Isolation, identification, and characterisation of fungi from a platinum mine

by

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DECLARATION

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ABSTRACT

Mycohydrometallurgy is the application of fungi and their metabolites such as organic acids (OAs) for the extraction and recovery of metals through mechanisms such as bioleaching. The purpose of this study was to identify and characterise fungal isolates from a platinum mine to determine potential fungal candidates for bioleaching of a platinum concentrate. Fungi were isolated from platinum mining samples using traditional microbiological techniques and sixty-six isolates were identified based on their internal transcribed spacer (ITS) regions using Sanger sequencing, as members of either *Ascomycota*, *Basidiomycota* or *Mucoromycota*. Qualitative OA screening was conducted by inoculating isolates into Potato Dextrose Agar containing a pH indicator to select OA producing isolates. These isolates were quantitatively screened using high-performance liquid chromatography and showed the production of acetic, butyric, formic, lactic, propionic, and indole-3-acetic acid. Based on their OA production, *Penicillium* sp., *Rhizopus microsporus,* and *Aspergillus terreus* underwent tolerance tests to copper, chromium, and nickel. Their overall tolerance was low, suggesting that they are promising candidates for two-step direct bioleaching as it involves the growth of fungi prior to their exposure to the metal-containing solid material.

Dedicated to my parents and sister, for their love and support.

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NOMENCLATURE

%	percentage
^{o}C	degree Celsius
<	less than
≥	greater than or equal to
µB	microbar
µg/ml	microgram per millilitre
µl	microlitre
µm	micrometre
Al	aluminium
ANOVA	analysis of variance
As	arsenic
Au	gold
AU	acid unitage
BLAST	Basic Local Alignment Search Tool
$C_6H_8O_7$	citric acid
Cd	cadmium
CFU	colony-forming unit
CFU/g	colony-forming unit per gram
CFU/ml	colony-forming unit per millilitre
Co	cobalt
Cr	chromium
CTAB	cetyltrimethylammonium bromide
Cu	copper
$CuCl_2.2H_2O$	copper (II) chloride dihydrate
EDTA	ethylenediaminetetraacetic acid
Eh	redox potential
Fe	iron
FeO	ferrous oxide

FeS	iron sulphide
g	gravities
g	gram
g/l	gram per litre
H	hydrogen
H_2SO_4	sulphuric acid
Hg	mercury
HNO_3	nitric acid
HPLC	high-performance liquid chromatography
IAA	indole-3-acetic acid
Ir	iridium
ITS	internal transcribed spacer
$K_2Cr_2O_7$	potassium dichromate
$K_2Pt(II)Cl_4$	potassium tetrachloroplatinate
$K_2Pt(IV)Cl_6$	potassium hexachloroplatinate
kg	kilogram
LMWOAs	low molecular weight organic acids
M	molar
mg/l	milligram per litre
mg/ml	milligram per millilitre
MIC	minimum inhibitory concentration
min	minute
ml	millilitre
ml/min	millilitre per minute
mM	millimolar
mm	millimetre
Mn	manganese
mV	millivolt
n	number of replicates
NaCl	sodium chloride

NCBI	National Center for Biotechnology Information
ng/µl	nanogram per microlitre
Ni	nickel
$NiCl_2.6H_2O$	nickel (II) chloride hexahydrate
NiO	nickel (II) oxide
$NiSO_4$	nickel sulphate
nm	nanometre
O_2	oxygen
OA	organic acid
Os	osmium
Pb	lead
PCR	polymerase chain reaction
Pd	palladium
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
PGMs	platinum group metals
ppm	parts per million
Pt	platinum
$PtCl_4^{2-}$	tetrachloroplatinate
$PtCl_6^{2-}$	hexachloroplatinate
$Pt(NH_3)_4Cl_2^-$	tetraamminedichloroplatinum(iv)
Rh	rhodium
rpm	revolutions per minute
Ru	ruthenium
S	sulphur
SO_4^{-2}	sulphate
Sn	tin
SO_2	sulphur dioxide
TE	tris - ethylenediaminetetraacetic acid
TI	tolerance index

UG2	Upper Group 2
Zn	zinc

1 INTRODUCTION

1.1 Introduction

Platinum (Pt) is one of the rarest and most valuable metals on earth and is therefore labelled as a precious metal. It is the most well-known metal in the six-member family of platinum group metals (PGMs), where the remaining five members are ruthenium (Ru), osmium (Os), rhodium (Rh), iridium (Ir), and palladium (Pd) (Vermaak, 1995). Platinum is the only competitor for gold (Au) in terms of investment and the manufacturing of jewellery due to its malleability and resistance to fading (Jones, 2005). However, PGMs are also of great importance in the chemical and industrial industry for their use as catalysts and catalytic converters, respectively (Rao and Reddi, 2000). They also have important applications as catalysts in the production of petroleum and other fuels and chemicals from crude oil (Rao and Reddi, 2000). In the industrial sector, their application as catalytic converters are important for exhaust control in transport vehicles (Rao and Reddi, 2000).

South Africa accounts for the largest production of PGMs in the world and as a result, it contributes significantly to the gross domestic profit of the country (Ndeddy Aka and Babalola, 2017). However, the mining and production of these precious metals come with their own challenges, which include a lack of economic, environmentally friendly, and effective processing technologies for ore, concentrate, tailings, waste, and near-surface oxidised ores (Hedrich *et al.*, 2020; Mudd, 2010; Thethwayo, 2018). Prior to the extraction of PGMs, the extraction of base metals such as copper (Cu), chromium (Cr), cobalt (Co), nickel (Ni), and iron (Fe) from PGM concentrate need to be considered; as these precious and base metals have similar geochemical behaviour and are usually concentrated together geologically (Hedrich *et al.*, 2020; Jones, 2005; Thethwayo, 2018).

Conventional technologies such as pyrometallurgy and hydrometallurgy, which have been used for the processing of PGM ore has led to ineffective extraction of base metals and PGMs and have high energy requirements (Mudd, 2010; Thethwayo, 2018). Therefore, where pyrometallurgy and hydrometallurgy are

unable to be efficiently applied, biohydrometallurgy could be the possible alternative. Biohydrometallurgy is the use of biological agents for the extraction and solubilisation of metals and is a more economic, effective, and environmentally-friendly removal and recovery process compared to conventional methods (Johnson, 2014). Bioleaching is a biohydrometallurgical process which has been used in previous studies to recover or remove base metals from solid material using microorganisms such as bacteria and fungi and the metabolites which they produce e.g. organic acids (OAs) (Johnson, 2014). Some studies have used acquired strains of microorganisms while others have isolated microorganisms from the mining environment or contaminated soil on which they intend to perform bioleaching experiments. Bacteria have been isolated from soil samples from a platinum mine tailings dam and have been considered as candidates for bioleaching of metal-contaminated soil (Ndeddy Aka and Babalola, 2017). However, studies on the isolation of fungi from a platinum mine have not been conducted. The studies which have been performed on the isolation of fungi involve their isolation from various other mining and metal-contaminated sites. The genera *Aspergillus* and *Penicillium* have often been isolated from such sites and were found to have adaptive qualities, such as their ability to produce extracellular metabolites, which aids in their tolerance to high concentrations of heavy metals (Deng *et al.*, 2012; Ghosh and Paul, 2015). The tolerance to heavy metals is partially due to their production of OAs which solubilise and chelate metals from complexes (Din *et al*., 2020; Sazanova *et al.*, 2015). As a result, *Aspergillus niger* has been extensively used in biohydrometallurgical studies due to its known ability to produce OAs (Din *et al.*, 2020).

1.2 Research Problem

Fungi and bacteria have been isolated from mining and metal-contaminated sites for their use in the bioleaching of base metals. However, only bacteria have been isolated from platinum mines specifically and there have not been any studies thus far involving the isolation of fungi from a platinum. There is also a need for the

identification of more fungal candidates which have a high base metal bioleaching efficiency and potential to bioleach PGMs from PGM materials. Therefore, the identification and characterisation of fungi isolated from a platinum mine is important to determine fungal candidates for the bioleaching of base metals and PGMs from PGM materials.

1.3 Hypothesis, Aims and Objectives

This study hypothesised that fungi will be isolated from platinum mine samples, from the genera *Aspergillus* and *Penicillium* due to their frequent isolation from mining environments. Isolates from these genera have the ability to produce OAs which assists in their adaptation to the heavy metal concentrated environments present in a mine. Therefore, the second hypothesis was that the isolates identified in the study will produce OAs.

This study aimed to identify and characterise fungal isolates from a platinum mine to determine potential fungal candidates for bioleaching of platinum concentrate.

The objectives were:

(i) To isolate and enumerate fungi from soil, slurry, and liquid samples retrieved from a platinum mine using traditional microbiological techniques.

(ii) To identify the fungal isolates using molecular techniques such as genomic DNA extraction, polymerase chain reaction (PCR), agarose gel electrophoresis, and Sanger sequencing.

(iii) To characterise the fungal isolates based on their OA production using qualitative and quantitative methods.

(iv) To characterise selected isolates on their heavy metal tolerance by determining their heavy metal tolerance index for selected heavy metals.

2 LITERATURE REVIEW

2.1 General Introduction

The literature review will first discuss the world's PGM reserves, followed by traditional processing of PGM ore in South Africa and the challenges faced with the mining of PGMs in the country. Thereafter, the isolation of fungal isolates for mycohydrometallurgical processes and the mechanisms which these isolates use to carry out such processes will be reviewed. This will be followed by a review of the different steps involved in fungal bioleaching. Finally, the isolation of microorganisms from a platinum mine from previous studies will be discussed and the use of microorganisms for base metal and platinum bioleaching from platinum sources will be evaluated.

2.2 Mining of Platinum Group Metals (PGMs)

2.2.1 PGM reserves

South Africa and Russia are the world leaders in platinum production, however, Russia produces less than a fifth of platinum compared to South Africa (Aleksandrova and O'Connor, 2020). Platinum is a member of the family of PGMs which also consists of Ru, Os, Rh, Ir, and Pd. Figure 2.1 summarises the world's PGM reserves and shows that South Africa accounts for over 90% of the world's reserves (Garside, 2021).

The main deposits of PGM ore in South Africa are located in the north of the country, in the Bushveld Igneous Complex, which contains the Merensky Reef, Upper Group 2 (UG2) Reef, and Platreef (Thethwayo, 2018). Since 1925, the Merensky Reef has been the main source for PGMs, however, due to the depletion of the Merensky resources, the UG2 Reef is now responsible for most of the platinum-bearing ore in South Africa (Thethwayo, 2018). The Platreef is lower in PGM values but higher in base metal values and as a result, it was the last of the three ore bodies to be exploited (Thethwayo, 2018).

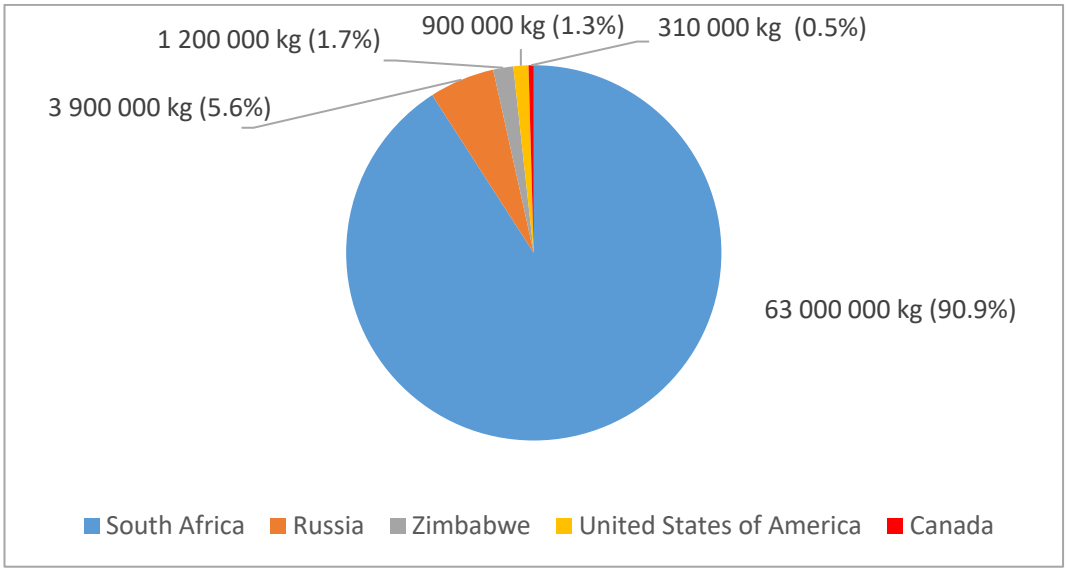

Figure 2.1: The world's estimated reserves of platinum group metals (PGMs) (Garside, 2021).

2.2.2 Processing of PGMs

The traditional processing of PGM ores in South Africa involves comminution, flotation, smelting, converting, base metal refinery, and precious metal refinery (Figure 2.2). Comminution results in the reduction of the ore size through the crushing and milling of the ore which is then treated using gravity separators (Safarzadeh *et al.*, 2018). The produced fines are treated using flotation cells to separate it into tailings and sulphide-rich PGM concentrate (Thethwayo, 2018). Smelting is then used to separate the valuable (sulphide) minerals from the gangue (oxide and silicate) minerals (Jones, 1999). The gangue is discarded as slag and the sulphide minerals are further treated in the converting process where sulphur (S) and iron sulphide (FeS) are oxidised to sulphur dioxide (SO_2) and ferrous oxide (FeO), respectively (Jones, 1999). SO_2 and FeO are removed as an off-gas and slag, respectively (Thethwayo, 2018). The converter matte then undergoes base metal refinery which uses processes such as pressure oxidation which results in the separation of base metals from PGM residue (Bezuidenhout *et al.*, 2013). The base metal-free concentrate then undergoes precious metal refinery where the

individual metals are separated using solution extraction and precipitation methods (Safarzadeh *et al.*, 2018).

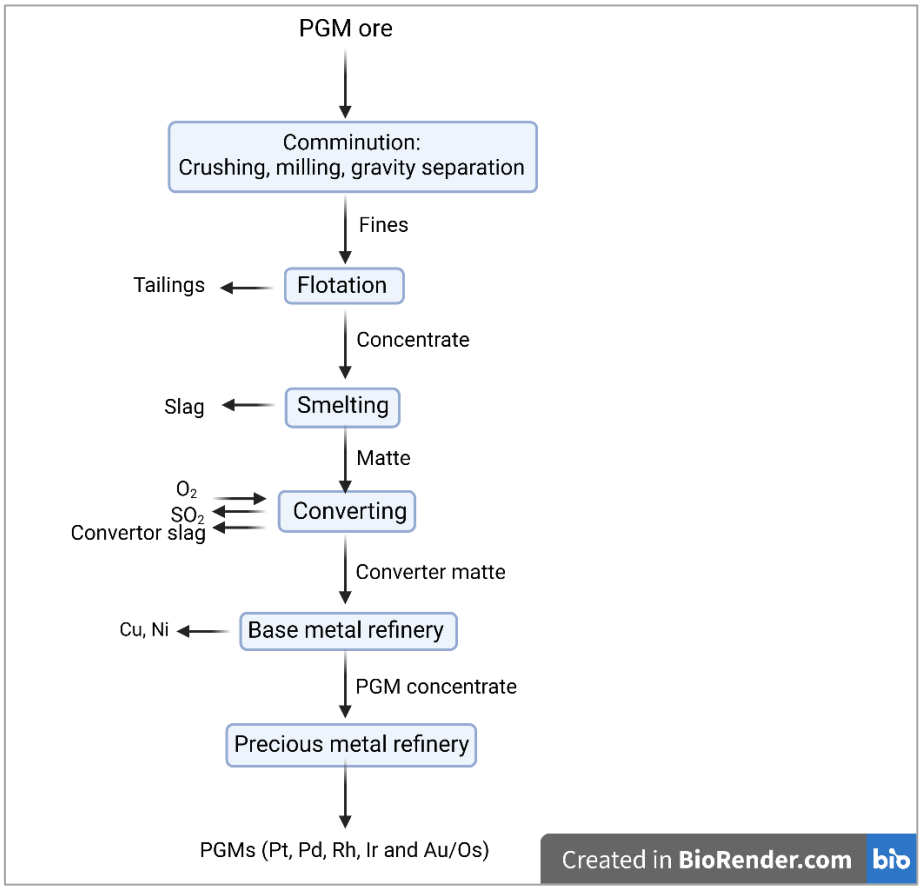

Figure 2.2: The traditional processing of platinum group metal (PGM) ore in South Africa (Thethwayo, 2018).

2.2.3 Challenges of mining PGMs

The processing of PGM ore results in high energy consumption and despite lower energy consumption by open-pit mines, such as the Mogalakwena mine (24°0' South, 28°55' East), the open-pit mine produces large volumes of waste rock (Mudd, 2010). The large volumes of the waste rock and tailings produced require management to prevent it from having a negative impact on the environment and communities near the mines (Mudd, 2010). Curtis (2008) highlighted how important the managing of mine waste is due to the study conducted on the impact of the Anglo Platinum mine on local communities. It was found that their

water resources were contaminated due to mining activities, and contained high concentrations of total dissolved salts, such as sulphate and nitrate (Curtis, 2008). Therefore, an environmentally friendly and effective remediation process needs to be developed. The same is required to process and further process the near-surface oxidised ore from the open-pit mines and slag wastes from smelters, respectively (Hedrich *et al.*, 2020; Mudd, 2010). Without a feasible and effective method, the processing of this ore and waste will not take place and the possible extraction of valuable metals will not occur.

As mentioned previously, there has been an increase in exploitation of the UG2 Reef due to the depletion of the Merensky Reef, however, problems arise with the use of ore from the UG2 Reef due to its high chromite content (60%) (Thethwayo, 2018). This is due to the concentrate produced after flotation having a high chrome content which smelters are not equipped to handle (Thethwayo, 2018). Therefore, an alternate method to smelting needs to be considered to ensure efficient processing of the concentrate.

2.3 Mycohydrometallurgy

2.3.1 Introduction

Pyrohydrometallurgy and hydrometallurgy are two types of extractive metallurgical processes (Anderson, 2016). Pyrometallurgy involves using high temperatures to carry out smelting and refining operations to extract metals from their minerals, whereas hydrometallurgy uses aqueous solutions to separate the metals (Anderson, 2016). These processes result in low metal recovery, high operating costs, and have a negative environmental impact; therefore, alternative extractive metallurgical processes have been explored (Guezennec *et al*., 2014). Microorganisms such as bacteria and fungi have been studied for their use in biohydrometallurgy, which is a branch of extractive metallurgy which involves the use of biological systems and aqueous chemistry to recover metals from substances such as ores, concentrate, and waste materials (Ofori-Sarpong *et al.*,

2010). Extensive research has been conducted on the use of bacteria in this field, however, there are fewer studies on the use of fungi. Mycology is the branch of biology which focuses on the study of fungi, therefore, the term "mycohydrometallurgy" describes the link between mycology and hydrometallurgy and is defined as the application of fungi for the extraction and recovery of metals (Ofori-Sarpong *et al.*, 2010).

The first step in developing a mycohydrometallurgical process is the selection of fungi. The fungi selected can either be acquired or indigenous to the mining environment from where the solid material, which will be used for bioleaching, is obtained. The advantage of isolating indigenous fungi is that they would have acquired traits which allow them to adapt to e.g., the mining or metal-contaminated environment they were isolated from (Din *et al.*, 2020).

2.3.2 Fungi in mining environments

Fungi are found in various environments and the fungal community vary depending on the pH, redox potential (Eh), moisture content, and temperature of the environment from which they are isolated (Brockett *et al*., 2012; Gagnon *et al.*, 2020). Additionally, a parameter that also affects the fungal community is the presence of heavy metals (Gagnon *et al.*, 2020). Table 2.1 lists studies that have been performed regarding the isolation and identification of fungi from mining environments, where fungal strains were isolated from ore, soil, tailings, or overburden samples. These strains were identified due to their ability to tolerate heavy metals, produce OAs or solubilise phosphates, as most studies screen fungal isolates for these characteristics prior to their identification. Therefore, although a greater number of fungi may be isolated from a sample, the strains which are usually identified are those which exhibit characteristics relevant to the specific study. From Table 2.1, the species which are commonly isolated belong to the genera of *Aspergillus* and *Penicillium.* This is due to the variety of heavy metals which they acquire tolerance to and due to the OAs which they produce, as these characteristics are important when considering the development of a

mycohydrometallurgical process. The studies in Table 2.1 have either isolated fungi for bioprocessing or bioremediation purposes. Bioremediation involves the use of microorganisms for the removal of heavy metals from contaminated water and soil (Iram *et al.*, 2013). The development of bioremediation methods is important due to the metal contamination which results from many mining sites and the effect it has on communities around it, such as the contamination of water sources (Curtis, 2008). Bioprocessing involves the use of microorganisms to obtain a higher-grade concentrate (Hedrich *et al.*, 2020). The use of bioprocessing methods is important in developing more environmentally friendly and feasible processing technologies. The mechanisms which fungi use to carry out bioremediation and bioprocessing will be discussed in Section 2.3.3.

Table 2.1 lists fungi that have been isolated and identified from various mines and their tolerance to selected base metals. However, fungi have not been isolated from platinum mines before and therefore when Argumedo-Delira *et al.* (2020) conducted a study on the tolerance of fungi to platinum, they used strains isolated from samples surrounding a landfill. The strains of *Aspergillus niger* MX5, *Trichoderma harzianum* MX2, *Fusarium oxysporum* MX17, *Hypocrea lixii* MXPE12, and *Fusarium solani* MXPE15 were found to be tolerant to a platinum concentration as high as 300 mg/L with little morphological changes observed compared to the control (Argumedo-Delira *et al.*, 2020). These findings allowed the authors to conclude that these strains could be used in the development of biotechnological processes for the recovery of platinum from primary and secondary sources.

Table 2.1: Fungi isolated from mining environments with acquired characteristics.

Fungal isolates	Mining site source	Characteristic	Reference
Trichoderma virens, T. harzianum, T. gamsii	Mine tailings - wastewater samples	Cr, Pb, Zn, Ni, Cu[1]	(Tansengco and Tejano, 2018)
Trichoderma saturnisporum	Mine tailings - wastewater samples	Cr, Pb, Ni, Cu	(Tansengco and Tejano, 2018)

Trichoderma ghanense, Rhizopus microsporus	Gold and gemstone mine site - soil samples	Cu, Pb, Fe, Cd, As	(Oladipo *et al.*, 2018)
Fomitopsis meliae	Gold mine site - soil samples	Cu, Pb, Fe	(Oladipo *et al.*, 2018)
Rhizopus oryzae	Gold mine tailings	Cr, Cu, As, Pb, Cd	(Sey and Belford, 2021)
Trametes versicolor	Gold mine tailings	Cr	(Sey and Belford, 2021)
Trichoderma viride, Aspergillus fumigatus, Trichophyton rubrum	Gold mine tailings	Cr, Cu	(Sey and Belford, 2021)
Penicillium chrysogenum	Smelting industry - soil sample	Cd, Cu, Pb, Zn	(Deng *et al.*, 2012)
Aspergillus sclerotiorum, Komagataella phaffi, Trichoderma harzianum	Nanjing lead-zinc mine - soil sample	Cd, Cr, Pb	(Liaquat *et al.*, 2020)
Aspergillus niger, A. aculeatus	Nanjing lead-zinc mine - soil sample	Cd, Cr	(Liaquat *et al.*, 2020)
Trichoderma atroviride	Nickle mining wastewater - soil and sediment samples	Cr, Co, Ni	(Hernahadini *et al.*, 2014)
Aspergillus niger, A. humicola, A. awamori, A. versicolor, A. phoenicus	Chromite mine - overburden dumps	Cr, Co, Ni, Fe	(Ghosh and Paul, 2015)
Penicillium	Iron ore	Phosphate solubiliser	(Adeleke *et al.*, 2010)
Alternaria, Epicoccum	Iron ore	-	(Adeleke *et al.*, 2010)
Aspergillus, Alternaria, Penicillium, Sterila, Trichoderma, Fusarium	Chhattisgarh region in India - soil samples taken from coal, iron ore and limestone mining sites, as well as from a steel plant.	Organic acid producers	(Gupta and Khan, 2018)
Aspergillus sp., *Penicillium* sp.	Limonite ore (low-grade nickel ore)	Ni	(Handayani and Suratman, 2017)

1 – Heavy metals to which the fungal isolate is tolerant.

2.3.3 Fungal bioleaching

There are four main mechanisms that fungi use for bioleaching namely, acidolysis, complexolysis, biosorption, and bioaccumulation (Dusengemungu *et al.*, 2021). However, acidolysis is the most rapid and frequently used bioleaching mechanism (Dusengemungu *et al.*, 2021).

2.3.3.1 Acidolysis

The conversion of organic compounds (e.g., glucose or sucrose) by enzymatic reactions in the cytosol and mitochondrion of heterotrophic fungal cells results in the production of OAs such as gluconic, citric and lactic acids (Dusengemungu *et al.*, 2021). Acidolysis is a process that utilises protons provided by these low molecular weight organic acids (LMWOAs) to protonate oxygen atoms of metal oxides (Bahafid *et al.*, 2017). The attachment of protons to the metal surface reduces the strength of bonds and results in the removal of metal ions from the surface and the production of water molecules due to the reaction of protons with the oxygen atoms (Bahafid *et al.*, 2017). An example of acidolysis can be seen in equation (1), which displays the reaction of protons with nickel (II) oxide (NiO) to produce nickel ions.

$$NiO + 2H^{+} \xrightarrow{acidolysis} Ni^{2+} + H_2O \qquad \text{(Equation 1)}$$

2.3.3.2 Complexolysis

Complexolysis takes place through the chelation mechanism and allows for the formation of complexing agents which leads to metal extraction (Dusengemungu *et al.*, 2021). It occurs due to the bond formed between metal ions and ligands (OAs), being stronger than the lattice bonds formed between the metal ions and solid particles (McKenzie *et al.*, 1987). Therefore, allowing the solubilisation of metal ions and enhancing the bioleaching process. Complexolysis also allows for the stabilisation of metal ions produced during acidolysis and reduces their (the

metal ions) toxic effect on the fungi (Srichandan *et al*., 2019). An example of complexation can be seen in equation (2), where nickel ions complex with citric acid.

$$Ni^{2+} + C_6H_8O_7 \xrightarrow{complexolysis} Ni(C_6H_5O_7)^- + 3H^+ \quad \text{(Equation 2)}$$

2.3.3.3 Biosorption

During biosorption, the metal ions which occur in the solution due to the production of OAs which dissolve the metal from the solid material, are adsorbed by the fungal biomass (Dusengemungu *et al.*, 2021). Thereby decreasing the quantity of metal in the solution. The reactions which are involved in the biosorption of metals are ion exchange, complexion, adsorption, and precipitation (Bahobil *et al*., 2017). With the use of a direct bioleaching method (Section 2.3.4), biosorption could take place and result in an increase in the bioleaching efficiency by the removal or recovery of base metals and PGMs.

2.3.3.4 Bioaccumulation

Bioaccumulation is the uptake of metals by the biomass of living organisms (Dusengemungu *et al.*, 2021). It takes place when metal ions accumulate or precipitate in vacuoles due to their transportation between the cell membrane and the cell of these organisms (Brandl *et al*., 2001). Unlike biosorption, bioaccumulation does not require the production of metabolites for the uptake of metals, however, the process is slower than biosorption. Fungal bioaccumulation is thought to take place due to the binding of metal ions to functional groups present in fungal mycelia, such as amine, phosphate, hydroxyl, carboxyl, and sulphate, which allows the metal ions to act as a cation exchanger (Dusengemungu *et al.*, 2021). An example of such a reaction can be seen in equation (3).

$$Ni^{2+} + SO_4^{-2} \xrightarrow{bioaccumulation} NiSO_4\,(s) \quad \text{(Equation 3)}$$

2.3.4 Indirect and direct bioleaching

There are different methods for bioleaching namely indirect and direct bioleaching (Handayani and Suratman, 2017). Indirect bioleaching occurs when the solid bioleaching material is exposed to the metabolites produced by microorganisms, without the microorganisms present in the bioleaching setup (Handayani and Suratman, 2017). Whereas direct bioleaching occurs in the presence of microorganisms and can be sub-divided into one-step and two-step bioleaching procedures (Brandl *et al*., 2001). One-step bioleaching takes place when the microorganism is inoculated into the media containing the solid material to be bioleached (Brandl *et al*., 2001; Deng *et al*., 2012). Two-step bioleaching involves the growth of the microorganism in growth media, followed by the addition of the solid material after exponential biomass growth occurs (Brandl *et al*., 2001; Deng *et al*., 2012). This allows the microorganisms a chance to grow and produce metabolites prior to being exposed to the solid material (Deng *et al*., 2012).

Handayani and Suratman (2017) compared indirect and direct bioleaching of low-grade nickel ore using species of *Aspergillus* and *Penicillium*. They found that direct bioleaching resulted in a 9% increase in the percentage of nickel extracted compared to indirect bioleaching for both *Aspergillus* sp. and *Penicillium* sp. (Handayani and Suratman, 2017). *Penicillium* sp. had a 9% lower bioleaching efficiency than *Aspergillus* sp. using the direct method, as they had a 48% and 57% bioleaching efficiency, respectively (Handayani and Suratman, 2017).

Brandl *et al.* (2001) determined the efficiency of *Thiobacillus thiooxidans*, *T. ferrooxidans*, *A. niger*, and *P. simplicissimum* to bioleach Cu, Ni, tin (Sn), aluminium (Al), lead (Pb), and zinc (Zn) from electronic waste using a one-step procedure. However, they suggested that a more efficient method would be two-step bioleaching to allow biomass growth to be separated from metal leaching. A study performed by Deng *et al.* (2012) compared one-step and two-step bioleaching using *P. chrysogenum* to bioleach heavy metals from contaminated soil. They found that two-step bioleaching had a leaching efficiency of 13%, 21%, 5%, and

14% greater than the one-step procedure for cadmium (Cd), Cu, Pb and Zn, respectively. Therefore, the bioleaching method which could result in the greatest efficiency to process PGM ores may be two-step bioleaching.

2.3.5 Biohydrometallurgical methods for base metal removal from PGM sources

The use of fungi in biohydrometallurgical processes for the removal of base metals from PGM sources has not been studied in previous research. However, the use of iron and sulphur oxidising bacteria has been investigated. Mwase *et al.* (2012) used a culture of *Metallosphaera hakonensis* to bioleach Cu (91.1%), Ni (98.5%) and Co (83.5%) from low-grade flotation concentrate obtained from Platreef ore. Due to the success of this experiment, they used the same culture to bioleach Cu, Ni and Co from crushed whole ore where 93%, 75% and 53% of each metal was removed, respectively (Mwase *et al.*, 2014). Hedrich *et al.* (2020) conducted bioleaching experiments on oxidised PGM ore from stockpiles of the Mogalakwena mine using acidophilic iron and sulphur oxidising bacteria and reported an 86% total base metal recovery of metals such as Co, Cu, Ni, and manganese (Mn).

2.3.6 Biohydrometallurgical methods for platinum recovery

Compared to the studies performed on the recovery or removal of base metals from ores, concentrate, waste materials, and contaminated soil, studies on the recovery of platinum using biohydrometallurgical methods are scarce. The studies which have been performed have used bacterial isolates which were acquired from culture collections or isolated from wastewater and not directly isolated from platinum mining sites or processes.

The bioleaching experiment performed by Hedrich *et al.* (2020) described in Section 2.3.6, resulted in the recovery of base metals, however, it also resulted in the release of platinum group minerals. Therefore, bioleaching resulted in the enhancement of the following chemical leaching process, HNO_3/NaCl and a

cyanide leach, which was used to extract PGMs. The direct extraction of platinum using bioleaching methods was shown in a study by Brandl *et al.* (2008) where cyanogenic bacteria were used to extract platinum (0.2%) from automobile catalytic converters. Maes *et al.* (2016) reported the extraction of 98% of Pt (II) and 97% of Pt (IV) from solutions of 100 mg/L of potassium tetrachloroplatinate ($K_2Pt(II)Cl_4$) and potassium hexachloroplatinate ($K_2Pt(IV)Cl_6$) using mixed cultures of *Halomonasae*, *Bacillaceae,* and *Idiomarinaceae,* which are families of halophilic bacteria. Therefore, this study demonstrated the platinum recovery potential of halophilic mixed cultures in acidic saline conditions (Maes *et al*., 2016). In 2017, Maes *et al.* (2017) studied the biosorption recovery of five Pt-complexes under acidic conditions using four Gram-positive species, *Shewanella oneidensis*, *Cupriavidus metallidurans*, *Geobacter metallireducens*, and *Pseudomonas stutzeri* and one Gram-negative species, *Bacillus toyonensis*. All species were found to completely recover platinum from $PtCl_4^{2-}$ and $PtCl_6^{2-}$, whereas only *S. oneidensis* and *C. metallidurans* recovered 99% of platinum from cisplatin (Maes *et al*., 2017). Carboplatin was partially recovered with a maximum recovery of 25% and no platinum recovery was observed for $Pt(NH_3)_4Cl_2$ (Maes *et al*., 2017).

2.3.7 Microorganisms isolated from a platinum mine

As explained in Section 2.3.2, there are no studies involving the isolation of fungi from a platinum mine. However, there are a few studies which have been performed that involve the isolation of bacteria from a platinum mine. Neddy Aka and Babalola (2017), isolated bacteria belonging to the genera *Pseudomonas*, *Bacillus*, *Alcaligenes* and *Proteus,* which displayed multiple tolerance to the heavy metals, Cr, Cd, and Ni, from a platinum mine in South Africa. Bacteria were also isolated from a platinum mine tailings dam in South Africa, where *Paenibacillus lautus* and *Stenotrophomonas maltophilia* were found in high abundance (Rauwane *et al.*, 2018). Therefore, the presence of these bacterial species in platinum mines may indicate their possible use for bioleaching, however, there are no studies which investigated the bioleaching efficiency of these species.

2.4 Summary

This study is aimed at identifying and characterising fungi isolated from a platinum mine. The characterisation of fungi based on their OA producing ability and heavy metal tolerance allows for the selection of indigenous fungal isolates to be used in biohydrometallurgical processes. Fungal bioleaching is such a process that uses fungi to assist in the recovery of metals from ore, concentrate, and waste materials.

The literature review in this chapter discussed the worlds PGM reserves and the traditional processing methods of PGMs, where a need for a more environmentally friendly, feasible and effective processing method was seen. Mycohydrometallurgy as a possible solution was then considered and previous literature which involved the identification of fungal isolates from mines and the use of fungi for mycohydrometallurgical processes to remove or recover base metals from solid material was reviewed. A need for the identification of fungi from a platinum mine for their possible use in bioleaching processes was seen as there have only been studies involving the isolation of bacteria from platinum mines and the use of bacteria for the bioleaching of platinum sources which have been conducted. These studies all occurred in South Africa, most likely due to the country containing the highest PGM reserves in the world. Therefore, the need for the identification of fungi from a platinum mine, especially from South Africa is apparent, in order for mycohydrometallurgical processes for the recovery of platinum to be developed.

3 ISOLATION AND IDENTIFICATION OF FUNGI

3.1 Introduction

The isolation and identification of microorganisms from mine and heavy metal contaminated environments are important to understand the diversity of microorganisms present in these environments. This is due to the presence of microorganisms which have developed adaptive qualities that allow them to tolerate these harsh heavy metal concentrated environments (Din *et al*., 2020). The isolation and identification of such adapted microorganisms are important to determine which isolates can be used in biohydrometallurgical processes to extract and solubilise heavy metals from ore, concentrate, and waste materials for bioprocessing and bioremediation purposes (Hedrich *et al*., 2020; Iram *et al*., 2013). The possible identification of fungal isolates which have not previously been identified in mining or metal-contaminated environments is also of interest.

Bacteria and fungi are examples of microorganisms which have been isolated from these environments, however, the studies involving the isolation of bacteria are far greater compared to fungi (Ofori-Sarpong *et al*., 2010). With regards to a platinum mine, bacteria have previously been isolated from such a mine, however, there are no known studies involving the isolation of fungi (Neddy Aka and Babalola, 2017; Rauwane *et al*., 2018). Additionally, the enumeration (total fungal count) of fungi from mine and metal-contaminated sites are rarely performed as most studies focus on the sole isolation of specific heavy metal tolerant fungi for their biosorption capabilities. However, the enumeration of fungi assists with obtaining a better understanding of the fungal diversity in the heavy metal concentrated environment.

There are two methods which have been used for the identification of microorganisms, namely, morphological, and molecular techniques. Molecular methods are usually preferred as more accurate results are obtained due to the possible identification of isolates to their species level (Raja *et al.*, 2017). When considering extracting DNA from fungi, the first step in performing molecular techniques is determining the use of an effective DNA extraction method due to

their strong cell wall being harder to lyse compared to other microorganisms (van Burik *et al.*, 1998). Additionally, the use of a DNA extraction kit is expensive and at times ineffective when dealing with multiple different fungal isolates (Umesha *et al.*, 2016).

Therefore, this study aimed to isolate and identify fungi from samples retrieved from a platinum mine using microbiological techniques and a feasible and effective DNA extraction method in order to obtain pure DNA for downstream applications, respectively.

3.2 Materials and Methods

3.2.1 Sample collection

A total of thirty-nine samples were collected from four regions at the Mogalakwena platinum mine (24°0' South, 28°55' East) namely: the concentrator plant, the pond outside of the concentrator plant, in-front of the crusher, and in-front of the concentrator. All the samples collected from the concentrator plant were liquid samples collected at different stages of the flotation process. The samples obtained from the remaining three regions were soil, slurry, or liquid samples taken from around the mine. The full list of sample sets is shown in Table 3.1. The samples were placed in either 50 ml sterile centrifuge tubes or a polyethylene bag and were transported in a polystyrene box, stored at 4^{o}C, and were analysed at the Industrial Microbiology and Biotechnology Laboratory (University of the Witwatersrand) within 24 hours of sampling.

3.2.2 Analysis of pH and redox potential

The pH and Eh in units of mV, for all samples, were measured (Crison - Model Basic 20- pH-Meter). To prevent contamination of the samples, liquid samples were analysed by aseptically transferring 2 ml of sample into a 15 ml centrifuge tube. Solid samples were analysed by aseptically transferring 1.0 g of sample into a 15 ml centrifuge tube and adding 10 ml of distilled water to the tube.

3.2.3 Microbiological characterisation

For the isolation of fungi from the samples, a 10^{-1} to 10^{-3} dilution series was performed aseptically with sterile saline (0.9%) being used as the diluent. Thereafter, aliquots of 100 µl from each dilution were spread plated onto Potato Dextrose Agar (PDA) (pH 5.6 ± 0.2) plates containing 1% chloramphenicol and incubated at 25°C for 7 days. The antibiotic, chloramphenicol, was added to inhibit bacterial growth. The results are represented as colony-forming units (CFU) per ml or per g of sample. Distinct fungal colonies were then sub-cultured until pure cultures were obtained. The cultures were maintained on PDA plates at 4°C.

3.2.4 Genomic DNA extraction

The genomic DNA extraction method was modified from protocols performed by Doyle and Doyle (1987) and van Burik *et al.* (1998). The sixty-six pure fungal isolates were inoculated into 10 ml of Sabouraud Dextrose Broth in a 15 ml centrifuge tube and incubated at 25°C for 7 days on a 200-rpm orbital shaker. The mycelia were transferred into 2 ml microcentrifuge tubes, a pellet of cells was obtained through centrifugation (MiniSpin microcentrifuge – Eppendorf) at 1 677 x *g* for 10 min and the supernatant was discarded. The tubes were then covered with parafilm, and tiny perforations were made. They were stored in the – 80°C freezer overnight and the pellets were lyophilised (VirTis BenchTop Pro Lyophilizer – SP Scientific) at 222 µB and – 80°C, for 24 hours. The lyophilised pellets were suspended in a 2 ml microcentrifuge tube containing; 500 µl extraction buffer [1 M Tris (pH 8.0), 5 M NaCl, 0.5 M EDTA and 2% CTAB in 1 L distilled H_2O] which was pre-heated at 65°C (Waterbath WNB 7 – Memmert), 1 µl β-mercaptoethanol, and four sterile glass beads (5 mm). This was vortexed thoroughly and cells were lysed by sonication (Scientech Ultrasonic Cleaner – 702) for 1 hour, at 55°C, and the high-frequency setting. The mixture was then transferred to a new tube and incubated at 65°C for 60 min. Thereafter, all steps were performed on ice. After incubation, 500 µl chloroform: isoamyl alcohol (24:1) was added and mixed by vigorous inversion. This mixture was centrifuged at 12 045 x *g*, 4°C, and for 10 min. The top

aqueous layer was extracted and transferred to a new tube and the chloroform: isoamyl alcohol step was repeated. The nucleic acid was precipitated with an equal volume of isopropanol and the tube was gently inverted until DNA precipitated. It was then centrifuged at 12 045 x *g*, 4^{o}C, and for 10 min and the supernatant was discarded. The pellet was washed in 500 µl ice-cold 70% ethanol, vortexed briefly and centrifuged at 12 045 x *g*, 4^{o}C, and for 10 min and the wash step was repeated. The pellet was air-dried and resuspended in 50 µl 1 x TE buffer containing 20 µg/ml RNase A.

3.2.5 Quantification of DNA

A Thermo Scientific™ NanoDrop™ 1000 Spectrophotometer was used to determine the concentration and purity of the extracted DNA at 260 nm. A concentration of ≥ 10 ng/µl was considered as acceptable. The ratio of absorbance of ≥ 1.8 at 260/280 nm was considered as acceptable and pure DNA.

3.2.6 Sanger sequencing

PCR, agarose gel electrophoresis, and Sanger sequencing were carried out at Inqaba Biotech (Pretoria, South Africa). Amplification of the internal transcribed spacer (ITS) region was performed using OneTaq® Quick-Load® 2X Master Mix (NEB, Catalogue No. M0486) with the fungal universal primer set ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'- TCCTCCGCTTATTGATATGC) (White *et al.*, 1990). The PCR products were run on an agarose gel and the bands were extracted using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research, Catalogue No. D4001). The fragments were sequenced in the forward and reverse direction (Nimagen, BrilliantDye™ Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000) and purified (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit™, Catalogue No. D4050). The purified fragments were analysed (ABI 3500xl Genetic Analyzer (Applied Biosystems, ThermoFisher Scientific)) for all isolates. The .ab1 files generated by the ABI 3500XL Genetic Analyzer were analysed using the CLC Bio Main Workbench v7.6 and the results were analysed using the Basic Local

Alignment Search Tool (BLAST) (National Center for Biotechnology Information (NCBI)).

3.3 Results and Discussion

3.3.1 Physicochemical characteristics and fungal counts

The pH of the samples obtained from the platinum mine ranged from slightly acidic (6.78) to alkaline (9.27) with the lowest pH being measured from sample 31 which was the liquid sample obtained from the pond outside of the concentrator plant and the highest pH was measured from sample 32 which was the soil sample taken from in-front of the crusher (Table 3.1). The Eh in the present study ranged from 26 to -103 mV (Table 3.1), with the lowest reducing environment being sample 31 and the highest reducing environment being sample 23 which was the dry soil sample obtained from the same region as sample 31.

The CFU/ml or CFU/g of sample ranged from 0 to 1.2×10^4 among all the samples retrieved from the mine (Table 3.1). The highest fungal count (1.2×10^4) was from sample 33 which was a soil sample taken from in-front of the crusher. The low fungal counts were observed from all the liquid samples obtained from the concentrator plant, with most samples from this region having 0 CFU/ml and the highest count (2.0×10^2 CFU/ml) being from sample 17 which was obtained from cleaner scavenger tail B. The soil, slurry, and liquid samples obtained from the three remaining regions, namely, the pond outside of the concentrator plant, in-front of the crusher, and in-front of the concentrator resulted in higher fungal counts, apart from sample 31 which was the liquid sample obtained from the pond outside of the concentrator plant, as it had a count of 2.0×10^2 CFU/ml which was the same as sample 17. These low fungal counts were also observed for the other two liquid samples from the same region (samples 29 and 30).

Table 3.1: Physicochemical characteristics and fungal counts of samples obtained from the Mogalakwena platinum mine.

Mine Sample no.	Sample name	Region in the mine where samples were obtained	pH	Eh (mV)	CFU/ml or CFU/g of sample
1	Rougher Feed	A[1]	8.69	-79	0
2	Rougher Feed	A	8.61	-81	1.0×10^2
3	Rougher Tail A	A	8.15	-47	0
4	Rougher Tail A	A	8.17	-45	0
5	Rougher Tail B	A	7.69	-27	0
6	Rougher Tail B	A	7.68	-28	0
7	Scavenger Feed	A	8.51	-71	0
8	Scavenger Feed	A	8.51	-67	0
9	Scavenger Tail A	A	7.35	-9	0
10	Scavenger Tail A	A	7.40	-11	1.0×10^2
11	Scavenger Tail B	A	8.55	-72	1.0×10^2
12	Scavenger Tail B	A	8.53	-79	1.0×10^2
13	Cleaner Feed	A	8.27	-59	1.0×10^2
14	Cleaner Feed	A	8.40	-66	0
15	Cleaner Scavenger Tail A	A	8.23	-55	0
16	Cleaner Scavenger Tail A	A	8.45	-66	0
17	Cleaner Scavenger Tail B	A	8.36	-63	2.0×10^2
18	Cleaner Scavenger Tail B	A	8.42	-64	1.0×10^2
19	Dry soil	B[2]	8.63	-99	1.2×10^3
20	Dry soil	B	9.11	-103	4.0×10^2
21	Dry soil	B	8.30	-64	1.2×10^3
22	Dry soil	B	8.25	-77	8.0×10^2
23	Slurry	B	7.27	-23	1.1×10^3
24	Slurry	B	6.96	3	1.4×10^3
25	Slurry	B	6.90	1	5.0×10^3
26	Slurry	B	7.53	-32	1.0×10^3
27	Slurry	B	6.87	8	1.1×10^3
28	Slurry	B	7.39	-18	1.0×10^4
29	Liquid	B	6.86	15	6.0×10^2
30	Liquid	B	6.90	8	3.0×10^2
31	Liquid	B	6.78	26	2.0×10^2
32	Soil	C[3]	9.27	-99	7.0×10^2
33	Soil	C	9.21	-100	1.2×10^4
34	Soil	C	8.96	-73	4.0×10^3
35	Soil	C	8.78	-60	2.1×10^3
36	Slurry	C	7.90	-48	5.0×10^3

37	Slurry	C	8.27	-65	3.0×10^3
38	Slurry	C	8.32	-65	3.0×10^3
39	Soil	D[4]	8.49	-47	1.6×10^3

1 – A: concentrator plant, 2 – B: pond outside of the concentrator plant, 3 – C: in-front of the crusher, 4 – D: in-front of the concentrator

There are only a few studies which have determined the fungal counts and pH of samples isolated from mining or metal-contaminated environments, with the Eh of samples not often being mentioned. However, pH and Eh are inversely correlated as explained by the oxidation of two molecules of water (losing four electrons) resulting in the production of O_2 and $4H^+$, therefore leading to acidification (Husson *et al.*, 2018). This is seen in the present study as the samples with pH values below 6 all had higher Eh values.

Oladipo *et al.* (2014) determined the fungal counts of samples obtained from gold and gemstone mines and determined the counts to range between 2.4×10^4 to 2.5×10^5 CFU/g of soil, with the pH ranging between 3.5 and 5.3, respectively. pH values in the acidic range, as seen in the mentioned study, have been found to result in a decrease in microbial growth due to an increase in metal dissolution which results in greater toxicity to microbes (Nwuche and Ugoji, 2008). However, this was not the case in the study by Tansengco and Tejano (2018) where they isolated fungi from wastewater retrieved from mine tailings, as they determined that higher fungal counts were observed in samples with lower pH values. In the present study, a correlation between pH and fungal counts is difficult to make as higher fungal counts were not observed in all samples with a higher pH. This is seen when comparing the pH and fungal counts of sample 32 (pH 9.27) and sample 33 (pH 9.21), as they both have a pH above 9. However, sample 32 has a much lower fungal count (7.0×10^2 CFU/g) compared to sample 33 (1.2×10^4 CFU/g). Therefore, the difference in fungal counts may be due to other factors not relating to pH.

Rath *et al.* (2010) isolated fungi from iron and chromite mine soil as well as from a control sample and determined the fungal counts to be 9.2, 9.9, and 18.3 CFU/g

of soil and the soil pH to be 5.1 ± 0.09, 5.4 ± 0.05, and 6.6 ± 0.04, respectively. They determined that these low counts could be due to low nutrients in the soil, more specifically, low levels of organic carbon which is essential for the growth of heterotrophs (Rath *et al.*, 2010). They also concluded that the acidic pH and high concentration of heavy metals in the soil could have resulted in extremely low fungal counts.

Therefore, in terms of the present study, the fungal counts of the samples retrieved from the concentrator plant could have been very low as they were retrieved from various processes that occur during flotation. During flotation, there is a severe lack of nutrients compared to the other three regions from which the samples were retrieved, and the fungi are exposed to high concentrations of heavy metals which could prevent or inhibit fungal growth. In terms of the fungal counts among the samples from the remaining three regions, the difference in fungal counts between the samples could be due to the difference in levels of organic carbon between the samples, the adaptation of the fungi to the heavy metal concentrations for the specific sample site, and the difference in the microbial community as a whole.

The spread plates for samples 13, 24, 33 and 39 obtained from the four different regions of the mine can be seen in Figure 3.1. The decrease in the number of colonies in terms of fungi, yeast, as well as bacteria resistant to the antibiotic used can be clearly seen from the 10^{-1} to the 10^{-3} spread plates. For sample 13 taken from the concentrator plant, the low colony counts, in general, can be clearly seen with only one fungal isolate appearing as a fluffy white colony in the lowest dilution at the edge of the plate. Sample 24 and sample 33 taken from the pond outside of the concentrator plant and in-front of the crusher, respectively, had many different types of filamentous fungal growth on their 10^{-1} spread plates, however, the types of colonies between the plates differed. This was seen by the different types of white fungal colonies as well as a yellow colony on sample 24's plate and the presence of black, white, brown, cream, and black sporulating fungal colonies on sample 33's plate.

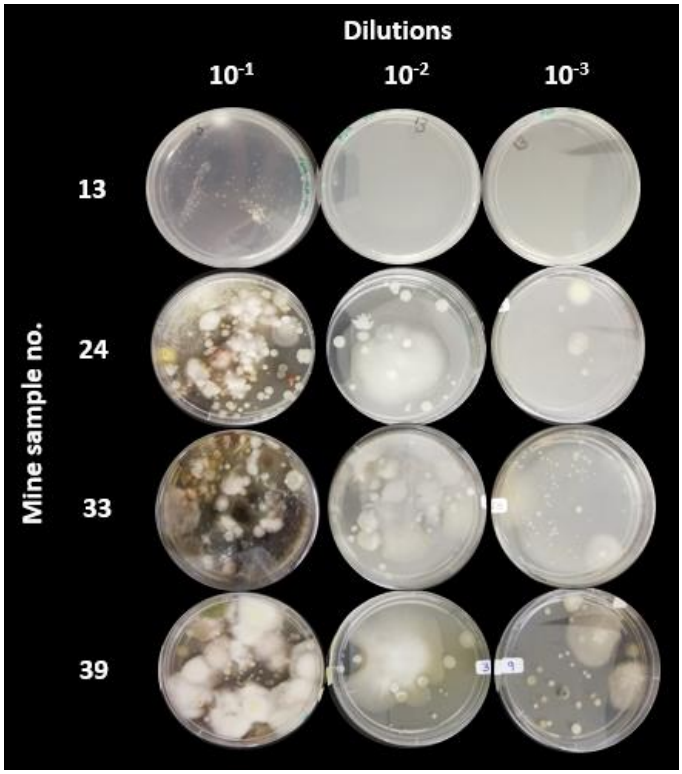

Figure 3.1: Spread plates of the mine samples. Dilutions 10^{-1} to 10^{-3} (left to right) for samples 13, 24, 33, and 39 obtained from the four different regions of the platinum mine.

The 10^{-1} spread plate for sample 39 mostly consisted of the growth of a white fluffy colony surrounding the plate with a greenish sporulating colony at one edge of the plate. The 10^{-2} spread plate for sample 39 also shows the interaction between an antibiotic-resistant bacterial colony with a filamentous fungal colony as a clear zone of growth inhibition of the fungal colony is observed due to the presence of the bacterial colony. Therefore, this could explain the low fungal counts of samples 20, 22, 29, 30, 31, and 32, as the microbial community as a whole may affect fungal growth negatively or positively (Boer *et al.*, 2005). The identification of microbial communities may be done by using a 16S ribosomal ribonucleic acid gene and ITS next-generation sequencing method to identify bacterial and fungal species present in the samples, respectively (Böhmer *et al.*, 2020).

After the repeated sub-culturing of distinct fungal colonies from the spread plates, sixty-six pure cultures of fungal isolates were obtained, and molecular techniques were used to identify these isolates.

3.3.2 Evaluation of DNA extraction method

The DNA extraction method used resulted in high concentrations and relatively good purity ratios of DNA (Table A1 – Appendix A). All isolates' DNA extracts resulted in a concentration between 39.8 and 1809.3 ng/µl, a A_{260}/A_{280} ratio between 1.85 and 2.29 and a A_{260}/A_{230} ratio between 1.15 and 2.78 (Table A1 – Appendix A). It's important to analyse the purity of DNA extracts to determine if the extraction method is sufficient and if contaminants will affect downstream applications such as PCR. The acceptable range for purity ratios is 1.8 to 2.0 for A_{260}/A_{280} and 1.8 to 2.2 for A_{260}/A_{230} (Olson and Morrow, 2012).

High A_{260}/A_{280} ratios indicate that RNA was co-extracted, however, this does not affect downstream applications, but it does result in an overestimation of the concentration of DNA (Li *et al.*, 2008). Therefore, the DNA concentration for some of the isolates in the present study e.g., isolate no. 30 and 57 which had the highest concentrations of DNA, might be lower than indicated due to RNA contamination since these two isolates also had the highest A_{260}/A_{280} ratios (Table A1 – Appendix A). Low A_{260}/A_{230} ratios are known to indicate protein and polysaccharide contamination which can affect the amplification of DNA during PCR. However, protein contamination would have resulted in a low A_{260}/A_{280} ratio and this was not observed for any of the isolates (Olson and Morrow, 2012). Therefore, the contaminants were likely polysaccharides. Despite the purity ratios not remaining within the acceptable ranges, this did not affect the amplification of DNA during PCR and the identification of the isolates was possible, therefore suggesting that the DNA extraction method used was successful.

3.3.3 Evaluation of identification method

The sixty-six fungal isolates were identified and are listed in Table 3.2. The identification of thirty-two of the isolates to their species level was possible with the primer set used, however, the identification of the remaining thirty-four isolates to their exact species level was not possible. For these thirty-four isolates, either the species name, genus, or family was not possible to deduce based on the sequencing results obtained.

Raja *et al.* (2017), conducted an extensive review on the methods used for fungal identification and concluded that, when possible, identification should occur using a combination of micromorphological, cultural, and molecular techniques. However, morphological identification leads to a variety of limitations which include the limited amount of morphological characteristics which can be used for identification and the frequent inability to identify the isolates according to their species level (Ko Ko *et al*., 2011; Raja *et al.*, 2017). With regards to molecular identification, the ITS region which was amplified in the present study is the most useful nuclear ribosomal gene for species-level identification (Raja *et al.*, 2017). However, it has been found to be ineffective when dealing with highly specious genera such as *Aspergillus, Cladosporium, Fusarium, Penicillium* and *Trichoderma* as these genera have limited or no barcode gaps in this region (Raja *et al.*, 2017; Samson *et al*., 2014)).

From the results in the present study, the mentioned genera were all identified and at least one of the isolates identified from each genus was not able to be identified to their exact species (Table 3.2). Raja *et al.* (2017), suggested the use of protein-coding genes to be used with or to replace ITS regions in species identification via barcoding due to the presence of intron regions which can evolve faster than ITS regions. Therefore, this could be a possible solution to identify the fungal isolates which could be selected for further studies for bioleaching purposes, to their species-level.

Table 3.2: Identification and classification of fungal isolates from the Mogalakwena platinum mine.

Phylum	Current name	Family	Mine sample no.	GenBank accession no.	Isolate no.
Asco-mycota	*Bartalinia sp., Bartalinia pondoensis*	*Bartaliniaceae*	33	MT477058.1, KJ767127.1	16
	Bartalinia pondoensis	*Bartaliniaceae*	34	JQ425386.1	23
	Clonostachys epichloe	*Bionectriaceae*	23	KJ780769.1	41
	Neoscytalidium dimidiatum	*Botryosphaeriaceae*	33	MF580799.1	33
	Tiarosporella graminis	*Botryosphaeriaceae*	23	KF531828.1	55
	Chaetomella raphigera	*Chaetomellaceae*	33	KF193633.1	62
	Chaetomium sp., Chaetomium strumarium	*Chaetomiaceae*	24	KX618205.1, KY558668.1	54
	Pseudothielavia terricola, Chrysocorona lucknowensis, Acrophialophora jodhpurensis	*Chaetomiaceae*	27	NG_069813.1, MH877835.1, MH873474.1	49
	Pseudothielavia terricola, Chrysocorona lucknowensis, Acrophialophora jodhpurensis	*Chaetomiaceae*	33	NG_069813.1, MH877835.1, MH873474.1	64
	Cladosporium anthropophilum, Cladosporium cladosporioides	*Davidiellaceae*	34	MN511353.1, MK813966.1	3
	Cladosporium sp., Cladosporium cladosporioides, Cladosporium westerdijkieae	*Davidiellaceae*	39	MK355726.1, MT466517.1, MF473314.1	11
	Cladosporium sp., Cladosporium cladosporioides, Cladosporium westerdijkieae	*Davidiellaceae*	29	MK355726.1, MT466517.1, MF473314.1	12
	Cladosporium sp., Cladosporium cladosporioides, Cladosporium westerdijkieae	*Davidiellaceae*	31	MK355726.1, MT466517.1, MF473314.1	28

Cladosporium tenuissimum	*Davidiellaceae*	28	MF473304.1	37
Pseudocoleophoma polygonicola	*Dictyosporiaceae*	35	MZ492974.1	63
Didymella sp., Phoma sp., Didymella americana	*Didymellaceae*	28	MG967669.1, KY790596.1, MK646045.1	43
Ectophoma multirostrata	*Didymellaceae*	34	MG897497.1	29
Epicoccum sorghinum	*Didymellaceae*	25	MN215621.1	45
Phoma sp.	*Didymellaceae*	27	JN207257.1	10
Phoma herbarum, Epicoccum sorghinum	*Didymellaceae*	27	MT420621.1, MN944541.1	27
Phoma herbarum, Epicoccum sorghinum	*Didymellaceae, Didymellaceae*	29	MT420621.1, MN944541.1	24
Phoma sp., Boeremia sp., Aspergillus niger	*Didymellaceae, Didymellaceae, Trichocomaceae*	30	MK066907.1, MH931265.1, KT963790.1	9
Phoma sp., Didymella pinodella	*Didymellaceae*	35	JN207257.1, MW784722.1	61
Phoma sp., Didymella sp., Didymella pinodella	*Didymellaceae*	19	JN207257.1, MH257388.1, MW784722.1	47
Phoma sp., Epicoccum phragmospora	*Didymellaceae*	28	JN207257.1, NR_165920.1	52
Stagonosporopsis cucurbitacearum	*Didymellaceae*	10	GU045304.1	22
Trichoderma longibrachiatum	*Hypocreaceae*	26	KT852813.1	46
Trichoderma sp., Trichoderma erinaceum, Trichoderma koningiopsis	*Hypocreaceae*	22	MK870709.1, KC884817.1, FR670342.1	56
Pyrenochaeta sp.	*Incertae sedis*	34	EU750693.1	4
Pyrenochaeta sp.	*Incertae sedis*	36	EU750693.1	5
Fusarium chlamydosporum	*Nectriaceae*	36	MG250446.1	20
Fusarium chlamydosporum	*Nectriaceae*	23	KX421422.1	50
Fusarium chlamydosporum	*Nectriaceae*	22	MG250446.1	51
Fusarium equiseti	*Nectriaceae*	23	MT626672.1	13
Fusarium equiseti	*Nectriaceae*	23	MK780235.1	21
Fusarium oxysporum	*Nectriaceae*	39	MK249867.1	48
Fusarium sp., Fusarium chlamydosporum	*Nectriaceae*	39	MH582472.1, KX421422.1	59

Fusarium sp., Fusarium solani, Fusarium phaseoli	*Nectriaceae*	39	MK640565.1, KX583231.1, KF717534.1	31
Alternaria alternata	*Pleosporaceae*	29	MK773579.1	35
Alternaria sp.	*Pleosporaceae*	36	MW784825.1	60
Alternaria sp., Alternaria alternata	*Pleosporaceae*	19	MK640569.1, MK311341.1	15
Bipolaris zeae	*Pleosporaceae*	23	MT505870.1	1
Bipolaris sp., Helminthosporium bondarzewii, Curvularia spicifera	*Pleosporaceae, Massarinaceae, Pleosporaceae*	21	MF590167.1, MH877988.1, MH875110.1	18
Curvularia beasleyi, Curvularia hawaiiensis	*Pleosporaceae*	28	MH414893.1, KC999927.1	8
Curvularia petersonii	*Pleosporaceae*	33	NR_158448.1	34
Exserohilum rostratum, Setosphaeria rostrata, Bipolaris maydis	*Pleosporaceae*	22	MT524320.1, LT837845.1, MH197141.1	36
Exserohilum rostratum, Setosphaeria rostrata, Bipolaris maydis	*Pleosporaceae*	20	MT524320.1, LT837845.1, MH197141.1	39
Periconia sp., Periconia macrospinosa	*Pleosporaceae*	10	HQ607981.1, MT658103.1	6
Neopyrenochaeta telephoni	*Pleosporineae*	35	KM516291.1	65
Westerdykella ornata	*Sporormiaceae*	24	NR_103587.1	2
Westerdykella sp., Westerdykella centenaria	*Sporormiaceae*	26	MG250473.1, NR_156002.1	44
Albifimbria viridis	*Stachybotryaceae*	35	MN625931.1	19
Stachybotrys microspora	*Stachybotryaceae*	32	MK956918.1	66
Aspergillus flavus	*Trichocomaceae*	29	MT645322.1	38
Aspergillus sp., Aspergillus costaricensis, Aspergillus tubingensis	*Trichocomaceae*	33	MT447519.1, MT558927.1, MN067891.1	40
Aspergillus terreus	*Trichocomaceae*	36	MT316343.1	17
Aspergillus terreus	*Trichocomaceae*	28	MT316343.1	58
Aspergillus welwitschiae, Aspergillus niger	*Trichocomaceae*	22	MK450669.1, MT620753.1	26
Penicillium sp., Penicillium magnielliptisporum	*Trichocomaceae*	39	MT424930.1, MK450730.1	14
Coprinellus radians	*Psathyrellaceae*	21	JN943117.1	25

Basidio-mycota	*Fomitopsis meliae*	*Fomitopsidaceae*	13	KT718002.1	32
	Peniophora sp.	*Peniophoraceae*	19	HQ607800.1	42
	Physisporinus sp., Vanderbylia fraxinea, Physisporinus vitreus	*Meripilaceae, Polyporaceae, Meripilaceae*	20	KU958554.1, KX081102.1, KU194320.1	7
	Terana caerulea	*Phanerochaetaceae*	20	KP134980.1	53
Mucoro-mycota	*Mucor fragilis*	*Mucoraceae*	35	MK910073.1	30
	Rhizopus microsporus	*Mucoraceae*	22	KJ417579.1	57

3.3.4 Identification of fungal isolates

Of the sixty-six isolates, 89.39% belonged to the phylum *Ascomycota*, 7.58% to *Basidiomycota*, and 3.03% to *Mucoromycota*. The pie chart in Figure 3.2 does not represent all sixty-six isolates, as only sixty-three isolates were used to represent the phyla and families of the isolates in the pie chart. This was due to the omission of isolates no. 7, 9, and 18 (Table 3.2), as the families to which they belong to were uncertain. The percentages of phyla in the present study are similar to the study performed by Gupta and Khan (2018), where the majority of isolates belonged to *Ascomycota* with a few belonging to *Mucoromycota*. In a study by Held *et al.* (2020), fungi were isolated from various samples from an underground iron ore mine where out of the 164 fungal isolates identified, 65.85% were *Ascomycota*, 18.90% were *Mucoromycota*, and 15.85% were *Basidiomycota*. Therefore, the findings in the present study regarding *Ascomycota* being the major group of fungi is similar to previous studies of fungi in mine and metal-contaminated sites (Al-Garni *et al.*, 2009; Bahobil *et al.*, 2017; Ezzouhri *et al.*, 2009; Gupta and Khan, 2018; Held *et al.*, 2020; Iram *et al.*, 2012; Siham, 2007). This is likely due to their organic acid (OA) production, biosorption or bioaccumulation abilities which helps them tolerate these heavy metal-containing environments.

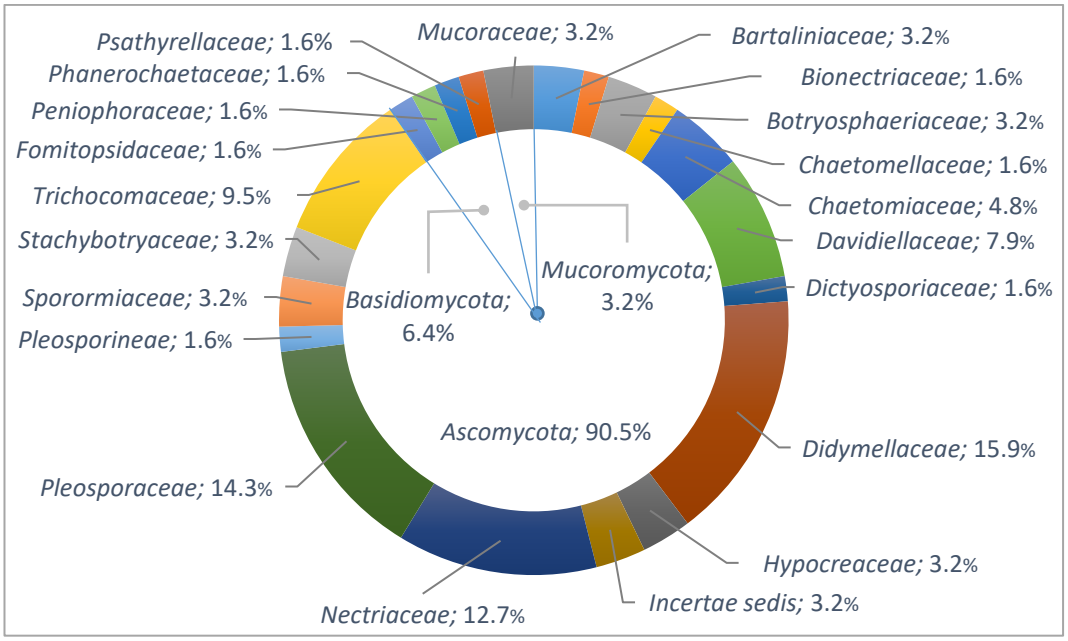

Figure 3.2: Percentage of sixty-three fungal isolates belonging to their respective families and phyla. There was more than one possibility for the families of isolates no. 7, 9, and 18, therefore these isolates were excluded from the pie chart. However, isolates no. 9 and 18 are *Ascomycota* and isolate 7 is a member of *Basidiomycota*. Therefore, when considering the phyla's percentages regarding the sixty-six isolates, 89.39% belonged to the phylum *Ascomycota,* 7.58% to *Basidiomycota*, and 3.03% to *Mucoromycota*.

The identified isolates were members of several different families of *Ascomycota*, *Basidiomycota*, and *Mucoromycota* (Table 3.2 and Figure 3.2). The genera of isolates no. 16, 23, 41, 33, 55, 62, 49, 64, 63, 29, 7, 53, 65, 2, 44, and 19 (Table 3.2) which are members of the families, *Bartaliniaceae, Bartaliniaceae, Bionectriaceae, Botryosphaeriaceae, Botryosphaeriaceae, Chaetomellaceae, Chaetomiaceae, Chaetomiaceae, Dictyosporiaceae, Didymellaceae, Meripilaceae/Polyporaceae, Phanerochaetaceae, Pleosporineae, Sporormiaceae, Sporormiaceae,* and *Stachybotryaceae*, respectively, have not been previously identified in a mine, metal-contaminated site, or in any relevant studies where fungal heavy metal tolerance or OA production was tested. Most of the isolates identified in the present study have been known to be endophytes or phytopathogens, therefore, these newly identified genera from the platinum mine implies that these isolates

were able to develop adaptive qualities to survive in such an environment which has not previously been reported for such genera.

3.3.4.1 *Ascomycota*

Isolate no. 54 (Table 3.2) is a member of the family *Chaetomiaceae*. Species of *Chaetomium* have been identified by Tulsiyan *et al.* (2017), where they isolated fungi from coal mine samples to determine fungal diversity and the optimum coal powder concentration for fungal growth. The amount of coal powder which the different species of *Chaetomium* could optimally grow in the presence of ranged between 5 g and 10 g per PDA plate. Shindia *et al*. (2006) isolated fungi from Egyptian soil and sugar cane waste samples to determine their gluconic acid production and found that *Chaetomium* sp. was not capable of producing the acid. Li *et al*. (2016) isolated fungi from plants taken from the wasteland and slag heap of a Pb-Zn mining site and determined fungal tolerance to Pb, Zn and Cd. In their study, *Chaetomium globosum* was identified from the wasteland and was found to only be tolerant to Pb and Cd (Li *et al.*, 2016).

There were five isolates identified as *Cladosporium*, namely isolates no. 3, 11, 12, 28 and 37 (Table 3.2), which are members of the family *Davidiellaceae*. *Cladosporium* spp. have also been isolated by Li *et al.* (2016) from the wasteland and slag heap and were found to be tolerant to Pb and Cd. *Cladosporium cladosporioides* and *C. herbarum* were isolated by Shindia *et al.* (2006) and were determined to not produce gluconic acid and *Cladosporium* spp. was identified by Tulsiyan *et al.* (2017) and its optimum growth was determined to be in the presence of 5 g per PDA plate of coal powder. Văcar *et al*. (2021) isolated and identified fungi from the rhizosphere of a plant located in a mercury (Hg) contaminated site and determined fungal tolerance to Cu, Cd, Hg, Pb, and Zn, to assess fungal Hg remediation capabilities. In their study, *Cladosporium* sp. was identified and determined to be tolerant to Cu and Hg (Văcar *et al.*, 2021). Al-Garni *et al*. (2009) isolated fungi from soil contaminated with industrial waste and determined fungal tolerance to Cd to select highly tolerant species for biosorption

of Cd from an aqueous solution. In their study, *C. cladosporioides* was identified and was found to be tolerant to Cd (Al-Garni *et al.*, 2009). *Cladosporium cladosporioides* was also isolated from samples from a sewage treatment plant, and it was determined to be tolerant to Cd and Hg (Bahobil *et al.*, 2017). Bahobil *et al*. (2017) aimed to determine fungal tolerance to the mentioned heavy metals to identify isolates with the greatest potential for the biosorption of Cd and Hg. Shankar and Sivakumar (2016), isolated fungi from leaf litter soil samples to determine their citric acid production and determined that *Cladosporium* sp. could produce this OA. Ngo *et al.* (2021) identified *Cladosporium tenuissimum* from the eyepieces of binoculars obtained from a museum. The identification of fungi in their study was important to develop modern glasses resistant to harmful fungi due to fungal deterioration of non-metallic materials (Ngo *et al.*, 2021).

Eleven possible isolates were members of the family *Didymellaceae*, namely isolates no. 22, 29, 43, 45, 10, 27, 47, 52, 61, 24, and possibly isolate 9 (Table 3.2). Strains of isolate no. 22 have not been identified from any relevant studies, however *Stagonosporopsis* sp. was identified in the study by Văcar *et al.* (2021) where it was determined to be tolerant to Cd, Hg, Pb, and Zn. *Phoma costaricensis* and *Didymella glomerata* were also isolated by Văcar *et al.* (2021) and were determined to be tolerant to Hg, Pb, and Zn and *P. costaricensis* was also found to tolerate Cu. Gupta and Khan (2018) identified fungal isolates from soil samples retrieved from various mine sites and a steel plant to determine fungal isolates capable of being used in biohydrometallurgical processes based on their OA production. In their study, species of *Phoma* were identified, however, they were not found to produce OAs (Gupta and Khan, 2018). Strains of isolate no. 45 have not been identified in any relevant studies, however, *Epicoccum nigram* and *Phoma* sp. were isolated by Li *et al.* (2016) where *E. nirgam* was determined to be tolerant to Pb and Cd whereas *Phoma* sp. was only tolerant to Cd. *Phoma herbarum* and *E. nigram* were identified from soapstone sculptures where their ability to produce the OAs, oxalic, malic, citric, acetic, butyric, fumaric and succinic acid, was evaluated (Boniek *et al.*, 2017). They were found to produce all the

aforementioned OAs which are associated with the deterioration of the sculptures (Boniek *et al.*, 2017).

Isolates no. 46 and 56 (Table 3.2) are members of the family *Hypocreaceae* and were both identified as part of the genus *Trichoderma*. This genus has often been isolated from mining sites. Species of *Trichoderma* have also been isolated in the previously mentioned studies (Al-Garni *et al.*, 2009; Bahobil *et al.,* 2017; Ngo *et al.,* 2021; Shindia *et al.,* 2006). In the study by Al-Garni *et al.* (2009), *T. longibrachiatum* was determined to be tolerant to Cd and Bahobil *et al.* (2017) determined *T. viride* to be tolerant to Hg. Ngo *et al.* (2021) isolated *T. koningiopsis* and Shindia *et al.* (2006) isolated species of *Trichoderma* which were determined to not produce gluconic acid.

There were two strains of *Pyrenochaeta sp.* identified, isolates no. 4 and 5 (Table 3.2). However, the family to which this genus is a member is unknown and hence it is referred to as *incertae sedis* (Kowalski and Bilański, 2021). *Pyrenochaeta sp.* has previously been identified as an Mn(II)-oxidising fungus from passive coal mine drainage systems (Santelli *et al.*, 2010).

A total of eight isolates were identified as *Fusarium*, namely isolates no. 13, 20. 21, 31, 48, 50, 51, and 59 (Table 3.2). *Fusarium* was the only genera identified as a member of the family *Nectriaceae*. Previous studies have also isolated and identified *Fusarium* species (Al-Garni *et al.*, 2009; Bahobil *et al.,* 2017; Li *et al.,* 2016; Văcar *et al.,* 2021; Shindia *et al.,* 2006; Gupta and Khan, 2018). *Fusarium oxysporum* and *F. chlamydosporum* were found to be tolerant to Cd (Al-Garni *et al*., 2009) and Hg (Bahobil *et al*., 2017), respectively. *Fusarium* sp. was determined to be tolerant to Pb, Zn, and Cd in the study by Li *et al.* (2016), and *F. solani*, *F. equiseti*, and *F. oxysporum* were determined to be tolerant to Cd, Cu, Hg, Pb, and Zn in the study by Văcar *et al.* (2021). Additionally, *F. solani* was identified by Shindia *et al.* (2006) and was found to not produce gluconic acid. In a study by Siham (2007), fungi were isolated from soil samples obtained from an Electric Meter manufacturing facility where fungal tolerance to Pb and Cu was

determined. *Fusarium equiseti* and *F. solani* were identified in the study and were determined to be tolerant to Pb and Cu (Siham, 2007). Ezzourhi *et al.* (2009) isolated fungi from heavy metal contaminated sites in the Moghogha river (Tangier, Morocco) and the isolates tolerance to Pb, Cr, Cu, Zn, and Cd was determined. *Fusarium* spp. were identified in their study and found to be tolerant to Pb, Cr, Cu, and Zn (Ezzouhri *et al.*, 2009). Fungi were isolated from soil irrigated with industrial wastewater in the study by Iram *et al.* (2012) and the isolates tolerance to Cr and Pb was determined. From the study, *Fusarium* spp. were identified and were found to be tolerant to both the metals (Iram *et al.*, 2012).

There were ten isolates identified which are possibly members of the family *Pleosporaceae*, isolates no. 1, 6, 8, 15, 34, 35, 36, 39, 60, and possibly isolate 18 (Table 3.2), as isolate no. 18 could either be a member of *Pleosporaceae* or *Massarinaceae*. Species of *Alternaria* and *Curvularia* are often isolated from heavy metal contaminated sites. Species of *Alternaria* have been isolated from the previously mentioned studies (Bahobil *et al.,* 2017; Ezzouhri *et al.,* 2009; Gupta and Khan, 2018; Li *et al.,* 2016; Shankar and Sivakumar, 2016; Shindia *et al.*, 2006; Siham, 2007). The species of *Curvularia* which were identified in the present study have not previously been isolated from mine or metal-contaminated sites to the best of my knowledge, however, a commonly isolated species of *Curvularia* is *C. lunata*. This species has been isolated in the previously mentioned studies (Bahobil *et al.,* 2017; Boniek *et al.,* 2017; Ngo *et al.*, 2021; Tulsiyan *et al*., 2017). *Helminthosporium* sp. has been isolated in the studies by Iram *et al.* (2012) and Shankar and Sivakumar (2016) where it was determined to be tolerant to Cr and Pb and to produce citric acid, respectively. Species from the genus *Bipolaris* have not been identified from mine or metal contaminated sites, however, *B. sorokiniana* was identified in the study by Boniek *et al.* (2017) and found to produce the OAs oxalic, malic, citric, acetic, butyric, fumaric, and succinic acid. Species that belong to the genus *Periconia*, including *P. macrospinosa* were also identified by Boniek *et al.* (2017) and were found to produce the mentioned OAs.

Setosphaeria rostrata was isolated in the study performed by Al-Garni *et al.* (2009) and was determined to be tolerant to Cd.

Isolate no. 66 (Table 3.2) is a member of the family *Stachybotryaceae*. Strains of this isolate have not previously been identified from mine or metal-contaminated sites. However, *Stachybotrys chartarum* has been isolated from tin mine tailings soil samples (Ding *et al.*, 2018).

Trichocomaceae is the last family which will be discussed that is a part of the phylum *Ascomycota*. The isolates no. 14, 17, 26, 38, 40, 58, and possibly isolate no. 9 (Table 3.2), are members of this family. The genera *Penicillium* and *Aspergillus* are often isolated from mine and metal-contaminated sites. The exact identity of isolate no. 14 was not specified as it could be *P. magnielliptisporum* or another species of *Penicillium*. *Penicillium magnielliptisporum* has not been isolated from any mine or metal contaminated site, however other species from this genus have been isolated in the previously mentioned studies (Al-Garni *et al.,* 2009; Bahobil *et al.,* 2017; Boniek *et al*., 2017; Ezzouhri *et al.*, 2009; Gupta and Khan, 2018; Li *et al.,* 2016; Ngo *et al.*, 2021; Shankar and Sivakumar, 2016; Shindia *et al.,* 2006; Siham, 2007; Tulsiyan *et al*., 2017; Văcar *et al*., 2021). They were determined to be tolerant to a variety of different heavy metals and were found to produce several OAs. *Aspergillus* is also known for its heavy metal tolerance and production of OAs and with regards to the species of *Aspergillus* which were identified in the present study, *A. welwitschiae* and *A. costaricensis* have not been identified from any mine or metal-contaminated sites. *Aspergillus niger* was also identified in the previously mentioned studies (Al-Garni *et al.*, 2009; Bahobil *et al.*, 2017; Ezzhouri *et al.*, 2009; Iram *et al.*, 2012; Shankar and Sivakumar, 2016; Shindia *et al.*, 2006; Siham, 2007). *Aspergillus terreus* was identified in the studies by Al-Garni *et al.* (2009) and Shindia *et al.* (2006), and *A. tubingensis* was isolated from metal contaminated soil in a study by Din *et al.* (2020) where it was determined to be tolerant to cobalt (Co), nickel (Ni) Cd, Cu, and Pb and to produce the OAs, oxalic, gluconic, and fumaric acid. *Aspergillus flavus* was isolated in the studies by Al-Garni *et al.* (2009), Iram *et al.* (2012), Shankar and Sivakumar (2016), and Shindia

et al. (2006). However, after a literature search on the isolate, certain strains were found to produce aflatoxins which are naturally occurring mycotoxins (Saito and Machida, 1999). Therefore, the use of isolate no. 38 in further experiments was halted due to the possible production of this mycotoxin. As a result, sixty-five isolates were used in further experiments, instead of sixty-six isolates.

3.3.4.2 *Basidiomycota*

Five *Basidiomycetes* were identified, and each was a member of a different family. Isolates no. 32, 25, 42, and 53 (Table 3.2) are members of the families *Fomitopsidaceae*, *Psathyrellaceae*, *Peniophoraceae*, and *Phanerochaetaceae*, respectively. Isolate no. 7 (Table 3.2) is possibly a member of the family *Meripilaceae* or *Polyporaceae*. These five isolates are all wood-decaying fungi (Bakir *et al.*, 2021; Cartabia *et al.*, 2021; Mahawaththage Dona *et al.*, 2019; Oliver *et al.*, 2010; Patel *et al.*, 2021; Wolfaardt *et al.*, 2004). A strain of isolate no. 32 was previously identified from a gold mine site where it was determined to be tolerant to Cu, Pb, and Fe (Oladipo *et al.*, 2018). A strain of isolate no. 42 has previously been isolated from soil contaminated with Pb from a shooting range and it was found to produce oxalic, citric, malonic, and formic acid and determined to solubilise Pb (Sullivan *et al.*, 2012). A strain of isolate no. 25 has been identified in the study by Boniek *et al.*, (2017) and determined to produce oxalic, malic, citric, acetic, butyric, fumaric, and succinic acid. It has also been identified in the study by Ngo *et al.* (2021).

3.3.4.3 *Mucoromycota*

Isolates no. 30 and 57 (Table 3.2) are both members of the family *Mucoraceae*. Strains of isolate no. 30 have not previously been identified in mining environments, however, species from the genus *Mucor* have been isolated in the previously mentioned studies (Bahobil *et al.*, 2017; Gupta and Khan, 2018; Li *et al.*, 2016; Shindia *et al.,* 2006; Siham, 2007). *Mucor sp*. was also isolated in a study by Zahoor *et al.* (2017) from plants grown on heavy metal contaminated soil and

was identified due to its tolerance to multiple heavy metals which include, Mn, Zn, Cr, Co, and Cu. A strain of isolate no. 57 was also identified by Oladipo *et al*. (2018) where it was isolated from a gemstone mining site and found to tolerate Cu, Pb, Fe, Cd, and arsenic (As).

3.4 Summary and Conclusions

To determine the physicochemical and fungal characteristics of the samples obtained from a platinum mine, the pH and redox potential of all samples were measured, and fungi were enumerated. The pH of the samples ranged from slightly acidic (6.78) to alkaline (9.27) and the Eh ranged from 26 to -103 mV, implying that the sample environment was a highly reducing environment. The fungal counts among all the samples retrieved from the mine ranged between 0 to 1.2 x 10^4 CFU/ml or CFU/g of sample.

It was concluded that the effect of pH on fungal growth was difficult to infer as similar pH's of samples taken from the same region had drastically different fungal counts, therefore, the differences in fungal counts could be due to the variation of heavy metal concentrations, nutrient availability, and microbial communities among all the samples. To verify this, future work could involve determining the nutrient concentrations, heavy metal concentration and the microbial community of the samples from the mine.

In terms of the identification of the pure fungal cultures obtained after repeated sub-culturing, the majority of isolates identified were members of the phylum *Ascomycota* which corresponded to previous studies involving the isolation of fungi from mine and metal-contaminated sites. The large number of *Ascomycetes* is likely due to their ability to tolerate heavy metals. Several isolates which were identified had not previously been identified in any mine, metal-contaminated site, or in any relevant studies where fungal heavy metal tolerance or OA production was tested. However, species from the genera *Cladosporium, Fusarium*, *Trichoderma*, *Penicillium,* and *Aspergillus* were identified in the present

study which corresponded to various studies involving fungi from mine and metal-contaminated sites.

4 CHARACTERISATION OF FUNGI

4.1 Introduction

Bioleaching is a mycohydrometallurgical process which fungi use to extract and solubilise heavy metals from ore, concentrate, and waste materials (Ofori-Sarpong *et al*., 2010). The main mechanism of fungal bioleaching is acidolysis which takes place due to the production of LMWOAs by fungi (Dusengemungu *et al*., 2021). Fungi have been isolated from mine and metal-contaminated environments and have exhibited this OA-producing characteristic due to its role in fungal adaption to heavy metal concentrated environments (Din *et al*., 2020). Therefore, the characterisation of fungi isolated from a mine based on their OA production is important to determine which isolates have the potential to be used in bioleaching (Gupta and Khan, 2018).

Additional mechanisms which fungi use for bioleaching are biosorption and bioaccumulation which require the presence of fungal biomass to occur (Dusengemungu *et al*., 2021). Therefore, understanding fungal heavy metal tolerance capabilities are important in determining the bioleaching method which should be considered as either an indirect or direct approach could be taken, where the latter requires fungal exposure to the solid material undergoing bioleaching (Valix and Loon, 2003).

Due to the OA production by fungal isolates from a platinum mine not previously being studied, the present study aimed to qualitatively determine OA production of sixty-five fungal isolates from a platinum mine, to quantitatively determine the production of LMWOAs of selected isolates and to determine the heavy metal tolerance of high OA-producing isolates.

4.2 Materials and Methods

4.2.1 Qualitative screening

Sixty-five fungal isolates from a platinum mine were identified and are listed in Table 3.2. To determine their capability of producing OAs, qualitative OA screening

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 and 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).

4.2.2 Quantitative screening

The thirteen isolates which resulted in an AU value of ≥ 1 were further characterised to identify and quantify the OAs which they produce. The isolates were inoculated in 20 ml of Potato Dextrose Broth (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. After the incubation period, 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% H_2SO_4) 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.

4.2.3 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 with PGMs in PGM concentrate. Cu and Ni were used as chloride salts, $CuCl_2.2H_2O$ and $NiCl_2.6H_2O$, respectively, and Cr was used as $K_2Cr_2O_7$ (Ghosh and Paul, 2015). A 10 000 mg/L stock solution of these

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 25^{o}C for 7 days. The TI of the isolates to each metal was determined according to the methodology followed by Oladipo *et al*. (2018).

4.2.4 Statistical analysis

One-way analysis of variance (ANOVA) followed by a comparison using Duncan's Multiple Range Test at $P < 0.05$ was used to compare means for the acid unitage values and heavy metal TI between isolates ($n = 3$).

4.3 Results and Discussion

4.3.1 pH indicator test

The pH indicator test allowed for the quick screening of OA producing isolates and the results for the sixty-five isolates is shown in Figure 4.1. Out of the sixty-five fungal isolates, fifteen showed a positive result due to the formation of a yellow zone around the colony indicating a decrease in pH due to OA production. The AU values calculated for the fifteen isolates are shown in Table 4.1 and are representative of the quantity of OAs produced relative to the size of the colony. The isolates with an AU value of ≥ 1 were selected for further characterisation as this indicated that these isolates produced the highest amount of OAs. The method used for qualitative screening has been used in a previous study to determine which of the three OA producing *Aspergillus* species isolated in the study, should be selected for further characterisation and be used for bioleaching (Din *et al.*, 2020). The strain which had the highest AU value along with the greatest pH decrease and highest OA concentration was then selected and identified as *Aspergillus tubingensis* (Din *et al.*, 2020). The qualitative test in the

present study proved to be an efficient and quick screening method for the identification of OA producing isolates out of a large number of isolates tested.

The OA production by the genera of isolates no. 1, 2, 4, 5, 7, 16, 19, 22, 23, 29, 33, 36, 39, 41, 44, 49, 53, 55, 62, 63, 64, 65, and 66 (Table 3.2) has not previously been reported. Therefore, the ability of these isolates to produce OAs was unknown, however, isolates no. 7, 53, and 62 displayed this ability. The AU value for isolates no. 7 and 53 was significantly lower than the other thirteen isolates as it was below 1, therefore, these isolates were not considered for further characterisation. However, the AU values for isolate no. 62 was above 1 and was therefore selected for further characterisation.

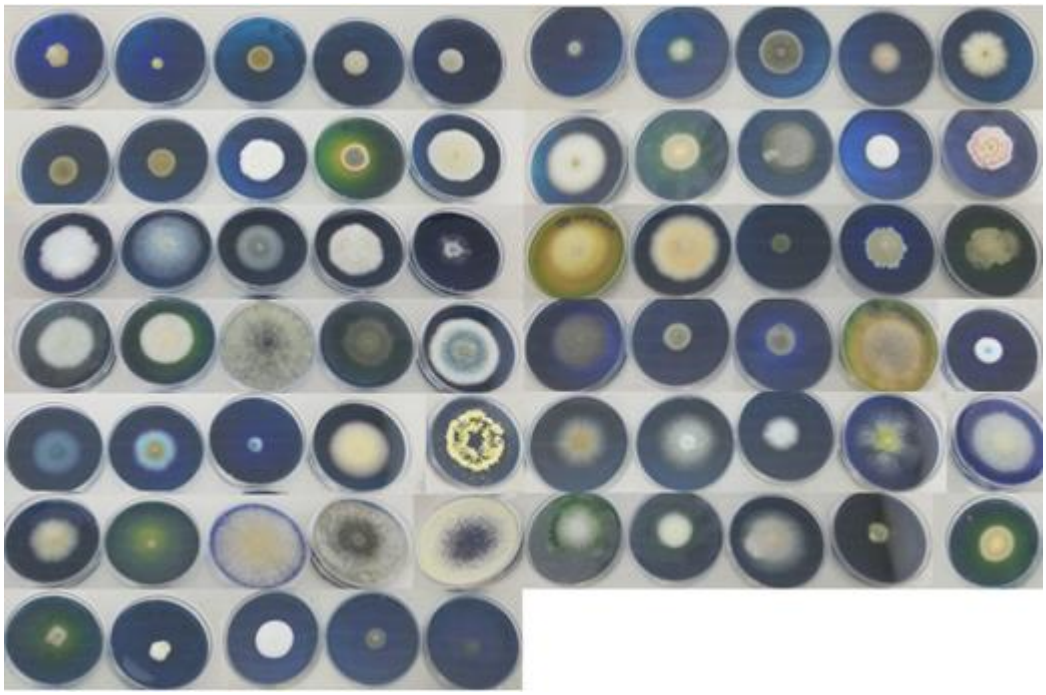

Figure 4.1: Qualitative screening for organic acid (OA) production. Fungal isolate numbers are arranged from 1 – 37 and 39 – 66 (From left to right – top to bottom). The yellow zone formed due to OA production can be clearly seen for isolates no. 14, 17, 26, 30, 32, 40, 53, 57, 61, and 62. However, Isolates no. 7, 31, 34, 58, and 60, also resulted in the formation of yellow zones which are difficult to observe in the figure.

4.3.1.1 *Ascomycota*

The results obtained (Figure 4.1) for isolate no. 54 (Table 3.2) which is part of the genus *Chaetomium* corresponded to those obtained in the study by Shindia *et al*. (2006). The study indicated that the total acidity of two species of the genus was not detected due to the absence of OA production (Shindia *et al.*, 2006). The same was observed in the present study as a yellow zone was not formed by the colony.

In previous studies, the species of *Cladosporium* identified showed the least amount of total acidity compared to all identified isolates, or acidity was not detected (Ahmed *et al.*, 2015; Shindia *et al.*, 2006). Therefore, the results in the present study corresponds to the mentioned studies as none of the *Cladosporium* isolates (Table 3.2) resulted in a yellow zone formation.

The only isolate which was a member of the *Didymellaceae* family (Table 3.2) that displayed OA production was isolate no. 61. There are a few studies which have determined that species of *Phoma* were capable of producing OAs, such as the studies by Boniek *et al*. (2017) and Palmer *et al*. (1991). Isolate no. 9 which was identified as either *Phoma* sp., *Boeremia* sp., or *Aspergillus niger* is likely not *A. niger* due to all other *Aspergillus* isolates (Table 3.2) tested having displayed OA production.

The *Trichoderma* isolates (Table 3.2) produced negative results, despite their frequent isolation from mine and metal-contaminated sites. Therefore indicating that their tolerance to heavy metals is likely due to their bioaccumulation abilities.

The production of OAs by *Fusarium chlamydosporum* and *F. equiseti* has not previously been investigated. However, due to the occurrence of *Fusarium* species in multiple studies involving their isolation from metal-contaminated environments and the study of *Fusarium* species for their OA production, the production of OAs by *Fusarium* isolates (Table 3.2) was expected (Ahmed *et al.*, 2015; Akintokun *et al*., 2007; Al-Garni *et al.*, 2009; Ezzouhri *et al.*, 2009; Farooq *et al*., 2015; Iram *et al.*, 2012; Li *et al.*, 2016; Shindia *et al.*, 2006; Siham, 2007; Văcar

et al., 2021). However, despite the results from previous studies, only one *Fusarium* isolate indicated OA production, namely, isolate no. 31 (Table 3.2). Previous studies which involved the quantification of OAs from *Fusarium solani* showed high amounts of acidity in the medium the isolate was grown in (Ahmed *et al.*, 2015; Shindia *et al.*, 2006). Therefore, the results ontained for isolate no. 31, corresponded to the mentioned studies and the other *Fusarium* isolates may not have produced OAs as the pH, temperature, and medium they were grown in may not have been optimal for OA production by these strains of *Fusarium*.

Species of *Periconia*, including *P. macrospinosa,* were identified in a previous study and determined to produce several OAs, however, this was not the case for isolate no. 25 (Table 3.2) in the present study (Boniek *et al.*, 2017). The OA production for the *Curvularia* isolates identified has not been previously tested. However, isolate no. 34 (Table 3.2) did produce a slightly yellow zone and had an AU value of 1.16 ± 0.08 (Table 4.1). The species of *Curvularia* which has commonly indicated its OA production is *C. lunata,* which was not isolated in the present study (Boniek *et al.*, 2017; Farooq *et al.*, 2015; Ngo *et al.*, 2021). Species of *Alternaria* have also frequently indicated their OA production, including *Alternaria alternata*, however, in the present study, the only OA-producing *Alternaria* isolate was isolate no. 60 (Table 3.2) (Farooq *et al.*, 2015; Palmer *et al.*, 1991; Shankar and Sivakumar, 2016). Low levels of total acidity which were similar to those displayed by *Cladosporium* in previous studies, were also displayed by *Alternaria* species (Ahmed *et al.*, 2015; Shindia *et al.*, 2006). Therefore, although *Alternaria* has been found to produce OAs, the levels of OA production may be low which could explain the results for isolates no. 15 and 35 (Table 3.2).

The five *Trichocomaceae* isolates (Table 3.2) all displayed OA production. This was expected due to the number of studies which have isolated species of *Aspergillus* and *Penicillium* from mine and metal-contaminated sites and have determined their OA production (Ahmed *et al.*, 2015; Akintokun *et al.*, 2007; Palmer *et al.*, 1991; Shankar and Sivakumar, 2016; Shindia *et al.*, 2006).

4.3.1.2 *Basidiomycota*

Despite *Corpinellus radians* OA producing ability in previous studies, isolate no. 25 (Table 3.2) did not display this ability (Boniek *et al.*, 2017; Ngo *et al.*, 2021). This was also observed for isolate no. 42 (Table 3.2), whereas in a previous study, *Peniophora* sp. produced over 10 mM of OAs (Sullivan *et al.*, 2012). Isolate no. 32 (Table 3.2) has not been previously tested for its OA production but displayed its production in the present study with an AU value of 1.16 ± 0.02 (Table 4.1). A different species from the same genera, *Fomitopsis palustris,* has previously been tested for its ability to produce oxalic acid and was found to produce high levels of the acid (Choi *et al.*, 2014).

4.3.1.3 *Mucoromycota*

The results obtained for isolates no. 30 and 57 (Table 3.2) corresponded with those of different species from the genera *Mucor* and *Rhizopus* from previous studies. As *Mucor racemosus* and *Rhizopus oryzae* have been found to produce high amounts of OAs (Ahmed *et al.*, 2015; Akintokun *et al.*, 2007; Farooq *et al.*, 2015; Shindia *et al.*, 2006).

Table 4.1: Acid unitage (AU) values calculated for the fifteen organic acid-producing fungal isolates.

Isolate no.	Isolate name	Colony zone (mm)	Yellow zone (mm)	Acid unitage (AU)
7	*Physisporinus sp., Vanderbylia fraxinea, P. vitreus*	24.67 ± 1.15	20 ± 6.93	0.82 ± 0.31 e
14	*Penicillium sp., P. magnielliptisporum*	31.83 ± 3.25	65.33 ± 10.41	2.05 ± 0.14 a
17	*Aspergillus terreus*	39.17 ± 0.29	48.33 ± 0.58	1.23 ± 0.01 cd
26	*Aspergillus welwitschiae, A. niger*	64.33 ± 2.08	85 ± 0	1.32 ± 0.04 bc
30	*Mucor fragilis*	41.33 ± 8.08	51.67 ± 5.86	1.26 ± 0.13 cd
31	*Fusarium sp., F. solani, F. phaseoli*	55.67 ± 1.53	59 ± 2	1.06 ± 0.01 d
32	*Fomitopsis meliae*	53.67 ± 4.16	62.33 ± 5.51	1.16 ± 0.02 cd
34	*Curvularia petersonii*	52.5 ± 3.28	61 ± 7	1.16 ± 0.08 cd
40	*Aspergillus sp., A. costaricensis, A. tubingensis*	66.33 ± 0.58	84 ± 1.73	1.27 ± 0.02 cd
53	*Terana caerulea*	85 ± 0	57 ± 13.08	0.67 ± 0.16 e
57	*Rhizopus microsporus*	30.5 ± 8.05	68.33 ± 16.78	2.25 ± 0.10 a
58	*Aspergillus terreus*	40.83 ± 4.54	50 ± 5.57	1.22 ± 0 cd
60	*Alternaria sp.*	24.33 ± 2.52	31 ± 4.58	1.27 ± 0.05 cd
61	*Phoma sp., Didymella pinodella*	40.67 ± 1.53	63 ± 9.54	1.53 ± 0.20 b
62	*Chaetomella raphigera*	25.33 ± 4.04	38 ± 3.46	1.51 ± 0.10 b

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

4.3.2 High-performance liquid chromatography

The HPLC chromatogram for the standards is shown in Figure B1 (Appendix B) and the pH values measured and HPLC results for the thirteen selected isolates are shown in Table 4.2. HPLC analysis of the samples resulted in unknown peaks in the chromatogram of all isolates samples and an example of this is shown by isolate no. 58 in Figure B2 (Appendix B) where four unknown peaks were detected. In future studies, the OAs which could also potentially be detected could be fumaric, malic, oxalic, succinic, and tartaric acid as fungi have also been known to produce these OAs. Therefore, one of these OAs were likely produced and resulted in these unknown peaks (Liaud *et al.*, 2014). Isolate no. 26 resulted in the greatest change in pH from 4.8 to 3.07 and the lowest change in pH was measured for isolate no. 40 from 4.8 to 4.28. With regards to the HPLC results, all isolates produced IAA and the highest amount of IAA was detected from isolate no. 32. Isolate no. 57 was the only isolate which produced butyric acid (10.7 mM) and acetic acid (1.18 mM) and none of the isolates produced citric and gluconic acid. Isolates no. 14, 17, 26, 30, 31, 34, 40, 58, 60, and 61, produced lactic acid with the highest amount (9.7 mM) being produced by isolate no. 58. Propionic acid was produced by isolates no. 14, 30, 31, 60, and 61 and the highest amount (1.57 mM) was produced by isolate no. 14. Formic acid was produced by the mentioned isolates which produced propionic acid, including isolate no. 57 and the highest amount of formic acid (2.15 mM) was produced by isolate no. 30.

OA production varies between genera, species and different strains from the same species and factors which influence OA production include, growth media, carbon source, pH, temperature, and incubation period for fungal growth (Dusengemungu *et al.*, 2021). Table 4.3 summarises studies which also tested for the OAs quantified in the present study and the concentrations of the acids produced by the fungal strains in the mentioned studies were converted to mM to compare the concentrations to the present study.

The production of gluconic and citric acid was not detected for any of the isolates tested in the present study, however, Table 4.3 lists fungi that belong to the genera *Alternaria, Aspergillus, Curvularia,* and *Penicillium* whose production of the mentioned OAs was detected. From the isolates listed in Table 4.3, the difference in OA production among the same strain of fungi exposed to different growth media, as well as the difference among different strains of the same species grown in the same media can be clearly seen. For example, in the study by Akintokun *et al.* (2007), *Aspergillus niger*, *A. terreus,* and *Penicillium chernestinum* produced varying concentrations of gluconic acid when exposed to phosphate rock and tri-calcium phosphate (Table 4.3). When exposed to phosphate rock, all three fungi produced citric acid, however, none of the fungi produced citric acid when exposed to tri-calcium phosphate (Table 4.3). Another example of varying OA production by the same strain exposed to different growth media and parameters can be seen for *Penicillium* sp. strain JM-2008 (Table 4.3) (Sullivan *et al.*, 2012). The difference in OA production among different strains of the same species can be seen in Table 4.3, when comparing the OA production by two strains of *C. lunata* in the study by Boniek *et al.* (2017). Their concentrations of butyric, acetic, and citric acid differed despite being of the same species and being grown in the same medium and exposed to the same parameters.

The isolates in Table 4.2 which all produced IAA, are either endophytes or phytopathogenic fungi. These types of fungi are known for their production of IAA due to the stimulation of plant growth when produced by endophytic fungi or to cause disease and interfere with the plants' defence system when produced by phytopathogenic fungi. In 2018, Numponsak *et al.* conducted a study where they determined IAA production by the endophytic fungi *Colletotrichum fructicola*, where it was determined to produce 6.88 ± 0.87 mM of IAA (Table 4.3). They compared this concentration to previous studies involving the production of IAA by fungi and found that this was the highest amount of IAA produced among all studies at the time (Numponsak *et al.*, 2018).

Table 4.2: The pH and organic acid (OA) concentration (mM) of Potato Dextrose Broth (pH 4.8) inoculated with fungal isolates after 7 days of incubation at 25°C.

Isolate no.	Isolate name	pH	Organic acid concentration (mM)							
			Indole-3-acetic	Lactic	Butyric	Propionic	Formic	Acetic	Citric	Gluconic
14	*Penicillium sp., P. magnielliptisporum*	3.75	88.7	0.53	-	1.57	0.18	-	-	-
17	*Aspergillus terreus*	4.01	65.73	6.06	-	-	-	-	-	-
26	*Aspergillus welwitschiae, A. niger*	3.07	81.88	1.13	-	-	-	-	-	-
30	*Mucor fragilis*	3.8	43.2	3.18	-	0.06	2.15	-	-	-
31	*Fusarium sp., F. solani, F. phaseoli*	4.17	65.2	2.15	-	1.41	1.87	-	-	-
32	*Fomitopsis meliae*	3.64	103.31	-	-	-	-	-	-	-
34	*Curvularia petersonii*	3.81	46.15	1.47	-	-	-	-	-	-
40	*Aspergillus sp., A. costaricensis, A. tubingensis*	4.28	77.05	0.52	-	-	-	-	-	-
57	*Rhizopus microsporus*	3.26	65.05	-	10.7	-	1.4	1.18	-	-
58	*Aspergillus terreus*	3.98	64.53	9.7	-	-	-	-	-	-
60	*Alternaria sp.*	4.16	66.66	0.45	-	0.66	1.67	-	-	-
61	*Phoma sp., Didymella pinodella*	3.92	70.73	7.67	-	0.01	0.05	-	-	-
62	*Chaetomella raphigera*	4.04	94.18	-	-	-	-	-	-	-

" – " OA not detected

However, in 2020, a study was performed where *Acrocalymma* sp. was determined to produce 180.72 mM of IAA (Table 4.3) (Silveira *et al.*, 2020). Despite IAA production in the present study being lower than Silveira *et al.* (2020), the concentration of IAA produced by all the isolates in the present study can still be regarded as high, as most studies involving the quantification of IAA from fungi produce much lower concentrations which range from 0.0027 mM to 1.78 mM (Gusmiaty *et al.*, 2019; Khan *et al.*, 2021; Syamsia *et al.*, 2021; Wulandari and Suryantini, 2019). The present study is also the first known report of IAA production by species of the genera *Chaetomella*, *Fomitopsis*, and *Mucor*.

Propionic acid is usually known to be produced by bacteria, such as *Propionibacterium freudenreichii*. A strain of this bacteria has been found to produce propionic acid at a concentration as high as 1152.81 mM during growth in an expanded bed adsorption bioreactor (Wang *et al.*, 2020). Liaud *et al.* (2014) conducted a study where they determined the production of various OAs among multiple *Ascomycota* and *Basidiomycota* and determined that *A. niger* (BRFM422) produced the highest amount of propionic acid, 2.7 mM (Table 4.3). In the present study, none of the *Aspergillus* species tested produced propionic acid, however, isolate no. 14 produced the OA at a concentration of 1.57 mM (Table 4.2) and therefore showed promising results for use in bioleaching. Very high concentrations of propionic acid have previously been used as a fungistatic or fungicide and an example of such a study is by Dijksterhuis *et al.* (2019), where they determined that concentrations between 6.1 and 31 mM affect the growth of poultry feed specific fungi. Therefore, this is likely why propionic acid was not produced in such high amounts in the present study as very high concentrations may inhibit fungal growth.

To the best of my knowledge, the production of butyric and acetic acid from *Rhizopus microsporus* has not previously been reported. However, Table 4.3 lists other genera which have previously been tested for these OAs. When comparing the concentration of butyric acid produced by isolate no. 57 to those listed in Table 4.3, isolate no. 57 produced this OA in much higher amounts which is promising

for bioleaching purposes. Despite isolate no. 57 producing a lower concentration of acetic acid than some of the isolates listed in Table 4.3, it was the only isolate to have produced acetic acid in the present study and the concentration produced was similar to most of the acetic acid concentrations in Table 4.3. Therefore, this isolates butyric and acetic acid production makes it a promising fungal candidate for bioleaching.

The concentration of formic acid produced by isolates no. 14, 30, 31, 57, 60, and 61 was lower compared to the listed *Penicillium* and *Aspergillus* strains in Table 4.3. However, isolate no. 60 did produce a higher concentration compared to *Alternaria* sp. in Table 4.3 and the isolates which showed promising results for formic acid production were isolates no. 30, 31, 57, and 60. The concentration of lactic acid produced by isolates no. 17, 30, 31, 34, 58, and 61 was all higher compared to those listed in Table 4.3, with isolates no, 17, 58, and 61 having the highest concentrations. Therefore, these three isolates showed promising results for use in bioleaching in terms of their lactic acid production.

Considering the HPLC results for the thirteen isolates, isolates no. 14, 57, and 58 are promising isolates for further experiments leading to bioleaching. However, the OA production by all thirteen isolates may be improved by determining the optimum growth conditions for the isolates. Alternatively, experimentation with a mixed culture of the high OA producing fungal isolates can be performed to determine if the total OA production in terms of the type of OA and concentration, may be increased.

Table 4.3: Studies involving the quantification of organic acids (OAs) produced by fungal isolates.

Fungi name	Organic acid concentration (mM)								Growth medium and incubation parameters	Reference
	IAA	Lac	But	Pro	For	Ace	Cit	Glu		
Colletotrichum fructicola	6.88±0.87								PDB with L-tryptophan[1], 26 days[2], 30℃[3], pH 6[4]	(Numponsak *et al.,* 2018)
Acrocalymma sp.	180.72								PDB with L-tryptophan, 8 days, 26℃	(Silveira *et al.,* 2020)
Alternaria sp.		0.35			0.25		0.001	-	PYGV, 8 days, 25℃	(Palmer *et al.,* 1991)
Phoma sp.		0.17			0.23		-	-	PYGV, 8 days, 25℃	(Palmer *et al.,* 1991)
Aspergillus niger		0.48				1.05	2.76	1.02	Basal medium with phosphate rock, 14 days, 20℃, pH 7	(Akintokun *et al.,* 2007)
Aspergillus terreus		0.43				0.93	2.57	1.72	Basal medium with phosphate rock, 14 days, 20℃, pH 7	(Akintokun *et al.,* 2007)
Penicillium chernestinum		0.41				0.68	1.66	0.76	Basal medium with phosphate rock, 14 days, 20℃, pH 7	(Akintokun *et al.,* 2007)
Aspergillus niger		0.82				1.42	-	2.36	Basal medium with tri-calcium phosphate, 14 days, 20℃, pH 7	(Akintokun *et al.,* 2007)
Aspergillus terreus		0.65				2.28	-	2.42	Basal medium with tri-calcium phosphate, 14 days, 20℃, pH 7	(Akintokun *et al.,* 2007)
Penicillium chernestinum		1.26				0.99	-	2.04	Basal medium with tri-calcium phosphate, 14 days, 20℃, pH 7	(Akintokun *et al.,* 2007)
Curvularia lunata (HQ607915.1)			0.10			1.21	0.21		Mo medium, 1st day of stationary phase, 27℃, pH 5.8	(Boniek *et al.,* 2017)
Curvularia lunata (JQ936202.1)			0.06			0.73	0.69		Mo medium, 1st day of stationary phase, 27℃, pH 5.8	(Boniek *et al.,* 2017)
Penicillium sp. strain JM-2008					9.5		0.5		Czapek Dox, 5 days, 30℃, pH 7	(Sullivan *et al.,* 2012)
Penicillium sp. strain JM-2008					-		-		Malt extract, 5 days, 30℃, pH 5	(Sullivan *et al.,* 2012)
Fusarium solani								-	Modified Czapek Dox, 7 days, 28℃, pH 6	(Shinidia *et al.,* 2006)
Alternaria alternata								5.1	Modified Czapek Dox, 7 days, 28℃, pH 6	(Shinidia *et al.,* 2006)
Aspergillus niger (BRFM428)		1.11							Fermentation medium with glucose, 6 days, 30℃, pH 5.5	(Liaud *et al.,* 2014)
Aspergillus flavus (BRFM821)			2.27						Fermentation medium with glucose, 6 days, 30℃, pH 5.5	(Liaud *et al.,* 2014)
Aspergillus niger (BRFM422)				2.7					Fermentation medium with glucose, 6 days, 30℃, pH 5.5	(Liaud *et al.,* 2014)
Aspergillus flavipes (BRFM456)					71.66				Fermentation medium with glucose, 6 days, 30℃, pH 5.5	(Liaud *et al.,* 2014)
Aspergillus niger (BRFM428)						6.66			Fermentation medium with glucose, 6 days, 30℃, pH 5.5	(Liaud *et al.,* 2014)
Aspergillus niger (BRFM422)							10.47		Fermentation medium with glucose, 6 days, 30℃, pH 5.5	(Liaud *et al.,* 2014)
Aspergillus niger (BRFM431)								18.86	Fermentation medium with glucose, 6 days, 30℃, pH 5.5	(Liaud *et al.,* 2014)

Blank spaces indicate that production of that specific OA was not tested. " – " Indicates the OA was not detected. IAA – indole-3-acetic acid, Lac – lactic acid, But –butyric acid, Pro – propionic acid, For – formic acid, Ace – acetic acid, Cit – citric acid, Glu – gluconic acid. 1 – growth medium, 2 – incubation period, 3 – incubation temperature, 4 – pH of growth medium.

4.3.3 Morphological changes and heavy metal tolerance index (TI)

4.3.3.1 Morphology

The morphological changes observed and heavy metal TI for isolates no. 14, 57, and 58 grown on metal supplemented plates, are depicted in Figures 4.2 and 4.3, respectively. In the metal supplemented plates, morphological changes of the three fungal isolates, which include a change in colour and growth, were observed, as well as pigmentations in the culture medium for isolate no. 58 (Figure 4.2).

In the control plate, isolate no. 14 produced mostly orange colonies with yellow on the edges of the colony, whereas its exposure to Cu and Ni resulted in the production of mostly black/grey colonies with white/cream or white/yellowish edges, respectively. Isolate no. 14's growth was severely inhibited when exposed to Cr and its spore colour was white/grey. The only morphological change observed for isolate no. 57 was a change in the size of the colony. When exposed to Cu, isolate no. 57 grew the same as in the control plate with its white-cottony colony covering the entirety of the plate, however, its growth was inhibited when exposed to Ni, and severely inhibited when exposed to Cr.

Isolate no. 58 produced mostly white and yellowish spores observed in the control plate. When exposed to Cu and Ni, isolate no. 58 resulted in a change in spore colour as well as pigment production, however, its exposure to Cr did not result in these changes as only growth inhibition was observed. When exposed to Cu, the spores were brown and denser around the region of inoculation and a gradual change in pigmentation from dark brown to brown to light brown, was observed. Its exposure to Ni resulted in yellow spore production and a yellow/light brown pigmentation around the colony.

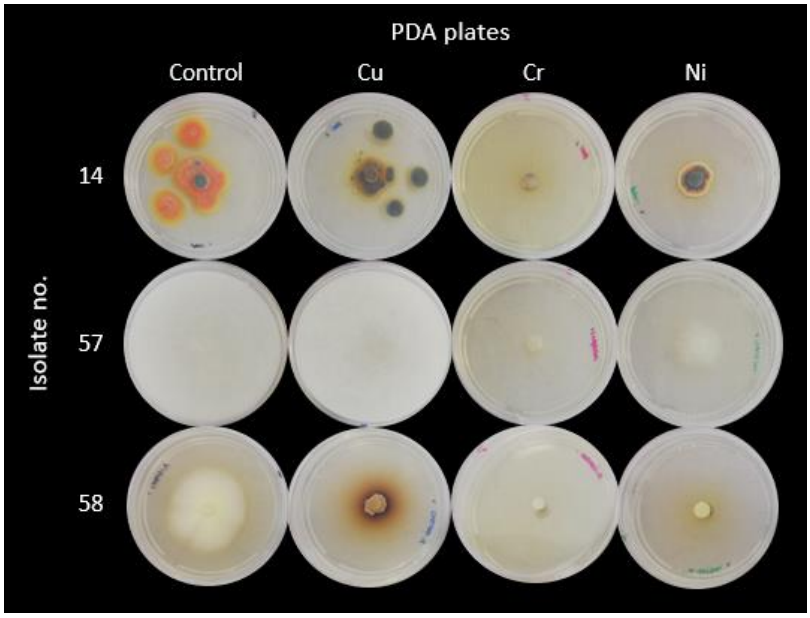

Figure 4.2: Morphological changes of fungal isolates exposed to heavy metals. *Penicillium sp., P. magnielliptisporum* (14); *Rhizopus microsporus* (57); and *Aspergillus terreus* (58) grown on Potato Dextrose Agar (PDA) plates supplemented with 200 mg/L of copper (Cu), chromium (Cr), and nickel (Ni) compared to growth on the control PDA plate incubated at 25°C for 7 days.

Morphological changes and pigment production has previously been reported in fungi exposed to heavy metals such as Cu, Ni, Cr, Cd, Zn, and Pb, where growth inhibition was observed by the fungi due to metallic stress (Baldrian, 2003; Eliseo and Bitacura, 2021; Liaquat *et al.*, 2020). Changes in the size and colour of colonies were previously observed when *Rhizopus* sp. was exposed to 5 mM of Cu as the colony became lighter in colour and its growth was inhibited (Eliseo and Bitacura, 2021). Liaquat *et al.* (2020), investigated the morphological changes of the multimetal tolerant strains of *Aspergillus sclerotiorum*, *A. aculeatus*, *A. niger*, *Komagataella phaffi*, and *Trichoderma harzianum* when grown in media supplemented with 1 500 ppm of Cd, Cr, or Pb. Changes in the colour of the colonies and growth inhibition were observed among the colonies as well as an orange pigment production on the edge of the colonies in all metal supplemented plates (Liaquat *et al.*, 2020). It was previously reported that pigment production occurs due to the precipitation of metal ions on the fungal cell wall and it has also been related to the survival mechanisms used by fungi to reduce metal stress,

while the colour changes of the colonies were linked to metal sequestration (Gruhn and Miller, 1991; Kowshik and Nazareth, 2000; Liaquat *et al.*, 2020).

4.3.3.2 Tolerance index (TI)

The TI for isolates no. 14, 57, and 58 to Cu were all significantly different ($P < 0.05$) with isolate no. 57 depicting the highest tolerance. The TI for isolates no. 14 and 57 to Cr was not significantly different, however, they were significantly different to the TI of isolate no. 58 to Cr, the same was observed for the isolates TI to Ni.

The metal tolerance of *Aspergillus* sp., *Penicillium* sp., and *Rhizopus* sp. has been previously investigated. In the study by Ezzouhri *et al.* (2009), strains of *Penicillium* sp. were all determined to have a high tolerance to Cu when grown in solid media containing 1 mM of the metal. This contrasts the results in the present study as isolate no. 14 had a low tolerance to Cu (Figure 4.3). However, similar TI values to the present study were observed for three strains of *Penicillium* sp. exposed to 1 mM of Cu in solid media (Sharma *et al.*, 2011). In the same study by Sharma *et al.* (2011), one strain of *Aspergillus* sp. was found to have a high tolerance to 1 mM of Cu and another strain was found to have a very low tolerance to the metal. Tolerance is dependent on the isolate and its site of isolation, therefore, the difference in tolerance among the two strains could be explained by the heterogeneity of pollution from the site the isolates originated from (Sharma *et al*., 2011). Oladipo *et al.* (2016) and Oladipo *et al.* (2018), investigated the tolerance of *A. terreus* and *R. microsporus* to varying concentrations of Cu in solid media over 13 days, respectively. *Aspergillus terreus* was found to have a high tolerance when exposed to 250 mg/L of Cu, however, at 1000 mg/L its tolerance was low, however, *R. microsporus* tolerance remained high at 1000 mg/L (Oladipo *et al.*, 2016, 2018). The tolerance of isolate no. 57 in the present study (Figure 4.3), corresponded to *R. microsporus* in the mentioned studies, however, isolate no. 58 had a very low tolerance to Cu compared to *A. terreus* in the study by Oladipo *et al.* (2016).

In the study by Ezzouhri *et al.* (2009), the strains of *Penicillium* sp. were investigated for their tolerance to 1 mM of Cr and all strains displayed a high tolerance to the metal, as opposed to isolate no. 14 in the present study. Ahmad *et al.* (2006) determined the minimum inhibitory concentration (MIC) of five strains of *Penicillium* sp. and found that the MIC of the strains exposed to Cr varied from 300 to 600 mg/L. In the same study, the MIC for strains of *Aspergillus* sp. and *Rhizopus* sp. to Cr was also determined and found to be 300 and 500 mg/L, respectively. In another study by Ahmad *et al.* (2005), *Aspergillus* sp. and *Rhizopus* sp. were found to have a MIC of 200 and 400 mg/L to Cr, respectively. Different isolation sites were used in the study by Ahmad *et al*. (2006) and Ahmad *et al*. (2005), therefore, the isolates were exposed to different conditions which could explain the difference in the MIC of similar species. It was previously reported that *A. terreus* was resistant to 15 mg/L of Cr in liquid media (Abdullahi and Machido, 2018). Therefore, the results in the present study do not correspond to previous studies involving the genera of these isolates as all three isolates displayed very low tolerance to Cr. As mentioned previously, tolerance is dependent on the isolate and site of isolation. Therefore, different strains could respond differently to the same environment or may respond differently due to the difference in the sites they were isolated from.

The TI of *Penicillium* spp. and *Aspergillus* spp. to 1 mM of Ni was also investigated in the study by Sharma *et al.* (2011), where it was determined that the strains of *Penicillium* sp. and *Aspergillus* sp. both had a low to moderate tolerance to Ni. The TI of isolate no. 14 in the present study had a low tolerance to Ni, however, the TI of isolate no. 58 was very low. There are no previous studies for the TI of isolate no. 57 to be compared to, however, as was described for Cr, the MIC of *Penicillium* sp., *Aspergillus* sp., and *Rhizopus* sp. to Ni was also determined by Ahmad *et al.* (2006). The MIC for *Penicillium* spp. varied from 400 to 850 mg/L, *Aspergillus* spp. varied from 300 to 400 mg/L, and the MIC for *Rhizopus* sp. was 850 mg/L. In the study by Ahmad *et al.* (2005), *Aspergillus* sp. also had a MIC of 400 mg/L to Ni, however, *Rhizopus* sp. had a MIC of 400 mg/L.

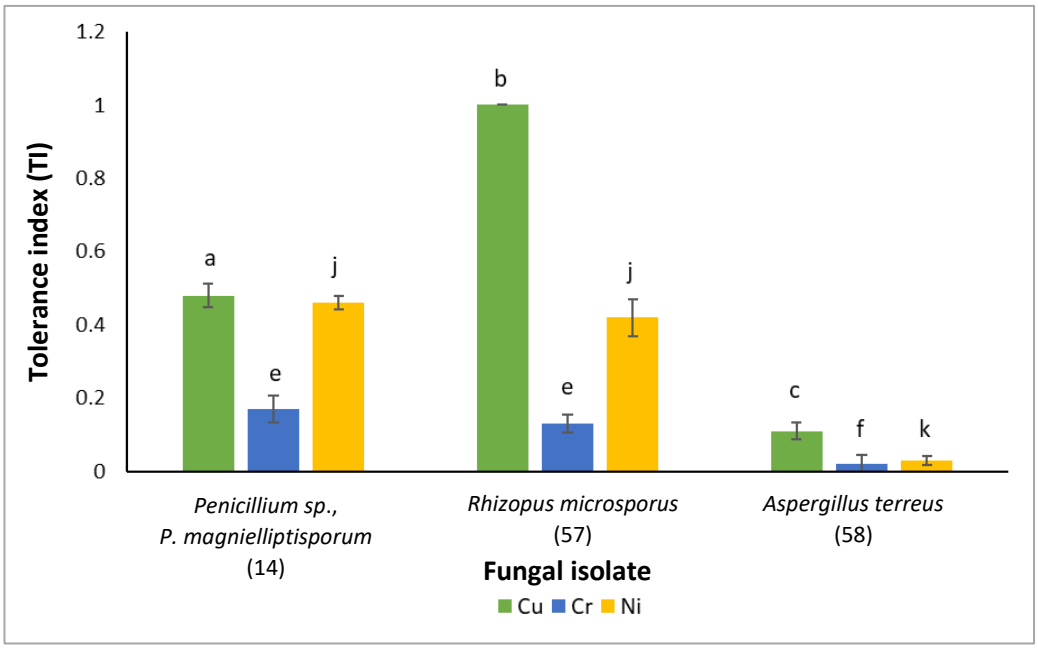

Figure 4.3: Heavy metal tolerance index (TI) of fungal isolates. *Penicillium sp., P. magnielliptisporum* (14); *Rhizopus microsporus* (57); and *Aspergillus terreus* (58) exposed to 200 mg/L of copper (Cu), chromium (Cr), and nickel (Ni). Different letters over bars with the same colour indicates a significant difference in tolerance as analysed by Duncan's Multiple Range Test at $P < 0.05$ (n = 3, mean ± standard error).

When considering the previous studies which investigated the TI of fungi to Cu, Cr, and Ni, the only isolate in the present study which had a promising TI was isolate no. 57 which displayed its high tolerance to Cu. Despite this, the TI to Cu ranged from very low to low for isolates no. 14 and 58 and the same was observed for the TI to Cr and Ni among all three isolates. Therefore, these isolates may not have acquired a high tolerance to the tested heavy metals when exposed to the environment from which they were isolated. Previous studies have resorted to tolerance training to increase fungal tolerance to specific heavy metals to utilise the fungi for direct bioleaching (Anahid *et al.*, 2011; Valix and Loon, 2003). This training involves the repeated sub-culturing of fungi exposed to increasing concentrations of heavy metals to obtain a highly metal tolerant fungal species for biosorption and bioaccumulation of metal ions during bioleaching (Anahid *et al.*, 2011). This would be beneficial for one-step direct bioleaching; however, two-step direct bioleaching could be the method used as it involves the growth of fungi prior

to their exposure to the metal-containing solid material. This allows for fungal biomass growth to occur without inhibition by metal exposure which could therefore result in the occurrence of biosorption and bioaccumulation and an increase in bioleaching efficiency.

4.4 Summary and Conclusions

The qualitative OA screening test resulted in a positive result for fifteen out of the sixty-five isolates tested, namely, isolates no. 7 (*Physisporinus sp., Vanderbylia fraxinea, P. vitreus*), 14 (*Penicillium* sp., *P. magnielliptisporum*), 17 (*Aspergillus terreus*), 26 (*Aspergillus welwitschiae, A. niger*), 30 (*Mucor fragilis*), 31 (*Fusarium* sp*., F. solani, F. phaseoli*), 32 (*Fomitopsis meliae*), 34 (*Curvularia petersonii*), 40 (*Aspergillus* sp*., A. costaricensis, A. tubingensis*), 53 (*Terana caerulea*), 57 (*Rhizopus microsporus*), 58 (*Aspergillus terreus*), 60 (*Alternaria* sp.), 61 (*Phoma* sp., *Didymella pinodella*), and 62 (*Chaetomella raphigera*). After calculating the AU values for these isolates, isolates no. 7 and 53 were not selected for further screening due to their significantly lower AU values compared to the other isolates.

HPLC analysis of the remaining thirteen selected isolates resulted in high amounts of IAA being detected by all isolates compared to most studies. Butyric and acetic acid were only detected by isolate no. 57 and the production of lactic acid, propionic acid and formic acid were detected by various isolates. None of the isolates produced citric and gluconic acid, however, exposing the isolates to various pH's, carbon sources, temperatures and a longer incubation period may result in the detection of these OAs by the fungi. There were also unknown peaks present in the chromatograms for samples of all isolates and future work could involve the identification and quantification of the OAs tested for in the present study in addition to OAs such as fumaric, malic, oxalic, succinic, or tartaric acid, which likely caused one of the unknown peaks as fungi are known to also produce these OAs.

Isolates no. 14, 57, and 58 were selected for heavy metal tolerance tests to Cu, Cr, and Ni due to their high production of OAs, and high tolerance was only observed by isolate no. 57 to Cu. Isolates no. 14 and 58 had a low and very low tolerance to Cu, respectively, and all three isolates either had a low or very low tolerance to Cr and Ni. The results from this test indicated that if these isolates were to be used in one-step direct bioleaching, they would have to undergo tolerance training to the heavy metals in order for their growth and subsequent OA production to take place for bioleaching to occur. Alternatively, two-step direct bioleaching may be the method used as this will allow the fungi to grow exponentially for biomass formation and OA production to occur prior to exposure to the heavy metals during bioleaching.

5 CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

5.1.1 Introduction

The main aims of this study were to isolate and identify fungi from a platinum mine using traditional microbiological techniques and molecular techniques, respectively, and to characterise the fungal isolates based on their OA production using qualitative and quantitative approaches. An additional aim included the characterisation of selected fungal isolates based on their heavy metal tolerance to Cu, Cr, and Ni. This is the first known study investigating the fungal community in a platinum mine.

5.1.2 Traditional microbiological isolation of fungi

The isolation of filamentous fungi from various samples obtained from different regions of a platinum mine resulted in fungal counts ranging between 0 to 1.2 x 10^4 CFU/ml or CFU/g of sample. The pH of the samples ranged from slightly acidic (6.78) to alkaline (9.27) and the redox potential (Eh) measured ranged from 26 to -103 mV which implied that the sample environments were highly reducing. It was concluded that the differences in fungal counts among the samples could be due to the variation of heavy metal concentrations, nutrient availability, and microbial communities, as samples with similar pH values had a large variation between their fungal counts.

5.1.3 Molecular identification of fungi

Sixty-six fungal isolates were identified and 89.39% belonged to the phylum *Ascomycota*, 7.58% to *Basidiomycota*, and 3.03% to *Mucoromycota*. The large percentage of *Ascomycota* identified corresponded to previous studies involving fungi from mine and metal-contaminated sites. Most of these studies focus on isolating heavy metal tolerant fungi and therefore, the large number of *Ascomycetes* identified in the present study is likely due to the genera of this phylum having the ability to tolerate harsh mining environments. Interestingly,

several isolates identified in the present study had not previously been identified in any mine, metal-contaminated site, or in any relevant studies where fungal heavy metal tolerance or OA production was tested. Namely, isolates no. 2 (*Westerdykella ornata*), 7 (*Physisporinus* sp., *Vanderbylia fraxinea*, *P. vitreus*), 16 (*Bartalinia* sp., *B. pondoensis*), 19 (*Albifimbria viridis*), 23 (*Bartalinia pondoensis*), 29 (*Ectophoma multirostrata*), 33 (*Neoscytalidium dimidiatum*), 41 (*Clonostachys epichloe*), 44 (*Westerdykella* sp., *W. centenaria*), 49 (*Pseudothielavia terricola*, *Chrysocorona lucknowensis*, *Acrophialophora jodhpurensis*), 53 (*Terana caerulea*), 55 (*Tiarosporella graminis*), 62 (*Chaetomella raphigera*), 63 (*Pseudocoleophoma polygonicola*), 64 (*Pseudothielavia terricola*, *Chrysocorona lucknowensis*, *Acrophialophora jodhpurensis*), and 65 (*Neopyrenochaeta telephoni*). Therefore, the present study provided knowledge on new isolates present in such environments, however, the results for other isolates such as *Aspergillus*, *Cladosporium*, *Fusarium*, *Penicillium* and *Trichoderma*, corresponded to previous studies involving fungi from mine and metal-contaminated sites.

5.1.4 Qualitative organic acid characterisation

The pH indicator test allowed for the determination of the OA-producing ability of sixty-five isolates resulting in fifteen OA producing isolates being identified, namely, isolates no. 7, 14 (*Penicillium* sp., *P. magnielliptisporum*), 17 (*Aspergillus terreus*), 26 (*Aspergillus welwitschiae, A. niger*), 30 (*Mucor fragilis*), 31 (*Fusarium* sp*., F. solani, F. phaseoli*), 32 (*Fomitopsis meliae*), 34 (*Curvularia petersonii*), 40 (*Aspergillus* sp*., A. costaricensis, A. tubingensis*), 53, 57 (*Rhizopus microsporus*), 58 (*Aspergillus terreus*), 60 (*Alternaria* sp.), 61 (*Phoma* sp., *Didymella pinodella*), and 62. However, the AU values for isolates no. 7 and 53 were significantly lower than the other isolates and were therefore not considered for further characterisation.

5.1.5 Quantitative organic acid characterisation

HPLC analysis of the thirteen selected isolates resulted in high amounts of IAA being detected by all isolates compared to most studies. Gluconic and citric acid was not detected for any of the isolates and butyric and acetic acid were only detected for isolate no. 57. The production of lactic, propionic, and formic acid was detected by various isolates with some producing a much higher concentration than others. There were also unknown peaks present in the chromatograms of some of the isolates which indicated that the isolates could produce other LMWOAs.

5.1.6 Heavy metal tolerance characterisation

Isolates no. 14, 57, and 58 were selected for heavy metal tolerance tests due to their high production of OAs. High tolerance was only observed by isolate no. 57 to Cu. Isolates no. 14 and 58 had a low and very low tolerance to Cu, respectively, and all three isolates either had a low or very low tolerance to Cr and Ni. Therefore, concluding that these three isolates have a low tolerance to the heavy metals when comparing their species tolerance to these heavy metals in previous studies.

5.1.7 Contribution to the research field

The study has contributed to the knowledge of fungal isolates present in mine and metal-contaminated sites as several isolates were identified which have not previously been identified from such sites. The study has also resulted in the identification and quantification of LMWOAs produced by fungal isolates from the genera *Alternaria*, *Aspergillus*, *Chaetomella*, *Curvularia*, *Fomitopsis*, *Fusarium*, *Mucor*, *Penicillium*, *Phoma/Didymella,* and *Rhizopus*. This allowed for the selection of three isolates, namely, isolates no. 14, 57, and 58, for heavy metal tolerance tests due to their production of LMWOAs. Due to their overall low tolerance, it was concluded that isolates no. 14, 57, and 58 may be used for the bioleaching of platinum sources using a two-step bioleaching process.

5.2 Recommendations

Based on the findings of the present study, the following recommendations for future studies are proposed.

Optimum growth conditions

The fungal isolates which produced the highest OAs can be selected for further studies involving the determination of their optimum growth conditions for the greatest amount of OA production. The OAs detected for can also be expanded due to the presence of unknown peaks in the isolates HPLC chromatograms in the present study. Isolates could also be inoculated as mixed cultures to determine if a higher concentration of OA will be produced.

Direct bioleaching

Conducting direct bioleaching using the two-step method could be the way forward. Alternatively, tolerance training of fungal isolates to heavy metals can result in their use in one-step bioleaching.

Metabolomics

Mass spectrometry-based metabolomics could be used to obtain a better understanding of fungal bioleaching mechanisms. This technology would allow for the accurate detection of multiple different organic molecules and the comparison of relative concentrations among various bioleaching conditions.

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APPENDICES

APPENDIX A

NANODROP READINGS

Table A1: NanoDrop readings for the sixty-six fungal isolates.

Fungal isolate no.	A_{260}/A_{280}	A_{260}/A_{230}	ng/µl
1	2.15	2.15	329
2	2.13	2.27	293.1
3	2.14	2.29	142.4
4	2.13	2.29	303.8
5	2.04	2.28	538.4
6	2.14	2.42	279
7	2.07	1.98	60.1
8	2	1.62	325.1
9	2.14	2.33	433.5
10	2.1	2.24	370.5
11	2.1	1.92	226.5
12	1.9	1.59	252.2
13	2.04	2.14	536.3
14	1.85	1.15	244.2
15	2.09	2.39	525.3
16	2.11	2.17	1038.4
17	2.12	2.28	299.5
18	2.12	2.04	518.4
19	2.21	2.38	410.2
20	2.12	2.09	268.3
21	2.05	2.02	374.8
22	2.11	2.26	1320.4
23	2.16	2.41	661.4
24	2.06	2.29	1036.1
25	2.09	1.91	213.8
26	2.09	2.18	189.6
27	2.08	2.15	811.4
28	2.13	2.19	401.5
29	1.96	2.01	1087.1
30	2.27	2.44	1809.3
31	2.14	2.24	675.8
32	2.18	2.35	258.2
33	2.05	2.25	539.3
34	2.23	2.42	130.9
35	2.12	2.24	1003.4
36	1.85	1.37	563.3
37	2.26	2.03	63.9
38	2.1	2.03	585.4

39	2.07	1.19	60.6
40	2.27	2.32	39.8
41	1.95	2.02	408.5
42	2.13	2.44	460.3
43	2.19	2.46	1343.4
44	2.14	2.36	249
45	2.17	2.51	560.5
46	2.16	2.42	279.7
47	2.16	2.22	1046.2
48	2.12	2	532.1
49	2.25	2.42	929.1
50	2.12	2.1	504.8
51	2.2	2.27	1055.6
52	2.14	2.26	560.5
53	2.1	2.39	431.5
54	2.03	2.19	295.2
55	2.03	2.18	446.3
56	2.04	1.6	189.1
57	2.29	2.78	1739.2
58	2.08	2.33	469
59	2.06	2.14	363.9
60	2.27	1.93	62.3
61	2.14	2.58	104.8
62	2.23	2.52	83.4
63	2.15	2.27	183.2
64	2.15	2.26	451
65	2.08	2.15	460.6
66	2.18	2.13	56.5

APPENDIX B

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY CHROMATOGRAMS

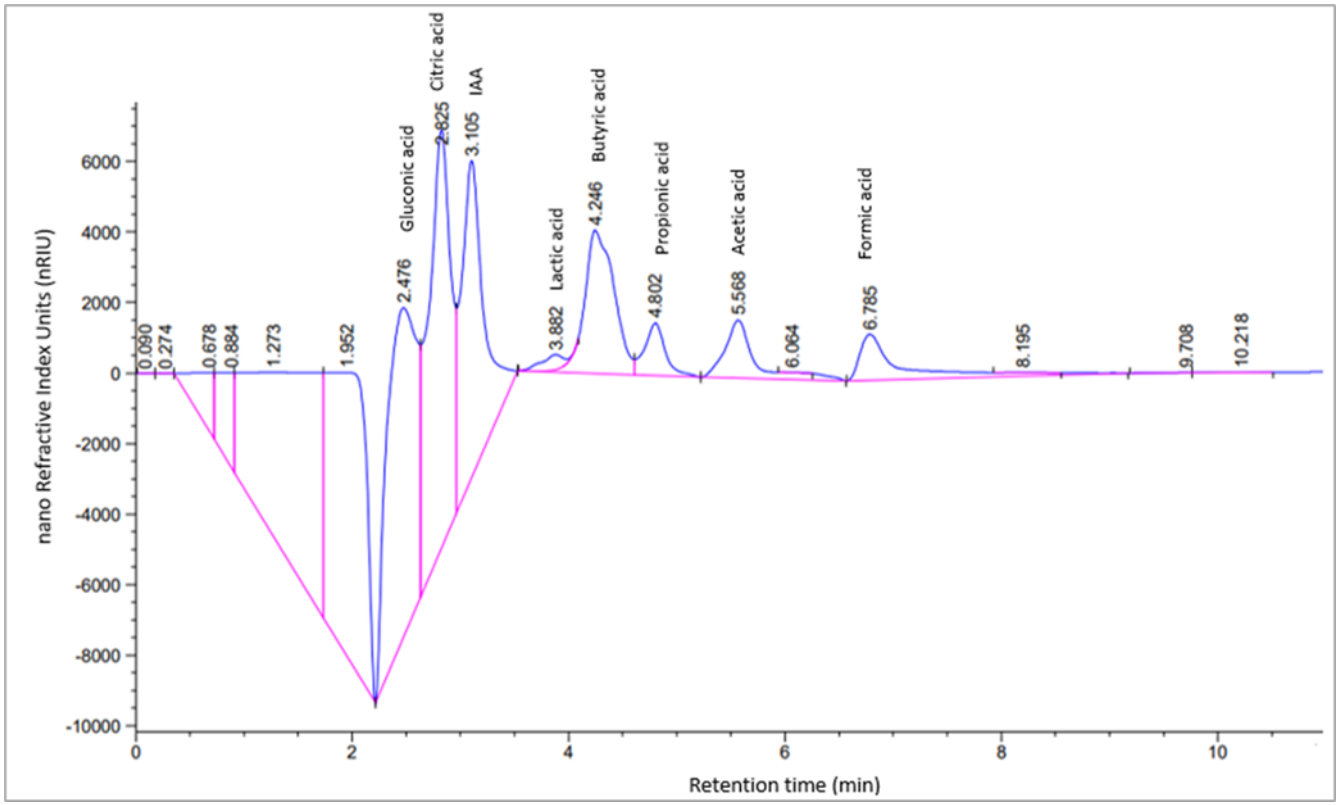

Figure B1: High-performance liquid chromatography (HPLC) chromatogram showing the peaks for the 2 mM standards of gluconic acid, citric acid, indole-3-acetic acid (IAA), lactic acid, butyric acid, propionic acid, acetic acid, and formic acid.

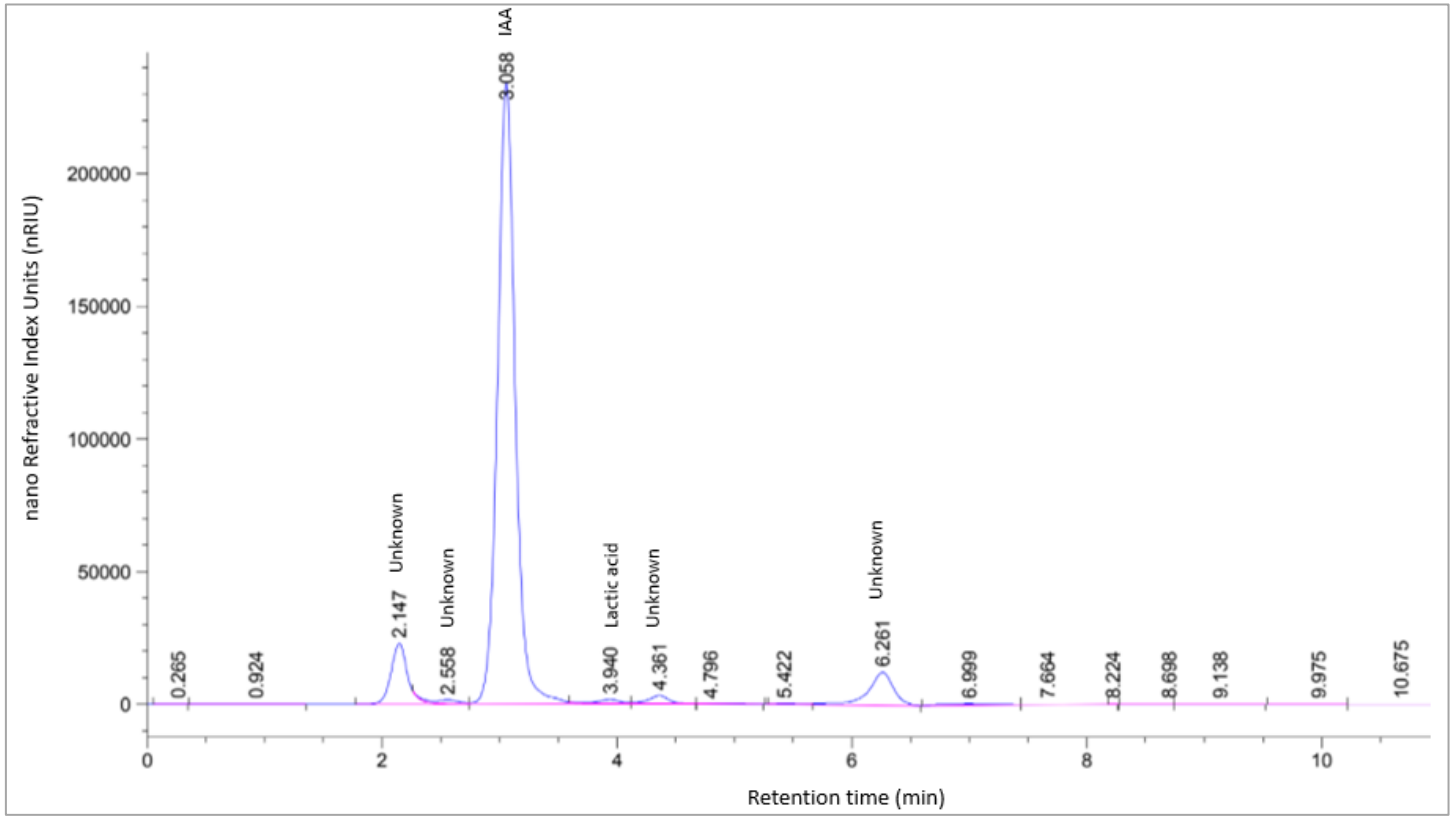

Figure B2: High-performance liquid chromatography (HPLC) analysis for organic acids in the culture supernatant of isolate no. 58 (*Aspergillus terreus*) at 7 days of incubation in potato dextrose broth. The corresponding peaks detected in the culture medium were of indole-3-acetic acid (IAA) and lactic acid including four unknown peaks with retention times (min) of 2.1, 2.6, 4.4, and 6.3.