLC. Figure 4.7 illustrates that citric acid was the primary organic acid produced by all four isolates, followed

by oxalic acid. Propionic acid was only quantified for T*richoderma*. When comparing citric acid production, it become evident that within 24 hours, *Trichoderma* produced 350 g/l of citric acid, surpassing the other three isolates.

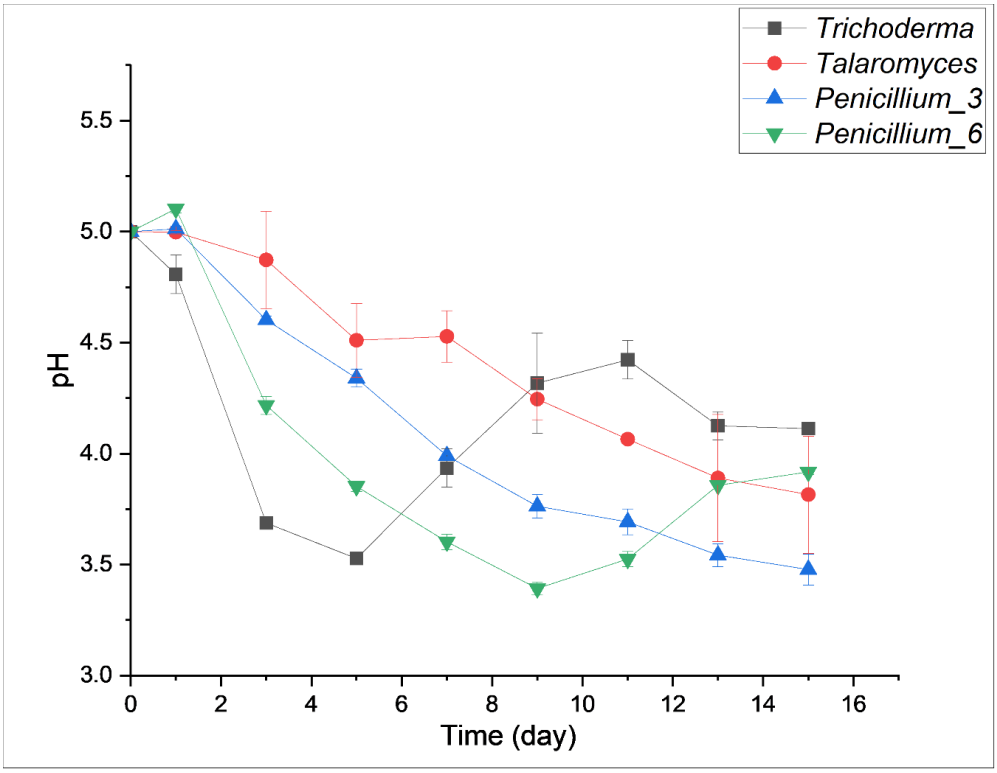

Figure 4.6: Organic acid production by *Trichoderma*, *Talaromyces*, *Penicillium_3* and *Penicillium_6* with glucose as the carbon source after 15 days. Error bars represent ±SD; n=3.

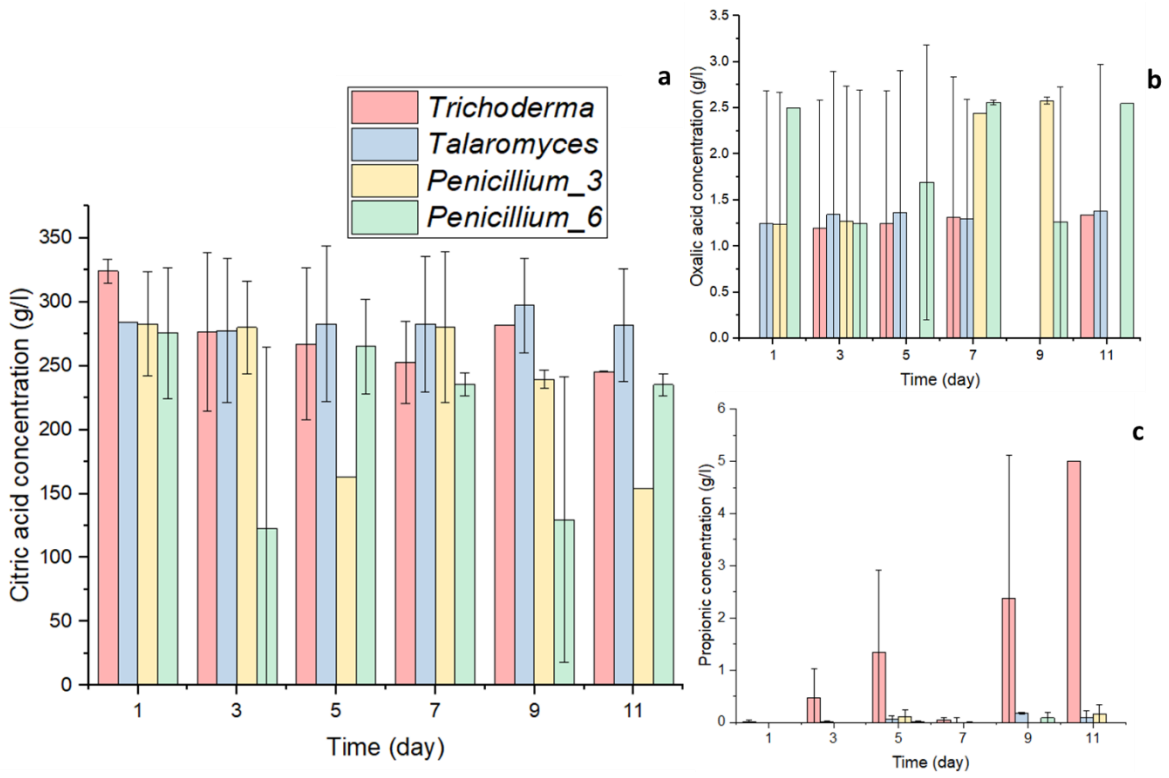

Figure 4.7: Organic acid produced by *Trichoderma, Talaromyces, Penicillium_3* and *Penicillium_6,* quantified using HPLC**. a-** citric acid, **b-** oxalic acid and **c** – propionic acid.

4.3.3.3 Compounds/Metabolites screening

To gain insights into the up-regulated pathways during organic acid production, we employed GC-MS to identify metabolites/compounds contributing to organic acid production, especially citric acid production. The same samples used for the above were analyzed for metabolites/compounds. Figure 4.8 displays the different chemical compounds that were identified. Kyoto Encyclopedia of Genes and Genomes (KEGG) was used for compound identification and determination of the pathways they are related to. Most of the compounds were associated with pathways related to the citric acid cycle (highlighted in blue), lipid biosynthesis (purple), and carbohydrate metabolism (orange). Compounds linked to carbohydrate metabolism were observed to be more abundant in the early growth stages of all four isolates, with *Talaromyces* exhibiting the most pronounced increase.

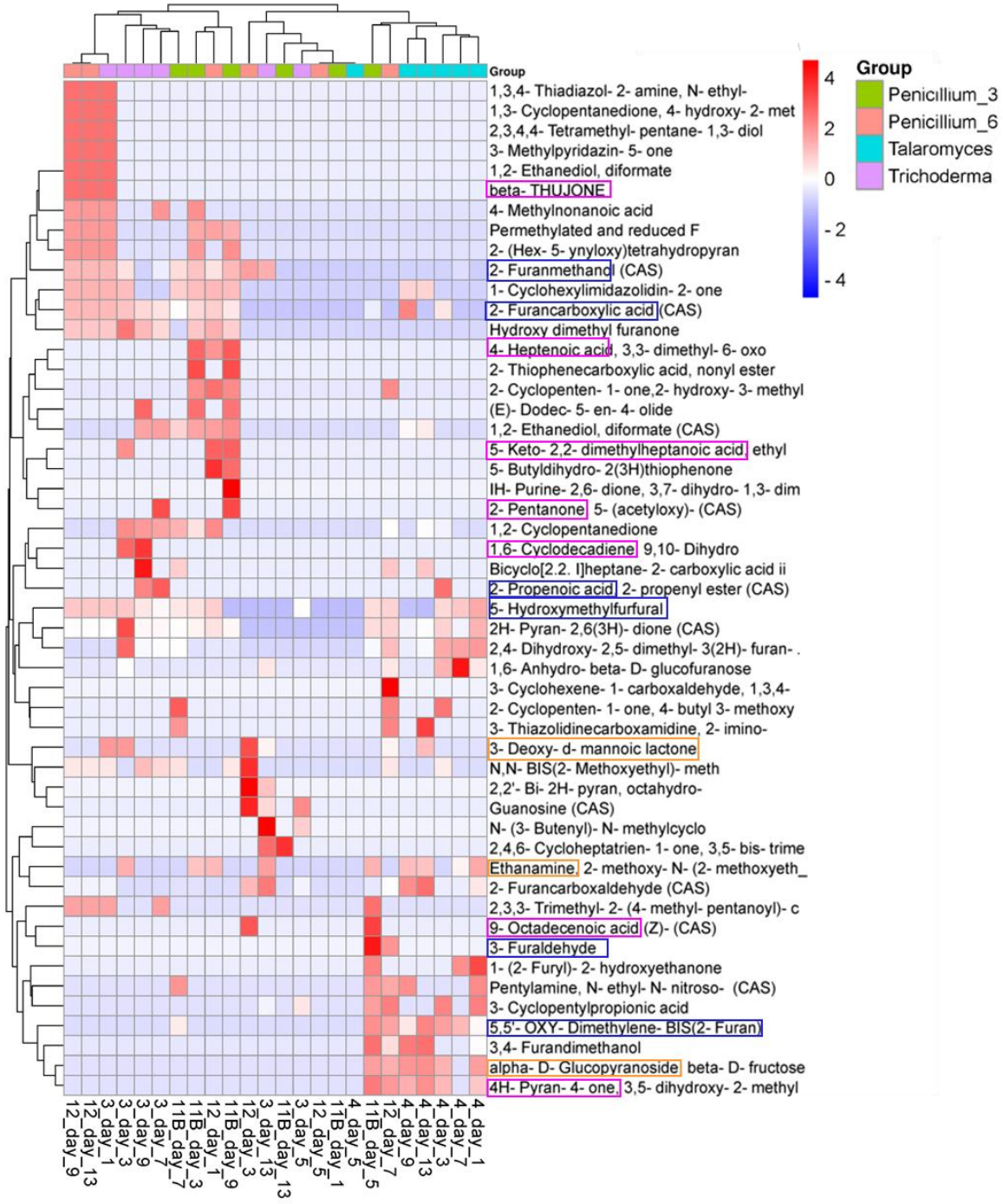

Figure 4.8: The heatmap depicts the average levels of identified metabolites detected during the growth of *Trichoderma*, *Talaromyces*, *Penicillium*_3, and *Penicillium_6*. Columns represent sample collection days for each isolate, while rows represent different metabolites. Metabolites highlighted in blue are associated with the citric acid cycle, those in purple pertain to lipids, and those in orange are related to carbohydrate metabolism.

Compounds that eventually contribute to the citric acid cycle were also observed, which explains why all four isolates produced citric acid as the primary organic acid. These

compounds include 2-Furan, 2-Furanmethanol, 2-Furancarboxylic acid, 3-Furaldehyde and 5-Hydroxymethylfurfural; all of which are furan derivatives except 3-Furaldehyde (Dutta, 2021; der Hoeven *et al.,* 1989). Figure 4.9a shows that they are part of furfural degradation metabolism, as highlighted in red. Three of these compounds were observed to be abundant and were compared among the four isolates, as shown in Figures 4.9b, c and d. *Talaromyces* had the highest abundance of 2-furan, followed by *Penicillium_3*. While 2-Furanmethanol was abundant in all isolates except *Talaromyces*. 5-Hydroxymethylfurfural was abundant in all the four isolates.

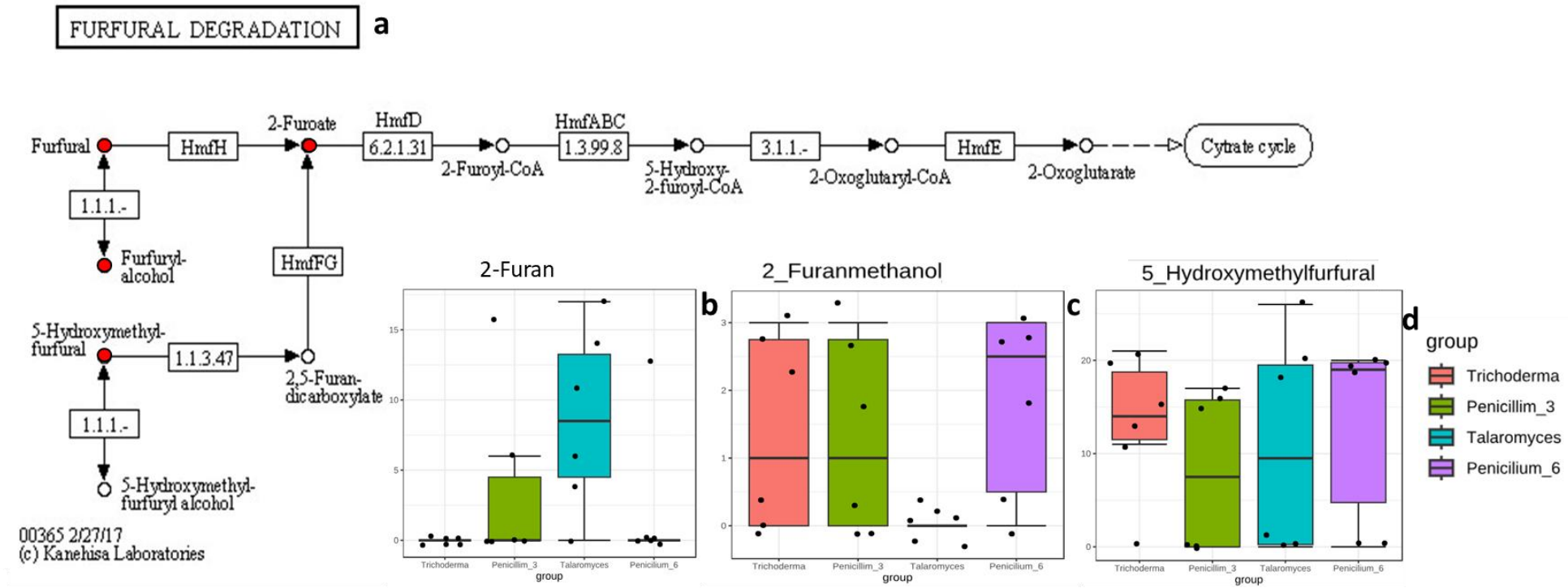

Figure 4.9: a-depicts the furfural degradation pathway, with the four compounds highlighted in red. **b-d**: The Box plots represent the most abundant metabolites and show their comparison among the four isolates, which eventually contributes to the citric acid cycle.

4.4 Discussion

In the current study, mine tailing samples, representing an extreme environment, were used to isolate fungi. Eight different genera were identified and all of them have been reported or linked to bioleaching related studies (Yin *et al.,* 2023; Tansengco *et al.,* 2018; Etemadzadeh *et al.,* 2016; Martinez *et al.,* 2015; Vásquez *et al.,* 2015; Amiri *et al.,* 2011). Thus, confirming the importance of isolating potential bioleaching from extreme environments.

Qualitative and quantitative assay: organic acid and metal tolerance

The ability of a fungi to cause a decrease in pH during growth is linked to the production of organic acid. Thus, the isolates were screened for organic acid production, as one of the most important qualities for potential bioleaching fungi. A lowest pH during the growth of *Trichoderma*, *Talaromyces*, *Penicillium_3* and *Penicillium_6* was observed, and the isolates were selected for further analysis. Of the 4, *Trichoderma* showed higher metal tolerance (Figure 4.2). Its growth at 300 mg/l of Al and Zn, as well as 100 mg/l of Ni exceeded that of the controls without metals, suggesting an enhanced metal tolerance ability, possibly due to various mechanisms that allow the fungus to thrive in the presence of these metals such as metal uptake and sequestration as well as stress responses (Moore *et al.,* 2008; Bellion *et al.,* 2006). A TI of less than 1 was observed for *Talaromyces, Penicillium_3* and *Penicillium_6.* This implies that these isolates are susceptible to the toxicity of the metals. At TI<1 a sublethal effect was observed since the organisms did not die but showed characteristics of impaired growth as compared to the controls (Appendix A4.5), which may result from reduced or impaired metabolic functions (Lobos *et al.,* 2021). It is important to note that petri dish assays have limitations regarding biomass production. For example, fungal growth may primarily focus on hyphal extension with limited biomass accumulation. This makes it challenging to accurately assess the effect of metals on growth. For more accurate measurements, broth can be used instead of agar, where dried cell weight can be used as a measure (Alavi et al., 2021; Bahaloo-Horeh et al., 2018). While the growth of all four isolates was affected as metal concentration increased, cell growth was not inhibited until extremely high concentration (such as 1600 mg/l of Al) was used. This indicates the acclimatization potential of the isolates, meaning that the four isolates may adapt to even higher concentrations of the metals over time. This acclimatization effect was reported by Yang *et al.* (2009), where the two un-adapted *A. niger* strains were susceptible to Al and Fe, however after six months of adaptation, the

organism could tolerate up to 3500 mg/l Al and 700 mg/l Fe. This acclimatization potential can also be represented by the near zero TI for *Peniccilim*_3 and 6 when exposed to Cr.

Growth rates and effects of metal on fungal growth phases

In bioleaching, one of the challenges lies in the slow kinetics, which can be attributed to the growth and activities of fungi. Therefore, identifying isolates with high growth rates under metal exposure is crucial. From the growth rate results, it was observed that *Trichoderma* exhibited higher growth rates compared to the control without added metal, while the opposite trend was noticed at the highest metal concentrations (Figure 4.3). According to Bahaloo-Horeh *et al.* (2018) and Jang & Valix (2017), microorganisms tend to respond to heavy metals in a biphasic manner, with low metal concentrations stimulating growth and higher metal concentrations inhibiting it. The high growth rates exhibited by *Trichoderma* at metal concentrations where it has a TI >1 indicate its suitability as a bioleaching candidate.

A five-growth phase behaviour of fungi under metal stress was used to understand the effect of metals on the fungal growth (Figure 4.1). For a fungus to exhibit good tolerance towards a specific metal, it should demonstrate a higher TI (>1) at a similar growth phase, in this case, phase d. However, *Trichoderma* which demonstrated TI>1did not follow the five-growth phase behaviour while others did (Figure 4.4). In a study by Rose & Devi (2018), this observation was reported at a metal concentration in which the organism has TI <1, however in our results, this was observed at TI >1 and TI <1 for *Trichoderma* and at TI< 1 for the other isolates. According to Valix & Loon (2003), a higher TI in fungi is linked to the initial rapid fungal growth seen in phase b and the relatively low death rate noted in phase c. Ther other three isolates had a TI<1 even at the lowest concentration and this can be attributed to the accelerated growth in phase b while in phase c a continuous decline in growth rate was observed. Furthermore, a prolonged lag phase as seen in *Talaromyces* and *Penicillium_3* also demonstrate the susceptibility of these isolates to metal where their growth rates and activity are affected.

Carbon source utilisation: glucose, sucrose and fructose

Organic acid production is mainly dependent on availability of carbon source and from literature it is important to identify carbon source best utilized by the organism in question (Shen *et al.,* 2023; Din *et al.,* 2020; Chaerun *et al.,* 2017). In addition to using pH, calculated molarity of organic acid was used to select the best utilized carbon source. However, there was no substantial difference in terms of the calculated molarity between the different carbon sources used while pH difference was observed (Figure 4.5). This suggests that the molarity of

the acids produced may not scale directly with pH. This phenomenon is likely due to the production of multiple acids by each organism when utilizing different carbon sources. Each acid has a unique effect on pH, and when titrated, some acids will be monoprotic, while others are weak acids, only partially dissociating (Park *et al.*, 2017). Nevertheless, regardless of their identity and characteristics, all acids will still have an effect on pH. In this case based on the lowest pH observed, glucose was selected as the best utilized carbon source.

Comparison of organic acid production using glucose as the carbon source

Comparison of the 4 isolates showed that *Trichoderma* reached the lowest pH at the lowest time (Figure 4.6). This rapid decline in pH is indicative of *Trichoderma*'s rapid metabolic activities, suggesting that the provided carbon source was rapidly converted into organic acids and other metabolic products. Our findings align with a study conducted by Shah *et al.* (2020), who investigated pH and organic acid produced during the growth of *A. niger*. Their study reported a similar phenomenon, where an increase in *A. niger'*s biomass coincided with a decrease in pH. Notably, in this study by Shah et al. (2020), a strong negative correlation between biomass and pH was observed, highlighting the important role of metabolic activities in driving the production of organic acids. Using HPLC, citric acid, oxalic acid and propionic acids were quantified (Figure 4.7). Comparison of citric acid, which was produced as the main organic acid, showed that the highest concentration was reached by *Trichoderma* in a shortest time. This further confirms *Trichoderma*'s fast metabolism, which also explains the quicker attainment of the lowest pH. A fast metabolism implies that *Trichoderma* was able to utilize the carbon source within five days, as evidenced by the observed increase in pH after day 5. This increase may be due to a shift in metabolic processes from producing organic acids to utilizing them for energy or the production of secondary metabolites hence the rise in pH after day 5. Subsequently, the citric acid concentration in the other three isolates exceeded that of *Trichoderma* after day 7, elucidating why their lowest pH was observed after day 9. When it came to oxalic acid, *Penicillium_3* and *Penicillium_6* recorded the highest oxalic acid produced.

The purpose of this study was to screen fungal isolates for their bioleaching potential. In bioleaching, organic acids serve as the primary leaching agents, with citric and oxalic acid being major contributors through processes such as acidolysis and complexolysis, which involve chelation reactions (Dusengemungu *et al.*, 2021; Pathak *et al.*, 2021b). Citric acid is typically generated via the citric acid cycle, whereas oxalic acid and propionic acid follow

distinct pathways. The prevalence of citric acid production across all isolates suggests their metabolic processes are inclined toward the citric acid cycle. This alignment with the citric acid cycle could be attributed to its role in cellular respiration, the primary energy production pathway. GC-MS was used to identify metabolite/compound produced during the production of organic acids listed above, mainly citric acid (Figure 4.8). The higher levels of carbohydrate metabolites produced in the early growth stages were observed, which may be connected to biomass formation and energy production, crucial for cell functioning (Zuo *et al.,* 2021). Additionally, pathways leading to lipid metabolite production, primarily fatty acyls, were also abundant. Lipids are primarily associated with energy storage (Lin & Heitman, 2005). Since the isolates were grown glucose as a carbon source, excess glucose could have been converted into fatty acids and stored as lipids for later ATP generation.

Compounds such as 2-Furan, 2-Furanmethanol and 5-Hydroxymethylfurfural were found in abundance (Figure 4.9). 2-Furan also known as furan, is a compound biologically produced by fungi as a secondary metabolite, which functions among others as a defence mechanism (Chen *et al.,* 2021; Luo *et al.,* 2017). 2-Furanmethanol is also a secondary metabolite with antimicrobial activities (Al-Rashdi *et al.,* 2022). 5-Hydroxymethylfurfural on the other hand is known for its antioxidant activities, that is, providing protection to cells against free radicals (Zhu *et al.,* 2022). The question arises as to why these fungal isolates produced these compounds in abundance. One possible explanation may be due to excess carbon influx, where the high concentration of glucose led to a metabolic overflow (Legiša & Mattey, 2007). In such a scenario, the excess carbon from metabolic pathways like glycolysis and gluconeogenesis could surpass the capacity of downstream pathways such as the citric acid cycle. Consequently, a metabolic diversion will occur, resulting in the production of these compounds in addition to lipid metabolism (Liu *et al.,* 2023; Morin *et al.,* 2011). As illustrated in Figure 4.9a, they are involved in furfural degradation metabolism, ultimately contributing to the citric acid cycle (citrate cycle). This was explained in a metabolic analysis study of *Penicillium* by Yang *et al.* (2021), stating that the increase of 2-Furoic acid in the furfural degradation pathway, produces more α-ketoglutarate (also known as oxoglutarate), which is an important intermediate in citric acid cycle. Thus, it may represent another metabolic pathway related to energy storage in fungi.

4.5 Conclusion

This study identified *Trichoderma* as a promising candidate for bioleaching due to its metal tolerance, fast growth rates, and organic acid production. Metabolic profiling of the four fungal isolates revealed that the citric acid cycle is the predominant pathway involved in organic acid production, with glucose as the carbon source. Other pathways related to lipid biosynthesis and carbohydrate metabolism also play important roles. The production of compounds that are part of furfural degradation metabolism may represent another metabolic pathway related to energy storage in fungi.

Although the three non-*Trichoderma* isolates showed low metal tolerance, they may still be useful for indirect/spent media bioleaching, where only their organic acids interact with the metals. Overall, bioleaching experiments are required to test the organisms' bioleaching ability, whether through direct bioleaching in the case of *Trichoderma* or spent media bioleaching for the other three isolates.

Future studies

In the future, researchers may further explore the molecular mechanisms that are involved in bioleaching processes with the fungal isolates that have been identified. Transcriptomic, proteomic, or metabolomic analyses to can be used to identify and characterize specific genes, proteins, and metabolites that play a role in metal tolerance, organic acid production, and the overall efficiency of bioleaching. Optimizing the conditions for bioleaching is necessary in order to improve the efficiency of fungal isolates. This may involve optimizing factors such as pH, temperature, nutrient levels, and metal concentrations to increase the production of organic acids and solubilization of metals.

Beyond bioleaching, the organic acids produced by the fungal isolates may have potential applications in other biotechnological processes. Further research could investigate the possibility of using these organic acids for metal recovery from electronic waste, soil remediation, or as starting materials for creating more valuable chemicals.

4.6 References

Adeleke, Rasheed, Eugene Cloete, and D. K. (2010). Isolation and identification of iron ore - solubilising fungus. *South African Journal of Science*, *106*(9), 1–6. https://doi.org/10.4102/sajs.v106i9/10.254

Alavi, N., Partovi, K., Majlessi, M., Rashidi, M., & Alimohammadi, M. (2021). Bioleaching of metals from cellphones batteries by a co-fungus medium in presence of carbon materials. *Bioresource Technology Reports*, *15*. https://doi.org/10.1016/j.biteb.2021.100768

Al-Rashdi, F. K. H., Al-Sadi, A. M., Al-Riyamy, B. Z., Maharachchikumbura, S. S. N., Al-Sabahi, J. N., & Velazhahan, R. (2022). Endophytic fungi from the medicinal plant Aloe dhufarensis Lavranos exhibit antagonistic potential against phytopathogenic fungi. *South African Journal of Botany*, *147*, 1078–1085. https://doi.org/10.1016/j.sajb.2020.05.022

Amiri, F., Mousavi, S. M., & Yaghmaei, S. (2011). Enhancement of bioleaching of a spent Ni/Mo hydroprocessing catalyst by Penicillium simplicissimum. *Separation and Purification Technology*, *80*(3), 566–576. https://doi.org/10.1016/j.seppur.2011.06.012

Anahid, S., Yaghmaei, S., & Ghobadinejad, Z. (2011). Heavy metal tolerance of fungi. *Scientia Iranica*, *18*(3 C), 502–508. https://doi.org/10.1016/j.scient.2011.05.015

Bahaloo-Horeh, N., Mousavi, S. M., & Baniasadi, M. (2018). Use of adapted metal tolerant Aspergillus niger to enhance bioleaching efficiency of valuable metals from spent lithium-ion mobile phone batteries. *Journal of Cleaner Production*, *197*, 1546–1557. https://doi.org/10.1016/j.jclepro.2018.06.299

Barratt RW, Johnson GB, Ogata WN. (1965). Wild-type and mutant stocks of Aspergillus nidulans. *Genetics 52*(1):233–246

Bellion, M., Courbot, M., Jacob, C., Blaudez, D., & Chalot, M. (2006). Extracellular and cellular mechanisms sustaining metal tolerance in ectomycorrhizal fungi. In *FEMS Microbiology Letters* (Vol. 254, Issue 2, pp. 173–181). https://doi.org/10.1111/j.1574-6968.2005.00044.x

Brisson, V. L., Zhuang, W. Q., & Alvarez-Cohen, L. (2020). Metabolomic Analysis Reveals Contributions of Citric and Citramalic Acids to Rare Earth Bioleaching by a Paecilomyces Fungus. *Frontiers in Microbiology*, *10*. https://doi.org/10.3389/fmicb.2019.03008

Brown, R. M., Mirkouei, A., Reed, D., & Thompson, V. (2023). Current nature-based biological practices for rare earth elements extraction and recovery: Bioleaching and biosorption. In

Renewable and Sustainable Energy Reviews (Vol. 173). Elsevier Ltd. https://doi.org/10.1016/j.rser.2022.113099

Chaerun, S. K., Sulistyo, R. S., Minwal, W. P., & Mubarok, M. Z. (2017). Indirect bioleaching of low-grade nickel limonite and saprolite ores using fungal metabolic organic acids generated by Aspergillus niger. *Hydrometallurgy*, *174*, 29–37. https://doi.org/10.1016/j.hydromet.2017.08.006

Chen, A., Hao, X., Xiao, Y., Zou, K., Liu, H., & Liu, X. (2019). *Responses of Acidithiobacillus thiooxidans A01 to Individual and Joint Nickel (Ni 2 +) and Ferric (Fe 3 +)*. 1–12. https://doi.org/10.3390/min9020082

Chen, S., Cai, R., Liu, Z., Cui, H., & She, Z. (2021). Secondary metabolites from mangrove-associated fungi: source, chemistry and bioactivities. In *Natural Product Reports* (Vol. 39, Issue 3, pp. 560–595). Royal Society of Chemistry. https://doi.org/10.1039/d1np00041a

der Hoeven, van, M Mutat, J. C., & Kallio, H. (1989). Food Chem. In *Gazz. Chim. Ital* (Vol. 37). https://pubs.acs.org/sharingguidelines

Din, G., Hassan, A., Rafiq, M., Hasan, F., Badshah, M., Khan, S., Chen, G., Ripp, S., & Shah, A. A. (2020). Characterization of Organic Acid Producing Aspergillus tubingensis FMS1 and its Role in Metals Leaching from Soil. *Geomicrobiology Journal*, *37*(4), 336–344. https://doi.org/10.1080/01490451.2019.1701585

Dusengemungu, L., Kasali, G., Gwanama, C., & Mubemba, B. (2021). Overview of fungal bioleaching of metals. *Environmental Advances*, *5*, 100083. https://doi.org/10.1016/j.envadv.2021.100083

Dutta, S. (2021). Valorization of biomass-derived furfurals: reactivity patterns, synthetic strategies, and applications. In *Biomass Conversion and Biorefinery*. Springer Science and Business Media Deutschland GmbH. https://doi.org/10.1007/s13399-021-01924-w

Etemadzadeh, S. S., Emtiazi, G., & Etemadifar, Z. (2016). Heterotrophic Bioleaching of Sulfur, Iron, and Silicon Impurities from Coal by Fusarium oxysporum FE and Exophiala spinifera FM with Growing and Resting Cells. *Current Microbiology*, *72*(6), 707–715. https://doi.org/10.1007/s00284-016-1008-x

Faraji, F., Golmohammadzadeh, R., Rashchi, F., & Alimardani, N. (2018). Fungal bioleaching of WPCBs using Aspergillus niger: Observation, optimization and kinetics. *Journal of Environmental Management*, *217*, 775–787. https://doi.org/10.1016/j.jenvman.2018.04.043

Ilyas, S., Bhatti, H. N., Bhatti, I. A., Sheikh, M. A., & Ghauri, A. (2010). Bioleaching of metal ions from low grade sulphide ore : Process optimization by using orthogonal experimental array design. *African Journal of Biotechnology*, *9*(19), 2801–2810. https://doi.org/10.5897/AJB10.026

Jang, H. C., & Valix, M. (2017). Overcoming the bacteriostatic effects of heavy metals on Acidithiobacillus thiooxidans for direct bioleaching of saprolitic Ni laterite ores. *Hydrometallurgy*, *168*, 21–25. https://doi.org/10.1016/j.hydromet.2016.08.016

Katoh K, Rozewicki J, Yamada KD. (2019). MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. *Brief Bioinform*. *19;20*(4):1160-1166. doi: 10.1093/bib/bbx108.

Keshavarz, S., Faraji, F., Rashchi, F., & Mokmeli, M. (2021). Bioleaching of manganese from a low-grade pyrolusite ore using Aspergillus niger: Process optimization and kinetic studies. *Journal of Environmental Management*, *285*. https://doi.org/10.1016/j.jenvman.2021.112153

Kolenčík, M., Urík, M., Čerňanský, S., Molnárová, M., & Matúš, P. (2013). *LEACHING OF ZINC, CADMIUM, LEAD AND COPPER FROM ELECTRONIC SCRAP USING ORGANIC ACIDS AND THE ASPERGILLUS NIGER STRAIN*.

Le, L., Tang, J., Ryan, D., & Valix, M. (2006). Bioleaching nickel laterite ores using multi-metal tolerant Aspergillus foetidus organism. *Minerals Engineering*, *19*(12), 1259–1265. https://doi.org/10.1016/j.mineng.2006.02.006

Legiša, M., & Mattey, M. (2007). Changes in primary metabolism leading to citric acid overflow in Aspergillus niger. In *Biotechnology Letters* (Vol. 29, Issue 2, pp. 181–190). https://doi.org/10.1007/s10529-006-9235-z

Lin, X., & Heitman, J. (2005). Chlamydospore formation during hyphal growth in Cryptococcus neoformans. *Eukaryotic Cell*, *4*(10), 1746–1754. https://doi.org/10.1128/EC.4.10.1746-1754.2005

Liu, H., Jin, Y., Zhang, R., Ning, Y., Yu, Y., Xu, P., Deng, L., & Wang, F. (2023). Recent advances and perspectives on production of value-added organic acids through metabolic engineering.

In *Biotechnology Advances* (Vol. 62). Elsevier Inc. https://doi.org/10.1016/j.biotechadv.2022.108076

Lobos, A., Harwood, V. J., Scott, K. M., & Cunningham, J. A. (2021). Tolerance of three fungal species to lithium and cobalt: Implications for bioleaching of spent rechargeable Li-ion batteries. *Journal of Applied Microbiology*, *131*(2), 743–755. https://doi.org/10.1111/jam.14947

Luo, Z., Ren, H., Mousa, J. J., Rangel, D. E. N., Zhang, Y., Bruner, S. D., & Keyhani, N. O. (2017). The PacC transcription factor regulates secondary metabolite production and stress response, but has only minor effects on virulence in the insect pathogenic fungus Beauveria bassiana. *Environmental Microbiology*, *19*(2), 788–802. https://doi.org/10.1111/1462-2920.13648

Mani, D., & Kumar, C. (2014). Biotechnological advances in bioremediation of heavy metals contaminated ecosystems: An overview with special reference to phytoremediation. In *International Journal of Environmental Science and Technology* (Vol. 11, Issue 3, pp. 843–872). Center for Environmental and Energy Research and Studies. https://doi.org/10.1007/s13762-013-0299-8

Martínez P, Gálvez S, Ohtsuka N, Budinich M, Cortés MP, Serpell C, Nakahigashi K, Hirayama A, Tomita M, Soga T, Martínez S, Maass A, P. P. (2012). Metabolomic study of Chilean biomining bacteria Acidithiobacillus ferrooxidans strain Wenelen and Acidithiobacillus thiooxidans strain Licanantay. *Metabolomics*, *9*(1), 247–257. https://doi.org/10.1007/s11306-012-0443-3

Martinez, P., Vera, M., Bobadilla-Fazzini, R. A., Haghshenas, D. F., Alamdari, E. K., Bonakdarpour, B., Darvishi, D., Nasernejad, B., Elissawy, A., Ebada, S. S., & Ashour, M. L. (2015). Omics on bioleaching: current and future impacts. *Applied Microbiology and Biotechnology*, *99*(20), 8337–8350. https://doi.org/10.1007/s00253-015-6903-8

Mishra, S., Panda, S., Akcil, A., & Dembele, S. (2023). Biotechnological Avenues in Mineral Processing: Fundamentals, Applications and Advances in Bioleaching and Bio-beneficiation. In *Mineral Processing and Extractive Metallurgy Review* (Vol. 44, Issue 1, pp. 22–51). Taylor and Francis Ltd. https://doi.org/10.1080/08827508.2021.1998043

Moazzam, P., Boroumand, Y., Rabiei, P., Baghbaderani, S. S., Mokarian, P., Mohagheghian, F., Mohammed, L. J., & Razmjou, A. (2021). Lithium bioleaching: An emerging approach for the

recovery of Li from spent lithium ion batteries. In *Chemosphere* (Vol. 277). Elsevier Ltd. https://doi.org/10.1016/j.chemosphere.2021.130196

Moore, B. A., Duncan, J. R., & Burgess, J. E. (2008). Fungal bioaccumulation of copper, nickel, gold and platinum. *Minerals Engineering*, *21*(1), 55–60. https://doi.org/10.1016/j.mineng.2007.08.005

Morin, N., Cescut, J., Beopoulos, A., Lelandais, G., Le Berre, V., Uribelarrea, J. L., Molina-Jouve, C., & Nicaud, J. M. (2011). Transcriptomic analyses during the transition from biomass production to lipid accumulation in the oleaginous yeast Yarrowia lipolytica. *PLoS ONE*, *6*(11). https://doi.org/10.1371/journal.pone.0027966

Muddanna, M. H., & Baral, S. S. (2019). Leaching of nickel and vanadium from the spent fluid catalytic cracking catalyst by reconnoitering the potential of Aspergillus niger associating with chemical leaching. *Journal of Environmental Chemical Engineering*, *7*(2). https://doi.org/10.1016/j.jece.2019.103025

Narayanasamy, M., Dhanasekaran, D., Vinothini, G., & Thajuddin, N. (2018). Extraction and recovery of precious metals from electronic waste printed circuit boards by bioleaching acidophilic fungi. *International Journal of Environmental Science and Technology*, *15*(1), 119–132. https://doi.org/10.1007/s13762-017-1372-5

Nkuna, R., Ijoma, G. N., & Matambo, T. S. (2022). Applying EDTA in Chelating Excess Metal Ions to Improve Downstream DNA Recovery from Mine Tailings for Long-Read Amplicon Sequencing of Acidophilic Fungi Communities. *Journal of Fungi*, *8*(5). https://doi.org/10.3390/jof8050419

Park, L. K. E., Liu, J., Yiacoumi, S., Borole, A. P., & Tsouris, C. (2017). Contribution of acidic components to the total acid number (TAN) of bio-oil. *Fuel*, *200*, 171–181. https://doi.org/10.1016/j.fuel.2017.03.022

Pathak, A., Kothari, R., Vinoba, M., Habibi, N., & Tyagi, V. V. (2021b). Fungal bioleaching of metals from refinery spent catalysts: A critical review of current research, challenges, and future directions. *Journal of Environmental Management*, *280*(November 2020), 111789. https://doi.org/10.1016/j.jenvman.2020.111789

Pathak, A., Morrison, L., & Healy, M. G. (2017). Catalytic potential of selected metal ions for bioleaching, and potential techno-economic and environmental issues: A critical review. *Bioresource Technology*, *229*, 211–221. https://doi.org/10.1016/j.biortech.2017.01.001

Pathak, A., Vinoba, M., & Kothari, R. (2021a). Emerging role of organic acids in leaching of valuable metals from refinery-spent hydroprocessing catalysts, and potential techno-economic challenges: A review. *Critical Reviews in Environmental Science and Technology*, *51*(1), 1–43. https://doi.org/10.1080/10643389.2019.1709399

Qayyum, S., Meng, K., Pervez, S., Nawaz, F., & Peng, C. (2019). Optimization of pH, temperature and carbon source for bioleaching of heavy metals by Aspergillus flavus isolated from contaminated soil. *Main Group Metal Chemistry*, *42*(1), 1–7. https://doi.org/10.1515/mgmc-2018-0038

Rose, P. K., & Devi, R. (2018). Heavy metal tolerance and adaptability assessment of indigenous filamentous fungi isolated from industrial wastewater and sludge samples. *Beni-Suef University Journal of Basic and Applied Sciences*, *7*(4), 688–694. https://doi.org/10.1016/j.bjbas.2018.08.001

Roy, J. J., Cao, B., & Madhavi, S. (2021). A review on the recycling of spent lithium-ion batteries (LIBs) by the bioleaching approach. In *Chemosphere* (Vol. 282). Elsevier Ltd. https://doi.org/10.1016/j.chemosphere.2021.130944

Russo, F., Ceci, A., Pinzari, F., Siciliano, A., Guida, M., Malusà, E., Tartanus, M., Miszczak, A., Maggi, O., & Persiani, A. M. (2019). Bioremediation of dichlorodiphenyltrichloroethane (DDT)- contaminated agricultural soils: Potential of two autochthonous saprotrophic fungal strains. *Applied and Environmental Microbiology*, *85*(21). https://doi.org/10.1128/AEM.01720-19

Sajjad, W., Zheng, G., Din, G., Ma, X., Rafiq, M., & Xu, W. (2019). Metals Extraction from Sulfide Ores with Microorganisms: The Bioleaching Technology and Recent Developments. In *Transactions of the Indian Institute of Metals* (Vol. 72, Issue 3, pp. 559–579). Springer. https://doi.org/10.1007/s12666-018-1516-4

Santhiya, D., & Ting, Y. P. (2005). Bioleaching of spent refinery processing catalyst using Aspergillus niger with high-yield oxalic acid. *Journal of Biotechnology*, *116*(2), 171–184. https://doi.org/10.1016/j.jbiotec.2004.10.011

Sarkodie, E. K., Jiang, L., Li, K., Yang, J., Guo, Z., Shi, J., Deng, Y., Liu, H., Jiang, H., Liang, Y., Yin, H., & Liu, X. (2022). A review on the bioleaching of toxic metal(loid)s from contaminated soil: Insight into the mechanism of action and the role of influencing factors. In *Frontiers in Microbiology* (Vol. 13). Frontiers Media S.A. https://doi.org/10.3389/fmicb.2022.1049277

Shah, S. S., Palmieri, M. C., Sponchiado, S. R. P., & Bevilaqua, D. (2020). Environmentally sustainable and cost-effective bioleaching of aluminum from low-grade bauxite ore using marine-derived Aspergillus niger. *Hydrometallurgy*, *195*(May), 105368. https://doi.org/10.1016/j.hydromet.2020.105368

Shaikh, Z., & Qureshi, P. (2013). Screening and Isolation of organic acid producers from samples of diverse habitats. In *Int.J.Curr.Microbiol.App.Sci* (Vol. 2, Issue 9). http://www.ijcmas.com

Shekhar Samanta, N., Das, P. P., Dhara, S., & Purkait, M. K. (2023). An Overview of Precious Metal Recovery from Steel Industry Slag: Recovery Strategy and Utilization. In *Industrial and Engineering Chemistry Research*. American Chemical Society. https://doi.org/10.1021/acs.iecr.3c00604

Shen, L., Zhou, H., Shi, Q., Meng, X., Zhao, Y., Qiu, G., Zhang, X., Yu, H., He, X., He, H., & Zhao, H. (2023). Comparative chemical and non-contact bioleaching of ion-adsorption type rare earth ore using ammonium sulfate and metabolites of Aspergillus niger and Yarrowia lipolytica to rationalise the role of organic acids for sustainable processing. *Hydrometallurgy*, *216*. https://doi.org/10.1016/j.hydromet.2023.106019

Shourie, A., & Vijayalakshmi, U. (2022). Fungal Diversity and Its Role in Mycoremediation. In *Geomicrobiology Journal* (Vol. 39, Issues 3–5, pp. 426–444). Taylor and Francis Ltd. https://doi.org/10.1080/01490451.2022.2032883

Tansengco, M., Tejano, J., Coronado, F., Gacho, C., & Barcelo, J. (2018). Heavy metal tolerance and removal capacity of trichoderma species isolated from mine tailings in Itogon, Benguet. *Environment and Natural Resources Journal*, *16*(1), 39–57. https://doi.org/10.14456/ennrj.2018.5

Thavamani, P., Samkumar, R. A., Satheesh, V., Subashchandrabose, S. R., Ramadass, K., Naidu, R., Venkateswarlu, K., & Megharaj, M. (2017). Microbes from mined sites: Harnessing their potential for reclamation of derelict mine sites. In *Environmental Pollution* (Vol. 230, pp. 495–505). Elsevier Ltd. https://doi.org/10.1016/j.envpol.2017.06.056

Valix, M., & Loon, L. O. (2003). Adaptive tolerance behaviour of fungi in heavy metals. *Minerals Engineering*, *16*(3), 193–198. https://doi.org/10.1016/S0892-6875(03)00004-9

Valix, M., Usai, F., & Malik, R. (2001). Fungal bio-leaching of low grade laterite ores. *Minerals Engineering*, *14*(2), 197–203. https://doi.org/10.1016/S0892-6875(00)00175-8

Vásquez, C., Colmenárez, Y., Morales-Sánchez, J., Valera, N., Sandoval, M. F., & Balza, D. (2015). *Current and Potential Use of Phytophagous Mites as Biological Control Agent of Weeds*. *July*.

Wösten, H. A. B. (2019). Filamentous fungi for the production of enzymes, chemicals and materials. In *Current Opinion in Biotechnology* (Vol. 59, pp. 65–70). Elsevier Ltd. https://doi.org/10.1016/j.copbio.2019.02.010

Wu, H. Y., & Ting, Y. P. (2006). Metal extraction from municipal solid waste (MSW) incinerator fly ash - Chemical leaching and fungal bioleaching. *Enzyme and Microbial Technology*, *38*(6), 839–847. https://doi.org/10.1016/j.enzmictec.2005.08.012

Yang, J., Wang, Q., Wang, Q., & Wu, T. (2009). Heavy metals extraction from municipal solid waste incineration fly ash using adapted metal tolerant Aspergillus niger. *Bioresource Technology*, *100*(1), 254–260. https://doi.org/10.1016/j.biortech.2008.05.026

Yang, Q., Qian, X., Routledge, M. N., Wu, X., Shi, Y., Zhu, Q., & Zhang, H. (2021). Metabonomics analysis of postharvest citrus response to Penicillium digitatum infection. *LWT*, *152*. https://doi.org/10.1016/j.lwt.2021.112371

Yin, X., Shan, X., Shao, W., Liu, L., Liang, G., Zhou, Q., & Lin, W. (2023). Copper from Waste Printed Circuit Boards Was Effectively Bioleached Using Newly Isolated Microorganisms and Subsequently Recovered by Microbial Fuel Cell. *Sustainability*, *15*(13), 9996. https://doi.org/10.3390/su15139996

Zhu, A., Zheng, F., Zhang, W., Li, L., Li, Y., Hu, H., Wu, Y., Bao, W., Li, G., Wang, Q., & Li, H. (2022). Oxidation and Antioxidation of Natural Products in the Model Organism Caenorhabditis elegans. In *Antioxidants* (Vol. 11, Issue 4). MDPI. https://doi.org/10.3390/antiox11040705

Zuo, J., Tang, J., Lu, M., Zhou, Z., Li, Y., Tian, H., Liu, E., Gao, B., Liu, T., & Shao, P. (2021). Glycolysis Rate-Limiting Enzymes: Novel Potential Regulators of Rheumatoid Arthritis Pathogenesis. In *Frontiers in Immunology* (Vol. 12). Frontiers Media S.A. https://doi.org/10.3389/fimmu.2021.779787

CHAPER 5: BIOLEACHING STUDIES BY *TRICHODERMA ASPERELLUM*: METAL RECOVEY, ORGANIC ACID PRODUCTIONS AND TRANSCRIPTOMICS

Objective

Chapter 4 evaluated the bioleaching capabilities of four fungal isolates, assessing their growth rates, metal tolerance, and organic acid production. Among the four isolates, *Trichoderma asperellum* (*T. asperellum*) emerged as the most promising bioleaching fungus. This chapter evaluated the bioleaching capabilities of the fungus *T. asperellum* using different metal-containing samples, such as tailings, its natural habitat, and ore, a distinct environment. The objective of this chapter is to gain insights into the bioleaching ability, specifically metal tolerance and organic acid production, at the genetic level by employing a combined transcriptomics and metabolomics approach.

Publication status

This chapter has been submitted to Journal of Environmental Management, see appendix A5.1.

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Abstract

Bioleaching is a biotechnological process that use fungi to extract metals from ores and tailings/waste. This study evaluated the genetic responses of the fungus *T. asperellum* during bioleaching of ore and tailing samples. One-step, two-step and spent media bioleaching processes were compared. From HPLC results, oxalic acid, citric acid and propionic acids were quantified, however, oxalic acid was the main organic acid involved in bioleaching of metals. Metal recovery exhibited variations between ore and tailing samples and among different bioleaching approaches. Notably, the two-step bioleaching process for both tailing and ore resulted in the highest recovery of zinc (>54%) and nickel (>60%), respectively. Oxalic acid is known to efficiently bioleach metals such as Zn amongst others. The differences in the highest metal recovered by the same organism were attributed to nickel's tendency to precipitate as nickel oxalate when oxalic acid is the main bioleaching agent, while its efficient recovery in ore bioleaching was correlated with the presence of manganese. Additional metals such as Co, Mn, Mg, Cu, and As were also successfully recovered. Transcriptomic analysis unveiled substantial upregulation of genes associated with biological processes, cellular components, and molecular functions. Notably, genes involved in cell membrane structure and function were consistently upregulated, indicating *T. asperellum*'s capacity to adapt to the environmental stresses encountered during metal bioleaching. These findings expand our comprehension of

the multifaceted mechanisms dictating varying metal recovery rates in diverse bioleaching processes.

Keyword words: Bioleaching, organic acid, differentially expressed genes, metal recovery.

5.1 Introduction

In recent years, there has been a growing interest in evaluating the use of filamentous fungi in bioleaching due to the inherent challenges associated with the process, including slow kinetics, low yield, and extreme conditions (Dusengemungu *et al.,* 2021; Moazzam *et al.,* 2021; Pathak *et al.,* 2017). Furthermore, the demand for an effective bioleaching process is also amplified by the environmental impact of conventional methods observed from mining activities that threatens the continuous use of natural resources such as soil and water (Farjana *et al.,* 2019; Yang *et al.,* 2019; Pourret *et al.,* 2015; Huang *et al.,* 2010). Bioleaching is a low-cost and energy-efficient method that offers a simplified approach without the need for specialized equipment, unlike traditional methods (Moazzam *et al.,* 2021; Opare *et al.,* 2021; Borja *et al.,* 2016). The recent exploitation of low-grade ore resulting from rapid depletion of high-grade ore reserves also contributed to the growing demand for an efficient bioleaching process because in conventional methods higher energy input is required as the metal content in the ore decreases (Sajjad *et al.,* 2019). Filamentous fungi play a crucial role in bioleaching by providing another notable advantage when dealing with low-grade ores that contain multiple metals, since filamentous fungi can simultaneously bioleach multiple metals, unlike conventional methods (Pathak *et al.,* 2021). However, despite these advantages, the above-mentioned challenges impede the commercial application of this process.

The challenges encountered in the bioleaching process are attributed to the reliance on biological activities of microorganisms. These activities can be influenced by various factors, including pH levels, high concentrations of heavy metals, and essential nutrients (Arshadi *et al.,* 2020; Sajjad *et al.,* 2019; Gu *et al.,* 2018). As a result, optimization studies normally involve screening for potential organism(s) that can function better under extreme conditions expected during bioleaching. One such organism is the fungus *Trichoderma asperellum,* which was isolated from mine tailing and selected for this study based on its metal tolerance and organic acid production. Thus, examples of optimisation strategies include isolation of fungi from extreme habitat, testing their heavy metal tolerance or adapting them to increasing concentration of heavy metals, finding optimal growth conditions (pH and Temperature), as well as their carbon source utilisation which leads to the production of organic acids (Din *et*

al., 2020; Bahaloo-Horeh *et al.,* 2018; Oladipo *et al.,* 2018). The success of one such strategy, namely metal tolerance adaptation, was demonstrated in a study conducted by Bahaloo-Horeh *et al.* (2018). They observed that subjecting *A. niger* to serial adaptation from 0.3% to 1% of spent battery powder (which contains metals) enhanced the fungus's tolerance to metals as compared to un-adapted *A. niger*. Similar results were reported by Shah *et al.* (2020). Once the metal tolerance is acquired, it allows fungi to withstand and thrive in environments with elevated metal concentrations. This tolerance to metals is often accompanied or results from an upregulation of genes associated with metal chelation, metal transport, and organic acid production (Chen *et al.,* 2023; Dey *et al.,* 2022). The increased expression of these genes leads to improved production and secretion of organic acids, including oxalic acid, citric acid, and gluconic acid. The study by Bahaloo-Horeh *et al.* (2018), also observed that the adaptation to metals also resulted in an improved production of organic acids, which ultimately enhanced the efficiency of bioleaching compared to un-adapted *A. niger*. It should be noted that in fungi, bioleaching revolves around the production of organic acids which are responsible for metal solubilization through mechanisms such as acidolysis and complexolysis (Dusengemungu *et al.,* 2021; Pathak *et al.,* 2021, 2017; Faraji *et al.,* 2018; Pinzari *et al.,* 2012; Xu & Ting, 2009). Therefore, understanding the regulation and optimization of organic acid production as well as metal tolerance in fungi during bioleaching is essential for enhancing the process.

In this study, the bioleaching ability of *T. asperellum,* was evaluated for metals contained in tailings, a habitat to which the organism is adapted. This ability was compared to its bioleaching performance for metals present in ore. The focus of the study was to compare the solubilization of metals, production of organic acids, and the expression of genes under different bioleaching strategies (one-step, two-step, and spent media bioleaching). By studying the genetic and molecular mechanisms underlying organic acid production and metal tolerance in fungi, researchers can identify key genes and metabolic pathways involved. This knowledge can be applied to improve the efficiency of the process and develop strategies for optimizing metal extraction from mineral resources.

5.2 Methodology

5.2.1 Mine tailing chromite ore characterization

Tailings samples were collected from different dumps around Krugersdorp, Gauteng province South Africa as described in chapter 3 section 3.2.1. The ore was obtained from open cast mine in Limpopo Province, South Africa. The tailings were ground in a pestle and mortar to break lumps. The initial metal composition of the two different samples were quantified using

inductively coupled plasma atomic emission spectroscopy (ICP-AES) (Shimadzu ICPE-9820, Gauteng, South Africa) by ICP-AES, as described in chapter 3 section 3.2.2.

5.2.2 Bioleaching assay

The fungal isolate *T. asperellum* was isolated from mine tailing samples, along with other fungi, it was selected for bioleaching assay in this chapter based on its high metal tolerance and fast metabolism in organic acid production from the experiments conducted in chapter 4. In this study, the bioleaching ability of *T. asperellum* was evaluated on tailings and ore. The tailing represents a habit for this organism (the organism was isolated from tailings), while ore represent a different type of sample in which the bioleaching ability of *T. asperellum* was evaluated. To prevent indigenous microorganisms from the samples from influencing the bioleaching experiment, the fine samples were autoclaved three times at 112°C for 30 minutes (Horeh *et al.,* 2016; Martínez *et al.,* 2012)

5.2.2.1 Bioleaching media and inoculum preparation

Potato dextrose agar was used to grow *T. asperellum* isolate and after 5 days of incubation at 30°C, the agar plate was covered with dH_2O, and spores were collected. The collected spores were then serially diluted up to 10^7 (vortexing in between) for use in bioleaching experiments (Krishnamoorthy *et al.,* 2021; Faraji *et al.,* 2018). Bioleaching media, GYEM, listed in Table 3.1 (Chapter 3) was prepared and supplemented with 100 g/l of glucose (Amiri *et al.,* 2011; Kishimoto & Tano, 1987).

5.2.2.2 Bioleaching experimental set-up

In bioleaching assays by fungi, there are three main procedures: one-step, two-step, and spent medium. These approaches, as reported by several researchers (Krishnamoorthy *et al.,* 2021; Faraji *et al.,* 2018; Bahaloo- Horeh *et al.,* 2017; Horeh *et al.,* 2016) will be compared in this study.

All bioleaching experiments were carried out in triplicates in 500 ml Duran Schott bottles containing 400 ml of sterile bioleaching media. Twenty grams of sterilized tailing or ore was used. This was determined by an initial screening test where the fungus *T. asperellum* was grown in different amounts (5-30g) of the samples and the pH was measured as compared to the fungi grown in a media only (Appendix A5.2). All reaction bottles were incubated at 30°C in a shaking incubator at 120 RPM as described by Faraji *et al.,* (2018). Three bioleaching approaches, i.e., one-step, two-step, and spent media were used to bioleach the various metals present in the tailings and ore samples. In one step approach, the fungal spores were inoculated

to the bioleaching media, along with the addition of the tailing or ore. This was incubated for 31 days. In two-step approach, the fungal spores were grown in the bioleaching media, and the pH was monitored daily. The tailings or ore were added once a significant drop in pH was observed (Krishnamoorthy *et al.,* 2021; Faraji *et al.,* 2018) and then followed by incubation for 31 days. In the last approach, spent media bioleaching, the fungal spores were grown in bioleaching media for 14 days. The media with cells was filtered using sterile 0.2 µm filters, to remove cells and the filtered solution containing organic acids, referred to as spent media, was mixed with either tailings or ore samples. This was incubated for 7 days in a shaking incubator. Two controls were employed: (1) a positive control containing bioleaching media inoculated with fungal spores, and (2) a negative control containing uninoculated bioleaching media.

In one-step and two-step, the pH was monitored every other day, while samples for RNA, metal and organic acid analysis (15 ml in total) were collected every 7 days. Every collection day, in both one-step and two-steps, bioleaching media (equal amounts as the samples removed) was added to each reaction bottle (Krishnamoorthy *et al.,* 2021). The choice was made due to the nature of sampling process, which is destructive sampling process. Removal of the sample for further analysis may disrupt the overall activity of the fungi and the bioleaching process. However, adding the same volume of bioleaching media that was removed to the system could help maintain the appropriate carbon source concentration and pH in the leaching solution. The collected samples were stored in -80˚C freezer, until further analysis. For spent media bioleaching, after tailing or ore samples were added, the pH was measured every day and samples for metal and organic acid analysis were also collected every day. ICP-AES was conducted as explained in section 5.2.1, except that the sample were in liquid form and there was no need for digestion.

The recovery of metals was calculated by equation 5.1 (Alavi *et al.,* 2021 and Bahaloo- Horeh *et al.,* 2017):

$$\text{Metal recovery \%} = \frac{C_S \, x \, V_S}{C_F \, x \, M_F} 100 \qquad (5.1)$$

where C_S = metal concentration in the leach liquor (mg/l), V_S = the volume of the bioleaching medium (L), C_F = metal content of the tailing/ ore samples (mg/g) and M_F =mass of the tailing/ore used.

5.2.3 HPLC analysis

The collected samples for organic acid quantification using HPLC, were filtered using 0.2 µm filters. The samples were analyzed at the University of the Witwatersrand, South Africa. The procedure is as follows: the standard solutions were injected into the HPLC (Agilent 1200 series) followed by samples. The injection volume was 20 µl, with a flow rate of 1 ml/min and 0.2 ml of sulfuric acid in 1 L of deionised water was used as mobile phase. The separation of components took place in a BioRad fermentation column at a temperature of 65°C. The separated components were then detected by Refractive index column and the profile was observed. The quantification and detection were then performed using Agilent CDS Open Lab software Rev c.01.10 (287).

5.2.4 RNA extraction

Samples collected from bioleaching assay were centrifuged at 10 000 RPM to pellets the fungal cells and the supernatant was discarded. The cells were resuspended in 8 µg/µl EDTA for metal ion stabilisation as described in Chapter 3, section 3.3.2 (Nkuna *et al.,* 2022). The Zymo research Quick-RNA™ MiniPrep Plus Kit (Zymo Research Corp, Irvine, California) was used to extract RNA from treated fungal cells, following manufacture's instruction. The 260/280 ratio of the extracted RNA, which represent RNA purity was measured using NanoDrop™ (Thermo Scientific™, Waltham United States). The RNA samples were sent to Inqaba Biotech (Gauteng, South Africa) on dry ice for RNA-Seq using ILLUMINA MiSeq. The RNA MiSeq method involved conversion of total extracted RNA cDNA synthesis using reverse transcription. Ribosomal RNA depletion was performed to enrich for mRNA molecules. Next, library preparation was carried out, including adapter ligation and PCR amplification. Quality control checks were conducted before sequencing on the Illumina MiSeq platform, generating high-throughput RNA sequencing data for downstream analysis.

5.2.4.1 RNA sequence analysis

The RNA sequences were received from Inqaba Biotech as Fastq files. The raw RNA sequence reads were inspected for quality using FastQC (version 0.11.5), followed by trimming sequence of low-quality reads using Trimmomatic (version 0.39) (Bolger *et al.,* 2014). Summarised quality control is represented in Table 5.1. *De novo* assembly of the quality-controlled reads was performed with Trinity v2.15.1 (Haas *et al.,* 2013; Grabherr *et al.,* 2011). The quality of assembled sequences was evaluated using BUSCO and N50, where the former assesses the completeness of an assembly while the latter is a statistical measure were a higher N50 value indicates longer and more complete transcripts (Manni *et al.,* 2021; Haas *et al.,* 2013).

Differentially expressed transcripts were extracted using DESeq2 from Trinity output and heatmap was generated. Trinotate v4.0.1 (swissprot_blastx, swissprot_blastp and TrinotatePFAM) was used for functional annotation of the assembled transcripts. The gene identity number from sprot_Top_BLASTX_hit in the Trinotate output were used for gene enrichment on ShinyGO 0.77 (http://bioinformatics.sdstate.edu/go/).

Table 5.1: Overview of Illumina RNA-seq data quality

Sample groups	Average total clean reads	Average clean bases (Mbp)	Average GC%
Control	1039269	149.67	52.67
One step ore	929885	134.24	52.80
Two step ore	2529094	125.37	52.67
One step tailing	3880038	137.27	52.00
Two step ore	2357572	136.46	51.80

5.3 Results

The fungus *T. asperellum* was used for bioleaching of metals from tailing and ore using three bioleaching approaches.

5.3.1 Changes in pH

One-step bioleaching: The pH decreased consistently during the first 7 days, followed by fluctuations in the range of 3.5-4 for tailings and ±4 for ore (Figure 5.1a). The pH of tailings samples was lower than that of ore throughout the experiment. *Two-step bioleaching:* Twenty-four hours after adding samples to be leached, the pH increased from 3.7 to around ±4.3 for tailings and from 4 to around 4.5-5 for ore. The pH remained within these ranges for 21-24 days (Figure 5.1b). In the last 6-10 days of the experiment, the pH of both tailings and ore decreased continuously. Spent media: The pH measured on day 14 (before addition of tailings and ore) was around 5 and remained within the range of 5-5.5 after addition of samples.

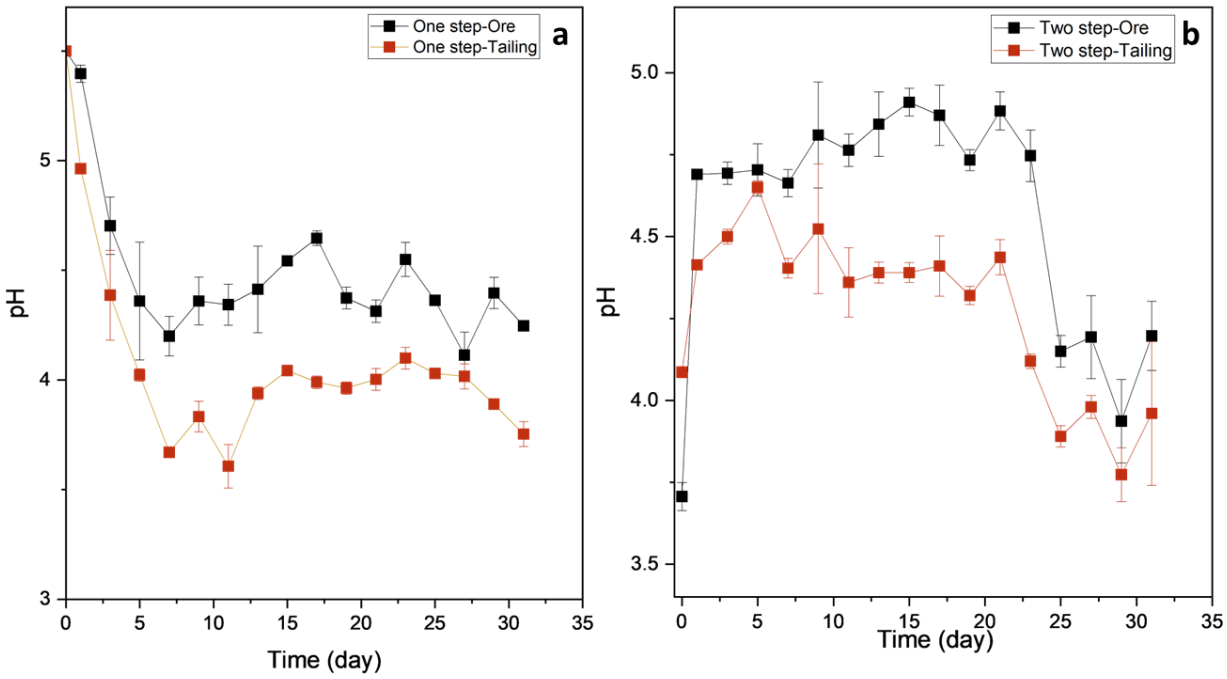


Figure 5.1: pH measurements between different bioleaching steps. **a** – one-step bioleaching, **b** – two-step bioleaching.

5.3.2 Bioleaching mechanisms.

Initial metal analysis in tailing and ore was conducted using ICP-AES as indicated in Table 5.2. *One-step bioleaching:* Higher recovery across most metals in tailings. Tailings showed significant recovery for several metals (Co, As, Mg, Zn), while ore only showed notable recovery for Mg and As (Figure 5.2a and b). *Two-step bioleaching*: Similar bioleaching efficiency for both tailings and ore, but higher overall recovery in ore for many metals (Ni, Mn, Zn, Cu, Mg). Tailings still had good recovery for specific metals (Zn, Co, Ni). *Spent media leaching*: Again, higher recovery in tailings for majority of metals (Co, Ni, Cu) compared to ore, except for Mg.

Table 5.2: ICP-AES quantified metal concentration of tailings and ore samples.

Sample	Metals (% W/W)									
	Cr	**Ni**	**As**	**Al**	**Co**	**Cu**	**Fe**	**Mg**	**Mn**	**Zn**
Tailing	5.4±0072	4.5±0.0573	5.3±0.0872	572.3±0.0111	0.9±0.0190	2.5±0.030	616.9±0.0067	107.5±0.091	6.1±0.0180	7.2±0.012
Ore	15.8±0.044	2.7±0.0062	2.9±0.0051	56.2±0.0011	0.0	1.2±0.033	165.2±0.0211	62.8±0.0028	2.2±0.0037	23.0±0.033

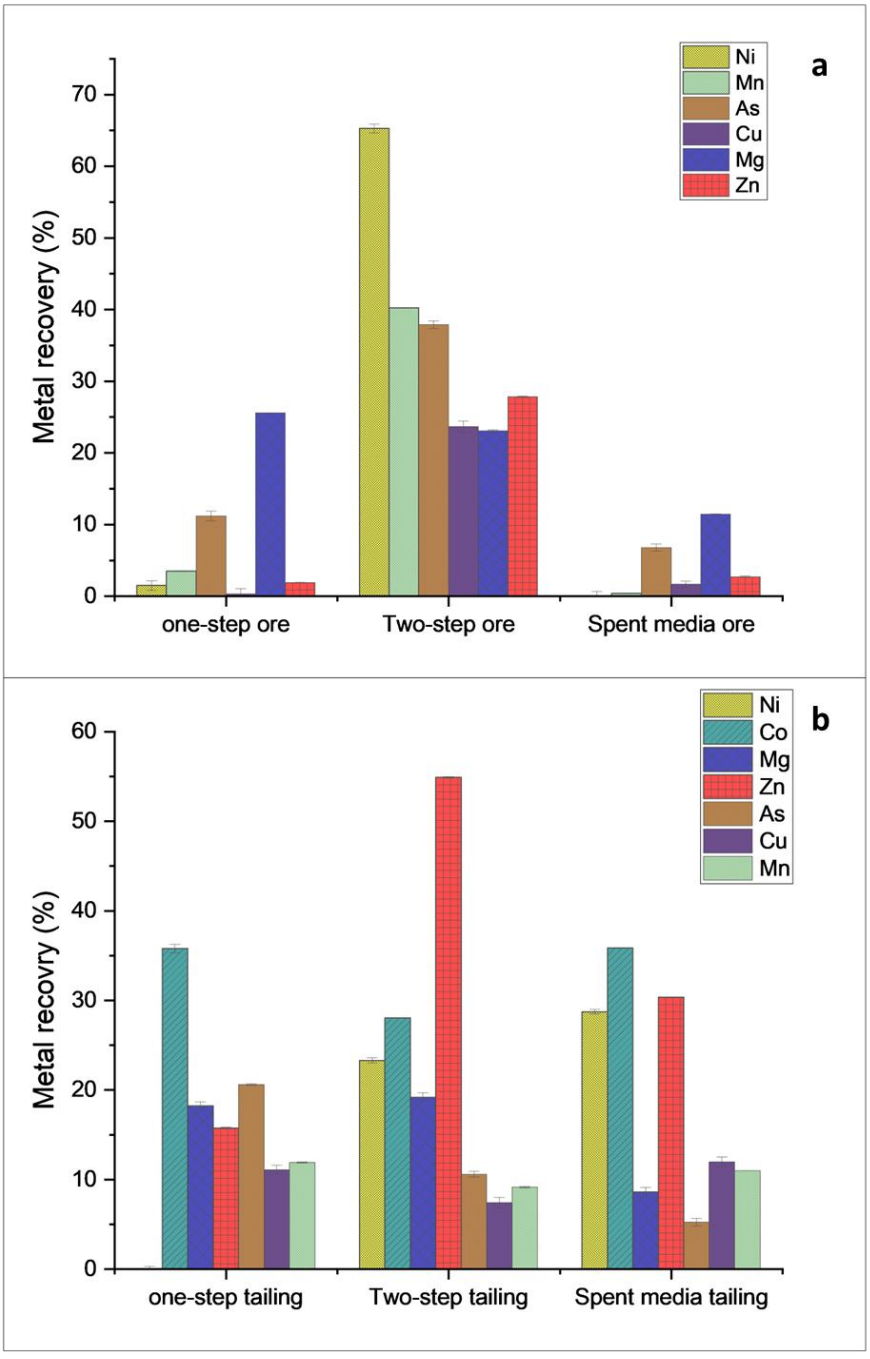


Figure 5.2: Comparison of percentage of metals recovered from ore (**a**) and tailing (**b**) using three bioleaching steps, i.e., one-step, two-step and spent media.

5.3.3 Organic acid production

Results from high performance liquid chromatography shows that *T. asperellum* produced oxalic acid ($C_2H_2O_4$) and citric acid ($C_6H_8O_7$) during various bioleaching processes (Figure 5.3a and b). *T. asperellum* produced oxalic acid as the main organic acid, while citric acid was produced in low quantities (Figure 5.3a – d). In both one-step and two-step bioleaching of

tailing samples, *T. asperellum* produced high levels of oxalic acid, averaging 672.52 mg/l in one-step and 930.13 mg/l in two-step bioleaching. In Figure 5.3b, the organic acid (~800 mg/l oxalic acid) produced in two-step before addition of metal containing samples was quantified and plotted under day 0

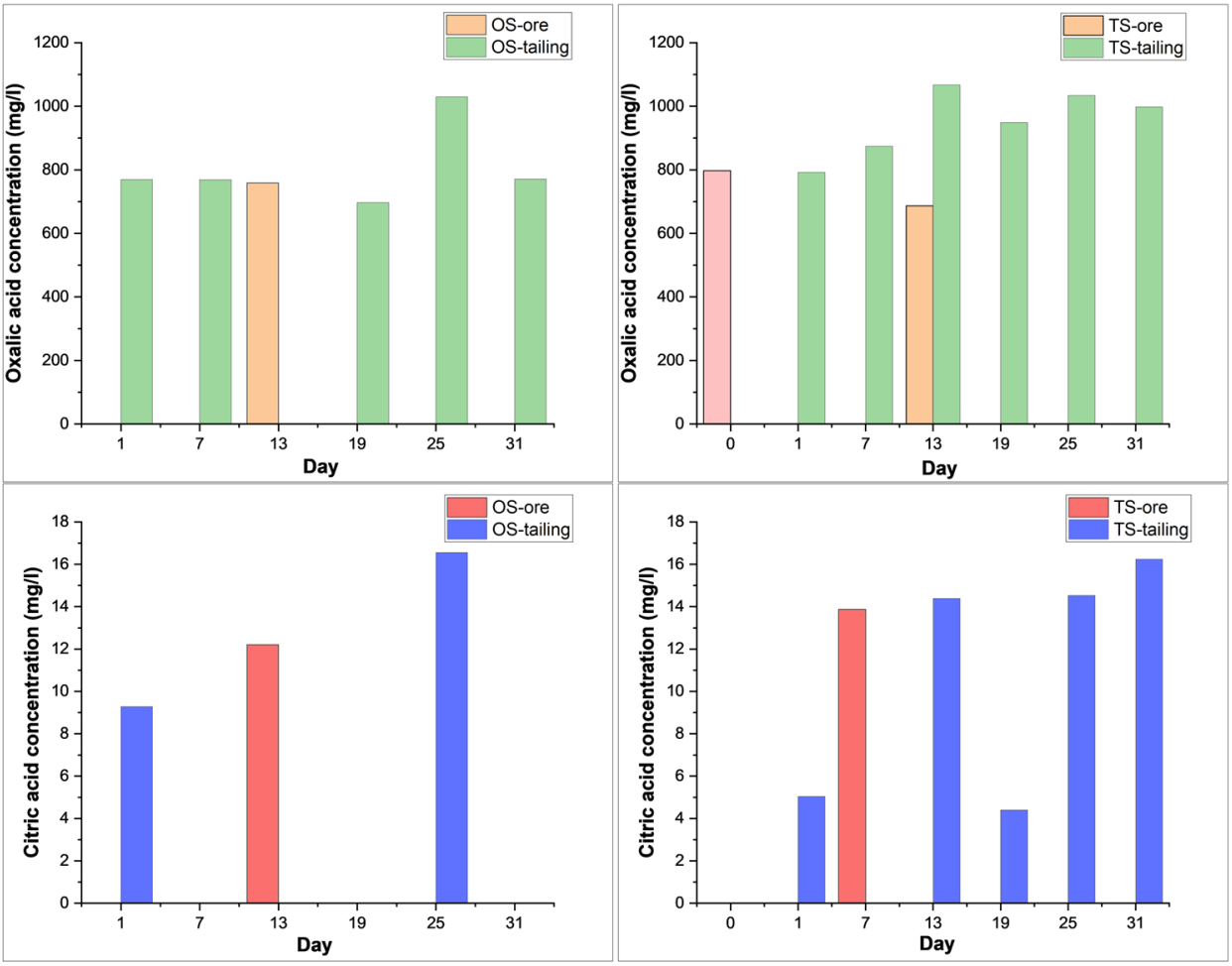


Figure 5.3. Comparison of organic acid quantified using HPLC. a – one step and b -two step for oxalic acid, c – one step and d two step for citric acid

5.3.4 RNA-Seq data analysis

Differential expression: to determine the differentially expressed gene in *T. asperellum* during one-step and two-step bioleaching of ore and tailing, DESeq2 (R/Bioconductor package) software was used to analyze the differential expression of log2-transformed FPKM values obtained from Trinity. The differentially expressed genes (DEGs) were then clustered as indicted in Figure 5.4, where yellow represent upregulated genes and purple downregulated genes. The heatmap shows that most of the up-regulated genes were observed in tailing samples (both one-step and two-step) though some genes of one-step ore were up-regulated, most are

down-regulated. Two-step ore on the hand shows similar profile to the control were all DEGs are downregulated.

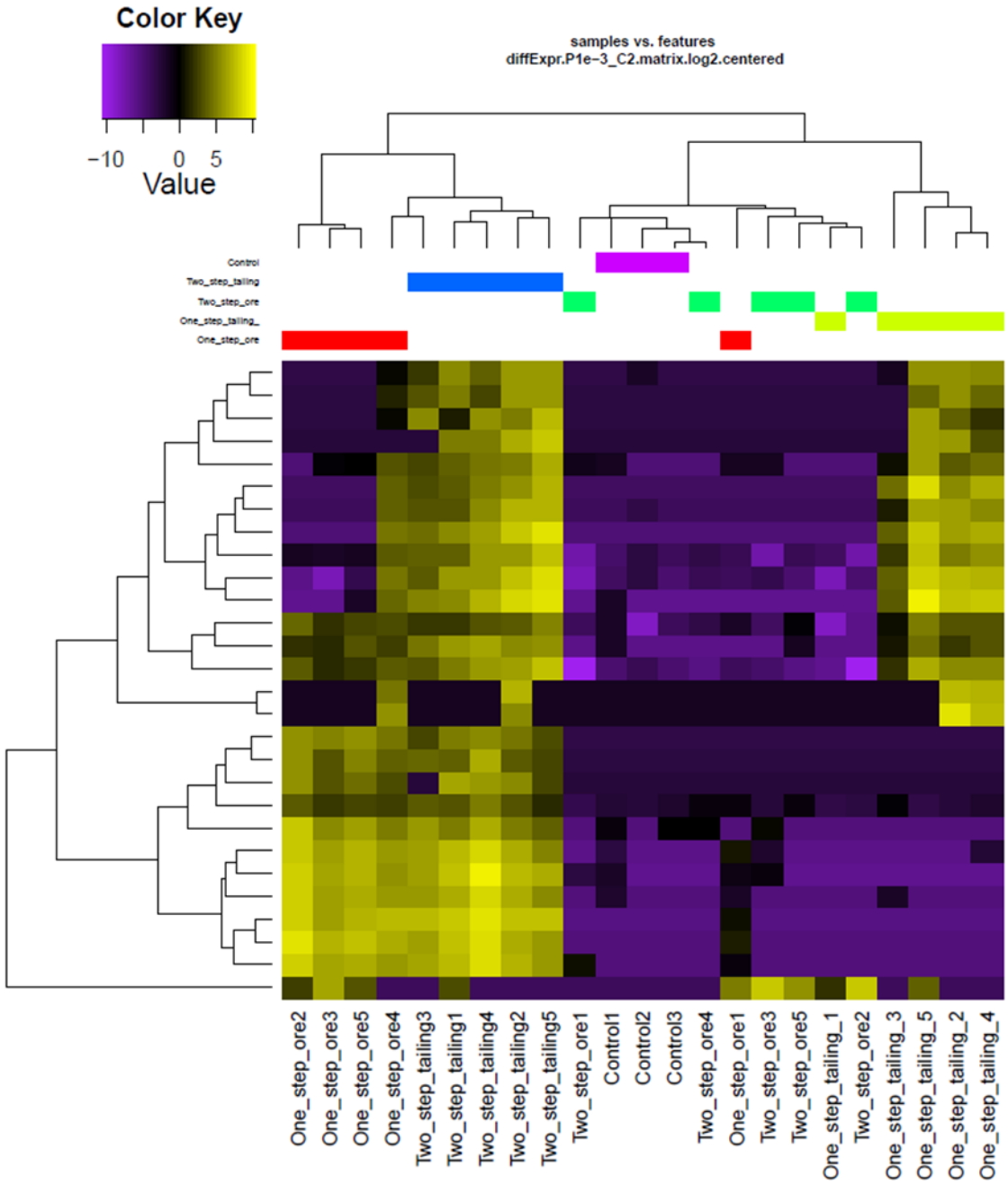


Figure 5.4: Hierarchical cluster analysis of the differentially expressed genes in *T. asperellum* during one-step and two step bioleaching processes of ore and a tailing. Control samples (*T. asperellum* grown in bioleaching media without tailing or ore samples) are also included. The expression values are plotted a logarithmic scale, with the mean value of each feature subtracted from all of its value in that row. Upregulated genes are shown in yellow and downregulated genes as purple.

5.3.4.1 Pathway analysis

Functional enrichment analysis was performed on the above DEGs, and it revealed significant genes for biological processes (BP), cellular component (CC) and molecular function (MF) for *T. asperellum* during bioleaching of tailing and ore (Figure 5.5a). Of the enriched genes, most are involved in biological process related to transport and maintenance of balance between iron ions within the cell (iron transport and homeostasis) as seen in Figure 5.5a. Enriched genes belonging to cellular components include those that related to cellular and structural aspects of the cell membrane. The relationship between enriched genes is shown in Figure 5.5b, where pathways sharing >20% genes are connected. In this plot, a connection between significantly enriched gene sets (darker colour) that are involved in biological function and cellular component is shown. To list a few, Figure 5b shows an enrichment of a set of genes involved in plasma membrane- an outer part of the cell, intrinsic component of membrane – intrinsic membrane proteins embedded in the cell membrane with roles such as adhesion, transport and signalling, transmembrane transport- involved in the movement of substances across the cell membrane.

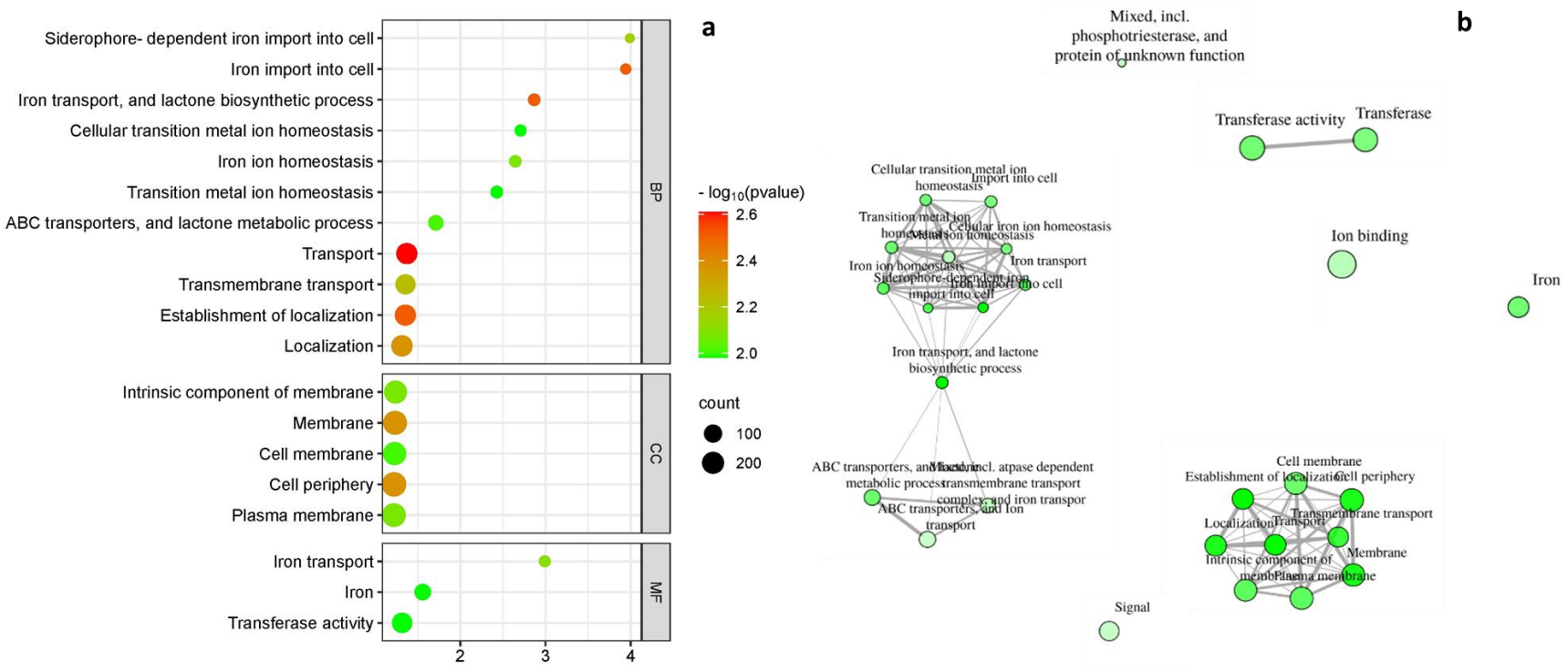


Figure 5.5: a - Gene enrichment dot plot for *T. asperellum* gene expressed during bioleaching (The gene ratio versus the enriched pathway). The colour scale indicates different thresholds of the p-value, and the size of the dot indicates the number of genes corresponding to each pathway. The Fisher test was performed to indicate the significance of gene enrichment (FDR < 0.05). **b-** Interactive plot showing the relationship between enriched pathways. Darker nodes represent more significantly enriched gene sets.

Differentially expressed genes with functions related to the cell membrane were compared among the different bioleaching experiments and the control. Comparison between the samples was decided based on the points listed in Figure 5.6. The results of the comparisons were presented using MA plots, which shows up- and down-regulated genes.

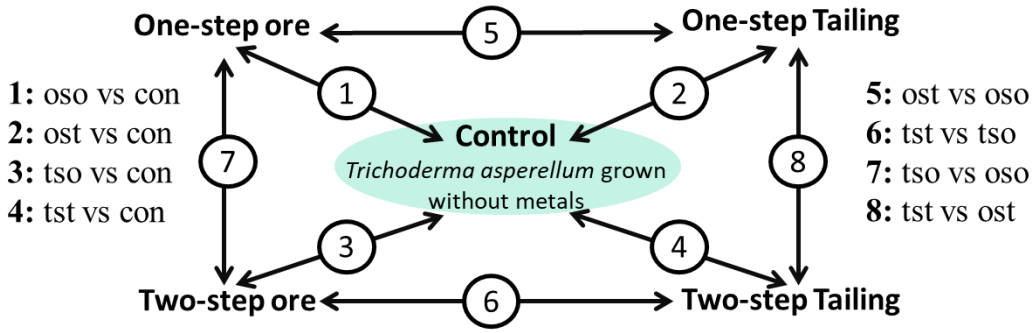
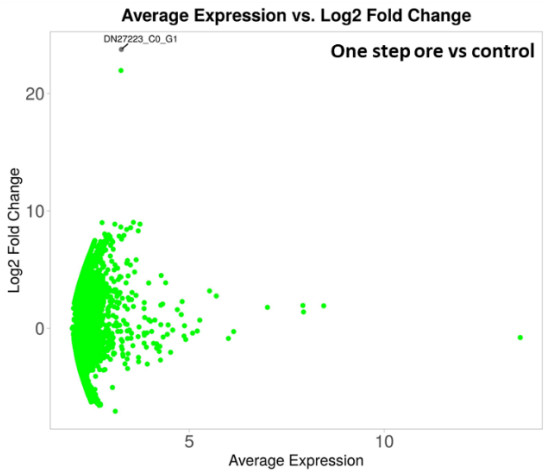
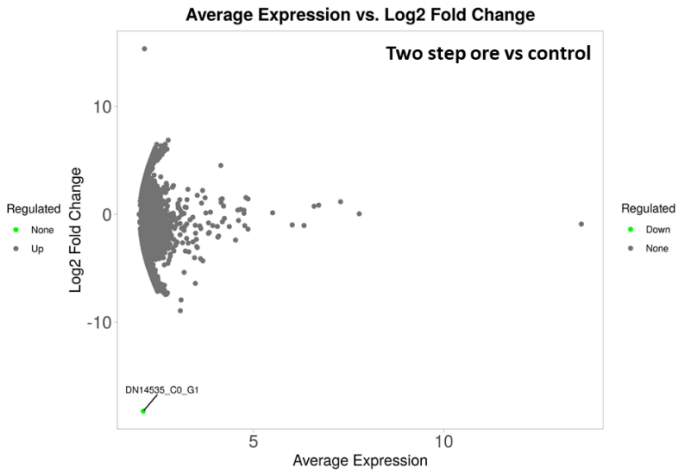
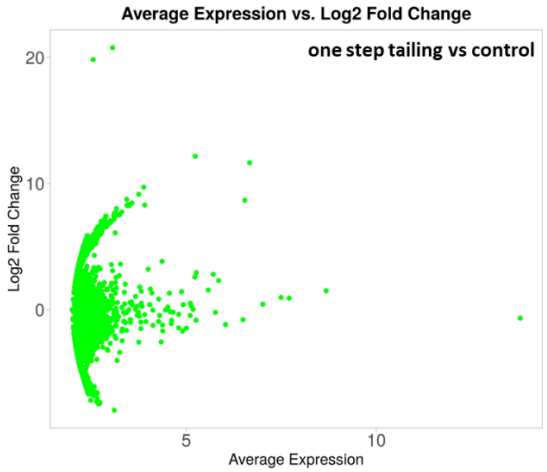


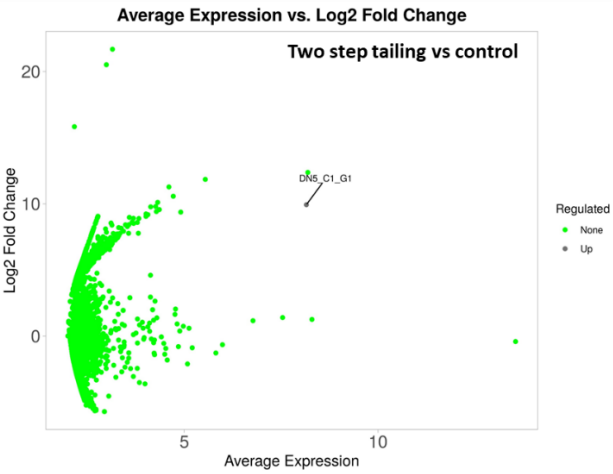
Figure 5.6: Comparison of differenctially expressed genes between one-step, two-step bileaching (for tailing and ore) and control.

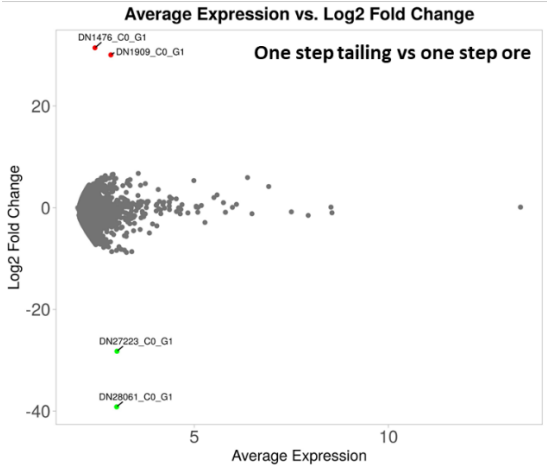
Trinity was used for *de novo* assembly of the transcript, followed by Trinotate for functional annotation. The differentially expressed genes on the MA plots are labeled with Trinity IDs (e.g., DN14535_C0_G1). These Trinity IDs will be used along with gene ontology numbers obtained from KEGG during Trinotate functional annotation. In Figure 5.7, the MA plots represent comparisons of the log fold change of expression in one sample compared to another. For example, one MA plot compares the log fold change of expression in one-step ore to the log fold change of expression in the control. The DEGs are labeled on each MA plot with an indication of whether they are upregulated or downregulated. A common observation among the 8 comparison groups is that most of the DEGs are related to the function of cell membrane. No differentially expressed genes related to organic acid production were observed. Another common observation is that the comparion between groups 1- 4 (bioleaching types vs control) showed fewer DEGs. Whereas comparions between groups 5-8 (amongs the different bioleaching types) has more DEGs, especially comparison of two-step tailing to two-step ore (the two bioleaching processess with the highest recovery of metals).

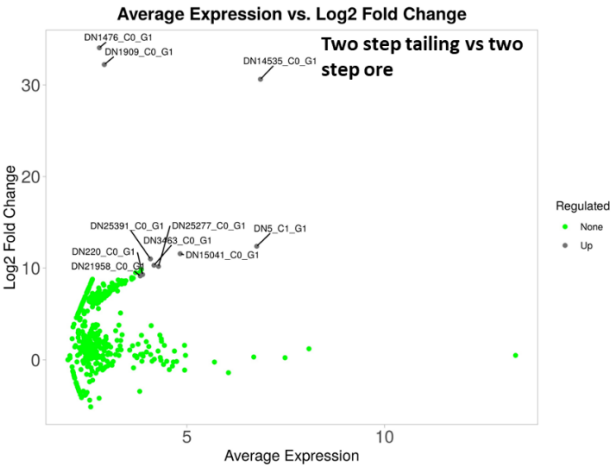

Average Expression vs. Log2 Fold Change
One step ore vs control
DN27223_C0_G1
Log2 Fold Change
Average Expression
Regulated
None
Up

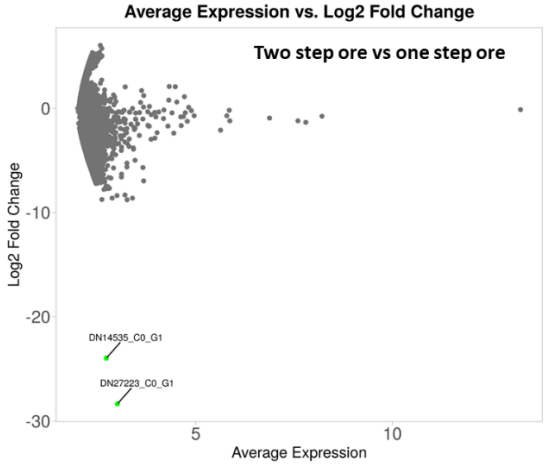

Average Expression vs. Log2 Fold Change
Two step ore vs control
DN14535_C0_G1
Log2 Fold Change
Average Expression
Regulated
Down
None


Average Expression vs. Log2 Fold Change
one step tailing vs control
Log2 Fold Change
Average Expression
Regulated
None


Average Expression vs. Log2 Fold Change
Two step tailing vs control
DN5_C1_G1
Log2 Fold Change
Average Expression
Regulated
None
Up


Average Expression vs. Log2 Fold Change
One step tailing vs one step ore
DN1476_C0_G1
DN1909_C0_G1
DN27223_C0_G1
DN28061_C0_G1
Log2 Fold Change
Average Expression
Regulated
Down
None
Up


Average Expression vs. Log2 Fold Change
Two step tailing vs two step ore
DN1476_C0_G1
DN1909_C0_G1
DN14535_C0_G1
DN25391_C0_G1
DN25277_C0_G1
DN5_C1_G1
DN3483_C0_G1
DN220_C0_G1
DN21958_C0_G1
DN15041_C0_G1
Log2 Fold Change
Average Expression
Regulated
None
Up


Average Expression vs. Log2 Fold Change
Two step ore vs one step ore
DN14535_C0_G1
DN27223_C0_G1
Log2 Fold Change
Average Expression
Regulated
Down
None

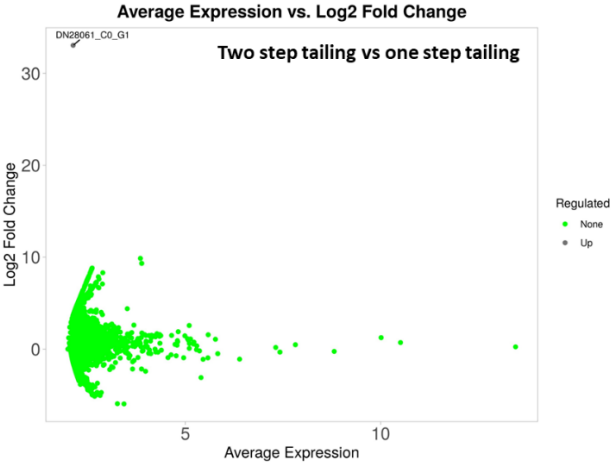

Average Expression vs. Log2 Fold Change
Two step tailing vs one step tailing
DN28061_C0_G1
Log2 Fold Change
Average Expression
Regulated
None
Up

Figure 5.7: MA plot of the differentially expressed genes of compared groups. The values were calculated as the log fold change of expression in sample A compared to sample B (as indicted in Figure 5.6.). Genes that differentially expressed are labelled as either up or downregulated.

5.4 Discussion

In the present study, the bioleaching potential of a candidate fungus was assessed using different metal-containing samples and bioleaching conditions. The primary objective was to elucidate the bioleaching capacity and metal tolerance at a functional level, employing transcriptomics and metabolomics to unravel the mechanisms underlying metal recovery and tolerance during bioleaching.

Changes in pH

Organic acids are part of secondary metabolites which directly result in pH changes (Horeh et al. 2016). Thus, the continuous decrease and fluctuation in pH during the bioleaching experiment. It was observed that the fungus *T. asperellum* reached the lowest pH in just 5 days in the absence of metal-containing samples (before addition of metals for two-step), compared to 7 days in one-step bioleaching. This highlights a drawback of the one-step process, where mixing samples and cells at the same time may impede the growth rate and subsequently the activity of the organism (Elahian *et al.,* 2020). The overall comparison of the two metal containing samples shows that the bioleaching of tailings had the lowest pH in both one-step and two-step. Similar results were observed by Krishnamoorthy *et al.* (2021) and Marappa *et al*. (2020). Low pH enhances metal solubilization; therefore, two-step bioleaching is characterized as the most efficient metal leaching process compared to one-step leaching in most studies (Arshadi *et al.,* 2020; Srichandan *et al.,* 2019; Xu *et al.,* 2014; Qu & Lian, 2013).

Bioleaching mechanism

Overall, the bioleaching of tailing samples was efficient in all the bioleaching processes. This is likely due to the lower pH observed in tailing samples. According to McCarthy & Blum (2018), pH is a crucial factor that enhances the activity of fungi. The low pH in tailings bioleaching creates an acidic environment that is ideal for the growth and activity of acidophilic organisms like *T. asperellum*. Hence, higher metal recovery was observed in all tailings bioleaching experiments. One-step ore bioleaching had the lowest metal recovery. This is likely because of the adaptation process to metals in ore, where the growth rate was delayed, and this may have negatively affected the metabolic activities of *T. asperellum* (Xu and Ting, 2009). In

addition, the difference in chemical composition between tailing and ore may have also contributed to the higher metal recovery in one-step tailing bioleaching. Tailings are reported to contain more organic matter than ore, which may influence the leaching process (Bolaños-Benítez *et al.,* 2018). Organic matter can sometimes positively impact the process by serving as a nutrient source and exhibiting chelating abilities (Bolaños-Benítez *et al.,* 2018). In two-step, the bioleaching ability of *T. asperellum* was confirmed, where the highest recovery of Ni (>65%) and Zn (>54%) for both ore and tailing samples, respectively, was observed. This difference in the highest metal recovered may also be due to the difference in chemical composition between tailing and ore.

Organic acid production

In the context of fungi, bioleaching is an indirect process in which organic acids produced by fungi interact with metal compounds to solubilize them. There are three main mechanisms by which this can occur: acidolysis, complexolysis, and redoxolysis and bioaccumulation (subsequent mechanism that occur after the three) (Alavi *et al.,* 2021; Bahaloo-Horeh *et al.,* 2018; Xia *et al.,* 2018; Asghari *et al.,* 2014). Acidolysis is the key mechanism, involving the protonation of oxygen atoms on the surface of the metal compound (Xia *et al.,* 2018). The metal is separated from the surface of the metal compound once the oxygen and protons are combined with water. The protons are provided by the organic acids produced by the fungi. Although acidolysis is the key mechanism, it is complemented by complexolysis, in which the metal ions released during acidolysis are stabilized (Burgstaller & Schinner, 1993). *T. asperellum* produced oxalic acid ($C_2H_2O_4$) and citric acid ($C_6H_8O_7$) during various bioleaching processes. The reaction of oxalic acid ($C_2H_2O_4$) and citric acid ($C_6H_8O_7$) during various bioleaching processes (Figure 5.3a and b). The reactions between these two organic acids and metals through acidolysis and complexolysis are represented below (Alavi *et al.,* 2021; Xia *et al.,* 2018).

Oxalic acid:

$C_2H_2O_4 \rightarrow C_2HO_4^- + H^+$ (pK_{a1} = 1.25)

$C_2HO_4^- \rightarrow C_2O_4^{2-} + H^+$ (pK_{a2} = 4.14)

Citric acid:

$C_6H_8O_7^- \rightarrow C_6H_7O_7^- + H^+$ (pK_{a1} = 3.09)

$C_6H_7O_7^{2-} \rightarrow C_6H_6O_7^{2-} + H^+$ (pK_{a2} = 4.75)

$C_6H_6O_7^{2-} \rightarrow C_6H_5O_7^{3-} + H^+$ (pK_{a3} = 6.40)

It was observed that *T. asperrellum* produced high levels of oxalic acid in both one-step and two-step bioleaching. Similar results were reported by Alavi *et al.* (2021), where in both one-step and two-step bioleaching processes oxalic acid was the highest. One of the reasons they gave for high oxalic acid, that relate to the current study is that the presence of nickel contributes to the production of oxalic acid (Alavi *et al.*, 2021; Santhiya & Ting, 2005). In terms of metal recovery, Faraji *et al.* (2018) and Krężel & Maret (2016) observed that high oxalic acid concentrations are associated with high bioleaching efficiency of zinc. Consequently, the two-step bioleaching process for tailings in the current study demonstrated higher bioleaching efficiency of zinc (>54%). Low bioleaching of metals such as copper and nickel was observed in both methods for tailing. This could be due to the precipitation of copper and nickel as oxalates in the presence of high oxalic acid concentrations (Tzeferis & Agatzini-Leonardou, 1994). Faraji *et al. (2018)* reported similar findings with oxalic acid, where *A. niger* demonstrated high bioleaching efficiency of zinc but low bioleaching efficiency of copper and nickel. High oxalic acid production poses a limitation for bioleaching of copper or nickel, as it precipitates these metals as insoluble oxalates, significantly decreasing bioleaching efficiency.

Contrary to the results of tailing leaching, two-step ore leaching resulted in high recovery of nickel (>65%), even though oxalic acid was present in high concentration (686.72 mg/L). This raises the question of why nickel precipitation did not occur, as explained for tailing. In addition to being efficient at leaching zinc, oxalic acid is also known to leach many other metals, including nickel. As explained above, complexolysis complements acidolysis, where the organic acid (such as oxalic acid) acts as a chelator and forms complexes (ligand-induced metal solubilization) with the metal ions released by acidolysis. These metal complexes have higher solubility than the original mineral. However, a number of bioleaching conditions can cause these soluble complexes to precipitate, including increased pH, high oxalate ion concentration, and increased heat (Verma *et al.*, 2019). pH and temperature are unlikely to be the cause in this study, as the pH was still acidic (although slightly higher than in tailing) and the experiments were conducted at approximately constant temperature (30˚C). Therefore, the precipitation of nickel oxalate in tailing may have been caused by the high concentration of oxalate ions. Asadrokht & Zakeri (2022) observed that oxalic acid initially dissolved both iron and nickel from laterite ore, but that long leaching times allowed unstable nickel oxalate ions to precipitate as nickel oxalate dihydrate ($NiC_2O_4 \cdot 2H_2O$). The high recovery of nickel in two-step ore may have been influenced by the presence of manganese, as over 35% of manganese was recovered in two-step ore, while less than 10% manganese was recovered in two-step tailing. Manganese

can form complexes with organic acids like oxalic acid (Keshavarz *et al.*, 2021). This complexation can make manganese more soluble in the leachate. In the presence of manganese, oxalic acid may preferentially bind to manganese ions, leaving fewer oxalate ions available to form precipitates with nickel. This could explain why nickel does not precipitate as nickel oxalate in the ore samples. Ghosh & Paul (2016), observed that oxalic acid facilitated the solubilization of manganese from ferro-manganese nodules, and that the solubilization of manganese released nickel concomitantly. However, it should be noted that the presence of manganese can have either a positive or negative effect on the bioleaching of nickel. Overall, the high concentrations of oxalic acid observed suggest that the bioleaching processes were mainly carried out by oxalic acid, even though citric acid was also quantified.

Pathways analysis

Differentially expressed genes refers to genes that have different levels of expression in different conditions, such as in one-step versus two-step or one-step/two-step versus control. The up or down regulationa means that are either being transcribed more or less frequently in one condition compared to the other. These DEGs are important when it comes to understanding how *T. asperellum* respond to different metal containing samples and different bioleahcing processess. From the results it was observed that all DEGs are related to the function of cell membrane and none of the DEGs are related to organic acid prodcution. This may suggest that the production of organic acid in *T. asperellum* may not be affected (to some extend) by the presence of metals. This was confrimed in the MA plot of one-step tailing vs control (Figure 5.7) where no differntailly expressed genes were observed. Implying that there was no difference in terms of organic acid productction related genes between one-step tailing and control, though this one-step tailing leaching method was able to produce high oxalic acid (Figure 5.3) and also bioleach over 30% of cobalt (which was the highest) (Figure 5.2). Another common observation is that the comparison between bioleaching types and the control revealed fewer DEGs (Figure 5.6). This finding suggests that this fungus, isolated from tailing samples characterized by low pH and heavy metal content, possesses genes essential for bioleaching. This observation underscores the significance of isolating potential bioleaching fungi from extreme environments, such as tailing samples.

Comparions amongst the different bioleaching types showed more DEGs as compared to comparison of bioleaching types and control. Figure 5.7 compares the DEGs between one-step tailing and one-step ore, highlighting the upregulation of genes DN1476 and DN1909 in tailing

samples, with the following gene ontology-GO:0005789 for endoplasmic reticulum (ER) and GO:0000139 for endoplasmic reticulum (ER) membrane respectively. The ER is a vast membrane-bound compartment that permeates the cytoplasm of eukaryotic cells (Schwarz & Blower, 2016). It is enveloped by the ER membrane, a phospholipid bilayer. Both the ER membrane and the ER itself play crucial roles in cellular function (Phillips, 2016; Schwarz & Blower, 2016). The ER membrane acts as a barrier between the ER lumen and the cytoplasm, while the ER serves as the primary site for protein synthesis, folding, and transport (Phillips, 2016). In the context of metal tolerance or bioleaching, an augmented ER function might be necessary to counteract the stress induced by heavy metals or to support metabolic processes associated with bioleaching (Chen *et al.,* 2023; Emri *et al.,* 2021). Overall, the upregulation of ER-related genes signifies an adaptive response to environmental stress. This explains the higher bioleaching efficiency of one-step tailing compared to one-step ore. Metal-induced stress, inhibition of transcription factors, and mRNA degradation are potential mechanisms by which metal toxicity may have affected the activity of *T. asperellum* in one-step ore bioleaching (Ran *et al.,* 2023; Emri *et al.,* 2021).

The bioleaching recovery of two-step tailing and two-step ore yielded high metal recovery, with Zn recovery exceeding 54% and Ni recovery surpassing 62% for their respective processes. Notably, over 10 differentially expressed genes were upregulated in tailing compared to ore. This differential gene expression may explain the varying metal recoveries observed between the two processes, with ore bioleaching slightly more efficient than tailing bioleaching. Several of the upregulated genes were associated with functions related to the endoplasmic reticulum (GO:0005789), endoplasmic reticulum membrane (GO:0000139), carboxy-lyase activity (GO:0004586), plasma membrane (GO:0015221), integral component of plasma membrane (GO:0016020), iron ion binding (GO:0004222), RNA-directed DNA polymerase activity (GO:0009036), and lyase activity (GO:0071555). These upregulated genes in tailing likely play a role in the response to metal toxicity or other toxic compounds that may be present due to the sample type. Despite their high organic content, tailing samples represent a byproduct of metal separation processes, such as flotation. The flotation process typically employs various chemical reagents, including collectors, frothers, and depressants (Nakhaei & Irannajad, 2018). Residues of these reagents can persist in the tailings and may exert toxic effects to bioleaching microorganims (Xue *et al.,* 2023; Jafari *et al.,* 2018). Genes associated with detoxification and membrane transport, such as those involved in "plasma membrane" and "integral component of plasma membrane," may be upregulated in response to the presence of

these toxic chemicals (Lewis *et al.,* 2020). Essentially, the presence of potentially toxic compounds in tailings can establish a biochemical environment distinct from that of ore. This altered environment can trigger specific stress responses and adaptations by *T. asperellum*, leading to the upregulation of genes that are not as prominently expressed during ore bioleaching.

5.5 Conclusion

This study investigated the bioleaching capabilities of *T. asperellum.* The analysis of differentially expressed genes during the bioleaching of tailings and ore samples has yielded valuable insights into *T. asperellum*'s adaptive mechanisms and its remarkable ability to bioleach metals under diverse conditions. Several key conclusions can be drawn:

- The acidic environment observed in tailing bioleaching promotes metal recovery through two key mechanisms. Firstly, low pH directly enhances the solubility of target metals through protonation, facilitating their release from the mineral matrix. Secondly, acidic conditions favor the growth and activity of acidophilic microorganisms like *T. asperellum*
- Oxalic acid is crucial organic acid involved in bioleaching. High concentrations of oxalic acid are associated with enhanced bioleaching efficiency of metals like zinc and nickel. However, these elevated oxalic acid levels may lead to the precipitation of nickel and copper as oxalates. This precipitation can significantly reduce the overall metal recovery efficiency of metals such as copper and nickel.
- *T. asperellum*'s genetic responses to bioleaching conditions are influenced by both the sample type and the presence of potentially toxic compounds. For instance, tailing samples, with potential residues from metal separation processes like flotation, may have impacted *T. asperellum*'s gene expression patterns.
- Differentially expressed genes involved in the transport and maintenance of iron ions within the cell play a pivotal role in maintaining iron homeostasis during bioleaching. Genes associated with the structure and function of the cell membrane are consistently upregulated, highlighting their significance as the first line of defence against metal exposure.

In conclusion, the study highlights the intricate interplay of factors influencing bioleaching efficiency, including sample type, genetic responses, metal concentrations, and the presence of potentially toxic compounds. These findings provide valuable insights into the mechanisms

responsible for varying metal recovery observed in different bioleaching processes and highlight the remarkable adaptability of *T. asperellum* in extreme environments. These mechanisms include enhanced protein folding and stress response in the ER in one step tailing compared to one-step ore, potentially due to upregulated genes associated with this organelle. In tailing bioleaching, *T. asperellum* adapted to the presence of toxic chemicals through upregulated genes related to detoxification and metal handling, leading to a distinct biochemical environment, and potentially explaining varying metal recoveries.

5.6 References

Alavi, N., Partovi, K., Majlessi, M., Rashidi, M., & Alimohammadi, M. (2021). Bioleaching of metals from cellphones batteries by a co-fungus medium in presence of carbon materials. *Bioresource Technology Reports*, *15*. https://doi.org/10.1016/j.biteb.2021.100768

Amiri, F., Mousavi, S. M., & Yaghmaei, S. (2011). Enhancement of bioleaching of a spent Ni/Mo hydroprocessing catalyst by Penicillium simplicissimum. *Separation and Purification Technology*, *80*(3), 566–576. https://doi.org/10.1016/j.seppur.2011.06.012

Arshadi, M., Esmaeili, A., & Yaghmaei, S. (2020). Investigating critical parameters for bioremoval of heavy metals from computer printed circuit boards using the fungus Aspergillus niger. *Hydrometallurgy*, *197*. https://doi.org/10.1016/j.hydromet.2020.105464

Asadrokht, M., & Zakeri, A. (2022). Chemo-physical concentration of a Low-grade nickel laterite ore. *Minerals Engineering*, *178*. https://doi.org/10.1016/j.mineng.2022.107398

Asghari, I., Mousavi, S.M., Amiri, F., Tavassoli, S. (2014). Bioleaching of spent refinery catalysts: a review. *J. Ind. Eng. Chem*, *14*(7), 553–554. https://doi.org/10.1016/S1473-3099(14)70794-7

Athirah, S., Jamali, M., Afiza Badaluddin, N., Nataqain Baharum, S., Salim, J. M., Ahmad, A., & Taib, M. (2020). Trichoderma atroviride isolated from mangroves of the east coast of peninsular malaysia exhibited high tolerance against heavy metal cadmium. In *Malays. Appl. Biol* (Vol. 49, Issue 4). http://www.ncbi.nlm.nih.gov/blast.

Bahaloo-Horeh, N., Mousavi, S. M., & Baniasadi, M. (2018). Use of adapted metal tolerant Aspergillus niger to enhance bioleaching efficiency of valuable metals from spent lithium-ion mobile phone batteries. *Journal of Cleaner Production*, *197*, 1546–1557. https://doi.org/10.1016/j.jclepro.2018.06.299

Bolaños-Benítez, V., van Hullebusch, E. D., Lens, P. N. L., Quantin, C., van de Vossenberg, J., Subramanian, S., & Sivry, Y. (2018). (Bio)leaching behavior of chromite tailings. *Minerals*, *8*(6). https://doi.org/10.3390/min8060261

Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics*, *30*(15), 2114–2120. https://doi.org/10.1093/bioinformatics/btu170

Borja, D., Nguyen, K., Silva, R., Park, J., Gupta, V., Han, Y., Lee, Y., & Kim, H. (2016). Experiences and Future Challenges of Bioleaching Research in South Korea. *Minerals*, *6*(4), 128. https://doi.org/10.3390/min6040128

Burgstaller, W., & Schinner, F. (1993). Leaching of metals with fungi. In *Journal of Biotechnology* (Vol. 27).

Chen, G., Shi, H., Ding, H., Zhang, X., Gu, T., Zhu, M., & Tan, W. (2023). Multi-scale analysis of nickel ion tolerance mechanism for thermophilic Sulfobacillus thermosulfidooxidans in bioleaching. *Journal of Hazardous Materials*, *443*. https://doi.org/10.1016/j.jhazmat.2022.130245

Dey, P., Malik, A., Singh, D. K., Haange, S. B., von Bergen, M., & Jehmlich, N. (2022). Insight Into the Molecular Mechanisms Underpinning the Mycoremediation of Multiple Metals by Proteomic Technique. *Frontiers in Microbiology*, *13*. https://doi.org/10.3389/fmicb.2022.872576

Din, G., Hassan, A., Rafiq, M., Hasan, F., Badshah, M., Khan, S., Chen, G., Ripp, S., & Shah, A. A. (2020). Characterization of Organic Acid Producing Aspergillus tubingensis FMS1 and its Role in Metals Leaching from Soil. *Geomicrobiology Journal*, *37*(4), 336–344. https://doi.org/10.1080/01490451.2019.1701585

Dusengemungu, L., Kasali, G., Gwanama, C., & Mubemba, B. (2021). Overview of fungal bioleaching of metals. *Environmental Advances*, *5*, 100083. https://doi.org/10.1016/j.envadv.2021.100083

Elahian, F., Heidari, R., Charghan, V. R., Asadbeik, E., & Mirzaei, S. A. (2020). Genetically modified Pichia pastoris, a powerful resistant factory for gold and palladium bioleaching and nanostructure heavy metal biosynthesis. *Artificial Cells, Nanomedicine and Biotechnology*, *48*(1), 259–265. https://doi.org/10.1080/21691401.2019.1699832

Emri, T., Gila, B., Antal, K., Fekete, F., Moon, H., Yu, J.-H., & Pócsi, I. (2021). AtfA-Independent Adaptation to the Toxic Heavy Metal Cadmium in Aspergillus nidulans. *Microorganisms*, *9*(7), 1433. https://doi.org/10.3390/microorganisms9071433

Faraji, F., Golmohammadzadeh, R., Rashchi, F., & Alimardani, N. (2018). Fungal bioleaching of WPCBs using Aspergillus niger: Observation, optimization and kinetics. *Journal of Environmental Management*, *217*, 775–787. https://doi.org/10.1016/j.jenvman.2018.04.043

Farjana, S. H., Huda, N., Parvez Mahmud, M. A., & Saidur, R. (2019). A review on the impact of mining and mineral processing industries through life cycle assessment. In *Journal of Cleaner Production* (Vol. 231, pp. 1200–1217). Elsevier Ltd. https://doi.org/10.1016/j.jclepro.2019.05.264

Ghosh, S., & Paul, A. K. (2016). Bioleaching of nickel by Aspergillus humicola SKP102 isolated from Indian lateritic overburden. *Journal of Sustainable Mining*, *15*(3), 108–114. https://doi.org/10.1016/j.jsm.2016.11.002

Grabherr, M. G., Haas, B. J., Yassour, M., Levin, J. Z., Thompson, D. A., Amit, I., Adiconis, X., Fan, L., Raychowdhury, R., Zeng, Q., Chen, Z., Mauceli, E., Hacohen, N., Gnirke, A., Rhind, N., Di Palma, F., Birren, B. W., Nusbaum, C., Lindblad-Toh, K., … Regev, A. (2011). Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nature Biotechnology*, *29*(7), 644–652. https://doi.org/10.1038/nbt.1883

Gu, T., Rastegar, S. O., Mousavi, S. M., Li, M., & Zhou, M. (2018). Advances in bioleaching for recovery of metals and bioremediation of fuel ash and sewage sludge. In *Bioresource Technology* (Vol. 261, pp. 428–440). Elsevier Ltd. https://doi.org/10.1016/j.biortech.2018.04.033

Haas, B. J., Papanicolaou, A., Yassour, M., Grabherr, M., Blood, P. D., Bowden, J., Couger, M. B., Eccles, D., Li, B., Lieber, M., Macmanes, M. D., Ott, M., Orvis, J., Pochet, N., Strozzi, F., Weeks, N., Westerman, R., William, T., Dewey, C. N., … Regev, A. (2013). De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. *Nature Protocols*, *8*(8), 1494–1512. https://doi.org/10.1038/nprot.2013.084

Horeh, N. B., Mousavi, S. M., & Shojaosadati, S. A. (2016). Bioleaching of valuable metals from spent lithium-ion mobile phone batteries using Aspergillus Niger. *Journal of Power Sources*, *320*, 257–266. https://doi.org/10.1016/j.jpowsour.2016.04.104

Huang, X., Sillanpää, M., Gjessing, E. T., Peräniemi, S., & Vogt, R. D. (2010). Environmental impact of mining activities on the surface water quality in Tibet: Gyama valley. *Science of the Total Environment*, *408*(19), 4177–4184. https://doi.org/10.1016/j.scitotenv.2010.05.015

Jafari, M., Shafaei, S. Z., Abdollahi, H., Gharabaghi, M., & Chehreh Chelgani, S. (2018). Effect of Flotation Reagents on the Activity of L. Ferrooxidans. *Mineral Processing and Extractive Metallurgy Review*, *39*(1), 34–43. https://doi.org/10.1080/08827508.2017.1323748

Keshavarz, S., Faraji, F., Rashchi, F., & Mokmeli, M. (2021). Bioleaching of manganese from a low-grade pyrolusite ore using Aspergillus niger: Process optimization and kinetic studies. *Journal of Environmental Management*, *285*. https://doi.org/10.1016/j.jenvman.2021.112153

Kishimoto, N., & Tano, T. (1987). Acidophilic heterotrophic bacteria isolated from acidic mine drainage, sewage, and soils. *The Journal of General and Applied Microbiology*, *33*(1), 11–25. https://doi.org/10.2323/jgam.33.11

Krężel, A., & Maret, W. (2016). The biological inorganic chemistry of zinc ions. *Archives of Biochemistry and Biophysics*, *611*, 3–19. https://doi.org/10.1016/j.abb.2016.04.010

Krishnamoorthy, S., Ramakrishnan, G., & Dhandapani, B. (2021). Recovery of valuable metals from waste printed circuit boards using organic acids synthesised by Aspergillus niveus. *IET Nanobiotechnology*, *15*(2), 212–220. https://doi.org/10.1049/nbt2.12001

Lewis, R. W., Okubara, P. A., Fuerst, E. P., He, R., Gang, D., & Sullivan, T. S. (2020). Chronic Sublethal Aluminum Exposure and Avena fatua Caryopsis Colonization Influence Gene Expression of Fusarium avenaceum F.a.1. *Frontiers in Microbiology*, *11*. https://doi.org/10.3389/fmicb.2020.00051

Manni, M., Berkeley, M. R., Seppey, M., Simão, F. A., & Zdobnov, E. M. (2021). BUSCO Update: Novel and Streamlined Workflows along with Broader and Deeper Phylogenetic Coverage for Scoring of Eukaryotic, Prokaryotic, and Viral Genomes. *Molecular Biology and Evolution*, *38*(10), 4647–4654. https://doi.org/10.1093/molbev/msab199

Marappa, N., Ramachandran, L., Dharumadurai, D., & Nooruddin, T. (2020). Recovery of Gold and Other Precious Metal Resources from Environmental Polluted E-waste Printed Circuit Board by Bioleaching Frankia. *International Journal of Environmental Research*, *14*(2), 165–176. https://doi.org/10.1007/s41742-020-00254-5

Martínez P, Gálvez S, Ohtsuka N, Budinich M, Cortés MP, Serpell C, Nakahigashi K, Hirayama A, Tomita M, Soga T, Martínez S, Maass A, P. P. (2012). Metabolomic study of Chilean biomining bacteria Acidithiobacillus ferrooxidans strain Wenelen and Acidithiobacillus thiooxidans strain Licanantay. *Metabolomics*, *9*(1), 247–257. https://doi.org/10.1007/s11306-012-0443-3

McCarthy, S., Ai, C., & Blum, P. (2018). Enhancement of Metallosphaera sedula Bioleaching by Targeted Recombination and Adaptive Laboratory Evolution. In *Advances in Applied*

Microbiology (Vol. 104, pp. 135–165). Academic Press Inc. https://doi.org/10.1016/bs.aambs.2018.03.002

Mishra, N., Khan, S. S., & Sundari, S. K. (2016). Native isolate of Trichoderma: a biocontrol agent with unique stress tolerance properties. *World Journal of Microbiology and Biotechnology*, *32*(8). https://doi.org/10.1007/s11274-016-2086-4

Moazzam, P., Boroumand, Y., Rabiei, P., Baghbaderani, S. S., Mokarian, P., Mohagheghian, F., Mohammed, L. J., & Razmjou, A. (2021). Lithium bioleaching: An emerging approach for the recovery of Li from spent lithium ion batteries. In *Chemosphere* (Vol. 277). Elsevier Ltd. https://doi.org/10.1016/j.chemosphere.2021.130196

Nakhaei, F., & Irannajad, M. (2018). Reagents types in flotation of iron oxide minerals: A review. In *Mineral Processing and Extractive Metallurgy Review* (Vol. 39, Issue 2, pp. 89–124). Taylor and Francis Inc. https://doi.org/10.1080/08827508.2017.1391245

Nkuna, R., Ijoma, G. N., & Matambo, T. S. (2022). Applying EDTA in Chelating Excess Metal Ions to Improve Downstream DNA Recovery from Mine Tailings for Long-Read Amplicon Sequencing of Acidophilic Fungi Communities. *Journal of Fungi*, *8*(5). https://doi.org/10.3390/jof8050419

Oladipo, O. G., Awotoye, O. O., Olayinka, A., Bezuidenhout, C. C., & Maboeta, M. S. (2018). Heavy metal tolerance traits of filamentous fungi isolated from gold and gemstone mining sites. *Brazilian Journal of Microbiology*, *49*(1), 29–37. https://doi.org/10.1016/j.bjm.2017.06.003

Opare, E. O., Struhs, E., & Mirkouei, A. (2021). A comparative state-of-technology review and future directions for rare earth element separation. In *Renewable and Sustainable Energy Reviews* (Vol. 143). Elsevier Ltd. https://doi.org/10.1016/j.rser.2021.110917

Pathak, A., Kothari, R., Vinoba, M., Habibi, N., & Tyagi, V. V. (2021). Fungal bioleaching of metals from refinery spent catalysts: A critical review of current research, challenges, and future directions. *Journal of Environmental Management*, *280*(November 2020), 111789. https://doi.org/10.1016/j.jenvman.2020.111789

Pathak, A., Morrison, L., & Healy, M. G. (2017). Catalytic potential of selected metal ions for bioleaching, and potential techno-economic and environmental issues: A critical review. *Bioresource Technology*, *229*, 211–221. https://doi.org/10.1016/j.biortech.2017.01.001

Phillips, J. (2016). Climate change and surface mining: A review of environment-human interactions & their spatial dynamics. In *Applied Geography* (Vol. 74, pp. 95–108). Elsevier Ltd. https://doi.org/10.1016/j.apgeog.2016.07.001

Pinzari, F., Colaizzi, P., Maggi, O., Persiani, A. M., Schütz, R., & Rabin, I. (2012). Fungal bioleaching of mineral components in a twentieth-century illuminated parchment. *Analytical and Bioanalytical Chemistry*, *402*(4), 1541–1550. https://doi.org/10.1007/s00216-011-5263-1

Pourret, O., Lange, B., Bonhoure, J., Colinet, G., Decrée, S., Mahy, G., Séleck, M., Shutcha, M., & Faucon, M. P. (2015). Assessment of soil metal distribution and environmental impact of mining in Katanga (Democratic Republic of Congo). *Applied Geochemistry*, *64*, 43–55. https://doi.org/10.1016/j.apgeochem.2015.07.012

Qu, Y., & Lian, B. (2013). Bioleaching of rare earth and radioactive elements from red mud using Penicillium tricolor RM-10. *Bioresource Technology*, *136*, 16–23. https://doi.org/10.1016/j.biortech.2013.03.070

Ran, M., Zhao, G., Jiao, L., Gu, Z., Yang, K., Wang, L., Cao, X., Xu, L., Yan, J., Yan, Y., Xie, S., & Yang, M. (2023). Copper Ion Mediates Yeast-to-Hypha Transition in Yarrowia lipolytica. *Journal of Fungi*, *9*(2). https://doi.org/10.3390/jof9020249

Sajjad, W., Zheng, G., Din, G., Ma, X., Rafiq, M., & Xu, W. (2019). Metals Extraction from Sulfide Ores with Microorganisms: The Bioleaching Technology and Recent Developments. In *Transactions of the Indian Institute of Metals* (Vol. 72, Issue 3, pp. 559–579). Springer. https://doi.org/10.1007/s12666-018-1516-4

Santhiya, D., & Ting, Y. P. (2005). Bioleaching of spent refinery processing catalyst using Aspergillus niger with high-yield oxalic acid. *Journal of Biotechnology*, *116*(2), 171–184. https://doi.org/10.1016/j.jbiotec.2004.10.011

Schwarz, D. S., & Blower, M. D. (2016). The endoplasmic reticulum: Structure, function and response to cellular signaling. In *Cellular and Molecular Life Sciences* (Vol. 73, Issue 1, pp. 79–94). Birkhauser Verlag AG. https://doi.org/10.1007/s00018-015-2052-6

Shah, S., Cesar Palmieri, M., Regina Pombeiro Sponchiado, S., & Bevilaqua, D. (2020). Enhanced bio-recovery of aluminum from low-grade bauxite using adapted fungal strains. *Brazilian Journal of Microbiology* , *51*, 1909–1918. https://doi.org/10.1007/s42770-020-00342-w/Published

Srichandan, H., Mohapatra, R. K., Parhi, P. K., & Mishra, S. (2019). Bioleaching approach for extraction of metal values from secondary solid wastes: A critical review. *Hydrometallurgy*, *189*. https://doi.org/10.1016/j.hydromet.2019.105122

Tzeferis, P. G., & Agatzini-Leonardou, S. (1994). Leaching of nickel and iron from Greek non-sulphide nickeliferous ores by organic acids. In *Hydrometallurgy* (Vol. 36).

Verma, A., Kore, R., Corbin, D. R., & Shiflett, M. B. (2019). Metal Recovery Using Oxalate Chemistry: A Technical Review. *Industrial and Engineering Chemistry Research*, *58*(34), 15381–15393. https://doi.org/10.1021/acs.iecr.9b02598

Xia, M., Bao, P., Liu, A., Wang, M., Shen, L., Yu, R., Liu, Y., Chen, M., Li, J., Wu, X., Qiu, G., & Zeng, W. (2018). Bioleaching of low-grade waste printed circuit boards by mixed fungal culture and its community structure analysis. *Resources, Conservation and Recycling*, *136*(January), 267–275. https://doi.org/10.1016/j.resconrec.2018.05.001

Xu, T. J., Ramanathan, T., & Ting, Y. P. (2014). Bioleaching of incineration fly ash by Aspergillus niger - Precipitation of metallic salt crystals and morphological alteration of the fungus. *Biotechnology Reports*, *3*, 8–14. https://doi.org/10.1016/j.btre.2014.05.009

Xu, T. J., & Ting, Y. P. (2009). Fungal bioleaching of incineration fly ash: Metal extraction and modeling growth kinetics. *Enzyme and Microbial Technology*, *44*(5), 323–328. https://doi.org/10.1016/j.enzmictec.2009.01.006

Xue, Z., Feng, Y., Li, H., Zhu, Z., Xu, C., Ju, J., & Yang, Y. (2023). A comprehensive review on progresses of coal and minerals bioflotation in presence of microorganisms. In *Journal of Environmental Chemical Engineering* (Vol. 11, Issue 6). Elsevier Ltd. https://doi.org/10.1016/j.jece.2023.111182

Yang, B., Bai, Z., & Zhang, J. (n.d.). *Environmental impact of mining-associated carbon emissions and analysis of cleaner production strategies in China*. https://doi.org/10.1007/s11356-020-11551-z/Published

CHAPTER 6: GENERAL CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

The challenges experienced during bioleaching are mainly due to the activities of microbial communities being affected by the metal toxicity and other harsh condition related to bioleaching process. Accordingly, this study focused on process optimization through understanding functional activities taking place during metal exposure and bioleaching. In the first objective, we were able to troubleshoot challenges presented by metal ions in affecting recovery and downstream application of DNA. The recommendation from this chapter was later applied towards successful RNA extraction and subsequent analysis using EDTA and the recommended concentration range.

In a study where the interest is on the activity of microorganisms, it is important to isolate such organisms. In chapter 3, the profiling of the fungal community gave insights on the type of dominant genera and with the use of selective media we were able to isolate most of the profiled dominant genera as reported in chapter 4. Of the 15 different isolates, 12 had bioleaching potential, that is, metal tolerance and organic acid production. Thus, the use of tailings, which resembles bioleaching condition (extremely low pH and high heavy metal content) was confirmed as the source of phylogenetically diverse fungal community with bioleaching capability. Subsequent quantitative analysis led to the selection of four fungal isolates, *Trichoderma, Talaromyces, Penicillium_3* and *Penicillium_6* in which their metabolites were studies during growth and glucose metabolism. This was based on the importance of organic acids during bioleaching which is mainly dependent on the type of carbon source used. In addition to carbohydrate and lipids, which were eventually linked to citric acid production the upregulation of compounds such as 2-Furan, 2-Furanmethanol and 5-Hydroxymethylfurfural was observed. These compounds are involved in furfural degradation metabolism, ultimately contributing to the citric acid cycle (citrate cycle). This is important because when grown in high glucose source, the fungus diverted metabolic activities to circumvent the carbon overflow and in addition to lipid production, the fungus produced secondary metabolites that eventually can be converted to organic acid in case of carbon source depletion. Thus, it may represent another metabolic pathway related to energy storage in fungi. Comparison of results in chapter 4 lead to the selection of *T. asperellum* as a candidate for bioleaching. One limitation of this chapter is the use of agar to investigate the effects of metals on fungal growth. For future studies, methods employing broth are recommended, as they allow for the use of dried cell weight as a more accurate measure of metal impact on fungal growth.

T. asperellum is a promising fungus for bioleaching applications due to its high metal tolerance and organic acid production capacity. This study demonstrated that *T. asperellum* can efficiently bioleach metals from both tailings and ore samples, regardless of the sample type. Additionally, transcriptomics analysis revealed that *T. asperellum*'s metal tolerance is associated with upregulated genes involved in cell membrane structure and function. This suggests that *T asperellum* is able to tolerate high metal exposure by maintaining the integrity of its cell membrane. The observed upregulation of genes related to cell membrane activity suggests a crucial role in metal tolerance, highlighting a potential mechanism for bioleaching optimization. Furthermore, the study revealed that the presence of metals did not hinder organic acid production by *T. asperellum* since the genes related to organic acid production were not differentially expressed in the presence of metals. This suggest that *T. asperellum*'s metal tolerance does not disrupt its metabolic activities. This resilience in metabolic activities of organic acid, even in the presence of metals, highlights the robustness of *T. asperellum*'s bioleaching ability and its potential for practical applications. Overall, this study provides valuable insights into the bioleaching potential of *T. asperellum* and its underlying molecular mechanisms.

Recommendation:

This study makes significant progress in addressing the complex challenges encountered in bioleaching processes, particularly focusing on the complex relationship between microbial activities, metal toxicity, and harsh bioleaching conditions. The application of EDTA for successful DNA/RNA extraction, as outlined in this study, serves as a valuable methodological advancement. Further research can explore variations in metal ion exposure and their specific impacts on DNA/RNA extraction to further improve protocols especially for environmental samples characterized as challenging to obtain pure DNA/RNA.

Chapter 5 convincingly demonstrates several key aspects that support scalability such as high metal tolerance and organic acid production by *T. asperellum*. This tolerance is further supported by the upregulation of genes involved in cell membrane function, suggesting a robust mechanism for metal resistance. The bioleaching efficiency was obseerved across both tailings and ore samples, indicating *T. asperellum*'s adaptability to different feedstock materials. This adaptability is crucial for real-world applications with varying ore compositions. Transcriptomic analysis provides valuable insights into the underlying molecular mechanisms

of metal tolerance and organic acid production. This understanding can inform future optimization strategies for enhanced bioleaching performance.

Beyond these initial findings, further research is crucial to comprehensively assess and optimize the scalability of the process. Scaling up from laboratory-scale bioreactors to industrial-scale operations requires testing under larger-volume conditions. This would confirm the feasibility and cost-effectiveness of the process on a commercial scale. Future research should explore the specific roles of genes and pathways upregulated in this study, particularly those involved in cell membrane regulation. This knowledge can guide targeted genetic modifications to potentially enhance metal tolerance and bioleaching performance.

In conclusion, this study not only advances our understanding of the molecular mechanisms behind metal tolerance in *T. asperellum* but also lays the foundation for future bioleaching optimization strategies. By addressing the key points mentioned above, we can translate these promising findings into scalable and practical bioleaching technology with significant potential for the sustainable recovery of metals.

ADDENDUMS

Journal of *Fungi*

MDPI

Article

Applying EDTA in Chelating Excess Metal Ions to Improve Downstream DNA Recovery from Mine Tailings for Long-Read Amplicon Sequencing of Acidophilic Fungi Communities

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Abstract: The hostile environment of mine tailings contains unique microbial life capable of bioleaching. The metagenomic analysis of such an environment provides an in-depth understanding of the microbial life and its potential, especially in biomining operations. However, DNA recovery from samples collected in those environments is challenging due to the presence of metal ions that interfere with the DNA analysis. A varied concentration of EDTA (4–13 µg/µL) to chelate the metal ions of enriched tailing samples prior to DNA extraction was performed. The results show that 9 µg/µL of EDTA was effective in most samples. However, the increasing concentration of EDTA negatively affected the DNA recovery. The sequencing of the successfully extracted DNA revealed a diverse range of fungal genera, some of which have not been previously reported in tailing or bioleaching applications. The dominant genera include *Fodinomyces*, *Penicillium*, *Recurvomuces*, *Trichoderma*, and *Xenoacremonium*; their traits were determined using the FungalTraits database. This study demonstrates the need to include a preliminary metal-chelating step using EDTA before DNA extractions for samples collected from metal-rich environments. It further showed the need for optimization but provided a benchmark range, particularly for tailings. However, we caution that a further EDTA removal step from the extracted DNA should be included to avoid its interferences in downstream applications.

Keywords: bioleaching; DNA extraction; ethylene diamine tetraacetic acid (EDTA); fungi; mine tailing

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Citation: Nkuna, R.; Ijoma, G.N.; Matambo, T.S. Applying EDTA in Chelating Excess Metal Ions to Improve Downstream DNA Recovery from Mine Tailings for Long-Read Amplicon Sequencing of Acidophilic Fungi Communities. *J. Fungi* **2022**, *8*, 419. https://doi.org/10.3390/jof8050419

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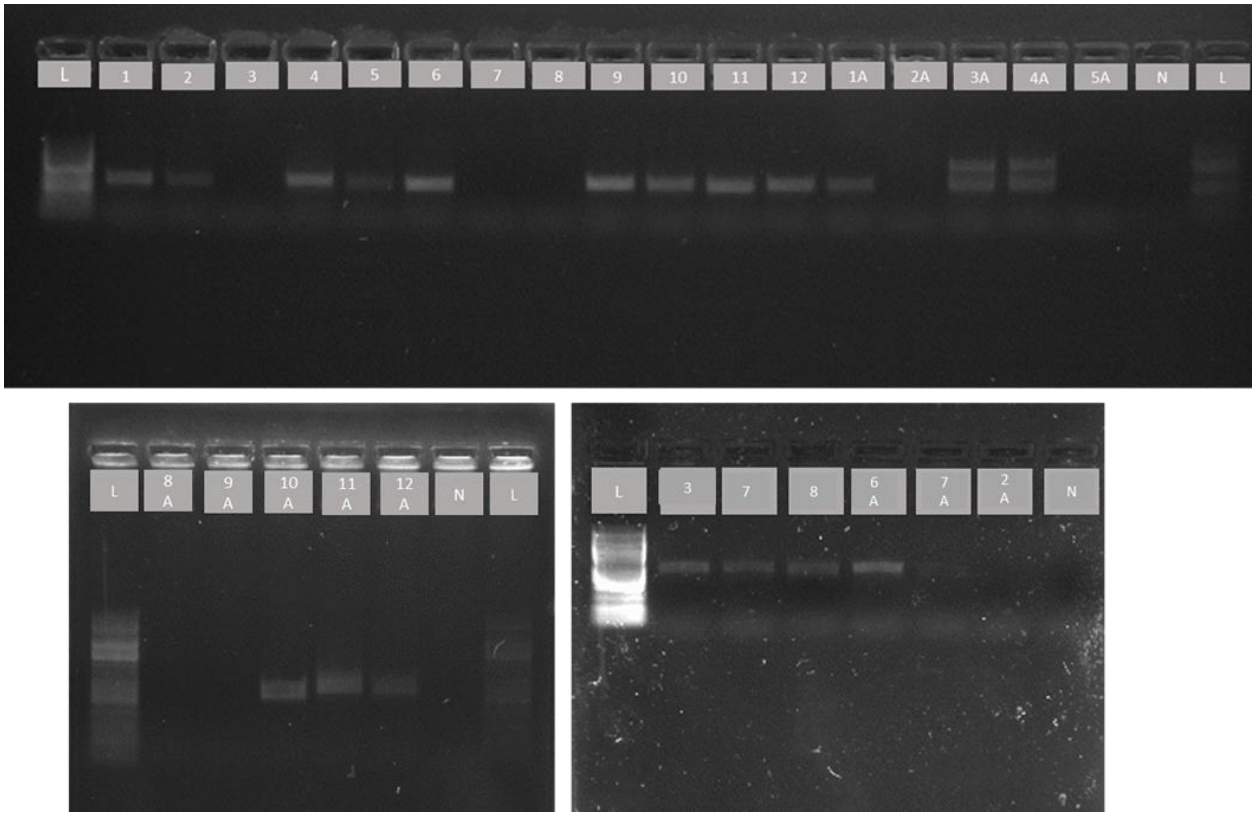
1. Introduction

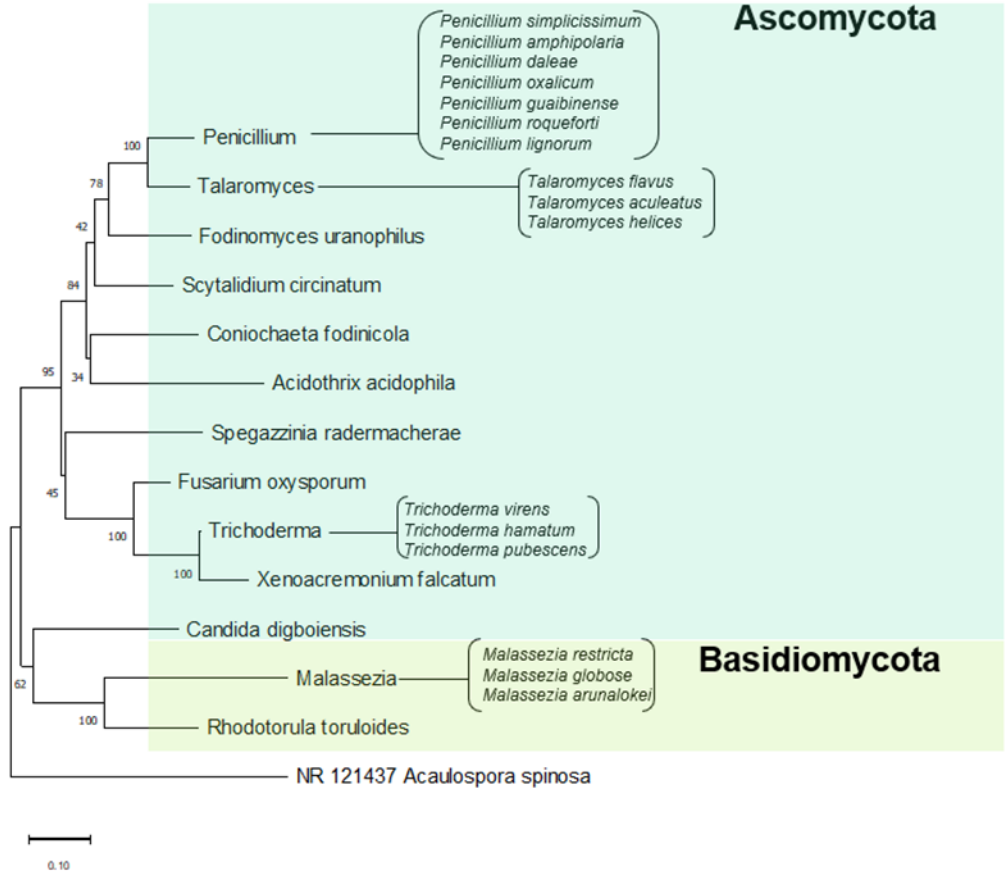
Mine tailings sites frequently contain distinct and phylogenetically diverse microbial communities that can be exploited for their specific metal-extraction capabilities (bioleaching) [1]. Despite the presence of both chemolithotrophic prokaryotes and heterotroph eukaryotes in these environments, the research is focused more on the chemolithotrophic members of the community in comparison to the eukaryotic members [2,3]. However, in recent years, the characteristics of the eukaryotic members, particularly their acid-tolerance, have piqued the interest of scholars and scientists in studying and applying them in bioleaching processes [4]. Depending on the research question, bioleaching research employs either a culture-dependent method or a culture-independent method or a combination of the two [5]. However, profiling such microbial communities relies on metagenomics approaches as they have been shown to be very useful in rapidly elucidating large sample areas. Metagenomic DNA isolated from tailing environments is a potential genetic resource from which the uncultured microbial species' phylogenetic affiliation can be determined, and their genetic potential can be explored by identifying novel genes with potential applications in bioleaching processes [6].

The foundation of various biological applications in microbial profiling depends on accurate and reliable DNA analysis. Separating pure DNA from cell matrix or biological

Appendix A3.1: Screenshot that shows that the manuscript published in Fungi Journal-2022



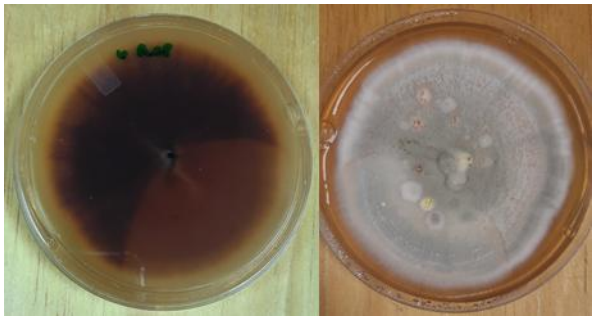
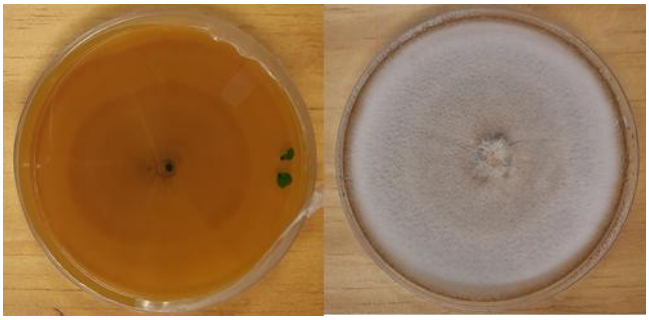
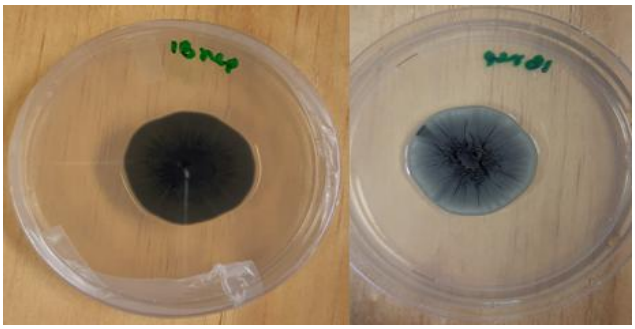
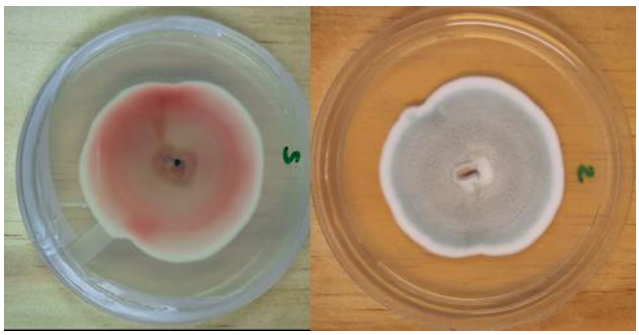
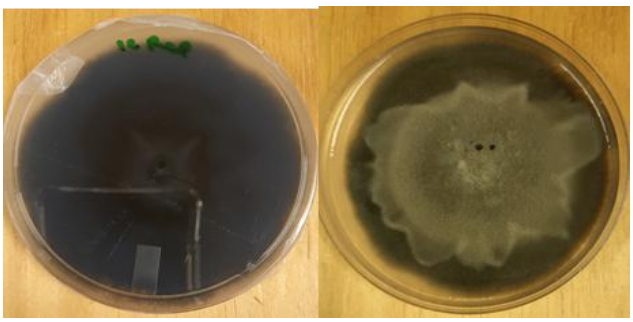
Appendix A3.2: Gel image showing samples with successful amplification of ITS region. 1-10 represent TM1, TM2, TM3, 1TA, 2TA, 3TA, 1TB, 2TB, 3TB, 1TC, 2TC, 3TC for S1 respectively and 1A to 10A represent TM1, TM2, TM3, 1TA, 2TA, 3TA, 1TB, 2TB, 3TB, 1TC, 2TC, 3TC for S2 respectively. L-ladder and N -Negative.



Appendix A3.3: Evolutionary relationships of taxa resolved to species level. The evolutionary relationships of taxa are resolved to the species level. The Neighbor-Joining method was used to infer the evolutionary history (Tanunchai *et al.*, 2022). Next to the branches is the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) (Põlme *et al.*, 2020). The outgroup was *Acaulospora spinosa*.

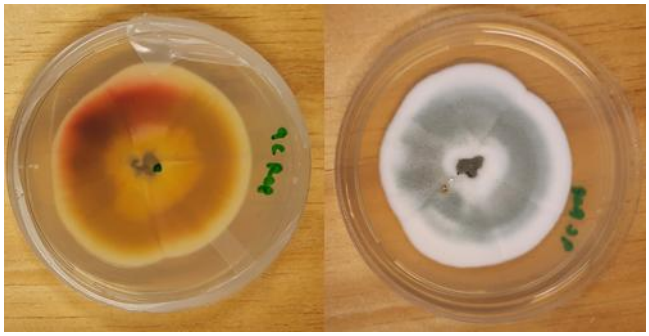
Appendix A4.1: Screenshot that shows that the manuscript is currently under review in Minerals Journal -Bioremediation of Contaminants in Mine Areas.

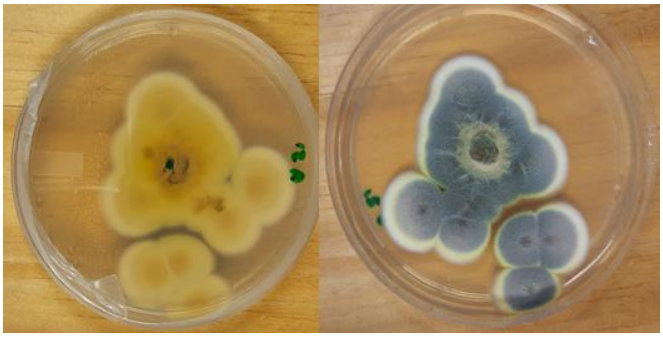
Appendix A4.2: Morphological characterization of 15 fungal isolates on PDA

Front	**Back**	**Front**	**Back**
Coniochaeta 		*Fusarium* 	
Talaromyces_1 		*Acidiella* 	
*Penicillium_*1 		*Acidomyces* 	
Talaromyces_2		*Penicillium_2*	

*Penicillium*_4

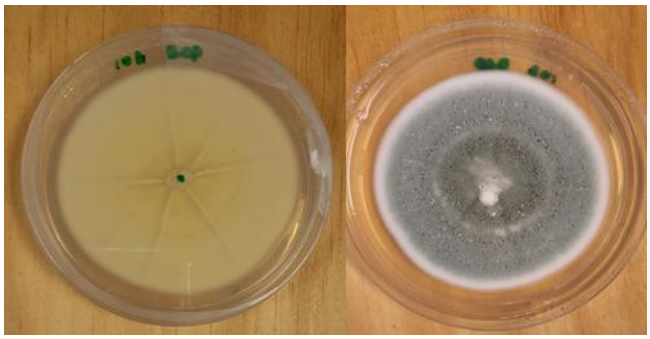
*Penicillium*_3

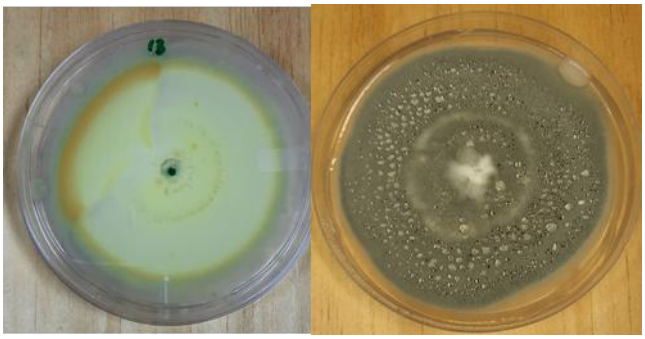




*Penicillium*_5

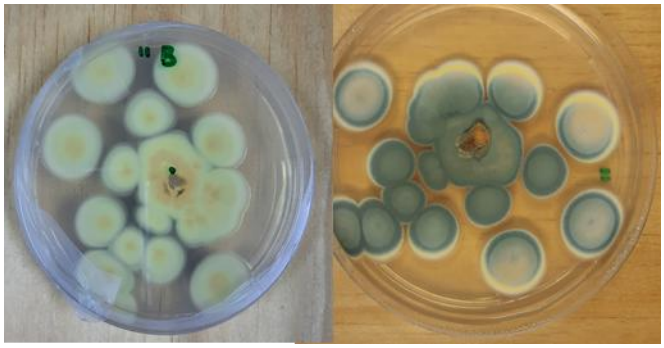
*Penicillium*_7

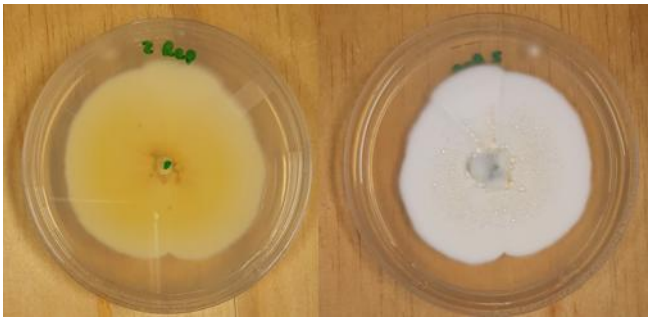




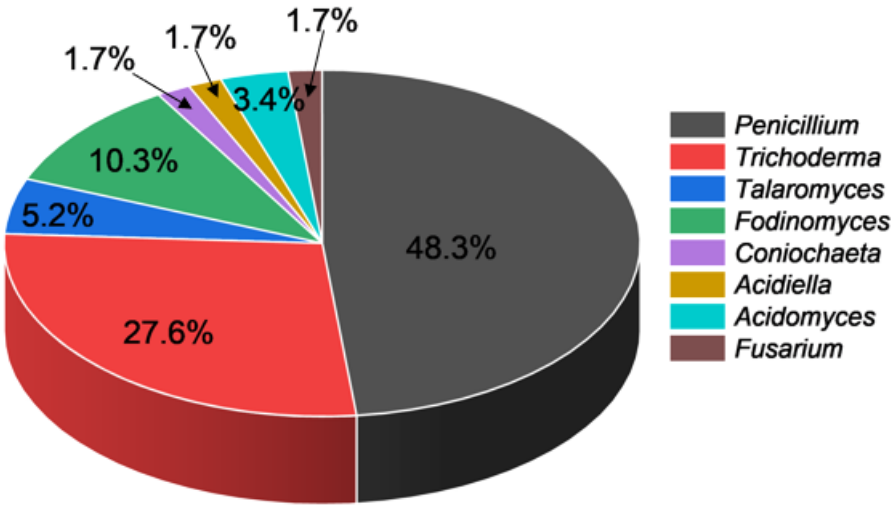
*Penicillium*_6

*Penicillium*_8





Trichoderma



Appendix A4.3: Relative abundance (number of isolates) of fungi isolated from the four tailing samples.

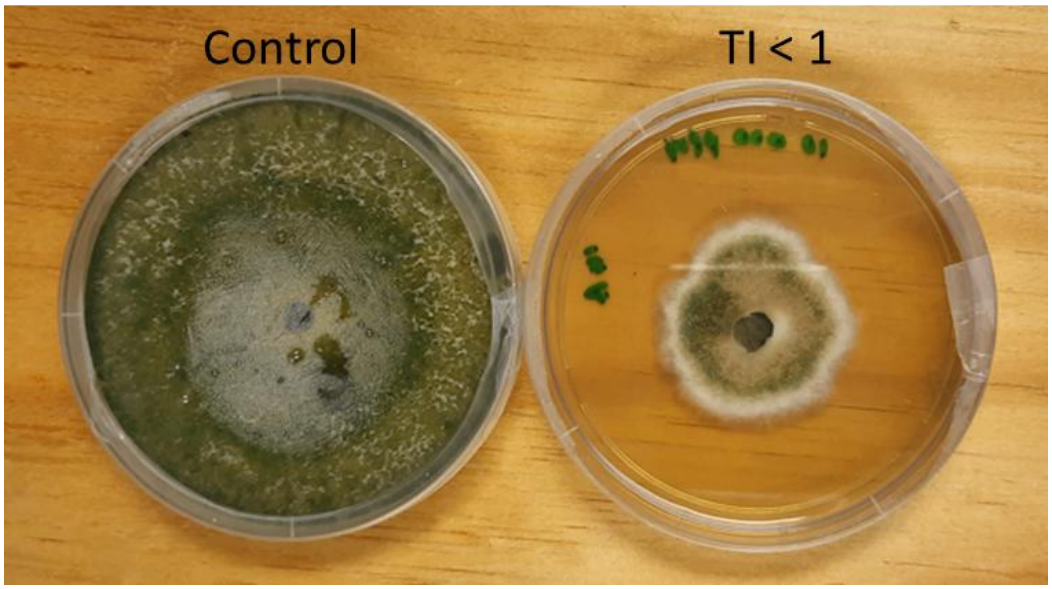
Appendix A4.4: Operational taxonomic units of fungal isolates, clustered at 97% similarity. The identity of the representative isolates is presented at a genus level. The accession number of each isolated is also listed.

Representative OTU	OTUs (accession number and sample ID)	Frequency	Genera
OL439087_S2L1B2	OL439087_S2L1B2, OL439115_S2L2B2, OL439090_S3L3B2, OL439088_S2L1B3, OL439106_L2A6, OL439102_S2MA6, OL439098_S3L3B2-1, OL439095_S3L1B2, OL439084_L2B2, OL439094_S3L1B1,	16	*Trichoderma*

	OL439089_S3L2B2, OL439081_S2L2B1, OL439097_S3L3B1, OL439082_S3L1B4, OL439086_S2L1A1, OL439099_S3L3B4		
OL439096_S3L1C2	OL439096_S3L1C2, OL439116_S2L3B5, OL439111_S2MC2, OL439066_L2A4, OL439079_MA3, OL439092_L3C3, OL439078_L2C1, OL439108_L2A3, OL439112_MA7, OL439112_L1A3	10	*Penicillium* sp. *(1)*
OL439093_S2MC2-1	OL439093_S2MC2-1, OL439123_S3L3B5, OL439114_S2L1B4, OL439077_S3MC3, OL439100_L2B2-1, OL439080_MB8, OL439083_S3L2B3, OL439067_L2B4	8	*Penicillium* sp. *(2)*
OL439070_L3C2	OL439070_L3C2, OL439125_S3MA1, OL439121_MB9, OL439122_MC5, OL439107_L1C2, OL439069_L3C1	6	*Penicillium* sp. *(3)*
OL439101_L3C4	OL439101_L3C4, OL439120_S3L2C2, OL439109_S2L2C2, OL439105_S3L2C3, OL439116_S2L2B3, OL439110_S2L2C3	6	*Fodinomyes*
OL439075_S2MA4	OL439075_S2MA4, OL439073_MC8, OL439071_MA4	3	*Talaromyces* sp. *(1)*
OL439104_S3L2C1	OL439104_S3L2C1, OL439103_S3L2C1-1	2	*Acidomyces*
OL439104_S2MC3	OL439104_S2MC3, OL439072_MA6	2	*Penicillium* sp. *(4)*
OL439091_S3L3C3	OL439091_S3L3C3	1	*Penicillium* sp. *(5)*

OL439085_L3B1	OL439085_L3B1	1	*Fusarium*
S2MA5	OL439118_S2MA5	1	*Penicillium* sp. *(6)*
OL439118_S3L1C1	OL439119_S3L1C1	1	*Coniochaeta.*
OL439074_S2MA1	OL439074_S2MA1	1	*Penicillium* sp. *(7)*
OL439124_S3L3B6	OL439124_S3L3B6	1	*Acidiella*
OL439068_L1B2	OL439068_L1B2	1	*Talaromyces (2)*

*The number in brackets (1-8) are used to differentiate different species of the same genera



Appendix A4.5: The images depict the growth of *Trichoderma* in GYEA without metal (control) and its growth in a high metal concentration where it is susceptible. Impaired growth is observed compared to control.

Appendix A5.1: Screenshot that shows that the manuscript is currently under review in Journal of Environmental management.

Appendix A5.2: pH values of *T. asperellum* grown in different amounts of ore and tailing samples after 10 days of incubation using GYEM. Initial pH was 5.5.

Sample amount (g)	Ore	Tailing
5 g	3.8	3.0
10 g	3.8	3.0
15 g	3.9	3.5
20 g	4.2	3.2
25 g	5.8	4.2
30 g	6.2	4.4