Identification and characterisation of bacteria isolated from a platinum mine

by

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DECLARATION

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ABSTRACT

Bioleaching involves the solubilisation of metals from solid insoluble substrates such as minerals using microorganisms. Bacteria isolated from mines could have properties that are desirable in the metal bioleaching field. These include iron and sulphur-oxidation and organic acid production. The aim of this study was to isolate, identify and characterise bacteria from a platinum mine. Bacteria were isolated from the concentrator plant of a platinum mine and enumerated using traditional microbiological techniques. The bacteria were then identified using Sanger sequencing and NCBI-BLAST. The colony morphology of the bacteria was noted, and the cellular morphology of the bacteria was analysed using gram staining. To find the optimum growth media for the bacteria, growth curves were conducted in Luria-Bertani, nutrient and 9K broth. Organic acid production by the bacteria was analysed using high-performance liquid chromatography. The results showed that the isolated indigenous bacteria grew well in soils with a pH between 6-9. The colony-forming units of bacteria from each sampling site showed that there was a substantial abundance of bacteria in and around the concentrator plant. A total of 50 bacterial isolates were identified and Proteobacteria was the dominant bacterial phylum. Six bacterial isolates namely *Bacillus* sp., *Pseudoxanthomonas mexicana*, *Pseudomonas stutzeri*, *Arthrobacter globiformis*, *Pseudomonas aeruginosa* and *Pseudomonas brassicacearum* had desirable bioleaching characteristics and were selected for further characterisation. The bacteria were able to produce gluconic, citric, lactic, propionic, butyric, formic, and indole-3-acetic acid. Therefore, they can potentially be used for the bioleaching of platinum group metals.

DEDICATION

Dedicated to my family and friends for being my rock.

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LIST OF SYMBOLS

°C	Degree Celsius
<	Less than
(-)	Negative
%	Percent
(+)	Positive

LIST OF ABBREVIATIONS

2,4-DAPG	2,4-Diacetylphloroglucinol
$(NH_4)_2SO_4$	Ammonium sulphate
ANOVA	Analysis of variance
AAS	Atomic Absorption Spectroscopy
BLAST	Basic Local Alignment Search Tool
$Ca(NO_3)_2$	Calcium nitrate
CFU	Colony-forming units
CFU/g	Colony-forming units per gram
CFU/mL	Colony-forming units per millilitre
DNA	Deoxyribonucleic acid
K_2HPO_4	Dipotassium hydrogen phosphate
EPS	Extracellular polysaccharide
Fe^{3+}	Ferric iron
Fe^{2+}	Ferrous iron
i.e.,	For example
g	gram
g/L	Grams per litre
Cr(VI)	Hexavalent chromium
HPLC	High-Performance Liquid Chromatography
H	Hydrogen
ID	Identity
IAA	Indole-3-acetic acid
IMBL	Industrial Microbiology and Biotechnology
Ir	Iridium
FeS_2	Iron disulphide
LB	Luria-Bertani
$MgSO_4$	Magnesium sulphate
µL	microlitres
µm	micromoles
μhr^{-1}	Micros per hour
mg/mL	Milligrams per millilitre
mL	millilitre
mM	millimolar
M	Molar
ng/g	Nanograms per gram
NCBI	National Center for Biotechnology
NGS	Next Generation Sequencing
NB	Nutrient Broth
100X	One hundred times
OD_{600}	Optical density at 600 nanometres wavelength
Os	Osmium
O_2	Oxygen
Pd	Palladium
Pt	Platinum

PGM	Platinum Group Metal
PCR	Polymerase Chain Reaction
KCl	Potassium chloride
p	Probability
rpm	Revolutions per minute
Rh	Rhodium
rDNA	Ribosomal Deoxyribonucleic acid
rRNA	Ribosomal ribonucleic acid
Ru	Ruthenium
NaCl	Sodium chloride
SO_4	Sulphate
H_2SO_4	Sulphuric acid
UG2	Upper Ground 2
H_2O	Water

CHAPTER ONE: INTRODUCTION

1.1 Introduction

1.1.1 General introduction and problem statement

Platinum group metals (PGM's) are a group of scarce metals that includes platinum, iridium, palladium, osmium, rhodium, and ruthenium. The Circular Economy of the European Union has declared that platinum is a scarce raw material (Keersemaker, 2020). There is a high demand for PGMs due to their desirable properties and applications in several industries, including the transportation and energy industries, such as for vehicle catalytic converters and fuel cells. An increase in the demand for more sustainable and environmentally friendly technologies in both the transportation and energy industries has also resulted in high demand for PGMs. These properties include thermal stability, resistance to corrosion and chemical inactivity (Granados-Fernández *et al.*, 2021). PGMs, more especially platinum, have a distinctive catalytical property that plays an important role in chemical and electrochemical processes that are important in catalytic converters found in vehicles (Yousif, 2019).

In the earth, platinum occurs at an average concentration of 0.4 ng/g and very few large economically prospective deposits have been identified, making it extremely rare (Reith *et al.*, 2014). Due to the high demand and rarity of platinum, it has become a commodity for several countries. The main natural deposits of PGMs are found in South Africa, Zimbabwe, and Russia (Oberthür, 2018), with South Africa having one of the largest PGM's industries in the world. The most commonly used metallurgical process for the recovery of PGMs from pristine ores is floatation and it has achieved platinum recoveries greater than 85 % (Sefako, Sekgarametso & Sibanda, 2017). In South Africa, PGMS are mined from the Bushveld Complex which consists of the Merensky reef, Upper Group 2 (UG2) reef and the Platreef (Cawthorn, 2010). The fast exhaustion of the Merensky reef has forced PGM producers to mine the upper group 2 (UG2) reef or the Platreef. During the milling of UG2 or Platreef ores, a low-quality concentrate with high chromite content is produced (Mwase, Petersen & Eksteen, 2012). This low-grade concentrate increases the smelter cost and decreases the smelter integrity because of the increase in chromite (Mwase, Petersen & Eksteen, 2014). Increased chromite in the

concentrate causes an accumulation of heat resistant chromite spinel layers in the furnaces which affect the functioning and availability of the furnaces (Valenta & Mapheto, 2010). This slows production down resulting in a financial loss. Consequently, the low-grade concentrate leads to an increase in processing cost and a decrease in PGM production.

Given that the recovery of PGMs is expensive, not sustainable, or environmentally friendly, alternative approaches such as biohydrometallurgical methods must be explored. PGMs are primarily contained in either PGM accessory minerals or in base metal sulphide minerals such as chalcopyrite ($CuFeS_2$), pyrrohotite (FeS) and pentandite [$(NiFe_9)S_8$] (Kraemer, Junge & Bau, 2017). These methods include bioleaching with sulphur and iron oxidising microorganisms as well as organic/inorganic acid-producing microorganisms (Schippers *et al.,* 2014; Kraemer *et al.,* 2015). Despite South Africa having several PGM mines very little work has been done on studying the microbial diversity at these mining sites. Microorganisms found at these PGMs mines could have characteristics that make them suitable for use in the bioleaching of PGMs. Bioleaching has thus far been applied on an industrial level in the recovery of nickel, copper, zinc, and cobalt from sulphide bearing ores (Hedrich *et al*., 2020). Mesophilic microorganisms such as *Acidithiobacillus ferrooxidans, Acidithiobacillus thiooxidans* and *Leptospirillum ferrooxidans* have been mainly used for the bioleaching of these metals (Simate, 2009; Nguyen *et al*., 2018). Two studies have been successfully carried out where a thermophilic microorganism, *Metallosphaera hakonensis*, has been used for the base metal bioleaching of a PGM low-grade concentrate and a coarse ore (Mwase, Petersen & Eksteen, 2012, 2014).

1.1.2 Research problem

The bacterial community and diversity found at several mine sites such as gold and copper mines have been extensively studied. However, very few studies have been done on the bacterial community and diversity found at PGM mines. It is theorised that organic acid-producing, and iron and sulphur-oxidising bacteria may be isolated from a PGM mine, in this study.

1.1.3 Research aims and objectives

This study aims to isolate, identify, and characterise bacteria from a platinum concentrator plant.

Objectives

- Isolation and enumeration of bacteria.
- Molecular identification of bacteria.
- Characterisation of bacteria.

CHAPTER 2: LITERATURE REVIEW

2.1 The platinum group metals mining industry

2.1.1 Platinum group metals

Platinum group metals (PGMs) are rare precious metals and consist of six metals namely: platinum (Pt), palladium (Pd), osmium (Os) rhodium, iridium (Ir) and ruthenium (Ru) (Seymour & O'Farrelly, 2012). In contrast to gold, which is primarily used in jewellery and dentistry, PGMs are used in a variety of industrial applications such as dental alloys, jewellery, catalytic converters, and thermocouples. The most commercially important metals within this group are platinum and palladium, with the automotive industry being their most substantial industrial application, where they are used in vehicle catalytic converter units (Gunn, 2013). These metals are extremely rare due to their low natural abundance and the complexity of their extraction and refining processes (Seymour & O'Farrelly, 2012).

The PGMs are transition metals and are chemically similar. They all have siderophile and chalcophile propensities where they can associate or bond with iron and sulphur and have also been shown to associate with nickel and copper (Seymour & O'Farrelly, 2012). The physical properties of the PGMs differ considerably, but they all show properties that are consistent with metals such as forming alloys, conducting heat and electricity and a certain degree of malleability and ductility (Seymour & O'Farrelly, 2012). Platinum is exceptionally resistant to corrosion due to its high oxidation potential, making it stable in the air even at high temperatures (Reith *et al.*, 2014). In addition to this, it is also soft and ductile. These properties make it a highly sort out metal with applications in dentistry, thermometers, jewellery, anticancer drugs, prosthetics, and catalytic converter units (Reith *et al*., 2014).

Platinum group metals are very rare and occur at mean concentration of 0.4 ng/g. As a result, there are very few minable deposits that have been identified (Reith *et al*., 2014). Currently, the major minable PGM deposits include the Bushveld Indigenous complex in South Africa, the Great Dyke mine in Zimbabwe, the Stillwater complex in the United States of America and placer deposits in Russia

(Birke *et al*., 2018). Placer deposits are mineral deposits, formed by surface weathering of primary rocks, that accumulate due to wind, water or ice action (Haldar, 2013). Approximately 90 % of the world's PGM deposits are found in South Africa. It is the largest producer of platinum in the world, with an approximate production of 80 % of the world's annual platinum production (Birke *et al*., 2018). Within the South African Bushveld Indigenous complex, platinum is mined from three sections, namely the Merensky Reef, UG2 Reef and Platreef.

2.1.2 Current platinum group mining methods: An overview

The South African platinum industry has many underground mines and a couple of opencast mines. Figure 2.1, shows the conventional steps involved in the beneficiation of PGMs which include; comminution, electrical smelting, converting, base metal refining and PGM refining and separation (Safarzadeh, Horton & Van Rythoven, 2018). It is important to note that the mineralogy of the ore will determine the beneficiation process and there are often deviations from and additions to this general process depending on the type of ore. Comminution involves the treatment of the PGM ore in primary and secondary crushers resulting in fine fragments or particles. It is then milled in rod and ball milling circuits (Thethwayo, 2018). There is a wide array of comminution circuits used in the PGM industry, with the two-staged circuit as the most common. Thereafter, the milled PGM ore undergoes flotation where it is treated using flotation cells and gravity separators. Flotation involves the separation of mineral particles according to their hydrophobicity (Sahu *et al*., 2022). The flotation circuit typically has a rougher stage and two cleaner stages (Cramer, 2001). The common reagents used during flotation are dithiophosphate and xanthate collectors with pH between 7.5-9 (Cramer, 2001). The resulting product from comminution is a sulphide-rich PGM concentrate. Iron and sulphur-oxidising bacteria can potentially be used to for the bioleaching of the sulphide-rich PGM concentrate. The dried concentrate is then sent to an electrical smelter where the oxide bearing gauge (silicates) is removed from the PGM rich sulphides using high temperatures between 600-900°C. The resulting matte can either be processed directly into converters or it can be granulated before it is converted, depending on the producer.

The resulting matte goes through a process called converting, where iron sulphide and sulphur are converted to ferrous oxide and sulphur oxide, respectively. The oxidation is performed through the treatment of the matte in Pierce-Smith converters or the Ausmelt process where the iron dioxide is separated as a fayalitic slag, and the sulphur dioxide is separated as an off-gas. After cooling the matte is then milled and treated in a base metal refinery that includes the removal of impurities such as zinc, lead and selenium, cobalt removal and recovery, copper and nickel electrowinning and the disposal of sulphur (Cramer, 2001). The final stage of platinum mining is purification which involves the use of several hydrometallurgical processes such as solvent extraction, leaching, ion exchange and molecular recognition technology (Cramer, 2008).

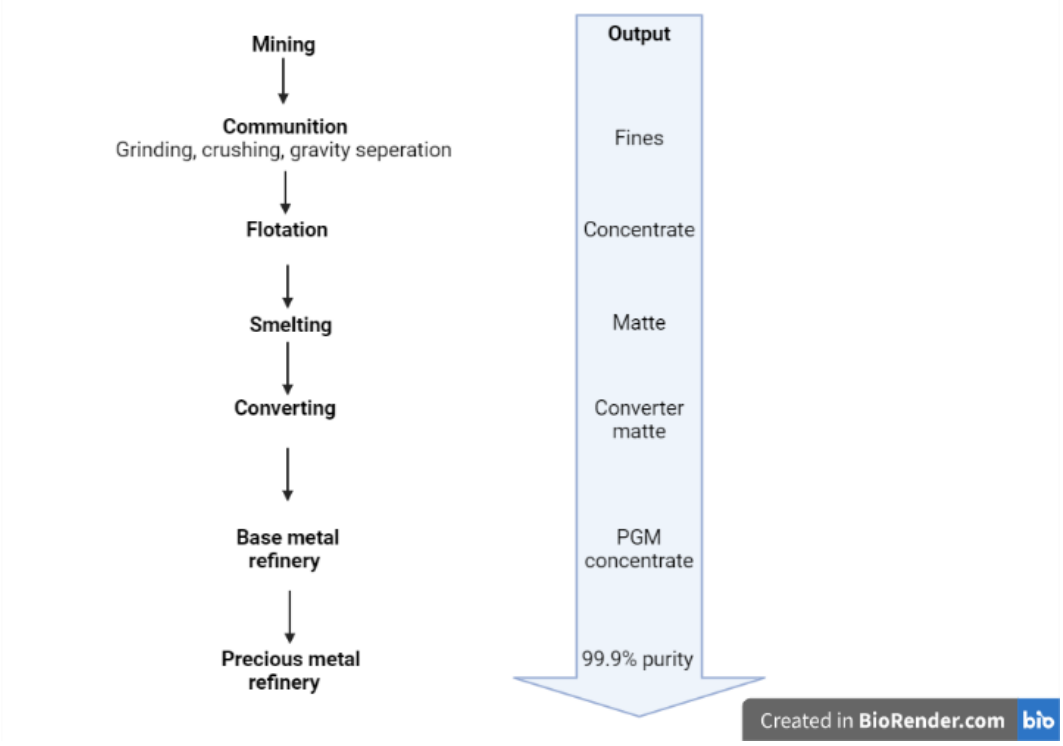


Figure 2.1: A general overview of PGM ore processing (Sinisalo & Lundström, 2018; Thethwayo, 2018).

2.1.3 An overview of the platinum group metal concentrate process

In the PGM mining industry, several flotation circuits are currently employed to concentrate the PGMs that have been mined, such as the "South African MF2/MF3" Flowsheet, Northam Flowsheet, Noril'sk Flowsheet, Stillwater Flowsheet (Cole & Ferron, 2002). The South African PGM mining industry mainly uses the "South African MF2/MF3" Flowsheet and Northam Flowsheet (O'Connor & Alexandrova, 2021). The type of flotation circuit used depends on the ore mineralogy of the mined PGM. The process of PGM concentrating begins with the PGM ore being fed into a semi-autogenous mill which grinds the ore, thus providing primary grinding (Cramer, 2001). Thereafter, the ground ore is processed into a ball mill, which provides secondary grinding. The ore is then put through a process called flash flotation where approximately 60% of the PGM is recovered (Cole & Ferron, 2002). Primary flotation takes place in a rougher or scavenger flotation bank. The rougher bank contains a cell/tank that can produce a final grade concentrate that does not require further processing in the concentrator (Cole & Ferron, 2002). The scavenger bank produces a concentrate that may need to undergo primary flotation again. The remaining rougher bank concentrate is then processed into the cleaner circuit which is a series of three column cells/tanks that each recover approximately 30% of PGMs (Cole & Ferron, 2002). The scavenger concentrate is either processed to the rougher bank or the cleaner circuit. The final concentrate is then made into a slurry through a concentrate thickener and is then subsequently processed to a smelter (Cramer, 2001). At this stage, the produced concentrate can potentially be bioleached using iron and sulphur oxidising or organic acid producing bacteria.

2.2 Bacterial diversity in mines

2.2.1 Introduction

The diversity of microorganisms found at various mines is dependent on several factors which include physiochemical factors such as pH, redox potential, and electrical conductivity. It is also dependent on soil structure, nutrients, temperature, heavy metal concentration, and the soil ecosystem (Marais, 2012). The dominant phyla of microorganisms commonly found at mining sites are Proteobacteria which includes *Pseudomonas* and *Acidovorax* species (Kamika & Momba, 2014; Pereira,

Vicentini & Ottoboni, 2014; Pradhan *et al*., 2022). Some of the bacterial species found at mining sites are extremophilic and can grow at a pH as low as 2 and at high heavy metal concentrations. The bacterial diversity at mining sites differs across regions and is primarily dependent on the type of extractive process used at a particular site (Fernandes *et al*., 2018).

2.2.2 Bacteria found at mining sites

The study of microorganisms at mining sites, which are considered complex environments, has become possible due to the application of PCR amplification of the 16S rRNA genes. Proteobacteria, Bacteroidetes, Acidobacteria and Firmicutes are dominantly found in mining sites (Thavamani *et al*., 2017; Fashola, Ngole-Jeme & Babalola, 2020). Figure 2.2 provides a brief overview of the bacterial diversity found at mining sites.

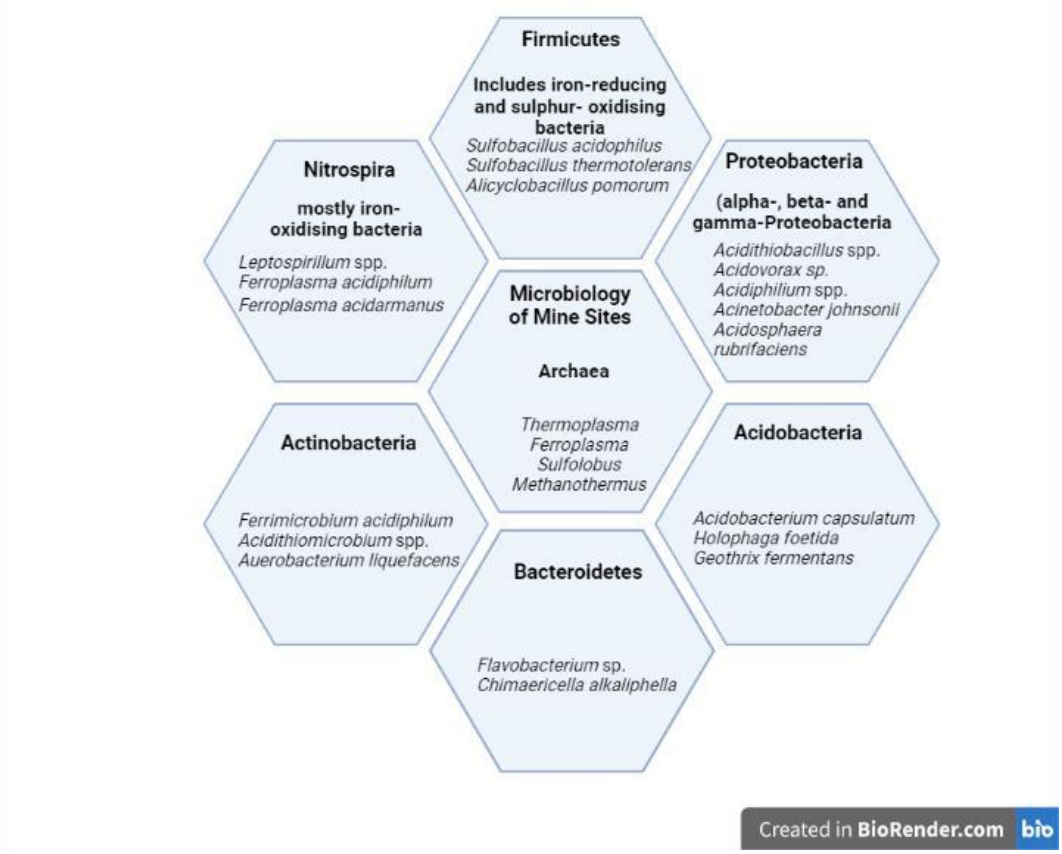


Figure 2.2: Bacterial and Archaeal diversity in terms of phyla found at mine sites (Thavamani *et al*., 2017).

Bacteria found at mine sites have adapted to the high heavy metal concentration characteristic of mine sites and are typically iron and sulphur-oxidising, as shown in Figure 2.2. This involves the oxidation of sulphur and ferrous iron to sulphate and ferric iron and the ions generated from these reactions are subsequently used by the bacteria as electron donors to obtain energy (Ranalli, Zanardini & Sorlini, 2009; Korehi *et al.*, 2013; Sultan & Faisal, 2014; Hassanshahian & Ghoebani, 2018). Examples of iron and sulphur-oxidising bacteria found at mine sites include *Acidithiobacillus thiooxidans*, *Acidithiobacillus ferrooxidans*, *Leptospirillum* sp. and *Sulfobacillus* sp.

Bacteria have been isolated and studied from several mines around the world including copper, iron, silver, coal, platinum, and gold mines. Table 2.1 provides a summary of the different bacteria isolated from various mines. Studies by He *et al*. (2007); Kuang *et al.*(2013) and Santofimia *et al*. (2013) that focused on analysing microbial communities at copper mines in China and Spain showed that the microbial diversity is directly affected by soil elements, pH, and temperature. Xie *et al*. (2007), also found that there was a diverse distribution of *A. ferrooxidans* and *Leptospirillum* sp. which are iron-oxidising bacteria. The distribution of these bacterial species can be attributed to the high concentration of elements such as copper, sulphur, and iron at the mine. *Acidithiobacillus ferrooxidans* is a widely studied bacterial species in studies of acid mine drainage and it has been considered as a model organism in bioleaching research. A study by Domingues *et al.*(2020), found that Firmicutes was the dominant phylum found at a copper mine in Brazil, followed by Proteobacteria and Actinobacteria, with bacteria from the *Bacillus*, *Lysinibacillus* and *Alcaligenes* genera as the most dominant. Pepper *et al.* (2012), found that Proteobacteria and Actinobacteria were the most dominant phyla found at an Arizona copper mine. These studies show that there is a variety of bacteria at copper mines.

Bacteria isolated from gold mines have also been shown to dominantly be from the Proteobacteria, Actinobacteria and Firmicutes phyla. These bacteria also exhibit heavy metal tolerance. *Bacillus cereus* and *Enterobacter asburiae* have been isolated from gold mine tailings and these bacteria exhibit high heavy metal

tolerance suggesting that they have developed specific mechanisms to endure heavy metal stress (Fashola *et al*., 2020). A study by Fashola *et al*. (2020), identified and characterised seventeen bacterial isolates with *Bacillus sp.* and *Enterobacter sp*. as the most dominant. Bacteria isolated from coal mines are also dominantly from the Proteobacteria, Firmicutes and Actinobacteria phyla, however, Adeleke, Cloete & Khasa (2012), found that Bacteroidetes was also a dominant phylum. These bacteria were able to produce organic acids that could assist in iron ore solubilisation. A study by Ekpa *et al.* (2017), isolated *Acidithiobacillus* , *Pseudomonas* and *Leptospirillum* bacterial species. These bacteria can oxidise ferrous iron thereby generating energy and can locally concentrate ferric iron by absorbing it to microbial surfaces. They are also mesophilic acidophiles and exhibit good bioleaching properties.

Table 2.1: Summary of the bacteria isolated from copper, gold, and coal mines.

Mines	Dominant microorganisms	Reference
Copper mine	Proteobacteria, Actinobacteria, Firmicutes, *Acidithiobacillus ferrooxidans*, *Leptospirillum sp*., *Bacillus sp*., *Lysininbacillus sp*. and *Alcaligenes*.	(Xie *et al*., 2007; Pepper *et al*., 2012; Domingues *et al*., 2020)
Gold mine	Proteobacteria, Actinobacteria, Firmicutes *Bacillus sp*., *Enterobacter sp*., *Bacillus subtilis*, *Bacillus cereus*, *Arthrobacter sp*.,	(Fashola, Ngole-Jeme & Babalola, 2020)
Coal mine	Proteobacteria, Actinobacteria, Firmicutes, Bacteroidetes, *Acidithiobacillus sp.*, *Pseudomonas sp.*, *Leptospirillum sp.*	(Adeleke, Cloete & Khasa, 2012; Ekpa *et al*., 2017)

2.2.3 Bacteria isolated from platinum mines

Several studies have focused on identifying and characterising bacteria isolated from platinum mines. These studies focused on heavy metal of the isolated bacteria. Marais (2012), investigated bacteria found at a platinum mine tailing dam. Nine species were identified, as shown in Table 2.2, with *Paenibacillus lautus* as the most widely isolated. Some of the *Paenibacillus* isolates identified in the study were able to produce endospores, which enables the bacteria to endure environmental stress. These bacterial strains have a high heavy metal tolerance with *Bacillus subtilis* showing the highest tolerance. A study by Rauwane (2008), also found *Paenibacillus lautus* to be the most abundant isolate found at a platinum mining site.

Table 2.2: Bacteria isolated from platinum mining sites.

Bacterial isolate	**Reference**
Paenibacillus lautus, Paenibacillus lautus strain DS19, *Paenibacillus* sp. C15, uncultured *Paenibacillaceae, Stenotrophomonas maltophilia, Bacillus* sp. KDNB5, *Bacillus cereus, Alcaligenes* sp. DJWH 146-2,	(Marais, 2012)
Bacillus barbaricus	(Rauwane, 2008)

2.3 Bacterial Bioleaching

2.3.1 Introduction

There is an increasing demand for PGMs due to an increase in their industrial applications. However, there is also a decrease in the easily exploited and highgrade PGM deposits. Therefore, the use of new biotechnological methods to recover PGMs from low-grade ores is essential to meeting this demand. One such biotechnological method is bioleaching. Bioleaching or microbial leaching can be defined as the process where metals are solubilized from an insoluble substrate (Schippers *et al*., 2014). This process normally occurs because of the metabolism of the microorganisms. Bioleaching provides a sustainable, and cost-effective alternative to conventional metallurgical extraction methods.

2.3.2 Bacteria used in bioleaching

The bacteria that are important in the bioleaching process involved with metal extraction can be divided according to their nutritional requirements i.e., chemolithotrophic and heterotrophic bacteria and their growth temperature i.e., mesophilic, and thermophilic bacteria.

2.3.2.1 Chemolithotrophic bacteria

Chemolithotrophic bacteria can produce energy through the oxidation of inorganic molecules through aerobic or anaerobic respiration for biosynthesis (Enrich-Prast *et al*., 2014). A major group of this category are iron and sulphur-oxidising bacteria. Chemolithotrophic bacteria commonly used for bioleaching include *A. ferrooxidans* which can oxidise iron and sulphur, *A. thiooxidans* and *A. caldus* which can oxidise sulphur and *L. ferrooxidans* and *Leptospirillum ferriphilum* which are iron oxidisers. Bioleaching using chemolithotrophic bacteria has been commercially applied for the extraction of copper (Brierley & Brierley, 2013; Yin *et al*., 2018), gold (Van Hille, Wyk & Harrison, 2011; Gericke, 2012) and nickel (Puhakka, Kaksonen & Riekkola-Vanhanen, 2007; Riekkola-Vanhanen, 2007).

2.3.2.2 Heterotrophic bacteria

Heterotrophic bacteria obtain energy and carbon from organic compounds (Van Aken, 2011). These bacteria produce metabolic by-products that can interact with the mineral surface resulting in metal dissolution. These by-products include

complexing organic acids such as citric, acetic, and oxalic acid, exopolysaccharides as well as proteins that can aid in metal solubilisation (Schippers *et al*., 2013). Heterotrophic bacteria that have been used for bioleaching include *Pseudomonas aeruginosa* and *Bacillus sp*. (Jain & Sharma, 2004; Irannajad *et al*., 2014; Barnett, Palumbo-Roe & Gregory, 2018). Heterotrophic bacteria have also been used for the bioleaching of copper and zinc (Shabani *et al*., 2013; Irannajad *et al.*, 2014).

2.3.2.3 Mesophilic bacteria

Mesophilic bacteria grow at temperatures between 25°C and 35°C. Examples of chemolithotrophic mesophilic bacteria used in bioleaching include *Acidithiobacillus* spp., which are aerobic, iron and sulphur-oxidising, these include *Acidithiobacillus caldus*, *albertis*, *acidophilus*, *concretivorus* and *prosperous* (Kumar e*t al*., 2019). These bacteria can grow at a low pH, making them suitable for bioleaching. *Leptospirillum* spp. are also mesophilic and can tolerate higher concentrations of molybdenum, silver and uranium when compared to *A. ferrooxidans* (Kumar *et al*., 2019). *Leptospirillum* spp. can grow between temperatures of 20°C and 45°C (Rawlings, 2013). However, bioleaching at higher temperatures is more desirable. Examples of mesophilic heterotrophic bacteria used in bioleaching include *Bacillus* spp. and *Pseudomonas* spp. These bacteria can grow between 20°C to 42°C (Chakravarty & Anderson, 2015; Kumar *et al*., 2019).

2.3.3 Mechanisms of bioleaching

Leaching reactions in metal leaching use ferric iron and protons from organic or inorganic acids to solubilise metals (Rawlings, 2005). Metal leaching relies on three processes, acidolysis, redoxylysis and complexolysis (Wu & Ting, 2006; Sethurajan, 2015). When microorganisms attach to a surface, they start forming an exopolysaccharide layer and within this layer the bio-oxidation reactions involved in bioleaching occur (Rawlings, 2005). The microorganisms provide both the space and leaching chemicals for bioleaching. In previous literature, bioleaching was described as either direct or indirect bioleaching as shown in Figure 2.3 (Bosecker, 1997; Sand *et al*., 1999; Tributsch, 1999). The direct mechanism involves the attachment of bacteria to the mineral and the indirect mechanism the bacteria oxidise iron resulting in mineral dissolution.

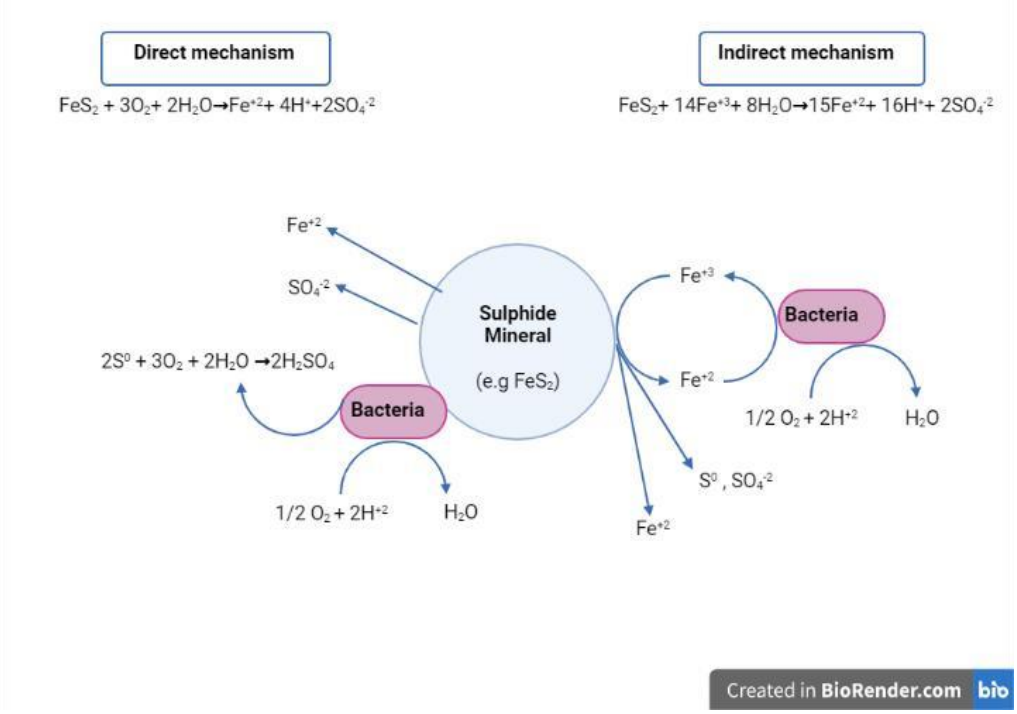


Figure 2.3: Bacterial mechanisms of bioleaching. In direct bioleaching, bacteria attach to the mineral surface and the oxidation of sulphur results in metal dissolution. In indirect bioleaching, bacteria consume oxygen and oxidise iron (Mishra & Rhee, 2014).

2.3.3.1 Redoxylysis

Bioleaching by chemolithotrophic bacteria is achieved through iron and sulphur oxidation. Mineral dissolution reactions and the oxidation intermediates differ depending on the mineralogy of the sulphide bearing mineral. Schippers & Sand (1999), proposed that there are two mechanisms involved in mineral dissolution, a thiosulphate mechanism, and a polysulphide mechanism. Metals like pyrite and molybdenite which are acid insoluble use the thiosulphate mechanism for oxidation and sphalerite, galena and chalcopyrite use the polysulphide mechanism (Tao & Dongwei, 2014). For the thiosulphate mechanism, thiosulphate is the primary intermediate and sulphate is the primary end-product, and the metal is solubilised when ferric iron acts on the metal sulphides (Schippers *et al.*, 2014). In the polysulphide mechanism, sulphur is the primary intermediate and the insoluble metal is acted on by both ferric iron and protons. The bacteria involved in the

solubilisation of the metals provide the protons for a proton attack in the form of sulphuric acid and re-oxidise the iron keeping it in its oxidised ferric form for oxidation of the mineral.

2.3.3.2 Organic acids

Organic acids produced by heterotrophic bacteria can solubilise metals such as copper and nickel. These acids are produced through the metabolic activity of bacteria where sugar substrates such as glucose and sucrose are converted into organic acids (Barnett, Palumbo-Roe & Gregory, 2018). Organic acids solubilise metals, as shown in Figure 2.4, through mineral acidolysis by providing protons (hydrogen) or by acting as complexing agents and complexing with the metals in the minerals (Giese, 2021). The metal dissolution reaction is catalysed by organic acids where the cation-oxygen bonds of the metal are weakened by the formation of metal-organic complexes. Organic acids also aid in metal dissolution by lowering the pH of the solution which is ideal for bioleaching (Barnett, Palumbo-Roe & Gregory, 2018). Bacteria that are used in organic acid bioleaching include *Bacillus* sp., *Pseudomonas* sp., and *Erwinia* sp. The organic acids that can potentially be used in bioleaching include but are not limited to citric, lactic, oxalic and gluconic acid. Several studies have shown that PGMs can be dissolved by organic acids (Mwase, 2009; Trinh *et al*., 2017). The use of organic acids in bioleaching is advantageous because they can be biodegraded easily, and they are cost-effective.

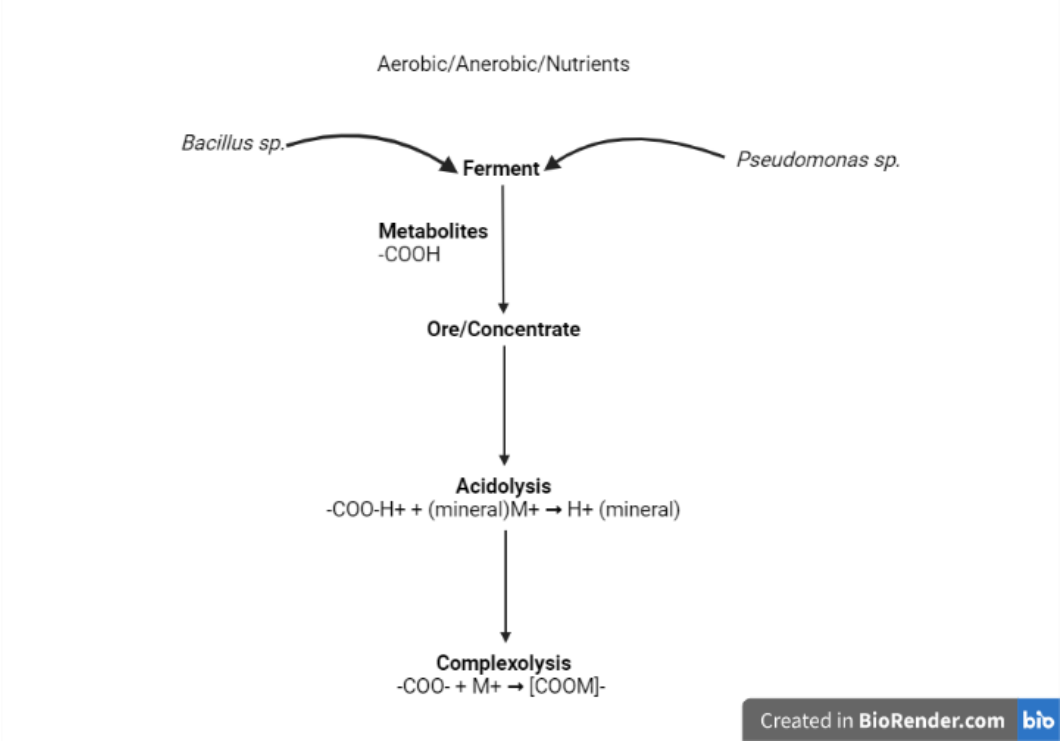


Figure 2.4: Overview of metal solubilisation by organic acids through acidolysis and complexolysis (Mwase, 2009).

2.4 Bacterial bioleaching of platinum group metal ores

Bioleaching has been accepted as a cost-effective, green, and sustainable alternative to traditional hydrometallurgical and pyrometallurgical extraction methods. To date, only gold sulphide ores have been bioleached on an industrial scale (Brierley, 2016), and on a lab scale. The bioleaching of sulphide platinum ore concentrates has been demonstrated (Mwase, Petersen & Eksteen, 2012, 2014). Mwase, Petersen & Eksteen (2012), conducted heap leaching using thermophilic and mesophilic bacteria to bioleach base metals and PGMs from a low-grade PGM concentrate. The experiment resulted in the extraction of 85% cobalt, 52% copper and 95% nickel after 30 days. The remaining concentrate from the heap leaching experiment was leached with cyanide resulting in the extraction of 46% rhodium, 20.3% platinum and 87% palladium. In 2014, Mwase, Petersen & Eksteen, studied a heap leaching method that was tested on crushed coarse platinum ore using iron and sulphur oxidising bacteria that are both thermophilic and acidophilic. The bioleaching

experiment resulted in the extraction of 75% nickel, 93% copper and 53% cobalt after 304 days. A subsequent cyanide leach resulted in the extraction of 90.3% gold, 57.8% platinum and 99.7% palladium after 60 days. Both studies showed that using bioleaching to extract PGMs from PGM ores or concentrates is a viable and effective alternative method to the costly and problematic smelter process.

CHAPTER THREE: IDENTIFICATION OF BACTERIA

3.1 Introduction

The number of studies that have identified and characterised bacteria from platinum mines are very few and have focused solely on bacteria from platinum mine tailings. The study of bacterial diversity at mine sites has become more common. Literature has shown that the microbial community found in heavy metal contaminated soils is complex and well-adapted (Halter *et al*., 2011; Gillan *et al*., 2015). The bacteria found at these sites play environmentally important roles such as biogeochemical cycling and heavy metal bioaccumulation (Fernandes *et al*., 2018). Thus, it is important to acquire comprehensive knowledge about these bacteria and their communities.

The increase in demand for bacteria capable of bioleaching and bioremediation has motivated studies on bacteria that inhabit mine sites. Most of the studies conducted on these bacteria have focused on their heavy metal tolerance/resistance (Wei *et al*., 2009; Marais, 2012; Ndeddy Aka & Babalola, 2017). This study aimed to isolate and identify bacteria from a platinum mine concentrator plant and its surroundings.

3.2 Methods and Materials

3.2.1 Sample collection

Thirty-nine samples were collected from the Anglo Platinum Mogalakwena Mine (23.9709° S, 28.9166° E), in Limpopo, South Africa. The samples were collected from the concentrator plant, the pond outside the concentrator plant and in front of the crusher and concentrator and consisted of a mixture of liquid, slurry, and soil samples. They were placed in labelled 50 mL Greiner Bio-One polypropylene conical centrifuge tubes except for sample 39 which was placed in a polypropylene plastic bag. The samples were transported to the laboratory (Industrial Microbiology and Biotechnology Lab, University of the Witwatersrand) and stored at 4°C.

3.2.2 Determination of sample pH

For the measurement of the soil samples, 1 g of each soil sample was suspended in 10 mL of autoclaved distilled water. The pH of the samples was measured using a calibrated glass electrode Crison pH-meter.

3.2.3 Isolation of bacteria

For the isolation of bacteria from the Anglo samples, a 10^{-1} - $10^{-4}$and 10^{-1} - 10^{-5} dilution series was performed for plating onto Luria-Bertani (LB) and 9K agar, respectively. Luria-Bertani agar was composed of 5 g/L yeast extract, 5 g/L NaCl, 10 g/L tryptone and 15 g/L agar. 9K agar was composed of 5 g/L $MgSO_4 \cdot 7H_2O$, 0.05 g/L $Ca(NO_3)_2$, 3 g/L $(NH_4)_2SO_4$, 0.5 g/L K_2HPO_4, 0.1 g/L KCl and 15 g/L of agar. For the dilution series, 1 g or 1 mL of each sample was suspended in 9 mL of 0.9% saline (NaCl) solution. Thereafter, aliquots of 100 µL from each dilution were spread plated onto their respectively labelled LB or 9K agar plates. The plates were incubated at 37°C for 48 hours. After the incubation period, the plates were observed, colonies were counted, and distinct colonies were subcultured, using the streak plate method until pure cultures were obtained.

3.2.4 Genomic DNA extraction

The extraction of DNA was performed on bacteria pure cultures isolated from the samples. Prior to DNA extraction, a colony of each bacterial isolate was resuspended in 300 µL of autoclaved distilled water, vortexed and 250 µL of the mixture was used for DNA extraction. DNA extraction was performed using the ZymoBiomics™ DNA Miniprep Kit (Catalog No D4301) as per manufacturer's protocol (Appendix B). The quantity and quality of the DNA was analysed using the NanoDrop® ND-1000 spectrophotometer and stored at 4°C until further analysis.

3.2.5 DNA sequencing

The DNA was sent to Inqaba Biotec™ (Pretoria, South Africa) where PCR, agarose gel electrophoresis and Sanger sequencing were carried out. The 16S target region was amplified using OneTaq® Quick-Load® 2X Master Mix (NEB) with the universal primers 27F and 1492R shown in Table 3.1 (Lane *et al*., 1991; Turner *et al*., 1999). The PCR products were electrophoresed on an agarose gel and extracted using a Zymoclean™ Gel DNA Recovery Kit. The extracted 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) to remove any impurities, unincorporated dNTPs, DNA polymerase enzymes and unbound primers. The ABI 3500xl Genetic Analyzer (Applied Biosystems, ThermoFisher Scientific) was used to analyse the purified fragments. The .ab files produced by the ABI 3500XL Genetic Analyzer were analysed by the CLC Bio Main Workbench v7.6 and the results were analysed through a Basic Local Alignment Search Tool (BLAST) search [National Centre for Biotechnology Information (NCBI)] **Table 3.1**: Universal primer set used for PCR amplification.

Name of Primer	Target	Sequence (5' to 3')
16S-27F	16S rDNA sequence	AGAGTTTGATCMTGGCTCAG
16S-1492R	16S rDNA sequence	CGGTTACCTTGTTACGACTT

3.3 Results and Discussion

3.3.1 Bacterial enumeration

Prior to bacterial isolation and enumeration, the pH of each sample was measured, and the results are shown in Table 3.2 below. The pH for the samples taken from the concentrator plant, which were all liquid samples, ranges from 7.35 to 8.69. For the samples from the pond outside the concentrator plant, the pH ranged from 6.39 to 9.11. The pH was between 7.90 and 9.27 for the samples taken from in front of the crusher. The soil sample collected from in front of the concentrator had a pH of 8.49. Therefore, the bacteria were isolated from liquid, slurry and soil samples with a pH that ranged from 6.39-9.11.

Soil chemistry is correlated to changes in the soil microorganisms' communities across different spaces. Studies have shown that the bacterial composition of microbial communities corresponds to soil pH (Shen *et al*., 2013; Qi *et al*., 2018; Cho *et al*., 2019).Therefore, the pH of the samples and isolation plates would influence the bacterial species isolated and their CFUs. The pH of the isolation plates was 7 therefore, acidophilic bacteria were not selected.

Table 3.2: Sampling sites within the Anglo Platinum Mogalakwena mine in Limpopo South Africa and the pH and colony forming units per gram/ milliliter of each sample.

Sample number	Sample	Site Collection point	Sample pH	CFU/mL or CFU/g of sample
1	Rougher Feed	Concentrator plant	8.69	8×10^3
2	Rougher Feed	Concentrator plant	8.61	5.2×10^4
3	Rougher Tail A	Concentrator plant	8.15	8.9×10^5
4	Rougher Tail A	Concentrator plant	8.17	2.6×10^5
5	Rougher Tail B	Concentrator plant	7.69	5.7×10^4
6	Rougher Tail B	Concentrator plant	7.68	2.04×10^5
7	Scavenger Feed	Concentrator plant	8.51	1.5×10^5
8	Scavenger Feed	Concentrator plant	8.51	1.8×10^5
9	Scavenger Tail A	Concentrator plant	7.35	4.4×10^5
10	Scavenger Tail A	Concentrator plant	7.40	1.9×10^4
11	Scavenger Tail B	Concentrator plant	8.55	1.4×10^5
12	Scavenger Tail B	Concentrator plant	8.53	2.3×10^5
13	Cleaner Feed	Concentrator plant	8.27	6×10^4
14	Cleaner Feed	Concentrator plant	8.40	6.8×10^4

Sample number	Sample	Site Collection point	Sample pH	CFU/mL or CFU/g of sample
15	Cleaner Scavenger Tail A	Concentrator plant	8.23	5.4×10^4
16	Cleaner Scavenger Tail A	Concentrator plant	8.45	5.6×10^4
17	Cleaner Scavenger Tail B	Concentrator plant	8.36	1.42×10^5
18	Cleaner Scavenger Tail B	Concentrator plant	8.42	8.9×10^4
19	Dry soil	Pond outside of the plant	8.63	1.33×10^5
20	Dry soil	Pond outside of the plant	9.11	8.2×10^5
21	Dry soil	Pond outside of the plant	8.30	1.3×10^5
22	Dry soil	Pond outside of the plant	8.25	7.8×10^4
23	Soil and liquid	Pond outside of the plant	7.27	6.6×10^5
24	Soil and liquid	Pond outside of the plant	6.96	9×10^3
25	Soil and liquid	Pond outside of the plant	6.90	1.43×10^4
26	Soil and liquid	Pond outside of the plant	7.53	4×10^4
27	Soil and liquid	Pond outside of the plant	6.87	7.2×10^3
28	Soil and liquid	Pond outside of the plant	7.39	1×10^5
29	Liquid	Pond outside of the plant	6.86	9.3×10^4
30	Liquid	Pond outside of the plant	6.90	7×10^4
31	Liquid	Pond outside of the plant	6.78	6×10^4
32	Soil	In front of the crusher	9.27	5×10^5
33	Soil	In front of the crusher	9.21	1.09×10^3
34	Soil	In front of the crusher	8.96	1.94×10^5
35	Soil	In front of the crusher	8.78	2.01×10^5
36	Soil and liquid	In front of the crusher	7.90	6.1×10^4
37	Soil and liquid	In front of the crusher	8.27	1.6×10^4
38	Soil and liquid	In front of the crusher	8.32	2.9×10^5
39	Soil	In front of the concentrator	8.49	4.5×10^6

Sample 39 (Table 3.2), taken in front of the concentrator plant, has the highest CFU followed by sample 3 from the concentrator plant and samples 20 and 23 from the pond outside the concentrator plant. Sample 27, from the pond outside the concentrator plant, had the lowest CFU followed by sample 1, from the concentrator plant and 24 from the pond outside the concentrator plant. In general, soil samples had the highest CFU's compared to the liquid and slurry samples.

Bacterial community composition is affected by the soil where the bacterial community is found. The high CFU's seen in sample 39 could be due to the nature of the sample since it is a soil sample. A study by Kemp and Aller (2004),

investigated the bacterial diversity in various environments including aquatic and soil environments using rDNA libraries. They found that the phylogenetic richness of soils seems to be higher than the aquatic environments, indicating that it had higher bacterial diversity. Several studies have shown that soil has one of the highest bacterial diversity compared to other environments (Thompson *et al*., 2017; Bahram *et al*., 2018; Louca *et al*., 2019) The higher CFUs of the soil samples compared to the liquid samples could also be attributed to the number of organic nutrients available in the soil compared to the water found at the concentrator plant.

The difference in CFU's between the soil and water samples could also be due to the storage of the samples. Ahammed (2003), showed that the storage at 4°C of bacterial water samples results in a decrease in the CFUs of the bacteria after 12 hours. A study by Lee *et al*. (2021), showed that the CFUs of bacteria in soil samples was not significantly affected when stored at 4°C over a period of time.

3.3.2 Identification of bacterial isolates

The DNA sequences obtained from sequencing the bacterial isolates were matched with sequences of a similar nature on the NCBI website using the BLAST tool. The BLAST query shown in Table 3.3 demonstrated that a total of 50 bacterial species were isolated from the concentrator plant and the areas surrounding the plant. Bacterial species from the genus Bacillus and Pseudomonas were dominant, however, there was a wide variety of different genera.

Table 3.3: Identity of bacteria and GenBank Accession numbers of closely related species or species with highest sequence similarity percentage.

Isolate no.	Mine sample no.	BLAST prediction	GenBank Accession	Read length	E-value	% Identity	Phylum
15	26	*Acidovorax* sp.	AB646302.1	1124	0	100	
26	32	*Pseudomonas aeruginosa*	HQ018745.1	870	0	78,04	
27	26	*Pseudomonas brassicacearum*	KT695844.1	1381	0	99,71	
29	3	*Pseudomonas stutzeri*	CP071899.1	1439	0	99,93	
30	1	*Pseudomonas alcaliphila*	CP016162.1	1435	0	99,16	

33	5	*Pseudomonas stutzeri*	MT027239.1	1435	0	99,93	
35	4	*Pseudoxanthomonas mexicana*	JX860406.1	1442	0	99,65	
36	13	*Pseudoxanthomonas mexicana*	CP060731.1	1445	0	99,93	
37	31	*Comamonas aquatica*	KJ395470.1	1426	0	99,93	
38	21	*Salmonella enterica*	CP053585.1	1441	0	97,43	
41	36	*Pseudomonas otitidis*	AP022642.1	855	0	99,88	
43	9	*Pseudoxanthomonas mexicana*	JX860406.1	1394	0	99,93	
45	14	*Alishewanella fetalis*	MN809397.1	1419	0	99,15	
46	6	*Pseudomonas alcaligenes*	KT998857.1	1440	0	99,93	
49	10	*Methylobacterium adhaesivum (98% match)*	AB698743.1	826	0	98,42	
7	25	*Bacillus* sp.	MN961108.1/ CP050183.1	1459/1458	0/0	99,86/99,93	Firmicutes
10	18	*Bacillus* sp.	CP053938.1/ CP053997.1	1457/2908	0/0	99,86/99.86	
16	3	*Staphylococcus capitis*	CP053957.1	1399	0	99,71	
25	24	*Bacillus* sp.	MT510153.1/ MT490862.1	1401/1402	0/0	100/100	
28	24	*Bacillus* sp.	MT534569.1/ MT527538.1	1229/1229	0/0	99,92/99,92	
31	15	*Bacillus* sp.	MT538261.1/ MT184866.1/ CP040334.1	1448/1448/1448	0/00	99,93/99,93/99,93	
32	37	*Bacillus* sp.	MN581187.1/ MN006152.1	1440/1440	0/0	100\100	
34	16	*Bacillus* sp.	CP053938.1/ CP053997.1	1447/1447	0/0	99,86/99,93	
39	38	*Bacillus halotolerans*	CP054584.1	1445	0	99,93	
40	30	*Paenibacillus alvei*	MT498465.1	1437	0	99,51	
42	34	*Bacillus* sp.	CP054568.1/ CP053997.1	1133/1133	0/0	99,74/99,74	
5	35	*Arthrobacter globiformis*	EU221407.1	803	0	100	Actinobacteria
9	39	*Georgenia satyanarayanai*	MN326714.1	1409	0	99,01	
17	22	*Rhodococcus* sp.	MK443065.1/ MG833299.1	1090/1086	0/0	99,91/100	

Isolate no.	Mine sample no.	BLAST prediction	GenBank Accession	Read length	E-value	% Identity	Phylum
18	5	*Rhodococcus pyridinivorans*	MN179918.1	1423	0	99,86	
20	35	*Arthrobacter globiformis*	EU221407.1	842	0	99,76	
48	7	*Pseudarthrobacter phenanthrenivorans*	MN658730.1	835	0	99,52	
50	23	*Pseudarthrobacter defluvii*	KY882049.1	835	0	99,28	
14	23	*Microbacterium* sp.	CP027929.1, CP018134.1, KX404917.1	1361;1361;1361	0;0;0	99,85; 99,85; 99,85	Actinomycetota
21	20	*Microbacterium* lacusdiani	MG947593.1	1426	0	99,79	
22	30	*Microbacterium foliorum*	KP208636.1	830	0	99,75	
23	22	*Leucobacter chromiiresistens*	NR_117509.1	1369	0	100	
24	19	*Nocardia cyriacigeorgica*	LR215973.1	1406	0	100	
44	20	*Nocardia farcinica*	MT317187.1	1417	0	100	
19	23	*Chryseobacterium takakiae*	KC560016.1	575	0	98,43	Bacteroidetes
47	39	*Mucilaginibacter polysacchareus*	KM019772.1	1359	0	99,93	

The Proteobacteria phylum consisted of bacterial species such as *Pseudomonas*, *Acidovorax* and *Pseudoxanthomonas* (Table 3.3). *Pseudomonas* species have been shown to grow in heavy metal contaminated soils, they require minimum nutrients and some get energy from iron and sulphur oxidation (facultative chemolithotrophic) (Williams, Surridge & Cloete, 2008; Ranalli, Zanardini & Sorlini, 2009; Choudhary & Sar, 2011; Dixit, 2018). *Acidovorax* species have also been shown to be heavy metal tolerant, making them suitable for use in bioremediation (Ghane, 2017). In this study, the Firmicutes phylum consisted of mostly *Bacillus* species. *Bacillus* species have been isolated from various mines worldwide including a South African platinum mine (Marais, 2012). Some *Bacillus* species can detoxify heavy metals (Syed & Chinthala, 2015) and oxidise sulphur

and iron (Zhou *et al*., 2018), enabling this species to inhabit environments with high metal concentrations such as a platinum concentrator plant.

The isolation method in this study selected for mesophilic bacteria (Table 3.3). Some of the bacterial species have not been previously reported as isolates from mines, this includes, *Chelatococcus daeguensis, Pseudoxanthomonas mexicana, Georgenia satyanarayanai, Staphylococcus capitis, Chryseobacterium takakiae, Microbacterium* sp., *Leucobacter chromiiresistens, Nocardia cyriacigeorgica, Comamonas aquatica, Salmonella enterica, Bacillus halotolerans, Paenibacillus alvei, Nocardia farcinica, Alishewanella fetalis, Mucilaginibacter polysacchareus, Pseudarthrobacter phenanthrenivorans, Methylobacterium adhaesivum and Pseudarthrobacter defluvii*. This could be because little research has been conducted on identifying and characterising bacteria from platinum mines.

Some of the bacteria such as *G. satyanarayanai* and *Microbacterium* sp. have been isolated from other environments and have bioleaching capabilities.
Georgenia satyanarayanai has been isolated from a soda lake in India and a study by Shivlata & Tulasi (2015), showed that it has the potential to be used for bioleaching of metals at alkaline sites. Three *Microbacterium* spp. were isolated in this study, from the pond outside the concentrator plant. *Microbacterium* spp. have been previously isolated from soils contaminated with heavy metals (Brown *et al*., 2012), and some species have been shown to reduce metals such as chromium and alter heavy metal motility in contaminated soils (Soni *et al.*, 2014; Kumar & Saini, 2019). It has been postulated that *M. adhaesivum* has the potential to solubilise zinc and produce iron-chelating compounds but more research is required (Yadav & Nath Yadav, 2018). Therefore, these isolated *G. satyanarayanai* and *Microbacterium* bacterial species can potentially be used for PGM bioleaching.

Several of the isolated bacteria have been previously isolated from mines such as copper, coal, iron, and platinum mines. This includes *Arthrobacter globiformis*, *Bacillus* sp., *Pseudomonas stutzeri, Acidovorax* sp., *Rhodococcus* sp., *Rhodococcus pyridinivorans, Pseudomonas aeruginosa, Pseudomonas brassicacearum,*

Pseudomonas alcaliphila, Pseudomonas otitidis and Pseudomonas alcaligenes. Four *P. stutzeri* bacterial species were isolated from the concentrator plant. Isolate 8 comes from the Rougher Feed, 13 from the Cleaner Scavenger Tail A, 29 and 33 from Rougher Tail A and B respectively. Palanivel *et al.* (2020), found that *P. stutzeri* has copper bioremoval and bioaccumulation properties. These properties are important during the base metal bioleaching of PGMs. Three *Acidovorax* sp. (11,12 and 15) were isolated from the pond outside of the concentrator plant. A study by Xie *et al*. (2007), on the microbial population at several acid mineral bioleaching systems at a copper mine found that *Acidovorax* sp. were present in these populations, thus suggesting that they have high metal tolerance and could potentially be used for bioleaching. *Acidovorax* sp. have also been shown to be present in the bacterial community involved in chalcopyrite bioleaching in a shake flask system (He *et al*., 2010).

Two *Rhodococcus* bacterial species have been identified in this study, one from the concentrator plant and another from the pond outside the concentrator plant. *Rhodococcus* species including *R. pyridinivorans* have been isolated from copper and gold mines and some of these species are also capable of copper biosorption (Neeratanaphan, Rungsunsombut & Phonimdaeng, 2015; Sulistinah, Riffiani & Sunarko, 2015; Baltazar *et al.*, 2019). *Pseudomonas aeruginosa* has low nutritional requirements and has been isolated from low nutrient environments including uranium mine waste and iron mine (Williams, Surridge & Cloete, 2008; Choudhary & Sar, 2011). In this study *P. aeruginosa* was isolated from a soil sample collected in front of the crusher. It has previously been shown to be tolerant to copper, nickel and chromium and has the potential for use in bioleaching and bioremediation (Abdelbary, Elgamal & Farrag, 2019).

Eight *Bacillus* sp. isolates were identified, isolates 31 and 34 come from the Cleaner Scavenger Tail A and isolate 10 from Cleaner Scavenger Tail B samples taken from the concentrator plant. *Bacillus* sp. isolates 7, 25 and 28 come from a soil and liquid sample taken from the pond outside the concentrator plant and isolates 32 and 42 come from a soil sample taken from in front of the crusher. *Bacillus* sp. was the

most identified bacterial species in this study and inhabited 3 out of the 4 collection sites. *Bacillus* sp. have previously been isolated from several areas including platinum, coal and chromite mines (Dhal *et al*., 2010; Marais, 2012; Rekha, Dhanalakshmi & Sudha, 2013; Banerjee e*t al*., 2019). One *P. brassicacearum* bacterial isolate was identified from the pond outside of the concentrator plant it has previously been isolated from copper mines (Hassanshahian *et al*., 2019). Two *A. globiformis* bacterial isolates were identified, both come from a soil sample taken from in front of the crusher. *A. globiformis* is commonly found in the rhizosphere of graminaceous crops and has been isolated from an abandoned mine (Sharma *et al*., 2016).

In addition to organic acid-producing bacteria, iron and sulphur oxidising bacteria can be used for metal bioleaching. *Pseudomonas aeruginosa*, *Bacillus* sp., *P. stutzeri* and *P. brassicacearum* can oxidise iron and sulphur (Mason & Kelly, 1988; Cornelis & Dingemans, 2013; Xia *et al*., 2017; Zhou *et al*., 2018; Hassanshahian *et al*., 2019). Six *P. mexicana* bacterial isolates were identified from the concentrator plant. Isolates 4 and 35 come from the Rougher Feed, isolates 2 and 43 from Scavenger Tail A, isolate 36 from the cleaner feed and isolate 3 from the Cleaner Scavenger Tail A. *Pseudomonas mexicana* has previously been isolated from an anaerobic digester and the rhizosphere of a leguminous plant can oxidise sulphur (Thierry *et al*., 2004; Ghosh & Roy, 2007; Krishnani *et al*., 2010). These bacterial isolates have the potential to be used for the bioleaching of PGMs through the oxidation of sulphur and iron.

3.4 Summary and conclusions

The bacteria prefer growing at a pH between 6-9. Therefore, bioleaching should be done between pH 6-9.-The soil samples had a higher number of bacteria compared to the liquid and slurry samples.

A total of 50 mesophilic bacterial isolates were identified from the platinum mine concentrator plant and its surrounding. The bacteria belong to five phyla namely, Proteobacteria, Actinobacteria, Firmicutes, Actinomycetota and Bacteroidetes, with

Proteobacteria being the most dominant. *Pseudomonas* and *Bacillus* species were the most dominant. Several of the isolated bacteria are sulphur oxidising bacteria such as *P. mexicana* and some are iron oxidisers such as *P. stutzeri* and *P. aeruginosa*. Some *Bacillus* sp. can oxidise both iron and sulphur. *Bacillus* sp., *P. mexicana, P. stutzeri, P. aeruginosa* and *P. brassicacearum* can produce organic acids. Several of the identified bacteria have previously been isolated from mines such as copper and platinum mines and these include *P. stutzeri, P. brassicacearum, P. aeruginosa* and *Bacillus* sp. Bioleaching experiments have been conducted using *Acidovorax* sp., *P. aeruginosa* and *P. brassicacearum*.

CHAPTER FOUR: CHARACTERISATION OF BACTERIA

4.1 Introduction

The characterisation of bacteria isolated from mines has focused on their heavy metal tolerance/resistance. However, the bacteria from these mines could have properties desirable for the metal bioleaching process. Bacteria have been used for biotechnological processes, due to their ability to grow fast and produce desirable metabolites. Before using bacteria for any biotechnological process, it is important to characterise the bacteria. This includes their preferred growth conditions, growth rate, and metabolite production.

When growing bacteria to produce metabolites it is important to note what medium results in optimum growth. There are many media available, and the composition of the media varies. Different media provide different carbon sources for the bacteria which can result in different growth rates and metabolite production (Carlson *et al.*, 2020).

Organic acids such as citric, lactic, butyric, propionic, gluconic and formic acids are gaining more attention for use in bioleaching. These acids are biodegradable and effective in metal dissolution (Pathak, Vinoba & Kothari, 2021). In some cases, the organic acid leaching efficiency has been shown to be greater than the typically used inorganic acids (Al-Sheeha *et al.*, 2013). The organic acids dissolve metals through complexation and acidolysis. Bacteria have been shown to produce these organic acids (Anastassiadis *et al.*, 2008; Delvasto *et al*., 2008; Juturu & Wu, 2016; Gonzalez-Garcia *et al.*, 2017). This study focused on characterising six bacterial isolates based on morphology, growth, and organic acid production.

4.2 Materials and Methods

4.2.1 Characterisation of cellular and colony morphology

A total of 50 bacterial isolates were identified, however multiples of the same bacterial species were identified (Table 3.3). In these cases, one of the repeated bacterial species was selected for characterisation, in total 31 bacterial isolates were characterised for cell and colony morphology. The cell and colony morphology were investigated as a general characterisation of the identified bacteria and to see whether the identified bacteria where different, morphologically, to already classified bacteria of the same species. The colony morphology of the bacteria isolated from the Anglo Platinum Mogalakwena Mine was determined. The bacteria grown on LB (pH 7) agar plates were analysed and the shape and colour of the colonies were recorded. The cellular morphology of each isolate was determined using the Gram staining technique according to Coico (2006).

4.2.2 Growth curves

Six bacterial isolates (Table 4.1), *Bacillus* sp., *P. mexicana*, *P. stutzeri*, *A. globiformis*, *P. aeruginosa* and *P. brassicacearum* were isolated from the Anglo Platinum Mogalakwena Mine. An extensive literature review (Table 4.1) was done, and these six bacterial species were selected for characterisation based on their potential to produce organic acids and oxidise iron and sulphur.

Table 4.1: Six bacterial isolates isolated from a platinum mine, the GenBank Accession numbers of closely related species or species with the highest sequence similarity percentage and studies showing their organic acid production and iron and sulphur oxidation capabilities.

Bacterial Isolate	GenBank Accession	Studies
Bacillus sp. (isolate 10)	CP053938.1/ CP053997.1	(Xia *et al*., 2017; Saeid, Prochownik & Dobrowolska-Iwanek, 2018; Zhou *et al*., 2018)
Pseudomonas stutzeri	LR134482.1	(Lalucat et al., 2006; Hao *et al*., 2007; Mahmood *et al*., 2009; Prasanna *et al*., 2010; Li, Li & Li, 2017)
Pseudoxanthomonas mexicana	MK942423.1	(Thierry *et al*., 2004; Krishnani *et al*., 2010)
Arthrobacter globiformis	EU221407.1	(Vijayalakshmi, 2011)
Pseudomonas aeruginosa	HQ018745.1	(Mason & Kelly, 1988; Sankaralingam *et al*., 2014)
Pseudomonas brassicacearum	KT695844.1	(Achouak *et al*., 2000; Kong & Glick, 2017; Hassanshahian *et al*., 2019)

To evaluate the growth and find the optimum growth media of the 6 selected bacterial isolates, growth curves were carried out in triplicates for 45 hours. The 6 isolates were each inoculated into 250 mL of Luria Bertani broth (5 g/L yeast extract, 5 g/L NaCl, 10 g/L tryptone), 9K broth [5 g/L $MgSO_4 \cdot 7H_2O$, 0.05 g/L $Ca(NO_3)_2$, 3 g/L $(NH_4)_2SO_4$, 0.5 g/L K_2HPO_4, 0.1 g/L KCl] and nutrient broth (16 g/L) (NB) and incubated at their respective growth temperatures. The growth media used was all at a pH of 7. The growth media and temperatures were selected based on the growth media used in the studies (Table 4.1) showing organic acid production and iron and sulphur oxidation. *Pseudomonas mexicana*, *P. aeruginosa*, *Bacillus* sp., and the mixed culture, which consisted of all the 6 selected bacterial isolates,

were incubated at 37°C (Thierry *et al*., 2004; Sakil Munna *et al*., 2015; Nur Indah Koni *et al*., 2017). A mixed culture was included to see how the bacteria would grow together. Studies have shown that bioleaching using a mixed culture of bacteria results is more efficient in bioleaching compared to using single cultures (Akcil, Ciftci & Deveci, 2007; Fu *et al.*, 2008; Peng *et al*., 2021) *Pseudomonas stutzeri* was incubated at 35°C (Lalucat *et al*., 2006) and *A. globiformis* and *P. brassicacearum* at 30°C (Achouak *et al*., 2000; Busse & Wieser, 2014). After 3 and 6 hours, 1 mL was sampled from each culture and the optical density (OD_{600}) was measured. One millilitre of LB, 9K and NB were used as blanks. After 20 hours and every 2 hours thereafter, the optical density was measured for 45 hours.

To test for organic acid production during the exponential phase, the 6 bacterial isolates were inoculated into 250 mL of nutrient broth (pH 7) and incubated at their respective temperatures for 16 hours. Thereafter, 1 mL was sampled for optical density to monitor bacterial growth and high-performance liquid chromatography (HPLC) analysis. The organic acids were not extracted prior to analysis, the broth containing the metabolites was analysed. The samples for HPLC analysis were centrifuged at 10000 rpm for 10 minutes. The supernatant was then filtered using 0.45 µm GVS cellulose acetate syringe filters and stored at 4°C until further analysis.

4.2.3 High-performance liquid chromatography

For the analysis of the organic acids, standards of gluconic, lactic, citric, acetic, butyric, propionic, formic, and indole-3-acetic acid were prepared with concentrations ranging from 1mM to 5 mM. The standards were filtered using 0.45 µm GVS cellulose acetate syringe filters. The organic acids were analysed using an Agilent-1200 series HPLC fitted with a Bio-rad fermentation column and refractive index detector. Separation was performed at a column temperature of 65°C and 20% H_2SO_4 was used as the eluent and was delivered at a rate of 1mL/min for 13 minutes.

4.2.4 Statistical analysis.

A one-way ANOVA was performed to analyse statistically significant differences between the mean growth of the 6 bacterial isolates in the three different media, namely 9K, LB and NB.

4.3 Results and Discussion

4.3.1 Phylogeny and Colony and cellular morphology

The Gram stain, cell shape and colony morphology of the isolated bacterial species are shown in Table 4.2 below.

Table 4.2: Morphological characteristics of the bacterial isolates grown on LB agar.

Bacterial Isolates	Gram stain	Cell shape	Colony morphology
Chelatococcus daeguensis	-	Rod	Circular, white opaque
Pseudoxanthomonas mexicana	-	Rod	Irregular, light brown
Arthrobacter globiformis	+	Rod/cocci	Circular, transparent white
Bacillus sp.	+	Rod	Circular, white
Pseudomonas stutzeri	-	Rod	Irregular, cream white
Georgenia satyanarayanai	+	Rod	Circular, yellow
Acidovorax sp.	-	Rod	Irregular, cream white
Microbacterium sp.	+	Rod	Irregular, cream white
Staphylococcus capitis	+	Cocci	Circular, white
Rhodococcus sp.	+	Rod/cocci	Circular, pale pink
Rhodococcus pyridinivorans	+	Rod/cocci	Circular, pale pink
Chryseobacterium takakiae	-	Rod	Circular, translucent yellow
Microbacterium lacusdiani	+	Rod	Circular, yellow
Microbacterium foliorum	+	Rod	Circular, pale yellow
Leucobacter chromiiresistens	+	Rod	Circular, yellow
Nocardia cyriacigeorgica	+	Rod	Circular, white
Pseudomonas aeruginosa	-	Rod	Circular, opaque white
Pseudomonas brassicacearum	-	Rod	Circular, white
Pseudomonas alcaliphila	-	Rod	Irregular, translucent white
Comamonas aquatica	-	Curved rod	Circular, white
Salmonella enterica	-	Rod	Irregular, translucent white
Bacillus halotolerans	+	Rod	Circular, cream white
Paenibacillus alvei	+	Rod	Circular, white
Pseudomonas otitidis	-	Rod	Irregular, cream white
Nocardia farcinica	+	Rod	Circular, cream white
Alishewanella fetalis	-	Rod	Circular, cream white
Pseudomonas alcaligenes	-	Rod	Circular, translucent white
Mucilaginibacter polysacchareus	-	Rod	Circular, light pink
Pseudarthrobacter phenanthrenivorans	+	Rod/cocci	Circular, cream white
Methylobacterium adhaesivum	-	Rod	Circular, light pink
Pseudarthrobacter defluvii	+	Rod/cocci	Circular, cream white

In this study, the number of Gram-positive and Gram-negative bacterial species was similar as shown in Table 4.2. Despite this, more Gram-negative bacterial species were isolated from the concentrator plant compared to the other sampling sites. The concentrator plant could have a higher concentration of heavy metals when compared to the other sampling sites as shown by a study performed by Ekosse (2006), which showed that the heavy metal concentration of the samples increased closer to the concentrator plant. This finding supports the theory that Gram-negative bacteria are more tolerant to heavy metals compared to their Gram-positive counterparts. Piotrowska-Seget, Cycoń & Kozdrój, (2005), found that metal tolerance has been more associated with Gram-positive bacteria such as those in the genera *Corynebacterium*, *Bacillus* and *Arthrobacter*. Several of the bacteria isolated in our study were identified as *Bacillus*, which corresponds with the findings of Ellis *et al*. (2003), that there was a higher abundance of *Bacillus* sp. in most soils contaminated with heavy metals.

Several studies have shown that Gram-negative bacteria are better adapted and more tolerant to heavy metal environments compared to Gram-positive bacteria (Hassen *et al.*, 1998; Wenderoth & Reber, 1999; Bennisse *et al.*, 2004). Benyehuda *et al*. (2003), stated that Gram-negative bacteria are more tolerant to heavy metals due to the complex structure of their cell wall, which forms a more effective barrier of permeability against the heavy metals thereby limiting the access of metals into the cells. They also argued that the surface structures that form the cell wall can interact with metal ions and detoxify the ions (Benyehuda *et al*., 2003). Another study by Mounaouer, Nesrine & Hassen (2014), also showed that Gram-negative bacteria are more heavy metal tolerant when compared to Gram-positive bacteria. It was stated that in addition to their cell wall, Gram-negative bacteria can produce low molecular weight proteins that can bind metals resulting in a conformational modification of the molecules into higher molecular weight molecules that are then unable to diffuse through the plasma membrane and enter the cell (Mounaouer, Nesrine & Hassen, 2014). Some Gram-negative bacteria do not have metal protein transporters which thus prevents or stops the entry of metals into the cell thus increasing its resistance or tolerance to heavy metals (Mounaouer, Nesrine & Hassen, 2014).

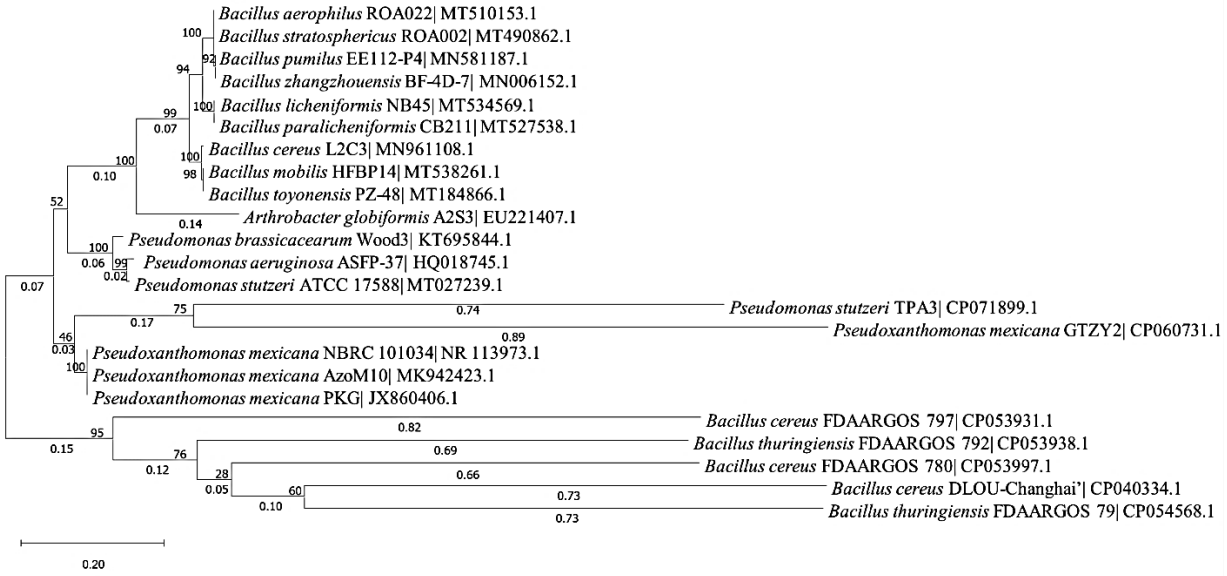


Figure 4.1: Phylogenetic analysis based on a partial sequence of the 16S rRNA gene inferred using the maximum likelihood method, showing the relationship of the six bacterial species and bacterial isolates of the same species.

Figure 4.1 shows the phylogenetic relationship of the six selected bacterial species and bacterial isolates of the same species identified in chapter 3. The six bacterial isolates from the present study showed a variety of morphological characteristics after incubation for 48 hours on nutrient agar as shown in Figure 4.2. The *Bacillus* sp. had circular, white colonies which is consistent with what was found in our study (Figure 4.2D). *Pseudoxanthomonas mexicana* had irregularly shaped, cream white colonies. *Pseudomonas aeruginosa* shown in Figure 4.2E, had irregularly shaped, opaque colonies. *Pseudomonas brassicacearum* had small white colonies as shown in Figure 4.2C. *Arthrobacter globiformis* has small white circular colonies (Figure 4.2A) and has previously been isolated from an abandoned mine (Sharma *et al.*, 2016). In Figure 4.1F, *P. stutzeri* has cream white wrinkle-like, irregularly shaped colonies.

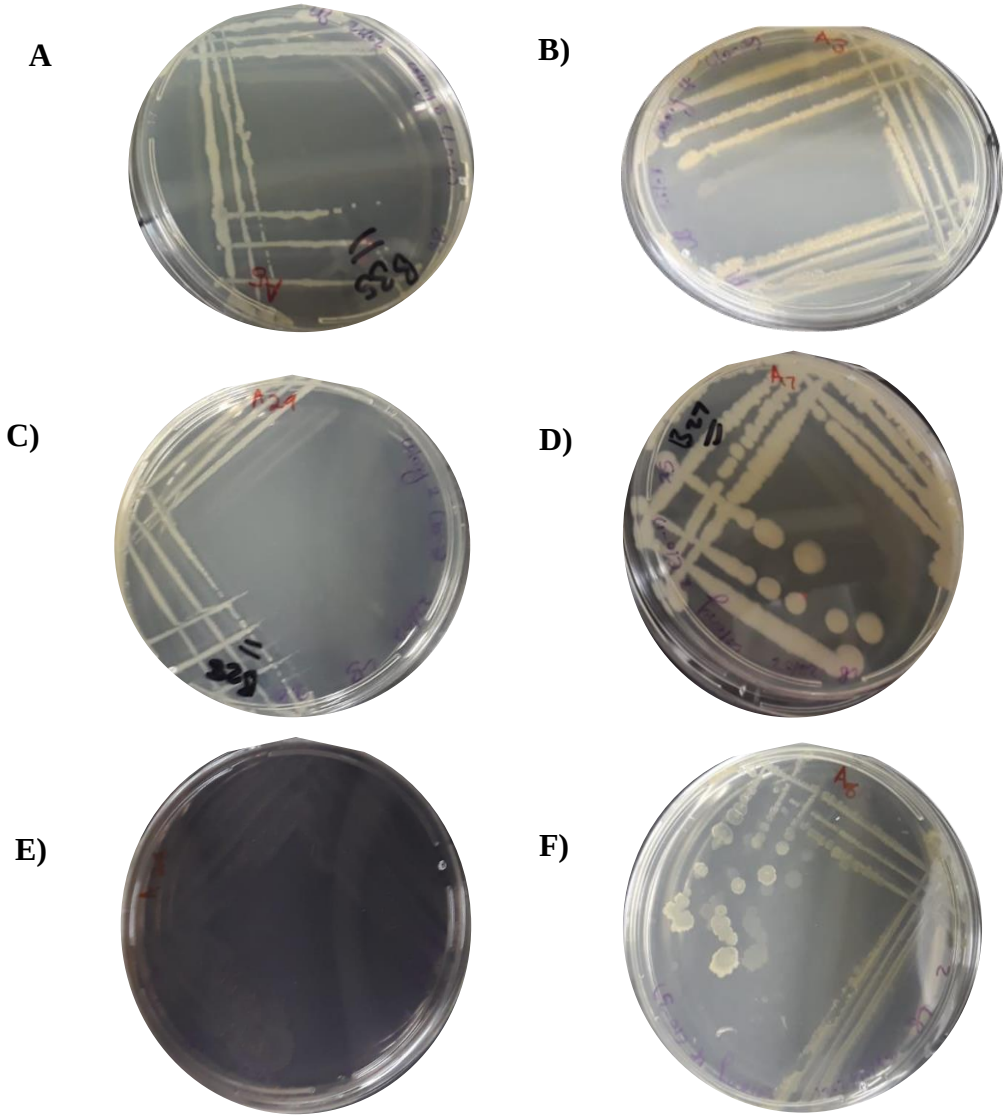


Figure 4.2: Morphological characteristics shown by the six bacterial isolates. The bacteria were identified as A) Arthrobacter *globiformis*, B) *Pseudoxanthomonas mexicana*, C) *Pseudomonas brassicacearum*, D) *Bacillus sp.*, E) *Pseudomonas aeruginosa*, and F) *Pseudomonas stutzeri.*

4.3.2 Growth curves

To determine which media is optimal for the growth of the 6 bacterial isolates, growth curves were conducted in 9K, LB and NB media. All 6 bacterial isolates were shown to grow best in NB media compared to 9K and LB media as shown in Figures 4.3, 4.4 and 4.5. There was little to no growth in 9K media and an

unsubstantial amount of growth in LB. For *Bacillus* sp., *P. stutzeri* and the mixed culture, the exponential phase was between hours 3 and 20. The exponential phase for *A. globiformis*, *P. mexicana* and *P. aeruginosa* was between 6 and 20 hours. *Pseudomonas brassicacearum* had slower growth in NB compared to the other isolates, with an exponential phase starting after 20 hours.

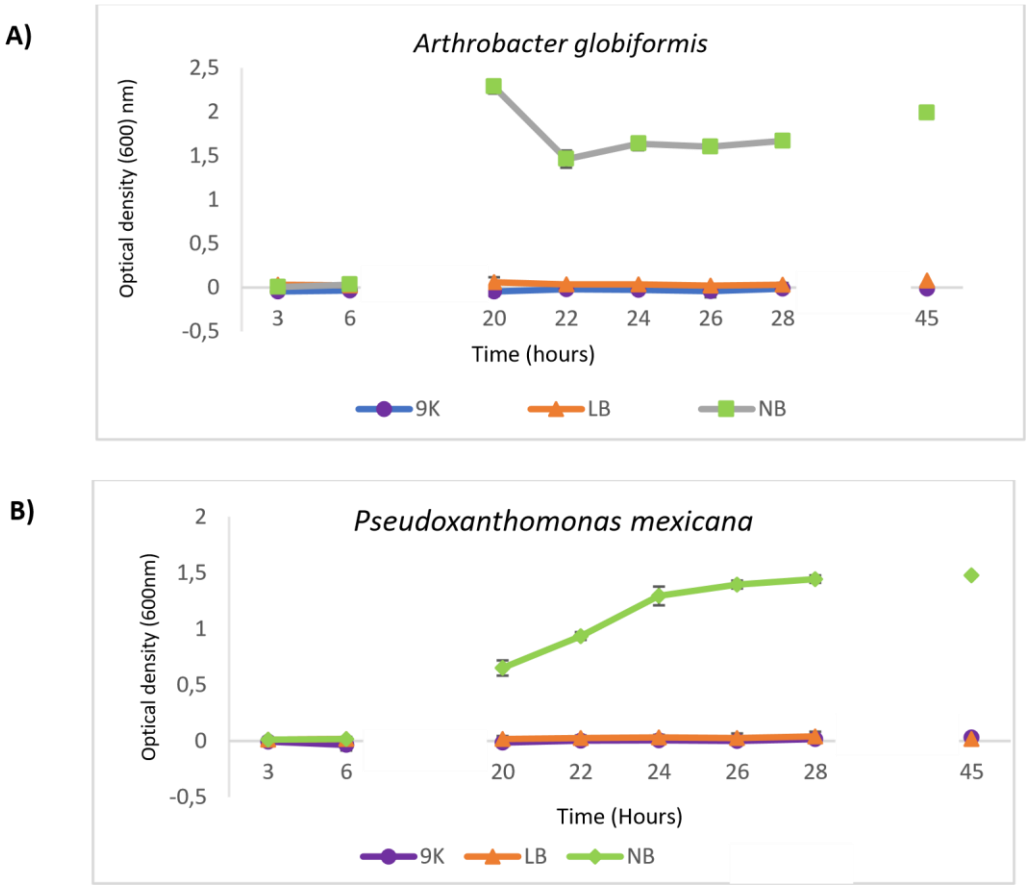


Figure 4.3: Growth curves of A) *Arthrobacter globiformis* and B) *Pseudoxanthomonas mexicana* grown in 9K, LB and NB broth. The error bars represent the standard deviations of the three bacterial replicates. The linear breaks on the x-axis indicate the missing sampling periods. The statistical significance was analysed using one-way ANOVA with $p < 0.05$. The bacteria showed the greatest growth in NB media and the least growth in 9K media.

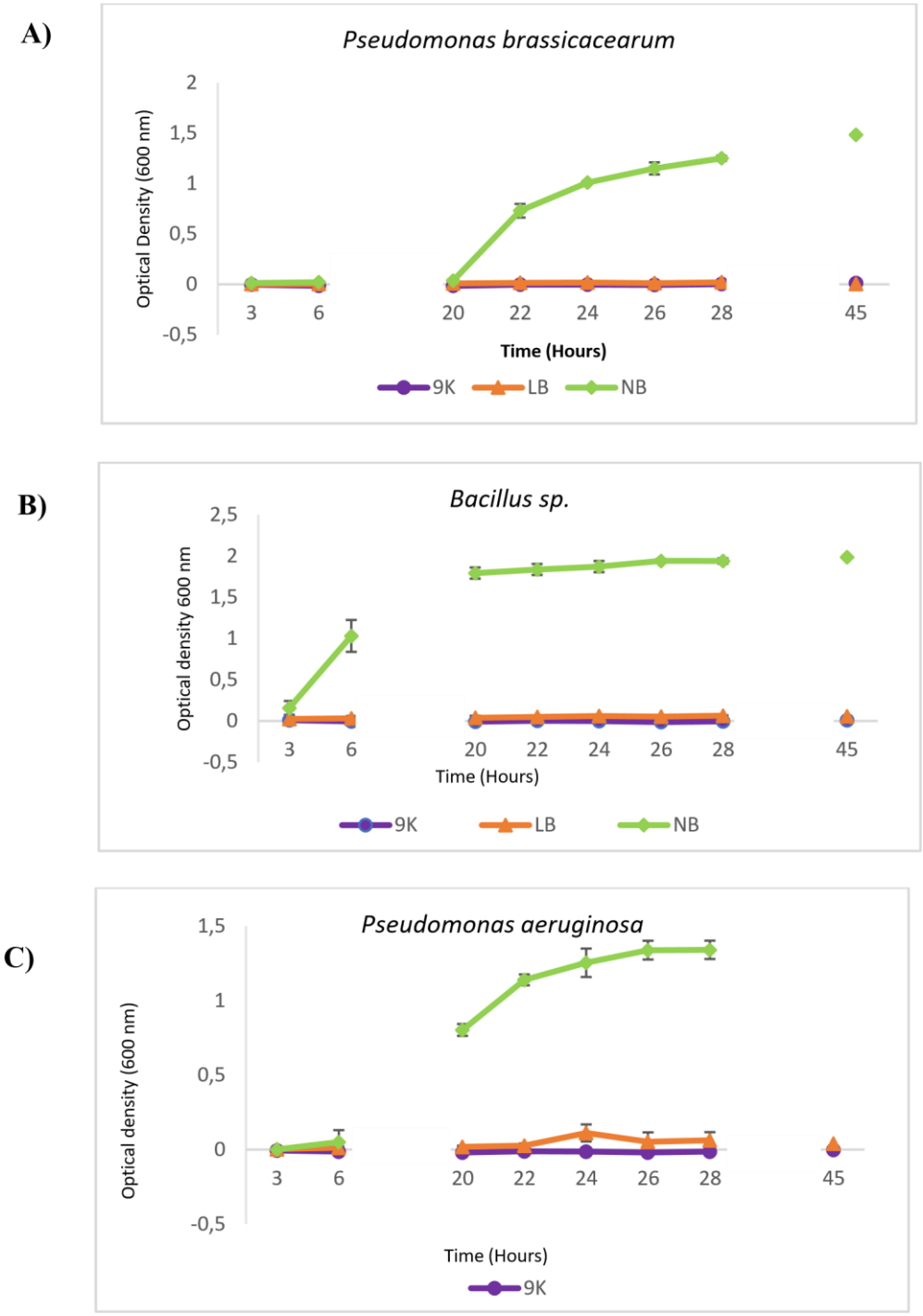
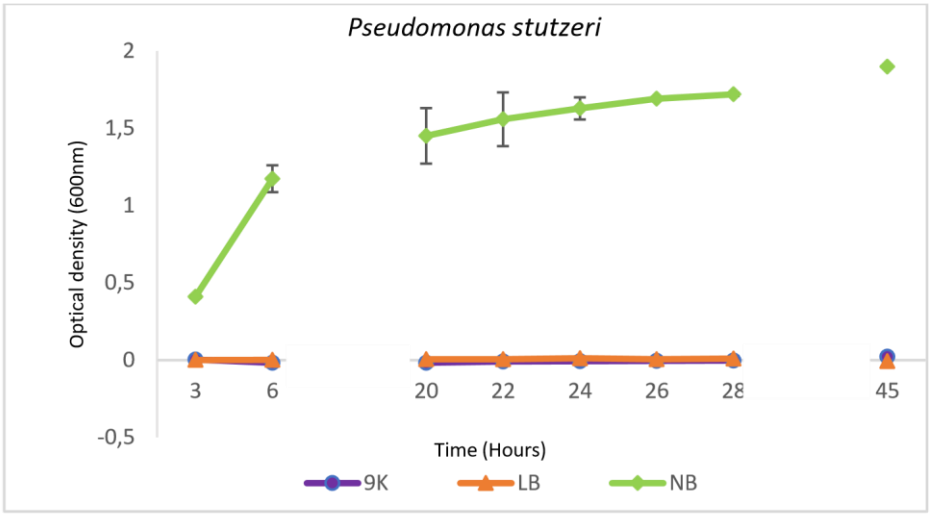


Figure 4.4: Growth curves of A) *Pseudomonas brassicacearum* and B) *Bacillus sp.* C) *Pseudomonas aeruginosa* grown in 9K, LB and NB broth. The error bars represent the standard deviations of the three bacterial replicates. The linear breaks on the x-axis indicate the missing sampling periods. The statistical significance was analysed using one-way ANOVA with $p < 0.05$. The bacteria showed the greatest growth in NB media and the least growth in 9K media.

A)



B)

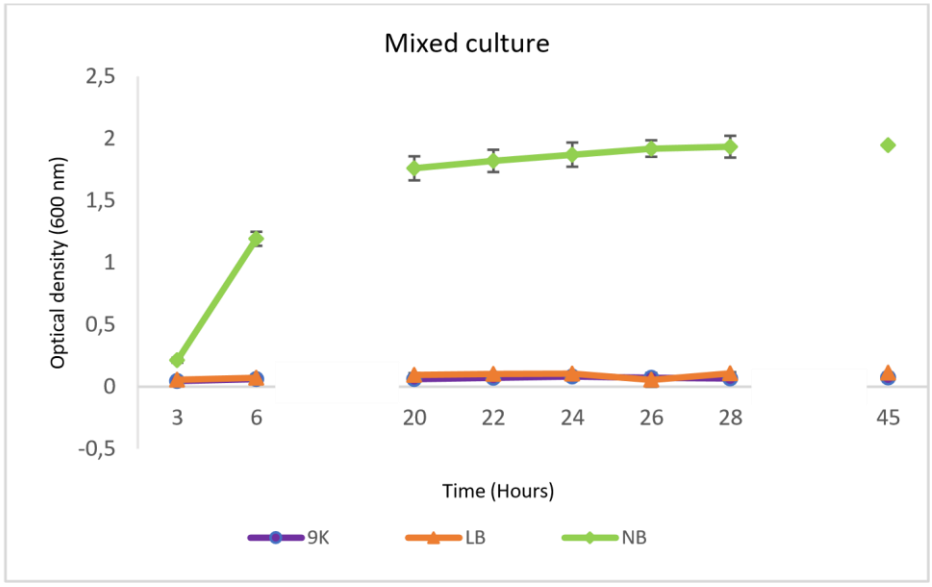


Figure 4.5: Growth curves of A) *Pseudomonas stutzeri* and B) mixed culture grown in 9K, LB and NB broth**.** The error bars represent the standard deviations of the three bacterial replicates. The linear breaks on the x-axis indicate the missing sampling periods. The statistical significance was analysed using one-way ANOVA with $p < 0.05$. The bacteria showed the greatest growth in NB media and the least growth in 9K media.

The growth rates of each of the 6 bacterial isolates were calculated during the exponential phase using the slope of the log of the growth curve. The following equation was used to calculate the respective bacterial growth rates: $\mu = \frac{2.303(\log OD_2 - \log OD_1)}{t_2 - t_1}$

$\mu = 2.303(\log OD_2 - \log \mu = \frac{2.303(\log OD_2 - \log OD_1)}{t_2 - t_1.}) / t_2 - t_1.$

Where μ is the growth rate, OD_1 is the initial optical density, OD_2 is the final optical density, t_1 is the initial time and t_2 is the final time.

The growth of each bacterial isolate is shown in Table 4.3.

Table 4.3: The growth rates of *Arthrobacter globiformis*, *Pseudoxanthomonas mexicana*, *Pseudomonas stutzeri*, *Pseudomonas brassicacearum*, *Bacillus sp.*, *Pseudomonas aeruginosa* and mixed culture grown in LB and NB media. The bacteria grown in the NB medium had the highest growth rate.

Bacteria	*Bacillus sp.*		*P. mexicana*		*P. stutzeri*		*A. globiformis*		*P. aeruginosa*		*P. brassicacearum*		Mixed culture	
Media	LB	NB	LB	NB	LB	NB	LB	NB	LB	NB	LB	NB	LB	NB
Growth rate (μ/hr)	0,015	0,039	0,002	0,256	0,069	0,350	0,069	0,304	0,027	0,197	0,071	0,449	0,021	0,028

The results show that NB is the optimum growth medium for the bacterial isolates when compared to LB and 9K. The composition of NB and LB differs significantly, with the main difference being that NB contains peptone and meat extract and LB contains tryptone. Tryptone is a mixture of peptides that is produced through the enzymatic digestion of casein by trypsin. Tryptone provides nitrogen in the form of amino acids which play a role in the synthesis of protein for the bacteria (Godbey, 2014). Meat peptone found in nutrient broth is produced through the enzymatic digestion of animal bovine tissue resulting in peptides and amino acids which provides a nitrogen source for microorganisms (Sanchez-Rosario & Johnson, 2021). The amino acid composition of tryptone and meat peptone differ, which can result in a difference in growth of the bacteria in the two different media. Although theoretically, amino acids can assist the growth of a variety of bacteria, the amount and distribution of amino acids can potentially alter the growth of bacteria.

Meat extract found in nutrient broth is produced from low-fat meat such as bovine heart and tendon content. It contains a combination of vitamins, peptides, amino

acids, and minerals (Pearson, 2003). Compared to peptone, meat extract does not undergo severe protein hydrolysis treatment, as a result, it can also provide phosphates and additional energy sources that might not be found in peptone. Little to no growth was observed for the bacterial isolates grown in 9K. 9K contains no organic compounds that the bacteria can use for energy, this shows that the bacterial isolates are heterotrophic and require an organic carbon source to grow.

It has been suggested that mixed bacterial cultures grow better than single bacterial cultures. In nature, bacteria flourish in compounded communities and the survival and fitness of a single bacterium is dependent on its interactions with other bacteria in the population (West *et al.*, 2006). Studies by (Paczia *et al.*, 2012; Fiore *et al.*, 2015) have shown that bacteria in communities cooperate metabolically, where the metabolites from one bacterial species are used as energy sources for another bacterial species in the population. In this study, the mixed culture did not grow better than the other bacterial isolates as shown by the growth rates in Table 4.3. This could be caused by some of the isolates producing antimicrobial compounds that inhibit the growth of the other bacterial isolates in the culture. *Pseudomonas stutzeri* has been shown to produce an antimicrobial compound, zafrin [4betamethyl-5, 6, 7, 8 tetrahydro-1 (4beta-H)-phenanthrenone], which has been shown to act against Gram-positive and Gram-negative bacteria (Uzair *et al.*, 2008). *Pseudomonas brassicacearum* produces hydrogen cyanide and the antimicrobial compound, 2,4-DAPG. This could result in the inhibition of bacterial growth (Zhou *et al.*, 2012).

The one-way ANOVA test was used to determine if there is a statistically significant difference between the mean growth of the bacterial isolates grown in the three different media. The p-value for all the bacterial isolates was less than 0.05. Therefore, there is a significant difference between the mean growth of the bacterial isolates grown in the different media. The media used have different compositions as explained previously, thus resulting in the difference in growth.

From the growth curves it was determined that the exponential phase of the bacterial isolates except for *P. brassicacearum* was approximately between 3 and 20 hours. The organic acid production and substrate consumption during the 16–22-hour

growth period of the bacterial isolates was tested. Figure 4.6 shows the optical density of the 6 bacterial isolates and the mixed culture. During this period *Bacillus* sp. has the highest optical density followed by the mixed culture. The optical densities of *A. globiformis*, *P. mexicana*, *P. brassicacearum* and *P. aeruginosa* are low compared to the other bacterial isolates.

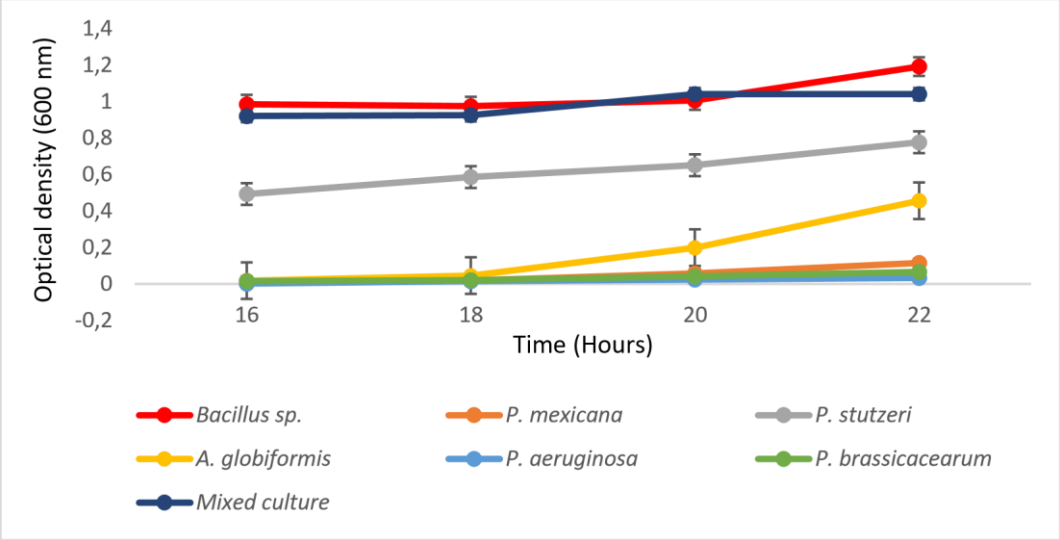


Figure 4.6: Optical density (600 nm) of *Bacillus* sp., *Pseudoxanthomonas mexicana*, *Pseudomonas stutzeri*, *Arthrobacter globiformis*, *Pseudomonas aeruginosa*, *Pseudomonas brassicacearum* and the mixed culture grown in nutrient broth. The error bars represent the standard deviations of the bacterial replicates.

Colony forming units of the 6 bacterial (Figure 4.7) were used to estimate the number of metabolically viable cells between 16 – 22 hours. The colonies of the mixed culture were too many to count (>300 colonies). *Bacillus* sp. has the highest CFUs followed by *P. stutzeri* this corresponds with the optical density values shown in Figure 4.6. However, after 22 hours all the bacterial isolates except *P. mexicana* had similar CFUs.

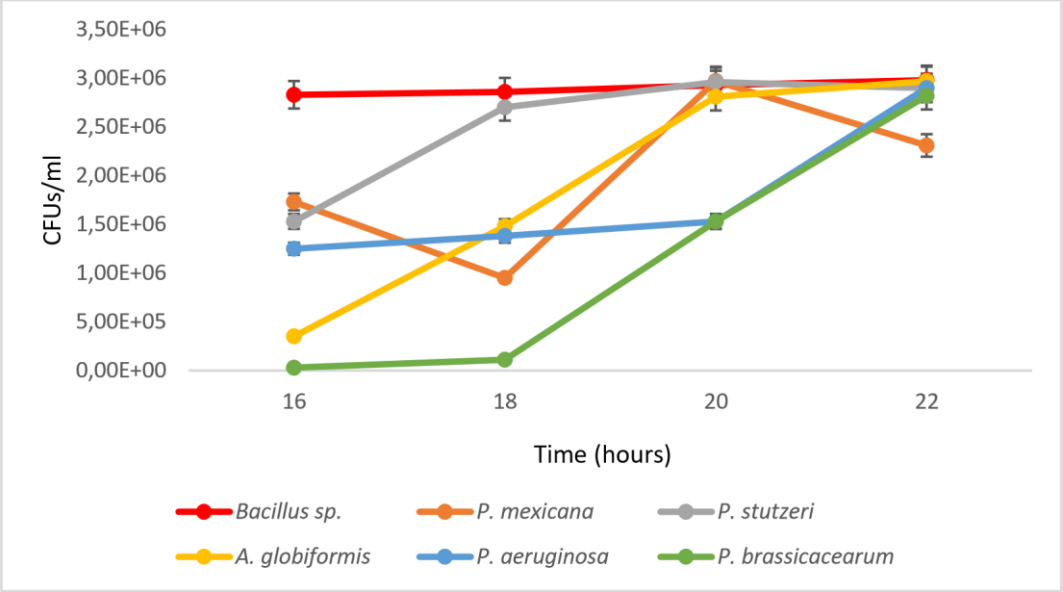


Figure 4.7: The colony forming units (CFUs) of *Bacillus* sp., *Pseudoxanthomonas mexicana*, *Pseudomonas stutzeri*, *Arthrobacter globiformis*, *Pseudomonas aeruginosa*, *Pseudomonas brassicacearum* grown in nutrient agar. The error bars represent the standard deviations of the bacterial replicates.

4.3.3 Organic acid production

The bacterial isolates were tested for organic acid production and the results are displayed in Table 4.4. All the bacterial isolates produced citric and lactic acid. Gluconic acid was only produced by *Bacillus* sp., *P. mexicana*, *P. stutzeri*, *A globiformis* and *P. aeruginosa*. Indole-3-acetic acid was produced by *Bacillus* sp., *P. mexicana* and *P. aeruginosa*. Butyric acid was produced by *P. mexicana* and *P. stutzeri*. Propionic acid was produced by *Bacillus* sp., *P. mexicana*, *A. globiformis*, and the mixed culture. Formic acid was produced by *P. mexicana*, *A. globiformis*, *P. aeruginosa* and the mixed culture. None of the bacterial isolates produced acetic acid.

Table 4.4: Organic acid production by the six bacterial isolates.

		Organic acid concentration (mM)						
Bacteria	**Time (Hours)**	**Gluconic acid**	**Citric acid**	**Indole-3acetic acid**	**Lactic acid**	**Butyric acid**	**Propionic acid**	**Formic acid**
Bacillus sp.	16	28,269±1,801		3,180±1,042	1,675±0,440			
	18	0,082±0.013						
	20		3,455±0,402		4,388±1,465			
	22		2,072±0,408		4,024±0,541		6,974±0,112	
P. mexicana	16	29,833±1,369		2,904+1,993	4,904±0,912	0,116±0.031		
	18		27,329±0,299		1,095±0,974	0,039±0,059		
	20		2,610±0,435		5,160±0,276		7,971±0,145	4,027±0,556
	22		2,506±0,211				8,174±0,871	
P. stutzeri	16	27,846±1,295						
	18					0,394±0,513		
	20							
	22		2,592±0,580		5,870±0,880			
A. globiformis	16	34,894±0,580					2,839±0,163	
	18				7.558±1,452			
	20		2,123±1,004		2,991±0,301		5,566±1,007	
	22		2,071±0,613		5,450±0,494		5,838±0,890	3,570±0,817
P. aeruginosa	16	30,173±1,611						
	18			4,162±1,722			0,914±0,515	8,644±0,702
	20		1,924±0,220		0,144±0,150		5,370±0,509	
	22		2,199±1,006		7,241±0,766		2,040±0,931	6,429±0,549
P. brassicacearum	16				2,700±0,460			
	18		0,003±0,004					
	20		3,078±1,016		16,178±1,363			
	22		2,471±0,556		9,982±0,182			
Mixed culture	16						2,301±0,631	
	18		2,821±0,258		9,307±0,850			
	20		1,985±0,300		18,973±0,085		4,877±0,283	
	22		3,705±0,859					0,103±0,126

1-Blank space indicates a concentration of 0 mM.

2- Acetic acid is not included in the table because none of the bacterial species produced it.

The use of organic acids for bioleaching has gained traction due to them being costeffective and economically friendly compared to chemical leaching. Organic acids are also biodegradable and result in little environmental pollution. The selected organic acids, except indole-3-acetic acid, have been used in bioleaching experiments. Gluconic acid has been used in the bioleaching of vanadium (Bellenberg *et al.*, 2021) and rare earth elements (Reed *et al.*, 2016). *Bacillus* sp., *P. mexicana*, *P. stutzeri*, *A. globiformis* and *P. aeruginosa* produced a gluconic acid at 16 hours. Little to no gluconic acid was detected in the 18–22-hour samples for these bacterial species therefore the presence of gluconic acid in the 16-hour sample seems to be an artifact. *Pseudomonas, Acetobacter* and *Gluconobacter* species have been shown to produce gluconic acid (Ramachandran *et al*., 2006). A study by Delvasto *et al*. (2008), found that a *Bacillus* species could produce 34 mM of gluconic acid after 7 days of growth. In our study *Bacillus* sp. produced 28,269 mM. An increase in gluconic acid production can be achieved by altering the growth temperature and medium. Nonetheless, these results show that the bacterial isolates have the potential to produce gluconic acid which can then be used in the bioleaching of PGMs.

Citric acid has been used in several bioleaching experiments. A study by Li *et al*. (2014), showed that the use of citric acid increased the metal leaching of copper tailings. Williams & Cloete (2010), investigated the use of microbial citric acid for the removal of phosphorus and potassium from an iron ore concentrate and found that 23.53% of phosphorus and potassium were removed. Another study by Barnett, Palumbo-Roe & Gregory (2018), used a *Bacillus* sp. that produced citric, gluconic and oxalic acid to bioleach rare earth elements and found that it was effective when compared to other leaching techniques. In our study, *P. mexicana* produced the most citric acid, however, to date no other known studies have shown that this bacterium can produce citric acid. *Bacillus, Pseudomonas* and *Arthrobacter* species have been shown previously to produce citric acid (Anastassiadis *et al*., 2008; Vyas & Gulati, 2009).

Indole-3-acetic (IAA) acid is a plant growth-promoting compound that is commonly produced by soil microorganisms found in the rhizosphere of plants

(Zhang *et al*., 2021).*Pseudomonas mexicana, Bacillus sp*. and *P. aeruginosa* from the current study produced IAA. The bacterial isolates have previously been isolated as endophytes which grow in close association with plants and produce plant growth-promoting compounds such as IAA (Liaqat & Eltem, 2016; Devi *et al*., 2017; Lopes *et al*., 2018).

Lactic acid has been used for the leaching of cobalt, nickel, lithium, and manganese from spent lithium-ion batteries with a recovery rate of up to 98.9% (Li *et al*., 2017). It has also been used, in conjunction with other organic acids for the dissolution of cobalt and nickel from limonite ores (Tang & Valix, 2004). *Pseudomonas mexicana* produced the most lactic acid followed by *A. globiformis*. The variance in lactic acid production for these bacterial species could be a result of the acidification of the medium during organic acid production. Lactic acid is commonly produced by lactic acid bacteria (LAB) which include *Lactobacillus*, *Leuconostoc* and *Lactococcus* species (Juturu & Wu, 2016). However, these bacteria are fastidious, have complex nutritional requirements and grow at low temperatures. *Bacillus* species have been shown to produce high amounts of lactic acid when grown at a high pH (Abedi & Hashemi, 2020). The increased production of lactic acid usually requires high temperatures because amylases, which hydrolyse starch to lactic acid, have increased activity at higher temperatures (Juturu & Wu, 2016). Thus, increasing the temperature and pH for the bacterial isolates could potentially result in higher lactic acid production.

Butyric acid has been used in the bioleaching of zinc (Wang *et al*., 2019), while acetic acid has been used for the leaching of scandium (Kiskira *et al*., 2021), zinc (Elomaa *et al*., 2019) and lead (Osasona, Oke & Adebayo, 2021). Butyric acid was only produced by two of the bacterial isolates in very low amounts. Butyric acid production is commonly an anaerobic process and is produced by strictly anaerobic bacteria such as *Clostridium* and *Butyrivibrio* species. The culture conditions favourable for butyric acid production include temperatures between 35-37°C and glucose, sucrose, and lactose as the preferred carbon sources. In this study, NB was used as the medium of choice and did not contain the necessary carbon sources for butyric acid production. The bacterial isolates were also grown at different

temperatures with *A. globiformis* and *P. brassicacearum* being grown at 30°C, which can account for these isolates not producing any butyric acid. The bacterial isolates were also grown in aerobic conditions, which is not favourable for butyric acid production and could have resulted in little to no butyric acid being produced.

Acetic acid was not produced by any of the bacterial isolates. Of the 6 bacterial isolates, only *Bacillus* sp. has been shown to produce acetic acid (Yan *et al*., 2013; Mumtaz *et al.*, 2019). Acetic acid production is typically an aerobic fermentative process and bacteria from the *Acetobacter* and *Gluconobacter* genera are the most commonly used (Xu, Shi & Jiang, 2011). The medium used to grow up the bacteria as well as the growth temperature could have resulted in no acetic acid production.

Formic acid has been used to enhance the bioleaching of copper from chalcopyrite concentrate (Arslanoğlu & Yaraş, 2020). The study also showed that formic acid could be used as a leaching agent for the leaching of precious metals containing ores. Formic acid was also used in the bioleaching of cobalt from copper/cobaltrich sulphatic mine tailings, with a recovery of 66% (Zhang *et al*., 2020). *Pseudomonas aeruginosa* produced the most formic acid in our study.

Propionic acid has been used in the chemical leaching of iron ore. It has also been used in combination with other organic acids for the bioleaching of cobalt and copper from lithium batteries (Urias *et al.*, 2021). *Pseudoxanthomonas mexicana* and *A. globiformis* produced the most propionic acid. There are no studies that have shown these bacteria to produce propionic acid. Propionic acid is produced by several bacteria including *Propionibacterium acidipropionici*, *Clostridium homopropionicum* and *Propionibacterium jensenii* (Ranaei *et al*., 2020). *Propionibacterium* is suggested as the best producer of propionic acid (GonzalezGarcia *et al.*, 2017). Wang, Jin & Yang (2015), showed that *P. acidipropionici* was able to produce up to 751.890 mM of propionic acid. *Pseudomonas mexicana* and *A. globiformis* produced a total of 16.145 and 14.243 mM of propionic acid respectively. When compared to *P. acidipropionici*, these concentrations are very low. This could be because these bacteria are not common propionic acid producers, and the culture conditions were not favourable for propionic acid production.

4.4 Summary and conclusions

For the morphology of the bacterial isolates, there was not a considerable difference in the number of Gram-positive and Gram-negative species. Most of the bacterial species were rod-shaped and only one was cocci shaped. The six selected bacterial species did not grow in 9K media and there was very little growth in LB broth. The bacteria grew best in NB. The mixed culture which was expected to have the fastest growth did not grow better than the individual bacterial isolates. The bacteria produced organic acids, with *P. aeruginosa* producing the most formic and indole3-acetic acid, *P. mexicana* producing the most lactic and propionic acid, *P. stutzeri* producing the most butyric acid, *A. globiformins* producing the most gluconic acid and the mixed culture producing the most citric acid.

From the growth curves, NB is the best medium to grow the bacteria in. The bacteria produce organic acids which can be used for bioleaching. A mixed culture of the bacteria that produced the most organic acids could be used during bioleaching. However, low amounts of organic acids are produced, and it would be necessary to explore ways to increase organic acid concentrations. Overall, the bacteria have great potential to be used in the bioleaching of PGMs.

CHAPTER 5: CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

This study aimed to isolate, identify, and characterise indigenous bacteria from a platinum mine concentrator plant and its surroundings. The bacteria were identified using molecular techniques and characterised morphologically, by Gram-staining and metabolically through growth curves, analysing and organic acid production. This is the first known study to identify and characterise bacteria from a platinum mine concentrator plant.

An estimation of the abundance of bacteria found at the concentrator plant was made using colony-forming units (CFUs). There was a substantial abundance of bacteria found at the concentrator plant. The pH of the soil where the bacteria inhabit has an influence on the number of bacteria found in that soil. This study showed that the isolated indigenous bacteria preferred growing in soils with a pH between 6-9. Therefore, when growing the bacteria up in the laboratory the media should be at a pH between 6-9.

The molecular identification of the bacteria showed that there were 50 different bacterial isolates. This suggests that the bacteria found at the concentrator plant and its surrounding are quite diverse and it also shows that these bacteria can adapt to unfavourable environments such as heavy metal contaminated environments. Bacteria from the Proteobacteria phylum such as *Pseudomonas, Pseudoxanthomonas* and *Acidovorax* species were the most dominant. Several of the indigenous bacteria such as *P. aeruginosa*, *Bacillus sp.*, *P. brassicacearum*, *P. stutzeri* and *P. mexicana* had some properties that are desirable for bioleaching such as iron and sulphur oxidation and organic acid production.

Of the 50 identified bacterial isolates, six were selected for further characterisation namely, *P. aeruginosa*, *P. mexicana*, *A. globiformis*, *P. stutzeri*, *P. brassicacearum* and *Bacillus sp*. These bacteria grew well in NB and reached the exponential phase within six hours, except *P. brassicacearum* which reached the exponential phase after 20 hours. These bacteria produced gluconic, citric, lactic, propionic, and formic acid which have been shown to be effective in bioleaching. In conclusion, these six indigenous bacteria have great potential to be used in bioleaching.

5.2 Recommendations

This study has shown that bacteria isolated from a platinum mine can be used to produce organic acids. It is therefore recommended that these bacteria be used for organic acid production for bioleaching purposes. It is also suggested that more organic acid production tests using these bacteria are conducted using different culture conditions.

Based on literature, few of the isolated bacteria can oxidise iron and sulphur. It is recommended that these bacteria be tested, to determine whether they are iron and sulphur-oxidising bacteria

The bacteria were isolated from a platinum mine concentrator plant and its surroundings. The presence and concentration of heavy metals at the concentrator plant and its surrounding should be measured. Thereafter performing metal tolerance tests for these bacteria is recommended.

APPENDIX A

A1 Preparation of media used in the isolation and enumeration of bacterial species.

A1.1 Luria-Bertani Agar Medium

Composition

Yeast Extract	5 g/L
Sodium Chloride	5 g/L
Tryptone	10 g/L
Agar	15 g/L

Suspend in 1 L of distilled water

Autoclave at 121°C for 15 minutes

A1.2 9K Agar Medium

Composition

$MgSO_4 \cdot 7H_2O$	5 g/L
$Ca(NO_3)_2$	0.05 g/L
$(NH_4)_2SO_4$	3 g/L
K_2HPO_4	0.5 g/L
KCl	0.1 g/L
Agar	15 g/L

Suspend in 1 L of distilled water

Autoclave at 121°C for 15 minutes

A2 Preparation of culture medium used for the characterisation of the six bacterial isolates.

A2.1 Luria-Bertani Agar Medium

Composition

Yeast Extract	5 g/L
Sodium Chloride	5 g/L
Tryptone	10 g/L

Suspend in 1 L of distilled water

Autoclave at 121°C for 15 minutes

A2.2 Nutrient Broth (Merck)

Composition

Meat Extract	1 g/L
Yeast Extract	2 g/L
5 g/L Peptone	5 g/L
Sodium Chloride	8g/L

Suspend 16 g in 1 L of distilled water

Autoclave at 121°C for 15 minutes

A2.3 9K Broth Medium

Composition

$MgSO_4 \cdot 7H_2O$	5 g/L
$Ca(NO_3)_2$	0.05 g/L
$(NH_4)_2SO_4$	3 g/L
K_2HPO_4	0.5 g/L
KCl	0.1 g/L

Suspend in 1 L of distilled water

Autoclave at 121°C for 15 minutes

APPENDIX B

B1 DNA Extraction Protocol

1. Add sample to a **ZR BashingBead™ Lysis Tubes (0.1 & 0.5 mm)**. Add 750 µL **ZymoBIOMICS™ Lysis Solution** to the tube and cap tightly. ***Note****: For samples stored and lysed in* ***DNA/RNA Shield™ Lysis Tubes****, do not add ZymoBIOMICS™ Lysis Solution and proceed to Step 2.*

Sample Type	**Maximum Input**
Feces	200 mg
Soil	250 mg
Liquid Samples1 and Swab Collections2	250 µL
Cells (isotonic buffer, *e.g*., PBS)	50-100 mg (wet weight) (109 bacterial and 108 yeast cells)
Samples in DNA/RNA Shield™,3	≤ 1 mL

2. Secure in a bead beater fitted with a 2 mL tube holder assembly and process using optimized beat beating conditions (speed and time) for your device.
3. Centrifuge the **ZR BashingBead™ Lysis Tubes (0.1 & 0.5 mm)** in a microcentrifuge at ≥ 10,000 x *g* for 1 minute.
4. Transfer up to 400 µL supernatant to the **Zymo-Spin™ III-F Filter** in a **Collection Tube** and centrifuge at 8,000 x *g* for 1 minute. Discard the Zymo-Spin™ III-F Filter.
5. Add 1,200 µL of **ZymoBIOMICS™ DNA Binding Buffer** to the filtrate in the Collection Tube from Step 4. Mix well.
6. Transfer 800 µL of the mixture from Step 5 to a **Zymo-Spin™ IICR Column** in a Collection Tube and centrifuge at 10,000 x *g* for 1 minute.

7. Discard the flow through from the Collection Tube and repeat Step 6.

8. Add 400 µL **ZymoBIOMICS™ DNA Wash Buffer 1** to the Zymo-Spin™ IICR Column in a new Collection Tube and centrifuge at 10,000 x *g* for 1 minute. Discard the flow-through.

9. Add 700 µL **ZymoBIOMICS™ DNA Wash Buffer 2** to the Zymo-Spin™ IICR Column in a Collection Tube and centrifuge at 10,000 x *g* for 1 minute. Discard the flow-through.

10. Add 200 µL **ZymoBIOMICS™ DNA Wash Buffer 2** to the Zymo-Spin™ IICR Column in a Collection Tube and centrifuge at 10,000 x *g* for 1 minute.

11. Transfer the Zymo-Spin™ IICR Column to a clean 1.5 mL microcentrifuge tube and add 100 µL (50 µL minimum) **ZymoBIOMICS™ DNase/RNase Free Water** directly to the column matrix and incubate for 1 minute. Centrifuge at 10,000 x *g* for 1 minute to elute the DNA5, 6.

12. Place a **Zymo-Spin™ III-HRC Filter** in a new Collection Tube and add 600 µL **ZymoBIOMICS™ HRC Prep Solution**. Centrifuge at 8,000 x *g* for 3 minutes.

13. Transfer the eluted DNA (Step 11) to a prepared Zymo-Spin™ III-HRC Filter in a clean 1.5 mL microcentrifuge tube and centrifuge at exactly 16,000 x *g* for 3 minutes.

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