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Analysis of indigenous filamentous fungal isolates for base metal bioleaching from a low-grade platinum metal ore concentrate

by

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Dissertation

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Declaration

I declare that this dissertation is my own, unaided work. It is being submitted for the Degree of Master of Science at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.



(Signature of candidate)

day of 10 January 2026 at Franklin Roosevelt Park, Randburg.

Abstract

This study aimed to investigate the potential of fungal species isolated from a platinum mine for bioleaching base metals from a platinum group metal (PGM) flotation concentrate, as an alternative to conventional, environmentally harmful processing methods. Platinum group metals are valuable for their diverse industrial applications, yet traditional extraction methods are energy-intensive and environmentally damaging. Fungal bioleaching, using organic acids produced by fungi, offers an eco-friendly alternative by facilitating the dissolution and extraction of metals from ores. However, there is a gap in the research on fungal species isolated from platinum mining environments, and their bioleaching capabilities remain underexplored. A previous study isolated and characterized 66 fungal species from a platinum mine, of which 32 were identified to species level, predominantly belonging to Ascomycota. Fourteen of these isolates were selected based on their significant production of organic acids, particularly oxalic acid, and their potential for bioleaching. The addition of sucrose as a supplemental carbon source enhanced spore production, growth rates and organic acid production, suggesting broader applications for these isolates in bioleaching. Screening experiments showed that *Fusarium* sp. and *Aspergillus niger* had the highest spore counts and consistent growth in Czapek yeast extract (CYE) medium, making them prime candidates for bioleaching. Spent medium leaching using *Fusarium* sp. resulted in the highest base metal extractions, particularly iron, nickel and cobalt, under low pH and high temperature conditions, which were more effective than one-step leaching. The one-step leaching experiments showed promising copper extraction, with *Fusarium* sp. demonstrating higher copper recovery compared to *A. niger*, although overall base metal extractions were lower than those achieved through spent medium leaching. These findings highlight the potential for enhancing bioleaching processes, particularly for copper, and suggest that longer leaching periods or more extreme conditions could further improve extraction rates. The hypothesis that bioleaching, driven by organic acids produced by fungi, can facilitate base metal dissolution from a low-grade PGM flotation concentrate was supported by these results. This study bridges the research gap on platinum mine-isolated fungi and their bioleaching potential. It was concluded that *Fusarium* sp. and *A. niger* are promising candidates for future bioleaching applications, and that CYE was an effective medium for promoting fungal growth

and organic acid production. Further investigation into fungal bioleaching and the application of more extreme leaching conditions could improve extraction efficiencies and provide a more sustainable method for PGM extraction.

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  أشهدُ أن لا إله إلا اللهُ وأشهدُ أن محمداً رسولُ اللهِ

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List of Abbreviations

Absorbance units – A

Acetonitrile – CAN

Acid unitage – AU

Adenosine triphosphatase – ATPase

Aluminium - Al

Analysis of variance – ANOVA

Arsenic – As

Cadmium - Cd

Calcium carbonate – $CaCo_3$

Calcium hydroxide – $Ca(OH)_2$

Calcium ion – Ca^{2+}

Centimetres squared – cm^2

Co-enzyme A – CoA

Colony-forming units per millilitre – CFU/mL

cubic metre per ton - m^3/t

Czapek yeast extract – CYE

Design of experiment – DOE

Dipotassium phosphate – K_2HPO_4

Distilled water – dH_2O

Environmental, social and governance – ESG

Ferrous sulphate – $FeSO_4$

Grams - g

Grams per tonne – g/t

Greenhouse gases – GHG

High performance liquid chromatography – HPLC

Hydrochloric acid – HCl

Hydrogen peroxide – H_2O_2

Inductively coupled optical emission spectroscopy – ICP OES

Industrial microbiology and biotechnology laboratory – IMBL

Iridium – Ir

Kilometre – Km

Kilowatt hours per meter squared - kWh/m^2

Lead – Pb

Lithium - Li

Magnesium sulphate – $MgSO_4$

Malt extract - ME

Manganese - Mn

Metal oxide – MeO

Microlitres - µL

Micrometre - µm

Milligrams per litre – mg/L

Millilitres – mL

Millimetre – mm

Millimolar – mM

Million litres per day – ML/day

Million tons – Mt

Molar – M

Molybdenum - Mo

Nanometres – nm

Nitric acid – HNO_3

Optical density – OD

Osmium – Os

Palladium – Pd

Parts per million - ppm

PGM - Platinum group metals

Platinum – Pt

Potassium chloride – KCl

Potassium dihydrogen phosphate – KH_2PO_4

Potato dextrose – PD

Printed circuit boards – PCBs

Pulp density – PD

Reverse phase – RP

Revolutions per minute – rpm

Rhodium – Rh

Roswell Park Memorial Institute – RPMI

Ruthenium - Ru

Selenium – Se

Silver - Ag

Sodium hydroxide – NaOH

Sodium nitrate – $NaNO_3$

Spores per millilitre – spores/mL

Square kilometres – km^2

Sulphuric acid – H_2SO_4

Sustainable development goals – SDGs

Tailing storage facilities – TSFs

Tellurium – Te

Ultraviolet – UV

United states geological survey – USGS

Upper group 2 – UG2

Uranium – U

V - Vanadium

Water supply system – WSS

Weight by volume – w/v

Zinc - Zn

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Chapter 1: Introduction

1.1 Introduction

The platinum group metals (PGMs) are a selection of rare metals comprising platinum (Pt), palladium (Pd), ruthenium (Ru), rhodium (Rh), iridium (Ir) and osmium (Os), all of which are widely sought after due to their high melting point, conductivity and corrosion resistance (Rao *et al.*, 2000). They are heavily utilised in the electrochemical, petrochemical and jewellery industries, with platinum being particularly sought after (Hughes *et al.*, 2021). PGMs as a whole are chemically inert and resistant to corrosion, allowing for biological compatibility and use in different healthcare treatments without major toxic effect. They are commonly utilised in the production of medical devices such as defibrillators and pacemakers, and in the makeup of anti-cancer drugs (Levine *et* al., 2010). One important aspects of PGMs are their catalytic activity, which is utilised by the automotive industry in the production of auto-catalysts (catalytic converters), a crucial component in the effort to control the rate of harmful vehicular carbon monoxide emissions into the atmosphere. Up to 90% of the worldwide PGM reserves are contained within the South African Bushveld Igneous complex, and it is the largest producer of PGMs in the world (Cawthorn, 2010). These reserves occur in igneous rock formations, which are types of rock rich in other minerals and base metals. As a result, PGMs often co-exist with certain base and precious metals (Rao *et al.*, 2000). These base metals can interfere with the final PGM yield and are usually separated during pre-treatment steps involving chemical leaching (Sefako *et al.*, 2019).

Conventional PGM processing routes involve energy intensive high-heat smelting procedures and environmentally-damaging, chemically intensive processing steps (Sefako *et al.*, 2019). Significant pressure is placed on the surrounding environment, with risks of water pollution, harmful emissions and loss of landscape (Cole, 2023). Significant pressure is also placed on the economy of the country, as rising costs and depleting ore reserves threaten to impact an industry that contributes substantially to South Africa's GDP (gross domestic profit) (Hughes *et al.*, 2021). There is a global push to introduce more eco-friendly and sustainable alternatives to conventional PGM processing. Bioleaching, a metal extraction technique utilising microorganisms in the dissolution and recovery of PGMs, metals and other interfering

elements from a source material without the need for chemical and energy intensive processes, has been proposed (Dusengemungu *et al*., 2021). There is a crucial gap in the industrial implementation of microbial bioleaching for platinum metal extraction.

A by-product of heterotrophic fungal metabolism is biogenic organic acid, such as carboxylic acid (Liaud *et al*., 2014). Glucose, as a carbon source, is consumed by the fungi leading to the production of carboxylic acids (Magnuson and Lasure, 2004). These acids aid the fungi in the breakdown of ore while decreasing the pH of the surrounding environment and can be utilised in the breakdown and extraction of metals (Dusengemungu *et al*., 2021). Protons introduced by the organic acids during acidification of a medium cause metals to leach from their original source into the solution, where they form metal chelates (Gadd, 2010). This process allows desired metals to be separated from unwanted material. Fungi are also capable of withstanding increasing concentrations of heavy metals (Fomina and Gadd, 2014). This ability enables them to continue growing during the bioleaching process. Fungal bioleaching can be achieved through direct contact between the fungi and the source material, or through indirect leaching. Indirect leaching is achieved by separating the organic acids from the fungal biomass and using the produced organic acids to bioleach metals (Bahaloo-Horeh and Mousavi, 2017). Several studies have been successful in applying fungal bioleaching to metal extraction on a lab scale, however not much research has been conducted on industrial scale application. Species of *Aspergillus* and *Penicillium*, which are from the phylum Ascomycota, are the most commonly utilized fungi for bioleaching. Research on fungal species outside the phylum Ascomycota, and platinum mine-isolated fungi specifically, is limited (Dusengemungu *et al*., 2021).

1.2 Problem Statement

There is a substantial research gap with regard to fungal bioleaching of PGMs, specifically concerning the characterisation and application of platinum-mine isolated fungi. Implementation of bioleaching on an industrial scale is dependent on closing this gap. The considerable environmental impact of traditional leaching methods, and depletion of natural resources as a result, necessitates rapid advancement in the area of bioleaching. Studies exist

on the isolation and identification of bacteria from platinum mines, however the same cannot be said for fungi. Fungi localised to a platinum mining environment may have a higher affinity for PGMs given their close proximity, which could increase the fungi's potential bioleaching efficiencies. Further research of a wider range of fungal species is also required, in order to discern those fungi with high bioleaching potential based on their growth and organic acid-producing capabilities. Therefore, characterisation of a large number of platinum-mine isolated fungal species is required, as well as assessment of their ability to bioleach metals.

1.3 Research Aims and Objectives

This study intends to bridge a gap in fungal bioleaching research by determining if fungal species isolated from a platinum mining environment are capable of bioleaching base metals from a PGM concentrate. The extraction of base metals from a PGM upper-ground 2 (UG2) flotation concentrate will be explored through two mechanisms of indigenous fungal-species based bioleaching processes: cell-free bioleaching (spent medium), reliant on the biogenic acids produced by the fungi and a direct, cell-dense inoculum-based one-step leaching. The ability of the isolated fungal species to induce the dissolution and separation of base metals from a PGM UG2 flotation concentrate must be investigated before further application of fungal PGM bioleaching.

Aims were set to be achieved through the following objectives:

- Characterisation of the growth patterns of 14 fungal isolates in different types of nutrient media
- Determination of the spore-producing capabilities of the 14 fungal isolates in different types of nutrient media
- Quantification of organic acids produced by the 14 fungal isolates through reverse-phase high performance liquid chromatography (HPLC)

- Screening of the 14 isolates based on growth and organic acid production to identify two isolates with ideal parameters that promote the dissolution of base metals from the PGM flotation concentrate
- Investigation of the base metal extraction capabilities of the two screened isolates through one-step and spent medium bioleaching experiments

1.4 Hypothesis

Fungal species have proved successful at the bioleaching of heavy metals from both primary and secondary sources (Dusengemungu *et al*., 2021). This is due to carboxylic organic acids, produced by fungi in the presence of a high concentration of a rapidly metabolizable carbon source. Chelating properties of these acids allow them to function in mineral dissolution. As a result, it is postulated that bioleaching using organic acids, produced by carbon-source supplemented fungal species, will result in the dissolution and separation of base metals from a low-grade PGM flotation concentrate.

Chapter 2: Literature Review

2.1 Platinum Group Metals

2.1.1 Discovery

Platinum, discovered in mid-sixteenth century Columbia, whose name derives from the source of the metal being named "*platina del Pinto"*, is the first of a series of metals defined as the platinum group metals (Rao *et al*., 1991). A relatively definitive account of the platinum metal was first published in southwest London in 1755 (Hartley, 1991). The discovery of palladium, rhodium, osmium and iridium would follow in 1803, nearly three centuries after the discovery of platinum. Palladium, named for the asteroid "Pallas", was discovered while attempting the refinement of platinum by W. H. Wollaston. Wollaston would also discover rhodium, naming it for the red colour of its salts from the Greek word *rhodes* (rose). Iridium, named after the Greek *iris* (rainbow) due to the range of colours of its salts, was discovered by Smithson Tennant when investigating material from the original "*platina"* that was insoluble in aqua regia, a corrosive mixture of nitric acid (HNO_3) and hydrochloric acid (HCl) used to dissolve platinum and palladium (Doğan *et al*., 2016). Osmium was also discovered by Tennant in this manner, who named it for its odorous tetroxide after the Greek *osme* (a smell) (Hartley, 1991). Karl Klaus isolated ruthenium in 1844 after dissolving, distilling and heating insoluble remnants from the treatment of platinum with aqua regia. Ruthenium is named for the Latin *Ruthenia* (Russia), its location of origin (Hartley, 1991).

2.1.2 Occurrence

Platinum group metals (PGMs) are characterised as having a low crustal abundance, with each metal comprising less than 2% by weight of the Earth's crust (Hartley, 1991). The estimated relative abundances of each metal in parts per million (ppm) are as follows: Pt 0.005 ppm, Pd 0.001 ppm, Rh 0.0004 ppm, Ir 0.0004 ppm, Os 0.0004 ppm and Ru 0.0004 ppm. PGMs frequently occur in either an iron-loving siderophile state or a sulphide-loving chalcophyllic

state, with mafic igneous rock, a silicate rock rich in magnesium and iron (Cross *et al*., 1902), and ultramafic rock being the most PGM-rich ore bodies (Hughes *et al*., 2021). The richest of these PGM-containing ore bodies occur in the South African Bushveld igneous complex, the Zimbabwean Hartley complex, the Australian Northern Territory, Sudbury (Ontario), the placer deposits in the Russian Ural Mountains as well as the Noril'sk nickel copper platinum group metal (Ni-Cu-PGM) sulphide deposits in the Russian Arctic, and the Still-water complex of Montana, USA (Rao *et al*., 2000). The PGM content in grams per tonne (g/t) of these different ore bodies however, vary, meaning the degree and type (primary/secondary) of PGM extraction is dependent on the concentration of PGMs within the specific ore body as well as the value of those PGMs. Within the Canadian Sudbury complex, PGM extraction is secondary to copper-nickel production given the low total ore body PGM content of 0.79 g/t, with only 75 g of PGMs obtained per tonne of nickel (Hartley, 1991). Within the Russian Noril'sk complex, PGMs are produced in conjunction with copper-nickel extraction, given the much richer 9.74 g/t total PGM content of its ore body (Hughes *et al*., 2021). Table 1 breaks down the total PGM content of each of the ore bodies contained within these reserves as well as the content of the individual PGMs (Hughes *et al*., 2021).

Table 1: Ore body platinum group metal content (grams/tonne) of worldwide platinum group metal reserves including total and individual platinum, palladium, ruthenium, osmium, iridium and rhodium reserves (Hughes *et al.*, 2021).

Ore Body	Pt g/t	Pd g/t	Rh g/t	Ru g/t	Ir g/t	Os g/t	Total PGM g/t	PGM Reserves Tonnes [a]
South Africa								**63,000**
Merensky Reef (H)	2.7	1.4	0.16	0.33	0.05	0.04	4.68	
- East Bushveld (M)	2.32	1.22	0.13	0.23	0.04	-		
- West Bushveld (M)	3.6	1.6	0.28	0.63	0.14	-		
Platreef (H)	1.9	1.9	0.12	0.14	0.04	0.03	4.13	
- North Bushveld (M)	0.82	1.1	0.08	-	-	-	2.0	
UG2 (H)	2.0	1.3	0.34	0.45	0.13	0.05	4.27	
- East Bushveld (M)	2.42	2.06	0.45	0.76	0.18	-		
- West Bushveld (M)	2.89	1.48	0.54	0.93	0.22	-		
Zimbabwe								**1200**
Hartely Complex	2.6	1.8	0.21	-	-	-	4.61	
USA								**900**
Stillwater Complex	3.3	11	0.6	0.36	0.21	-	15.47	
Canada								**310**
Sudbury Complex	0.3	0.4	0.03	0.04	0.01	0.01	0.79	
Lac-des Iles	0.2	2.3	-	-	-	-	2.5	
Russia								**3900**
Noril'sk	2.5	7	0.24	-	-	-	9.74	
Reserve (tonnes)	**32,374**	**24,433**	**3909**	**6731**	**1530**	**333**	**-**	**69,310**
Lifetime (years) [b]	**202**	**116**	**166**	**201**	**224**	**-**	**-**	**-**

PGMs are the primary product of the South African Bushveld complex, an area of nearly 40 000 square kilometres (km^2) separated into the Platreef, Upper Group 2 (UG2) reef and Merensky reef, with a total PGM content in g/t of 4.13, 4.27 and 4.68 respectively (Cawthorn, 2010). Altogether, the reefs contain 63 000 tonnes of total PGM reserves, 59 100 tonnes more

than the next reserve and nearly 90% of the known worldwide PGM reserves (Hughes *et al*., 2021). In contrast to Russia and Canada, ores within the bushveld complex primarily produce PGMs, with copper and nickel as the secondary products. The Merensky reef PGM composition is high at 60% Pt, 25% Pd, 4% Rh and 11% of Ru, Os and Ir, however the UG2 reef contains 300 – 750 million ounces more PGMs than Merensky while also having a lower copper and nickel content (Naldrett et al., 2011). The higher density of PGMs allows for greater PGM tonnage to volume ratios while the lower base metal content allows for fewer pre-treatment steps (Sefako *et al*., 2019). The mineral content of the Merensky reef includes several PGM-base metal alloys, namely: laurite [$(RuFeOsIrPt)S_2$], kotulskite [$(PtPdNi)(TeBiSb)_2$], Pt–Fe [Pt_3Fe], braggite [PtS], cooperite [(PtPdNi)S] and moncheite [$(PtPdNi)\ (TeBiSb)_2$] (Kyriakakis, 2005).

2.1.3 Production of Platinum Group Metals

PGM production occurs from primary sources (mining of ore bodies) and secondary sources (recycling of unprocessed low-grade ore, electronic components, automotive parts, etc.) (Kyriakakis, 2005). Primary PGM production in South Africa involves the direct smelting of PGM ore bodies to produce a PGM ore concentrate (Sefako *et al*., 2019). Milling of these ore bodies is followed by froth flotation, which separates minerals based on their hydrophobicity, producing a flotation concentrate with a 60 - 90% sulphide ore recovery and 100 – 1000 g/t PGM content (Kyriakakis, 2005). Following flotation is pyrometallurgical separation, where the concentrate is dried and smelted at temperatures exceeding 1450 ^{o}C to produce a Cu-Ni-Fe-S PGM fluid matte (Mudd, 2012). Leaching with sulphuric acid (H_2SO_4) in autoclaves with increased oxygenation is conducted to leach the Cu, Ni, Fe, S and other present metalloids such as arsenic (As), selenium (Se) and tellurium (Te) (Sefako *et al*., 2019). The Ni-Cu sulphide alloys are allowed to crystalise out of solution as the matte cools, while the Fe and S are separated by oxidation as a result of the oxygenation and forced into the spaces between the sulphide crystals. Crushing of the matte allows an Fe-PGM alloy to be separated through magnetism, followed by leaching of the Fe to produce a PGM-heavy concentrate (Kyriakakis *et al*., 2005). The usual PGM content of the concentrate following separation is shown in Table 2. The individual PGMs from the concentrate are separated by precious metal refining through

selective electrolytic and ion-exchange procedures (Hughes *et al*., 2005). The conventional processing route is shown in as described is shown in Figure 1.

Table 2: Range of individual platinum group metal content within the concentrate following base metal removal (Kyriakakis, 2005)

	Platinum	Palladium	Rhodium	Ruthenium	Iridium	Total PGM
Metal Content (%)	36-40	17-22	4-5	7-11	1.4-2	60-80

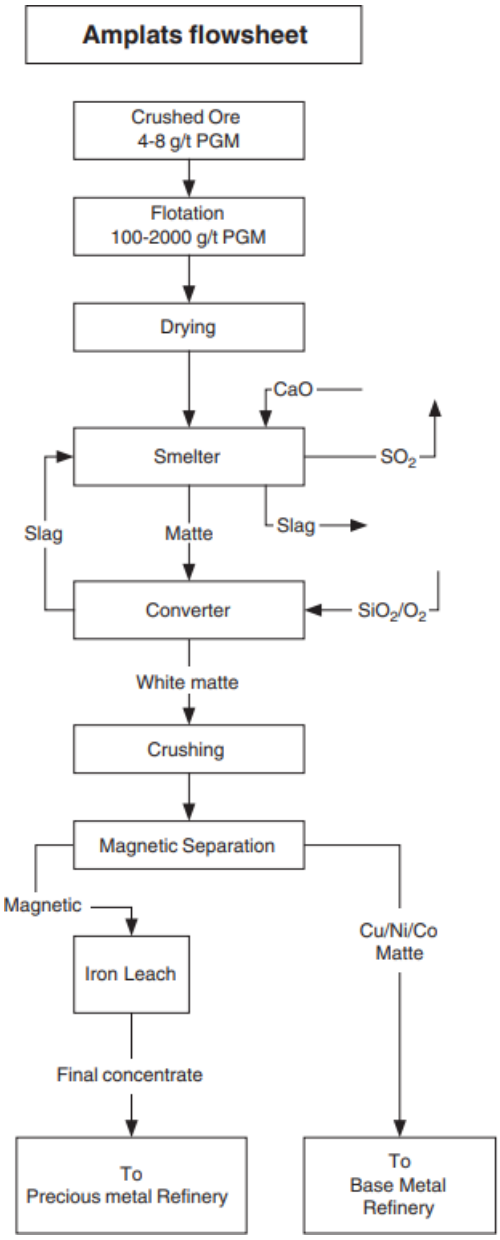


Figure 1: Traditional processing circuit in South Africa for the separation of platinum group metals from sulphidic ores for the production of a platinum group metal concentrate (Kyriakakis, 2005)

Secondary PGM processing involves the extraction of Pt, Pd, Ru, Ir and Rh from recycled electronic waste, spent automotive catalytic converters (auto-catalysts), jewellery and manufacturing scrap (Hughes *et al*., 2021). Studies have been done on potential PGM extraction from mobile phones, automative printed circuit boards (PCBs) and automotive oxygen sensors (Cui and Zhang, 2008)**.** Hagelüken (2008) states that the potential PGM content of 80 g/t Pd, 130 g/t Pd and 2000 g/t Pt can be acquired from computer boards, mobile phones and auto-catalysts respectively. This is significantly higher than the <10 g/t PGM content found in primary ores (Cawthorn, 2010). Auto-catalysts are by far the largest contributor to PGM recycling, given the very high PGM content contained in the ceramic catalyst brick of the catalytic converter (Hagelüken, 2012). More than 80% of the PGM recovery in the USA in 2014 was from auto-catalysts, with more than 60% of the PGM recycling in Europe from auto-catalysts in 2012 (Hughes *et al*., 2021).

2.2 Challenges, Environmental Impacts and Sustainability

The impact of PGM mining is assessed through environmental, social and governance (ESG) risk factors, both to and by PGM mining operations, according to the United Nations Sustainable Development Goals (SDGs) (Cole, 2023). These SDGs encompass nine potential ESG risk areas involved with platinum mining, namely: energy, biodiversity, air quality, waste, community, social vulnerability, land use, governance and water. According to Lèbre and coworkers (2020), who quantified the probability and severity of socio-environmental impacts of mining, PGMs have the highest ESG risk of all transition minerals in the mining industry. Given that the vast majority of worldwide PGM production occurs in South Africa, the country is facing considerable ESG risks (Cole, 2023).

2.2.1 Water

In a study by Cole, (2023), the ESG risk factors of the South African Anglo Mogalakwena PGM mine were assessed. Water supply and management is seen as the biggest ESG risk by mining

corporations. The climate surrounding the Mogalakwena mine is semi-arid and hot, with low annual rainfall and high annual evaporation. This led to the development of water resource projects to provide bulk water supply to the mine, the surrounding mining communities and other domestic areas in the Mokopane and Polokwane regions. Of the 68.6 million litres per day (ML/day) of water provided by the Mogalakwena municipality to the mine, urban and rural areas, 40% is lost (Kamuti, 2022). Of the 66 ML/day of water used by the mine, only a 0.25 cubic metre per ton (m^3/t) water use efficiency is achieved. This is considerably lower than the 1.68 m^3/t achieved by other South African platinum mines, likely because Mogalakwena is the only large open pit mine (Kamuti, 2022). As a result, demand for bulk water supply in the area continues to grow (52% from 2015 to 2020) as mining continues and the communities expand, which has reduced the water quality in the area. Wastewater management and the Mokopane Water Supply System (WSS) were given a high risk and critical risk rating respectively, according to the South African Green Drop Report and Blue Drop Report in 2022 (Cole, 2023). The municipality is unable to manage the interdependency of the PGM mining operation and the growing mining community on its water supply, leading to a downturn on the quality of water and water management of the surrounding areas.

2.2.2 Energy/Emissions

Mining companies rank decarbonisation and climate change as the 2nd and 3rd highest ESG risks (Lèbre *et al*., 2020). Mining operations in South Africa are dependent on coal-fired power stations for their baseload electricity, which means they have a constant need for electricity. This significantly increases GHG (greenhouse gas) emissions while exacerbating the existing national energy crisis caused by poor management and slow implementation of renewable energy (Cole, 2023). Despite the access to electricity, semi-urban and rural areas surrounding Mogalakwena still rely on firewood for personal use and an income source, contributing to deforestation. As of 2021, South Africa, along with 29 other countries, has introduced a national strategy for net-zero carbon emissions by implementing large-scale renewable hydrogen projects (Department of Science and Innovation, 2021). The surrounding Mogalakwena area also has a high solar radiation potential of six kilowatt hours per meter squared (kWh/m^2), with solar power plants scheduled to be built as of 2024 (CSIR, 2019).

2.2.3 Waste

Rock, water and chemical slurries, known as mine tailings, are a considerable waste product of large-scale mining operations, with billions of tons produced annually (Cole, 2023). These tailings are often stored in either tailing storage facilities (TSFs) or mine tailing dams, which given their massive size, are a considerable disaster risk. TSFs are susceptible to climatic (rainfall, wind) and geological (tectonic movement) events of the surrounding area, while tailing dams are susceptible to catastrophic failures that are common for large-scale industrial installations of this nature (Owen *et al*., 2020). Globally, six separate TSFs failures were recorded between 2010 and 2019, leading to widespread destruction of agricultural land and ecosystems as well as considerable loss of life (>200 lives lost at the Córrego do Feijão mine tailings dam collapse in Brazil in 2019) (Roche et al., 2017)**.** The Mogalakwena mine currently utilizes three active TSFs, with current tailing volumes of the first two TSFs at 250 Mt (million tons), as well as eight waste rock dump sites (Cole, 2023). During 2022, 84.7 Mt of rock was extracted from Mogalakwena mine but only 13.9 Mt was processed, a ratio of 6:1 of waste rock produced (Cole, 2023). TSF failure was identified as a key risk to the Mogalakwena mine by the Global Tailing Portal, with two of its TSFs being labelled as major hazards for TSF failure as they are located within 1 kilometre (km) of 12 separate villages (Global Tailings Portal, 2023)

2.2.4 Challenges of Secondary Extraction (Recycling)

Given that the production routes of the automotive industry and the PGM content of its products are well known, PGM recycling from the auto industry should be well established (Hughes *et al*., 2021). The number of vehicles manufactured annually is also well known, including the number of vehicles currently in use. However, several factors around the recycling of vehicles as well the demand of certain PGMs makes predicting the amount of PGMs extracted from automotive sources difficult. Vehicle recovery for recycling is known to experience lag phases, where large proportions of the vehicles retired within any given year are scrapped rather than recycled. In 1998, 30% of all vehicles retired went unaccounted for according to the United States Geological Survey (USGS), with European countries presenting a similar recovery rate (Hagelüken *et al*., 2012). PGM recycling is also susceptible to the

growing lag phase between catalyst manufacturing and recycling, increasing from 13 to 18 years by 2016 (Hagelüken *et al*., 2012). The availability of secondary sources for PGM extraction decreases as vehicles last longer and fewer are recycled.

2.3 Alternative Extraction

Attempts to alleviate the challenges of metal extraction are not only driven by the impact that resource exploitation has on the current state of the environment, but also by the rapid depletion of valuable metal ore reserves (Dusengemungu *et al*., 2021). A large number of studies investigate alternative, eco-friendly and affordable methods of metal extraction from alternative sources such as low-grade ore, mine tailings, e-waste and spent catalysts (Dusengemungu *et al*., 2021; Ilyas *et al*., 2013; Kumar *et al*., 2013; Seh-Bardan *et al*., 2012). Given the growing demand for metal production, further investigation and implementation of alternative extraction methods from low-grade ore and waste sources are necessary. Bacterial bioleaching (using bacterial species such as *Acidithiobacillus* spp.) of certain metals is already a well-established industrial process (Liang and Gadd, 2017). However, a study by Sedlakova-Kadukova and coworkers (2020) has stated that the leaching kinetics of bacterial bioleaching are considerably slower than fungal bioleaching. The leaching of Li (lithium) by acidophilic bacteria took nearly 300 more days to achieve a higher percent extraction than heterotrophic fungi (Sedlakova-Kadukova *et al*., 2020). Bacteria also display a lower tolerance to pH changes and metal toxicity than fungi (Kumar and Yaashikaa, 2020). Numerous studies have supported the use of fungal microorganisms for low-grade mineral/metal bioleaching, given the ability of fungi to produce organic acids as well as tolerate exposure to different metals (Dusengemungu *et al*., 2021).

2.3.1 Fungal Organic Acid Production

One important ability of heterotrophic fungi is their degradation of lignocellulosic biomass, through the secretion of biodegradative enzymes (Ayele *et al*., 2021). By-products of the

biomass degradation, such as glucose, lead to the production of various low-molecular weight carboxylic organic acids by the fungal microorganisms (Dusengemungu *et al.*, 2021). Liaud and coworkers (2014) suggest that this carboxylic organic acid production provides a competitive advantage for fungi over other microorganisms by reducing the pH of the surrounding environment, whereas other functions may be tied to mineral weathering for nutrient supply. Acid production is also thought to play a role in cellulosic degradation, while also supporting fungal metal tolerance by detoxification through metal complexation (Liaud *et al.*, 2014). Karaffa and Kubicek (2020), state that organic acid production can only occur under specific conditions, such as availability of a highly metabolizable carbon source, sufficient aeration and a sub-optimal concentration of nitrates/phosphates to limit biomass production.

Organic acid production, triggered by a metabolizable carbon source, occurs within the cytosol and mitochondria of a fungal cell (Dusengemungu *et al.*, 2021). Of the carboxylic acids produced by fungi, the synthesis of monocarboxylic lactic acid occurs as a result of glycolysis, the synthesis of di- and tri- carboxylic acids (citric, acetic, succinic, malic, fumaric and oxalic) are products of the aerobic catabolism of glucose (at high concentrations) and the synthesis of gluconic acid occurs extracellularly and is then taken into the cell (Karaffa and Kubicek, 2020) (Figure 2). For the dicarboxylic acids (fumaric, malic, oxalic) produced during glucose catabolism, the presence of the enzyme pyruvate carboxylase is important and unique to fungal metabolism (Din *et al.*, 2020). No carbon dioxide (CO_2) is released during glucose catabolism, which is important for the synthesis of pyruvate-by-pyruvate carboxylase, as one carbon (C) is consumed per pyruvate synthesized. Therefore, in the presence of excess carbon and glucose, organic acid synthesis is able to yield twice as much dicarboxylic acid as glucose (Ayele *et al.*, 2021). Tricarboxylic acid synthesis also relies on pyruvate carboxylase, which utilizes CO_2 produced from the decarboxylation of acetyl-COA (co-enzyme A) to produce citrate and then citric acid (Fathollahzadeh *et al.*, 2019).

Eighty to ninety percent of industrial lactic acid production is achieved through homolactic fermentation (lactate production without CO_2 and ethanol as byproducts) using bacteria such as *Lactobacillus* spp. and also through the fermentation of corn starch with yeasts such as *Saccharomyces* and *Kluyveromyces* (Abedi and Hashemi, 2020). Lactic acid production by the filamentous fungi *Rhizopus oryzae* has been well documented, with a nearly 70% yield of lactic acid achieved from a glucose-rich medium. The maximum possible lactic acid yield is believed

to be twice that of the glucose taken in (two moles of lactic acid per one mole of glucose). However, this maximum yield is interrupted by the synthesis of byproducts such as ethanol (Karaffa and Kubicek, 2020). According to Abedi and Hashemi, (2020), fermentation with filamentous fungi for lactic acid production has several advantages over yeast and bacterial fermentation, namely: the use of chemically defined media with inorganic nutrient sources leading to more efficient purification steps, increased glucose metabolism leading to higher lactic acid yields and the production of pure L-lactic acid which allows for food application.

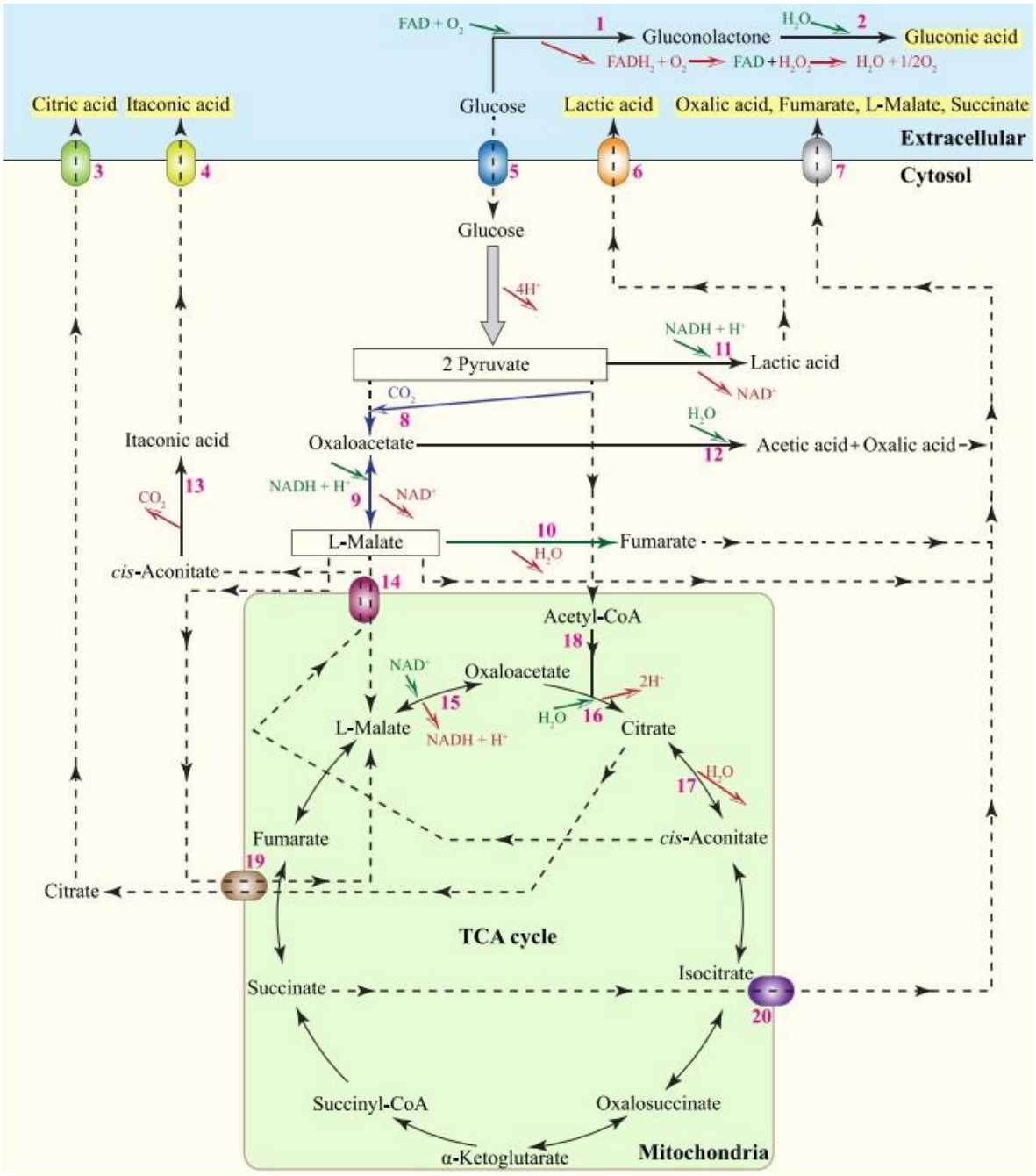


Figure 2: Points of synthesis in the glycolytic pathway for carboxylic organic acids following glucose uptake (Karaffa and Kubicek, 2020).

Dicarboxylic oxalic acid is the most widespread fungal organic acid produced, according to Palmieri and coworkers (2019), who state that oxalate/carboxylic acid production is present in many species of mycorrhizal, brown/white-rot, plant and mammalian pathogenic fungi. Because oxalic acid promotes biomass degradation and occurs at a wide pH range (2-6), it's production is widespread among different fungi. Despite this, oxalic acid has no

biotechnological uses given the toxicity of oxalate as a result of its strong calcium ion (Ca^{2+}) chelating ability (Choi *et al*., 2014). Another dicarboxylic acid, malic acid, is chemically produced for food applications, with its production also being observed in *Rhizopus* and *Aspergillus* species (Sahasrabudhe and Sankpal, 2001). The production of malate, following glycolysis, leads to the production of L-malic acid with the inclusion of calcium carbonate ($CaCO_3$) and has yields of up to 95% from *Aspergillus* species during fermentation. However, production of malic acid through fermentation does not meet food safety standards, given the presence of toxic and carcinogenic aflatoxins in species of *Aspergillus* (Dusengemungu *et al.,* 2021). Similar to malic acid, the production of dicarboxylic fumaric acid is seen in several *Rhizopus* species and requires high concentrations of glucose, carbon and $CaCO_3$ for increased yields. Fumaric acid production is also limited by the presence of ethanol as a by-product (Ruijter *et al*., 2002).

Tricarboxylic citric acid production in mid-19th century was achieved through large-scale extraction from citrus fruit, given that citric acid makes up nearly 8% of citrus fruit dry weight (Karaffa and Kubicek, 2020). By the late 19th and early 20th century, *Penicillium* and *Aspergillus* species were found to produce citric acid from sugar solutions (molasses, corn starch) in liquid culture (Książek, 2023). Production of citric acid, by *A. niger* specifically, was achieved by the separation of mycelia from the culture medium, precipitation of the citric acid out of the spent broth with calcium hydroxide ($Ca(OH)_2$) and the conversion back to citric acid with sulphuric acid (Fathollahzadeh *et al*., 2019). Metabolic citric acid production is achieved through glycolytic breakdown of a simple sugar (glucose, fructose) into two moles of pyruvate, then converted into acetyl-CoA (co-enzyme A) and oxaloacetate, before being condensed into citric acid in the mitochondria and released through the cell membrane. As with other organic acids, high yields of citric acid production are only achieved with an increased carbon supply (Książek, 2023).

Fungal production of gluconic acid is enzymatically driven via the dehydrogenation of glucose to lactone by glucose oxidase (Narisetty *et al*., 2023). The lactone is hydrolysed to form gluconic acid in a process that is either extracellular or semi-bound to the cell wall, depending on the fungal species. Production of glucose oxidase is triggered by high glucose concentrations but is limited by hydrogen peroxide (H_2O_2), which is released during the dehydrogenation of glucose. Certain fungi, such as *A. niger*, release enzymes that catalyse the

breakdown of H_2O_2 into oxygen and water, promoting glucose conversion into gluconic acid (Walaszczyk *et al.,* 2018). Gluconic acid is also a good carbon source specific to *A. niger* over other microorganisms. As a result, *A. niger* is most commonly used for gluconic acid production, capable of a 95% yield from a 35% weight by volume (w/v) glucose solution (Karaffa and Kubicek, 2020).

2.3.2 Fungal Bioleaching Mechanisms

Fungal bioleaching is separated into four mechanisms based on the interaction between fungal organic acid and target metals; acidolysis, complexolysis, bioaccumulation and chelation (Gadd, 2010). As highlighted above, heterotrophic microorganisms metabolise organic substances (e.g., glucose) for growth and energy production, releasing carboxylic organic acids and enzymes as a result (Magnuson and Lasure, 2004). These metabolites are able to solubilize metal into metal complexes through chelate formation, allowing for the separation of a target metal from its source (Fomina and Gadd, 2014).

2.3.2.1 Acidolysis

Metal solubilisation is achieved by the protonation of oxygen atoms on a metal-ore surface, decreasing the strength of the bonds and allowing for metal ions to be released from the ore surface into solution (Srichandan *et al*., 2019). The protonated oxygen atoms of a metal oxide (MeO) react with water to produce free metal ions, as shown in Equation 1.

$$MeO + 2H^+ \rightarrow Me^{2+} + H_2O \qquad \text{(Equation 1)}$$

Fungal organic acids are responsible for providing the protons that drive acidolysis, while increasing metal solubility by reducing the access that anions have to metal cations through the use of proton translocating ATPase (adenosine triphosphatase) (Dusengemungu *et al*., 2021). The organic acid proton supply also functions in the stabilisation of metal chelating complexes and is the limiting factor for the final amount of metal oxides produced (Suja *et al*., 2018).

2.3.2.2 Complexolysis

The metal ions released into solution by acidolysis are complexed into metal chelates by organic acid and amino acid ligands, which promote metal complexing and solubilisation (Srichandan *et al*., 2019), as seen in Equation 2.

$Fe^{3+} + 0.5HO_2C.CO_2H \rightarrow Fe(O_2C.CO_2)_3^{3-} + 6H^+$ (Equation 2)

The bonds formed between the metal-ligand complex are stronger than the bonds between metal and ore surface, promoting metal extraction/solubilisation from the solid ore, while the proton stabilisation of the metal complexes reduce the effect of metal toxicity on the fungal microorganism (Dusengemungu *et al*., 2021). Metal complexation has been shown to be specific to the organic ligand that is produced, for example, citric acid complexing with magnesium and oxalic acid complexing with iron (Fathollahzadeh *et al*., 2019).

2.3.2.3 Bioaccumulation

Bioaccumulation involves the intracellular uptake of solubilised metal ions into the fungal biomass, a process achieved by passive adsorption (Suja *et al*., 2018). The uptake is believed to be the result of functional groups (hydroxyl, carboxyl, etc.) present on the fungal mycelium, which bind metal ions through the exchange of cations and precipitate them out of solution, as shown in Equation 3 (Fathollahzadeh *et al*., 2019).

$Me^{2+} + HO_2C.CO_2H \rightarrow (Me(O_2C.CO_2))(s) + 6H^+$ (Equation 3)

2.3.2.4 Biosorption

Biosorption is similar to bioaccumulation, in that solubilised metal ions are adsorbed into the fungal biomass from solution (Suja *et al*., 2018). However, contrary to bioaccumulation, this process is driven by the organic acids produced (Fathollahzadeh *et al*., 2019). Metal ions that

were leached into solution by the organic acids are taken up by the fungal biomass independent of the functional groups present on the fungal mycelia (Dusengemungu *et al*., 2021).

2.3.3 Direct and Indirect Leaching Applications

2.3.3.1 One Step Leaching

Bioleaching can be performed by direct one-step, two-step or indirect cell-free spent medium leaching processes (Fathollahzadeh *et al*., 2019). One-step leaching involves growth of the fungi in the presence of the target leaching material, allowing for simultaneous metabolism of the nutrient substrate as the fungi grows and leaching of target metals from the ore/waste as result of the metabolites produced (Kumar and Yaashikaa, 2020). The leaching efficiency of one-step leaching may be reduced given that certain conditions required for fungal growth are sub-optimal for metal recovery, such as higher pH and lower temperatures (Srichandan *et al*., 2019). However, organic acid production differs when fungi are incubated in the presence of metal ores and waste materials. Seh-Bardan and coworkers (2019) found that oxalic and gluconic acid were produced in higher quantities by *Aspergillus fumigatus* during one-step leaching of mine tailings. This study also found that oxalic acid was the major leaching agent during one-step leaching processes, with up to 260 mM of oxalic acid produced by *A. fumigatus* at different tailings concentrations. Extractions of As, Fe, manganese (Mn) and zinc (Zn) by *A. fumigatus* were higher during one-step leaching than the extractions in two-step leaching (Seh-Bardan *et al*., 2019).

2.3.3.2 Two-Step Leaching

Two-step leaching involves growth of the fungi and metabolism of the nutrient substrate separate from the target leaching material, which may be subjected to a pre-treatment step (Bahaloo-Horeh and Mousavi, 2017). Pre-treatment is introduced before leaching to allow for

breakdown of the target ore and removal of unwanted elements, to enhance the ore's characteristics for leaching and improve its rate of dissolution (Yakoumis *et al*., 2021). Oxidative roasting treatments are used to form metal alloys and convert the metallic compounds into oxides, allowing for the removal of impurities which could hinder PGM recovery. Reductive roasting treatments allow for the removal of passivation layers surrounding the metal, which are formed during natural oxidation and shield the metals from chemical breakdown during leaching (Habashi, 1997). Once sufficient fungal growth is achieved, the fungi are introduced to the pre-treated target metal ore/waste material to leach the target metals (Kumar and Yaashikaa, 2020).

Two-step leaching efficiencies benefit from the optimisation of leaching conditions for metal extraction rather than fungal growth, while reducing any inhibitory effects by the target metals (Moazzam *et al*., 2021). Seh-Bardan and coworkers (2019) found citric acid to be the main leaching agent during *A. fumigatus* two-step leaching, at 8.5 mM concentration. However, despite this low concentration of citric acid, a 65% extraction of lead (Pb) was achieved, higher than the Pb extraction during one-step leaching. The leaching efficiency of two-step leaching is higher for certain metals, however the use of a second step makes two-step leaching less cost-effective compared to one-step.

2.3.3.3 Spent Medium Leaching

Indirect spent medium leaching involves the extraction of target metals in the absence of fungal cells and biomass, which have been centrifuged and filtered out of a fungal culture leaving behind a cell-free 'spent' medium (Lobos *et al*., 2021). Once fungi have reached the stationary phase of their growth, sufficient metabolite production is achieved. Following filtration, the target ore/waste material is added to the spent medium for metal extraction (Moazzam *et al*., 2021). Spent medium leaching efficiencies benefit from reduced processing times and simplified operating procedures, including the potential for improved leaching given the temperature and pH extremes that are possible in the absence of microorganisms. Spent medium leaching also allows for increased pulp densities, given that any inhibitory effect is irrelevant in the absence of microorganisms (Bahaloo-Horeh and Mousavi, 2017). A study by

Bahaloo-Horeh and Mousavi (2017), which compared one-step, two-step and spent medium leaching by *A. niger* from spent lithium batteries, found that the highest extractions of Co, Li, Mn, Al (aluminium), Cu and Ni were achieved using spent medium leaching.

2.3.4 Fungal Species Used for Bioleaching

As mentioned previously, several studies exist on the application of fungal bioleaching to different ore source materials. Table 3 lists different fungi and their capacity for metal extraction from low-grade ores, mine tailings, e-waste, solid wastes and spent catalysts. The most commonly used fungi are species of *Aspergillus*, most notably *A. niger*, and *Penicillium*. This is consistent with the study by Liaud and coworkers (2014) on the organic acid production of filamentous fungi. Of the 40 strains of Ascomycetes tested, especially species of *Aspergillus* and *Penicillium*, all were capable of producing organic acids (Liaud, *et al*., 2014). A study by Anahid and coworkers (2010), focusing on the heavy metal tolerance of the fungal species *A. foetidus, A. niger* and *P. simplicissimum*, found that these species were highly tolerant to increased concentrations of Mo and V (vanadium) in spent refinery catalysts, while being less so to Ni, Co and Zn. These findings are consistent with the studies by Santhiya and Ting (2006) and Amiri and coworkers (2011) shown in Table 3, where the bioleaching of spent catalysts by *A. niger* and *P. simplicissimum*, respectively, showed higher extractions of Mo (82.3 and 97.6%) than Ni (78.5 and 45.7%). *P. simplicissimum*, in conjunction with *A. niger,* was also able to extract more than 98% of Al from a low-grade bauxite ore according to a study by Shah and coworkers (2020). *A. foetidus* showed a similar extraction capacity for Al (88.43%) from spent catalysts, in a study by Das and coworkers (2019). *A. niger* was shown to be capable of increased extraction of Ni from low-grade ore in a study by Biswas and coworkers (2013), despite the findings of the study by Anahid and coworkers (2010). *A. niger* was capable of metal bioleaching across multiple heavy metal source materials.

Table 3: Fungal species used for the bioleaching of metals from different metal sources

Fungal Species	Source Material (Low-Grade Ore)	Extracted Metal	Extraction Capacity (%)	Reference
Penicillium notatum	Black Shale	Cu Co	49.29 53.51	Anjum *et al*., 2009
Aspergillus oryzae	Manganese	Mn	79	Mohanty *et al*., 2017
Aspergillus flavus *Aspergillus niger*	Chalcopyrite	Cu	77 71 – 74	Rao *et al*., 2002
Aspergillus niger *Penicillium funiculosum*	Laterite	Ni Co Ni Co	34.6 25 10.5 12	Valix *et al*., 2001
Aspergillus niger	Boehmite	Al	43.1	Urík *et al*., 2018
Penicillium citrunum	Manganese	Mn	64.58	Acharya *et al*., 2002
Aspergillus foetidus	Nickel laterite	Ni Co Fe Mg	28 31 16 30	Le *et al*., 2006
Aspergillus niger	Uranium (U)	U	80	Sun *et al*., 2020
Aspergillus niger *Penicillium simplicissimum*	Bauxite	Al	≥98	Shah *et al*., 2020
Aspergillus niger	Bauxite	Ni Co	80	Biswas *et al*., 2013
Aspergillus niger	Copper	Cu	60	
Aspergillus fumigatus	Gold	As Fe Mn Pb Zn	62 58 100 56 54	Seh-Bardan *et al*., 2012
Cladosporium sp. A *Cladosporium* sp. B	Silver	Mg Ag Mg Ag	74 67 58 40	Huerta-Rosas *et al*., 2020
Aspergillus niger	WEEE*	Cu, Sn Al, Ni, Pb	65 95%	Brandl *et al*., 1999
Penicillium simplicissimum	PCBs	Cu Ni	90 89	Arshadi *et al*., 2019
Aspergillus niger	PCBs	Au	42	Madrigal-Arias *et al*., 2015
Aspergillus niger	Fly ash	Cd Zn Cu	81 66 57	Brandl, 1996

		Pb Mn Al Cr, Fe, Ni	52 32 27 10	
Penicillium rubens	Soil	Cd	79	Khan *et al*., 2019
Penicillium chrysogenum	Soil	Cd Cu Pb Zn Mn Cr	74 59 24 55 57 25	Deng *et al*., 2013
Aspergillus niger	Red mud	Al	75	Vachon *et al*., 1994
Aspergillus niger	Refinery catalyst	Ni Mo Al	78.5 82.3 65.2	Santhiya and Ting, 2006
Aspergillus niger *Aspergillus foetidus*	Spent catalyst	Al Al	81 88.43	Das *et al*., 2019
Penicillium simplicissimum	Hydro-processing catalyst	Mo Ni	97.6 45.7	Amiri *et al*., 2011.
Aspergillus nomius	Batteries	Cu Pb Ni Fe	75.14 73.21 99.8 50.15	Chatterjee *et al*., 2020

*Waste electrical equipment

In the study by Huerta-Rosas and coworkers (2020), two strains of *Cladosporium* successfully extracted Mg and Ag (silver) from silver mine tailings. Along with the organic acid producing capabilities of Ascomycetes, *Cladosporium* was found to be capable of effectively lixiviating Ag and Mg from solid silver mine residues, resulting in 67 and 74% Ag and Mg extractions, respectively. These findings are consistent with a study done by Wang and coworkers (2022), which found that species of *Cladosporium* have very high adsorption and oxidation rates of heavy metals, with up to 99% of Mn (manganese) removal achieved. The resistance to environmental conditions, mechanical rigidity of the cell wall and particle accumulation characteristics of a filamentous fungi such as *Cladosporium* were believed to be the driving factors behind its heavy metal adsorption (Wang *et al*., 2022).

Research focusing on the application of fungal species to PGM bioleaching is limited, including research on platinum mine-isolated fungal species as a whole. Research on PGM bioleaching specifically is also limited, with recent studies focusing instead on the application of biogenic

cyanide from cyanogenic bacteria for PGM bioleaching (Chipise *et al*., 2023; Pathak *et al*., 2022). As seen in Table 3, research around the application of fungi to bioleaching is centred around species of the phylum Ascomycota. In a study by Argumero-Delira and coworkers (2020), strains of *Fusarium*, *A. niger*, *Hypocrea* and *Trichoderma* were found to be tolerant to increased concentrations of platinum (>300 mg/L), however the application of these fungi to platinum metal bioleaching was not explored further.

2.4 Conclusion

The aim of this study is the characterisation and application of platinum mine-isolated fungal species to the bioleaching of base metals from a PGM mine concentrate, based on their growth and organic acid producing capabilities. Fungal bioleaching represents a sustainable alternative to conventional PGM processing, which faces challenges such as depleting ore reserves and considerable environmental impact. This literature review outlined the current state of conventional PGM processing, from worldwide PGM reserves to conventional PGM processing pathways and its drawbacks. Significant risk is placed on the environmental, social and governance status of South Africa, given its role as the primary producer of PGMs in the world. Water contamination, energy consumption, emissions and waste production are significant challenges faced by the country as a result of its PGM processing. A decrease in the availability of secondary PGM sources, specifically catalytic converters, for recycling is a further roadblock to overall PGM production. The use of an alternative PGM extraction method was discussed as well as extraction from multiple sources such as low-grade ore and mine tailings. Fungal bioleaching was explored as a potential alternative for PGM processing. Fungi were found to be capable of biogenic organic acid production in the presence of a rapidly metabolizable carbon source, allowing for the successful solubilisation and extraction of metals as a result of metal ion complex formation. While there are several studies on the application of fungi to metal extraction, it was found that most of these studies focus only on the application of *Aspergillus* and *Penicillium* species to bioleaching. Research on the characterization of platinum mine-isolated fungal species and their application to PGM bioleaching is limited. Development in this area of research is critical to the implementation of alternative PGM processing pathways, such as fungal bioleaching, in South Africa, where PGM processing is at the forefront.

Chapter 3: Methods and Materials

3.1 Sample Collection

Thirty-nine samples were collected from the Anglo-Mogalakwena platinum mine (24°10'59"S, 29°1'0"E) located near the town of Mokopane, Limpopo, South Africa, within the Bushveld Igneous Complex. All samples were collected from the concentrator plant, exterior pond, in-front of the crusher and in-front of the concentrator within the mine. Liquid samples were taken from the concentrator plant throughout the flotation process, whereas soil, slurry and liquid samples were taken from the remaining sources. Samples were placed in 50 mL sterile centrifuge tubes or polyethylene bags and stored at 4^{o}C in a polystyrene box, before transport to the Industrial Microbiology and Biotechnology Laboratory (IMBL) at the University of the Witwatersrand. Analysis of the entire sample set was performed by Haripersad (2022). Each isolated fungal species was subject to qualitative organic acid screening with the pH indicator bromocresol green, to determine if the isolates were suitable for bioleaching. Isolates with acid unitage (AU) values ≥ 1 were considered to have sufficient organic acid production and were selected for the current study.

3.2 Isolate Screening

3.2.1 Substrate Analysis

The following three nutrient media were used: potato dextrose (Oxoid), malt extract (ME) (Thermo-Fisher Scientific) and Czapek yeast extract (CYE) (Himedia Labs). All media were prepared according to manufacturer's guidelines with distilled water (dH_2O). CYE was buffered to pH 6.5 with 1 M H_2SO_4 (ACE Chemicals) to allow agar to set. Media were prepared and sterilized by autoclavation (Biobase) at 121^{o}C for 15 min and stored at 4^{o}C. The recipes for each media can be found in Appendix A.

3.2.2 Microscopic Spore Counts

Spore suspensions for the selected isolates were prepared from 7-day-old PD, ME and CYE culture plates for growth comparison within the three different nutrient media. Culture plates were flooded with 20 mL (millilitres) of autoclaved 0.1% (w/v) Tween 80 (Sigma-Aldrich) and agitated with a sterilized glass spreader (Samson et al., 2010). Tween 80 was used as a wetting agent to disperse and suspend spores into solution. The spore counts were determined microscopically by haemocytometer cell counting (Pitt and Hocking, 2009). An aliquot of 10 µL was pipetted onto the surface of the haemocytometer and viewed microscopically under 100x magnification. Suspensions were adjusted by dilution with dH_2O to 1 x 10^7 spores/mL after counting (Samson *et al.*, 2010). The calculations for the spore counts can be found in Appendix B. Suspensions with inadequate spore counts (<10^3) were left undiluted. Spore suspensions were stored at 4°C and used for inoculation in subsequent experiments. Each experiment was conducted in triplicate.

3.3 Growth Curves

One hundred microliters of each spore suspension were inoculated into 50 mL of autoclaved PD, ME and CYE broth medium respectively (Meletiadis *et al.*, 2001). Each inoculum was stored in individual autoclaved 50 mL falcon tubes (Axiology) and incubated in a shaking incubator at 150 rpm (revolutions per minute) (Labotec) at 25°C for 7 days. Prior to absorbance measurements, cultures were homogenised by vortexing at maximum speed for 60 seconds to disrupt hyphal aggregates. The optical density (OD) (450 nm) of each culture was recorded at 24 h intervals from the onset of incubation on day 0 to day 7 (Banerjee *et al.*, 1993). One millilitre of inoculum was sampled from each tube at each time interval. Optical density readings over the 7 day incubation period were used to produce growth curves for all fourteen isolates and their respective growth media (Meletiadis *et al.*, 2001). A further 2 mL of inoculum was sampled at each 24hr interval and stored in 2 mL Eppendorf tubes (Axiology) for high performance liquid chromatography analysis (HPLC). Each experiment was conducted in triplicate.

3.4 Organic Acid Production - Analytical Methods

Organic acid production was quantified using reverse phase (RP) HPLC (Shimadzu LC-2050 series), as described by Reuter and coworkers (2015), with minor modifications to the injection volume (from 5 µl to 20 µl). To remove spores and mycelia, inoculum samples were filtered into 2 mL screw top HPLC vials (Agilent) using sterile 0.45 micrometre (µm) 25-millimetre (mm) nylon syringe filters (Axiology). The filtrates were analysed using an aqueous 5 µm, 250 x 4.6 mm C-18 analysis column (PerkinElmer Brownlee). Vacuum-filtered 20% (v/v) acetonitrile (ACN) was used as mobile phase 1 and potassium dihydrogen-phosphate (KH_2PO_4) (adjusted to pH 2.4 with phosphoric acid) as mobile phase 2. The HPLC method can be found in Table 21, Appendix C. Organic acids were detected via an ultraviolet (UV) detector at 210 nanometres (nm). A one molar stock solution of an oxalic acid reference standard was used to prepare six calibration levels (1, 10, 20, 50, 75 and 100 mM) through serial dilution of the stock solution. Each standard was syringe-filtered and run on the HPLC. Data was acquired using LabSolutions software (Shimadzu). Each experiment was conducted in triplicate.

3.5 Base Metal Extractions

The extraction of base metals was undertaken using spent medium and inoculum-based (one-step) leaching processes.

3.5.1 Spent Medium Bioleaching

Seven-day old fungal cultures were flooded with 10 mL of 0.1% (v/v) Tween 80 and agitated with a glass spreader to lift spores and enhance extracellular metabolite release from the fungal cells themselves into solution (Zhang *et al.*, 2012). The resulting suspension was aspirated into a sterile beaker and the flooding process repeated twice more. A total of 30 mL of suspension was lifted from each plate. Each suspension was filtered through sterile 0.45 µm 25 mm nylon syringe filters to produce a cell-free (spent) medium. A design of experiment (DOE) was setup using DesignExpert™ software to outline the experimental parameters based on the input variables of pH, pulp density and temperature (Montgomery, 2017). The DOE for

spent medium leaching is shown in Table 4. Identical spent medium leaching experiments were conducted for both *Aspergillus niger* and *Fusarium* sp. Table 5 below outlines the set variables for each parameter, ranging from the low (Level 1), to the mid-range (Centre Point) to the high (Level 2). The DOE was used in place of replicate experiments.

In 14 autoclaved 250 mL flasks, 98-, 98.5- and 99-mL aliquots of the spent medium were mixed with the UG2 flotation concentrate relative to the corresponding 2, 1.5 and 1 % (w/v) pulp densities. The relevant pH (1, 2 or 3) of each flask was established using 10% (w/v) H_2SO_4 and sodium hydroxide (NaOH) solutions. Each flask was incubated at the relevant temperature (50, 60 or 70^oC) in shaking incubators at 150 rpm for 7 days. The pH of each flask was measured and adjusted (to pH 1, 2 or 3) every 24 h with 10% (w/v) H_2SO_4 and NaOH. On days 1, 4 and 7; 1.5 mL of leachate was sampled from each flask, diluted 10x to 15 mL with dH_2O and filtered with 0.45 µm syringe filters. Additional spent medium was added to replace the extracted leachate and bring each flask volume from 98.5 mL back up to 100 mL. Samples were analysed for base metals present in solution through inductively coupled optical emission spectroscopy (ICP-OES) by UIS Analytical Services (Pretoria, South Africa).

Table 4: Design of experiment (DOE) for spent medium leaching experiments – Produced using DesignExpert™ software

Standard Run Order	Random Run Order	Controlled Factors		
		pH	Temperature (^oC)	P. density (% w/v)
1	13	1	50	1
2	12	3	50	1
3	11	1	50	2
4	10	3	50	2
5	9	1	70	1
6	8	3	70	1
7	7	1	70	2
8	6	3	70	2
9	0	2	60	1.5

Table 5: Effective factors breakdown for spent medium leaching

Controlled Factors	Level 1 (-1)	Centre Point (0)	Level 2 (+1)
pH (A)	1	2	3
Temperature (°C) (B)	50	60	70
Pulp density (% w/v) (C)	1	1.5	2
Agitation rate (rpm)	150		

3.5.2 One-Step Bioleaching

Spore suspensions were prepared identically to the preparation for the spore count experiments (section 3.2.3). However, each suspension was left unfiltered for one-step leaching experiments.

The contents of each flask were setup according to the DOE presented in Table 6. Identical one-step leaching experiments were conducted for both A. *niger* and *Fusarium* sp. Table 7 below outlines the set variables for each parameter, ranging from the low (Level 1), to the mid-range (Centre Point) to the high (Level 2). Aliquots of the cell suspension were added according to the percentage of inoculum designated for each flask. CYE broth was added to bring the final volume up to 100 mL. The relevant pH (1, 2 or 3) of each flask was established using 10% (v/v) sulphuric acid and sodium hydroxide solutions. Each flask was incubated at a consistent temperature of 30°C in shaking incubators at 150 rpm for 7 days. The pH of each flask was measured and adjusted (to pH 1, 2 or 3) every 24 h with 10% (v/v) H_2SO_4 and NaOH. On days 1, 4 and 7; 1.5 mL of leachate was sampled from each flask, diluted 10x to 15 mL with dH_2O and filtered with 0.45 µm syringe filters. Additional CYE broth was added to replace the extracted leachate and bring each flask back up to 100 mL. Samples were analysed for base metals present in solution through ICP-OES by UIS Analytical Services (Pretoria, South Africa). The DOE for one step leaching is shown in Table 6.

Table 6: Design of experiment (DOE) for one-step leaching experiments – Produced using DesignExpert™ software

Standard Run Order	Random Run Order	Controlled Factors		
		pH	Inoculum (% v/v)	P. density (% w/v)
1	13	4	2	1
2	12	9	2	1
3	11	4	10	1
4	10	9	10	1
5	9	4	2	2
6	8	9	2	2
7	7	4	10	2
8	6	9	10	2
9	0	6.5	6	1.5

Table 7: Effective factors breakdown for one-step leaching

Controlled Factors	**Level 1 (-1)**	**Centre Point (0)**	**Level 2 (+1)**
pH (A)	4	6.5	9
Inoculum dosage (% v/v) (B)	2	6	10
Pulp density (% w/v) (C)	1	1.5	2
Agitation rate (rpm)	150		
Temperature (°C)	30		

3.5.3 Abiotic Control

Based on the DOE's for spent medium and one-step leaching, identical flasks were prepared and run concurrently with the relevant base metal extraction experiments, in the absence of a spent medium and inoculum. Flasks were made up to 100 mL volume with CYE and the ore

concentrate at the relevant pulp density in grams (g). The pH (1, 2 or 3) of each flask was monitored daily and maintained with H_2SO_4 and NaOH. Sampling was identical to methods mentioned above. Abiotic control tests were performed for every single base metal extraction experiment.

3.5.4 Ambient pH Control

Based on the DOE's for spent medium and one-step leaching, identical flasks were prepared and run concurrently with the relevant base metal extraction experiments, without any prior adjustment to the ambient pH of the culture and the flask following the addition of spent medium, inoculum, culture medium and ore concentrate. Flasks were prepared according to the relevant DOE. Sampling was identical to methods mentioned above. Ambient pH control tests were performed for every single base metal extraction experiment.

3.5.5 Base Metal Content

The base metal (Co, Cr, Cu, Fe, Ni) content of the PGM flotation concentrate was determined through x-ray diffraction by UIS Analytical Services (Pretoria, South Africa).

3.6 Statistical Analysis

The statistical significance of the spore count, growth curve and organic acid production experiments was verified by a one-way analysis of variance (ANOVA) or Welch's t-test (α = 0.05) with $p < 0.05$ being considered significant. The test was performed on the mean result from independent triplicate runs (n=3) of each experiment (West, 2021). A DOE was set up in place of triplicate runs for all bioleaching experiments.

Chapter 4: Results and Discussion

4.1 Isolate Screening

All fungal isolates were obtained from the Anglo-Mogalakwena platinum mine, located in Mokopane, Limpopo. Isolation and characterisation of the fungi, achieved by Haripersad, (2022), produced 66 fungal species from the liquid, slurry and soil samples taken from the mine. The methods for isolation and characterisation can be found in Appendix D. The 66 fungi were characterized into the following phyla: Ascomycota (89.3%), Basidiomycota (7.58%) and Mucoromycota (3.03%). The phyla table (Table 21, Appendix E) presents all 66 fungal species sourced, isolated and sequenced by Haripersad, (2022) from Anglo-Mogalakwena platinum mine. The large number of Ascomycetes, compared to Mucoromycetes and Basidiomycetes, is consistent with a study by Held and coworkers (2020), where two thirds of the 164 fungal species isolated from an iron ore mine were Ascomycetes. This is likely due to the organic acid producing, and bioaccumulation characteristics, of Ascomycetes (Liaud *et al*., 2014).

Qualitative organic acid screening of the 66 isolates resulted in a total of 14 filamentous fungal isolates selected for this study, based on the production of low-molecular weight organic acids, shown in section 4.4. Each isolate was successful in producing organic acid and were characterized into the following genera: *Alternaria, Aspergillus, Chaetomella, Curvularia, Fomitopsis, Fusarium, Mucor, Penicillium, Phoma, Physisporinus* and *Rhizopus.* These isolates were investigated in the current study and are shown in Table 8.

Table 8: List of 14 screened and selected indigenous filamentous fungal isolates

Isolate number	Name of Fungal Isolate	GenBank accession no.
1	*Physisporinus* sp.	KU958554.1
2	*Penicillium* sp.	MT424930.1
3	*Aspergillus niger*	MT620753.1
4	*Mucor fragilis*	MK910073.1
5	*Fusarium* sp.	MK640565.1
6	*Fomitopsis meliae*	KT718002.1
7	*Curvularia petersonii*	NR_158448.1
8	*Aspergillus* sp.	MT447519.1
9	*Rhizopus microsporus*	KJ417579.1
10	*Aspergillus terreus* (Strain 1)	MT316343.1
11	*Alternaria* sp.	MW784825.1
12	*Phoma* sp.	JN207257.1
13	*Chaetomella raphigera*	KF193633.1
14	*Aspergillus terreus* (Strain 2)	MT316343.1

Of the 14 filamentous fungal isolates, species from the genera *Aspergillus* and *Penicillium* are known for their production of a wide range of organic acids at high concentrations (Dusengemungu *et al*., 2021). Several *Aspergillus* and *Penicillium* species are commonly used for the bioleaching of mine tailings, electronic waste, solid waste and low-grade ore, given their organic acid-producing capabilities. There are several studies highlighting organic acid production from *Penicillium* sp, *A. niger, A.* sp*, Phoma* sp*, A. terreus, Curvularia, Fusarium, Fomitopsis, Mucor* and *Rhizopus* (Akintokun *et al*., 2007; Boniek *et al*., 2017; Choi *et al*., 2014; Liaud *et al*., 2014; Palmer *et al*., 1991; Shinidia *et al*., 2006; Sullivan *et al*., 2012). *Physisporinus* sp. and *Chaetomella raphigera* are the only isolates with previously unreported organic acid production (Haripersad, 2022). Based on their isolation from a heavy-metal contaminated environment and their organic acid producing abilities, each of these isolates were determined to have a capacity for bioleaching.

4.2 Spore Counts

A quantitative assessment of spore production by each isolate was performed to assess their potential for biomass production. Spore production directly correlates to the isolate's ability to produce adequate amounts of biomass according to Wolken and coworkers (2003), which states that increased spore production leads to increased mycelial and vegetative cell

production. Wolken and coworkers (2003) also state that this leads to an increase in the specific activity of a spore-forming microorganism. For the current study, it was believed that higher spore production would lead to increased biomass production within the 7-day incubation period, which would directly increase the amounts of inoculum produced for bioleaching. For bioleaching, a single agar plate with increased amounts of biomass will continually produce inoculum following several wash steps with a surfactant such as Tween 80. Each bioleaching flasks requires either 2, 6, 10 or 100 ml, therefore isolates with increased biomass are more able to produce large amounts of inoculum, requiring fewer plates and fewer wash steps. For flasks made up with 2, 6 and 10 ml inoculum dosages, a single plate with enough biomass could produce enough inoculum for a full nine flask leaching run.

Based on a study done by Zain (2009), the three nutrient media selected for this study were potato dextrose (PD) , malt extract (ME) and Czapek yeast extract (CYE). PD was used to assess the growth of each isolate in the absence of a supplemental carbon source. ME (maltose) and CYE (sucrose) were used to determine the response of each isolate to a specific substrate/carbon source and the impact that substrate would have on spore production, and hence biomass production, downstream. The aim was to determine the substrate that produced the highest spore production from each isolate, to narrow down the list of 14 isolates and possibly identify the ideal substrate for subsequent bioleaching. The results of the spore count in spores/mL are shown in Table 9.

Table 9: Spore count of the 14 indigenous isolates when grown on potato dextrose, malt extract and Czapek yeast extract.

Isolate	Spore/millilitre (10^7)		
	Potato Dextrose	Malt Extract	Czapek Yeast Extract
Aspergillus terreus (strain 2)	6.07 ± 0.25	6.23 ± 0.27	6.55 ± 0.29
Aspergillus niger	1.81 ± 0.09	5.35 ± 0.22	6.40 ± 0.28
Mucor fragilis	5.76 ± 0.24	5.95 ± 0.25	6.12 ± 0.26
Fusarium sp.	0.82 ± 0.04	2.75 ± 0.11	5.20 ± 0.21
Aspergillus terreus (strain 1)	5.04 ± 0.21	5.43 ± 0.23	5.56 ± 0.23
Phoma sp.	5.76 ± 0.23	4.68 ± 0.19	3.46 ± 0.15
Penicillium sp.	2.44 ± 0.10	1.54 ± 0.07	3.33 ± 0.14
Physisporinus sp.	0.00 ± 0.00	0.00 ± 0.00	1.32 ± 0.06
Aspergillus sp.	1.32 ± 0.06	1.32 ± 0.06	2.81 ± 0.12
Alternaria sp.	0.00 ± 0.00	0.00 ± 0.00	0.97 ± 0.04

Spore counts presented as mean ± SE (n=3).
F. meliae, C. petersonii, C. raphigera and R. microsporus did not produce spore counts above 10^3

Of the 14 isolates; *A. terreus*, *A. niger*, *Fusarium* sp., *Penicillium* sp., *Physisporinus* sp., *Aspergillus* sp. and *Alternaria* sp. displayed an increase in spore production when cultured in CYE. CYE displayed a larger increase in spore production per isolate than ME, when compared to the spore production on PD. Zain (2009) notes that species of *Aspergillus*, specifically *A. terreus*, display a much higher spore production on CYE medium (80 x 10^5/ centimetres squared (cm^2)) and ME (72 x 10^5/ cm^2) compared to PD (45 x 10^5 / cm^2), as a result of their high sucrose and maltose content, respectively. *P. janthinellum,* also displayed higher spore production per cm^2 on yeast and ME, when compared to PD (Zain *et al.*, 2009).

Of the isolates displaying an increase in spore production on CYE (compared to PD), *A. niger* and *Fusarium* sp. displayed significant increases from 1.81 x 10^7 spore/mL to 6.40 x 10^7 ($p = 0.003 < \alpha = 0.05$) and 0.82 x 10^7 to 5.20 x 10^7 (p-value: $0.0024 < \alpha = 0.05$) spore/mL respectively. The difference in spore counts for *M. fragilis* and *A. terreus* were insignificant on CYE vs PD ($p = 0.066$ and $0.058 > \alpha = 0.05$, respectively) and CYE vs ME ($p = 0.330$ and $0.383 >$

α = 0.05, respectively). However, given their high spore counts, *M. fragilis* and *A. terreus,* including *A. niger* and *Fusarium* sp., were noted as potential candidates for bioleaching. There was an increase in spore production for most of the isolates on ME compared to PD, however these increases were not significant (p-value: 0.232 > α = 0.05). The response of each isolates spore production on CYE was noted for further experiments. Triplicate experiments displayed an insignificant difference in overall spore counts per mL (p-value: 0.4287 > α = 0.05) between each substrate.

4.3 Isolate Growth Curves and Substrate Analysis

An absorbance analysis was performed to assess the growth rate of each isolate in liquid PD, ME and CYE. Absorbance was measured at OD450 based on a study by Banerjee and coworkers (1993), which highlighted a linear correlation between OD450 and biomass dry weight in filamentous fungi. The relationship between OD450 and biomass accumulation allowed for rapid estimation of growth and biomass concentration in a liquid culture, in the absence of conventional dry weight measuring techniques (Banerjee *et al*., 1993). This practice was applied to the current study to allow for a rapid growth assessment of a large sample size, without resorting to measurement of biomass dry weight. The use of OD450 was confirmed in (Banerjee *et al*., 1993).

4.3.1 Potato Dextrose

PD, a general all-purpose medium (Zain *et al*., 2009), was used to assess the individual growth rates of the 14 isolates in the absence of a supplemental carbon source. Growth rate results using PD were used as the baseline for the growth curve (Figure 3) stage of this study, given the high sucrose and maltose content of the CYE and ME substrates, respectively.

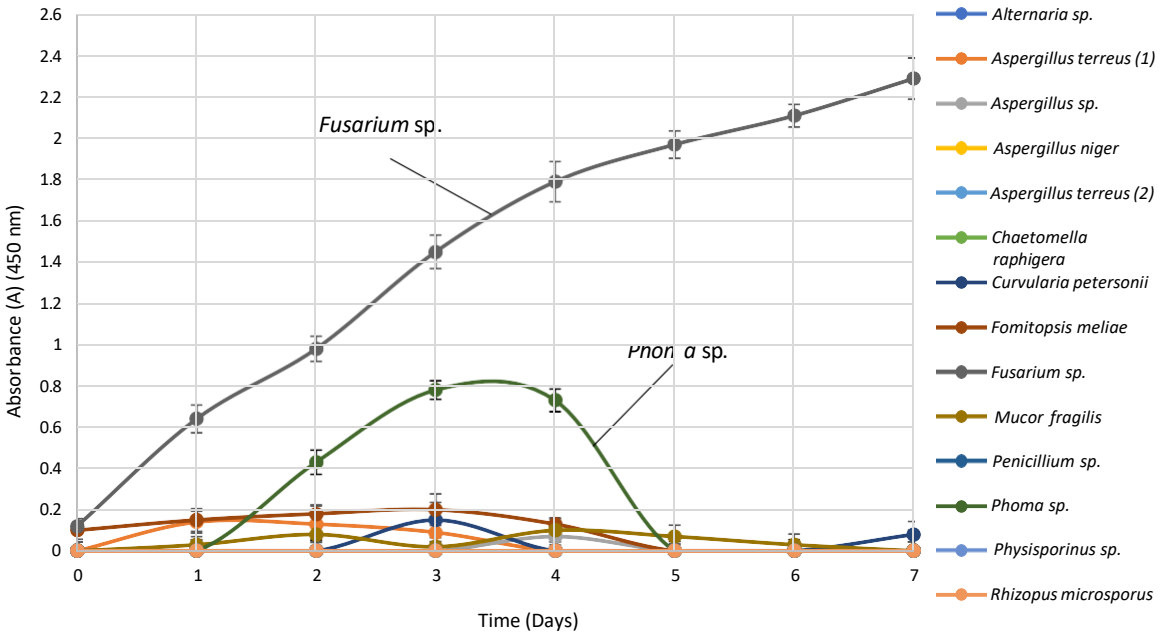


Figure 3: Growth curve displaying the changes in absorbance (450 nm) of the 14 fungal isolates throughout the 7-day growth period when cultured in potato dextrose. Isolates were shake-incubated (150 revolutions per minute) at 25°C for 7 days with absorbance measurements taken every 24 hours from 1 mL samples. (Mean ± SD, n=3).

Given that bioleaching is liquid-based, a growth curve analysis was done to assess the growth of each isolate in a liquid culture environment. A study by Bakratsas and coworkers (2021) states that cultivation of fungi in a liquid culture environment leads to higher mycelial growth and biomass production, but more importantly, leads to increased production of a wide variety of bioactive compounds. As both production of fungal inoculum and bioleaching occur within 7 days in the current study, only the isolates with a rapid growth response within the 7 day period were selected for bioleaching. Based on a study by Manan and Webb, (2018) which assess fungal growth in submerged fermentation through culture broth colour changes, it was believed that fungal growth could be assessed through changes in absorbance of the fungal liquid culture. An ideal growth curve for the current study was determined based on a study by Vrabl and coworkers (2019), which assessed filamentous fungal growth in batch culture and separates a growth curve into the lag phase, log (exponential) phase, stationary phase and death phase. A lag phase is characterised by a lack of growth at the onset of incubation, therefore given the 7 day incubation period, the lack of a lag phase beyond day 1 was considered an important factor. Isolates were considered for bioleaching based on the production of a clear log phase, which was determined to be a substantial increase in

absorbance (0 to >1 absorbance units (A)) as a result of exponential growth within the first 2 days, given that metabolic activity is highest during this phase (Vrabl *et al.*, 2019). Isolates were also considered for bioleaching based on the pattern of the stationary phase, which highlights a decline in the rate of growth due to diminishing nutrient supply (Vrabl *et al.*, 2019). Isolates with a stable stationary phase that shows no rapid decreases in absorbance, indicating the death phase, were considered for bioleaching together with the presence of a clear log phase. The highest absorbance achieved by an isolate was also considered, as it was believed this would lead to higher organic acid production. The use of changes in absorbance to assess fungal growth was supported in studies by Slowik and coworkers (2023) and Hameed and coworkers (2024).

The growth rate of *Fusarium* sp. (Figure 3) represents a nearly ideal growth curve, given the lack of a lag phase within the first day and a consistent rate of increase in absorbance all the way to day 7. Despite the lack of a distinct exponential growth phase within the first 2 days, there is a significant increase ($p = 0.0016 < \alpha = 0.05$) in absorbance from Day 0 to Day 2. *Fusarium* sp. maintains increasing growth through day 7, which agrees with the results of Mohsen and coworkers (2015). The work by Mohsen and coworkers (2015) also indicates that certain *Fusarium* spp. display maximum growth at a temperature of 25 ^{o}C and within a pH range of 4.5 to 8.5, the highest of which occurs at pH 7.5. The pH of PD (approximately 5.6) falls within this range (Zain *et al.*, 2009). A lag phase was expected around Day 5 to Day 7 given acidification of the broth medium during fungal growth, however a lack of a lag growth phase shows that any drop in pH was within the acceptable range for *Fusarium* sp. The lack of notable response by the remaining isolates means PD was not considered as the substrate for one-step leaching or the growth medium for spent medium leaching, despite the ideal growth rate of *Fusarium* sp. This result, excluding *Fusarium* sp. is consistent with those of Zain and coworkers (2009), which states that growth of certain filamentous fungal species, specifically those of the *Aspergillus* and *Penicillium* genera, are not affected considerably by PD. The growth response of *Fusarium* sp. in PD was noted for further experiments.

Phoma sp. was the only other isolate to display a change in absorbance throughout the incubation period. According to Bennett and Ponder (2018), the highest growth for *Phoma* sp. occurs at a pH of 5.5. The close proximity of this pH to the pH of the PD medium (pH 5.6) explains the initial change in absorbance. Acidification of the medium, possibly due to the

production of organic acids, could be the cause of the sudden decrease in growth of *Phoma* sp. from day 4 onwards. While the change in absorbance from day 1 to day 3 was significant ($p = 0.027 < \alpha = 0.05$), *Phoma* sp. was not chosen for bioleaching experiments given the lack of log phase, very short stationary phase and sudden death phase.

Given the overall lack of growth response from the isolates in PD, it was not considered as potential substrate for the growth of isolates for spent medium leaching and as a nutrient source/leaching substrate for one-step leaching. Triplicate experiments displayed an insignificant difference in absorbance (p-value: $0.2193 > \alpha = 0.05$) for each remaining isolate.

4.3.2 Malt Extract

ME is a nutrient rich medium capable of supporting a wide variety of microorganism (Black, 2020). Along with nitrogen, sodium chloride, calcium and magnesium, it contains a high concentration of maltose or ME to serve as a carbon source promoting the rapid growth of filamentous fungi and other microorganisms (Skaar and Stenwig, 1996). Secondary metabolite production in filamentous fungi is known to be stimulated in the presence of a significant carbon source, hence ME was selected as a potential substrate for the current study. ME was also selected for its buffering capacity, availability and relative affordability (Skaar and Stenwig, 1996).

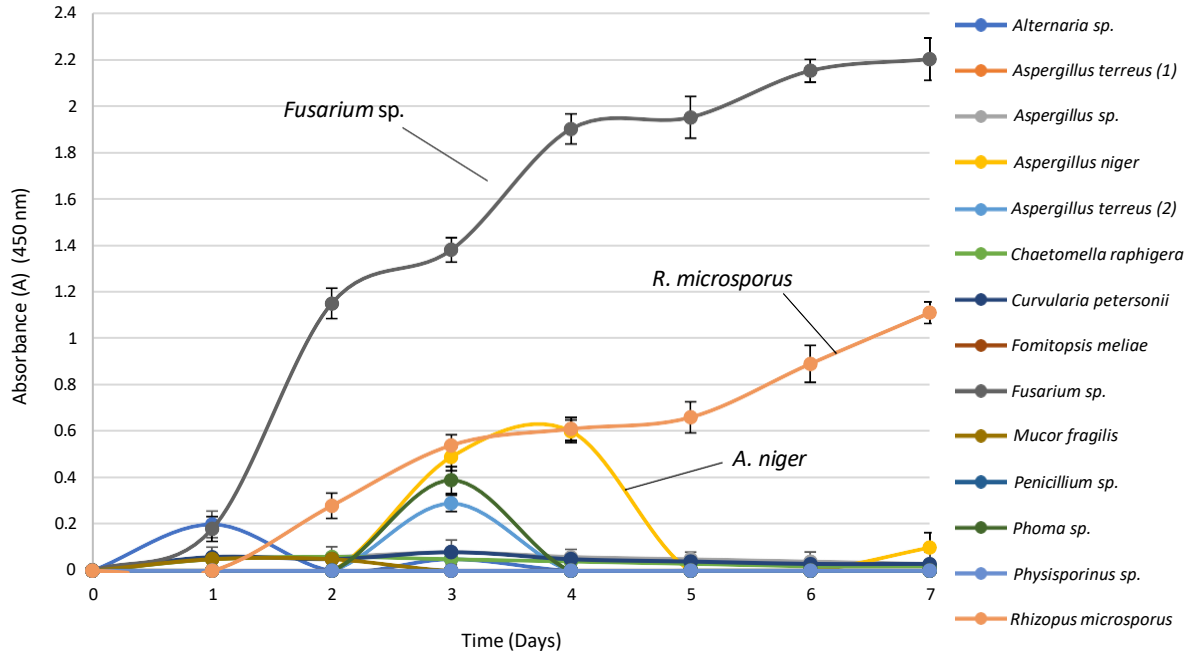


Figure 4: Growth curve displaying the changes in absorbance (450 nm) of the 14 fungal isolates throughout the 7-day growth period when cultured in malt extract. Isolates were shake-incubated (150 rpm) at 25°C for 7 days with absorbance measurements taken every 24 hours from 1 mL samples. (Mean ± SD, n = 3).

Similar to PD, ME (Figure 4) shows that *Fusarium* sp. had the most significant growth (p-value: 0.00033 < α = 0.05) when compared to the isolate with the next highest growth (*R. microsporus*). *Fusarium* sp. has the highest absorbance of any isolate during its log phase, stationary phase and has the highest overall absorbance. Both *Fusarium* sp. PD and ME curves displayed high absorbance, with the day 7 absorbance of *Fusarium* sp. at 2.2 AU in ME showing insignificant difference (p-value: 0.22 > α = 0.05) compared to its 2.3 AU in PD. Both curves also displayed consistent growth patterns throughout the incubation period. In ME, however, *Fusarium* sp. displayed a more prominent increase in absorbance from day 1 onwards, increasing from 0 AU between days 0 and 1, to approximately 1,15 AU by day 2 (compared to the 0 - 1 AU change within 2 days for *Fusarium* sp. in PD). Despite the initial change in absorbance being visible one day later in the incubation period than in PD, the growth curve in ME displays a distinct exponential growth phase which was absent from the growth of *Fusarium* sp. in PD. The improved response of *Fusarium* sp in ME, determined by the higher rate of increase in absorbance, can be attributed to the pH of the ME medium (pH 6.5) ('Mycology,' 2021). This pH is much closer to the ideal pH of 7.5 for growth of *Fusarium* sp, according to Mohsen and coworkers (2015). The reaction of *Fusarium* sp. grown in ME

compared to PD is consistent with the result seen in section 4.2. In ME, the increased growth of *Fusarium* sp. is consistent with the increased spore count it displayed compared to when it was grown in PD. Along with pH, this increase was attributed to the maltose content of the ME medium, acting as a supplemental carbon source (Zain *et al*., 2009). The response of *Fusarium* to a supplemented medium when compared to a standard medium (PD) is consistent with the work done by Mohsen and coworkers (2015). *Fusarium* sp. was again noted as a potential bioleaching candidate given its distinct log phase, stable stationary phase and high absorbance.

The isolate *R. microsporus* was the only other isolate to display a consistent growth pattern up until day 7 of the incubation period (Fig 4). Despite its overall absorbance being considerably lower than *Fusarium* sp, the absorbance of *R. microsporus* was significantly higher ($p < 0.0001$) when grown in ME compared to in PD. This is consistent with work done by Meletiadis and coworkers (2001), in which the growth rate of *R. microsporus* is three to four times higher on more specialised media (yeast nitrogen-base, Sabouraud) than on more general-purpose RPMI (Roswell Park Memorial Institute) media. PD is another general-purpose medium for the cultivation of a wide range of microorganisms, therefore it is unable to stimulate a higher growth response over a supplemented medium such as ME (Zain *et al*., 2009). *R. microsporus* was not considered for further bioleaching experiments given the lack of a log phase and low overall absorbance.

A. niger was another isolate to display a change in absorbance. As seen in Table 11, *A. niger* had a higher spore production when grown on ME than on PD. This is consistent with the work done by Zain and coworkers (2009), which shows increased growth of *Aspergillus* species in ME over PD. Significant changes in absorbance were only seen between days 2 and 3 (p-value: $0.00034 < \alpha = 0.05$), followed by a drastic decline in absorbance by day 5 (Fig 4).

Overall, ME medium was shown to induce a better growth response from certain isolates than PD due to its high maltose content. However, these were slight improvements are not significant enough (p-value: $0.143 > \alpha = 0.05$) for ME to be considered as a bioleaching substrate. Triplicate experiments displayed an insignificant difference in absorbance (p-value: $0.2701 > \alpha = 0.05$) for each remaining isolate.

4.3.3 Czapek Yeast Extract

CYE is a specialised sucrose-heavy, nitrate-, phosphate-, chloride- and sulphide-containing nutrient media, targeted at the isolation and growth of saprophytic soil fungi such as *Aspergillus* and *Penicillium* (Aryal, 2022). The CYE medium used in this study contained 30% (w/v) sucrose as its sole carbon source. Sodium nitrate ($NaNO_3$) is utilised in this media as a nitrogen source, dipotassium phosphate (K_2HPO_4) as a buffer, magnesium sulphate ($MgSO_4$) and ferrous sulphate ($FeSO_4$) to provide cations and potassium chloride (KCl) as an ion source (Aryal, 2022). This allows the medium to produce vigorous growth in saprophytic and filamentous fungal species while yielding significant mycelia and conidia production (Aryal, 2022).

As a result, CYE was selected for this study in order to determine its ability to induce significant biomass- and secondary metabolite production from each isolate for bioleaching. Its high nutrient and sucrose content were believed to make it an ideal substrate for the cultivation of fungi for bioleaching, and its buffering capacity was thought to be important for maintaining the pH of the media within the optimal range for each isolate.

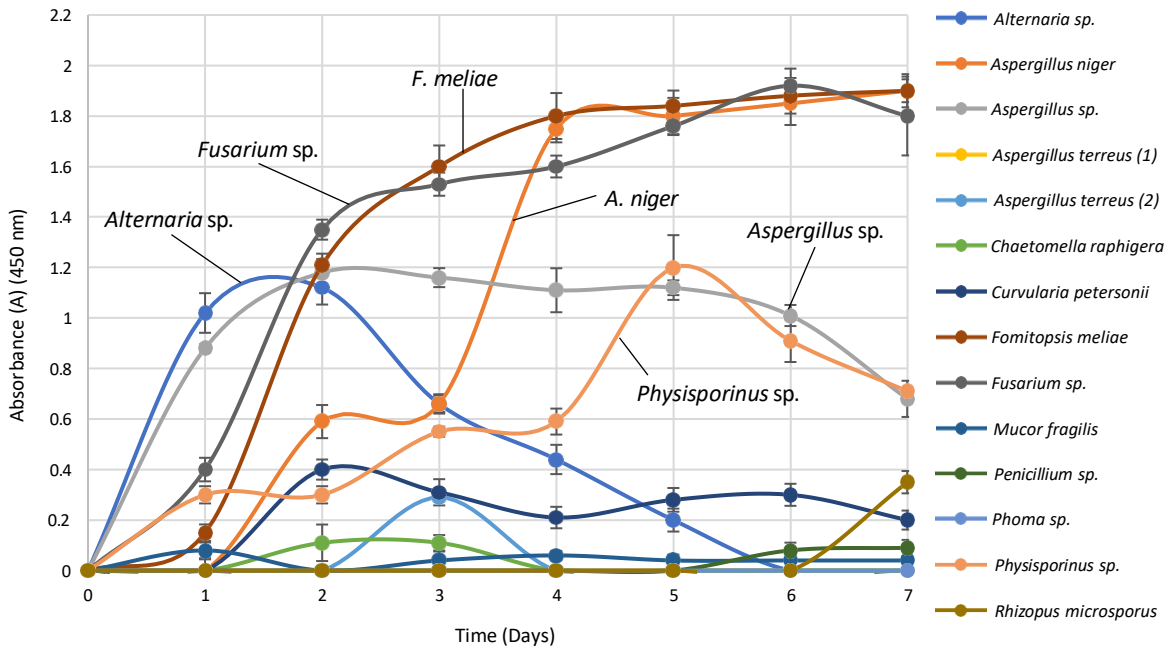


Figure 5: Growth curve displaying the changes in absorbance (450 nm) of the 14 fungal isolates throughout the 7-day growth period when cultured in Czapek yeast extract. Isolates were shake-incubated (150 rpm) at 25°C for 7 days with absorbance measurements taken every 24 hours from 1 mL samples (Mean ± SD, n=3).

As seen in Figure 5, the specialised CYE medium stimulated a much higher growth response in several of the isolates over the 7 day incubation period, including isolates that displayed no changes in absorbance when grown in PD and ME.

Fusarium sp. remained the most consistent isolate in terms of growth response to PD, ME and CYE, also displaying nearly identical growth to the one it produced in ME. Whereas the absorbance for *Fusarium* sp. on PD and ME reached 2.1 AU and higher, the absorbance for *Fusarium* sp. on CYE reached a maximum absorbance of only 1.9 AU on day 6, decreasing to 1.8 AU by day 7. This is consistent with work done by Mohsen and coworkers (2015), who states that the highest growth of certain *Fusarium* spp. (*F. sacchari, F. globosum and F. proliferatum*) occurs by the sixth day of incubation. However, the exponential growth phase of *Fusarium* sp. on CYE was the most promising. The difference in growth in PD, ME and CYE for *Fusarium* sp. was insignificant (p-value: $0.951 > \alpha = 0.05$). Changes in absorbance were seen immediately on day 0, reaching 0.4 AU by day 1 and reaching a high of 1.35 AU by day 2. This was the highest absorbance by *Fusarium* sp. on day 2 when using any of the substrate media, and the strongest exponential phase of any isolate on either of the three substrates. While both *Alternaria* sp. and *Aspergillus* sp. reached higher peaks in absorbance by day 1 than *Fusarium* sp, both curves failed to maintain their exponential phases, decreasing by day 2. Organic acid production has the potential to peak during the exponential growth phase of a 7 day incubation period, owing to the fungi's metabolism of the substrate for growth, while prioritizing the production of metabolites and energy over biomass (Liaud *et al*., 2014). The strong exponential phase, stable lag phase and high absorbance of *Fusarium* sp. was seen irrespective of the substrate tested, highlighting it as a promising candidate for bioleaching.

Another isolate with promising results was *F. meliae*. No reaction from *F. meliae* was seen in PD and ME, however the increased response from the isolate on CYE is consistent with work done by Choi and coworkers (2007), which states that the growth of certain species of *Fomitopsis* display favourable mycelial growth when supplemented with glucose and sucrose as carbon sources. The mycelial dry weight of *F. pinicola* was 0.79 g/L higher when supplemented with sucrose than with maltose (Choi *et al*., 2007), which could explain the contrasting growth curves from *F. meliae* in ME and CYE. The 30% (w/v) sucrose concentration in the CYE medium greatly improved the growth response of *F. meliae.* The effect of nitrogen sources on mycelial growth of *F. pinicola* was also tested by Choi and coworkers (2007), who

found that it was highest when yeast extract was used. Among the nitrogen sources tested was $NaNO_3$, which resulted in increased mycelial growth (Choi *et al*., 2007). The presence of both yeast extract and $NaNO_3$ as nitrogen sources in CYE would explain the increased growth from *F. meliae.* The growth response of the isolate *F. meliae* was noted for further bioleaching experiments.

Another isolate that was considered based on its growth curve was *A. niger* (Fig 5). Despite the lack of growth response by day 1, and low absorbance by day 2, the significant increase from day 3 onwards (p-value: $2.3 \times 10^{-5} < \alpha = 0.05$) highlights a strong exponential phase. Absorbance increased from 0.7 AU on day 3 to nearly 1.9 AU by day 4. This absorbance continued to increase, showing the highest absorbance of any of the other isolates on CYE by day 7. The increased growth response of *A. niger* is consistent with work done by Zain and coworkers (2009), which shows that the growth of *Aspergillus* species is higher on Czapek dox media than on ME and PD, both in terms of spore production and mycelial growth. The growth response of the isolate *A. niger* was noted for further experiments.

The growth curves of *Alternaria* sp. and *Physisporinus* sp. were not ideal (Fig 5). Despite early onset changes in absorbance and a strong exponential phase from *Alternaria* sp, the isolate failed to maintain a consistent increase in growth beyond day 2. *Physisporinus* sp. lacked a distinct exponential growth phase. Despite an increase in absorbance by day 4, the isolate peaked at an absorbance of only 1.1 AU by day 5 before tailing off significantly by day 7. The decrease in absorbance of *Aspergillus* sp. was not as drastic as both *Alternaria* sp. and *Physisporinus* sp, while also showing a strong exponential phase. Therefore, the growth of *Aspergillus* sp. was not immediately disregarded for further experimentation. Based on the work done on *Aspergillus* species by Zain and coworkers (2009), and the increased response seen from *A. niger*, it was believed that *Aspergillus* sp. might be a potential candidate for bioleaching experiments.

Given that CYE was able to induce an increased growth response from several more isolates (Fig 5), each displaying strong exponential phases, early onset of growth and high peak absorbances, it was selected as the as the ideal substrate for bioleaching over PD and ME. For spent medium leaching, CYE was determined to be the ideal substrate for the efficient cultivation of isolates in a timely manner while also promoting increased biomass production in order to produce large amounts of spent medium. For one-step leaching, the sucrose, yeast

extract and nitrogen content of the CYE medium make it the ideal substrate to promote growth of isolates during bioleaching. Triplicate experiments displayed an insignificant difference in absorbance (p-value: 0.1139 > α = 0.05) for each remaining isolate.

Following the growth response stage of the screening process, the potential isolates for bioleaching were *A. niger, Aspergillus* sp*, F. meliae* and *Fusarium* sp.

4.4 Organic Acid Production

The final stage of the screening process involved the quantification of organic acids produced by known organic acid-producing fungal isolates, in response to the CYE, PD and ME nutrient media over a 7 day incubation period. The organic acid producing capabilities of the above isolates were determined by qualitative analysis of organic acid production, performed by Haripersad (2022), in the form of a pH indicator test. Of the 66 isolates cultured on PD agar plates, which contained the pH indicator bromocresol green, 15 produced a positive result after 7 day incubation in the form of a yellow zone surrounding the fungal colony on the agar plate (Table 10). This confirms that organic acids were produced by those isolates, as a yellow colour change by bromocresol green indicates a drop in pH from 5.4 to 4 (Shaik and Qureshi, 2013). The size of the yellow zone relative to colony size on the agar plate is represented as values of acid unitage (AU), which shows the size of the yellow zone relative to the size of the colony (Din *et al*., 2020). Only isolates with AU values ≥ 1 were considered for the present study. An AU value below 1 was thought to show low organic acid production relative to the growth of the isolates. This study shows that AU results can be used to identify fungal isolates with potential for bioleaching. Of the 15 isolates with a positive result, *Terana caerulea* was the only isolate to have an AU value below 1, therefore it was disregarded for the current study.

Table 10: Previous study highlighting the 15 organic acid producing filamentous fungal isolates and the corresponding pH indicator tests results. Colony zone, yellow zone and acid unitage (AU) values calculated are shown.

Isolate No.	Fungal Isolate	Colony zone (mm)	Yellow zone (mm)	Acid unitage (AU)
1	*Physisporinus* sp.	24.76 ± 1.15	20.00 ± 6.93	1.13 ± 0.00
2	*Penicillium* sp.	31.83 ± 3.25	65.33 ± 10.41	2.05 ± 0.14
3	*Aspergillus niger*	64.33 ± 2.08	85.00 ± 0.00	1.32 ± 0.04
4	*Mucor fragilis*	41.33 ± 8.08	51.67 ± 5.86	1.26 ± 0.13
5	*Fusarium* sp.	55.67 ± 1.53	59.00 ± 2.00	1.06 ± 0.01
6	*Fomitopsis meliae*	53.67 ± 4.16	62.33 ± 5.51	1.16 ± 0.02
7	*Curvularia petersonii*	52.50 ± 3.28	61.00 ± 7.00	1.16 ± 0.08
8	*Aspergillus* sp.	66.33 ± 0.58	84.00 ± 1.73	1.27 ± 0.02
9	*Terana caerulea*	85.00 ± 0.00	57.00 ± 13.08	0.67 ± 0.16
10	*Rhizopus microsporus*	30.5 ± 8.05	68.33 ± 16.78	2.25 ± 0.10
11	*Aspergillus terreus* (Strain 1)	39.17 ± 0.29	48.33 ± 0.58	1.23 ± 0.01
12	*Alternaria* sp.	24.33 ± 2.52	31.00 ± 4.58	1.27 ± 0.05
13	*Phoma* sp.	40.67 ± 1.53	63.00 ± 9.54	1.53 ± 0.20
14	*Chaetomella raphigera*	25.33 ± 4.04	38.00 ± 3.46	1.51 ± 0.10
15	*Aspergillus terreus* (Strain 2)	40.83 ± 4.54	50.00 ± 5.57	1.22 ± 0.00

AU values are significantly different from each other as analysed by Duncan's Multiple Range Test at $P < 0.05$ (n = 3, means ± standard error).

The selection criteria for the remaining 14 isolates were based on the quantification of their organic acid production through HPLC, as well the results of the earlier spore count and growth curve experiments. The response of the isolates to each substrate as seen in sections 4.2 and 4.3, was verified by the amount of organic acid produced under the same conditions. Results of the organic acid production stage of experiments were used to determine the final two isolates to be used in the subsequent stages of bioleaching. Results displaying the concentration of oxalic acid in millimolar (mM) produced by the isolates during the 7-day incubation period are shown below in Table 11. Of the lactic, acetic, formic, butyric, citric, gluconic and oxalic acid standards tested, only oxalic acid produced quantifiable peaks. The HPLC chromatogram for the standards and a representative chromatogram of the one produced by the isolates are shown in Appendix F. Several studies highlight the ubiquitous production of strongly carboxylic, low-molecular weight oxalic acid (Graz, 2024; Palmieri *et al.*, 2018). This acid is frequently present within the fungal secrotome and drives processes relating to fungal bioweathering and biomineralization (Graz, 2024). A wide variety of fungal species produce oxalic acid, with calcium oxalate being one of the most common oxalate

minerals present within fungal hyphae (Palmieri *et al.*, 2018). This is evidenced by the above result, with 11 of the 14 fungal isolates producing quantifiable oxalic acid. According to Palmieri and coworkers (2018), oxalic acid production is dependent on nutrient availability, carbon source, nitrogen availability and pH, which explains the wide range of concentrations seen in Table 13. The variability of species among the fungal isolates and the complexity of molecular analysis could explain why oxalic acid was the only organic acid that was identified and quantified. A study by Din and coworkers (2020), involving the characterisation of organic acid producing species for bioleaching, showed that oxalic acid was produced in high concentrations (24 mM) by *Aspergillus* spp. in the presence of a sucrose carbon source. Based on this value and the results seen in Table 11, only isolates with ≥ 15 mM of oxalic acid produced by day 7 were considered for subsequent bioleaching experiments.

Table 11: Concentrations of oxalic acid during the 7-day growth period. Oxalic acid production on days 0-4 was negligible. The relevant substrate from which these results were achieved is also shown.

Isolate	Substrate	Concentration (mM)		
		Day 5	Day 6	Day 7
Rhizopus microsporus	CYE	7.575 ± 0.32	21.383 ± 0.88	15.971 ± 0.71
Physisporinus sp.	CYE	8.889 ± 0.36	21.179 ± 0.95	17.142 ± 0.74
Aspergillus niger	CYE	11.869 ± 0.49	17.912 ± 0.78	16.667 ± 0.71
Mucor fragilis	CYE	9.968 ± 0.41	17.887 ± 0.76	6.899 ± 0.29
Fusarium sp.	PD	12.967 ± 0.55	17.750 ± 0.73	18.166 ± 0.79
Curvularia petersonii	ME	8.235 ± 0.36	15.435 ± 0.64	17.093 ± 0.73
Aspergillus sp.	CYE	7.012 ± 0.28	13.779 ± 0.55	11.952 ± 0.52
Aspergillus terreus (Strain 1)	CYE	4.023 ± 0.17	12.195 ± 0.48	4.549 ± 0.19
Phoma sp.	PD	4.235 ± 0.18	10.593 ± 0.45	13.574 ± 0.56
Fomitopsis meliae	CYE	7.080 ± 0.31	7.060 ± 0.29	15.000 ± 0.61
Aspergillus terreus (Strain 2)	CYE	0.567 ± 0.02	2.051 ± 0.09	3.676 ± 0.16

Oxalic acid was not detected from day 0-4 for all species. Results are shown as mean ± SE (n=3)
Alternaria sp, *C. raphigera* and *Penicillium* sp. did not produce oxalic acid.

The total amount of oxalic acid produced peaked on day 6 for *R. microsporus, Physisporinus* sp*, A. niger, M. fragilis, Aspergillus* sp. and *A. terreus* (1), before decreasing by day 7. All six of these isolates were grown in CYE. Of the remaining isolates, namely, *Fusarium* sp*, C. petersonii, Phoma* sp*, F. meliae* and *A. terreus* (2), two were grown in PD (*Fusarium* sp. and *Phoma* sp) while one was grown in ME (*C. petersonii*). The highest concentration of oxalic acid produced, ranging from 21.383 to 17.750 mM, was seen on day 6 by isolates grown in CYE, with the exception of *Fusarium* sp. Each of these isolates showed an insignificant decrease in oxalic acid of 1.245 mM or more by day 7 (p-value: $0.37 > \alpha = 0.05$). *R. microsporus* showed a 25% decrease in oxalic acid by day 7. *Physisporinus* sp. saw a 19% decrease. *A. niger* showed a relatively small decrease at 7%, while *M. fragilis* showed the highest decrease of all isolates tested, at 61%. Of the remaining CYE isolates, *Aspergillus* sp. and *A. terreus* (1) showed a 13 and 63% decrease respectively, with *A. terreus* (1) the only one of the *Aspergillus* species to show a significant decrease (p-value: $0.00033 < \alpha = 0.05$) in oxalic acid concentration. Despite having the fourth highest oxalic acid concentration by day 6, *M. fragilis* was disregarded for bioleaching due to its decrease in oxalic acid to below 15 mM by day 7. The overall difference in oxalic acid concentration between days 5 and 6 was significant (p-value: $0.00023 < \alpha = 0.05$), whereas the overall difference between days 6 and 7 was not (p-value: $0.37 > \alpha = 0.05$).

This decrease in organic acid production was attributed to the high sucrose concentration of the CYE medium and the subsequent effect it has on the biomass production of fungal species. As seen in section 4.3, the CYE medium triggered increased growth responses from several isolates compared to the other substrates. This is directly related to increased biomass production according to work done by Meletiadis and coworkers (2001), Zain and coworkers (2009) and Choi and coworkers (2007). In the absence of a sufficient nutrient supply, certain fungi are known to prioritize biomass accumulation over metabolic processes, reducing the amount of primary and secondary metabolites produced (Liaud *et al*., 2014). As day 6 of the incubation period was reached, the reduced nutrient supply and increased biomass accumulation is likely to have triggered a decrease in oxalic acid concentration for isolates grown in CYE. Increased amounts of oxalic acid are also known to reduce the pH of the immediate environment (Palmieri *et al*., 2018). This would further reduce the rate at which the fungi grow and metabolise the nutrient substrate, leading to decreased oxalic acid production and lower concentrations of oxalic acid by day 7. Given that CYE was selected as

the ideal substrate for bioleaching, only isolates with minimal decreases in oxalic acid were considered for bioleaching experiments. Isolates with consistent oxalic acid concentrations across day 6 and 7 were also considered for bioleaching experiments. The high oxalic acid produced by *Fusarium* sp. in PD was consistent with the results seen in section 4.3. *Fusarium* sp. displayed the highest absorbance in PD, and in the absence of a supplemental carbon source, was likely able to induce high oxalic production without the increased biomass and eventual decrease in oxalic acid concentration. *Fusarium* sp. also produced the highest oxalic acid concentration of each isolate on day 4.

No oxalic acid production was detected by *Alternaria* sp*, C. raphigera* and *Penicillium* sp. The lack of organic acid production by *Alternaria* is consistent with a study by Ahmed and coworkers (2015), which states that although organic production can be found in *Alternaria* spp, their overall production is very low. The lack of organic acid production by *Chaetomella* is consistent with a study done by Shindia and coworkers (2006), which highlights an absence of organic acid production by species of the genus *Chaetomium*. Oxalic acid production by *Penicillium* spp. has rarely been reported, with several studies only showing oxalic acid production from *P. oxalicum* (Ikotun, 1984; Liao *et al*., 2019; Zhang *et al*., 2020). A study by Currie and Thom (1915), did show oxalic production by another species of *Penicillium*, however this was only in trace amounts.

Of the final shortlist of isolates from section 4.3.3 above (*A. niger, Aspergillus* sp.*, F. meliae* and *Fusarium* sp), only *Fusarium* sp. and *A. niger* were able to produce high concentrations of oxalic acid. Despite increasing by 53% on day 7, the overall concentration of oxalic acid produced by *F. meliae* was not high enough by day 6. *Aspergillus* sp. saw only a 13% decrease from day 6 onwards, however its overall concentration of oxalic acid was too low. Both these isolates were disregarded for bioleaching experiments. Based only on the oxalic acid production results, the isolates *R. microsporus*, *Physisporinus* sp*, A. niger, Fusarium* sp. and *C. petersonii* were shortlisted for bioleaching. It was also noted that the current study is the first to report production of any organic acid by *Physisporinus* sp.

4.5 Base Metal Extraction - One Step and Spent Medium Bioleaching

Based on the results from the spore counts, growth rate analysis and organic acid production, *Fusarium* sp. and *A. niger* were selected as the ideal isolates for bioleaching in the form of spent medium and one-step base metal extractions. Both isolates displayed high spore counts, which increased significantly when grown in CYE. *Fusarium* sp. displayed a consistently high growth rate in PD, ME and CYE, while the growth rate of *A. niger* increased significantly when grown in CYE. Despite not being the best performing isolates throughout all the screening experiments, *Fusarium* sp. and *A. niger* were the only isolates out of the 14 to perform consistently well across each screening experiment. The CYE substrate was chosen for bioleaching given that it promotes increased spore production, growth rate and organic acid production over the PD and ME substrates

For bioleaching, the efficacy of *A. niger* and *Fusarium* sp. at extracting base metals (Cobalt (Co), chromium (Cr), copper (Cu), iron (Fe) and nickel (Ni)) from a PGM flotation concentrate was assessed. The results of x-ray diffraction of the UG2 flotation concentrate are shown below in Table 12.

Table 12: Base metal content of the upper ground 2 (UG2) flotation concentrate.

Metal	Metal Content (g)
Cobalt	0.0010
Chromium	0.0206
Copper	0.0196
Iron	0.2400
Nickel	0.0342

The PGM flotation concentrate used in this study contained measurable quantities of base metals. Percentage extraction of base metals from all leaching experiments was calculated from the above values. The calculations can be found in Appendix B.

4.5.1 Spent Medium Bioleaching

Spent medium leaching was performed to assess the efficacy of base metal leaching by oxalic acid while allowing for extreme temperature and pH conditions that fall outside the

acceptable physiological range for fungal species (Meletiadis *et al*., 2001). Spent medium leaching utilizes a spent medium, which is a fungal inoculum that restricts further fungal growth and metabolism (Seh-Bardan *et al*., 2012). Fungi are cultivated on plates over a certain time period, whereafter the biomass is separated from the medium by centrifugation and filtration to produce a spent medium. Spent medium leaching utilizes only the metabolic products obtained during fungal growth, in the extraction of metals, rather than metabolic products produced in the presence of actively growing fungi. A study by Lobos and coworkers (2021) suggested that spent medium leaching may be optimal for fungal bioleaching as the lack of direct contact between fungi and the target metal can reduce toxic inhibitory effects caused by the metal. The aim of using spent medium leaching in the current study was to increase the efficiency of base metal leaching by oxalic acid within the 7 day leaching period. The leaching conditions for each corresponding flask in the spent medium leaching design of experiments are shown in Table 14.

The parameters for spent medium leaching, such as pulp density and leaching time, were determined based on several studies which performed extractions of base metals such as Fe, Cu, Ni, Co, and Cr from low-grade ores using filamentous fungal species (Abayomi *et al*., 2017; Biswas *et al*., 2013; Mulligan *et al*., 2004; Rao *et al*., 2002). The increased temperature and low pH parameters for spent medium leaching were selected based on a study by Biswas and coworkers (2013), which showed high Ni and Co extractions (80%) from a bauxite ore when leaching with an *A. niger* culture filtrate (spent medium) at temperatures of 60 and 70^{o}C and at a pH range between 1 and 3. The use of Tween 80 in the present study served to dislodge spores from the culture surface and extract extracellular metabolites secreted into the growth medium. A study by Tu and coworkers (2015) showed that Tween 80 significantly increased extracellular metabolite release in a fermentation environment (75.08% with regard to polymalic acid) from the saprophytic fungi *Aureobasidium pullulans*, by influencing membrane permeability. Other studies highlight an increase in mycelial cell membrane transport activity and enhanced extracellular metabolite release in the presence of Tween 80 (Liu and Wu, 2012; Zhang *et al*., 2012). The study by Liu and Wu (2012) highlights a significant increase in exopolysaccharide production as a result of the stimulation of exopolysaccharide release from the fungal cells by Tween 80, in a liquid culture environment. While the current study did not utilise fermentation or liquid culture for spent medium production, the ability for metabolite

recovery by Tween 80 observed above was believed to be sufficient when applied to agar-grown cultures.

4.5.1.1 Fusarium *sp*. *Spent Medium Bioleaching*

The results of inductively couple plasma optical emission spectroscopy (ICP-OES) were converted from mg/L according to the equation in Appendix C and presented below in Table 13 as the percentage of base metals extracted (%) from the concentrate.

Table 13: Percentage extraction of base metals by *Fusarium* sp. under spent medium conditions (pH, temperature (°C) and pulp density (%w/v)) at Days 1, 4 and 7. Flask number (1-9) is also displayed.

No.	Conditions (pH, Temperature, Pulp Density)	Day	Cobalt (%)	Chromium (%)	Copper (%)	Iron (%)	Nickel (%)
1	- pH 1 - 50°C - 1% (w/v)	1	5.00 ± 0.22	3.35 ± 0.12	4.85 ± 0.20	6.29 ± 0.24	4.77 ± 0.19
		4	14.00 ± 0.50	4.81 ± 0.21	5.00 ± 0.21	9.83 ± 0.39	6.81 ± 0.21
		7	20.00 ± 0.85	4.61 ± 0.20	4.85 ± 0.22	9.96 ± 0.45	8.54 ± 0.29
2	- pH 3 - 50°C - 1% (w/v)	1	5.00 ± 0.21	2.77 ± 0.11	3.16 ± 0.14	3.60 ± 0.12	3.22 ± 0.12
		4	5.00 ± 0.22	3.50 ± 0.15	4.53 ± 0.20	6.98 ± 0.31	2.47 ± 0.10
		7	6.00 ± 0.19	3.74 ± 0.13	3.27 ± 0.13	8.92 ± 0.31	1.87 ± 0.08
3	- pH 1 - 50°C - 2% (w/v)	1	10.00 ± 0.35	6.12 ± 0.23	9.13 ± 0.33	13.25 ± 0.55	8.27 ± 0.29
		4	13.00 ± 0.57	8.50 ± 0.36	9.69 ± 0.31	19.23 ± 0.66	13.77 ± 0.58
		7	16.00 ± 0.64	8.30 ± 0.34	9.10 ± 0.33	20.94 ± 0.71	18.80 ± 0.69
4	- pH 3 - 50°C - 2% (w/v)	1	11.00 ± 0.38	5.78 ± 0.22	7.30 ± 0.25	14.13 ± 0.44	7.31 ± 0.28
		4	11.00 ± 0.39	7.04 ± 0.30	7.35 ± 0.29	16.33 ± 0.66	8.77 ± 0.35
		7	14.00 ± 0.47	7.72 ± 0.33	7.40 ± 0.28	19.04 ± 0.68	11.70 ± 0.40
5	- pH 1 - 70°C - 1% (w/v)	1	7.00 ± 0.23	3.69 ± 0.13	4.80 ± 0.15	7.67 ± 0.28	4.68 ± 0.18
		4	12.00 ± 0.39	3.93 ± 0.14	4.39 ± 0.16	7.79 ± 0.35	5.20 ± 0.16
		7	19.00 ± 0.85	4.37 ± 0.15	4.85 ± 0.21	9.75 ± 0.34	6.90 ± 0.23
6	- pH 3 - 70°C - 1% (w/v)	1	5.00 ± 0.18	4.17 ± 0.18	3.83 ± 0.15	8.88 ± 0.38	4.04 ± 0.16
		4	9.00 ± 0.28	5.15 ± 0.19	4.44 ± 0.14	12.79 ± 0.49	8.92 ± 0.40
		7	15.00 ± 0.56	6.50 ± 0.25	5.26 ± 0.16	15.54 ± 0.50	16.02 ± 0.57
7	- pH 1 - 70°C - 2% (w/v)	1	18.00 ± 0.41	6.55 ± 0.24	5.83 ± 0.19	14.33 ± 0.49	8.48 ± 0.32
		4	17.00 ± 0.56	7.28 ± 0.25	8.47 ± 0.36	14.58 ± 0.46	9.36 ± 0.35
		7	23.00 ± 0.76	8.45 ± 0.38	9.44 ± 0.42	24.58 ± 0.83	24.36 ± 0.86
8	- pH 3 - 70°C - 2% (w/v)	1	5.00 ± 0.19	8.59 ± 0.33	7.70 ± 0.28	9.08 ± 0.39	8.10 ± 0.32
		4	11.00 ± 0.43	4.24 ± 0.19	4.26 ± 0.19	16.25 ± 0.68	10.05 ± 0.39
		7	15.00 ± 0.65	9.90 ± 0.33	9.01 ± 0.39	17.29 ± 0.67	13.54 ± 0.45
9	- pH 2 - 60°C - 1.5% (w/v)	1	4.40 ± 0.14	1.20 ± 0.08	4.07 ± 0.13	2.68 ± 0.09	3.90 ± 0.12
		4	5.80 ± 0.22	1.71 ± 0.08	5.35 ± 0.22	3.16 ± 0.13	4.00 ± 0.14
		7	5.20 ± 0.18	1.89 ± 0.08	5.73 ± 0.22	4.32 ± 0.17	4.57 ± 0.16

Values are expressed as mean ± standard error of replicates (n=3)

An observable amount of base metals was leached from the concentrate only 1 day into the leaching period. By day 1, flask 7, with the leaching conditions of pH 1, 70 ^{o}C and 2% (w/v) pulp density, leached the highest amounts of Co (18%), Fe (14.33%) and Ni (8.48%). Flask 4 (pH 2 and 50 ^{o}C) leached a nearly identical amount of Fe (14.13%). At pH 1, flask 3 leached a slightly lower amount of Fe (13.25%). Based on flasks 3 and 4, more Fe leaches at a higher pH (pH 5). However, in this case the leaching of Fe is higher at a lower pH (pH 1) when the temperature is higher (70^{o}C), as seen in flask 7. Cobalt displays the same extraction behaviour as Fe as seen in flasks 3, 4 and 7. Copper is the only metal to have its highest day 1 extraction at 50 ^{o}C, while Cr is the only metal to have its highest day 1 extraction at pH 3. The highest base metal extractions on day 1 was only seen at 2% (w/v) pulp densities, regardless of pH or temperature. High extraction of the base metals was attributed to the high concentration of oxalic acid present from the beginning of the leaching period. Spent medium leaching allows for high concentrations of oxalic acid to be present from the onset of incubation, allowing for base metal extraction to begin immediately (Dusengemungu *et al*., 2021). Based on the results from day 1, the optimal parameters for base metal extraction are pH 1, 70 ^{o}C and pulp density of 2% (w/v).

By day 4, the percentage of Cr extracted (8.50%) increased at the lower pH (pH 1) and lower temperature (50^{o}C) of flask 3 since day 1 (6.12%), however it decreased from the extraction percentage seen in the higher pH (pH 3) and higher temperature (70^{o}C) of flask 8 on day 1 (8.59%). Flask 7 extracted the highest amount of Co (17%) on day 4, but this was an insignificant change (p-value: 0.073 > α = 0.05) from day 1 . Iron extraction in flask 7 remained unchanged between days 1 and 4 but saw a 6% increase in flask 3. Conversely to what was seen on day 1, Fe extracted better at the lower temperature (50^{o}C) of flask 3 on day 4. Nickel extraction increased overall but increased more at the lower pH (pH 1) and temperature (50^{o}C). Copper extraction increased to 9.69% since day 1 (9.13%). Overall, the highest day 4 extractions were seen in flasks 3, 4 and 7, and at 2% (w/v) pulp densities. Flask 1 was the only flask at a 1% (w/v) pulp density to show a significant increase in base metal extraction by day 4 (p-value: 0.0013 < α = 0.05), extracting 14% of Co at both low temperature (50^{o}C) and low pH (pH 1). While *Fusarium* sp. showed a higher leaching capacity for Co on day 1, its leaching capacity of Fe was higher in five of the nine flasks on day 4. Only flasks 1, 5, 7 and 9 extracted more Co than Fe, with each flask having a low pH (pH 2 in the case of flask 9). *Fusarium* sp.

may have a higher capacity for leaching Co at very low pH based on these results. Fe extraction was the second highest (16.33%) in flask 4, displaying the capacity for *Fusarium* to extract Fe at lower temperatures regardless of the high pH. Based on the results of day 4, base metal extraction by *Fusarium* sp. was still higher at pH 1 and a temperature of 70 ^{o}C, however, temperature was shown to have specific effect on the extraction of Co, Ni and Fe.

Higher extractions of Co (23%), Fe (24.58%) and Ni (24.36%) were observed by day 7. All flasks, except flask 7, continued to extract more Co than Fe at pH 1. Flask 7 showed the most significant increase in extraction of Co, Fe and Ni from day 4 to 7 (p-value: 0.00060, 0.00028 and 0.00024 < α = 0.05, respectively), with the extractions increasing by 6, 10 and 10.59%, respectively. Fe and Ni showed nearly identical extraction in flask 3, 6, 7 and 9, highlighting the capacity of *Fusarium* sp. to extract both metals at equal concentrations. However, higher Fe extraction was evident from day 1 (Fe 14.33%, Ni 8.48%), while comparable Ni extraction was only seen on day 7 (Fe 24.58%, Ni 24.36%). This is consistent with work done by Suja and coworkers (2018), which showed low initial Ni extraction that increased (7 - 74%) after longer (7+ days) bioleaching periods. The increased Co and Ni extraction seen from spent medium leaching is also consistent with work done by Cameron and coworkers (2021), which showed that the extraction of Ni (90%) and Co (80%) was higher at temperatures greater than 40 ^{o}C and within a pH range of 1.6 to 3. The extreme leaching conditions permitted by spent medium leaching could have promoted further extraction of Co and Ni. Iron consistently showed the highest extraction on days 1, 4 and 7 by *Fusarium* sp. The extractions of Fe seen by *Fusarium* sp. are consistent with work done by Alibhai and coworkers (1993), which shows that *Aspergillus* and *Penicillium* spp, in the presence of a sucrose carbon source, have a 25 to 35% Fe leaching capacity when leaching from a finely-ground ore. Alibhai and coworkers (1993) also states that roughly the same extraction of Fe and Ni was achieved through oxalic acid leaching, while also showing a 55 to 65% leaching capacity of Ni and Co. This is confirmed based on the above results which highlight that *Fusarium* may maintain a similar leaching capacity to species of *Penicillium* and *Aspergillus*. The extractions of Cu were not consistent with the high leaching capacities of other fungal species when leaching from a low-grade ore. Studies by Mulligan and coworkers (2004) and Anjum and coworkers (2009) show increased Cu extractions (68 and 49.29%, respectively) when leaching from low-grade ore with *A. niger* and *P. notatum*, respectively. Both these studies, however, conducted leaching for extended

time periods, at 14 and 33 days, respectively. This along with the low Cr extraction, could highlight a lack of Cu and Cr leaching capacity by *Fusarium*, as well as the need for longer leaching periods.

4.5.1.2 Aspergillus niger *Spent Medium Bioleaching*

The experimental design for spent medium leaching with *A. niger* was identical to the experimental design for *Fusarium* sp. spent medium leaching. Results are shown below in Table 14.

Table 14: Percentage extraction of base metals by *A. niger* under spent medium conditions (pH, temperature (°C) and pulp density (%w/v)) at Days 1, 4 and 7. Flask number (1-9) is also displayed.

No.	Conditions (pH, Temperature, Pulp Density)	Day	Cobalt (%)	Chromium (%)	Copper (%)	Iron (%)	Nickel (%)
1	- pH 1 - 50°C - 1% (w/v)	1	9.00 ± 0.32	2.52 ± 0.09	2.86 ± 0.12	6.46 ± 0.29	2.95 ± 0.11
		4	14.00 ± 0.53	3.20 ± 0.12	2.91 ± 0.13	11.54 ± 0.51	3.86 ± 0.14
		7	21.00 ± 0.89	3.83 ± 0.16	4.54 ± 0.15	13.78 ± 0.49	12.04 ± 0.53
2	- pH 3 - 50°C - 1% (w/v)	1	5.00 ± 0.21	3.16 ± 0.12	3.42 ± 0.13	6.88 ± 0.28	5.09 ± 0.23
		4	14.00 ± 0.47	3.11 ± 0.12	3.37 ± 0.13	7.58 ± 0.25	6.29 ± 0.28
		7	12.00 ± 0.44	4.17 ± 0.15	3.57 ± 0.11	10.17 ± 0.35	9.30 ± 0.32
3	- pH 1 - 50°C - 2% (w/v)	1	14.00 ± 0.57	5.29 ± 0.23	5.92 ± 0.23	14.46 ± 0.45	8.76 ± 0.33
		4	21.00 ± 0.65	6.70 ± 0.27	6.17 ± 0.27	18.51 ± 0.57	9.87 ± 0.33
		7	27.00 ± 1.10	7.48 ± 0.32	7.04 ± 0.22	24.79 ± 1.02	23.51 ± 0.96
4	- pH 3 - 50°C - 2% (w/v)	1	14.00 ± 0.44	5.10 ± 0.22	6.73 ± 0.23	12.91 ± 0.42	9.36 ± 0.38
		4	15.00 ± 0.47	6.70 ± 0.22	6.17 ± 0.27	15.33 ± 0.66	8.51 ± 0.34
		7	16.00 ± 0.55	7.09 ± 0.23	6.48 ± 0.23	24.52 ± 0.78	21.94 ± 0.84
5	- pH 1 - 70°C - 1% (w/v)	1	12.00 ± 0.51	3.54 ± 0.12	2.96 ± 0.10	8.96 ± 0.28	4.76 ± 0.20
		4	17.00 ± 0.64	4.02 ± 0.14	3.23 ± 0.12	13.49 ± 0.41	7.34 ± 0.29
		7	22.00 ± 0.84	5.68 ± 0.20	4.29 ± 0.18	17.83 ± 0.62	14.67 ± 0.55
6	- pH 3 - 70°C - 1% (w/v)	1	10.00 ± 0.38	3.74 ± 0.12	2.65 ± 0.10	7.88 ± 0.24	6.81 ± 0.27
		4	12.00 ± 0.54	3.88 ± 0.17	2.70 ± 0.10	9.21 ± 0.32	9.33 ± 0.38
		7	19.00 ± 0.78	4.56 ± 0.17	2.65 ± 0.09	11.17 ± 0.43	15.23 ± 0.67
7	- pH 1 - 70°C - 2% (w/v)	1	17.00 ± 0.60	6.58 ± 0.23	6.97 ± 0.22	19.89 ± 0.76	12.53 ± 0.44
		4	23.00 ± 0.74	9.47 ± 0.42	7.50 ± 0.23	24.62 ± 0.85	15.82 ± 0.66
		7	29.00 ± 1.24	8.45 ± 0.33	5.46 ± 0.17	29.45 ± 1.23	26.76 ± 1.05
8	- pH 3 - 70°C - 2% (w/v)	1	15.00 ± 0.46	7.04 ± 0.22	4.90 ± 0.19	15.25 ± 0.67	10.50 ± 0.42
		4	19.00 ± 0.69	10.25 ± 0.41	7.81 ± 0.27	19.38 ± 0.69	17.88 ± 0.63
		7	20.00 ± 0.90	12.03 ± 0.44	7.30 ± 0.23	22.03 ± 0.98	11.30 ± 0.36
9	- pH 2 - 60°C - 1.5% (w/v)	1	8.60 ± 0.33	1.76 ± 0.08	4.90 ± 0.20	4.93 ± 0.22	4.98 ± 0.16
		4	8.60 ± 0.27	1.92 ± 0.08	5.20 ± 0.23	3.43 ± 0.12	3.44 ± 0.10
		7	6.60 ± 0.25	1.92 ± 0.09	5.24 ± 0.18	5.31 ± 0.18	4.88 ± 0.16

Values are expressed as mean ± standard error of replicates (n=3)

Initial extractions on day 1 are higher than the extractions seen on day 1 by *Fusarium* sp. (Table 13). Iron extraction by *A. niger* was insignificantly higher on day 1 (19.89%) than *Fusarium* sp. on day 4 (19,21%) (p-value: 0.195 > α = 0.05), while Ni was significantly higher for the same period (12.53 - 13.77%) (p-value: 0.046 < α = 0.05). This highlights an increased capacity for extraction by *A. niger* within the first 24 hours. Cobalt saw a 1% increase in extraction by *A. niger* on day 1 (18 – 19%). Cobalt and Fe continued to extract at a similar rate (19 and 19.89%, respectively). The increased Fe, Ni and Co extractions are consistent with a study done by Alibhai and coworkers (1993), which highlights similar extractions (25-35%) of Fe, Ni and Co by *Aspergillus*. However, the low Cu extraction (6.97%) observed in the current study does not agree with the results of a study done by Mulligan (2004), which states that *A. niger* has a higher capacity for Cu extraction (68%) from a low-grade ore, rather than Ni (34%), in a chemical leaching setup. The low Cu and Cr extractions seen from both *A. niger* and *Fusarium* sp. could be attributed to consequences of spent medium leaching. Similarly to leaching with *Fusarium* sp, extractions by *A. niger* were higher at a pulp density of 2% (w/v).

Iron (24.62%), Co (23%) and Cr (9.47%) extractions by *A. niger* on day 4 are not significantly different to the extractions by *Fusarium* seen on day 7 (Table 13) (24.58, 23 and 9.90%, respectively) (p-value: 0.97, 1.00 and 0.37 > α = 0.05, respectively). Despite Cr maintaining a low extraction rate, these results highlight the faster leaching capabilities of *A. niger* compared to *Fusarium* sp. Copper extraction (7.50%) on day 4 by *A. niger* was slightly lower than the extraction by *Fusarium* sp. on day 4 (9.69%), highlighting a slower capacity for Cu leaching by *A. niger*. Whereas *Fusarium* sp. saw higher extractions at 50 ^{o}C on day 4, the highest extractions by *A. niger* were only seen at 70 ^{o}C on day 4. The highest extractions were once again seen at a pulp density of 2% (w/v).

The day 7 extractions were the highest extractions of Fe (29.45%), Ni (26.76%) and Co (29%) achieved by spent medium leaching at this point. Nickel extractions continued to increase between days 4 and 7, as seen in *Fusarium* spent medium leaching (Table 13). While the similar extraction of Ni and Fe by oxalic acid leaching is consistent with a study done by Alibhai and coworkers (1993), neither metal was extracted at their proposed leaching capacities by *Aspergillus*. *Aspergillus* spp. are known to have a 55 to 60% leaching capacity for Ni and Co, however these extractions are only seen after a period of 48 days (Alibhai *et al.*, 1993). The lower-than-expected Ni and Co extractions could be attributed to the shortened leaching

period in this study, however both *A. niger* and *Fusarium* sp. have displayed Ni and Co leaching capacity. Iron extraction falls within its 25 to 30% leaching capacity by *A. niger,* proposed in the study by Alibhai and coworkers (1993). While higher extractions were seen at a 1% (w/v) pulp density in this instance, the highest extractions were still seen at a pulp density of 2% (w/v). The overall extractions by *A. niger* were not significantly different to *Fusarium* sp. spent medium extractions (p-value: $0.95 > \alpha = 0.05$).

4.5.1.3 Spent Medium Abiotic Control Bioleaching

Leaching was conducted under abiotic conditions to assess the impact that leaching parameters (pH, temperature) have on the base metal extractions, without the inclusion of oxalic acid-containing spent medium, from the flotation concentrate. The abiotic control flasks were run under identical conditions to the spent medium DOE, without *A. niger* or *Fusarium* sp. spent medium. Results for the spent medium abiotic control tests are shown below in Table 15.

Table 15: Percentage extraction of base metals during abiotic leaching under spent medium conditions (pH, temperature (°C) and pulp density (%w/v)) at Days 1, 4 and 7. Flask number (1-9) is also displayed.

No.	Conditions (pH, Temperature, Pulp Density)	Day	Cobalt (%)	Chromium (%)	Copper (%)	Iron (%)	Nickel (%)
1	- pH 1 - 50°C - 1% (w/v)	1	6 ± 0.25	4.32 ± 0.13	5.87 ± 0.24	10.33 ± 0.37	6.55 ± 0.21
		4	10 ± 0.37	4.9 ± 0.16	7.45 ± 0.31	11.92 ± 0.5	10.38 ± 0.45
		7	13 ± 0.56	5.05 ± 0.2	7.6 ± 0.28	13.17 ± 0.43	12.84 ± 0.47
2	- pH 3 - 50°C - 1% (w/v)	1	5 ± 0.2	0.29 ± 0.08	2.9 ± 0.11	2.08 ± 0.08	2.46 ± 0.08
		4	5 ± 0.16	0.39 ± 0.08	2.76 ± 0.09	1.07 ± 0.08	2.92 ± 0.1
		7	5 ± 0.22	0.24 ± 0.08	2.6 ± 0.08	0.02 ± 0.08	0.15 ± 0.08
3	- pH 1 - 50°C - 2% (w/v)	1	12 ± 0.5	6.6 ± 0.23	9.59 ± 0.38	15.63 ± 0.58	9.97 ± 0.33
		4	19 ± 0.79	7.82 ± 0.29	10.46 ± 0.39	19.79 ± 0.64	18.45 ± 0.79
		7	22 ± 0.7	7.28 ± 0.24	9.59 ± 0.37	19.53 ± 0.73	20.29 ± 0.84
4	- pH 3 - 50°C - 2% (w/v)	1	5 ± 0.18	0.24 ± 0.08	3.01 ± 0.12	0.5 ± 0.08	3.54 ± 0.14
		4	7 ± 0.25	0.24 ± 0.08	3.37 ± 0.13	0.63 ± 0.08	4.42 ± 0.16
		7	6 ± 0.26	0.24 ± 0.08	3.21 ± 0.12	0.6 ± 0.08	4.01 ± 0.16
5	- pH 1 - 70°C - 1% (w/v)	1	7 ± 0.28	3.88 ± 0.16	5.31 ± 0.2	9.13 ± 0.33	6.14 ± 0.24
		4	14 ± 0.59	4.85 ± 0.18	6.63 ± 0.23	12.46 ± 0.43	13.98 ± 0.56
		7	18 ± 0.66	5.1 ± 0.22	7.09 ± 0.22	13.88 ± 0.55	17.46 ± 0.55
6	- pH 3 - 70°C - 1% (w/v)	1	5 ± 0.17	0.24 ± 0.08	2.6 ± 0.1	0.2 ± 0.08	0.15 ± 0.08
		4	5 ± 0.19	0.29 ± 0.08	2.09 ± 0.08	0.7 ± 0.08	2.54 ± 0.08
		7	5 ± 0.15	0.24 ± 0.08	2.09 ± 0.08	0.76 ± 0.08	2.66 ± 0.1
7	- pH 1 - 70°C - 2% (w/v)	1	5 ± 1.15	0.37 ± 0.37	0.24 ± 0.5	0.00 ± 0.00	0.82 ± 0.9
		4	27 ± 1.34	8.88 ± 0.32	11.79 ± 0.4	22.92 ± 1.06	25.82 ± 1.02
		7	6 ± 0.25	0.24 ± 0.08	3.16 ± 0.1	0.41 ± 0.08	3.86 ± 0.12
8	- pH 3 - 70°C - 2% (w/v)	1	9 ± 0.32	0.34 ± 0.08	3.88 ± 0.13	0.91 ± 0.08	5.38 ± 0.21
		4	9 ± 0.45	0.49 ± 0.08	3.93 ± 0.14	1.07 ± 0.08	5.96 ± 0.19
		7	3 ± 0.13	0.01 ± 0.08	0.21 ± 0.08	0.03 ± 0.08	0.0 ± 0.08
9	- pH 2 - 60°C - 1.5% (w/v)	1	4 ± 0.17	0.01 ± 0.08	1.29 ± 0.08	0.05 ± 0.08	0.12 ± 0.08
		4	4 ± 0.13	0.01 ± 0.08	2.33 ± 0.1	3.1 ± 0.11	2.26 ± 0.08
		7	4 ± 0.15	0.32 ± 0.08	2.33 ± 0.09	3.1 ± 0.11	2.26 ± 0.09

Values are expressed as mean ± standard error of replicates (n=3)

Abiotic spent medium leaching, by day 1, only showed effective leaching of all five base metals in flasks 1, 3 and 5 (Cr 6.60%, Cu 9.59%, Fe 15.63%, Co 12%, Ni 9.97%). The extraction percentages of flask 3 are roughly consistent with the extractions seen from *A. niger* on day 1 (Cr 7.04%, Cu 6.97%, Fe 19.89%, Co 19%, Ni 12.53%), however those extractions were seen under the higher temperature (70°C) conditions of flask 7. Despite having the highest extractions during spent medium leaching, flask 7 from abiotic leaching shows no noticeable extractions apart from Co, which was also extracted at a significantly lower percentage (5%) compared to *A. niger* (17%) and *Fusarium* sp. (18%) flask 7 extractions on day 1. Cobalt was the only metal to extract at all conditions of abiotic leaching, while flask 7 was the only flask

set at pH 1 that failed to show increased extractions of all five base metals. The increased extractions seen in flasks 1, 3 and 5, all of which were set and maintained at pH 1, are consistent with Hosseini and coworkers (2017), who showed that the leaching kinetics of base metals from a low-grade flotation concentrate increase at higher temperatures and higher concentrations of sulphuric acid. Within the first day, abiotic/sulphuric acid leaching is not as consistent or effective across different temperature and pH levels as oxalic acid.

Day 4 saw a significant increase in Co (27%) ($p = 0.0057$), Cr (8.88%) ($p = 0.011$), Cu (11.79%) ($p = 0.0091$), Fe (22.92%) ($p = 0.0041$) and Ni (25.82%) ($p = 0.0055$) extractions in flask 7. The extractions of all five base metals except Ni were roughly consistent with their extractions seen during *A. niger* day 4 spent medium leaching (Co 23%, Cr 9.47%, Fe 24.62%, Cu 7.50%) . Iron, Ni and Co extractions, in flask 7 exclusively, were nearly as high as the highest spent medium extractions, seen from *A. niger* on day 7 (Table 14). Despite not showing any increased extractions on day 1, the high temperature (70^{o}C), low pH (pH 1) conditions of flask 7 remained the most successful at extracting high amounts of Fe, Ni and Co. Nonetheless, the roughly consistent extractions seen from the abiotic control and spent medium tests suggest that the concentration of inorganic sulphuric acid is a major contributing factor to the extractions seen during spent medium leaching. However, contrary to *A. niger* and *Fusarium* sp. spent medium leaching, abiotic leaching continues to be ineffective at leaching base metals at the higher pH (pH 3).

By day 7, Ni and Co extractions continued to increase rapidly. The high Ni (31.58%) and Co (34%) extraction remains consistent with the works of Hosseini and coworkers (2017). The high temperature (70^{o}C) and high concentration of sulphuric acid in flask 7 facilitated the highest extractions of any single base metal from all leaching experiments thus far. Iron extraction (24.25%) didn't increase significantly (p-value: $0.120 > \alpha = 0.05$) , while failing to reach the same extraction percentages achieved on day 7 of *A. niger* (29%) (Table 14) and *Fusarium* sp. (24%) (Table 13) spent medium leaching. Cobalt, Fe and Ni extractions were significantly lower in flask 1 (13, 13.17, 12.84%, respectively), flask 3 (22, 19.25, 20.29%, respectively) and flask 5 (18, 13.88, 17.46%, respectively) (p-value < 0.0001). Flask 3 produced the highest extractions of the three, highlighting pulp density as the limiting factor after pH, followed by temperature. Flask 8 was run at a pulp density of 2% (w/v) and temperature of 70^{o}C but was unable to overcome its high pH (pH 3) and produce increased extraction.

Based on the results of the abiotic control tests, at low pH, leaching of base metals from the flotation concentrate by *A. niger* and *Fusarium* sp. is not significantly different (p-value: 0.0125 < α = 0.05) from chemical leaching. The base metal extraction percentages from abiotic leaching, specifically in flasks 1, 3, 5 and 7, are similar to the extractions seen in spent medium leaching (Tables 13 and 14). Therefore, the impact that the fungi-produced oxalic acid has on base metal extractions, under low pH and high temperature conditions, is unclear. However, spent medium leaching did show consistent extraction across each flask, whereas abiotic leaching showed nearly no leaching at higher pH. The extractions from flasks 2, 4, 6 and 8 (all pH 3) were significantly lower than both *A*. *niger* (p-value: 0.0045 < α = 0.05) and *Fusarium* sp. (p-value: 0.0132 < α = 0.05) extractions under the same conditions. This displays a strong leaching capacity for abiotic chemical leaching only at low pH (pH 1), whereas spent medium leaching by *Fusarium* sp. and *A. niger* is effective across a wide range of pH and temperature conditions.

4.5.1.4 Ambient pH Spent Medium Bioleaching

Ambient pH control tests were conducted to assess the leaching capacity of *A. niger* and *Fusarium* sp. in the absence of inorganic H_2SO_4 and NaOH. Acid and alkaline leaching is already well established on an industrial scale for the treatment of ores and the extraction of high concentrations of metals from mine tailings and secondary wastes (Calgaro *et al.*, 2015). Given the efficacy of chemical leaching, particularly with H_2SO_4, control tests were conducted on the ambient pH of the spent medium when washed from *Fusarium* sp. and *A. niger* CYE plates. The results of the ambient pH control tests are shown in Table 16 as average base metal extraction on days 1, 4 and 7. The ambient pH of the flasks for *Fusarium* sp. and *A. niger were* pH ± 5.30 and pH ± 3.50, respectively.

Table 16: Percentage extraction of base metals by *A. niger* and *Fusarium* sp. during ambient pH control leaching under spent medium conditions (temperature (°C) and pulp density (%w/v)) at Days 1, 4 and 7.

Isolate	Day	Cobalt (%)	Chromium (%)	Copper (%)	Iron (%)	Nickel (%)
Fusarium sp.	1	3.11 ± 0.14	1.01 ± 0.05	3.46 ± 0.11	2.16 ± 0.07	3.23 ± 0.15
	4	3.89 ± 0.13	1.39 ± 0.05	4.39 ± 0.21	2.46 ± 0.11	3.40 ± 0.14
	7	3.57 ± 0.16	3.38 ± 0.16	3.96 ± 0.12	3.52 ± 0.15	3.64 ± 0.18
A. niger	1	4.22 ± 0.20	1.13 ± 0.05	2.12 ± 0.11	2.23 ± 0.09	2.11 ± 0.07
	4	5.21 ± 0.20	3.33 ± 0.16	2.28 ± 0.08	5.10 ± 0.25	4.76 ± 0.22
	7	6.58 ± 0.22	4.78 ± 0.20	3.52 ± 0.16	7.36 ± 0.35	6.35 ± 0.28

Values are expressed as mean ± standard error of replicates (n=3)

The results in Table 16 suggest that while leaching does occur at the ambient pH of an *A. niger* and *Fusarium* sp. spent medium, extractions are substantially lower when pH is not adjusted with inorganic acid. The concentration of oxalic acid produced by *Fusarium* sp. was not high enough to bring the pH down to within the ideal range of 1.6 to 3 for spent medium leaching, outlined in a study by Cameron and coworkers (2021). This could be a result of the increased biomass produced by *Fusarium* sp. during cultivation in the sucrose-heavy CYE medium, which leads to lower oxalic acid production according to a study done by Strasser and coworkers (1994). Ambient pH control tests suggest that base metal leaching from the flotation concentrate may be more effective through a combination of fungal spent medium and inorganic sulphuric acid leaching. It should be noted that *Fusarium* sp. has consistently shown a higher capacity for Cu extraction than *A. niger.*

Overall leaching by *A. niger* was more successful at ambient pH than *Fusarium* sp., but not significantly so (p-value: 0.0898 > α = 0.50). Ambient pH leaching also fails to be nearly as effective as leaching with inorganic acids (Tables 13 – 15). The lower ambient pH of *A. niger* (pH 3.50) facilitated better extractions from each metal compared to Fusarium sp. but was still outside the ideal pH range for spent medium leaching (Cameron *et al*., 2021). Despite the low extraction numbers, *A. niger* continues to show an affinity for Fe extraction over other base metals from the flotation concentrate. This is in contradiction with a study done by Mulligan and coworkers (2004), which states that the dissolution of Fe from a low-grade ore concentrate was minimal when compared to other base metals such zinc (Zn), Cu and Ni. Of the leaching experiments conducted so far, *A. niger* has been the most successful at Fe extraction from the low-grade flotation concentrate. Despite being less effective at Cu leaching than *Fusarium* sp, this difference in Cu extraction was insignificant (p-value: 0.080 >

α = 0.05). Overall leaching could be more effective with a combination of spent medium and sulphuric acid leaching, as seen from the results of the ambient pH tests. Ambient pH extractions were significantly lower than *Fusarium* sp. (p-value: 0.0019 < α = 0.05) and *A. niger* (p-value: 0.0080 < α = 0.05) spent medium leaching extractions, which suggest that the extractions seen during spent medium leaching was not due to passive leaching at ambient pH.

4.5.2 Base Metal Extraction - One-Step Bioleaching

One-step leaching was performed to assess the efficacy of base metal leaching when the flotation concentrate is in direct contact with actively metabolising fungal species during oxalic acid production. In one-step leaching, microorganisms are applied directly to the low-grade ore in the presence of a nutrient substrate, allowing for the synthesis of metabolic products and dissolution of metals from the ore to occur in a single step (Arya and Kumar., 2020). According to studies by Seh-Bardan and coworkers (2012) and Dusengemungu and coworkers (2021), oxalic acid concentration is highest during one-step bioleaching with *Aspergillus*, and that the capacity for base metal extraction by *Aspergillus* species is higher in one-step bioleaching compared to spent medium. The parameters for one-step leaching were determined based on the studies by Seh-Bardan and coworkers (2012) and Dusengemungu and coworkers (2021), including studies by Qayyum and coworkers (2019) and Amiri and coworkers (2011) which assessed the one-step leaching by *Aspergillus* spp. As the fungi grows and metabolises the CYE medium, organic acids are produced that begin to leach metals from the flotation concentrate.

4.5.2.1 Fusarium *sp*. *One-Step Bioleaching*

The results of ICP-OES were converted from mg/L according to the equation in Appendix C and presented in Table 17 as the percentage of base metals extracted (%) from the flotation concentrate.

Table 17: Percentage extraction of base metals by *Fusarium* sp. under one-step leaching conditions (pH, inoculum dosage (%v/v) and pulp density (%w/v)) at Days 1, 4 and 7. Flask number (1-9) is also displayed.

No.	Conditions (pH, Inoculum dosage, Pulp density)	Day	Cobalt (%)	Chromium (%)	Copper (%)	Iron (%)	Nickel (%)
1	- pH 4 - 2% (v/v) - 1% (w/v)	1	1.00 ± 0.04	0.10 ± 0.01	2.43 ± 0.08	0.01 ± 0.01	0.00 ± 0.00
		4	3.00 ± 0.15	0.81 ± 0.03	6.71 ± 0.25	0.41 ± 0.02	0.10 ± 0.01
		7	0.00 ± 0.00	0.57 ± 0.03	15.81 ± 0.74	0.07 ± 0.01	0.15 ± 0.01
2	- pH 9 - 2% (v/v) - 1% (w/v)	1	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
		4	1.00 ± 0.03	0.11 ± 0.01	0.17 ± 0.01	0.17 ± 0.01	0.13 ± 0.01
		7	1.00 ± 0.03	0.00 ± 0.00	0.25 ± 0.01	0.00 ± 0.00	0.45 ± 0.02
3	- pH 4 - 10% (v/v) - 1% (w/v)	1	1.00 ± 0.04	0.49 ± 0.02	1.34 ± 0.04	0.14 ± 0.01	0.05 ± 0.01
		4	4.00 ± 0.17	1.38 ± 0.05	7.07 ± 0.23	0.11 ± 0.23	0.12 ± 0.01
		7	4.00 ± 0.19	1.57 ± 0.06	13.63 ± 0.60	0.68 ± 0.03	0.41 ± 0.01
4	- pH 9 - 10% (v/v) - 1% (w/v)	1	1.00 ± 0.04	0.00 ± 0.00	0.00 ± 0.00	0.26 ± 0.01	0.00 ± 0.00
		4	2.00 ± 0.10	0.10 ± 0.01	0.12 ± 0.01	1.31 ± 0.05	0.00 ± 0.00
		7	1.00 ± 0.04	0.00 ± 0.00	3.37 ± 0.13	0.78 ± 0.01	0.07 ± 0.01
5	- pH 4 - 2% (v/v) - 2% (w/v)	1	3.00 ± 0.09	0.14 ± 0.01	0.00 ± 0.00	0.72 ± 0.02	0.11 ± 0.01
		4	7.00 ± 0.17	0.85 ± 0.04	4.00 ± 0.26	0.46 ± 0.45	0.01 ± 0.06
		7	5.00 ± 0.20	0.31 ± 0.01	7.09 ± 0.28	0.35 ± 0.01	1.16 ± 0.04
6	- pH 9 - 2% (v/v) - 2% (w/v)	1	1.00 ± 0.05	0.06 ± 0.01	0.00 ± 0.00	0.08 ± 0.01	0.09 ± 0.01
		4	1.00 ± 0.04	0.00 ± 0.00	0.16 ± 0.01	0.09 ± 0.01	0.09 ± 0.01
		7	1.00 ± 0.04	0.24 ± 0.01	2.09 ± 0.09	0.22 ± 0.01	0.09 ± 0.01
7	- pH 4 - 10% (v/v) - 2% (w/v)	1	2.00 ± 0.09	1.27 ± 0.05	0.28 ± 0.01	0.09 ± 0.01	0.03 ± 0.01
		4	4.00 ± 0.13	1.54 ± 0.07	7.53 ± 0.30	0.72 ± 0.02	0.17 ± 0.01
		7	5.00 ± 0.22	1.51 ± 0.07	10.73 ± 0.36	0.00 ± 0.00	0.21 ± 0.01
8	- pH 9 - 10% (v/v) - 2% (w/v)	1	1.00 ± 0.03	0.02 ± 0.01	0.23 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
		4	1.00 ± 0.05	0.04 ± 0.01	1.01 ± 0.04	0.20 ± 0.01	0.03 ± 0.01
		7	1.00 ± 0.05	0.20 ± 0.01	2.29 ± 0.09	0.03 ± 0.01	0.02 ± 0.01
9	- pH 6.5 - 6% (v/v) - 1.5% (w/v)	1	0.00 ± 0.00	0.00 ± 0.00	0.10 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
		4	0.00 ± 0.00	0.00 ± 0.00	0.10 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
		7	0.00 ± 0.00	0.17 ± 0.01	0.63 ± 0.02	0.12 ± 0.01	1.13 ± 0.05

Values are expressed as mean ± standard error of replicates (n=3)

Day 1 of *Fusarium* sp. one-step leaching showed ineffective base metal extraction, while also being the first leaching stage to extract Cu (2.43%) and Cr (1.27%) at higher percentages than Fe and Ni. Flasks 1, 3, 5 and 7 were all run at pH 4 (based on the DOE parameters), while displaying the highest extractions relative to the remaining flasks. The low extractions seen on day 1 can be attributed to the period of time between inoculation, fungal growth and production of metabolites, seen in one-step leaching (Mpinga *et al.*, 2015). Oxalic acid is only produced as the fungi grow and metabolise the nutrient media, therefore, the low extractions by day 1 are a result of smaller amounts of oxalic acid which were produced and thus were incapable of effective bioleaching. Despite the lower overall extractions, *Fusarium* sp. showed

a leaching capacity for Co, Cu and Cr at non-ideal conditions. Inoculum dosage and pulp density did not impact overall extractions on day 1.

By day 4, both Co (7%) (flask 5) and Cu (7.53%) (flask 7) extractions increased significantly (p-value: 0.00056 and 0.00031 < α = 0.50, respectively). Day 4 Cu extractions by *Fusarium* sp. one-step leaching were nearly as high as the Cu extractions (7.81%) seen from day 7 of the *A. niger* spent medium leaching (Table 14), further highlighting *Fusarium*'s increased capacity for Cu dissolution. Cobalt was extracted at a similar rate to Cu, however this extraction was not comparable to previous leaching experiments. According to a study done by Dusengemungu and coworkers (2021), species of *Fusarium* were isolated from Cu mine tailings in Xinjiang Province, China and were increasingly tolerant to higher concentrations of Cu. This could explain the increased capacity for Cu leaching by *Fusarium* sp. Metal toxicity impacting growth of *Fusarium* sp, as a result of increased non-Cu metal concentrations, could explain the low extractions of Fe, Ni and Cr (Dusengemungu *et al*., 2021). Oxalic acid production is highest at a pH range between 4 and 8, according to a study done by Andersen and coworkers (2009). Flasks 1, 3, 5 and 7 fall within this range, which explains the extractions seen from these flasks as well as the lack of extractions seen from the remaining flasks. The pH of each flask continues to be the only deciding factor with regard to base metal extractions, with pulp density and inoculum dosage having minimal effect.

By day 7, Co (5%) (flask 5) and Fe (0.71%) (flask 4) extraction decreased. This can be attributed to fungal biosorption by live *Fusarium* biomass. *Fusarium* spp. have a heavy metal removal capacity of up to 42% at pH 6, according to a study done by Ayele and coworkers (2021). Removal of Fe and Co occurred at a pH of 9 and 4, respectively, which suggests a capacity for biosorption by *Fusarium* at a wider pH range as well as a capacity for biosorption of base metals. Nickel (1.16%) and Cr (1.57%) extraction increased insignificantly , with *Fusarium* sp. continuing to show a low leaching capacity for either metal in one-step leaching. Copper extraction at pH 4 doubled by day 7 of one-step leaching, while also being extracted at a higher percentage than any previous leaching experiments. Extraction of Cu at these conditions are consistent with studies done by Anjum and coworkers (2009) and Rao and coworkers (2002), which highlights nearly 50% Cu extraction, from low-grade ore, by filamentous fungal species within a temperature range of 28 to 35^{o}C. A study by Dusengemungu and coworkers (2021) also highlights 60% Cu extraction from mine tailings at a pH range of 3.5 to 3.7. However, these

studies were conducted for extended leaching periods (≥ 20 days), well above the 7 day one-step leaching period of the current study. These results highlight a capacity for Cu leaching by *Fusarium* sp., effective at a pH of 4 and a 2% (v/v) inoculum dosage. Longer leaching periods may be required for increased extraction rates. Overall, *Fusarium* sp. one-step leaching from the flotation concentrate was ineffective for all base metals tested except Cu. Copper leaching by *Fusarium* sp. was significantly higher than Cr ($p = 0.008 < \alpha = 0.05$), Fe ($p = 0.014 < \alpha = 0.05$) and Ni ($p= 0.006 < \alpha = 0.05$), but not so for Co ($p = 0.195 > \alpha = 0.05$).

4.5.2.2 Aspergillus niger *One-Step Bioleaching*

The setup for one-step leaching with *A. niger* was identical to the setup for *Fusarium* sp. one-step leaching. Results are shown below in Table 18.

Table 18: Percentage extraction of base metals by *A. niger* under one-step leaching conditions (pH, inoculum dosage (%v/v) and pulp density (%w/v)) at Days 1, 4 and 7. Flask number (1-9) is also displayed.

No.	Conditions (pH, Inoculum dosage, Pulp density)	Day	Cobalt (%)	Chromium (%)	Copper (%)	Iron (%)	Nickel (%)
1	- pH 4 - 2% (v/v) - 1% (w/v)	1	0.00 ± 0.00	0.00 ± 0.00	0.10 ± 0.01	0.37 ± 0.04	0.23 ± 0.03
		4	0.00 ± 0.00	0.10 ± 0.01	8.87 ± 1.25	2.80 ± 0.35	0.91 ± 0.08
		7	1.00 ± 0.07	0.29 ± 0.02	12.19 ± 1.79	1.38 ± 0.12	0.53 ± 0.07
2	- pH 9 - 2% (v/v) - 1% (w/v)	1	1.00 ± 0.10	0.00 ± 0.00	0.09 ± 0.01	2.11 ± 0.16	0.09 ± 0.00
		4	1.00 ± 0.14	0.00 ± 0.00	6.19 ± 0.45	1.23 ± 0.08	0.13 ± 0.01
		7	0.00 ± 0.00	0.15 ± 0.01	13.98 ± 1.94	5.54 ± 0.78	0.79 ± 0.06
3	- pH 4 - 10% (v/v) - 1% (w/v)	1	0.00 ± 0.00	0.00 ± 0.00	0.87 ± 1.15	1.81 ± 0.17	0.88 ± 0.12
		4	1.00 ± 0.07	0.29 ± 0.02	9.89 ± 1.23	2.27 ± 0.12	2.31 ± 0.32
		7	1.00 ± 0.10	0.44 ± 0.04	9.63 ± 1.46	0.68 ± 0.05	0.41 ± 0.03
4	- pH 9 - 10% (v/v) - 1% (w/v)	1	3.00 ± 0.43	0.00 ± 0.00	0.26 ± 0.03	1.41 ± 0.20	1.32 ± 0.11
		4	6.00 ± 0.33	0.00 ± 0.00	0.12 ± 0.01	0.72 ± 0.10	0.90 ± 0.05
		7	1.00 ± 0.14	0.10 ± 0.01	0.09 ± 0.00	0.16 ± 0.01	0.79 ± 0.05
5	- pH 4 - 2% (v/v) - 2% (w/v)	1	1.00 ± 0.10	0.14 ± 0.03	4.11 ± 0.38	0.22 ± 0.01	0.37 ± 0.03
		4	5.00 ± 0.62	0.00 ± 0.00	7.09 ± 0.60	0.35 ± 0.03	1.16 ± 0.15
		7	1.00 ± 0.09	0.31 ± 0.03	3.35 ± 0.26	0.15 ± 0.01	0.23 ± 0.02
6	- pH 9 - 2% (v/v) - 2% (w/v)	1	2.00 ± 0.30	0.00 ± 0.00	0.36 ± 0.02	0.25 ± 0.03	0.32 ± 0.05
		4	1.00 ± 0.06	0.06 ± 0.01	2.55 ± 0.35	2.01 ± 0.27	2.13 ± 0.16
		7	1.00 ± 0.07	0.24 ± 0.03	2.65 ± 0.27	2.95 ± 0.26	0.93 ± 0.06
7	- pH 4 - 10% (v/v) - 2% (w/v)	1	0.00 ± 0.00	0.00 ± 0.00	2.14 ± 0.20	0.49 ± 0.05	0.85 ± 0.06
		4	1.00 ± 0.08	0.06 ± 0.01	2.50 ± 0.28	0.63 ± 0.05	1.46 ± 0.08
		7	1.00 ± 0.14	0.24 ± 0.03	2.50 ± 0.27	0.63 ± 0.08	1.46 ± 0.15
8	- pH 9 - 10% (v/v) - 2% (w/v)	1	0.00 ± 0.00	0.00 ± 0.00	2.30 ± 0.25	0.48 ± 0.05	1.11 ± 0.08
		4	0.00 ± 0.00	0.00 ± 0.00	2.29 ± 0.24	1.22 ± 0.13	0.50 ± 0.05
		7	0.00 ± 0.00	0.00 ± 0.00	2.16 ± 0.23	1.02 ± 0.06	0.00 ± 0.00
9	- pH 6.5 - 6% (v/v) - 1.5% (w/v)	1	3.00 ± 0.31	0.00 ± 0.00	3.77 ± 0.45	0.46 ± 0.07	0.50 ± 0.07
		4	0.00 ± 0.00	0.00 ± 0.00	1.42 ± 0.15	0.50 ± 0.05	0.00 ± 0.00
		7	1.00 ± 0.10	1.50 ± 0.22	2.04 ± 0.30	4.54 ± 0.58	2.16 ± 0.13

Values are expressed as mean ± standard error of replicates (n=3)

Day 1 extraction by *A. niger* was equally as ineffective as *Fusarium* sp. day 1 one-step extractions (Table 17) (p-value: 0.495 > α = 0.05). Cobalt extraction on day 1 (3%) was identical between the two isolates, however, where *Fusarium* sp. failed to extract Fe and Ni on day 1, *A. niger* failed to extract Cu and Cr. The Fe (0.49%), Ni (1.32%) and Co (3%) extractions are consistent with the affinity for Fe, Ni and Co leaching seen by *A. niger* in spent medium leaching (Section 4.5.1). These results are also consistent with work done by Alibhai and coworkers (1993), which states that *Aspergillus* spp. can leach Fe, Co and Ni while in the presence of a sucrose carbon source. Despite the low initial extractions, *A. niger* seems to

follow the same leaching pattern as seen in spent medium leaching. Contrary to *Fusarium* sp., higher extractions are seen at the high pH (pH 9) of flasks 4, 6, 8 and 9.

Day 4 extractions by *A. niger* saw similar increases in Cu (9.87%) and Co (6%) extractions between days 1 and 4, when compared to *Fusarium* sp. Minor increases in Fe (2.80%), Ni (2.13%) and Cr (0.29%) extraction also follow a similar pattern. Whereas Cu extraction was exclusively seen at a pH of 4 for *Fusarium* sp., *A. niger* extractions are more consistent at a wider range of conditions (pH 4, 9; inoculum dosage 2, 10% (v/v)). Copper, Fe and Ni extractions were observed in every flask during *A. niger* one-step leaching. The highest extractions, however, are still seen at a pH of 4. The Cu extractions observed in Table 18 are contrary to the results of *A. niger* spent medium leaching (Table 14), which showed a lower leaching capacity for Cu than other base metals. Dusengemungu and coworkers (2021) state that the recovery of Cu by *A. niger* is increased in the presence of physical substrates/biomass, which could explain the lower extraction of Cu by *A. niger* during the cell-free spent medium leaching. In the case of one-step leaching, these results concur with Mulligan and coworkers (2004), whose research highlighted an increased capacity for Cu leaching from a low-grade ore by *A. niger.*

Day 7 extractions showed a borderline significant increase in Cu (13.98%) (flask 2) extraction (p-value: $0.0504 \geq \alpha = 0.5$), with insignificant increases in Fe (4.54%) ($p = 0.099$), Ni (3.25%) ($p = 0.976$) and Cr (1.50%) ($p = 0.953$). Cobalt extraction decreased from day 4 to day 7 at double the rate observed during *Fusarium* sp. one-step leaching. This is consistent with a study done by Dusengemungu and coworkers (2020), which states that the capacity for Co biosorption is higher in *Aspergillus* than *Fusarium* spp. This study also states that the biosorption capacity is higher when using *A. niger* live biomass (Dusengemungu *et al*., 2020). The increased biomass produced by *A. niger* as a result of the sucrose-heavy CYE medium resulted in decreased extractions of Co by day 7 of the leaching period. The overall low extractions by *A. niger* during one-step leaching could also be a result of increased biomass production, which leads to lower oxalic acid production, according to Strasser and coworkers (1994). Attempts to supplement the growth and organic acid production of *A. niger* with a sucrose-heavy carbon source, may in-turn be hindering organic acid production in favour of biomass production, consequently leading to the increased biosorption of leached metals by day 7. Higher Cu extraction above the other base metals was not exclusive to either *A. niger* or *Fusarium* sp. and may highlight

a capacity for Cu extraction by one-step leaching in general from the flotation concentrate. Although minimal in certain cases, *A. niger* continues to show a leaching affinity for Fe and Ni over other base metals, across both one-step and spent medium leaching.

4.5.2.3 One-Step Abiotic Control Leaching

Leaching was conducted under abiotic conditions to assess the impact that leaching parameters (pH, pulp density (%w/v)) have on one-step base metal extractions from the flotation concentrate. The abiotic control flasks were run under identical conditions to the one-step DOE, without the inclusion of an *A. niger* or *Fusarium* sp. inoculum.

Table 19: Percentage extraction of base metals under abiotic one-step leaching conditions (pH and pulp density (%w/v)) at Days 1, 4 and 7. Flask number (1-9) is also displayed.

No.	Conditions (pH, Pulp density)	Day	Cobalt (%)	Chromium (%)	Copper (%)	Iron (%)	Nickel (%)
1	- pH 4 - 1% (w/v)	1	0.00 ± 0.05	0.00 ± 0.02	0.10 ± 0.05	0.37 ± 0.03	0.23 ± 0.02
		4	0.00 ± 0.06	0.15 ± 0.03	4.46 ± 0.10	1.48 ± 0.05	0.82 ± 0.04
		7	1.00 ± 0.07	0.44 ± 0.04	12.19 ± 0.15	2.21 ± 0.07	1.52 ± 0.05
2	- pH 9 - 1% (w/v)	1	1.00 ± 0.05	0.00 ± 0.02	0.00 ± 0.05	0.00 ± 0.03	0.53 ± 0.03
		4	2.00 ± 0.06	0.97 ± 0.04	5.15 ± 0.11	2.11 ± 0.06	2.11 ± 0.06
		7	2.00 ± 0.08	0.53 ± 0.05	1.68 ± 0.08	0.79 ± 0.05	2.08 ± 0.07
3	- pH 4 - 1% (w/v)	1	0.00 ± 0.04	0.00 ± 0.02	0.10 ± 0.05	0.09 ± 0.03	0.09 ± 0.02
		4	1.00 ± 0.05	0.10 ± 0.03	4.74 ± 0.10	1.81 ± 0.06	0.88 ± 0.05
		7	2.00 ± 0.07	0.24 ± 0.04	1.73 ± 0.09	2.78 ± 0.07	2.54 ± 0.07
4	- pH 9 - 1% (w/v)	1	1.00 ± 0.05	0.00 ± 0.02	0.26 ± 0.05	0.41 ± 0.04	1.32 ± 0.04
		4	2.00 ± 0.06	0.00 ± 0.03	4.39 ± 0.11	2.88 ± 0.07	2.54 ± 0.07
		7	2.00 ± 0.08	0.87 ± 0.05	5.61 ± 0.12	3.95 ± 0.08	1.58 ± 0.06
5	- pH 4 - 2% (w/v)	1	1.00 ± 0.05	0.00 ± 0.02	0.00 ± 0.05	0.00 ± 0.03	0.00 ± 0.02
		4	3.00 ± 0.07	0.14 ± 0.03	0.79 ± 0.09	0.95 ± 0.06	0.79 ± 0.05
		7	3.00 ± 0.09	1.50 ± 0.06	0.31 ± 0.08	5.25 ± 0.09	3.95 ± 0.08
6	- pH 9 - 2% (w/v)	1	1.00 ± 0.04	0.00 ± 0.02	0.15 ± 0.05	0.09 ± 0.03	0.09 ± 0.02
		4	2.00 ± 0.06	0.15 ± 0.03	5.71 ± 0.11	0.89 ± 0.06	1.58 ± 0.06
		7	3.00 ± 0.08	0.33 ± 0.04	4.85 ± 0.12	3.30 ± 0.08	2.69 ± 0.07
7	- pH 4 - 2% (w/v)	1	0.00 ± 0.05	0.00 ± 0.02	0.00 ± 0.05	0.33 ± 0.04	0.39 ± 0.03
		4	1.00 ± 0.06	0.00 ± 0.03	3.47 ± 0.10	2.22 ± 0.07	1.59 ± 0.06
		7	1.00 ± 0.07	0.58 ± 0.05	6.68 ± 0.13	1.83 ± 0.08	1.81 ± 0.07
8	- pH 9 - 2% (w/v)	1	0.00 ± 0.05	0.00 ± 0.02	0.05 ± 0.05	0.16 ± 0.03	0.53 ± 0.03
		4	1.00 ± 0.06	0.05 ± 0.03	3.72 ± 0.10	5.56 ± 0.08	0.53 ± 0.06
		7	2.00 ± 0.08	0.73 ± 0.05	2.86 ± 0.12	2.30 ± 0.08	1.99 ± 0.07
9	- pH 6.5 - 1.5% (w/v)	1	1.00 ± 0.05	0.00 ± 0.02	0.10 ± 0.05	0.37 ± 0.03	0.23 ± 0.02
		4	1.00 ± 0.06	0.10 ± 0.03	0.50 ± 0.09	1.46 ± 0.06	1.52 ± 0.06
		7	1.00 ± 0.07	0.73 ± 0.05	2.86 ± 0.11	3.10 ± 0.08	1.99 ± 0.07

Values are expressed as mean ± standard error of replicates (n=3)

Lower extractions were seen from abiotic control leaching on day 1, compared to *A. niger* and *Fusarium* one-step leaching experiments (Tables 17 and 18). The minimal extractions seen could be a result of the sulphuric acid used to adjust the pH of each flask, which according to a study done by Hosseini and coworkers (2017), promotes base metal extraction from a low-grade concentrate at increasing concentrations. Similarly, to spent medium abiotic leaching tests (Table 15), the absence of fungal metabolites/inoculum may lead to unhindered chemical leaching.

By day 4, Cu extraction increased significantly (p-value: $0.00056 < \alpha = 0.05$) overall, while increasing at a rate roughly consistent with, but less than, *A. niger* and *Fusarium* sp. one-step Cu leaching. One-step leaching of Cu, by day 4, seemed to occur independently of less effective conditions from previous leaching experiments, such as high pH, low temperature and lower pulp density. The percentage of Fe extracted in flask 8 (5.56%) is higher at this stage than all previous one-step leaching experiments, with its two highest day 4The extractions occurring at pH 9 (flasks 2 and 8). Chromium (0.97%), Co (2%) and Ni (1.58%) extractions did not increase substantially, with the leaching potential possibly limited by unfavourable leaching conditions (high pH and low temperature). Despite not extracting as effectively as the *A. niger* and *Fusarium* one-step leaching stages , the measurable and roughly consistent extractions seen from control leaching suggests that base metal extraction did occur in the absence of fungal inoculum. This suggests that one-step base metal extraction may be more efficient through a combination of inoculum-based and abiotic chemical leaching, at more ideal conditions.

By day 7, despite an increase in the maximum Cu extracted (6.68%) since day 4, overall Cu extraction decreased across each flask except flask 7. There was an increase in extraction of all base metals except Fe (5.25%), which only saw a minimal decrease. The highest extractions of Fe (5.25%), Ni (3.95%), Cr (1.50%) and Co (3%) are seen in flask 5 exclusively, which was run at pH 4 and a pulp density of 2% (w/v). This is consistent with earlier leaching experiments, which showed increased extractions at a 2% (w/v) pulp density and lower pH. Increased base metal extractions seen at these conditions agree with a study done by Dusengemungu and coworkers (2021). The overall extractions seen from abiotic one-step leaching suggest that base metal extraction by *A. niger* and *Fusarium* does not rely solely on the presence of fungal

inoculum and fungi-produced organic acid. Based on the results of one-step leaching and abiotic control leaching, Cu was the only base metal to be leached more effectively when in direct contact with an *A. niger* or *Fusarium* inoculum. The affinity for Cu leaching during one-step leaching conditions was noted. There was no significant difference between abiotic control, *A. niger* and *Fusarium* sp. one-step leaching (p-value: 0.598 > α = 0.05).

4.5.2.4 Ambient pH Control One-Step Bioleaching

Leaching was conducted at the unaltered pH of each flask when made up with *Fusarium* sp. and *A. niger* inoculum and liquid CYE media. The results of the one-step ambient pH control tests are shown in Table 20 as average base metal extraction over days 1, 4 and 7. The ambient pH of the flasks for *Fusarium* sp. and *A. niger were* pH ± 6.50 and pH ± 4.20, respectively.

Table 20: Percentage extraction of base metals by *A. niger* and *Fusarium* sp. during ambient control leaching under spent medium conditions (temperature (°C) and pulp density (%w/v)) at Days 1, 4 and 7.

Isolate	Day	Cobalt (%)	Chromium (%)	Copper (%)	Iron (%)	Nickel (%)
Fusarium sp.	1	0.03 ± 0.01	0.00 ± 0.00	1.38 ± 0.09	0.42 ± 0.03	0.32 ± 0.03
	4	0.23 ± 0.01	0.08 ± 0.01	2.13 ± 0.23	1.23 ± 0.13	0.86 ± 0.05
	7	0.25 ± 0.02	0.12 ± 0.01	1.65 ± 0.11	0.92 ± 0.08	0.84 ± 0.04
A. niger	1	1.56 ± 0.10	0.09 ± 0.01	2.69 ± 0.27	0.63 ± 0.04	0.57 ± 0.05
	4	1.22 ± 0.08	0.20 ± 0.02	2.57 ± 0.24	1.67 ± 0.10	1.36 ± 0.14
	7	0.89 ± 0.10	0.31 ± 0.03	1.83 ± 0.21	0.98 ± 0.08	1.32 ± 0.15

Values are expressed as mean ± standard error of replicates (n=3)

One-step base metal extractions were minimal with no significant increase by days 4 and 7 at the ambient pH of both *Fusarium* sp. (p-value: 0.223 > α = 0.05) and *A. niger* (p-value: 0.117 > α = 0.05). The trend of higher Cu extraction by *Fusarium* sp. over other base metals continued in the current experiment. Higher extractions on day 4 are consistent with the results of the one-step abiotic control tests (Table 19). Given that the pH of each flask was within the ideal pH range for biomass production, as mentioned previously, biosorption of the metals was triggered by live biomass leading to the decreased extractions seen on day 7 (Dusengemungu *et al*., 2020). The gradual decrease in Co extractions, from day 1 (1.56%) to 7 (0.89%), were consistent with the decrease in Co extractions from day 1 (3%) to 7 (2%)

observed during *A. niger* one-step leaching in section 4.5.2.2. Iron, Cu and Ni followed a similar declining trend, which was consistent with the extractions observed in one-step abiotic control leaching (section 4.5.2.3). Furthermore, the overall extractions for each base metal are lower than the extractions observed during abiotic control leaching. Given that the pH of each flask (± 4.20) is within the optimal range for fungal growth (Meletiadis *et al.,* 2001) , it is likely that leaching was limited by biomass production as observed during one-step ambient control leaching (section 4.5.2.4). The overall extractions at ambient pH highlight pH as the most significant factor for base metal extractions from the flotation concentrate, with ambient pH extractions being significantly lower than previous one-step leaching experiments ($p < 0.001$).

Chapter 5: Conclusions and Recommendations

5.1 Summary

Sixty six fungal species were isolated from a platinum mine in Limpopo, South Africa. Qualitative assessment of organic acid production showed that 14 of these isolates produced organic acids with an acid unitage (AU) value of 1 or greater, which indicated significant ($p < 0.05$) organic acid production relative to the growth of the isolate. Based on the aforementioned experiments, these 14 isolates were selected for the current study.

All 14 isolates were screened for spore production on potato dextrose (PD), malt extract (ME) and Czapek yeast extract (CYE) nutrient media to further determine which isolates, and which nutrient media, were best suited for bioleaching. The four species (*Aspergillus terreus, Aspergillus niger, Mucor fragilis, Fusarium* sp.*)* with the highest spore counts were Ascomycetes, with the first three (*A. terreus, A. niger* and *M. fragilis*) being the only isolates to have spore counts higher than 6.0×10^7 spores/mL. Spore production was also highest in CYE for every isolate except *Phoma* sp. This corresponded to previous studies (Zain *et al*., 2009; Aryal, 2022) which highlighted the increase in spore production induced by a sucrose-supplemented CYE nutrient media. It was concluded that the spore counts of *A. niger, A. terreus, M. fragilis* and *Fusarium* sp. made them potential candidates for bioleaching, given that increased spore production was linked to increased biomass production, which would allow for efficient mass-production of fungal inoculum for bioleaching purposes. It was also concluded that a sucrose medium such as CYE is more effective at promoting spore production than the maltose and dextrose of ME and PD agar, respectively.

Further screening was conducted on the growth rate of each isolate in a liquid PD, ME and CYE culture environment. The measurement of fungal growth by absorbance was performed using OD450 to allow for rapid assessment of growth without needing to perform analysis of biomass dry weight. Growth curves were produced based on absorbance and isolates were screened for 'ideal' growth curves, based on a distinct log phase within the first two days, a steadily increasing stationary phase, lack of a death phase and high absorbance by day 7. In PD, only *Fusarium* sp. produced an acceptable growth curve, given the rapid change in absorbance from day 0 and the constantly increasing absorbance (0 – 2.3 A) up to day 7.

Phoma sp. was the only other isolate to have a measurable change in absorbance (0 – 0.8 A), however the growth curve was inadequate given the lack of a distinct log phase followed by a sudden death phase. It was concluded that PD was not a suitable substrate for bioleaching as it was unable to stimulate an increased growth response from nearly all the isolates.

In ME, *Fusarium* sp. remained the only isolate to produce an acceptable growth curve, as a higher absorbance level was reached during the log phase on day 2. *Fusarium* sp. reached nearly the same absorbance level (2.2 A) as it did on PD (2.3 A), however it was concluded that the growth of *Fusarium* was improved in ME, given the stronger log phase (2.0 A by day 4 in ME versus 1.8 A by day 4 on PD). *Rhizopus microsporus* and *A. niger* also produced measurable growth curves, however neither were adequate for bioleaching given the low overall absorbance and lack of distinct log phase. It was concluded that ME was not a suitable substrate for bioleaching despite the slight increase in growth response from other isolates.

In CYE, a distinct change in absorbance was observed from multiple isolates. *Fusarium* sp. maintained its consistent growth pattern across all three substrates, showing the strongest log phase in CYE compared to PD and ME, but also showing the lowest absorbance in CYE of all three substrates by day 7. *Fomitopsis* was the only other isolate to produce an ideal growth curve, seen by the distinct log phase and high final absorbance. The log phase of *A. niger* was not ideal, given the lag in exponential growth between day 2 and 3 (0.6 - 0.7 A). However, the isolate was not disregarded from consideration for further experiments given the sudden increase in absorbance between days 3 and 4, along with achieving the highest overall absorbance between isolates grown in CYE. The only remaining isolate to be considered was *Aspergillus* sp*,* despite its decrease in absorbance (1.2 - 0.7 A) from day 3 to 7, given that it produced a distinct log phase. Previous research also highlights the bioleaching capacity of *Aspergillus* species (Dusengemungu *et al*., 2021). It was concluded that *Fusarium* sp*., Fomitopsis meliae*, *A. niger* and *A. terreus* were promising candidates for bioleaching and that CYE was once again best suited for bioleaching.

The final screening stage involved quantifying organic acid production by each isolate. Of the known fungi-produced organic acids which were screened for, only quantifiable peaks of oxalic acid were produced, and isolates were selected for bioleaching based on oxalic acid production of 15 mM concentrations or higher by day 7. *Curvularia petersonii, Fusarium* sp*., M. fragilis, F. meliae, A. niger, Physisporinus* sp. and *R. microsporus* all achieved 15 mM or

higher. The concentration of oxalic acid produced by these isolates was consistent with oxalic acid production seen in previous studies (Din *et al*., 2020; Graz, 2024; Palmieri *et al*., 2018) . *Mucor fragilis* was disregarded for further experiments given the drastic decrease in its oxalic acid production from day 6 (17.887 mM) to 7 (6.899 mM). It was concluded that *C. petersonii, Fusarium* sp.*, F. meliae, A. niger, Physisporinus* sp. and *R. microsporus* could be potential candidates for bioleaching given their produced oxalic acid concentrations. The highest oxalic acid concentrations (20+ mM) were produced by isolates grown in CYE, while eight of the 11 oxalic acid-producing isolates were grown in CYE. Therefore, it was concluded that CYE was the best substrate for bioleaching.

It was concluded that, from the 14 fungal isolates, *Fusarium* sp. and *A. niger* would be the most effective for bioleaching given that they consistently produced positive results throughout each screening experiment. The high spore production by both isolates allowed for increased biomass production, leading to easy production of large amounts of fungal inoculum within 7 days and rapid growth during one-step leaching. Their growth curves suggest that they would be more effective at producing organic acids within the 7 day leaching period, while quantitative analysis showed that they are capable of producing sufficient concentrations of oxalic acid for effective bioleaching.

Spent medium leaching by *Fusarium* sp. was found to be most effective at pH 1, a temperature of 70^{o}C and a pulp density of 2% (w/v). Iron (Fe), nickel (Ni) and cobalt (Co) were extracted more at these conditions, with Fe extraction the highest from day 1 to 7. Fe was more readily extracted by *Fusarium* sp. than any other base metal during spent medium leaching. A pulp density of 2% (w/v) resulted in higher extractions during days 1, 4 and 7. Spent medium leaching by *A. niger* produced higher initial extractions of each metal other than chromium (Cr) and copper (Cu), when compared to *Fusarium* sp. By day 4, Fe, Co and Cr extractions were higher than their extractions by *Fusarium* sp. on day 7. Fe, Ni and Co extractions (24.58; 24.36; 23%, respectively) were at their highest by day 7, despite not extracting at the proposed leaching capacity of *A. niger* (35%; 34%; 80%, respectively) based on previous studies (Biswas *et al*., 2013; Dusengemungu *et al*., 2021; Mulligan *et al*., 2004). The highest extractions overall were seen at a pulp density of 2% (w/v). Abiotic spent medium extractions on day 1 were consistent with *A. niger* day 1 extractions, however, these only occurred at a pH of 1, with extractions nearly non-existent at a higher pH. Co, Cu and Ni extractions were highest overall

by abiotic leaching, while Fe and Cr weren't as high compared to *Fusarium* sp. and *A. niger.* It was concluded that while the impact of chemical leaching is considerable at low pH during spent medium leaching, the abiotic control leaching shows *A. niger*- and *Fusarium* sp.-produced oxalic acid has a leaching capacity for base metals at a wide variety of temperatures and pH levels. From the ambient pH control tests, it was concluded that leaching is more effective during spent medium leaching in the presence of inorganic acid, and future studies could investigate base metal extraction from a PGM concentrate through a combination of spent medium and chemical leaching.

For one-step leaching, lower extractions were produced by *A. niger* and *Fusarium* sp.*,* compared to spent medium leaching, with nearly non-existent extractions of each base metal except Cu. The 15.81% Cu extraction by *Fusarium* on day 7 was the highest of any leaching stage, including leaching by *A. niger*. This highlighted the specific capacity for Cu leaching by *Fusarium* sp., as well as the affinity for Cu to extract during one-step leaching conditions compared to spent medium. Increased extractions by *Fusarium* sp. were only seen at the low pH (pH 4), with extractions being slightly higher from a combination of low pH and lower pulp density (1% w/v). *Fusarium* sp*.* produced nearly no extractions of the other base metals, with *A. niger* producing slightly higher extractions of Fe, Ni and Co. The Cu extractions by *A. niger* one-step leaching on day 7 were also higher than the extraction it achieved during spent medium leaching, once again showing that one-step leaching conditions promote better Cu extraction. Co and Fe extraction by *Fusarium* sp. decreased between days 4 and 7, which was attributed to biomass accumulation of the metals, which was verified by a previous study (Dusengemungu *et al*., 2020). During *A. niger* one-step leaching, only Co extraction decreased by day 7. The extractions seen from the abiotic control leaching were roughly consistent with *A. niger* one-step extractions except for Cu extraction. The ambient pH control tests also produced higher Cu extractions than any other base metals. It was concluded that extractions of Fe, Co, Cr and Ni from a PGM flotation concentrate by *A. niger* and *Fusarium* sp. were ineffective during one-step leaching, however, Cu was more effectively extracted from the PGM concentrate during one-step leaching by both *A. niger* and *Fusarium* sp. when compared to spent medium leaching.

5.2 Conclusion

Across screening and leaching experiments, CYE emerged as the most suitable medium. It consistently yielded the highest spore counts (supporting scale-up of inoculum production), the most effective growth curves for several isolates (supporting rapid production of culture material) and yielded ≥20 mM oxalic acid for most isolates (directly relating to leaching capacity). Among the 14 fungi, *Fusarium* sp. and *A. niger* are the best overall bioleaching candidates, by combining increased sporulation, robust liquid-culture growth curves with early, sustained biomass accumulation and reproducible oxalic-acid production at effective levels for bioleaching. With regard to base metal extractions, spent-medium leaching was most effective for the suite of base metals (especially Fe, Ni, Co)—with optimal conditions at pH 1, 70 °C, and 2% (w/v) pulp density—while one-step leaching under-performed for most metals except Cu. *Fusarium* sp. is more stable and consistent bioleaching agent, specifically with regard to Cu. *A. niger* yields higher extractions, specifically for Fe, Ni and Co, but is more variable at different conditions.

5.3 Recommendations

Growth characterisation and organic acid production

The spore count, growth rate and organic acid production of mine-isolated filamentous fungal species can be increased significantly by a supplemental carbon source such as sucrose, therefore, the wider applications of this group of isolates based on their increased growth and production of metabolites should be investigated further. Isolates should be cultivated in CYE to maximise spore and acid production.

Bioleaching

The base metal extraction capability of *Fusarium* sp. should be investigated further, given that it achieved nearly the same extractions when compared to commercially relevant *A. niger*. Spent medium leaching from the PGM concentrate can achieve base metal extraction, however, longer leaching periods and more extreme conditions should be investigated to determine if higher extractions can be achieved. Spent medium leaching is a more viable option for fungal bioleaching given the higher extractions achieved in this study, as well as the potential for high extractions given the extreme conditions allowed for in the absence of fungi in the leachant. The increased extraction of Cu during one-step leaching from the PGM concentrate should also be investigated. Spent-medium leaching should be deployed at pH 1, 70^{o}C and 2% (w/v) pulp density for broad metal recovery, with one-step leaching being utilised for targeted extraction of metals such as Cu.

5.4 Limitations

This study was conducted under controlled laboratory conditions. Therefore the bioleaching efficiencies observed may not inherently translate to industrial-scale systems, where parameters such as mineral heterogeneity and microbial competition may influence performance. Fungal growth was primarily assessed using absorbance (A) in place of biomass accumulation, which, while suitable for rapid screening and necessary in the current study, may not accurately represent true biomass accumulation and morphology in filamentous fungi. Organic acid quantification was limited to detectable oxalic acid. Metal extraction as a result of other organic acids or metabolites was not assessed. Additionally, all bioleaching experiments were conducted over relatively short timeframes from a singular source, which may not capture long-term leaching dynamics or variability in PGM concentrate composition. These limitations highlight the need for extended-duration studies, multi-acid analyses and scale-up of experimental parameters under more complex leaching conditions.

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Appendices

Appendix A

Nutrient Media Recipes

1. Czapek yeast extract:

30.00 g/L sucrose, 15.00 g/L agar, 5.00 g/L yeast extract, 1.00 g/L K_2HPO_4, 0,30 g/L $NaNO_3$, 0,05 KCl, 0,05 $MgSO_4$, 0,001 $FeSO_4$, 0,001 $ZnSO_4$, 0,0005 $CuSO_4$.

All reagents were mixed. For agar plates, pH was adjusted to 6.5 with H_2SO_4. Liquid CYE media was autoclaved before use and stored at 4°C.

2. Malt extract

15.00 g/L agar, 12.75 g/L maltose, 2,75 g/L dextrin, 2.35 g/L glycerol, 0.78 g/L peptone,

All reagents were mixed. Agar was excluded for liquid ME. Media was autoclaved before use and stored at 4°C.

3. Potato dextrose

20.00 g/L dextrose, 15.00 g/L agar, 4.00 potato infusion solids.

All reagents were mixed. Agar was excluded for liquid PD. Media was autoclaved before use and stored at 4°C.

Appendix B

Calculations

1. Spore count calculations

- Spores/mL = (n) x 10^4, where n = average count of cells per square of each quadrant counted.
- (A + B + C + D) = n

2. Base metal extraction percentage calculations

The results of ICP-OES were received in milligrams per litre (mg/L). The percentage extraction (%) was calculated by the following equation:

- Dissolved elements in liquid sample - DE (mg/L)
- Dilution Factor – DF
- Amount of element in grams – AE (g)
- Metal content of UG2 flotation concentrate – MC (g)

- $(DE \times DF) \div 1000 \times 0.1 = AE$

- $AE \div MC \times 100 = \%$

Appendix C

HPLC Method Parameters

Table 21: Outline of high performance liquid chromatography (HPLC) method parameters

Solvent Program		Time (min)	Flow rate (mL/min)	%A	%B	Curve
	1	Initial	1.5	80.0	20.0	-
	2	10.0	1.5	40.0	60.0	6
	3	12.5	1.5	40.0	60.0	6
	4	12.6	1.5	80.0	20.0	11
Analysis Time	12,5 min. 5-min injection delay time					
Pressure	2650 psi/177 bar					
Oven Temperature	30 °C					
Detection	210 nm					
Injection Volume	20 µl					
Sampling (Data) Rate	5 pts./sec					

Appendix D

Fungal Screening Method

Methodology by (Haripersad, 2022) for the isolation and screening of pure fungal cultures:

Qualitative screening

Sixty-six fungal isolates from a platinum mine were identified. Qualitative OA screening 45 was conducted. The isolates were inoculated in 39 g/L of PDA (pH 5.6 ± 0.2), which contained 0.2 g/L of the pH indicator bromocresol green. The plates were incubated at 25 ^{o}C for 7 days. Bromocresol green allows for a colour change from blue to yellow when the pH changes from 5.4 to 4.0. Acid unitage (AU) values for each colony were calculated by dividing the diameter of the yellow zone formed, by the diameter of the colony (Shaikh and Qureshi, 2013).

Quantitative screening

The thirteen isolates with an AU value of ≥ 1 were characterised to identify and quantify the OAs. The isolates were inoculated in 20 ml of PDB (24 g/L) at a pH of 4.8 and were incubated at 25 ^{o}C for 7 days on an orbital shaker set at 150 rpm. The culture was filtered through Whatman no.1 filter paper and 1 ml of the filtrate was centrifuged at 1 677 x g for 10 min (Shankar and Sivakumar, 2016). The supernatant was then filtered through a 0.45 µm syringe filter for high-performance liquid chromatography (HPLC) analysis (Din *et al.*, 2020). OAs were analysed using an HPLC (Agilent-1200 series) equipped with a BioRad fermentation column and refractive index detector. Separation occurred at a column temperature of 65 ^{o}C and the eluent (20% H2SO4) was delivered at a rate of 1 ml/min for 13 min. Standards of lactic acid, citric acid, acetic acid, butyric acid, formic acid, propionic acid, gluconic acid and indole-3-acetic acid (IAA) were prepared at concentrations ranging from 1 mM to 5 mM.

Heavy metal tolerance test

The morphological changes and tolerance index (TI) of isolates no. 14, 57, and 58 (Table 3.2), exposed to the heavy metals Cu, Cr, and Ni was determined. These isolates were selected due to their OA production, and these base metals were selected as they commonly occur together. Copper and Ni were used as chloride salts, CuCl2.2H2O and NiCl2.6H2O,

respectively, and Cr was used as K2Cr2O7 (Ghosh and Paul, 2015). A 10 000 mg/L stock solution of these 46 metals were prepared using sterile distilled water and the solution was filter sterilised (0.45 µm) (Din *et al*., 2020). Sterilised PDA was then supplemented individually with 200 mg/L of each metal salt and 1% chloramphenicol. A 6-day old culture of each isolate was used to extract 8 mm mycelial disks and inoculate it at the centre of the supplemented PDA plates and control plates (Oladipo *et al*., 2018). The plates were incubated at 25oC for 7 days. The TI of the isolates to each metal was determined according to the methodology followed by Oladipo *et al*. (2018).

Appendix E

Table 22: **Phyla Table - All sequenced microbial species isolated from Anglo-Mogalakwena platinum mine.** Species name, genus and family are not available for all isolates.

Phylum	Current name	Family	Mine sample no.	GenBank accession no.
Ascomycota	*Bartilinia sp.,*	*Bartaliniaceae*	33	MT477058.1,
	Bartilinia pondoensis			KJ767127.1
	Bartilinia pondoensis	*Bartaliniaceae*	34	JQ425386.1
	Clonostachys epichloe	*Bionectriaceae*	23	KJ780769.1
	Neoscytalidium dimidiatum	*Botryosphaeriaceae*	33	MF580799.1
	Tiarosporella graminis	*Botryosphaeriaceae*	23	KF531828.1
	Chaetomella raphigera	*Chaetomellaceae*	33	KF193633.1
	Chaetomium sp*.,*	*Chaetomiaceae*	24	KX618205.1,
	Chaetomium strumarium			KY558668.1
	Pseudothielavia terricola	*Chaetomiaceae*	27	NG_069813.1,
	Chrysocorona lucknowensis,			MH877835.1,
	Acrophialophora jodhpurensis			MH873474.1
	Pseudothielavia terricola	*Chaetomiaceae*	33	NG_069813.1,
	Chrysocorona lucknowensis,			MH877835.1,
	Acrophialophora jodhpurensis			MH873474.1
	Cladosporium anthropophilum,	*Davidiellaceae*	34	MN511353.1,
	Cladosporium cladosporioides			MK813966.1
	Cladosporium sp*.,*	*Davidiellaceae*	39	MK355726.1,

Cladosporium cladosporioides,			MT466517.1,
Cladosporium westerdijkieae			MF473314.1,
Cladosporium sp*.,*	*Davidiellaceae*	29	MK355726.1,
Cladosporium cladosporioides,			MT466517.1,
Cladosporium westerdijkieae			MF473314.1,
Cladosporium sp*.,*	*Davidiellaceae*	31	MK355726.1,
Cladosporium cladosporioides,			MT466517.1,
Cladosporium westerdijkieae			MF473314.1,
Cladosporium tenuissimum	*Davidiellaceae*	28	MF473304.1
Pseudocoleophoma polygonicola	*Dictysporiceae*	35	MZ492974.1
Didymella sp*.,*	*Didymellaceae*	28	MG967669.1,
Phoma sp*.,*			KY790596.1,
Didymella americana			MK646045.1
Ectophoma multirostrata	*Didymellaceae*	34	MG897497.1
Epicoccum sorghinum	*Didymellaceae*	25	MN215621.1
Phoma sp*.*	*Didymellaceae*	27	JN207257.1
Phoma herbarum,	*Didymellaceae*	27	MT420921.1,
Epicoccum sorghinum			MN944541.1
Phoma herbarum,	*Didymellaceae*	29	MT420621.1,
Epicoccum sorghinum	*Didymellaceae*		MN944541.1
Phoma sp*.,*	*Didymellaceae*	30	MK066907.1,
Boeremia sp*.,*	*Didymellaceae*		MH931265.1,
Aspergillus niger	*Trichocomaceae*		KT963790.1

Phoma sp.,	*Didymellaceae*	35	JN207257.1
Didymella pinodella			MW784722.1
Phoma sp.,	*Didymellaceae*	19	JN207257.1
Didymella sp.,			MH257388.1
Didymella pinodella			MW784722.1
Phoma sp.,	*Didymellaceae*	28	JN207257.1
Epicoccum phragmospora			NR_165920.1
Stagonosporopsis cucurbitacearum	*Didymellaceae*	10	GU045304.1
Trichoderma longibrachiatum	*Hypocreaceae*	26	KT852813.1
Trichoderma sp.,	*Hypocreaceae*	22	MK870709.1,
Trichoderma erinaceum,			KC884817.1,
Trichoderma koningiopsis			FR670342.1
Pyrenochaeta sp.	*Incertae sedis*	34	EU750693.1
Pyrenochaeta sp.	*Incertae sedis*	36	EU750693.1
Fusarium chlamydosporum	*Nectriaceae*	36	MG250446.1
Fusarium chlamydosporum	*Nectriaceae*	23	KX421422.1
Fusarium chlamydosporum	*Nectriaceae*	22	MG250446.1
Fusarium equiseti	*Nectriaceae*	23	MT626672.1
Fusarium equiseti	*Nectriaceae*	23	MK780235.1
Fusarium oxysporum	*Nectriaceae*	39	MK249867.1
Fusarium sp.,	*Nectriaceae*	39	MH582472.1,
Fusarium chlamydosporum			KX421422.1
Fusarium sp.,	*Nectriaceae*	39	MK640565.1,

Fusarium solani.,			KX583231.1,
Fusarium phaseoli			KF717534.1
Alternaria alternata	*Pleosporaceae*	29	MK773579.1
Alternaria sp.	*Pleosporaceae*	36	MW784825.1
Alternaria sp.*,*	*Pleosporaceae*	19	MK640569.1,
Alternaria alternata			MK311341.1
Bipolaris zeae	*Pleosporaceae*	23	MT505870.1
Bipolaris sp.*,*	*Pleosporaceae*	21	MF590167.1,
Helminthosporium bondarzewii	*Massarinaceae Pleosporaceae*		MH877988.1,
Curvularia spicifera			MH875110.1
Curvularia beasleyi,	*Pleosporaceae*	28	MH414893.1,
Curvularia hawaiiensis			KC999927.1
Curvularia petersonii	*Pleosporaceae*	33	NR_158448.1
Exserohilum rostratum,	*Pleosporaceae*	22	MT524320.1
Setosphaeria rostrata,			LT837845.1,
Bipolaris maydis			MH197141.1
Exserohilum rostratum,	*Pleosporaceae*	20	MT524320.1,
Setosphaeria rostrata,			LT837845.1,
Bipolaris maydis			MH197141.1
Periconia sp.*,*	*Pleosporaceae*	10	HQ607981.1,
Periconia macrospinosa			MT658103.1
Neopyrenochaeta telephoni	*Pleosporaceae*	35	KM516291.1
Westerdykella ornata	*Sporormiaceae*	24	NR_103587.1
Westerdykella sp.*,*	*Sporormiaceae*	26	MG250473.1,
Westerdykella centenaria			NR_156002.1

	Albifimbria viridis	*Stachybotrycaea*	35	MN625931.1
	Stachybotrys microspora	*Stachybotrycaea*	32	MK956918.1
	Aspergillus flavus	*Trichomaceae*	29	MT645322.1
	Aspergillus sp.,	*Trichomaceae*	33	MT447519.1,
	Aspergillus costaricensis,			MT558927.1,
	Aspergillus tubingensis			MN067891.1
	Aspergillus terreus	*Trichomaceae*	36	MT316343.1
	Aspergillus terreus	*Trichomaceae*	28	MT316343.1
	Aspergillus welwitschiae,	*Trichomaceae*	22	MK450669.1,
	Aspergillus niger			MT620753.1
	Penicillium sp.,	*Trichomaceae*	39	MT424930.1,
	Penicillium magnielliptisporum			MK450730.1
	Coprinellus radians	*Psathyrellaceae*	21	JN943117.1
Basidiomycota	*Fomitopsis meliae*	*Fomitopsidaceae*	13	KT718002.1
	Peniphora sp.	*Peniophoraceae*	19	HQ607800.1
	Physisporinus sp.,	*Meripilaceae,*	20	KU958554.1,
	Vandervylia fraxinea,	*Polyporaceae,*		KX081102.1,
	Physisporinus vitreus	*Meripilicaea*		KU194320.1
	Terana caerulea	*Phanerochaetaceae*	20	KP134980.1
Mucoromycota	*Mucor fragilis*	*Mucoraceae*	35	MK910073.1
	Rhizopus microsporus	*Mucoraceae*	22	KJ417579.1

Appendix F

HPLC Chromatograms

1. Oxalic acid chromatogram

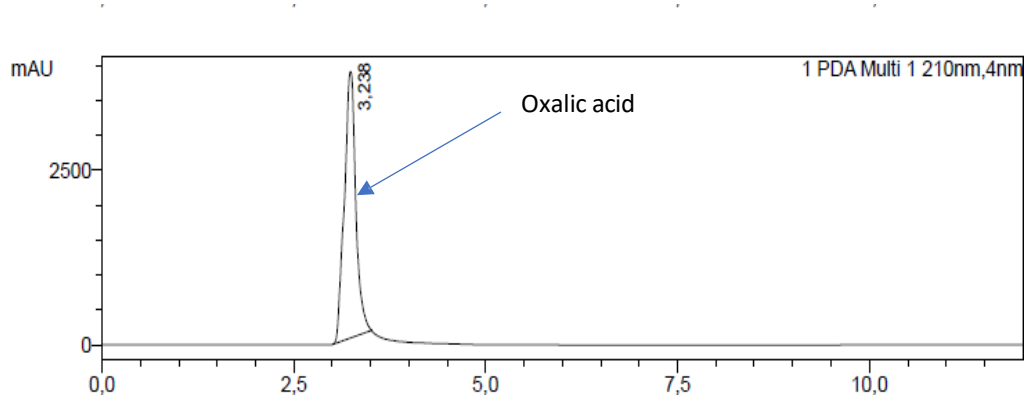


Figure 6: HPLC chromatogram of oxalic acid standard solution, detected at 210 nm using a PDA detector. A sharp peak was observed at a retention time of approximately 3.23 minutes, confirming the presence of oxalic acid under the applied conditions

2. Sample chromatogram

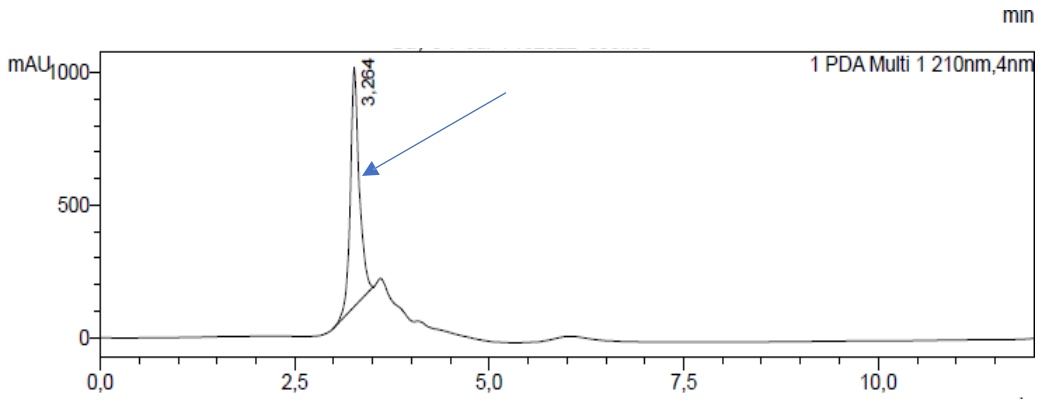


Figure 7: Example of an acceptable HPLC chromatogram used as a reference. The main peak appears at a retention time of approximately 3.26 minutes, detected at 210 nm with a PDA detector. The chromatographic profile demonstrates adequate peak shape, retention, and separation characteristics.