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THE USE OF *ASPERGILLUS NIGER* FOR THE REMOVAL OF POTASSIUM AND PHOSPHOROUS FROM THE IRON ORE OF THE SISHEN IRON ORE MINE, SOUTH AFRICA

by

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"Ek verklaar dat die verhandeling wat ek hiermee aan die Universiteit van Pretoria vir die PhD (Mikrobiologie)-graad voorlê, my eie werk is en nie vantevore deur my aan enige ander tersiêre inrigting vir enige graad voorgelê is nie."

"I certify that the thesis hereby submitted to the University of Pretoria for the degree of PhD (Microbiology) is my own work and has not previously been submitted by me in respect of a degree at any other tertiary institution."

Signature: _____

Date: _____

This thesis is dedicated to my wife, Julie, and daughter, Mackenzie

"We must not forget that when radium was discovered no one knew that it would prove useful in hospitals. The work was one of pure science. And this is proof that scientific work must not be considered from the point of view of the direct usefulness of it. It must be done for itself, for the beauty of science, and then there is always the chance that a scientific discovery may become like the radium, a benefit for humanity."

Marie Curie (1867-1934)

Lecture at Vassar College, May 14, 1921

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LIST OF ABBREVIATIONS

Acetyl-CoA	-	Acetyl-coenzyme A
AGI	-	Acute Gastrointestinal Illness
ARI	-	Acute Respiratory Illness
ATP	-	Adenosine Tri-phosphate
BIF's	-	Banded Iron Formations
bp	-	Base Pair
CI	-	Consistency Index
CO_2	-	Carbon Dioxide
$CuFeS_2$	-	Chalcopyrite
CuS	-	Copper Sulphide (Covellite)
Cu_2S	-	Chalcocite
$CuSO_4$	-	Copper Sulphate
dH_2O	-	Distilled Water
DNA	-	Deoxyribonucleic Acid
Fe	-	Iron
FeS_2	-	Iron Pyrite
$FeSO_4$	-	Ferrous Sulphate
$Fe_2(SO_4)_3$	-	Ferric Sulphate
H_2SO_4	-	Sulphuric Acid
HIV	-	Human Immunodeficiency Virus
K	-	Potassium
KCl	-	Potassium Chloride
K_2O	-	Potassium Oxide
$K_2O.Al_2O_3.4SiO_2$	-	Leucite
$K_2O.Al_2O_3.6SiO_2$	-	Silicide
$K_2O.SiO_2$	-	Potassium Silicate
Mg-ADP	-	Magnesium-Adenosine Diphosphate
Mg-ATP	-	Magnesium-Adenosine Triphosphate
$MgCl_2$	-	Magnesium Chloride
mm	-	Millimetre
mM	-	Millimolar

mmol	-	Millimole
Mt	-	Metric Tonnes
NADH	-	Nicotinamide adenine dinucleotide
ND	-	Not Detected
NH_4^+	-	Ammonium Ion
NiS	-	Nickel Sulphide
$NiSO_4$	-	Nickel Sulphate
OF	-	Oxidation-fermentation
P	-	Phosphorous
PCR	-	Polymerase Chain Reaction
rDNA	-	Ribosomal Deoxyribonucleic Acid
RI	-	Retention Index
RNA	-	Ribonucleic Acid
SO_2	-	Sulphur Dioxide
SX-EW	-	Solvent Extraction-Electrowinning
TAE	-	Tris-Acetate-EDTA
TBR	-	Tree Bisection-Reconnection
TCA	-	Tricarboxylic Acid
Tris-HCl	-	Tris-Hydrogen Chloride
µℓ	-	Microlitre
µmol	-	Micromole
UO_2	-	Uranium Dioxide (Uraninite)
UO_3	-	Uranium Trioxide
USA	-	United States of America
ZnS	-	Zinc Sulphide
$ZnSO_4$	-	Zinc Sulphate

LIST OF PUBLICATIONS, SUBMITTED MANUSCRIPTS AND CONFERENCE CONTRIBUTIONS

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2006 **WILLIAMS PJ, GEYER H, SURRIDGE AKJ, KATABUA J and CLOETE TE.** Bacterial population study of the industrial wastewater and iron ore of the Sishen Iron Ore Mine. 14th Biennial SASM Conference, CSIR Convention Centre, Pretoria, South Africa, 9-12 April 2006.

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SUMMARY

As global technologies and civilisation have advanced, the utilization of diverse mineral products has increased, resulting in the progressive depletion of high-grade mineral deposits. Consequently, metal production has to rely more on the use of lower-grade or complex ores, as well as metal extraction from mining and industrial wastes. Certain substances, such as phosphorous (P) and potassium (K), contained within the iron ore have a detrimental effect on the smelting process during steel manufacturing, resulting in the international steel making companies charging penalties when purchasing iron ore concentrates containing high concentrations of P and K. It has, therefore, become necessary to develop an economically viable and environmentally friendly process to reduce the high P and K concentrations contained in the iron ore concentrate of the Sishen Iron Ore Mine, resulting in the minimizing of the penalties charged by the steel making companies. During this study no microbial bioleaching candidates could be isolated from the aquatic environment in order to develop an economical process to remove the P and K from the iron ore concentrate.

The most likely candidate for the removal of P and K from the iron ore concentrate would seem to be *Acidithiobacillus ferrooxidans* that was isolated from the iron ore concentrate, however, this bacterium produces sulphuric acid, which is undesired in the iron ore industry due its corrosive properties. Furthermore, the P and K contained in the iron ore is in a non-sulphidic phase, and therefore, conventional bioleaching processes are not viable for their removal from the ore. Metals in certain non-sulphide minerals, such as the iron ore concentrate of the Sishen Iron Ore Mine may be solubilised by a process of complexation using microbially produced inorganic or organic acids. Chemical leaching of the iron ore concentrate using citric acid proved to be more efficient than "heap leaching", as more P and K was removed from the iron ore concentrate, as well as in a shorter time frame. The results of the chemical leaching suggested that a 1M citric acid leaching solution be used at 60ºC for 5 days for the chemical leaching process, as the most P and K is removed from the iron ore concentrate using these leaching conditions. The possibility to use *A. niger* as a bioleaching microorganism was also investigated, due to its ability to produce organic acids such as citric acid, which has the ability to remove P and K from the iron ore concentrate by chemical leaching. Compared to chemical leaching, which requires high concentrations of citric acid and/or high leaching temperatures, bioleaching using *A. niger* offers a more economical method with similar efficiency for the removal of P and K from the iron ore concentrate of the Sishen Iron Ore Mine. The most economically viable process for the removal of P and K from the iron ore concentrate, although not as efficient as chemical leaching using citric acid at high temperature, proved to be the bioleaching process using *A. niger* with a bioleaching pulp density of 33% at 30ºC for 10 days. This is the first report of the use of *A. niger* for the use in any bioleaching process.

CHAPTER 1

INTRODUCTION

As global technologies and civilisation have advanced, the utilization of diverse mineral products has increased, resulting in the progressive depletion of high-grade mineral deposits (Jian and Sharma, 2004). Consequently, metal production has to rely more on the use of lower-grade or complex ores, as well as metal extraction from mining and industrial wastes (Torma, 1986; Ehrlich, 1999; Costa *et al*., 2003). Since 1986, depletion of the richer iron ore deposits (>60% Fe; <0.24% K) worldwide necessitated the processing of lower-quality iron ore (<60% Fe; >0.24% K) (Personal communication[*]). Impurities, such as phosphorous (P) and potassium (K) contained within the lower-quality iron ore have a detrimental effect on the steel-making process, and therefore, steel-making plants charge penalties when purchasing iron ore with P and K levels exceeding 0.24%.

Smelting in blast furnaces is adversely affected by alkali's, such as K (Yusfin *et al*., 1999). Compounds of alkali metals are deposited on the surface of the coke, where they act as a catalyst in the gasification of carbon in the presence of CO_2 (Yusfin *et al*., 1999). The coke strength is reduced as a result of the gasification reaction occurring at lower temperatures (Yusfin *et al*., 1999). In addition to speeding up the gasification reaction, K present in the pores and cracks of the coke leads to the formation of $K_2O.SiO_2$ and $K_2O.Al_2O_3.2SiO_2$, leading to an increase in the volume of the coke and its subsequent fracture (Yusfin *et al*., 1999). K compounds, in particular, are the major cause of the destruction of the refractory lining of blast furnaces in the lower part of the stack, the bosh, and in some cases the tuyere zone of the hearth (Yusfin *et al*., 1999). K penetrates the monolithic alumosilicate lining in these regions, resulting in the formation of new minerals, such as silicide ($K_2O.Al_2O_3.6SiO_2$) or leucite ($K_2O.Al_2O_3.4SiO_2$) and the

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rearrangement of the crystalline lattice of the refractories, which is accompanied by a reduction in their softening point and mechanical strength (Yusfin *et al*., 1999). The result is the creation of stresses that cause cracks to form in the refractory lining, followed by the subsequent destruction of the lining (Yusfin *et al*., 1999). Therefore, most steel-making companies in the leading industrial nations have established limits on the concentration of alkali's (such as K_2O) in the charge material without changing the smelting practice (Yusfin *et al*., 1999). These limits on the alkali levels range from 0.25 % mass in Japan to 0.55 % mass in Switzerland (Yusfin *et al*., 1999).

In the past, the lower quality ore (<60% Fe; >0.24% K) has been blended with high quality ore (>60% Fe; <0.24% K) to "dilute" the P and K in the final iron ore product, which is exported to the steel-making plants (Personal communication[*]). Similar practices have been reported from other parts of the world, such as the Hamersley Province in Australia, where low-phosphorous ore (0.05% P) is blended with high-phosphorous ore (0.10% P), the former being the major component of the blend (Dukino *et al*., 2000). To date, blending of different quality iron ores has minimised the penalties charged by steel-making companies. However, the ratio of low-quality ore (<60% Fe; >0.24% K) to high-quality ore (>60% Fe; <0.24% K) is on the increase, and thus it is becoming an escalating problem within the economic functioning of the Sishen Iron Ore Mine.

A population study of the process- and ground water, as well as the iron ore of the mine would give insight into the microorganisms present in the mine environment. It might be possible to utilise existing microorganisms present in the environment to design and optimise an economically viable biotechnology process for the removal of P and K from the iron ore. Several microorganisms are capable of converting nutrients available in the environment to products, such as inorganic and organic acids, that can be used in industrial processes (Gupta and Sharma, 2002; Lesniak *et al*., 2002). Microorganisms can produce organic and inorganic acids, i.e. certain *Acidithiobacillus* spp. are capable of producing sulphuric acid (H_2SO_4), and A*spergillus niger* and certain *Penicillium* spp. are

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able to produce citric acid (Cunningham and Kuiack, 1992; Krebs *et al*., 1997; Alvarez-Vasquez *et al*., 2000; Gupta and Sharma, 2002; Lesniak *et al*., 2002; El-Holi and Al-Delaimy, 2003). These microbially produced acids may be valuable in a leaching process to reduce the P and K content of the iron ore.

During a previous study whereby a range of inorganic- and organic acids were tested for the removal of P and K from the iron ore of the Sishen Iron Ore Mine, it was discovered that citric acid (2-hydroxypropane-1,2,3-tricarboxylic acid) proved to be the best leaching agent for the removal of P and K without a major reduction in the iron content (0.46%) of the iron ore concentrate. Citric acid is an intermediate in the tricarboxylic acid (TCA) cycle, which is widely used in the food, beverage, pharmaceutical and cosmetic industries, but also has other applications in the textile, electroplating and bioremediation industries (Wang and Liu, 1996; Tran *et al*., 1998; Ates *et al*., 2002). The most popular microorganism for the large-scale production of citric acid is the white-rot fungus *Aspergillus niger*, due to its high citric acid productivity at low pH without the secretion of toxic metabolites (Kim, 2004). A possible process for the removal of P and K from the iron ore concentrate, may therefore, involve the fungal production of citric acid that can subsequently be used as a chemical leaching agent for the removal of P and K. In addition, it may be possible to use heap leaching technology, whereby the iron ore concentrate is directly inoculated with *A. niger*. The P and K contained within the iron ore concentrate may act as the sole source of these limiting growth factors required for fungal growth, which may consequently result in the selective removal of P and K from the iron ore concentrate by the fungus.

It has become important to develop an economically viable and environmentally friendly process to reduce the high P and K levels contained in the ore to improve the quality of iron ore that is being exported from the Sishen Iron Ore Mine. Currently such an economically viable biotechnological process for the reduction of P and K present in iron ore does not exist, and would therefore give Sishen Iron Ore Mine a competitive advantage in the international iron ore arena.

Introduction

The objectives of this study were to:

1. Investigate the microbial community of the water from the process dam, water flowing into the slime dam, water flowing from the slime dam and the ground water of the Sishen Iron Ore Mine.
2. Investigate the microbial community associated with the iron ore concentrate of the Sishen Iron Ore Mine.
3. Conduct a chemical analysis of the water from the process dam, water flowing into the slime dam, water flowing from the slime dam and the ground water of the mine, as well as chemically characterise the final iron ore concentrate from the Sishen Iron Ore Mine.
4. Evaluate a range of commercially available acids and oxidative chemicals for their chemical leaching ability.
5. Compare solid substrate- and submerged fermentation for the production of citric acid by *A. niger*.
6. Conduct chemical leaching of P and K from the iron ore concentrate using the citric acid produced by *A. niger*.
7. Conduct "heap leaching" of the iron ore concentrate using *A. niger*.
8. Propose an economically viable biotechnology process for the removal of P and K from the iron ore concentrate of Sishen Iron Ore Mine.

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CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Sishen Iron Ore Mine is situated in the Northern Province of South Africa approximately 280km north-west of Kimberley. This mine is one of the largest open cast mines in the world, yielding 25 million tonnes of high-grade hematite iron ore per annum. The iron ore concentrate that is exported to various steel making companies worldwide, contains phosphorous (P) and (K), which adversely affects the smelting process in blast furnaces. Therefore, the steel making companies charge penalties when purchasing iron ore concentrates with a P and K content above certain levels. The limit on the allowable amount of P and K is determined by the steel making companies and range from 0.25% mass in Japan to 0.55% mass in Switzerland.

In the past, lower quality iron ore has been blended with high quality iron ore in an attempt to "dilute" the P and K, however, the low quality iron ore stockpiles are increasing, and thus, becoming an escalating problem within the economical functioning of the mine. Therefore, it has become important to develop an economically viable process to reduce the P and K levels in the iron ore concentrate, in order to minimize the penalties charged by the international steel making companies. Bioleaching may provide a low-cost, environmentally friendly process technology for the removal of P and K from the iron ore concentrate of the Sishen Iron Ore Mine.

Conventional bioleaching/biooxidation refers to the microbial conversion of insoluble metals into soluble forms. Natural bioleaching has been known since A.D. 162, while the commercial application of bacterial leaching began in the 1950's at the Kennecott Utah Copper Company's Bingham Canyon Mine near Salt Lake City, Utah, USA. Various bioleaching technologies have since been developed, using microbes to catalyse the oxidation of sulphide minerals from ores that are otherwise not

processable by conventional pyrometallurgical techniques. Various metals, such as cobalt, copper, nickel, uranium and zinc, are extracted using bioleaching technology.

Certain substances in non-sulphide minerals, such as the P and K in the iron ore concentrate of the Sishen Iron Ore Mine, may be solubilised by a process of complexation using organic acids, such as citric and oxalic acid. Citric acid contains several carboxyl groups, which tend to donate protons (H^+), resulting in negatively charged carboxyl groups that are capable of forming stable complexes with several cations. Therefore, it would be possible that these negatively charged carboxyl groups might form stable complexes with the positively charged K cations present, and at the same time the release of H^+ ions may result in a hydrolysis reaction involving the P contained in the iron ore, resulting in the subsequent P and K removal from the iron ore concentrate.

Citric acid is an intermediate in the tricarboxylic acid (TCA) cycle, and can be produced by solid substrate- or submerged fermentation, the latter being used for the majority of the worldwide citric acid production. *Aspergillus niger* is the most popular microorganism for the large-scale production of citric acid due to its high citric acid productivity at low pH without the secretion of toxic metabolites. Citric acid production involves the catabolic production of pyruvate and Acetyl-coenzyme A (Acetyl-CoA) from hexoses by glycolysis, followed by the formation of citric acid by the TCA cycle.

The production of citric acid by *A. niger*, coupled with the chemical leaching of the iron ore concentrate using the produced acid, may be a suitable process for the removal of P and K from the iron ore concentrate of the Sishen Iron Ore Mine.

2.2 Iron Ore Deposits in the Northern Cape Province of South Africa

The largest known resources of high-grade hematite ore on the Southern African sub-continent can be found at the Palaeo-Preterozoic Transvaal Supergroup in the Northern Cape Province of South Africa (Carney and Mienie, 2003). The presence of excavations in the Northern Cape has been recorded from as far back as 1804 (Cairncross and Dixon, 1995), while mining activities in the Postmasburg area date

back to 2000 BC (Carney and Mienie, 2003). In 1945 the first commercial-scale exploitation of hematite ore commenced after the potential of the iron resources in this region was recognised (Carney and Mienie, 2003). Kumba Iron Ore, Ltd extracts hematite ore at the Sishen Iron Ore Mine for local and international markets (Figure 2.1) (Carney and Mienie, 2003).

2.2.1 Regional Geology

Superior-type banded iron formations (BIF's) of the Transvaal Supergroup crop out along the western margin of the Kaapvaal craton in the Northern Cape Province (Carney and Mienie, 2003). These BIF's consist of a range of distinctive hills, stretching for 400 km from Prieska in the south to Pomfret in the north (Carney and Mienie, 2003). The Postmasburg and Sishen areas are host to the bulk of the high-grade hematite ore (Carney and Mienie, 2003). The iron ore and associated lithologies of the Transvaal and Olifantshoek Supergroups crop out along an acruate belt for approximately 60 km, defining a regional anticlinal structure known as the Maremane anticline (Carney and Mienie, 2003). The Sishen Iron Ore Mine is located at the northern end of the Maremane anticline where the bulk of the hematite ores lie buried beneath younger cover lithologies, which strike north-south and plunge away from the centre of the anticline (Carney and Mienie, 2003). Laminated and massive ores constitute the bulk of the resource at the Sishen deposit, which are non-uniformly overlain by conglomerates, shales, flagstone and quartzite, termed the Gamagara Subgroup (South African Committee for Stratigraphy, 1995). Diamictite of the Makganyene Formation and lavas of the Ongeluk Formation have been thrust over the sedimentary rocks of the Gamagara Subgroup, followed by erosion by later events (Carney and Mienie, 2003). The erosional unconformities have been covered by tillite of the Dywka Group and clay and calcrete of the Kalahari Group (Carney and Mienie, 2003).

FIGURE 2.1

Location of the Sishen Iron Ore Mine in the Northern Cape Province of South Africa (Map modified from www.places.co.za)

2.2.2 The Sishen Iron Ore Mine

Sishen Iron Ore Mine (Figure 2.2) was established in 1953 and is situated approximately 280km north-west of Kimberley (Figure 2.1), and is one of the seven largest open cast mines in the world, with an open pit of approximately 11km long, 1.5 km wide and 400m deep (Personal communication*). The crushing and sorting plant is capable of processing in excess of 30 million tonnes of raw iron ore per

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annum that in turn yields 25 million tons of high-grade hematite iron ore, which is exported to various parts of the world via Saldanha Bay on the South African west coast. China is currently the largest market for the Sishen iron ore concentrate, although British Steel is the largest single customer (Personal communication*).

FIGURE 2.2

The Sishen Iron Ore Mine Situated in the Northern Cape Province of South Africa

The bulk of the iron ore resource of the Sishen Iron Ore Mine comprises of laminated and massive ores belonging to the Asbestos Hills Subgroup (Carney and Mienie, 2003). These orebodies are intensely folded and faulted, with a regional dip of 11º in a north-westerly direction (Carney and Mienie, 2003). The mine consists of a single elongated pit of ~10 km long and ~1.5 km wide with a mineable reserve of 895 metric tonnes (Mt) (Carney and Mienie, 2003). This deposit is capable of producing high-grade ore with an average iron content of 64.83%, P content of 0.073% and K content of 0.232% (Carney and Mienie, 2003).

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2.2.3 Potassium and Phosphorous Bearing Ores of the Sishen Iron Ore Mine

To date, muscovite remains the only K-containing mineral in the iron ore of the Sishen Iron Ore Mine, while the P bearing minerals are apatite, goyazite and woodhouseite (Personal communication*).

2.2.3.1 Muscovite

Muscovite (Table 2.1), also known as potash mica, is frequently found in igneous, metamorphic and detrital sedimentary rocks, and has a layered structure of aluminium silicate sheets, which are weekly bonded together by K^+ ions (Amethyst Galleries' Mineral Gallery, 1996; Wikipedia, 2006d). The K^+ ions are responsible for the perfect cleavage of muscovite, producing thin sheets or flakes that are highly flexible and elastic (Figure 2.3) (Amethyst Galleries' Mineral Gallery, 1996).

TABLE 2.1

Classification of Muscovite

Chemical Name	Potassium Aluminium Silicate (Hydroxide, Fluoride)
Chemical Formula	$KAl_2(AlSi_3O_{10})(F, OH)_2$
Class	Silicates
Subclass	Phyllosilicates
Group	Micas
Mohs Hardness	2 - 2.5
Specific Gravity	2.76 – 3

Various colours of muscovite exist, ranging from colourless to shades of grey, brown, green, yellow, or rarely violet or red (Amethyst Galleries' Mineral Gallery, 1996; Wikipedia, 2006d). Due to the colour variations, as well as often being translucent, muscovite crystals accompany various valuable minerals such as tourmaline, topaz, beryl and almandine, although muscovite is not often valuable as a mineral specimen (Amethyst Galleries' Mineral Gallery, 1996). Two valuable varieties of muscovite include the rare yellow five pointed stars, known as Star Muscovite (Figure 2.4), and

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a deep green variety due to colouration by chromium impurities, known as fuchsite (Figure 2.4) (Amethyst Galleries' Mineral Gallery, 1996).

FIGURE 2.3

Flexible and Elastic Sheet of Muscovite (Brazilian Rockhounds, 2005)

Muscovite is of importance in the manufacturing of fireproofing, insulating materials, lubricants, as well as electrical components due to the high heat and electrical insulating properties it has (Amethyst Galleries' Mineral Gallery, 1996; Wikipedia, 2006d). The name of muscovite is derived from Muscovy-glass, which is the name formerly used for the mineral due to its use for windows in Russia, and it was also used for the manufacturing of kitchen oven windows before synthetic materials replaced them (Amethyst Galleries' Mineral Gallery, 1996; Wikipedia, 2006d).

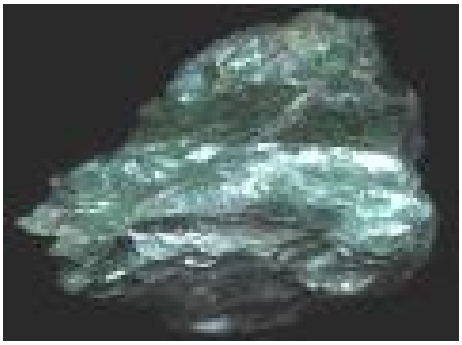

FIGURE 2.4

Yellow five pointed stars, known as Star Muscovite (left) (Wright's Rock Shop, 2005), and Green Muscovite, known as Fuchsite (Copyright [2005] by Andrew Alden, reproduced under educational fair use, http://geology.about.com)

2.2.3.2 Apatite

The name Apatite is derived from a Greek word meaning "to deceive", as it is very similar to other more valuable minerals such as olivine, peridot and beryl (Amethyst Galleries' Mineral Gallery, 1996). Apatite (Table 2.2) is a group of phosphate minerals, and can be classified as three different minerals, namely hydroxylapatite, fluorapatite and chlorapatite (Amethyst Galleries' Mineral Gallery, 1996; Wikipedia, 2006a). Fluorine, chlorine and hydroxyl ions are able to freely substitute one another within the crystal lattice, as all three elements are usually present in each specimen of apatite (Amethyst Galleries' Mineral Gallery, 1996). Apatite usually exists as small cryptocrystalline fragments and is widely distributed in igneous-, sedimentary- and metamorphic rocks, however, it can also exist as large crystals in certain contact metamorphic rocks, which can be cut as gems (Figure 2.5) (Amethyst Galleries' Mineral Gallery, 1996; Scandinavian Mineral Gallery, 1998).

Apatite also exists and is produced by biological systems. Hydroxylapatite is a major constituent of tooth enamel, as well as bone material (Amethyst Galleries' Mineral Gallery, 1996). When fluoridated water is ingested, hydroxyl ions are exchanged with fluoride ions, resulting in the formation of fluorapatite, which is stringer than hydroxylapatite, thus strengthening teeth and bone material (Amethyst Galleries' Mineral Gallery, 1996).

TABLE 2.2 **Classification of Apatite**	
Chemical Name	Calcium (Fluoro, Chloro, Hydroxyl) Phosphate
Chemical Formula	$Ca_5(PO_4)_3(OH, F, Cl)$
Class	Phosphates
Group	Apatite
Mohs Hardness	5
Specific Gravity	3.17 – 3.23

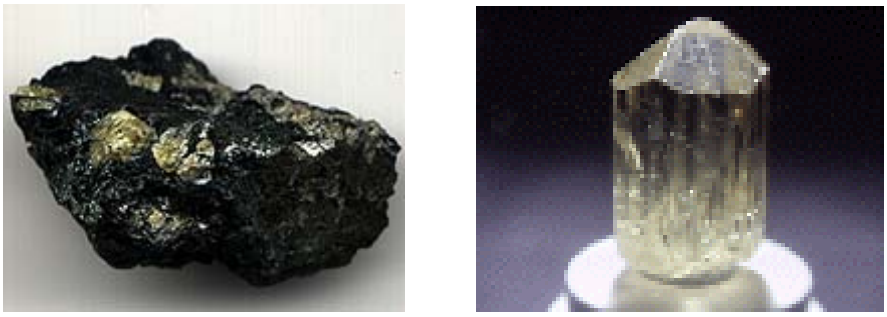

FIGURE 2.5

Chlorapatite from Altermark, Norway (left), and an apatite gemstone (right) (Amethyst Galleries' Mineral Gallery, 1996; Scandinavian Mineral Gallery, 1998)

2.2.3.3 Woodhouseite

Woodhouseite (Table 2.3) is a rare mineral, which forms flesh-coloured to colourless psuedocubic rhombohedrons (Figure 2.6) (Amethyst Galleries' Mineral Gallery, 1999). Woodhouseite is formed in quartz veins where it is frequently associated with topaz, tourmaline, andalusite and svanbergite, and is difficult to classify because it has both a sulphate anion group and a phosphate anion group (Amethyst Galleries' Mineral Gallery, 1999).

TABLE 2.3 Classification of Woodhouseite	
Chemical Name	Calcium Aluminium Phosphate Sulphate Hydroxide
Chemical Formula	$CaAl_3PO_4SO_4(OH)_6$
Class	Sulphates (sometimes Phosphates)
Group	Beudantite
Mohs Hardness	4.5
Specific Gravity	3

FIGURE 2.6

A cluster of quartz crystals coated with a honey-coloured dusting of woodhouseite crystals (Amethyst Galleries' Mineral Gallery, 1999)

2.2.3.4 Goyazite

Rhombohedral goyazite crystals occur as distinct particles dispersed in a matrix (Mineralogy Database, 2005). Goyazite crystals are transparent and range in colour from lemon yellow and pink to colourless (Figure 2.7) (Mineralogy Database, 2005). This mineral, named after the province of Goyaz, Brazil, is also known as bowmanite, hamlinite or lusungite (Table 2.4) (Mineralogy Database, 2005).

TABLE 2.4 Classification of Goyazite	
Chemical Formula	$SrAl_3(PO_4)_2(OH)_5.H_2O$
Subclass	Anhydrous Phosphates, Arseniates, Vanadates
Group	Crandallite
Mohs Hardness	4.5 - 5 (Flourite-Apatite)
Specific Gravity	3.22

FIGURE 2.7

Flat, tabular crystals of goyazite on dolomite (Paulin, 2005)

2.3 Blast Furnace Technology

The research into the effect of alkalis on blast furnace smelting peaked during the 1970's (Yusfin *et al*., 1999). This period is characterised by the construction of powerful blast furnaces, as well as the trend to use large quantities of iron ore pellets in the charge (Yusfin *et al*., 1999). At the same time, the problems related to the alkali activity during smelting became so sensitive, that a commission was organised by the "Ernkontorens" Metallurgical Society in Switzerland in 1972 to investigate the problem (Yusfin *et al*., 1999).

2.3.1 The Basic Functioning of a Blast Furnace

A diagrammatic representation of the functioning of a blast furnace is shown in Figure 2.8. Blast furnaces are used during the steel making process to chemically

reduce and physically convert iron oxides into liquid iron, known as "hot metal" (Ricketts, 2005). The blast furnace is built in the form of a tall chimney-like structure lined with refractory brick, where iron ore (iron oxide), coke (carbonaceous material), and limestone flux are dumped into the top of the furnace, while preheated air is blown into the bottom of the furnace (Ricketts, 2005). The raw materials require 6 to 8 hours to descend to the bottom of the furnace where they become the final product of liquid slag and liquid iron, known as "pig" iron, which are drained from the furnace at regular intervals (Ricketts, 2005). The preheated air, which was blown into the bottom of the furnace, ascends to the top in 6 to 8 seconds after going through several chemical reactions (Ricketts, 2005).

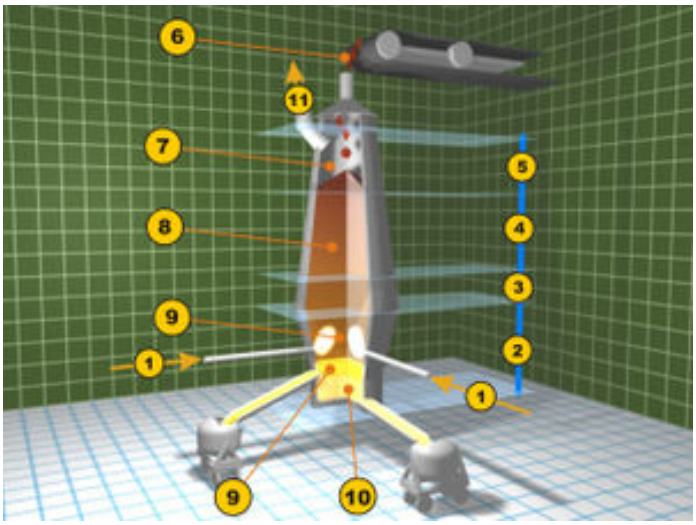

FIGURE 2.8

Diagrammatic representation of the functioning of a blast furnace (http://en.wikipedia.org/wiki/Blast_furnace#Chemistry, 2007)

1) Hot blast from Cowper stoves (Tuyere zone); 2) Melting zone (bosh); 3) Reduction zone of ferrous oxide (barrel); 4) Reduction zone of ferric oxide (stack); 5) pre-heating zone (throat); 6) Feed of ore, limestone and coke; 7) Exhaust gasses; 8) Column of ore, limestone and coke; 9) Removal of slag; 10) Tapping of molten pig ore; 11) Collection of waste gasses

In a blast furnace there are numerous chemical reactions that produce the desired final molten iron, which can be summarised as follows (Ricketts, 2005):

1) $3Fe_2O_3 + CO \rightarrow CO_2 + 2Fe_3O_4$ (Begins at 455ºC)
2) $Fe_3O_4 + CO \rightarrow CO_2 + 3FeO$ (Begins at 590ºC)
3) $FeO + CO \rightarrow CO_2 + Fe$ (Begins at 700ºC)

As the iron oxides undergo these chemical reactions, they begin to melt, resulting in the liquid iron trickling through the coke to the bottom of the furnace (Ricketts, 2005). The coke descends to level where the preheated air or hot blast enters the blast furnace, where it is ignited to generate heat by the following reaction (Ricketts, 2005):

$C + O_2 \rightarrow CO_2 + Heat$

The abovementioned reaction takes place in the presence of excess carbon at a high temperature, resulting in the CO_2 being reduced to carbon monoxide (required for the reduction of iron oxides) as follows (Ricketts, 2005):

$CO_2 + C \rightarrow 2CO$

The decomposition of the limestone flux results in the production of calcium oxide that removes sulphur from the iron ore, as well as silica (SiO_2), aluminium (Al_2O_3), magnesium (MgO), and calcium (CaO), which becomes part of the slag (Ricketts, 2005). Hot dirty gas is produced in the furnace during the iron making process, which exit at the top of the furnace where particulate matter is removed and the gas cooled (Ricketts, 2005). The gas is used as a fuel in the "hot blast stoves" used to preheat the air entering the blast furnace, while any excess gas is moved to the boiler house where it is used to generate steam (Ricketts, 2005).

Although the efficiency of blast furnaces is constantly evolving, the chemical and physical reactions remain the same (Ricketts, 2005). According to the American Iron and Steel Institute, "Blast furnaces will survive into the next millennium because the larger, efficient furnaces can produce hot metal at costs competitive with other iron making technologies" (Ricketts, 2005). However, one of the biggest drawbacks of the

blast furnaces is the inevitable CO_2 production as the iron is reduced from the iron oxides by carbon (Ricketts, 2005).

2.3.2 The Effect of Alkalis on the Functioning of the Blast Furnace

Smelting in blast furnaces is adversely affected by alkalis, in particular by K (Yusfin *et al*., 1999). These alkalis enter the blast furnace with the charge materials (Yusfin *et al*., 1999). The main sources of alkalis are not usually the iron ore or their concentrates, but instead the fluxes and strengthening additions used for making the pellets for the blast furnace charge (Yusfin *et al*., 1999). The alkali content of the limestone flux may reach 0.2 – 0.6%, while that of the coke may fluctuate from 0.04 – 0.25% (Yusfin *et al*., 1999). These alkalis are deposited on the surface of the coke, where they act as a catalyst and intensify the gasification of carbon in the presence of CO_2 (Yusfin *et al*., 1999). The gasification reaction is shifted toward lower temperatures resulting in the reduction in the strength of the coke (Yusfin *et al*., 1999). In addition to accelerating the gasification process, K present in the pores and cracks of the coke leads to the formation of compounds such as potassium silicate ($K_2O.SiO_2$) and potassium alumosilicate ($K_2O.Al_2O_3.2SiO_2$), resulting in an increase in the volume of the coke and its subsequent fracture (Yusfin *et al*., 1999).

The action of K is the major reason for the destruction of the refractory lining of blast furnaces in the lower part of the stack, the bosh, and in some cases the tuyere zone of the hearth (Figure 2.8) (Yusfin *et al*., 1999). Within these regions, K actively penetrates the monolithic alumosilicate lining, which leads to the formation of new minerals such as silicide ($K_2O.Al_2O_3.6SiO_2$) or leucite ($K_2O.Al_2O_3.4SiO_2$) (Yusfin *et al*., 1999). The rearrangement of the crystalline lattice of the refractories takes place, which is accompanied by a reduction in their softening point and mechanical strength, resulting in the creation of stresses that cause cracks to form (Yusfin *et al*., 1999). In the tuyere zone, the situation is aggravated by the formation of a condensed phase based on KCN (Yusfin *et al*., 1999). Condensation of alkali compounds on the surface of iron oxide pellets leads to their fracture and a deterioration in the gas permeability of the stock, as well as the rapid disintegration of the iron oxide pellets (Yusfin *et al*., 1999). In addition, the viscosity of the basic blast furnace slags are adversely affected by the presence of alkalis, which promotes the further formation of

alkaline alumosilicate groups such as $K_2O.Al_2O_3.2SiO_2$ in the slag melt (Yusfin *et al.*, 1999).

Most steel making companies in the leading industrial nations have established a limiting alkali load for blast furnaces, i.e. have set limits on the amounts of alkalis that may be present in the charge materials without changing the smelting practice (Table 2.5) (Yusfin *et al.*, 1999). Therefore, it is becoming increasingly important to remove these alkalis from the charge materials before they enter the blast furnace. The main methods that can be used to reduce the alkali content in blast furnaces are:

* Reducing the content of alkalis in the charge materials;
* Removing top dust and blast furnace slag from the sintering-machine charge;
* Operating the furnace on acid slags; and
* Increasing the volume of slag, especially in furnaces in which the slag volume is already low.

TABLE 2.5

Allowable Alkali load on the Blast Furnaces of Companies in Several of the Leading Industrial Nations (Yusfin *et al.*, 1999)

Nation	Company	Allowable Alkali Load kg.tonne^{-1} Pig Iron
Canada	STIKLO, Dofasko	3.0
Japan	Kawasaki, Seitetsu, Kobe Seikose, Sin Nippon Seitetsu, Nippon Kokan	2.5 – 3.1
United States of America	Ellenwood Steel, Jones and Laughlin Steel, United Sates Steel	3.2 – 4.5
England	British Steel	3.5
Germany	August Thyssen-Hütte	4.0
Switzerland	Grenges	5.5

Removing the P and K from the iron ore concentrate would, therefore, reduce the content of alkalis in the charge material entering the blast furnaces of the steel making companies to whom Kumba Iron Ore, Ltd. export iron ore concentrate. Bioleaching

may provide a low-cost, environmentally friendly processing technology for the removal of P and K from the iron ore concentrate of the Sishen Iron Ore Mine.

2.4 Conventional Bioleaching

Various technologies developed in the last couple of decades include microbial use for the extraction of different valuable metals, which is commonly known as bioleaching (Gilbertson, 2000; Rohwerder *et al*., 2002). Bioleaching refers to the microbial conversion of insoluble metals (e.g. metal sulphides such as CuS, NiS and ZnS) into soluble forms (usually metal sulphates such as $CuSO_4$, $NiSO_4$ and $ZnSO_4$). Due to the many oxidation reactions that occur during bioleaching, it can also be referred to as biooxidation (Sand, 2001; Rawlings, 2002, Roberto, 2002).

2.4.1 Historical Overview of Bioleaching

The phenomenon of natural bioleaching has been known since ancient times. Galen, a naturalist and physician from Pergamum, reported the utilisation of *in-situ* copper leaching in A.D. 162 from the island of Cyprus, making this one of the earliest records of the effects of bioleaching (Constantinou, 1992). Cupriferous solutions were collected from the mine water of the Skouriotissa mines, followed by concentration by evaporation, leaving behind crystallised copper sulphate (Constantinou, 1992). Pliny the Elder (A.D. 23-73) discussed the *vitreolus quasi vitrum*, a glass-like substance discovered on rocks, in his treatise on natural history (Dresher, 2004). Recent findings have revealed evidence, which predates these accounts of historical bioleaching practices (Dresher, 2004).

The natural leaching of copper and the formation of ‘gall springs’ during the East Han Dynasty (B.C. 206-A.D. 220) in China have been recorded (Dresher, 2004). This process was also recorded as being used during the Song Dynasty (A.D. 960-1271) (Dresher, 2004). During this process, also known as cementation, copper was precipitated from solution by dipping iron into the solution, a process that was used as far back as B.C. 150 in China (Dicinoski *et* al., 1998; Dresher, 2004).

Iron-rich acidic water draining from abandoned coal and metal mines, as well as unmined mineralised areas provide more evidence of natural bioleaching (Dresher, 2004). The production of acid mine drainage due to mining activities have in the past resulted in the naming of rivers such as Tinto, Tintillo and Aguas Teñidas, referring to the characteristic colouration of the rivers (Dresher, 2004). Industrial scale heap leaching, involving roasting of copper and iron sulphides, was conducted at the Rio Tinto mine (Spain) in the late 1700's, but was prohibited by law in 1888 due to the serious environmental damage caused by the clouds of sulphur dioxide, resulting in acid mine drainage (Gilbertson, 2000; Dresher, 2004). Heap leaching of copper, without the prohibited roasting of copper and iron sulphides, was continued with success until the 1970's (Gilbertson, 2000; Dresher, 2004). The success of the heap leaching of copper was attributed in 1947 to the presence of bacteria (Colmer and Hinkle, 1947).

The commercial application of bacterial leaching began in the 1950's at the Kennecott Utah Copper Company's Bingham Canyon Mine near Salt Lake City, Utah, USA. This process started after it became evident that blue copper-containing solutions were running out of waste piles containing copper sulphate minerals (Dresher, 2004). Due to the absence of powerful oxidising agents and acid, investigations were started, which revealed that the natural occurring bacteria were responsible for the oxidation of the iron sulphides in the piles (Malouf and Prater, 1961). The bacteria were given the names *Thiobacillus ferrooxidans* for their ability to oxidise iron sulphides, and *Thiobacillus thiooxidans* for their ability to oxidise sulphur to yield sulphuric acid (Malouf and Prater, 1961).

2.4.2 Development of the Bioleaching Industry

Bioleaching occurs as a natural process whereby microorganisms assist in the slow weathering of sulphidic orebodies, and thus, by using this concept for the extraction of metals from ores is simply the attachment of a natural process to commercial purposes (Gilbertson, 2000; Rawlings, 2002; Rohwerder *et al*., 2002). Various technologies developed in the last couple of decades include the microbial ability to catalyse the oxidation of sulphide minerals (Table 2.6) for the subsequent extraction of different valuable metals from ores that are otherwise not processable by

pyrometallurgical techniques, as well as for the extraction of metals from low-grade ores (Rawlings, 2002; Rohwerder *et al*., 2002; Dresher, 2004). This phenomenon, known as bioleaching, is distinguished from conventional acid leaching wherein only oxidised minerals are leached (Rohwerder *et* al., 2002; Dresher, 2004). During bioleaching, insoluble metals (e.g. metal sulphides such as CuS, NiS and ZnS) are converted into soluble forms (usually metal sulphates such as $CuSO_4$, $NiSO_4$ and $ZnSO_4$) due to the oxidation catalysed by microorganisms (Sand, 2001; Rawlings, 2002, Roberto, 2002). Previously only mesophilic bacteria were considered to be important during bioleaching, but the use of moderately and extremely thermophilic bacteria have become attractive during bioleaching (Johnson, 1998; Norris *et al*., 2000).

TABLE 2.6

Major Mineral Sulphides Present in Nature (Glazer and Nikaido, 1995)

Mineral	Chemical Formula	Name
Arsenic (As)	AsFeS	Arsenopyrite
Cobalt (Co)	$CuCo_2S_4$	Carrolite
Copper (Cu)	$CuFeS_2$	Chalcopyrite
	Cu_2S	Chalcocite
	CuS	Covellite
	Cu_5FeS_4	Bornite
Iron (Fe)	FeS_2	Pyrite, Marcasite
Lead (Pb)	PbS	Galena
Molybdenum (Mo)	MoS_2	Molybdenite
Nickel (Ni)	NiS	Millerite
	FeNiS	Pentlandite
Silver (Ag)	AgS	Argenite
Zinc (Zn)	ZnS	Sphalerite

Various metals are extracted in this manner, which include cobalt, copper, nickel, uranium and zinc (Olson *et al*., 2003; Rawlings *et al*., 2003). The recovery of gold and silver, however, applies the microbial activity for the removal of interfering metal sulphides from the ore prior to cyanidation treatment (Rohwerder *et al*., 2003). In this

case the term biooxidation should be used due to the fact that the bioleached metals are not recovered during the process (Rohwerder *et al*., 2003). Both bioleaching and biooxidation processes could be referred to in general as biomining (Bosecker, 1997; Rawlings, 2002; Rohwerder *et al*., 2002; Olson *et al*., 2003).

The type of resource to be processed determines which bioleaching process is to be used (Dresher, 2004). Currently three different types of bioleaching processes exist, depending on the raw material to be processed. A widely used process is dump leaching, whereby waste rock, low-grade ore or concentrator tailings are leached at the site of disposal (Dresher, 2004). Another process that is increasingly being used is heap leaching, which involves the leaching of newly mined material (intermediate grade ore, oxides and secondary sulphides) by depositing the material on an impervious natural surface or pad (Dresher, 2004). The material may be leached as mined or alternatively may be partially crushed and mixed with an acid prior to the depositing on the heap. The third process, agitated leaching, involves placing intermediate- to high-grade chalcopyrite concentrates in a tank, where it is leached using mechanical agitation (Dresher, 2004). Agitated leaching, however, is only in the experimental stage and is not commercially used.

Microorganisms are used during bioleaching to catalyse the oxidation reaction of iron sulphides to create ferric sulphate and sulphuric acid (Dresher, 2004). During copper extraction, ferric sulphate is responsible for the oxidation of copper sulphide minerals, followed by the leaching of the copper by the sulphuric acid (Dresher, 2004). In the case of uranium extraction, tetravalent uranium oxide is oxidised by ferric sulphate, rendering hexavalent uranium oxide, which is leached by the sulphuric acid (Dresher, 2004). The biooxidation of refractory gold ores involve bacteria that are able to oxidise a iron sulphide matrix in which the gold particles are embedded, resulting in the availability of the gold particles for cyanide leaching (Dresher, 2004). The desulphurisation of coal also involves bacteria for the oxidation of the pyrite contaminant present in coal, resulting in the solubilization of sulphur as ferric sulphate (Dresher, 2004).

Bioleaching offers several advantages for the extraction of base metals, such as:

1. The use of naturally occurring components (microorganisms, water and air).
2. Extendibility with a single reactor or a series of reactors.
3. Stirred tanks are simple to operate and maintain.
4. Relatively low pressures and temperatures prevail in the system.
5. No dust and SO_2 production.
6. Arsenic can be handled and disposed of in a stable form.
7. Capital costs are generally lower than conventional smelting and refining processes.
8. In the case of copper it is a compatible technology with current existing solvent extraction-electrowinning (SX-EW) plants.
9. Quick start-ups are possible, such as the BIOX® refractory gold plants.
10. The ability to economically process run-of-the-mine low-grade sulphide ores.
11. The ability to process ores that may not be feasible to be smelted due to the negative impact it may present on the environment (Gilbertson, 2000; Dresher, 2004).

However, bioleaching will not completely replace smelting for the following reasons:

1. Bioleaching does not recover precious metals in the ore, which are often an important component in the profitability of the operation.
2. The smelter requires the production of acid for conventional leaching, as well as to supplement bioleaching in the case that the ore body is high in acid consuming minerals. Certain acids, such as sulphuric acid are in short supply and expensive to deliver to remote locations where these operations normally exist.
3. Some ore bodies are not sufficiently high in acid consuming minerals, causing residual acid that is generated to have to be neutralised during the leaching process. In such a case smelting remains the only economically viable processing means (Dresher, 2004).

The use of microbes during ore processing has several advantages over conventional physicochemical methods, such as the fact that it is more environmentally friendly,

less energy is required and sulphur dioxide and other environmentally harmful gasses are not emitted (Rawlings, 2002). Furthermore, the wastes created by physicochemical mining procedures may lead to unfavourable acid and metal pollution (acid rock/mine drainage) when exposed to water and air through biological leaching (Colmer and Hinkle, 1947; Rawlings, 2002).

2.4.3 Microbiology of Bioleaching

Due to the substrate limitations that exist in mining environments, it was previously thought that these environments would have a low diversity of microbial flora (Rohwerder *et al*., 2003). Through extensive research conducted by Johnson (1998), as well as Hallberg and Johnson (2001), it has become evident that this is in fact not the case. At least 11 recognized prokaryotic divisions have shown to exist at acid mine drainage sites (Baker and Banfield, 2003). A variety of bacteria found in mining environments are able to play a role in bioleaching of metal sulphides, which include thermophilic microorganisms, heterotrophic bacteria as well as members of the genera *Leptospirillum* and *Acidithiobacillus* (Glazer and Nikaido, 1995).

The biology of microorganisms involved in bioleaching is becoming more complex due to increasing data on 16S rDNA gene sequences, enabling the description of new species of leaching bacteria and the reclassification of known species (Rohwerder *et al*., 2003). In the past only mesophilic bacteria were considered to be important for bioleaching, but nowadays genera of moderately and extremely thermophilic bacteria have also become attractive (Johnson, 1998; Norris *et al*., 2000). Furthermore, the direct enzymatic oxidation of the sulphur moiety of heavy metal sulphides, as described by Sand *et al*. (1995), does not exist (Rohwerder *et al*., 2003). Instead, the "indirect mechanism", i.e., non-enzymatic metal sulphide oxidation by iron(III) ions, combined with enzymatic re-oxidation of the resulting iron (II) ions, remains and now comprises two sub-mechanisms, namely contact and non-contact mechanisms (Sand *et al*., 2001; Rawlings, 2002). The contact mechanism involves the attachment of bacterial cells to the surface of the sulphide minerals, leading to the dissolution of the sulphide minerals as a result of the electrochemical processes, which takes place at the interface between the bacterial cell wall and the mineral sulphide surface (Sand *et al*., 2001; Rawlings, 2002). The non-contact mechanism is exerted by planktonic

bacteria, which are responsible for the oxidation of iron(II) ions in solution (Sand *et al*., 2001; Rawlings, 2002). The resulting iron(III) ions come into contact with the sulphide mineral surfaces where oxidation takes place (Sand *et al*., 2001; Takai *et al*., 2001; Rawlings, 2002). Both contact- and non-contact mechanisms involve bacteria, which contribute to mineral dissolution by generation of the oxidizing agent, iron(III) ions, and by the subsequent oxidizing of the sulphur compounds resulting from the dissolution (Sand *et al*., 2001; Rawlings, 2002).

2.4.3.1 The Diversity Among Leaching Bacteria

The predominant bioleaching microorganisms are extremely acidophilic bacteria, which are able to oxidise either inorganic sulphur compounds and/or iron(II) ions (Rohwerder *et al*., 2003). The classical leaching bacteria belong to the genus *Acidithiobacillus* (formerly *Thiobacillus*) (Kelly and Wood, 2000). The mesophilic *At. ferrooxidans* and *At. thiooxidans* are the first isolates of extremely acidophilic sulphur and/or iron(II)-oxidizing bacteria, and together with the moderately thermophilic *At. caldus* they belong to the Gram-negative γ-proteobacteria (Kelly and Wood, 2000). Other proteobacteria used for leaching include species of the genus *Acidiphilium*, such as *Ac. acidophilum* (Hiraishi *et al*., 1998), as well as members of the genus *Leptospirillum*, which belong to a new bacterial division (Hippe, 2000; Coram and Rawlings, 2002). In addition, some Gram-positive leaching bacteria have also been described, which include moderately thermophilic members of the genera *Acidimicrobium*, *Ferromicrobium* and *Sulfobacillus* (Clark and Norris, 1996; Norris *et al*., 1996; Johnson and Roberto, 1997). *Sulfolobales*, a group of extremely thermophilic, sulphur- and iron(II)-oxidizing archaebacteria, have also been implicated in leaching processes and include genera such as *Sulfolobus*, *Acidianus*, *Metallosphaera* and S*ulfurisphaera* (Fuchs *et al*., 1995; Fuchs *et al*., 1996; Kurosawa *et al*., 1998; Norris *et al*., 2000). Two species of mesoacidophilic iron(II) oxidizing archaebacteria have also been reported to play a role in bioleaching, namely *Ferroplasma acidiphilum* and *F. acidarmanus* (Edwards *et al*., 2000; Golyshina *et al*., 2000).

Bioleaching bacteria include species with an extremely limited substrate spectrum, in particular *L. ferrooxidans* and *L. ferriphilum*, which are only able to grow by

aerobically oxidizing iron(II) ions (Rohwerder *et al*., 2003). In contrast, *At. ferrooxidans* has a broad metabolic capacity, allowing this bacterium to live on the oxidation of reduced sulphur compounds, as well as the oxidation of molecular hydrogen, formic acid and iron(II) ions (Rohwerder *et al*., 2003). In addition, *At. ferrooxidans* is able to grow anaerobically by oxidation of sulphur compounds or hydrogen coupled with iron(III) ion reduction (Das *et al*., 1992; Pronk *et al*., 1992; Ohmura *et al*., 2002). At least 11 different cytochromes of the *c* type have been identified in the genome of *At. ferrooxidans*, suggesting the application of electron acceptors other than oxygen (Yarzábal *et al*., 2002).

Further metabolic diversity among leaching bacteria has been found with respect to their carbon assimilation pathways (Rohwerder *et al*., 2003). *Acidithiobacillus* spp. and *Leptospirillum* spp. are only able to grow chemolithoautotrophically, whereas *Acidiphilium acidophilum* and *Acidimicrobium ferrooxidans* are able to grow autotrophically with sulphur and iron(II) compounds, heterotrophically with glucose or yeast extract, and mixotrophically with all of these substrates (Clark and Norris, 1996; Hiraishi *et al*., 1998). An obligate mixotrophic iron(II)-oxidizing bacterium is *Ferromicrobium acidophilus* (Johnson and Roberto, 1997), while some *Sulfobacillus* spp. show poor chemolithotrophic growth, as do many thermophilic *Sulfolobales* (Johnson, 1998). Several *Acidiphilium* spp. and *Acidisphaera rubrifaciens* contain pigments, which may indicate the ability for photosynthetic activity (Hiraishi *et al*., 1998; Hiraishi *et al*., 2000; Hiraishi and Shimada, 2001).

2.4.3.2 Attachment of Leaching Bacteria to Sulphide Minerals

The attachment of leaching bacteria to the sulphidic energy source and the subsequent biofilm formation are prerequisites for mineral dissolution in natural environments, as well as industrial operations (Ruiz *et al*., 2007). These processes are mediated by extracellular polymeric substances (EPS), comprising polysaccharides, proteins and DNA (Vandevivere and Kirchman, 1993; Gehrke *et al*., 1998; Sand and Gehrke, 2006). The EPS mediates the attachment to a metal sulphide surface where it concentrates iron(III) ions by complexation through uronic acids or other residues at the mineral surface, thus, allowing an oxidative attack on the sulphide mineral (Sand and Gehrke, 2006). In the case of *At. ferrooxidans* R1 and pyrite, it was demonstrated

that produced EPS consisted of glucose, rhamnose, fucose, xylose, mannose, C_{12}-C_{20} saturated fatty acids, glucuronic acid and iron(III) ions (Gehrke *et al*., 1998; Gehrke *et al*., 2001). The attachment occurs due to mainly electrostatic interaction of the positively charged cells with negatively charged pyrite (Solari *et al*., 1992; Blake *et al*., 1994). In contrast, hydrophobic interactions do not play a role in the attachment of the bacteria to metal sulphide surfaces (Gehrke *et al*., 1998; Sampson *et al*., 2000). Cells that are grown on elemental sulphur do not grow on pyrite due to a considerably modified EPS composition (Rohwerder *et al*., 2003). Sulphur-grown EPS contains no complexed iron(III) ions or other positively charged groups, and less sugars and uronic acids, while it contains more fatty acids than pyrite-grown EPS (Rohwerder *et al*., 2003). Therefore, hydrophobic interactions play a role during the attachment of *At. ferrooxidans* to sulphur (Gehrke *et al*., 1998). This suggests that the leaching bacteria are able to adapt the composition and amount of their EPS according to the growth substrate (Rohwerder *et al*., 2003). In addition, the EPS serves as the reaction space where the biooxidation reactions take place (Sand *et al*., 1995; Gehrke *et al*., 1998; Tributsch, 2001; Rohwerder *et al*., 2003).

2.4.3.3 The Effect of Temperature on Bioleaching

Bioleaching processes have been operated at a range of temperatures from ambient to 80ºC (Rawlings *et al*., 2003). The types of iron- and sulphur oxidizing bacteria used for bioleaching practices differ depending on the temperature range of the process (Rawlings, 2005). The types of leaching bacteria found in processes operating from ambient to 40ºC tend to be similar irrespective of the mineral being treated, as are those within the temperature ranges 45-55ºC and 75-80ºC (Rawlings, 2005). Mineral solubilization processes are exothermic, and therefore, during some bioleaching processes cooling is required to keep the process at the optimum leaching temperature (Rawlings, 2005). Higher leaching temperatures generally lead to a much higher rates of chemical reactions, and in the case of minerals such as chalcopyrite, temperatures of 75-80ºC are required for copper extraction to take place at an economically viable rate (Rawlings, 2005).

2.4.4 Industrial Bioleaching Processes

Many industrial bioleaching operations, such as dump leaching and heap leaching, are currently in use worldwide. Dump leaching and heap leaching are almost identical processes, however, in the case of dump leaching, the ore is taken directly from the mine and stacked on a leach pad, whereas in the case of heap leaching, the ore is crushed into finer particles before it is heaped on a leach pad (Wikipedia, 2007a; Wikipedia, 2007b). During both processes the stacked ore is irrigated with leaching solution, which percolates through the heap and leaches out the valuable metals (Wikipedia, 2007a; Wikipedia, 2007b).

The early development and application of the concept of bioleaching was conducted in the United States, but since many other countries, such as Chile, have joined this revolutionising concept in the mining industry (Dresher, 2004).

2.4.4.1 The BIOX® Process

The Biological Oxidation (BIOX®) Process for the pre-treatment of refractory sulphide ores was commercialised in 1988, following extensive research conducted at Billiton Process Research (formerly GENCOR Process Research) in the 1970's and 1980's (Van Aswegen and Marais, 1998). This process offers many advantages over other conventional refractory treatment processes, such as roasting and pressure oxidation, which includes the following:

1. the bacterial cultures are able to withstand the fluctuations experienced on an operating plant,
2. the process is suited for operation in remote areas due to its simplicity,
3. the scale-up capability of the process has been demonstrated, and thus the process may be applied to large refractory ore bodies, and
4. due to the neutralisation of the plant effluents, precipitates are produced which comply with the most stringent environmental regulations.

In 1988, when it was decided to commercialise the technology on a licensing basis, the only operating BIOX® plant was the 10 tonne per day demonstration plant located

at Fairview Mine in South Africa (Van Aswegen and Marais, 1998). Commercial BIOX® plants which have been, or in the process of being commissioned are listed in Table 2.7.

The BIOX® process involves a mixed population of bacteria, namely *Acidithiobacillus ferrooxidans, Acidithiobacillus thiooxidans* and *Leptospirillum ferrooxidans*, which are able to break down the sulphide mineral matrix in the ore, resulting in the liberation of the occluded gold for subsequent cyanidation (Van Aswegen and Marais, 1998). The thickened flotation concentrate is continuously fed to a series of aerated stirred tank reactors (Van Aswegen and Marais, 1998). Parameters such as pH and temperature are controlled within narrow ranges to maintain the right balance of bacteria in order to achieve the optimum rate of oxidation (Van Aswegen and Marais, 1998). The optimum operating parameters of the BIOX® process are listed in Table 2.8.

TABLE 2.7

Commercial BIOX® Plants (Van Aswegen and Marais, 1998)

Location	Construction date	Capacity (t/day)
Fairview, South Africa	1984	55
Sao Bento, Brazil	1990/1991	150
Harbour Lights, Australia*	1992	40
Wiluna Mine, Australia	1993	158
Ashanti's Sansu, Ghana	1994	960
Tamboraque, Peru**	1997	60
Amantaytau, Uzbekistan***	?	2 055
Olympias, Greece***	?	668
Fosterville, Australia***	?	120

*Not in operation since 1994 due to the closing of the mine

**Decommissioned in 2000, but currently being recommissioned

***Future Developments

TABLE 2.8 Optimal Operating Parameters of the BIOX® Process	
Temperature	40-45ºC
pH	1.2-1.6
Percentage Solids in Feed	20%
Dissolved Oxygen	>2ppm
Retention Time	4-6 days
Nutrients	Fertilizer type ammonium, potassium and phosphorous salts

The bacteria attach to the metal sulphide surfaces in the ore, resulting in the increased oxidation of the sulphide minerals (Van Aswegen and Marais, 1998). The oxidation reactions that occur during the process are exothermic, and thus, circulating cooling water and removing the excess heat by cooling towers, cool the reactors (Van Aswegen and Marais, 1998). Limestone or sulphuric acid is added to keep the pH of the slurry within the optimum range, while large volumes of air is injected and dispersed in the slurry (Van Aswegen and Marais, 1998). The air contains sufficient oxygen required for sulphide mineral oxidation, as well as carbon (in the form of carbon dioxide) required to maintain microbial growth (Van Aswegen and Marais, 1998). In addition to the carbon dioxide introduced by injecting air into the slurry, carbonate minerals or limestone can be added to maintain sufficient levels of carbon necessary for microbial growth (Van Aswegen and Marais, 1998).

The overall residence time in the biooxidation reactors ranges between 4 and 6 days depending on the type of ore that is being processed, i.e. a shorter residence time can be expected when arsenopyrite is processed as compared to pyrite, because the oxidation rate of the former is faster than that of pyrite (Van Aswegen and Marais, 1998). Following the oxidation process, the BIOX® product is washed in a counter-current decantation circuit, followed by neutralisation of the solution by adding limestone and/or lime (Van Aswegen and Marais, 1998). Gold is eventually recovered from the washed BIOX® in a conventional cyanidation plant (Van Aswegen and Marais, 1998).

2.4.4.2 The GEOCOAT™ Process

Copper has been extracted from chalcopyrite for centuries using conventional pyrometallurgical methods (Harvey *et al*., 2002). Although these methods are relatively simple, they have certain disadvantages, such as high capital investments, operating costs and a negative impact on the environment (Harvey *et al*., 2002). In addition, these methods are inflexible when it comes to treating complex metal sulphides (Harvey *et al*., 2002). Therefore, dump- and stirred tank leaching using bacteria in a thermophilic system was developed (Brierley, 2000; Harvey *et al*., 2002). Commercial bioheap operations currently in operation are listed in Table 2.9.

The GEOCOAT™ process was developed by GeoBiotics, Inc. and involves heap leaching for the extraction of copper concentrates from chalcopyrite ores (Harvey *et al*., 2002). With the advantages of stirred tank systems and the simplicity of conventional heap leaching, this process provides an economical solution to the bioleaching of chalcopyrite ores (Harvey *et al*., 2002).

TABLE 2.9

Commercial Bioheap Leach Operations Currently in Operation (Harvey *et al*., 2002)

Location	Capacity (t/day)	Years in Operation
Lo Aguirre, Chile	16 000	1980-Present
Cerro Colorado, Chile	16 000	1993-Present
Girrilambone, Australia	2 000	1993-Present
Ivan, Chile	1 500	1994-Present
Quebrada Blanca, Chile	17 300	1994-Present
Andacollo, Chile	16 000	1996-Present
Dos Amigos, Chile	3 000	1996-Present
Cerro Verde, Peru	32 000	1996-Present
Zaldivar, Chile	~20 000	1998-Present
S&K Copper, Myanmar	15 000	1998-Present

Copper-bearing sulphide minerals are concentrated by flotation and thickened, resulting in a concentrate slurry (Harvey *et al*., 2002). A solid substrate, usually crushed, screened support rock, is thinly coated with the concentrate slurry, followed by stacking on a lined pad, where it is allowed to biooxidise (Johansson *et* al., 1999; Harvey *et al*., 2002). The biooxidation heap is inoculated with naturally occurring sulphide-oxidising bacteria, depending on the desired operation temperature (Johansson *et* al., 1999; Harvey *et al*., 2002). Moderate thermophiles, such as *Acidithiobacillus caldus* and *Sulfobacillus thermosulfidooxidans*, as well as extreme thermophiles, such as *Acidianus brierleyi*, *Acidianus infernus*, *Metallosphaera sedula*, *Sulfolobus acidocaldarius*, *Sulfolobus shibatae* and *Sulfolobus metallicus*, are frequently used for the GEOCOAT™ process (Harvey *et al*., 2002). The biooxidation heap is irrigated with leaching solution consisting of sulphuric acid, ferric iron and nutrients, while the heap is aerated by low-pressure blowers through a perforated pipe network at the base of the heap (Johansson *et* al., 1999; Harvey *et al*., 2002). As the biooxidation progresses, the sulphides are oxidized and the resulting soluble copper, iron arsenic and sulphate are removed from the heap by the recirculating solution, followed by neutralisation and conventional recovery methods (Harvey *et al*., 2002).

2.4.4.3 The BioCOP™ Process

The BioCOP™ process was developed by BHP Billiton, Ltd. and is conducted in an aerated stirred reactor containing dilute sulphuric acid and hyperthermophilic microorganisms, which are able to metabolise at temperatures between 60ºC and 90ºC. Limestone is added to the process to maintain the pH of the solution, as well as to provide the microorganisms with carbon dioxide needed for bacterial growth. The copper concentrate is added to the reactor and the leaching of chalcopyrite concentrates is complete within 10 days (Batty and Rorke, 2006).

2.4.4.4 The BacTech/Mintek Process

The BacTech/Mintek process involves a series of countercurrent reactors, the Circox™ bioreactor and the BAR™ (BacTech Aerated Reactor). The two proprietary bioreactors are in the experimental stage in Mexico. The Circox™ was originally developed for the bioremediation of municipal sewage and industrial wastewater.

Paques Bio Systems B.V. of the Netherlands holds the licence for the Circox™ bioreactor. The Circox™ bioreactor uses an airlift to circulate the solids within the reactor. Thermophilic microorganisms are used in the process, which operate between 25ºC and 55ºC and a pH of 0.5 to 2.5 is maintained within the reactor. Ambient air used to airlift the suspended solids within the reactor, is also responsible for adding carbon dioxide to the process. The nutrients for the microorganisms are added to the leaching liquor and the retention time is in the order of 30 days (Van Staden *et al*., 2003).

2.5 Complexation of Non-Sulphide Minerals

Metals in certain non-sulphide minerals, such as the iron ore concentrate of the Sishen Iron Ore Mine, may be solubilised by a process of complexation using organic acids, such as citric and oxalic acid (Rawlings, 2005). These organic acids are typically produced by certain types of fungi, such as *A. niger* (Jianlong, 2000; Vandenberghe *et al*., 2000; Rawlings, 2005). Citric acid contains several carboxyl groups, which tend to donate protons (H^+), resulting in negatively charged carboxyl groups that are capable of forming stable complexes with several cations (Sayer and Gadd, 2001). Therefore, it would be possible that these negatively charged carboxyl groups might form stable complexes with the positively charged K cations present. The H^+ ions released by the carboxyl groups on the other hand may be involved in a hydrolysis reaction, whereby they may react with the PO_4^{3-} ions, resulting in the subsequent P and K removal from the iron ore concentrate.

In contrast to bioleaching of sulphidic minerals using chemolithoautotrophic bacteria, which is the most studied and commercially exploitable aspect of mineral biotechnology today, there is a scarcity of literature on the dissolution of non-sulphidic minerals, such as oxides, silicate, carbonate and hydroxide minerals (Jain and Sharma, 2004). Complexation of non-sulphidic minerals involves the use of heterotrophic microorganisms, which require an organic carbon source as a source of energy and carbon for their growth (Jain and Sharma, 2004). As non-sulphidic ores generally contain no energy source for these microorganisms to utilize, an energy source must be added to the system (Jain and Sharma, 2004).

Heterotrophic microorganisms produce metabolic by-products that may interact with a mineral surface (Jain and Sharma, 2004). In addition to forming several organic acids such as acetic, citric, oxalic and keto-gluconic acid (Castro *et al*., 2000; Natarajan and Deo, 2001), heterotrophic microorganisms also produce exopolysaccharides, amino acids and proteins which are also able to solubilise metals via a variety of mechanisms (Welch and Vandevivere, 1995; Welch *et al*., 1999). However, organic acids have the advantage of producing both protons and a metal complexing organic acid anion (Gadd, 1999). Heterotrophic microorganisms also have other mechanisms that enable them to effectively leach non-sulphidic minerals. These mechanisms include bio-reduction, acidification, complexolysis and alkalinisation.

Bio-reduction involves microorganisms which are able to solubilise minerals, such as limonite, goethite or hematite, by reduction (Ehrlich, 1986; Ferris *et al*., 1989; Jain and Sharma, 2004). Ghiorse (1988) proposed that the production of oxalic acid by a fungus can affect the reduction of Fe (III) to Fe (II), thus increasing iron solubility. A process for the biological reduction of iron ore using *Pseudomonas* sp. has been developed (Hoffman *et al*., 1989), while Rusin *et al*. (1994) suggested a process for bioremediation of heavy metal contaminated soil using an iron-reducing *Bacillus* strain.

Acidification may result either from the formation of an acidic metabolite or from a preferential utilization of alkaline substrate (Jain and Sharma, 2004). It has been found that lowering the pH to less than 5 resulted in an increased dissolution rate of many silicate and aluminium silicate minerals (Welch and Ullman, 1996). Among the organic acids, 2-ketogluconic acid produced by some bacteria and citric acid and oxalic acid produced by some fungi have been shown to be effective in the dissolution of silicates by furnishing protons that help in breaking Si-O and Al-O bonds through protonation and catalysis (Welch and Ullman, 1996; Vandevivere *et al*., 1994; Drever and Stillings, 1997).

Complexolysis is a process that utilizes microbially formed complexing and chelating agents that mobilize mineral constituents (Fe, Al, Cu, Zn, Ni, Mn, Ca, Mg, etc.) (Jain and Sharma, 2004). Microorganisms are able to produce and excrete organic ligands

as a result of fermentation and degradation of organic macromolecules (Tzeferis and Agatzini-Leonardou, 1994; Paris *et al.,* 1996; Gadd, 1999). These organic ligands are able to increase the rates of mineral weathering by forming stable soluble metal-organic complexes in solution, resulting in increased mineral solubility (Amerhein and Surez, 1988; Bennett *et al*., 1988; Wieland *et al*., 1988). In addition, microbes are also able to produce extracellular polysaccharides, which are able to enhance mineral dissolution by complexing with ions in solution, or they can inhibit dissolution by irreversibly binding to reactive sites on the mineral surface (Welch and Vandevivere, 1995; Welch and Ullman, 1999; Welch *et al*., 1999). A further mechanism of metal solubilisation is the production of iron-chelating siderophores that specifically solubilise Fe (III) (Liermann *et al*., 2000).

Bio-solubilisation of silicates is also possible through alkalinisation of the media. Avakyan (1985) demonstrated the release of silicon from nepheline, plagioclase or quartz by using the bacteria *Sarcina ureae*. *S. ureae* produces ammonia, which results in the high alkalinisation of the medium. Under these conditions the Si-O bond is disrupted, resulting in the solubilisation of the mineral (Jain and Sharma, 2004).

Factors that affect the bioleaching process include the microbial population characteristics as well as various physicochemical parameters and the properties of the mineral to be leached (Brandl, 2001). Heterotrophic bioleaching is affected by the size of the microbial population, its metal tolerance and adaptation abilities to the mineral environment (Jain and Sharma, 2004). As an example, enhanced nickel extraction with *Aspergillus niger* and better cobalt extraction with *Penicillium funiculosum* was achieved from low-grade laterite ores (Valix *et al*., 2001b). Inoculum density has also been found to affect the bioleaching rate of minerals. A 50% enhancement in the rate of zinc extraction from filter dust was achieved on doubling the size of the inoculums of *P. simplicissimum* (Burgstaller *et al*., 1992). The rate of leaching is affected by the toxicity of certain metals, and therefore, the use of metal tolerant species enhances the rate of leaching (Tzeferis *et al*., 1994; Valix *et al*., 2001a). Physicochemical parameters, such as temperature, pH, oxygen supply, stirring rate and nutritional composition of the medium have a direct influence on the leaching efficiency of microbes (Jain and Sharma, 2004). The properties of the mineral to be leached also affect the leaching process. Pulp density of the solid to be

leached, particle size, mineralogical composition, effect of pre-treatment, surface area and hydrophobicity of the solids are major factors in determining the rate and extent of leaching (Jain and Sharma, 2004).

In addition to the extraction of metals from non-sulphidic ores and industrial residues, bioleaching with heterotrophic microorganisms can also be used to remove undesirable metal impurities from ores (biobeneficiation), as well as to detoxify soil, sediment and waste material polluted with heavy metals (Jain and Sharma, 2004).

There are both advantages and disadvantages of using heterotrophic microorganisms for bioleaching. Leaching by heterotrophs to solubilise metals from minerals is possible at high pH, while metal leaching by most of the autotrophic bacteria is possible only in acidic conditions (Burgstaller and Schinner, 1993; Krebs *et al*., 1997). The more neutral pH range at which heterotrophic microorganisms grow allows for easier microbial contamination to occur, and process sterilisation is costly and also presents technical problems for large scale operations (Jain and Sharma, 2004). Another cost consideration is the need for an organic carbon source for the growth of the heterotrophic microorganisms and the subsequent production of leaching agents. If cheap organic wastes generated in agriculture, in the food industry or in biotechnological processes can be used as growth substrates, leaching with fungi may be economic on an industrial scale (Jain and Sharma, 2004).

The identification, characterisation, selection, and development of bioprocesses for industrial and commercial applications require interdisciplinary cooperation between microbiologists, chemists, metallurgists and engineers. Heterotrophic leaching is extremely promising for the development of extraction technologies for non-sulphidic ores (Jian and Sharma, 2004).

2.5.1 Citric Acid Production by *Aspergillus niger*

Citric acid (2-hydroxypropane-1,2,3-tricarboxylic acid) (Figure 2.9) is an intermediate in the TCA cycle (also known as the citric acid cycle or Krebs cycle) (Figure 2.10), and is an important commercial product with global production reaching the 1.4 million ton/yr range (Graff, 2006). The majority of citric acid is produced by

submerged fermentation using the white rot fungus, *A. niger* (Jianlong, 2000; Vandenberghe *et al*., 2000). Citric acid is widely used in the food, beverage, pharmaceutical, chemical and cosmetic industries, and finds other applications in textiles, electroplating and bioremediation (Wang and Liu, 1996; Tran *et al*., 1998; Ates *et al*., 2002). In the United Kingdom, citric acid has been used as a buffer to increase the solubility of brown heroin. Single-use citric acid sachets have been used as an inducement in order to get heroin users to exchange their dirty needles for clean needles in an attempt to decrease the spread of HIV and hepatitis (Garden *et al*., 2003). Due to all the different applications, the volume of citric acid production by fermentation is continually increasing to keep up with the demand (Jianlong and Ping, 1998).

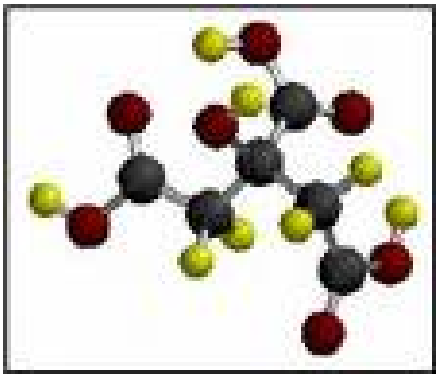

FIGURE 2.9

Molecular structure of citric acid (2-hydroxypropane-1,2,3-tricarboxylic acid) (Wikipedia 2006b)

Aspergillus niger is the most popular microorganism for the large-scale production of citric acid due to its high citric acid productivity at low pH without the secretion of toxic metabolites (Kim, 2004; Legiša and Mattey, 2007). In nature, *A. niger* grows on natural plant debris, such as leaves and on fruit (Legiša and Mattey, 2007). Most plant materials contain low levels of free carbohydrates (less than 100 µmol), whereas fruits may contain up to 500 mmol of sugars (Wrolstad and Shallenberger, 1981), and therefore, similar high levels of carbohydrates are implemented in the citric acid fermentation process (Legiša and Mattey, 2007).

Citric acid production by *A. niger* involves two main metabolic pathways, namely: 1) the catabolic pathway of hexoses to pyruvate and Acetyl-CoA by glycolysis, and 2) citric acid formation by the TCA cycle (Alvares-Vasquez *et al*., 2000).

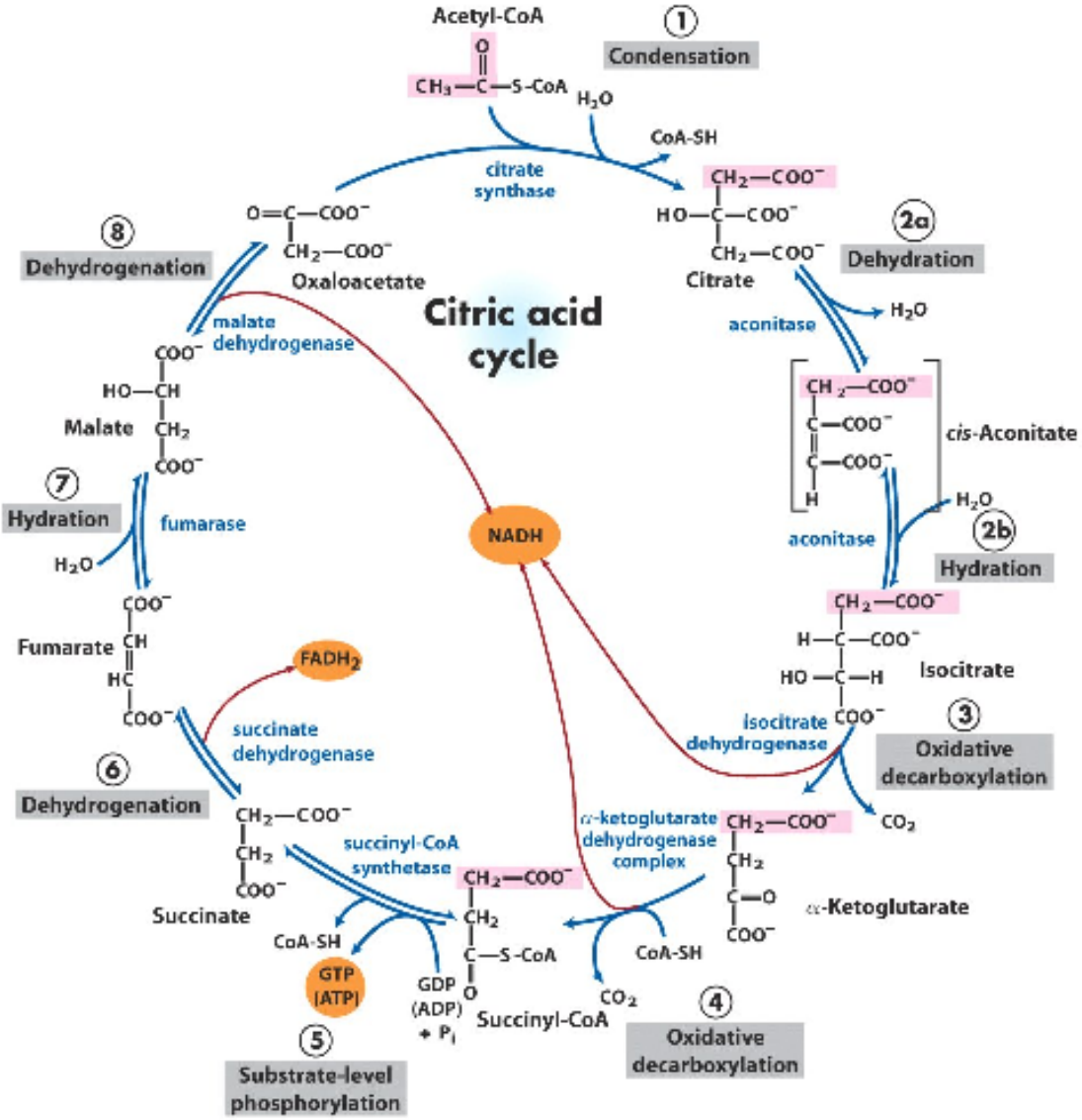

FIGURE 2.10

Major Intermediates of the Tricarboxylic Acid Cycle (Wikipedia, 2006c)

Glucose plays an important role in the production rate of citric acid, as its initial supply limits the glucolytic reactions of *A. niger* (Torres, 1994a; Torres, 1994b). Citric acid is excreted from *A. niger* cells in response to unfavourable intracellular conditions (Legiša and Mattey, 2007). This results in increased levels of TCA, such as citric acid and oxalic acid, during the growth of the fungus in high glucose

concentration media (Legiša and Mattey, 2007). It is documented that there are three main metabolic events that replenish TCA intermediates and predispose the fungal cells to product formation:

1. Fast uptake of glucose through simple diffusion,
2. Unrestricted metabolic flow through glycolysis, resulting in the availability of precursors for TCA intermediate synthesis, and
3. Uncoupled NADH re-oxidation resulting in lower levels of ATP and thus decreased anabolic reactions (Legiša and Mattey, 2007).

2.5.2 Uptake of Glucose Based on Passive Diffusion

Hexoses are commonly used in industry as the carbon source of choice for citric acid fermentation, since *A. niger* grow well on glucose and fructose (Hondmann and Visser, 1994). The hexoses are hydrolysed to monomers by invertase during the germination of the fungal spores (Legiša and Mattey, 2007). Although *A. niger* contains both high and low affinity carriers, which facilitate the uptake of glucose into the cell, it is documented that the glucose uptake rate and glucose concentration in citric acid producing systems is due to a simple diffusion model, rather than facilitated diffusion or active transport (Wayman and Mattey, 2000). This could be expected as both the high affinity carrier (encoded for by the sugar transporter gene, *mst*A) and low affinity carrier (presumed to be encoded for by the expression of the *mst*E gene) are inhibited at low pH and in the presence of citrate (VanKuyk *et al*., 2004; Forment *et al*., 2006; Legiša and Mattey, 2007). In contrast, citric acid producing systems showed no response to citric acid levels (Wayman and Mattey, 2000).

2.5.3 Regulation of Glycolysis

Physiological studies of *A. niger* during citric acid accumulation have indicated that no citric acid could be detected in the medium up to 24 h of growth in a batch system (Legiša and Mattey, 1986a). During the second day of fermentation, however, a relatively slow excretion rate was recorded, which was followed by a sudden increase in productivity thereafter (Ruijter *et al*., 1997; Papagianni *et al*., 2005). Abnormal spore germination in the form of bulbous cells can be observed during the early stages

of fungal growth, followed by the formation of highly branched filamentous hyphae after approximately 24 h (Legiša and Mattey, 2007). Legiša and colleagues (1981) showed that the filamentous hyphae, representing the major part of the biomass, are responsible for the citric acid excretion into the fermentation media.

The pentose phosphate pathway is predominant during the germination of the fungal spores, followed by a switch to glycolysis before citric acid is excreted (Legiša and Mattey, 1986b; Röhr *et al*., 1987). During these initial phases of fungal growth polyols are formed in addition to the accumulation of glucosamine (Röhr *et al*., 1987; Papagianni *et al*., 2005). As a result of this transient glucosamine accumulation in the medium, as well as the intermediate accumulation and later partial re-consumption of various polyols (glycerol, arabitol, erythritol and mannitol), quantitative balances indicate that more hexoses are taken up by the fungus than can be accounted for by the production of biomass, CO_2 and citrate during the first stage of fermentation, while more citrate is excreted during the later phase than sugar uptake would theoretically allow (Röhr *et al*., 1987; Papagianni *et al*., 2005). Polyols, mostly synthesised by the intermediates of the pentose phosphate pathway, may play an important role as osmoregulators in *A. niger* cells during growth in high sugar concentration media (Legiša and Mattey, 1986b; Röhr *et al*., 1987; Hondmann and Visser, 1994; Legiša and Kidrič, 1989).

The production phase of citric acid accumulation is initiated after approximately 24 h and accelerates after 40-50 h of growth in a batch system (Papagianni *et al*., 2005). During this phase the direct conversion of hexoses to pyruvate via glycolysis becomes predominant (Legiša and Mattey, 1986b). The mechanism causing the shift of glucose degradation from the pentose phosphate pathway to glycolysis is not yet fully understood (Legiša and Mattey, 2007). The central part of hexose metabolism is regulated at several levels:

1. At the transcription level,
2. By regulating the activity of allosteric enzymes by specific effectors, and
3. By post-translational modification (Mesojednik and Legiša, 2005; Mlakar and Legiša, 2006; Legiša and Mattey, 2007).

Panneman and colleagues (1998) showed that the transfer of the fungal mycelia to media with various carbon sources stimulated the synthesis of hexokinase, glucokinase and pyruvate kinase. The most pronounced effect on the initiation of transcription was observed in the case of glucose and fructose. In addition, it was found that the presence of a metabolisable carbon source was not sufficient for the expression of hexokinase and glucokinase genes in *A. niger*, but that active carbon metabolism was also required (Panneman *et al*., 1998).

2.5.3.1 Hexokinase and Glucokinase

The genes for hexokinase and glucokinase have been isolated from *A. niger* and the kinetics parameters have been determined (Panneman *et al*., 1996; Panneman *et al*., 1998). The proteins encoded for by the *A. niger* glucokinase gene *glk*A and hexokinase gene *hxk*A, respectively, both show similarity to other eukaryotic glucokinase and hexokinase proteins, in particular to the *Saccharomyces cerevisiae* glucokinase protein and the hexokinase proteins of budding yeasts (Panneman *et al*., 1996; Panneman *et al*., 1998). The hexokinase and glucokinase enzymes of *A. niger* are responsible for catalysing the hexose phosphorylation step (Legiša and Mattey, 2007). The hexokinase and glucokinase contribution towards the glucose phosphorylation was discovered to be dependant on the intracellular pH, as well as the glucose concentration in the medium (Panneman *et al*., 1998). At a pH of 7.5 it was found that the glucokinase activity was predominant, while at a pH of 6.5 and a glucose concentration above 0.5 mM the hexokinase activity became predominant (Panneman *et al*., 1998). The glucokinase and hexokinase genes are both expressed constitutively during active carbon metabolism, however, wider substrate specificity was observed with hexokinase than with glucokinase (Panneman *et al*., 1996; Panneman *et al*., 1998).

2.5.3.2 6-Phosphofructo-1-kinase

ATP-dependent 6-phosphofructose-1-kinase (*Pfk*1) is the second allosteric enzyme of the glycolytic pathway, which plays a crucial role in controlling metabolic flux in eukaryotes by catalysing the second essentially irreversible reaction of glycolysis, the

phosphorylation of fructose 6-phosphate using Mg-ATP to form fructose 1,6-biphosphate and releasing Mg-ADP (Legiša and Mattey, 2007).

2.5.4 Fermentation Conditions Affecting Citric Acid Production

Medium composition, such as carbon, nitrogen, P and K, plays an important role in the growth and metabolism of microorganisms, and therefore, optimising the medium composition may enhance the production of citric acid by *A. niger*.

Sucrose, fructose and glucose are the carbon sources of choice for the production of citric acid by *A. niger* (Sassi *et al*., 1991). Apple peels and pomace, grape pomace, banana extract, sugar cane bagasse and sugar beet molasses have been used in the past for the production of citric acid (Ngadi and Correia, 1992; Wang, 1998; Gutierrez-Correa *et al*., 1999). Of these carbon substrates, glucose is readily used by *A. niger*, as it does not need any modification to be metabolised (Kim, 2004). An increase in the glucose flux through glycolysis causes the over-production of citric acid during solid substrate fermentation, however, low concentrations of glucose may lead to the production of oxalic acid (Habison *et al*., 1983; Röhr and Kubicek, 1981; Alvarez-Vasquez *et al*., 2000; Leangon *et al*., 2000).

The effect of nitrogen on citric acid production has been studied extensively (Papagianni *et al*., 2005). Protein catabolism, as a result of manganese deficiency, leads to a high intracellular ammonium (NH_4^+) concentration, causing the inhibition of the enzyme phosphofructokinase (Röhr and Kubicek, 1981; Habison *et al*., 1983). Phosphofructokinase is an essential enzyme in the conversion of glucose and fructose to pyruvate, and therefore, its inhibition leads to a flux through glycolysis and the formation of citric acid (Röhr and Kubicek, 1981; Habison *et al*., 1983). Nitrogen and phosphate concentrations are low in media designed for citric acid fermentation by *A. niger* (Papagianni *et al*., 2005). In defined media, nitrogen can be supplied as ammonium sulphate and ammonium nitrate, resulting in a decrease in pH, which is a prerequisite of citric acid fermentation (Papagianni *et al*., 2005). Exogenous addition of NH_4^+ during citric acid fermentation was found to stimulate the rate of citrate production (Choe and Yoo, 1991; Yigitoglu and McNeil, 1992). The exogenous P concentration, like in the case of nitrogen, plays an important role in citric acid

fermentation (Mirminachi *et al*., 2002). The limiting concentration of P can be supplied as KH_2PO_4 or K_2HPO_4, inducing higher citric acid production and yield (Mirminachi *et al*., 2002).

Citric acid production is influenced by a number of culture conditions, and therefore, it is important to study the influence that the physical and chemical environments have on citric acid fermentation (Jianlong and Ping, 1998). Fungal cells show signs of adverse growth and metabolic production when cultivated under unfavourable temperatures (Ellaiah *et al*., 2003). Under higher temperatures than optimal, enzyme denaturation and inhibition, excess moisture losses and the ceasing of growth occurs, while at lower temperatures metabolic activity decreases (Adinarayana *et al*., 2003). The optimal temperature for the production of citric acid by *A. niger* ranges from 28 to 40ºC, depending on the fungal strain used (Roukas, 2000; Papagianni *et al*., 2005; Papagianni and Mattey, 2006).

Most filamentous fungi grow well under slightly acidic conditions ranging from pH 3 to 6, while some are able to grow at a pH below 2 in order to compete more efficiently with bacteria (Fawole and Odunfa, 2003). For citric acid production by *A. niger*, an initial pH range from 2 to 6 is required for solid substrate and submerged fermentation (Watanabe *et al*., 1998; Adham, 2002; Lesniak *et al*., 2002).

Inoculum density plays an important role during citric acid fermentation. A high inoculum density leads to population over-crowding, higher nutrient competition and rapid exhaustion of nutrients, while a lower inoculum density leads to a decrease in metabolite production, as well as an increase in contamination risk as a result of an insufficient cell population (Kota and Sridhar, 1999). For citric acid fermentation by *A. niger*, an inoculum density between 1×10^4 to 1×10^9 spores.ml^{-1} was found suitable (Favela-Torres *et al*., 1998; Ruijter *et al*., 2000; Adham, 2002; Papagianni *et al*., 2005; Papagianni and Mattey, 2006).

2.6 Recommendations

Bioleaching offers several advantages over conventional physicochemical methods for the removal of P and K from the iron ore concentrate of the Sishen Iron Ore Mine.

Bioleaching offers more environmentally friendly processes, less energy is required for operation and sulphur dioxide and other environmentally harmful gasses are not emitted (Rawlings, 2002). The P and K contained within the iron ore concentrate are both non-sulphidic minerals, and therefore, conventional bioleaching may not be a suitable candidate for their removal from the ore. However, it is known that non-sulphidic minerals, such as the P and K contained within the iron ore concentrate, may be solubilised by complexation using organic acids, such as citric acid (Rawlings, 2005). Citric acid contains several carboxyl groups, which tend to donate protons (H^+), resulting in negatively charged carboxyl groups that are capable of forming stable complexes with several cations, such as K present in the iron ore, and at the same time the release of H^+ ions may result in a hydrolysis reaction involving the P contained in the iron ore, resulting in the subsequent P and K removal from the iron ore concentrate of the Sishen Iron Ore Mine.

Therefore, the following recommendations are suggested for the removal of P and K from the iron ore concentrate of the Sishen Iron Ore Mine:

1. A microbial community study of the water resources and iron ore concentrate must be conducted to investigate the possibility of using microorganisms already present, and thus adapted to the extreme environment of the Sishen Iron Ore Mine, for the development of a viable process to remove the P and K from the iron ore concentrate,
2. A range of inorganic- and organic acids must be evaluated for their potential to chemically remove P and K from the iron ore concentrate,
3. The production of citric acid by *A. niger* must be evaluated to determine the most economically viable acid fermentation process,
4. The chemical leachability of the P and K from the iron ore concentrate using different citric acid concentrations and leaching temperatures must be determined.
5. The results of the abovementioned recommendations must be taken into account to propose an economically viable biotechnology process for the removal of the P and K from the iron ore concentrate of the Sishen Iron Mine.

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CHAPTER 3

Microbial Community Study of the Process- and Groundwater of the Sishen Iron Ore-Mine, South Africa

The editorial style of the *Water SA* was followed in this chapter

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3.1 Abstract

Investigating the microbial community of the Sishen Iron-Ore Mine in South Africa has become a topic of interest. Micro-organisms could prove to be useful in bioleaching processes, resulting in the minimization of the negative impact that certain substances, such as phosphorous (P) and potassium (K), have on the economic functioning of the mine. The objective of this investigation was, therefore, to determine which micro-organisms were indigenously present in the process- and groundwater systems of the mine. Groundwater samples and three different process water samples were collected from the mine, followed by chemical- and microbial community analyses. Microbial inhibition was observed in all the process water samples due to the relatively high levels of copper, chromium and zinc present. *Aeromonas hydrophila* proved to be the dominant bacterial species in all the process water samples, whereas *Pseudomonas aeruginosa* and *Herbaspirillum* spp. were observed in the ground water of the mine. None of the isolated microorganisms have been implicated in bioleaching practices, and therefore, these organisms will not be included as candidates for the removal of P and K from the iron ore of the Sishen Iron-Ore Mine.

Key Words: Sishen Iron Ore Mine, microbial community, process water, groundwater, bioleaching

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3.2 Introduction

The depletion of high-quality iron-ore (>60% Fe; <0.24% K) deposits necessitates the processing of lower-quality iron-ore (<60% Fe; >0.24% K) (Jain and Sharma, 2004; Taljaard, 2005). Impurities, such as P and K contained within the lower-quality iron-ore have a detrimental effect on the steel-making process, and therefore, steel-making plants charge penalties when purchasing iron-ore with P and K levels exceeding 0.24% (Yusfin *et al*., 1999). In the past, low-quality iron-ore concentrate has been blended with high-quality iron-ore, in an attempt to 'dilute' the P and K contained within the export iron-ore concentrate of the mine (Dukino *et al*., 2000). However, the low-quality iron ore stockpiles of the Sishen Iron-Ore Mine are increasing, and therefore, it is essential to develop an economically and environmentally friendly process to reduce the high P and K concentrations of the iron-ore concentrate.

Micro-organisms could prove to be useful in the removal of the P and K from the iron-ore, as they may have novel metabolic properties, which could enable them to produce acids that may prove invaluable when applied in industrial practice (Gupta and Sharma, 2002; Lesniak *et al*., 2002). It is essential to determine which micro-organisms are indigenous to the mine environment before strategising how best to employ them to industrial advantage. Therefore, there has been an increasing interest in the microbial community of the Sishen Iron-Ore Mine environment.

It is hypothesised that indigenous micro-organisms already living in the Sishen Iron-Ore Mine environment are capable of using the P and K in the iron-ore as structural components for their cell walls and membranes, as well as many other metabolic processes, such as organic acid production, since the environment selects for them to do so. The purpose of this investigation was to determine which micro-organisms are indigenously present in the process- and groundwater of the Sishen Iron-Ore Mine, as well as to determine the microbial diversity. To date no information regarding the microbial community of the Sishen Iron-Ore Mine's aquatic environment exists.

3.3 Materials and Methods

3.3.1 Sample Selection and Processing

Process water samples (10 ℓ) were collected in sterile containers at three different sampling points of the Sishen Iron-Ore Mine. The sampling points included water from the process dam, water flowing into the slimes dam, and water flowing from the slimes dam. In addition, a groundwater sample was collected from a borehole located within the Sishen Iron-Ore Mine. The samples were stored at 4°C until processing.

3.3.2 Chemical Analysis of the Process- and Groundwater

The pH and turbidity, as well as the levels of ammonium, hydrogen sulphide, nitrates, nitrites, total phosphorous, potassium, free chlorine, fluoride, copper, chromium, iron, manganese and zinc were determined for all water samples by spectrophotometry using the Spectroquant® Photometer SQ 118 (Merck, Darmstadt, Germany). Spectroquant® test kits (Merck) for each of the abovementioned parameters were used according to the manufacturers instructions. The pH of the water samples was measured using a Beckman Φ34 pH meter (Beckman Coulter, Inc., Fullerton, CA, USA).

3.3.3 *Total Plate Counts of the Process- and Groundwater*

Heterotrophic plate counts of the process- and groundwater of the mine were conducted using the pour plate method (Health Protection Agency, 2004a). A dilution series for each water sample was prepared in sterile test tubes using distilled water (dH_2O) (Health Protection Agency, 2004b). One millilitre of each dilution was pipetted into a 90 mm Petri dish (Concorde Plastics, Johannesburg, South Africa), followed by the addition of 20 mℓ of liquid (50°C) Standard Nutrient Agar (Appendix A) to each Petri dish. Once the agar had solidified, the agar plates were incubated for 48 h at 28°C. Each process- and groundwater sample was analysed in triplicate. Following the incubation period, the bacterial colonies were enumerated and the Simpson's index of diversity ($1\text{-}D = 1\text{-}\Sigma pi^2$), as well as the Equitability index ($E_D = D/D_{max}$) calculated (D: Simpson's diversity index; pi: proportion of species made up of the ith species; D_{max}: the maximum value D could assume if individuals in the community were completely evenly distributed).

3.3.4 Preparation of Pure Cultures

Pure cultures of each morphological distinct bacterial colony, which was isolated on the standard plate count agar plates, were prepared. Each colony was inoculated separately onto agar plates containing solidified Standard Nutrient Agar (Appendix A). The agar plates were incubated for 48 h at 28°C in order to obtain single bacterial colonies. The procedure was repeated, followed by the bacterial identification. Suspensions from the pure cultures isolated from the groundwater sample were prepared, using sterile dH_2O, for molecular analysis.

3.3.5 Bacterial Identification of the Bacteria Isolated from the Process Water

Bacteria isolated from the process water samples were Gram-stained according to the method described by the Health Protection Agency (2007). Oxidation-fermentation (OF) analysis was performed by the Hugh-Leifson test (Health Protection Agency, 2004c), using OF basal medium (Appendix A), supplemented with a 10% filter-sterilized solution of D (+) glucose (Merck), lactose (Merck) and sucrose (Merck). The oxidase test using *N*, *N*, *N*', *N*'-tetramethyl-*p*-phenylenediamine (Aldrich Chemical Co, Milwaukee, Wisconsin) was performed on all isolated bacteria (Health Protection Agency, 2004d). Finally, the bacterial species were identified using the API 20E and 20NE identification systems as described by the manufacturer (Analytab Products, Plainview, NY).

3.3.6 16S Polymerase Chain Reaction for the Amplification of Bacterial DNA from the Groundwater Sample

A 16S Polymerase Chain Reaction (PCR) was performed by amplifying a portion of the 16S eubacterial gene from the bacterial suspensions prepared from the pure cultures of bacteria isolated from the groundwater sample. The following primers were used for DNA amplification:

PRUN518r: 5'-ATT-ACC-GCG-GCT-GCT-GG-3' (Siciliano *et al*., 2003),

PA8f-GC: 5'-CGC-CCG-CCG-CGC-GCG-GCG-GGC-GGG-GCG-GGG-GCA-CGG-GGG-GAG-AGT-TTG-ATC-CTG-GCT-CAG-3' (Fjellbirkeland *et al*., 2001).

All PCR reagents were manufactured by Bio-Rad Laboratories (Hercules, CA, USA), unless otherwise stated. The PCR reaction was performed in a reaction volume of 25 µℓ containing, 150 mM KCl, 30 mM Tris-HCl (pH 9.0), 0.3% Triton X-100, 50 mM $MgCl_2$, 10 µM PCR nucleotide mix, 5 pmol primer PRUN518r (Whitehead Scientific, Cape Town, South Africa), 5 pmol primer PA8f-GC (Whitehead Scientific), 1.5 units of Taq polymerase and 0.5 µℓ bacterial suspension. Denaturation of extracted DNA at 95ºC for 10 min was followed by 35 cycles of denaturation at 94ºC for 30 s, annealing at 51ºC for 30 s, and extension at 72ºC for 1 min (Bio-Rad Thermal Cycler, Bio-Rad Laboratories). A final extension at 72ºC for 10 min concluded the PCR amplification of the DNA. A reaction containing no DNA template was included as a negative control. The amplified PCR products were separated using 1% agarose gel electrophoresis in Tris-Acetate-EDTA (TAE) buffer.

3.3.7. Sequence Analysis of the Bacterial DNA from the Groundwater Sample

Sequences of the 16S eubacterial gene of the rDNA operon were obtained using primer PRUN518r. The sequences reported in this study were compared to 16S eubacterial gene sequences present in the GenBank database by using the BLAST program of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). Matching hits with e-values closest to 0.0 were chosen for alignment. Reported and reference sequences were aligned using CLUSTAL X version 1.8 (ftp://ftp-igbmc.u-stras-bg.fr/pub/ClustalX/) (Thompson et al., 1997) and inserted gaps were treated as missing data. Ambiguously aligned regions were excluded from the data set before analysis. Phylogenetic analysis was based on parsimony using PAUP 4.0b8 (Phylogenetic Analysis Using Parsimony) (Swofford, 2000). Heuristic searches were made with random addition of sequences (1 000 replicates), tree bisection-reconnection (TBR), branch swapping and MULPAR effective and MaxTrees set to auto-increase. Evaluating tree length distributions over 100 randomly generated trees assessed phylogenetic signal in the data sets. The consistency (CI) and retention indices (RI) were determined for all data sets. Characters were re-weighted to the CI, and only informative characters were included, while missing, ambiguous and constant characters were excluded. Phylogenetic trees were rooted with *Bacillus subtilis* as outgroup to the remaining taxa.

Bootstrap analyses were conducted, retaining groups with 70% consistency, to determine confidence in branching points (1 000 replicates) for the most parsimonious trees generated.

3.4 Results and Discussion

The chemical analysis of the process- and groundwater samples of the Sishen Iron Ore Mine is reported in Table 3.1. The pH of all water samples ranged between 7.25 and 7.80. The water flowing to the slimes dam contained the highest concentrations of copper (0.72 mg.ℓ^{-1}), chromium (0.24 mg.ℓ^{-1}) and zinc (0.44 mg.ℓ^{-1}). This water also contained ammonium (0.23 mg.ℓ^{-1}), hydrogen sulphide (0.34 mg.ℓ^{-1}), high levels of nitrates (>90.0 mg.ℓ^{-1}), nitrites (>3.0 mg.ℓ^{-1}), phosphorous (2.4 mg.ℓ^{-1}), free chlorine (0.6 mg.ℓ^{-1}), fluoride (0.89 mg.ℓ^{-1}), iron (0.52 mg.ℓ^{-1}) and manganese (0.8 mg.ℓ^{-1}). The water collected from the process dam contained lower concentrations of copper (0.29 mg.ℓ^{-1}), chromium (0.20 mg.ℓ^{-1}) and zinc (0.31 mg.ℓ^{-1}), compared to the water flowing to the slimes dam, while it contained the highest concentrations of ammonium (0.39 mg.ℓ^{-1}), hydrogen sulphide (0.41 mg.ℓ^{-1}), phosphorous (3.4 mg.ℓ^{-1}), free chlorine (0.8 mg.ℓ^{-1}), fluoride (0.97 mg.ℓ^{-1}), iron (0.66 mg.ℓ^{-1}) and manganese (1.3 mg.ℓ^{-1}), as well as high levels of nitrates (>90.0 mg.ℓ^{-1}) and nitrites (>3.0 mg.ℓ^{-1}).

In contrast, the water collected from the slimes dam contained only high levels of nitrates (>90.0 mg.ℓ^{-1}), nitrites (2.0 mg.ℓ^{-1}) and fluoride (0.74 mg.ℓ^{-1}). These results indicate that the slimes dam is functioning correctly by precipitating elements such as copper, chromium, iron, manganese and zinc. The groundwater, however, only showed traces of nitrates (10.5 mg.ℓ^{-1}) and high levels of nitrites (>3.0 mg.ℓ^{-1}) in the water, with all other elements below the detection limits, indicating that the groundwater table remains isolated from contamination with heavy metals and other chemical compounds and elements, which may arise from the mining process.

TABLE 3.1

Chemical analysis of the process- and groundwater samples of the Sishen Iron-Ore Mine

Parameters	Process Dam	Water to the Slimes Dam	Water from the Slimes Dam	GroundWater
pH	7.54	7.61	7.80	7.25
Turbidity	54 NTU	14 NTU	1 NTU	23 NTU
Ammonium	0.39 mg.ℓ^{-1}	0.23 mg.ℓ^{-1}	ND	ND
Hydrogen Sulphide	0.41 mg.ℓ^{-1}	0.34 mg.ℓ^{-1}	ND	ND
Nitrates	>90.0 mg.ℓ^{-1}	>90.0 mg.ℓ^{-11}	>90.0 mg.ℓ^{-1}	10.5 mg.ℓ^{-1}
Nitrites	>3.0 mg.ℓ^{-1}	>3.0 mg.ℓ^{-1}	2.0 mg.ℓ^{-1}	>3.0 mg.ℓ^{-1}
Total Phosphorous	3.4 mg.ℓ^{-1}	2.4 mg.ℓ^{-1}	ND	ND
Potassium	ND	ND	ND	ND
Free Chlorine	0.8 mg.ℓ^{-1}	0.6 mg.ℓ^{-1}	ND	ND
Fluoride	0.97 mg.ℓ^{-1}	0.89 mg.ℓ^{-1}	0.74 mg.ℓ^{-1}	ND
Copper	0.29 mg.ℓ^{-1}	0.72 mg.ℓ^{-1}	ND	ND
Chromium	0.20 mg.ℓ^{-1}	0.24 mg.ℓ^{-1}	ND	ND
Iron	0.66 mg.ℓ^{-1}	0.52 mg.ℓ^{-1}	ND	ND
Manganese	1.3 mg.ℓ^{-1}	0.8 mg.ℓ^{-1}	ND	ND
Zinc	0.31 mg.ℓ^{-1}	0.44 mg.ℓ^{-1}	ND	ND

*ND – Not Detected

Table 3.2 illustrates the average plate counts obtained for each dilution of the process- and groundwater samples. When the bacterial counts for each dilution of the process water samples were compared to one another, it became evident that the bacterial growth was inhibited in all the undiluted samples. However, no inhibition effect was observed in the groundwater collected from the mine. The inhibitory effect that heavy metals have on bacterial growth is well documented (Gordon *et al.*, 1994; Yenigün *et al.*, 1996), and therefore, it can be assumed that the inhibitory effect observed in this study is most likely as a result of the copper, chromium and zinc contained in the process water of the mine.

TABLE 3.2

The average bacterial counts (cfu.mℓ^{-1}) obtained for the different dilutions of the process- and groundwater samples of the Sishen Iron-Ore Mine

Sample	Undiluted	1 log	2 log	3 log
Process Dam	1.1×10^2	5.13×10^3	2.17×10^3	2.0×10^3
To Slime Dam	0	5.53×10^2	4.67×10^2	1.33×10^3
From Slime Dam	2.97×10^2	9.23×10^2	6.0×10^2	0
Ground Water	2.66×10^1	0	0	0

As the copper, chromium and zinc were diluted in the dilution series, the inhibitory effect decreased in all the process water samples. The inhibitory effect was diminished at a 3-log dilution in the water flowing to the slimes dam, compared to a 1-log dilution in the water from the process dam and the water flowing from the slimes dam. This indicates that the level of the substance(s) responsible for the inhibitory effect must have been significantly higher in the water flowing to the slimes dam than the other two water sources. The total amount of copper, chromium and zinc in the water flowing to the slimes dam (1.4 mg.ℓ^{-1}) was significantly higher than observed in both the water from the process dam (0.8 mg.ℓ^{-1}) and the water flowing from the slimes dam (~0.0 mg.ℓ^{-1}), as well as the groundwater (~0.0 mg.ℓ^{-1}), confirming that these heavy metals were indeed responsible for the bacterial inhibitory effect observed during this study.

Bacteria isolated and identified by API analysis from the different process water samples collected at the Sishen Iron-Ore Mine, are listed in Table 3.3. *Aeromonas hydrophila* was found to be the dominant bacterial species in all the process water samples from the mine.

TABLE 3.3
Bacteria isolated and identified from the different process water samples collected at the Sishen Iron-Ore Mine

Identification of Isolated Bacteria	Average Bacterial Count (cfu.mℓ^{-1})
Water from the Process Dam	
Aeromonas hydrophila	4.67×10^3
Alcaligenes faecalis	3.21×10^2
Brevundimonas vesicularis	1.39×10^2
Acinetobacter junii	0.33×10^1
Water flowing from the Slimes Dam	
Aeromonas hydrophila	7.99×10^2
Pantoea spp.	7.01×10^1
Flavobacterium meningosepticum	5.35×10^1
Water flowing to the Slimes Dam	
Aeromonas hydrophila	1.08×10^3
Chryseomonas luteola	1.72×10^2
Enterobacter sakazakii	8.60×10^1

Comparing the Simpson's Index of Diversity (1-*D*) calculated for the three process water samples, it is evident that the bacterial diversity is greatest in the water flowing to the slimes dam (0.3279), followed by the water flowing from the slimes dam (0.2415) and water from the process dam (0.1677). Although the species richness of the water from the process dam is the highest (4), the population is dominated by *A. hydrophila*. The species richness of both the water flowing to and from the slimes dam was found to be 3, and the bacterial population was dominated to a lesser extent by *A. hydrophila* compared to the water from the process dam. This suggests that the species are more evenly distributed in both the water flowing to and from the slimes dam, compared to the water from the process dam.

Aeromonas hydrophila is a Gram-negative ubiquitous aquatic bacterium, which has been isolated from a wide range of water sources, such as river water, drinking water, as well as water distribution pipe biofilms (Havelaar *et al*., 1990; Chauret *et al*., 2001; Lynch *et al*., 2002; Bomo *et al*., 2004; Canals *et al*., 2006). *A. hydrophila* has been found to persist in chlorinated drinking water supplies as a result of biofilm production within distribution pipe systems (Fernandez *et al*., 2000; Bomo *et al*., 2004). *A. hydrophila* is able to produce cytotoxins and enterotoxins that are often associated with acute gastroenteritis, as well as wound infections in humans, and less commonly associated with septicaemia of immunocompromised patients (Janda and Abbott, 1998; Fernandez *et al*., 2000). In addition, *A. hydrophila* could also be pathogenic to fish, reptiles and amphibians, causing hemorrhagic septicemia (Fernandez *et al*., 2000). The pathogenicity of *A. hydrophila* has been associated with toxins, proteases, outer membrane proteins, lipopolysaccharides and flagella (Merino *et al*., 1996; Negueras *et al*., 2000; Rabaan *et al*., 2001; Canals *et al*., 2006). *Alcaligenes faecalis* is a heterotrophic nitrifying bacterium, which is commonly found in wastewater treatment systems where it is used for the removal of nitrogen from wastewater (Nishio *et al*., 1998; Kim *et al*., 2004; Joo *et al*., 2007). The phenol-degrading ability of *Alcaligenes faecalis* in wastewater sediments has also been documented (Tong *et al*., 1998). *Brevundimonas* (formerly *Pseudomonas*) *vesicularis* is an aerobic, non-sporulating and non-fermenting Gram-negative bacillus (Segers *et al*., 1994; Gilligan *et al*., 2003), which has been isolated from soil, bottled mineral water and hydrotherapy pools (Aspinall and Graham, 1989; Morais and da Costa, 1990; Davis *et al*., 1994). *Brevundimonas vesicularis* is an opportunistic human pathogen, which has mostly been associated with infections due to the immunocompromised state caused by underlying diseases, such as autoimmune disorders associated with long-term steroid use, end-stage renal disease treated by haemodialysis, and sickle cell anemia with functional asplenia (Gilad *et al*., 2000; Chi *et al*., 2004; Choi *et al*., 2006; Sofer *et al*., 2007). *Pantoea* spp. belong to the *Enterobacteriaceae* family and are important pathogens causing intestinal and systemic illness in humans and animals, and is commonly found in domestic wastewater sludge (Vacca *et al*., 2005; Chale-Matsau and Snyman, 2006). *Chryseobacterium* (formerly *Flavobacterium*) *meningosepticum* is an opportunistic pathogen often responsible for nosocomial

infections associated with water systems in hospitals (Squier *et al*., 2000). *Chryseobacterium meningosepticum,* which is typically found in water and soil, generally has low pathogenicity, but may be of clinical importance in immunocompromised patients, often causing neonatal meningitis with a high mortality rate (Ratner, 1984; Sheridan *et al*., 1993). *Chryseomonas luteola* has been implicated in the biosorption of chromium from industrial wastewater (Ozdemir and Baysal, 2004). *Chryseomonas luteola* was isolated from the water flowing to the slimes dam, containing the highest levels of chromium (0.24 mg.ℓ^{-1}) (Table 3.1). Therefore, the possibility exists to use *C. luteola* during water treatment to remove the chromium from the wastewater of the mine.

DNA was successfully extracted from all pure cultures isolated from the groundwater samples collected from the Sishen Iron-Ore Mine. The PCR of total DNA extracted from the pure cultures isolated from the groundwater samples yielded a ca. 500bp fragment of PCR product on a 1% TAE agarose gel. No protein contamination in the wells of the gel or RNA contamination smears below the DNA bands were observed. The phylogenetic analysis of the 16S eubacterial gene of the rDNA operon of the bacteria isolated from the groundwater of the mine is illustrated in Figure 3.1. The phylogenetic analysis indicated that 5 of the bacterial isolates from the groundwater samples have a strong sequence comparison with *Pseudomonas aeruginosa* (Verce *et al*., 2000) and a further 7 bacterial isolates a strong sequence comparison with a *Herbaspirillum* species (Probian *et al*., 2003).

Herbaspirillum species have previously been isolated from groundwater systems (Connon *et al*., 2005). These micro-organisms are able to exist in groundwater systems where nitrogen limitation prevails, due to their ability to fix nitrogen (Elbeltagy *et al*., 2001; Kirchhof *et al*., 2001; Connon *et al*., 2005). The fact that *Herbaspirillum magnetovibrio* was identified to survive in the iron-ore (Williams and Cloete, 2008) (Chapter 4), and that nitrogen (~11 mg.ℓ^{-1}) is limited in the ground-water, may explain why this bacterium is able to exist in the groundwater of the Sishen Iron-Ore Mine.

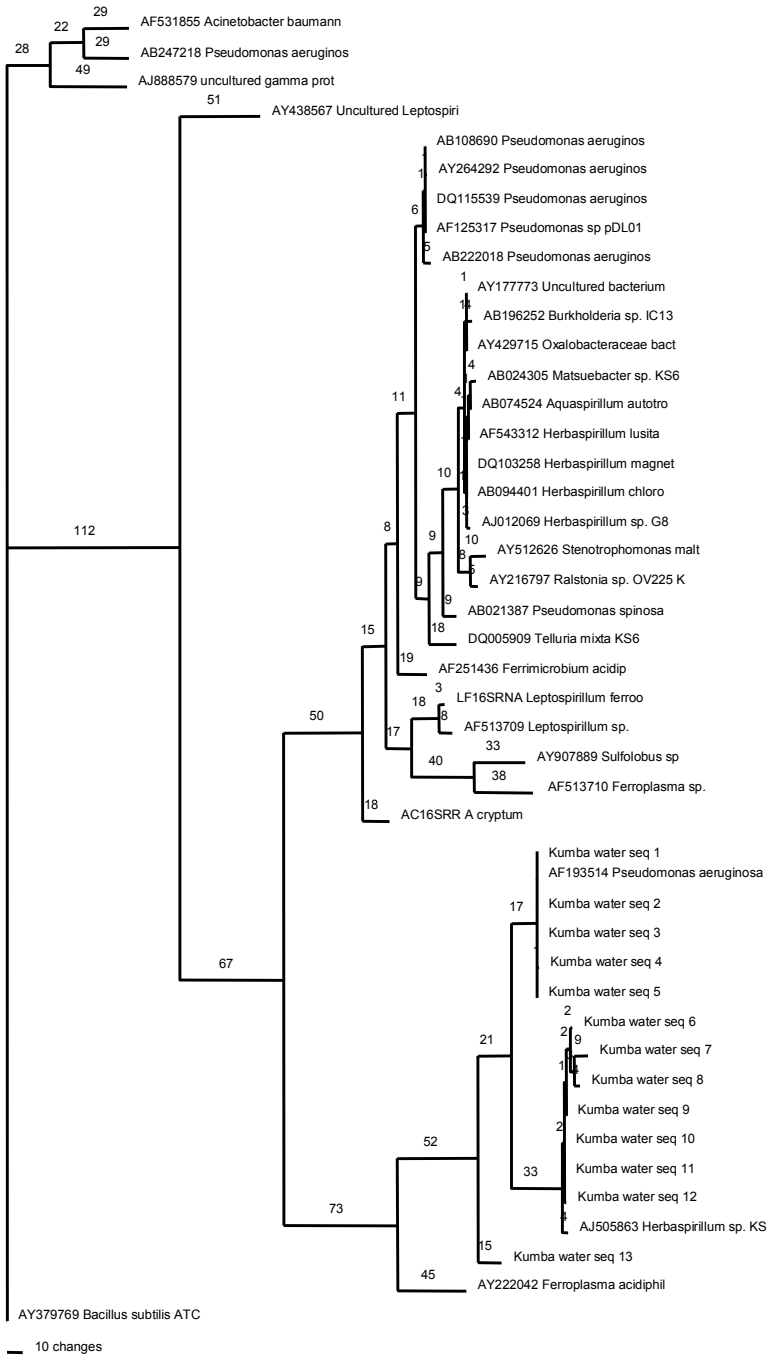

FIGURE 3.1

Phylogenetic analysis of the 16S Eubacterial gene of the rDNA operon of the bacteria isolated from the groundwater of the Sishen Iron-Ore Mine

Pseudomonas aeruginosa is a Gram-negative rod-like bacterium, which is ubiquitous in soil and water, and commonly detected in great amounts in sewage contaminated by humans and animals, although its main habitat remains controversial (Pellett *et al*., 1983; Römling *et al*., 1994; Todar, 2004). In nature, *P. aeruginosa* may be found in surface biofilms, or in a planktonic form, actively swimming by means of a single polar flagellum (Sauer *et al*., 2002; Todar, 2004). Taking into account the low nutritional content in the groundwater of the Sishen Iron-Ore Mine (Table 3.1), it is not surprising to isolate *P. aeruginosa,* as this bacterial species has very simple nutritional requirements (Todar, 2004). *Pseudomonas aeruginosa* is an opportunistic pathogen to humans and a major cause of nosocomial infection (Khan and Cerniglia, 1994; Römling *et al*., 1994), where it may cause urinary tract infections, acute respiratory illness (ARI), dermatitis, soft tissue infections, bacteraemia, bone and joint infections, acute gastrointestinal illness (AGI) and a variety of systemic infections, particularly in immunocompromised patients (Fegan *et al*., 1990; Hirarkata *et al*., 1991; Furuya *et al*., 1993; Todar, 2004; Morbidity and Mortality Weekly Report, 2006).

3.5 Conclusions

Except for *C. luteola* which may be used for the biosorption of chromium from waste water, there is no indication of bioleaching properties for any of the microorganisms isolated in the process- and groundwater systems, and therefore, they should be excluded as bioleaching candidates for the removal of undesirable substances from the iron-ore of the Sishen Iron-Ore Mine.

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CHAPTER 4

Microbial Community Study of the Iron Ore Concentrate of the Sishen Iron Ore Mine, South Africa

The editorial style of the *Water SA* was followed in this chapter

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4.1 Abstract

As a result of the advancing global technologies and civilisation, there has been a progressive depletion of high-grade mineral deposits. Consequently, it has become increasingly important to process lower-grade ores. Phosphorous (P) and particular potassium (K) contained in the iron ore concentrates of the Sishen Iron Ore Mine have a detrimental effect on the steel making process, whereby these alkali's cause cracks to form in the refractory lining of blast furnaces. It is initially essential to determine which microbes are indigenously present at the Sishen Iron Ore Mine before strategising how best to employ them to industrial advantage. Therefore, the objective of this study was to determine which microorganisms are indigenous to the iron ore and soil of the Sishen Iron Ore Mine. The bacterial 16S PCR and fungal ITS PCR revealed several bacterial and fungal species present in the Sishen Iron Ore Mine environment. According to phylogeny, the bacterial isolates were closely related to *Herbaspirillum* species, as well as *Acidithiobacillus ferrooxidans*, while the fungal isolates were closely related to *Aureobasidium pullulans*, *Phaeosphaeria nodorum*, *Aspergillus fumigatus*, and *Candida parapsilosis*. Isolating *A. fumigatus* from the iron ore/soil of the mine may indicate that *A. niger*, the most common fungi used for the production of citric acid, can adapt to the stringent mine environment. This would allow the application of *A. niger* for the production of citric acid, which may be used for the chemical leaching of the P and K from the iron ore concentrate of the Sishen Iron Ore Mine.

Key Words: Citric acid, Iron ore concentrate, Microbial community, Phosphorous, Potassium, Sishen Iron Ore Mine

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4.2 Introduction

The depletion of high quality iron ore (>60% Fe; <0.24% K) deposits necessitates processing of lower quality iron ore (<60% Fe; >0.24% K) (Jain and Sharma, 2004; Corne Taljaard, Personal communication). Elements such as phosphorous (P) and potassium (K) contained within the lower quality iron ore concentrates have a detrimental effect on steel making process, leading to penalties charged by the steel processing plants when purchasing iron ore concentrates containing high concentrations of P and K (Yusfin *et al*., 1999). In an attempt to minimise these penalties charged by the steel making companies, low quality ore concentrates has been blended with high quality iron ore concentrates to "dilute" the P and K in the export iron ore concentrate of the mine (Dukino *et al*., 2000). This has also been the practice in Hamersley, Australia, where low-P iron ore (0.05% P), being the major component, was blended with high-P iron ore (0.10% P) before export to the steel making companies (Dukino *et al*. 2000). However, the low quality iron ore stockpiles of the Sishen Iron Ore Mine are increasing, and it is therefore essential to develop an economical, environmentally friendly process to treat the iron ore concentrates that contain high concentrations of P and K.

Microorganisms are capable of converting nutrients in their surrounding environment to biochemical compounds, such as organic acids, required by their metabolism (Gupta and Sharma, 2002; Lesniak *et al*., 2002). The processes by which this happens can be invaluable when applied in industrial practice. For example, *Acidithiobacillus ferrooxidans* and A*t. thiooxidans* are capable of converting ferrous sulphate ($FeSO_4$) to ferric sulphate [$Fe_2(SO_4)_3$], while producing sulphuric acid (H_2SO_4) (Glazer and Nikaido, 1995; Rawlings, 2002). Ferric sulphate and H_2SO_4 both play an important role during the overall leaching of certain minerals from ore bodies (Glazer and Nikaido, 1995). Ferric sulphate is a strong oxidising agent, which is reduced to ferrous sulphate ($FeSO_4$), leading to the oxidation of certain insoluble metal sulphides to the corresponding soluble metal sulphate (Glazer and Nikaido, 1995). Sulphuric acid is responsible for maintaining the low pH in the environment in order to maintain the optimal metabolic activity of the bacteria in the system (Glazer and Nikaido, 1995).

It is plausible that microbes indigenously present in the iron-ore and surrounding soil are able to utilise P and K in their membrane and cell wall as structural components, as well as in many other microbiological metabolic processes. It is hypothesised that microbes already living and growing in soils and iron-ore at the Sishen mine are capable of this metabolism since their environment favours the appropriate required conditions. The purpose of this investigation was to determine which microbes are indigenously present in the iron ore and soil of the Sishen Iron Ore Mine before strategising how best to employ them to industrial advantage. To date no information regarding the microbial community present in the iron ore concentrates and soil of the Sishen Iron Ore Mine exists.

Some microorganisms are difficult to culture due to their different growth requirements and physiology, limiting simultaneous cultivation of several species (Widmer *et al*., 1999). Denaturing Gradient Gel Electrophoresis (DGGE) is an ideal molecular method for monitoring microbial community ecology since it is PCR based and does not employ classical methods of culturing the microbes within a sample. DGGE relies on variation in genetic sequence of a specific amplified region to differentiate between species within microbial communities (Banks and Alleman 2002; Koizumi *et al.* 2002). By determining the closest relatives of unknown microorganisms, the known characteristics can be inferred upon them (Ueda *et al.* 1995). Most commonly, 16S rRNA genes are used to give an overall indication of the bacterial species composition of a sample, while the Internal Transcribed Spacer region (ITS) is used in the case of fungal species composition.

4.3 Materials and Methods

4.3.1 Sample Selection

Iron ore concentrate samples (export product) were received for bacterial community analysis from the Sishen Iron Ore Mine. In addition, iron ore mixed with soil was collected at various locations within the mine for fungal community analysis.

4.3.2 Bacterial Isolation

Iron ore samples were prepared for DNA extraction by enrichment cultures. Briefly, the enrichment cultures consisted of 100 g of iron ore inoculated into 1 *l* of Nutrient Broth (Appendix A) (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) in 2 *l* Erlenmeyer flasks. The flasks and their contents were incubated at 25ºC for 24 h. The resulting suspension was used for DNA extraction.

4.3.3 Fungal Isolation

Fungi were isolated from the soil/iron ore by adding 100 g of each sample to 100 m*l* of sterile distilled water (dH_2O) in a different 500 m*l* Erlenmeyer flask. The flasks and their contents were incubated at 25ºC for 48 h. One millilitre of the suspension was plated onto 20 g.l^{-1} water agar (Merck, Darmstadt, Germany) in 60 mm Petri-dishes (Concorde Plastics, Johannesburg, South Africa). The Petri-dishes were incubated at 25ºC and monitored at 24 h intervals for fungal spore formation. Single fungal spores were then selected and transferred onto half-strength Potato Dextrose Agar (PDA) (Appendix A) (Merck) in 60 mm Petri-dishes (Concorde Plastics). The Petri-dishes were incubated at 25ºC for approximately 2 weeks.

4.3.4 Bacterial DNA Extraction

Bacterial DNA was extracted from the enrichment culture suspensions using the CTAB method (Doyle and Doyle, 1987; Cullings, 1992). Briefly, 1.5 m*l* of culture suspension was centrifuged to form a compact pellet, followed by the discarding of the supernatant. The pellet was resuspended in 567 µ*l* of Tris-EDTA (TE) buffer (Appendix A). Thirty microlitres of Sodium Dodecyl Sulphate (SDS) and 3 µ*l* of Proteinase K (20 mg.l^{-1}) was added and mixed, followed by incubation at 37ºC for 1 h. After incubation 100 µ*l* of 5 M Sodium Chloride (NaC*l*) (Appendix A) was added and mixed thoroughly, followed by the addition of 80 µ*l* of the CTAB/NaC*l* solution (Appendix A). The contents of the tubes were mixed thoroughly and incubated at 65ºC for 10 min. This was followed by mixing with an equal volume of chloroform/isoamyl alcohol, followed by centrifuging for 5 min. The supernatant was transferred to a new Eppendorf tube, followed by mixing with an equal volume of phenol/chloroform/isoamyl alcohol and centrifugation for 5 min. The supernatant was transferred to a new Eppendorf tube, followed by DNA precipitation by the

addition of a 0.6 volume of isopropanol. The DNA precipitate was collected by centrifugation for 15 min. The precipitated DNA was washed with 70% ethanol to remove the residual CTAB and salts, followed by centrifugation to form a pellet of DNA. The supernatant was discarded and the DNA allowed to dry in a vacuum dryer. The DNA was dissolved in 100 µ*l* of TE and stored at –20ºC for molecular analysis.

4.3.5 Fungal DNA Extraction

DNA was extracted from the fungal cultures using the sterile river sand method (Surridge *et al*., 2003). Briefly, fungal mycelium was placed in Eppendorf tubes, followed by the addition of sterile river sand to facilitate the disruption of the mycelia wall. Five hundred microlitres of DNA extraction buffer (DEB) was added to each tube and the samples ground. A further 200 µ*l* of DEB was added to each tube, followed by mixing of the samples in each tube. This was followed by the addition of 500 µ*l* of phenol and 300 µ*l* of chloroform, after which the tubes were vortexed. The tubes were centrifuged at 1 000 rpm for 60 min, followed by transferring of the upper aqueous phase to a new Eppendorf tube. Five hundred microlitres of phenol and 500 µ*l* of chloroform was added to each tube, followed by centrifugation at 10 000 rpm for 10 min. This phenol/chloroform step was repeated until the interphase was clean. The upper aqueous phase was transferred to a new Eppendorf tube, followed by the addition of 500 µ*l* of chloroform. The tubes were centrifuged at 11 000 rpm for 5 min, and the resulting aqueous phase transferred to a new Eppendorf tube. Twice the volume of 100% ethanol was added and mixed, followed by incubation at 4ºC overnight. The tubes were centrifuged at 11 000 rpm for 30 min and the supernatant discarded. The pellet containing DNA was washed by adding 500 µ*l* of 70% ethanol, followed by centrifugation at 11 000 rpm for 5 min. The supernatant was removed and the tubes containing the DNA vacuum dried for 30 min. The DNA was dissolved in 100 µ*l* of sterile Sabax water and stored at –20ºC for molecular analysis.

4.3.6 16S Polymerase Chain Reaction for Amplification of Bacterial DNA from the Iron Ore

A 16S Polymerase Chain Reaction (PCR) was performed by amplifying a portion of the 16S eubacterial gene from the bacterial DNA extracted from the enriched culture suspensions. The following primers were used for DNA amplification:

K: PRUN518r: 5'-ATT-ACC-GCG-GCT-GCT-GG-3' (Siciliano *et al*., 2003), M: PA8f-GC: 5'-CGC-CCG-CCG-CGC-GCG-GCG-GGC-GGG-GCG-GGG-GCA-CGG-GGG-GAG-AGT-TTG-ATC-CTG-GCT-CAG-3' (Fjellbirkeland *et al*., 2001).

All PCR reagents were manufactured by Bio-Rad Laboratories (Hercules, CA, USA), unless otherwise stated. The PCR reaction was performed in a reaction volume of 25 µl containing, 150 mM KCl, 30 mM Tris-HCl (pH 9.0), 0.3% Triton X-100, 50 mM $MgCl_2$, 10 µM PCR nucleotide mix, 5 pmol primer PRUN518r (Whitehead Scientific, Cape Town, South Africa), 5 pmol primer PA8f-GC (Whitehead Scientific), 1.5 units of Taq polymerase and 0.5 µl bacterial suspension. Denaturation of extracted DNA at 95ºC for 10 min was followed by 35 cycles of denaturation at 94ºC for 30 sec, annealing at 51ºC for 30 sec, and extension at 72ºC for 1 min (Bio-Rad Thermal Cycler, Bio-Rad Laboratories). A final extension at 72ºC for 10 min concluded the PCR amplification of the DNA. A reaction containing no DNA template was included as a negative control. The amplified PCR products were separated using 1% agarose gel electrophoresis in Tris-Acetate-EDTA (TAE) buffer.

4.3.7 Denaturing Gradient Gel Electrophoresis (DGGE)

The 16S PCR products were subjected to denaturing gradient gel electrophoresis (DGGE) according to the method described by Muyzer *et al*. (1993). Briefly, 10 µℓ containing *ca*. 250 ng of the 16S PCR products was loaded per lane onto a 25-55% denaturing gradient gel. The gel was run at 70V for 17 h at a constant temperature of 60ºC. Image analysis was performed using the Gel2K program and fingerprints were analysed in a cluster investigation using CLUST.

4.3.8 Internal Transcribed Spacer (ITS) Region Polymerase Chain Reaction for Amplification of Fungal DNA from the Iron Ore/Soil Samples

A portion of the Internal Transcribed Spacer (ITS) gene sequence of the DNA from each fungal isolate was subjected to PCR using the following primer set (White *et al*., 1990):

ITS1: 5'-CAT-CGA-GAA-GTT-CGA-GAA-GG-3'

ITS4: 5'-TAC-TTG-AAG-GAA-CCC-TTA-CC-3'

All PCR reagents were manufactured by Bio-Rad Laboratories (Hercules, CA, USA), unless otherwise stated. The PCR reaction was performed in a reaction volume of 25 µl containing, 150 mM KCl, 30 mM Tris-HCl (pH 9.0), 0.3% Triton X-100, 50 mM $MgCl_2$, 10 µM PCR nucleotide mix, 5 pmol primer ITS1 (Whitehead Scientific, Cape Town, South Africa), 5 pmol primer ITS4 (Whitehead Scientific), 1.5 units of Taq polymerase and 0.5 µl bacterial suspension. Denaturation of extracted DNA at 92ºC for 10 min was followed by 30 cycles of denaturation at 92ºC for 1 min, annealing at 50ºC for 1 min, and extension at 72ºC for 1 min (Bio-Rad Thermal Cycler, Bio-Rad Laboratories). A final extension at 72ºC for 10 min concluded the PCR amplification of the DNA. A reaction containing no DNA template was included as a negative control. The amplified PCR products were separated using 1% agarose gel electrophoresis in Tris-Acetate-EDTA (TAE) buffer.

4.3.9 Sequence Analysis of the Bacterial and Fungal DNA from the Iron Ore/Soil

Sequencing the PCR products from the 16S PCR and ITS PCR using the K and ITS1 primers above, respectively, provided tentative species identification. Each isolate was sequenced in an Eppendorf tube containing 1 µ*l* of clean PCR product, 2 µ*l* "Big Dye" (Roche) sequence mix, 0.32 µ*l* primer and 1.68 µ*l* filter-sterilised dH_2O. The sequence PCR products were cleaned by the addition of 15 µ*l* of sterile dH_2O, followed by the transfer of the entire volume to a 0.5 µ*l* Eppendorf sequencing tube. Two microlitres of 3 M sodium acetate and 50 µ*l* of 95% ethanol were added to each sequencing tube and allowed to stand on ice for 10 min, followed by centrifugation at 10 000 rpm for 30 min. The supernatant was removed, followed by washing of the DNA with 150 µ*l* of 70% ethanol. After centrifugation at 10 000 rpm for 5 min, the supernatant was aspirated and the DNA pellet vacuum dried for 10 min. DNA sequences were determined using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit using AmpliTaq® DNA Polymerase (Applied Biosystems, UK). Partial sequences of the 16S eubacterial gene, as well as of the ITS region of the rDNA of the bacteria and fungi, respectively, were obtained using the K and ITS1 primers, respectively. Nucleotide sequence order was confirmed by

comparison with the sequences obtained from the M and ITS4 primers, respectively, of the corresponding samples. The sequences reported in this study were compared to similar sequences present in the GenBank database by using the BLAST program of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov), and matching hits, with e-values closest to 0.0 indicating an exact match, were selected for alignment. Sequences were aligned with the CLUSTALX version 1.83 program (Thompson *et al.* 1997) and inserted gaps were treated as missing data. Ambiguously aligned regions were excluded from the data set before analysis. Phylogenetic trees were generated using the CLUSTALX version 1.83 program. The robustness of the trees was determined by bootstrap resampling of the multiple-sequence alignments (1 000 sets) with the CLUSTALX version 1.83 program. The graphical output of the phylogenetic trees was created with the TREEVIEW version 1.6.6 program (University of Glasgow; http://taxonomy.zoology.gla.ac.uk).

4.4 Results and Discussion

An attempt was made to isolate bacterial DNA directly from the iron ore concentrate by adding iron ore to sterilised dH_2O, followed by vigorous shaking using a vortex. This proved, however, to be ineffective as no bacterial identification was possible due to a very low DNA quantity in the sample. Therefore, it was decided to use enrichment techniques in attempt to obtain efficient DNA quantities for molecular identification of the bacterial species present in the iron ore concentrate samples. The shortcoming of this method is that certain heterotrophic bacteria may proliferate to such an extent that possible autotrophic bacteria may be suppressed, with the consequence that they may not be detected. The DNA extracted from the iron ore enrichment cultures using the CTAB method proved to be of a high quality, displaying no protein and no RNA contamination (Figure 4.1). The 16S PCR of the DNA extracted from the iron ore enrichment cultures yielded a *ca*. 500bp band on a 1.5% TAE agarose gel (Figure 4.2).

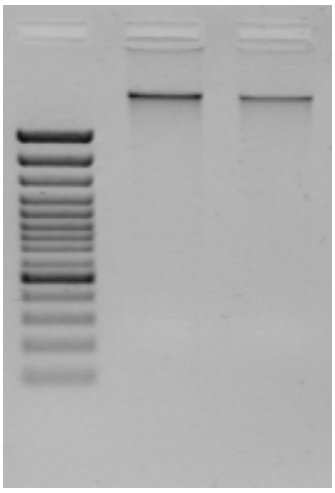

FIGURE 4.1

1.5% TAE agarose gel showing DNA extracted from two iron-ore samples using the CTAB method

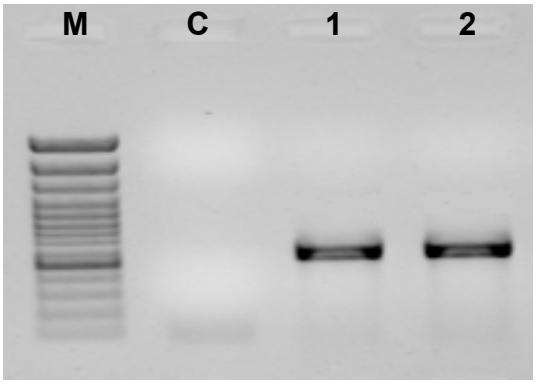

FIGURE 4.2

1.5% TAE agarose gel showing 16S PCR products of *ca.* 500bp (M = 100bp DNA marker, C = negative control, 1 & 2: iron ore samples

The DGGE with the 16S rDNA PCR products from the duplicate iron ore enriched samples resulted in a gel displaying a similar banded fingerprint pattern with some higher colour intensity (Figure 4.3). This duplication indicated reproducibility of results on DGGE gels and that some bacterial species within the sample form the dominant portion of the population indicated by darker banding (Figure 4.3). Each band on the gel is theoretically representative of only one distinct species.

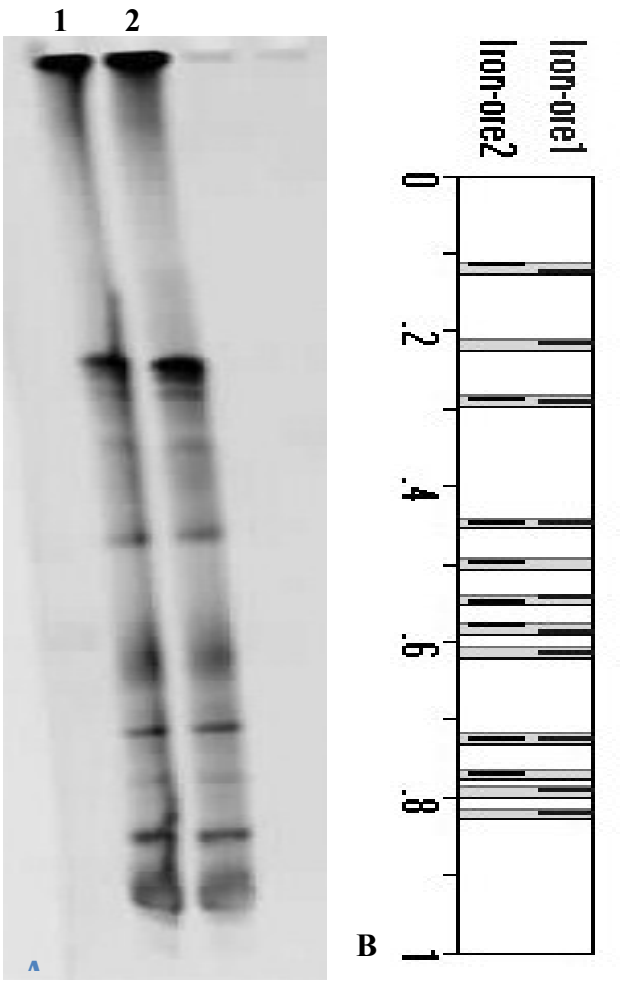

FIGURE 4.3

A: Denaturing gradient gel fingerprints resulting from assessing bacterial diversity between communities detected from iron-ore samples, using a gradient of 25-55%; B: Graphic representation of the denaturing gradient gel determining bacterial community richness and diversity

The DGGE gel showed clear multiple banding, forming a fingerprint, in each lane. However, only 12 bands were observed across both of the duplicate samples run, indicating 12 bacterial species present. This is most probably due to the extreme environment of the iron ore. Two dense, dominant bands were displayed in both of the duplicate fingerprints, which indicated that these species are prominent in the iron

ore. According to phylogeny and blast results these bands were both closely related to *Herbaspirillum* species (Figure 4.4).

Many of the *Herbaspirillum* species are known to be soil inhabitants associated with iron ore soils or extremophiles associated with chlorophenol degradation (Im *et al*. 2003, Cannon *et al*. 2005). According to literature linked to blast results on GenBank, *Herbaspirillum magnetovibrio* is an example of a novel magnetotactic bacterial species found in iron ore soil (Gao *et al*. 2005). One of their chief functions is nitrogen fixation and root nodule formation in nutrient limited soils (Kniemeyer *et al.* 1999, Probian *et al*. 2003, Valverde *et al*. 2003). All but one of the sequences found in the enriched iron ore concentrate were close matches to *Herbaspirillum* species, while KS2 is a closer match to *Acidithiobacillus ferrooxidans* (formerly known as *Thiobacillus ferrooxidans*) (Figure 4.4). It seems that most of the species present in the sample are to be expected in iron ore and its surrounding soils.

The most likely candidate for bioleaching is *Acidithiobacillus ferrooxidans*. This bacterial species is well known for its bioleaching properties, and is widely used in the industry for this purpose. *Acidithiobacillus ferrooxidans* is acknowledged as being responsible for iron and inorganic sulphur compound oxidation in areas such as mine tailings and coal deposits (Horan 1999). *Acidithiobacillus ferrooxidans* is assumed to be obligately aerobic, however, under anaerobic conditions it can be grown on elemental sulphur using ferric iron as an electron acceptor (Das *et al*., 1992; Pronk *et al*., 1992; Ohmura *et al*., 2002). It has been documented that *At. ferrooxidans* could also obtain energy from oxidising Cu^{+}, Se^{2-}, tetrathionate, molecular hydrogen, formic acid, antimony compounds, uranium compounds, and molybdenum compounds (Horan 1999). Thus, *At. ferrooxidans* can be considered a facultative anaerobe that plays an important role within iron and sulphur cycles in acidic environments such as in the mining industry (Horan 1999). This ability to grow in oxygen deficient environments implies useful biotechnology in bioleaching processes where anaerobic conditions may exist.

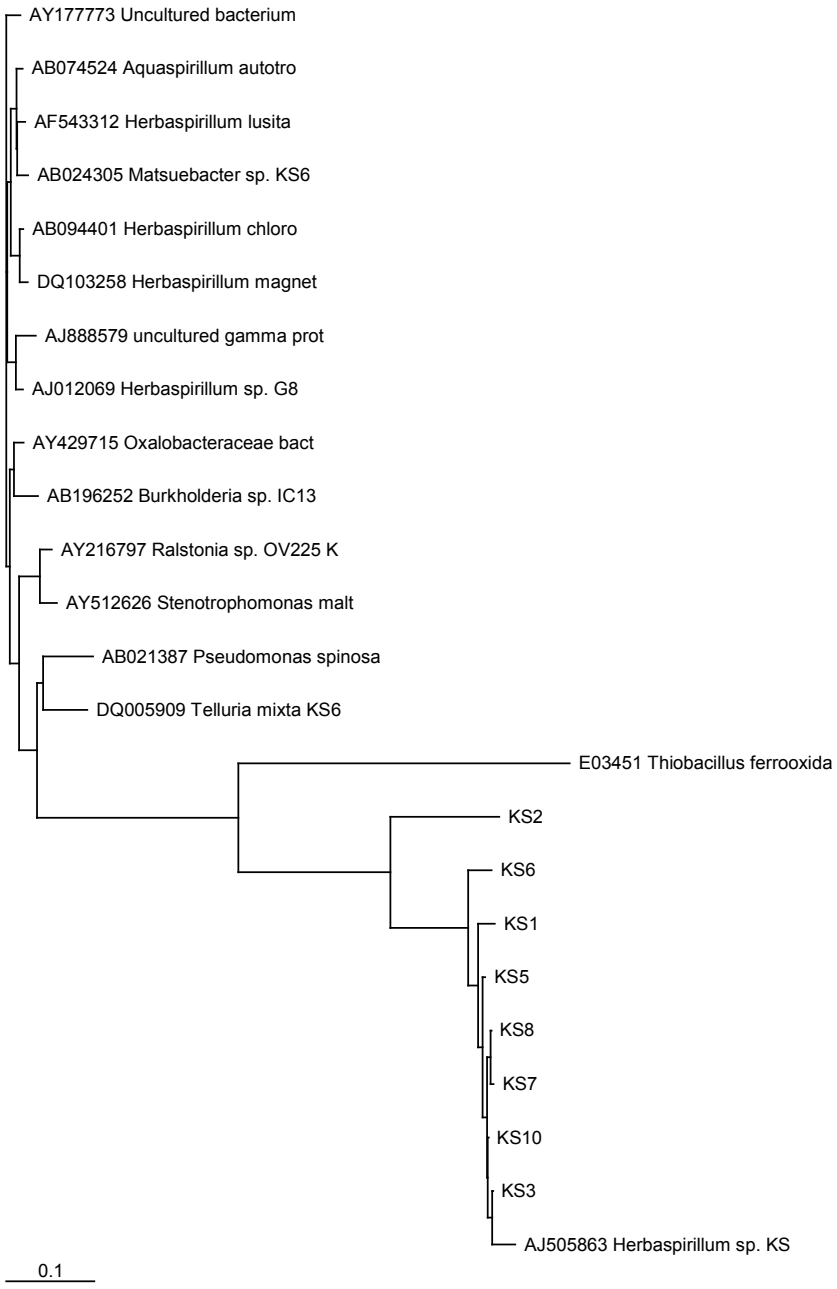

FIGURE 4.4

Phylogenetic analysis of the 16S Eubacterial gene of the rDNA operon of the bacteria isolated from the enriched iron ore concentrate of the Sishen Iron Ore Mine

DNA was successfully extracted from all fungal pure cultures isolated from the iron ore/soil of the Sishen Iron Ore Mine. The ITS PCR DNA extracted from the fungal

pure cultures yielded a *ca.* 700bp fragment of PCR product on a 1.5% TAE agarose gel (Figure 4.5).

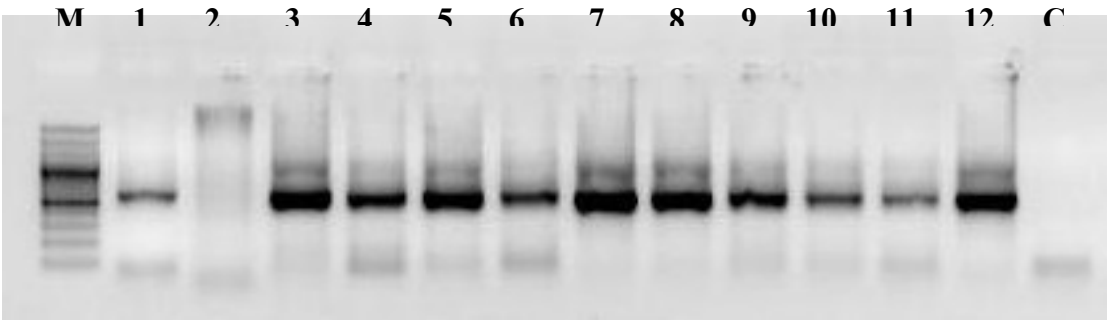

FIGURE 4.5

1.5% TAE agarose gel showing the ITS PCR products of *ca.* 700bp (M = 100bp DNA marker, 1-12 = fungal culture PCR amplicons, C = negative control)

The phylogenetic analysis of the ITS gene of the fungi isolated from the iron ore/soil of the mine is illustrated in Figure 4.6. The phylogenetic analysis indicated that one of the fungal isolates (KIOL1) from the iron ore/soil had a strong sequence identity with *Aureobasidium pullulans*, while another fungal isolate (KIOL2) had a strong sequence identity with *Aspergillus fumigatus* (Figure 4.6). The fungal isolates KIOL4 and KIOL6, as well as KIOL3 and KIOL5 were found to be similar to one another, having strong sequence identities to *Phaeosphaeria nodorum* and *Candida parapsilosis*, respectively (Figure 4.6).

Aureobasidium pullulans is a dimorphic fungus, which is commonly distributed throughout the environment, especially in soil and plants (Krogh *et al*., 1998; Yoshikawa *et al*., 2006). This fungus is able to grow well in the presence of high concentrations of saccharides, and produces polysaccharides (pullulans and β-glucan), oligosaccharides (FOS and isomaltooligosaccharides) and various carbohydrate-degrading enzymes (Deshpande *et al*., 1992; Yun *et al*., 1994; Hamada *et al*., 2000). *Aureobasidium pullulans* may be a potential candidate for bioremediation of heavy metal contaminated environments, as this fungus has been shown to assimilate certain heavy metals, such as zinc, cobalt, cadmium and copper (Gadd *et al*., 1987; Krogh *et al*., 1998). Furthermore, *A. pullulans* has been used for the commercial production of

xylanases, used for the enzyme-aided bleaching in the pulp and paper industry (Viikari *et al*., 1994).

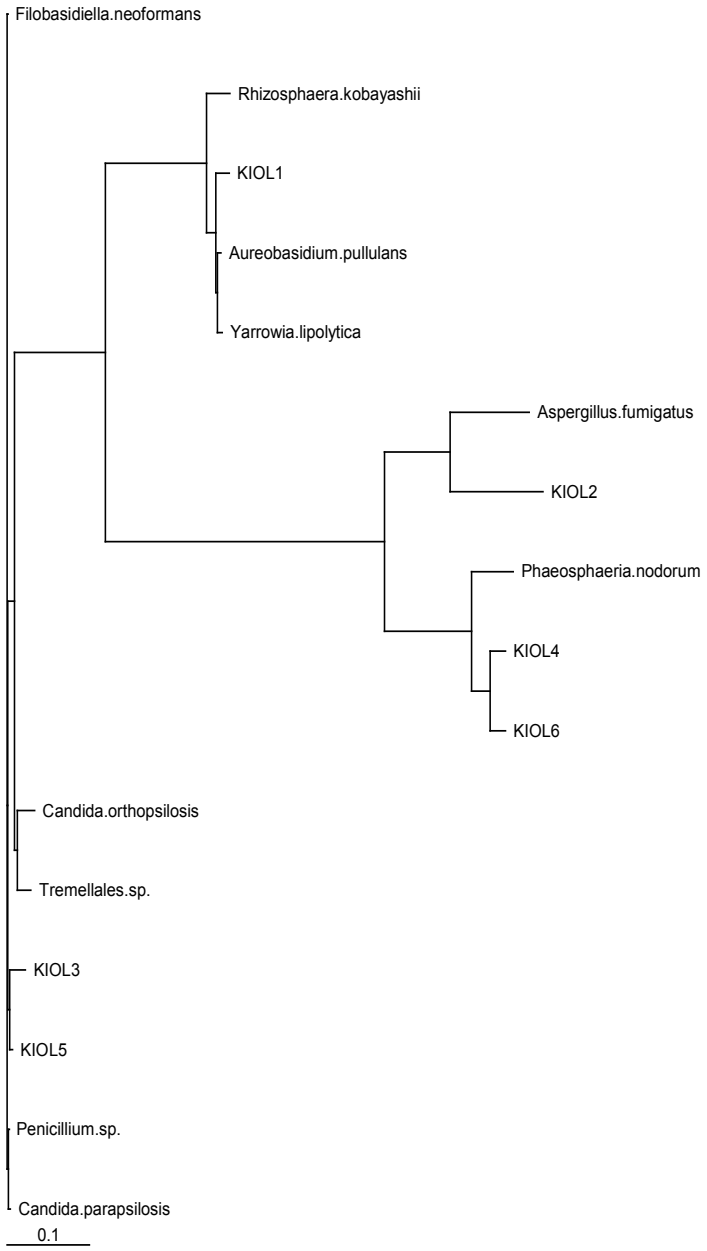

FIGURE 4.6

Phylogenetic analysis of the ITS gene of the fungi isolated from the iron ore/soil of the Sishen Iron Ore

Aspergillus fumigatus is frequently found in the environment, especially soil, and the fungus is able to be airborne in the form of conidia (Goto *et al*., 1998; Weig *et al*., 2001; Pastor *et al*., 2006). Humans frequently inhale the conidia of *A. fumigatus*, however, only a small proportion of the exposed individuals develop clinical manifestations (Weig *et al*., 2001). The most susceptible individuals suffer from immunosuppression, caused by therapeutics, leukopenia or chronic granulomatous disease, resulting in invasive pulmonary or disseminated aspergillosis (Weig *et al*., 2001). Another human pathogen, *Candida parapsilosis*, has emerged as an important cause of human candidiasis, often responsible for pathological lesions of the nails (Gautret *et al*., 2000; Krcmery and Barnes, 2002; Kiffer-Moreira *et al*., 2007). In addition, the heterothallic ascomycete, *Phaeosphaeria nodorum*, is a common necrotrophic plant pathogen causing leaf blotch and glume blotch diseases on wheat (*Triticum aestivum*) (Lalaoui *et al*., 2000; Stukenbrock *et al*., 2006). Neither *A. fumigatus* nor *C. parapsilosis* or *P. nodorum* have been reported to have industrial biotechnology application potential.

4.5 Conclusions

The most likely candidate for the removal of P and K from the iron ore concentrate would seem to be *Acidithiobacillus ferrooxidans*, however, this bacterium produces sulphuric acid, which is undesired in the iron ore industry due its corrosive properties. Furthermore, the P and K contained in the iron ore is in a non-sulphidic phase, and therefore, the use of organic acids, such as citric acid, for the removal of these contaminants may have potential (Rawlings, 2005). These organic acids are typically produced by certain types of fungi, such as *Aspergillus niger* (Jianlong, 2000; Vandenberghe *et al*., 2000; Rawlings, 2005). Although *A. niger* was not isolated from the iron ore/soil of the Sishen Iron Ore Mine, isolating *A. fumigatus* may indicate that *A. niger* may have the potential to adapt to the environment that prevails at the mine. This would allow the application of *A. niger* for the production of citric acid, which in turn may be used for the removal of P and K from the iron ore concentrate.

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CHAPTER 5

Chemical Leaching of Iron Ore Using a Range of Acids and Oxidative Chemicals

The editorial style of the *Water SA* was followed in this chapter

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5.1 Abstract

It has become important to process lower quality or complex iron ores due to the advancing global technologies and civilization. Phosphorous (P) and particular potassium (K) contained in the iron ore concentrates of the Sishen Iron Ore Mine have a detrimental effect on the steel making process, and therefore, it is becoming increasingly important to remove these alkalis from the charge materials before they enter the blast furnace. The P and K contained within the iron ore concentrate are in non-sulphidic phases, and therefore, these contaminants may be solubilised by a process of complexation using microbially produced inorganic or organic acids (Rawlings, 2005). Therefore, the objective of this study was to determine which acid would be considered to be the best leaching agent for the removal of P and K from the iron ore concentrate of the Sishen Iron Ore Mine. Citric acid proved to be the best leaching candidate for the removal of K from the iron ore, reducing the K content by 21.16%, while sulphuric acid reduced the P content by 27.58%, however, sulphuric acid is the most corrosive of the acids tested, removing 1.14% of the iron from the ore. Although no P was removed from the iron ore when using citric acid, further analyses are needed to determine whether higher leaching temperatures would play a role in the removal of P from the iron ore.

Key words: Phosphorous, potassium, iron ore concentrate, citric acid

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5.2 Introduction

As a result of the advancing global technologies and civilisation, there has been a progressive depletion of high-grade mineral deposits (Jian and Sharma, 2004).

Consequently, it has become increasingly important to process lower-grade or complex ores, as well as industrial and mining wastes (Torma, 1986). Elements such as phosphorous (P) and potassium (K) contained within the lower quality iron ore have a detrimental effect on steel making process, whereby these alkali's cause cracks to form in the refractory lining of blast furnaces (Yusfin *et al*., 1999). As a result the steel making companies charge penalties when purchasing iron ore that contains P and K above certain concentrations. Most steel making companies in the leading industrial nations have established limits on the concentrations of P and K that may be present in the charge materials without changing the smelting practice (Yusfin *et al*., 1999). Therefore, it is becoming increasingly important to remove these alkalis from the charge materials before they enter the blast furnace (Yusfin *et al*., 1999).

Conventional bioleaching involves the oxidation of insoluble metal sulphides to form corresponding soluble metal sulphates, enabling their removal from complex ore bodies (Rohwerder *et al*., 2002; Rawlings, 2005). The P and K contained within the iron ore concentrate are, however, in non-sulphidic phases, and therefore, conventional bioleaching processes are not viable for their removal from the ore. Metals in certain non-sulphide minerals, such as the iron ore concentrate of the Sishen Iron Ore Mine may be solubilised by a process of complexation using microbially produced inorganic or organic acids (Rawlings, 2005). Therefore, the objective of this study was to determine which acid would be considered to be the best leaching agent for the removal of P and K from the iron ore concentrate of the Sishen Iron Ore Mine.

5.3 Materials and Methods

5.3.1 Sample Selection

Iron ore samples (10 kg) of a –5+1 mm particle size were received from Sishen Iron Ore Mine for leaching analysis. The samples were stored in a dry environment for analysis.

5.3.2 Phosphorous and Potassium Phase Determination of the Untreated Iron Ore

The phases in which the P and K are present within the iron ore were determined by X-Ray Diffraction (XRD) analysis conducted at the Department of Geology, University of Pretoria, South Africa, as well as Kumba Resources, Ltd.

5.3.3 Chemical Preparation of Leaching Agents

All leaching chemicals used in this study were acquired from Merck, Darmstadt, Germany, with the exception of propionic acid, which was acquired from British Drug Houses, Ltd, Poole, UK. The leaching chemicals used during this study are listed in Table 5.1. 1 M leaching solutions were prepared by diluting the concentrated form of all chemicals in distilled water. The leaching solutions were stored at 25ºC until use.

TABLE 5.1
Leaching Chemicals for the Removal of P and K from the Iron Ore of the Sishen Iron Ore Mine

Chemical	Chemical Formula
Acetic Acid	$C_2H_4O_2$
Citric Acid	$C_6H_8O_7$
Ferric Sulphate	$Fe_2(SO_4)_3$
Hydrochloric Acid	HCl
Lactic Acid	$C_3H_6O_3$
Oxalic Acid	$C_2H_2O_4$
Propionic Acid	$C_3H_6O_2$
Sulphuric Acid	H_2SO_4

5.3.4 Chemical Leaching of Iron Ore

All leaching experiments were conducted in 250 ml Erlenmeyer flasks. Briefly, 100 ml of each of the leaching solutions was added to 100 g of iron ore in separate 250 ml glass Erlenmeyer flasks. A 100 g iron ore sample containing 100 ml of distilled water was used as a negative control. The flasks were incubated at 25ºC for 5 days. After the incubation the leaching solutions were removed and the iron ore washed five times with

distilled water. The treated iron ore samples were dried in an oven at 150ºC for 24 h. The iron ore samples were stored in a dry environment for chemical analysis.

5.4 Results and Discussion

The chemical composition of the iron ore of Sishen Iron Ore Mine is given in Table 5.2. The average weight % of P and K in the iron ore was found to be 0.14 for both elements as detected by XRF spectrometry. These weight percentages, however, were too low to be detected by XRD analysis at the Department of Geology, University of Pretoria. Through personal communication with a mineralogist at HQP Research and Development, Kumba Resources, Ltd, it was discovered that the only K bearing phase detected thus far in the iron ore of Sishen Iron Ore Mine, has been muscovite, while P has been detected in various phases in the iron ore, including apatite, goyazite and woodhouseite.

TABLE 5.2
Major Elemental Composition of the Iron Ore of the Sishen Iron Ore Mine

Major Elements	Mass %
Silica (SiO_2)	0.89
Titanium (TiO_2)	0.08
Aluminium (Al_2O_3)	1.18
Iron (Fe_2O_3)	96.90
Manganese (MnO)	0.02
Magnesium (MgO)	0.12
Calcium (CaO)	0.07
Sodium (Na_2O)	0.08
Potassium (K_2O)	0.14
Phosphorous (P_2O_5)	0.14
Chromium (Cr_2O_3)	0.01

The average chemical composition of the iron ore after treatment with the various leaching solutions is given in Table 5.3. Citric acid proved to be the best leaching candidate for the removal of K from the iron ore, reducing the K content by 21.42%, while sulphuric acid reduced the P content by 24.43%. Due to data scatter as a result of sample and assay variation however, the actual decrease in K using citric acid may be as low as 7.69%, while the decrease in P using sulphuric acid may be as low as 9.40%. Acetic acid and sulphuric acid proved to have the highest Fe-corroding properties, reducing the Fe content by 1.14% and 1.00% respectively.

TABLE 5.3
Average Chemical Composition of the Iron Ore after Treatment with the Various 1M Leaching Solutions for 5 Days

Major Elements	$C_2H_4O_2$	$C_6H_8O_7$	$Fe_2(SO_4)_3$	HCl	$C_3H_6O_3$	$C_2H_2O_4$	$C_3H_6O_2$	H_2SO_4
SiO_2	1.52	1.14	0.59	0.94	1.36	1.12	0.46	1.19
TiO_2	0.07	0.07	0.08	0.07	0.08	0.07	0.07	0.06
Al_2O_3	1.02	1.05	1.38	1.32	1.09	1.10	1.21	1.11
Fe_2O_3	95.93	96.45	97.21	97.15	96.29	96.39	97.70	96.14
MnO	0.02	0.02	<0.01	<0.01	0.02	0.02	<0.01	<0.01
MgO	0.10	0.10	0.08	0.13	0.12	0.12	0.10	0.07
CaO	0.06	0.05	0.04	<0.03	0.05	0.08	0.05	<0.03
Na_2O	0.07	0.07	<0.05	<0.05	0.06	0.05	<0.05	<0.05
K_2O	0.13	0.11	0.14	0.15	0.14	0.13	0.14	0.13
P_2O_5	0.13	0.14	0.14	0.12	0.13	0.16	0.14	0.10
Cr_2O_3	<0.01	<0.01	<0.01	<0.01	0.01	0.01	<0.01	<0.01

5.5 Conclusions

Citric acid seemed to be the best leaching candidate for the removal of P and K from the iron ore of the Sishen Iron Ore Mine. Although no P was removed by citric acid during this investigation, further analyses are needed to determine whether higher leaching

temperatures would play a role in the removal of P from the iron ore. Organic acids, such as citric acid produced by *Aspergillus niger*, possess several carboxyl groups, and therefore can act as chelating agents (Sayer and Gadd, 2001). These carboxyl groups tend to donate protons (H^+), resulting in negatively charged carboxyl groups that are capable of forming stable complexes with several metal cations, such as copper and zinc (Sayer and Gadd, 2001). Therefore, it is possible that these negatively charged carboxyl groups are able to form stable complexes with the K^+ cations present, while the H^+ cations are able to react with the negatively charged phosphorous, resulting in their removal from the iron ore concentrate.

5.6 References

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CHAPTER 6

The Production and Use of Citric Acid for the Removal of Phosphorous and Potassium from the Iron Ore Concentrate of the Sishen Iron Ore Mine, South Africa

The editorial style of *Hydrometallurgy* was followed in this chapter

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6.1 Abstract

The depletion of the richer iron ore worldwide has made it necessary to process lower quality iron ore. Certain substances, such as phosphorous (P) and potassium (K), contained within the iron ore have a detrimental effect on the smelting process during steel manufacturing. Therefore, international steel making companies charge penalties when purchasing iron ore concentrates containing high concentrations of P and K. To date, lower quality iron ore has been blended with high quality iron ore in an attempt to alleviate the P and K concentrations in the export iron ore product, however, the ratio of low quality iron ore to high quality iron ore is increasing, and thus becoming an escalating problem within the economic functioning of the Sishen Iron Ore Mine. It has, therefore, become necessary to develop an economically viable and environmentally friendly process to reduce the high P and K concentrations contained in the iron ore concentrate of the Sishen Iron Ore Mine, resulting in the minimizing of the penalties charged by the steel making companies. During this study solid substrate- and submerged fermentation using *Aspergillus niger* were compared for the production of citric acid, which is to be used for the chemical leaching of P and K from the iron ore concentrate. It was found that submerged fermentation proved to be more economical and efficient, producing a maximum citric acid concentration of 102.3 $g.l^{-1}$ in 96 h of fermentation. "Heap leaching" simulation experiments was found to be uneconomical due to the addition of fungal growth medium every 5 days as a result of growth factor depletion within this time, however, this process removed 17.65% K and no P from the iron ore concentrate. In contrast, chemical leaching of P and K from the iron ore

concentrate proved to be most efficient when using a 1M citric acid leaching solution at 60ºC, removing 35.29% P and 23.53% K contained within the iron ore concentrate. Therefore, the most economical and efficient process for the removal of P and K from the iron ore concentrate of the Sishen Iron Ore Mine, involves a two-stage process whereby citric acid is produced by *A. niger*, followed by the chemical leaching of the P and K from the iron ore concentrate using a 1M citric acid leaching solution at 60ºC.

Key words: Iron ore concentrate, phosphorous, potassium, solid substrate citric acid fermentation, submerged citric acid fermentation, heap leaching, chemical leaching

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6.2 Introduction

The depletion of the richer iron ore deposits worldwide, as a result of the advancing global technologies and civilisation, necessitated the processing of lower quality iron ore (Jian and Sharma, 2004). The phosphorous (P) and potassium (K) contained within the lower quality iron ore have a detrimental effect on the smelting process during steel making in blast furnaces (Yusfin *et al*., 1999). These compounds are deposited on the surface of the coke, where they act as a catalyst in the gasification of carbon in the presence of carbon dioxide (CO_2) (Yusfin *et al*., 1999). The presence of especially K in the coke leads to the formation of $K_2O.SiO_2$ and $K_2O.Al_2O_3.2SiO_2$, which leads to an increase in the coke volume and its subsequent fracture (Yusfin *et al*., 1999). In addition, K penetrates the monolithic alumosilicate lining of the furnace, resulting in the formation of silicide or leucite, and the subsequent rearrangement of the crystalline lattice of the refractories (Yusfin *et al*., 1999). This results in the creation of stresses that cause cracks to form in the refractory lining, leading to its subsequent destruction (Yusfin *et al*., 1999). Therefore, as a result of the negative financial impact due to the destruction of the refractory lining, the steel making companies charge penalties when purchasing iron ore concentrates containing high concentrations of P and K. The limits on alkali concentrations range from 0.25% mass in Japan to 0.55% mass in Switzerland (Yusfin *et al*., 1999).

In the past, lower quality iron ore was blended with high quality ore to "dilute" the P and K concentrations in the final iron ore product, which is exported to the international steel making plants. Similar practices have been reported from other parts of the world, such as the Hamersley Province in Australia where low-phosphorous ore (0.05% P) is blended with high-phosphorous ore (0.10% P), the former being the major component of the blend (Dukino *et al*., 2000). To date, the blending of different quality iron ores has minimised the penalties charged by the steel making companies, however, the ratio of low quality iron ore to high quality iron ore is increasing, and thus becoming an escalating problem within the economic functioning of the Sishen Iron Ore Mine. It has, therefore, become important to develop an economically viable and environmentally friendly process to reduce the high P and K concentrations contained in the iron ore concentrate to improve the quality of ore that is being exported to the international steel making companies.

During a previous study whereby a range of inorganic- and organic acids were tested for the removal of P and K from the iron ore concentrate of the Sishen Iron Ore Mine, it was discovered that citric acid (2-hydroxypropane-1,2,3-tricarboxylic acid) proved to be the best leaching agent for the removal of P and K without a major reduction in the iron content (0.46%) of the ore (Chapter 5). Citric acid is an intermediate in the tricarboxylic acid (TCA) cycle, which is widely used in the food, beverage, pharmaceutical and cosmetic industries, but also has other applications in the textile, electroplating and bioremediation industries (Wang and Liu, 1996; Tran *et al*., 1998; Ates *et al*., 2002). The most popular microorganism for the large-scale production of citric acid is the white-rot fungus *Aspergillus niger*, due to its high citric acid productivity at low pH without the secretion of toxic metabolites (Kim, 2004). Citric acid production by *A. niger* involves two main metabolic pathways, namely: 1) the catabolic pathway of hexoses to pyruvate and Acetyl-coenzyme A by glycolysis, and 2) citric acid formation by the TCA cycle (Alvares-Vasquez *et al*., 2000). During cell propagation and maintenance, complete oxidation of glucose leads to the production of adenosine triphosphate (ATP), CO_2 and H_2O (Jianlong, 2000). Depending on the growth conditions and concentrations of end

products, *A. niger* is able to control when to stop the full respiration process during the TCA cycle, resulting in the production of citric acid (Jianlong, 2000).

Citric acid contains three carboxyl groups, which tend to donate protons (H^+), resulting in negatively charged carboxyl groups that are capable of forming stable complexes with several cations (Sayer and Gadd, 2001). Therefore, it would be possible that these negatively charged carboxyl groups might form stable complexes with the positively charged K cations present, resulting in its removal from the iron ore concentrate. Therefore, the objective of this study was to develop a "bioleaching" process, whereby citric acid is produced and used to remove the P and K from the iron ore concentrate of the Sishen Iron Mine. In addition, a heap leaching process was investigated whereby *A. niger* was directly applied to the iron ore concentrate of the Sishen Iron Ore Mine.

6.3 Materials and Methods

6.3.1 Microorganism and Preparation of Inoculum

A freeze-dried sample of *Aspergillus niger* NRRL 567 was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and stored at 4ºC. *A. niger* spores were produced on Potato Dextrose Agar (PDA) (Merck Laboratories, Darmstadt, Germany) (Appendix A) at 30ºC and were sub-cultured at bi-weekly intervals. Spores were harvested after 7 days of incubation by adding 10 ml of 0.1% Tween 80 (Merck) solution to each plate. Spore inoculates of 1.0×10^7 spores.ml^{-1} were prepared using a haemocytometer.

6.3.2 Solid Substrate

Sphagnum Peat Moss (PM) (Schultz Company, Mississauga, Ontario, Canada) supplemented with glucose was used to simulate a sugar-rich by-product. The PM was sterilised in an autoclave at 121ºC for 15 min. The PM was wetted to moisture content (MC) of 80% wet weight with deionised water supplemented with various salts and glucose (Considine *et al.*, 1987).

6.3.3 Basal Salt Solution for Solid Substrate Fermentation

The basal salt solution used to wet the dried Peat Moss (DPM) and to provide basic nutrients to the fungal culture contained the following (Appendix A): 3.84 g.l^{-1} $(NH_4)_2SO_4$, 10.98 g.l^{-1} KH_2PO_4, 1.01 g.l^{-1} NaCl, 1.01 g.l^{-1} $MgSO_4.7H_2O$ and 1.01 g.l^{-1} $FeSO_4.7H_2O$ (Kim, 2004). After the addition to PM, the solution gave the following salt concentration in terms of kg DPM: 15.36 g $(NH_4)_2SO_4$, 43.92 g KH_2PO_4, 4.04 g NaCl, 4.04 g $MgSO_4.7H_2O$ and 4.04 g $FeSO_4.7H_2O$.

6.3.4 Fermentation Medium for Submerged Fermentation

The fermentation medium for the submerged fermentation was prepared using distilled water (dH_2O) and contained the following (Appendix A): 150 g.l^{-1} D-glucose, 2.5 g.l^{-1} $(NH_4)_2SO_4$, 0.5 g.l^{-1} $MgSO_4.7H_2O$, 2.0 KH_2PO_4, 0.1 × 10^{-3} g.l^{-1} $Fe_2(SO)_4.24H_2O$, 0.1 × 10^{-3} g.l^{-1} $ZnSO_4.7H_2O$ and 0.06 × 10^{-3} g.l^{-1} $CuSO_4.5H_2O$.

6.3.5 Fermentation Conditions for Solid Substrate Fermentation

Solid substrate fermentation was conducted in 500 ml Erlenmeyer flasks holding 25 g of DPM wetted with 100ml of the basal salt solution and 967.88 g.kg^{-1} DPM glucose (Merck) (Kim, 2004). The solid substrate was inoculated with 1ml of *A. niger* NRRL 567 inoculum (1.0 × 10^7 spores.ml^{-1}). The Erlenmeyer flasks and their contents were incubated at 30ºC for 6 days. The fermentation procedures were conducted in duplicate.

6.3.6 Fermentation Conditions for Submerged Fermentation

Submerged fermentation was conducted in 500 ml Erlenmeyer flasks. Briefly, 250 ml of fermentation medium was inoculated with 1 ml of *A. niger* NRRL 567 inoculum (1.0 × 10^7 spores.ml^{-1}). The Erlenmeyer flasks and their contents were incubated at 30ºC for 6 days. The fermentation procedures were conducted in duplicate.

6.3.7 Analytical Procedure

For each sampling procedure of the solid substrate fermentation, 5 g of wet sample of PM was harvested from each flask and placed in 50ml of dH_2O, followed by incubation in a shake incubator for 60 min at 150 revolutions per min (rpm) and 25ºC. The supernatant

was filtered through a 0.45µm syringe filter (Millipore), followed by the analyses for pH and citric acid quantification. The sampling of the submerged fermentation entailed the aseptic extraction of 5 ml of the fermentation medium from each flask to be used for analyses. The extracted medium was filtered through a 0.45µm syringe filter (Millipore), followed by the analyses for pH and citric acid quantification. The abovementioned analyses were performed daily for 6 days.

6.3.8 Citric Acid Concentration Analysis

Citric acid concentrations were determined by spectrophotometry at 420nm after adding pyridine and acetic anhydride as described by Marier and Boulet (1958). Briefly, 1 ml of the sample was added to a test tube containing 1.3 ml of pyridine (C_5H_5N) (Merck), followed by the addition of 5.7 ml of acetic anhydride ($C_4H_6O_3$) (Merck). The contents of the test tube was mixed by swirling the tube and immediately placed in a constant-temperature (22ºC) water bath. Colour development was allowed for 30 min, followed by reading the colour intensity at 420nm with the blank set on 100% transmission. The citric acid concentration was determined by referring to a standard curve for citric acid concentration. Citric acid concentrations were expressed per kg of DPM for solid substrate fermentation and $g.l^{-1}$ for submerged fermentation.

6.3.9 Chemical Leaching of Iron Ore Concentrate with Citric Acid

The produced citric acid was used for the chemical leaching of the iron ore concentrate. A 1M concentration of citric acid was added to a 40g sample of iron ore concentrate (–5+1mm particle size) in a 250ml Erlenmeyer flask. 40g of iron ore concentrate containing 40 ml of distilled water was used as a negative control. The flasks were incubated at 25ºC for 5 days. In addition, the leaching procedure was conducted using different concentrations (0.25M, 0.5M, 0.75M and 1M) of citric acid at a leaching temperature of 60ºC for 5 days. After the incubation the leaching solutions were removed and the iron ore concentrate washed five times with distilled water. The treated iron ore concentrate samples were dried in an oven at 150ºC for 24 h. The iron ore concentrate samples were stored in a dry environment for chemical analysis.

6.3.10 "Heap Leaching" of Iron Ore Concentrate Using Aspergillus niger

A "heap leaching" simulation experiment was conducted in 500 m*l* Erlenmeyer flasks. Briefly, spore suspensions were prepared as in 6.3.1. The fermentation medium used during these experiments was the same as used for submerged citric acid fermentation (6.3.4). Iron ore concentrate (500g) was placed in each Erlenmeyer flask and mixed with 50 m*l* of fermentation medium inoculated with 1 m*l* of the spore inoculum (1.0×10^7 spores.ml^{-1}). The flasks and their contents were incubated at 30ºC for a period of 40 days.

6.3.11 Chemical Analysis of the Treated Iron Ore Concentrate

The chemical content of the treated iron ore concentrate was analysed by the Department of Geology, University of Pretoria, Pretoria, South Africa, using XRF spectrometry of the major elements. Briefly, the samples were ground to <75µm in a Tungsten Carbide milling vessel, roasted at 1000ºC, followed by the fusing of 1g of sample and 9g $Li_2B_4O_7$ into a glass bead. Major element analysis was executed on the fused bead using a ARL9400XP+ spectrometer. Another aliquot of the sample was pressed into a powder briquette for trace element analysis.

6.4 Results and Discussion

Previous research has suggested that citric acid is excreted from *A. niger* cells in response to unfavourable intracellular conditions that lead to increased levels of tricarboxylic acids through anaplerotic pathways during growth in a high glucose concentration environment (Legiša and Mattey, 2007). It is suggested that polyols, in particular glycerol, may play an important role as osmoregulators in *A. niger* cells, which explains the ability to grow in environments where high glucose concentrations prevail (Legiša and Kidrič, 1989). Therefore, a high initial glucose concentration of 967.88 g.kg^{-1} DPM and 150 g.*l*$^{-1}$ was used for solid substrate- and submerged fermentation respectively, to ensure that citric acid is excreted from the *A. niger* mycelia.

Solid substrate- and submerged citric acid production by *A. niger* NRRL 567 at 30ºC is illustrated in Figure 6.1 and 6.2 respectively. Citric acid excretion commenced after approximately 24 h during the solid substrate fermentation, compared to 48 h during submerged fermentation. This may be attributed to the abnormal spore germination in the form of bulbous cells during the early stages of growth, followed by a sudden change in morphology to highly branched filamentous hyphae, which are responsible for the citric acid overflow (Legiša *et al*., 1981). During the germination of the fungal spores the pentose phosphate pathway is predominant, followed by a switch to glycolysis before the onset of citric acid excretion (Legiša and Mattey, 1986a; Röhr *et al*., 1987).

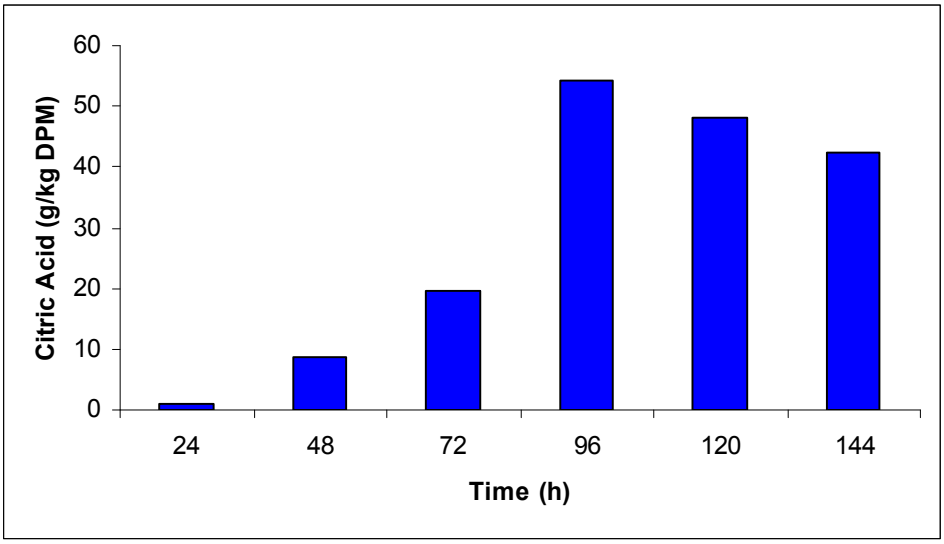

FIGURE 6.1

Diagrammatic Representation of the Solid Substrate Citric Acid Production from PM by *Aspergillus niger* NRRL 567 grown at 30ºC

An increase in citric acid productivity was observed until maximisation at approximately 96 h (54.2 $g.kg^{-1}$ DPM and 102.3 $g.l^{-1}$ respectively for solid substrate- and submerged fermentation) (Figures 6.1 and 6.2). Similar observations have been recorded, where little or no citric acid could be detected in the medium during the first 24 h of fermentation, while a relatively slow excretion rate was observed during the second day

of fermentation, followed by a sudden increase in citric acid productivity thereafter (Röhr and Kubicek, 1981; Legiša and Mattey, 1986b; Ruijter *et al*., 1997; Papagianni *et al*., 2005). During the productive phase of citric acid accumulation, the direct conversion of hexoses to pyruvate via glycolysis becomes predominant, starting after approximately 24 h and accelerating after 40-50 h of growth in a batch system (Legiša and Mattey, 1986a). It is, however, not clearly understood what the mechanism causing the shift of glucose degradation from the pentose phosphate pathway to glycolysis is (Legiša and Mattey, 2007).

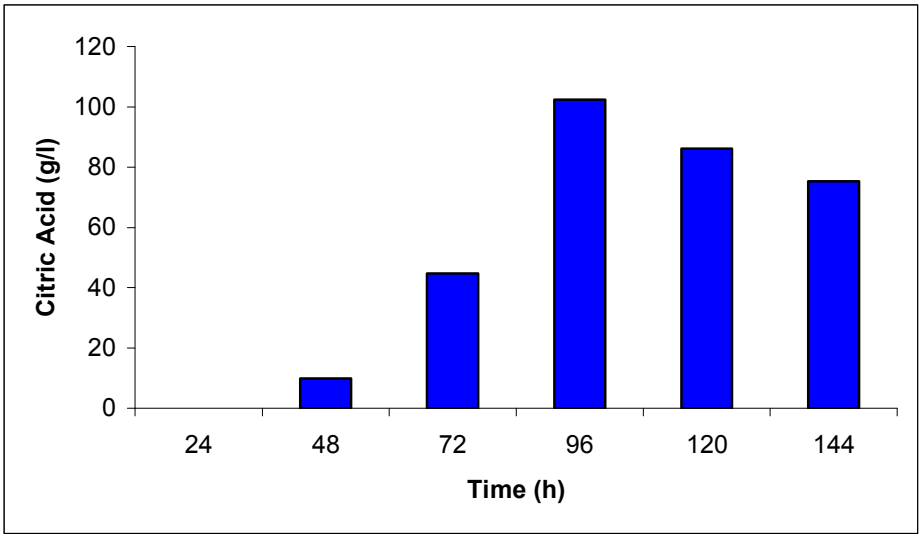

FIGURE 6.2

Diagrammatic Representation of the Submerged Citric Acid Production by *Aspergillus niger* NRRL 567 grown at 30ºC

To attain increased citric acid yields, nitrogen in the medium must be limiting (Kristianen and Sinclair, 1978). Citric acid production is only established once the nitrogen in the medium is depleted (Papagianni *et al*., 2005). The bulk of the ammonium is removed from the medium between 20 and 25 h, followed by the release of protons into the fermentation medium (Papagianni *et al*., 2005). In the early stage of citric acid production (<24 h), it seems that a chain of events is established where fungal growth

leads to ammonium uptake, which leads to proton release (Papagianni *et al*., 2005). Papagianni and colleagues (2005) suggested that a nitrogen compound must be produced and excreted by the mycelium, as the increase in biomass cannot be due to an accumulation of ammonia in the biomass of the fungus. It was subsequently reported that the nitrogen compound in question was in fact glucosamine, as identified by HPLC analysis (Papagianni *et al*., 2005). Following the detection of glucosamine, Papagianni and colleagues (2005) also investigated the formation, release and fate of the aminated compound throughout the fermentation process. It was found that the highest detected concentration of glucosamine in the medium was attained when using optimal concentrations of glucose and ammonium for citric acid production. Glucosamine excretion started at approximately 15 h of fermentation, followed by the start of citric acid production at about 24 h. It appears that the sudden increase in citric acid production is preceded by the sudden increase in glucosamine excretion into the medium by *A. niger*. Glucosamine appeared to be stable in the medium until approximately 85 h, where after it was reduced and subsequently depleted at 126 h of fermentation. This correlates with the citric acid production peak of 54.2 $g.kg^{-1}$ DPM at 96 h for solid substrate fermentation and 102.3 $g.l^{-1}$ for submerged fermentation, followed by the decline of citric acid production thereafter (Figures 6.1 and 6.2). Therefore, when comparing the fate of glucosamine and citric acid production during the fermentation process, it appears that glucosamine plays a direct role in citric acid production. Although the direct role of glucosamine during fermentation is unclear, it is suggested that it acts as a storage compound and is utilised by the fungus during the course of fermentation (Papagianni *et al*., 2005).

The initial pH decreased proportionally with the increase in citric acid concentration during both solid substrate- and submerged fermentation. In both fermentation processes the pH decreased during the first 96 h of fermentation as a result of citric acid production during this period. The maximum decrease of the pH of 1.34 and 2.17 respectively during solid substrate- and submerged fermentation was reached after 96 h, which correlates with the maximum citric acid concentrations achieved at this point during fermentation. In both solid substrate- and submerged fermentation the pH increased after

96 h due to the decreased citric acid concentrations in the fermentation media. During both fermentation processes it is evident that the evolution of the pH of the PM occurred in parallel with the citric acid concentration during the fermentation process. Therefore, the pH evolution may be used as an indirect indicator of citric acid production during the fermentation process.

The chemical composition of the untreated iron ore concentrate of the Sishen Iron Ore Mine is given in Table 6.1. XRF spectrometry of the iron ore concentrate revealed a P concentration of 0.17 % mass, a K concentration of 0.17 % mass and a Fe concentration of 96.90 % mass.

TABLE 6.1
Chemical Composition of the Untreated Iron Ore Concentrate of the Sishen Iron Ore Mine

Major Elements (Oxides)	**% Mass**
Silica (SiO_2)	1.89
Titanium (TiO_2)	0.08
Aluminium (Al_2O_3)	1.18
Iron (Fe_2O_3)	96.90
Potassium (K_2O)	0.17
Phosphorous (P_2O_5)	0.17

The average percentage removal of the major elements from the iron ore concentrate of the Sishen Iron Ore Mine by chemical leaching at for 5 days using different concentrations of citric acid is given in Table 6.2. Based on the results listed in Table 6.2 it is evident that the leaching temperature and the citric acid concentration plays an important role during the chemical leaching of the P and K from the iron ore concentrate. Leaching at 30ºC using a 1M citric acid leaching solution resulted in a 17.65% removal of both phosphorous and potassium, while at 60ºC a 35.29% and 23.53% removal of phosphorous and potassium, respectively, occurred (Table 6.2). Using a 1M citric acid leaching solution at 60ºC also resulted in the increased removal of silica (15.87%),

aluminium (18.64%) and iron (0.92%) as compared to leaching at 30ºC, whereby only 11.02% of aluminium, 0.46% of iron and no silica was removed from the iron ore concentrate (Table 6.2). Therefore, it is clear that chemical leaching of P and K from the iron ore concentrate occurred more efficiently at a higher temperature (60ºC), which is most likely due to the fact that chemical reactions occur more efficiently at higher temperatures.

TABLE 6.2
The Average Percentage Removal of the Major Elements from the Iron Ore Concentrate by Chemical Leaching for 5 Days Using Different Concentrations of Citric Acid

Major Elements	1M Citric Acid; 30ºC	0.25M Citric Acid; 60ºC	0.5M Citric Acid; 60ºC	0.75M Citric Acid; 60ºC	1M Citric Acid; 60ºC
Silica (SiO_2)	0.00	0.00	0.00	7.41	15.87
Titanium (TiO_2)	12.50	0.00	0.00	12.50	12.50
Aluminium (Al_2O_3)	11.02	0.00	3.39	13.56	18.64
Iron (Fe_2O_3)	0.46	0.00	0.42	0.89	0.92
Potassium (K_2O)	17.65	5.88	5.88	17.65	23.53
Phosphorous (P_2O_5)	17.65	0.00	23.53	35.29	35.29

Citric acid concentrations of 0.25M and 0.5M resulted in the removal of only 5.88±5.88% of K, while at citric acid concentrations of 0.75M and 1M the K removal increased to 17.65±5.88% and 23.53±5.88%, respectively. The decrease of K using citric acid concentrations of 0.25M and 0.5M, however, could be as a result of data scatter due to sampling and assay variation, and thus may be regarded as insignificant. The actual percentage decrease of K using citric acid concentrations of 0.75M and 1M may also be lower than recorded in Table 6.2 as a result of data scatter. When taking the data scatter into account, the actual decrease in K may be as low as 6.88% and 12.5% using a citric acid concentration of 0.75M and 1M respectively.

Similar results were observed for P removal, whereby 23.53±3.40% P was removed when using a citric acid concentration of 0.5M, compared to 35.29±5.88% P removal when 0.75M and 1M citric acid concentrations were used. The actual P removal, however, may be as low as 11.33% when using a citric acid concentration of 0.5M and 20.0% when using citric acid concentrations of 0.75M and 1M. When comparing the chemical leaching process at 60ºC on the iron ore concentrate, it was evident that the process occurs more efficiently at higher citric acid concentrations. The presence of a higher negatively charged carboxyl group concentration at higher citric acid concentrations leads to the formation of more stable complexes with the K cations present, resulting in a higher removal of K from the iron ore concentrate. Phosphorous is removed as a result of hydrolysis whereby the H^+ cations, which are released from the carboxyl groups of citric acid, form stable complexes with the negatively charged P, resulting in its removal from the iron ore concentrate. Higher citric acid concentrations result in higher H^+ cation concentrations, and therefore, more P can be removed from the iron ore concentrate.

Other parameters that must be taken into account during chemical leaching of P and K from the iron ore concentrate are contact time and particle size of the iron ore concentrate. During this investigation it was found that the maximum P and K removal from the iron ore concentrate occurred after 5 days of chemical leaching, with no increase in P and K removal thereafter. The particle size of the iron ore concentrate is a limiting factor during chemical leaching, and determines how much of the P and K, contained within the iron ore particles, are actually exposed to acid attack, which takes place during the chemical leaching process. As the particle size of the iron ore concentrate decreases, the surface area of the particles are increased, therefore, exposing a larger amount of the P and K contained within the iron ore concentrate to acid attack. Therefore, if treating iron ore with a smaller particle size than –5+1mm, more P and K may be removed from the iron ore concentrate, however, the iron ore concentrate with the particle size of –5+1mm is the final iron ore concentrate product exported from the Sishen Iron Ore Mine, and therefore, it was used during this study to investigate the removal of P and K from the iron ore concentrate using the smallest marketable particle size from the mine.

The heap leaching simulation experiments using *A. niger* for the removal of P and K from the iron ore concentrate of the Sishen Iron Ore Mine yielded various problems. *Aspergillus niger* was only able to proliferate on the surface of the iron ore concentrate layer in the flask, possibly due to the anaerobic conditions which may prevail within the iron ore concentrate layer. Therefore, oxygen would have to be introduced throughout the iron ore heap when using this type of technology. In addition, after a period of 5 days the fungus started to sporulate, indicating the depletion of growth factors needed for fungal growth to occur. After the addition of more growth medium to the inoculated iron ore concentrate, fungal growth recommenced, followed by sporulation 5 days thereafter. Therefore, heap leaching technology would require the addition of fungal growth medium every 5 days, making this type of technology uneconomical due to the high costs of growth factors such as glucose.

During the 40 day heap leaching simulation, the pH of the leachate solution decreased from 4.00 to 3.42, indicating that a low concentration of citric acid was produced due to the limiting of the carbon source (glucose) in the system. XRF analysis revealed that 17.65% K and no P was removed from the iron ore concentrate during the 40 day period. Thus, compared to chemical leaching of the iron ore concentrate using a 1M concentration of citric acid produced by *A. niger*, heap leaching technology is less efficient as less P (0% vs 35.29%) and K (17.65% vs 23.53%) is removed over a much longer contact time.

6.5 Conclusions

During solid substrate- and submerged fermentation using *A. niger* a high concentration of glucose was required to ensure that citric was excreted from the fungal mycelia. The maximum citric acid yield of 54.2 $g.kg^{-1}$ DPM by solid substrate fermentation and 102.3 $g.l^{-1}$ by submerged fermentation was attained after a period of 96 h in both processes. The fermentation media of both solid substrate- and submerged fermentation contained similar constituents, with the difference that the submerged fermentation medium contained less of each constituent. In addition, solid substrate fermentation required peat

moss as a solid substrate, which increases the costs incurred by using this fermentation process. Therefore, taking all the variable factors into account it is suggested that submerged fermentation be used for citric acid production by *A. niger*, as this fermentation process was found to be more economical and produced more citric acid in the same time.

Chemical leaching of the iron ore concentrate proved to be more efficient than "heap leaching", as more P and K was removed from the iron ore concentrate, as well as in a shorter time frame. In addition, using "heap leaching" technology proved to be uneconomical in this case, as growth medium will have to be added every 5 days to enhance the fungal growth. It is suggested that a 1M citric acid leaching solution be used at 60ºC for the chemical leaching process, as the most P and K is removed from the iron ore concentrate using these leaching conditions. Therefore, the most economical and efficient process for the removal of P and K from the iron ore concentrate of the Sishen Iron Ore Mine, involves a two-stage process whereby citric acid is produced by *A. niger*, followed by the chemical leaching of the P and K from the iron ore concentrate using a 1M citric acid leaching solution at 60ºC.

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CHAPTER 7

The Use of *Aspergillus niger* for the Removal of Phosphorous and Potassium from the Iron Ore Concentrate of the Sishen Iron Ore Mine, South Africa

The editorial style of the *Hydrometallurgy* was followed in this chapter

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7.1 Abstract

The depletion of the richer iron ore worldwide has made it necessary to process lower quality iron ore. Certain alkali substances, such as phosphorous (P) and potassium (K), contained within the iron ore have a detrimental effect on the smelting process during steel manufacturing. Therefore, international steel making companies charge penalties when purchasing iron ore concentrates containing high concentrations of P and K. It has, therefore, become necessary to develop an economically viable and environmentally friendly process to reduce the high P and K concentrations contained in the iron ore concentrate of the Sishen Iron Ore Mine. Conventional bioleaching refers to the microbial conversion of insoluble metals into soluble forms, however, the P and K contained within the iron ore concentrate are in non-sulphidic phases, and therefore, conventional bioleaching processes are not viable for their removal from the ore. Therefore, the use of an alternative bioleaching microorganism has to be investigated to remove the undesirable P and K from the iron ore concentrate. The objective of this study is, therefore, to investigate the possible use of *A. niger* in a bioleaching process for the economic removal of P and K from the iron ore concentrate of the Sishen Iron Ore Mine. During this study different pulp densities [bioleaching solution (ml)/iron ore (g)] were compared for the removal of P and K from the iron ore concentrate using *A. niger* as the bioleaching microorganism. A pulp density of 33% proved to be most efficient in terms of citric acid production, producing a maximum of 52.06 $g.l^{-1}$ of citric acid at 30ºC after 96 h. In addition, the 33% pulp density resulted in the maximum P and K removal of 23.53% and 17.65% respectively, and therefore, *A. niger* can be considered as a

suitable bioleaching candidate for the removal of P and K from the iron ore concentrate of the Sishen Iron Ore Mine.

Key words: Aspergillus niger, bioleaching, iron ore concentrate, phosphorous, potassium

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7.2 Introduction

The processing of lower quality iron ore has become necessary due to the rapid depletion of the richer iron ore bodies worldwide as a result of the advancing global technologies and civilisation (Jian and Sharma, 2004). Alkali compounds, such as P and K, contained within the lower quality iron ore have a detrimental effect on the smelting process during steel making in blast furnaces (Yusfin *et al*., 1999). These alkali compounds are deposited on the surface of the coke, where they act as a catalyst in the gasification of carbon in the presence of carbon dioxide (CO_2) (Yusfin *et al*., 1999). The presence of especially K in the coke leads to the formation of $K_2O.SiO_2$ and $K_2O.Al_2O_3.2SiO_2$, which leads to an increase in the coke volume and its subsequent fracture (Yusfin *et al*., 1999). In addition, K penetrates the monolithic alumosilicate lining of the furnace, resulting in the formation of silicide or leucite, and the subsequent rearrangement of the crystalline lattice of the refractories (Yusfin *et al*., 1999). This results in the creation of stresses that cause cracks to form in the refractory lining, leading to its subsequent destruction (Yusfin *et al*., 1999). Therefore, as a result of the negative financial impact due to the destruction of the refractory lining, the steel making companies charge penalties when purchasing iron ore concentrates containing high concentrations of P and K. The limits on alkali concentrations range from 0.25% mass in Japan to 0.55% mass in Switzerland (Yusfin *et al*., 1999).

In the past, lower quality iron ore was blended with high quality ore to "dilute" the P and K concentrations in the final iron ore product, which is exported to the international steel making plants (Dukino *et al*., 2000). To date, the blending of different quality iron ores has minimised the penalties charged by the steel making companies, however, the ratio of low quality iron ore to high quality iron ore is increasing, and thus becoming an

escalating problem within the economic functioning of the Sishen Iron Ore Mine. It has, therefore, become important to develop an economically viable and environmentally friendly process to reduce the high P and K concentrations contained within the iron ore concentrate to improve the quality of ore that is being exported to the international steel making companies.

Various technologies developed in the last couple of decades include microbial use for the extraction of different valuable metals, which is commonly known as bioleaching (Gilbertson, 2000; Rohwerder *et al*., 2002). Conventional bioleaching, also known as biooxidation, refers to the microbial conversion of insoluble metals (e.g. metal sulphides such as CuS, NiS and ZnS) into soluble forms (usually metal sulphates such as $CuSO_4$, $NiSO_4$ and $ZnSO_4$) (Sand, 2001; Rawlings, 2002, Roberto, 2002). The basic fundamentals of conventional bioleaching may, therefore, be used for the removal of P and K from the iron ore concentrate of the Sishen Iron Ore Mine. The P and K contained within the iron ore concentrate are, however, in non-sulphidic phases, and therefore, the use of an alternative bioleaching microorganism has to be investigated to remove the undesirable P and K from the iron ore concentrate.

Metals in certain non-sulphide minerals, such as the iron ore concentrate of the Sishen Iron Ore Mine, may be solubilised by a process of complexation using organic acids, such as citric and oxalic acid (Rawlings, 2005). These organic acids are typically produced by certain types of fungi, such as *A. niger* (Jianlong, 2000; Vandenberghe *et al*., 2000; Rawlings, 2005). Citric acid contains several carboxyl groups, which tend to donate protons (H^+), resulting in negatively charged carboxyl groups that are capable of forming stable complexes with several cations (Sayer and Gadd, 2001). Therefore, it would be possible that these negatively charged carboxyl groups might form stable complexes with the positively charged K cations present, and at the same time the release of H^+ ions may result in a hydrolysis reaction involving the P contained in the iron ore, resulting in the subsequent P and K removal from the iron ore concentrate.

The objective of this study is, therefore, to investigate the possible use of *A. niger* in a bioleaching process for the economic removal of P and K from the iron ore concentrate of the Sishen Iron Ore Mine.

7.3 Materials and Methods

7.3.1 Microorganism and Preparation of Inoculum

A freeze-dried sample of *Aspergillus niger* NRRL 567 was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and stored at 4ºC. *A. niger* spores were produced on Potato Dextrose Agar (PDA) (Merck Laboratories, Darmstadt, Germany) (Appendix A) at 30ºC and were sub-cultured at bi-weekly intervals. Spores were harvested after 7 days of incubation by adding 10 ml of 0.1% Tween 80 (Merck) solution to each plate. Spore inoculates of 1.0×10^7 spores.ml^{-1} were prepared using a haemocytometer.

7.3.2 Iron Ore Concentrate Sample Selection

Iron ore concentrate samples (-5+1mm particle size) were collected by Kumba Iron Ore, Ltd. at the Sishen Iron Ore Mine, South Africa.

7.3.3 Bioleaching Fermentation Medium

The fermentation medium was prepared using distilled water (dH_2O) and contained the following (Appendix A): 150 g.l^{-1} D-glucose, 2.5 g.l^{-1} $(NH_4)_2SO_4$, 0.5 g.l^{-1} $MgSO_4.7H_2O$, 2.0 KH_2PO_4, 0.1×10^{-3} g.l^{-1} $Fe_2(SO)_4.24H_2O$, 0.1×10^{-3} g.l^{-1} $ZnSO_4.7H_2O$ and 0.06×10^{-3} g.l^{-1} $CuSO_4.5H_2O$.

7.3.4 Bioleaching Conditions for the Removal of Phosphorous and Potassium from the Iron Ore Concentrate

Bioleaching was conducted in 1l Erlenmeyer flasks. Briefly, three separate 500 g iron ore concentrate samples were mixed with 100 ml, 250 ml and 500 ml of fermentation medium respectively. Each flask was inoculated with 1 ml of *A. niger* NRRL 567

inoculum (1.0×10^7 spores.ml^{-1}). The Erlenmeyer flasks and their contents were incubated at 30ºC for 10 days. The bioleaching procedures were conducted in duplicate.

7.3.5 Analytical Procedure

The analytical procedure entailed the aseptic extraction of 5 ml of the fermentation medium from each flask. The extracted medium was filtered through a 0.45µm syringe filter (Millipore), followed by the analyses for pH and citric acid quantification. The abovementioned analyses were performed daily for 10 days.

7.3.6 Citric Acid Concentration Analysis

Citric acid concentrations were determined by spectrophotometry at 420nm after adding pyridine and acetic anhydride as described by Marier and Boulet (1958). Briefly, 1 ml of the sample was added to a test tube containing 1.3 ml of pyridine (C_5H_5N) (Merck), followed by the addition of 5.7 ml of acetic anhydride ($C_4H_6O_3$) (Merck). The contents of the test tube was mixed by swirling the tube and immediately placed in a constant-temperature (22ºC) water bath. Colour development was allowed for 30 min, followed by reading the colour intensity at 420nm with the blank set on 100% transmission. The citric acid concentration was determined by referring to a standard curve for citric acid concentration. Citric acid concentrations were expressed in g.l^{-1}.

7.3.7 Chemical Analysis of the Treated Iron Ore Concentrate

The chemical content of the treated iron ore concentrate was analysed after the 10 day bioleaching process by the Department of Geology, University of Pretoria, Pretoria, South Africa, using XRF spectrometry of the major elements. Briefly, the samples were ground to <75µm in a Tungsten Carbide milling vessel, roasted at 1000ºC, followed by the fusing of 1g of sample and 9g $Li_2B_4O_7$ into a glass bead. Major element analysis was executed on the fused bead using a ARL9400XP+ spectrometer. Another aliquot of the sample was pressed into a powder briquette for trace element analysis.

7.4 Results and Discussion

The citric acid concentrations produced using different pulp densities [bioleaching solution (ml)/iron ore (g)] (1/1, 1/2, and 1/5) of fermentation medium is illustrated in Figure 7.1. It was evident that the presence of the iron ore concentrate in the fermentation flasks had a negative effect on the citric acid production, as lower concentrations of citric acid (30.45 – 52.06 $g.l^{-1}$) were detected compared to fermentation without the presence of iron ore concentrate, where the maximum citric acid concentration was 102.3 $g.l^{-1}$ after 96 h (Williams and Cloete, 2008). This phenomenon may be as a result of the inhibitory effect which iron may have on the metabolism of A. niger.

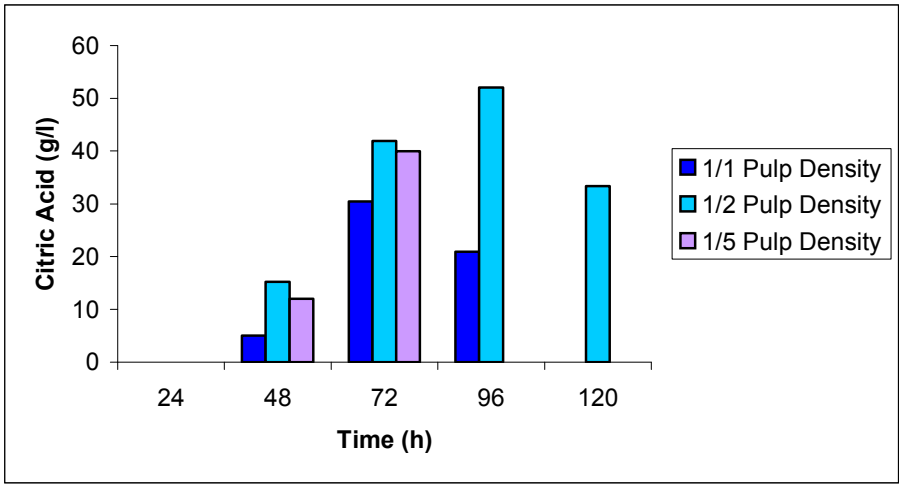

FIGURE 7.1

Diagrammatic Representation of the Citric Acid Production during Bioleaching by Aspergillus niger NRRL 567 at 30ºC

In addition, the pulp density also had an effect on the citric acid production. A 1/2 pulp density proved to be most efficient for citric acid production, producing a maximum citric acid concentration of 52.06 $g.l^{-1}$ after 96 h of fermentation, whereas the 1/1 and 1/5 pulp

densities produced maximum citric acid concentrations of 30.45 $g.l^{-1}$ and 39.99 $g.l^{-1}$ after 72 h respectively. No citric acid could be detected in the bioleaching solution after 96 h using a 1/5 pulp density compared to the 1/1 and 1/2 pulp densities where the citric acid disappeared from the solutions after 120 h and 144 h respectively. Similar patterns of citric acid reduction were observed during citric acid production by A. niger (Williams and Cloete, 2008).

The pH evolution using different pulp densities (1/1, 1/2, and 1/5) is illustrated in Figure 7.2. The pH of the bioleaching solutions using all three pulp densities rapidly decreased during the first 72 h of fermentation, followed by the pH stabilization from 96 h onwards. The pH of the 1/2 pulp density proved to reach the lowest levels of the pulp densities tested, stabilizing to an average of 1.79 from 120 h onwards, while the 1/1 and 1/5 pulp densities stabilized to an average of 2.24 and 2.38 in the same period. The maximum reduction in pH of 2.26 was achieved by using a pulp density of 1/2, while the maximum reduction of pH for the 1/1 and 1/5 pulp densities were 1.85 and 1.76 respectively.

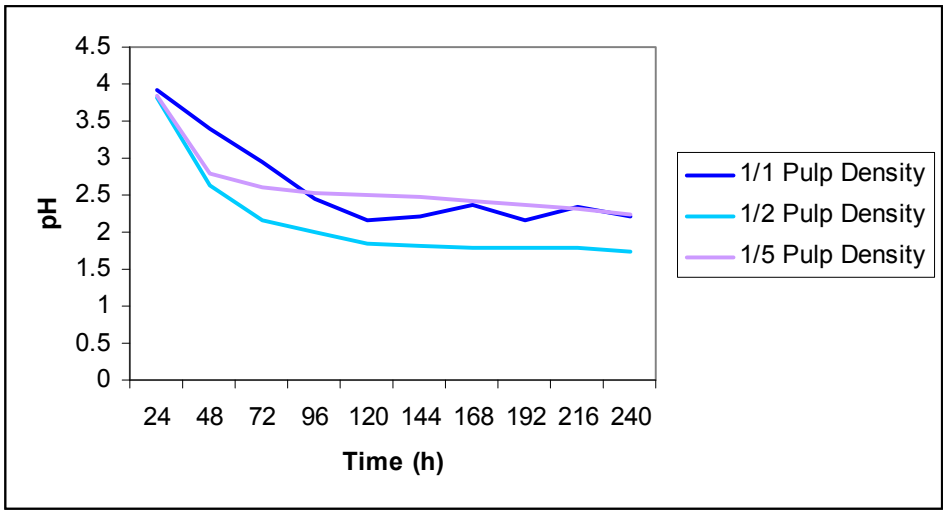

FIGURE 7.2

Diagrammatic Representation of the pH Evolution during the Bioleaching of the Iron Ore Concentrate of the Sishen Iron Ore Mine, South Africa

In addition to citric acid production, *Aspergillus niger* is able to produce other organic acids such as oxalic acid, which may explain the low pH throughout the bioleaching process, in spite of the citric acid depletion from the bioleaching solution after 96-144 h (Jianlong, 2000; Vandenberghe *et al*., 2000; Rawlings, 2005).

The chemical composition of the untreated iron ore concentrate of the Sishen Iron Ore Mine is given in Table 6.1. XRF spectrometry of the iron ore concentrate revealed a P concentration of 0.17 % mass, a K concentration of 0.17 % mass and a Fe concentration of 96.90 % mass.

TABLE 7.1
Chemical Composition of the Untreated Iron Ore Concentrate of the Sishen Iron Ore Mine

Major Elements (Oxides)	**% Mass**
Silica (SiO_2)	1.89
Titanium (TiO_2)	0.08
Aluminium (Al_2O_3)	1.18
Iron (Fe_2O_3)	96.90
Potassium (K_2O)	0.17
Phosphorous (P_2O_5)	0.17

The average percentage removal of the major elements from the iron ore concentrate of the Sishen Iron Ore Mine by bioleaching for 10 days using *A. niger* at different pulp densities is given in Table 7.2. The maximum P removal of 23.53±5.88% could be achieved using a pulp density of 1/2, while a maximum P removal of 11.76±6.79% and 5.88±5.88% was achieved using 1/1 and 1/5 pulp densities respectively. As a result of data scatter due to sampling and assay variation, however, the actual maximum P removal may be as low as 6.67% using a pulp density of 1/2. At the same time a maximum K removal of 17.65±5.88% was also achieved using a pulp density of 1/2, while only 5.88±5.88% K removal was possible for both the 1/1 and 1/5 pulp densities.

The maximum K removal however, may be as low as 6.25% using a pulp density of 1/2 as a result of data scatter due to sampling and assay variation. All bioleaching tests in this study proved to be relatively non-reactive towards the iron contained within the ore, as could be seen by the low levels (0.46% - 0.63%) of iron removal from the ore (Table 7.2).

TABLE 7.2
The Average Percentage Removal of the Major Elements from the Iron Ore Concentrate by Bioleaching for 10 Days Using *A. niger* at Different Pulp Densities

Major Elements	1/1 Pulp Density	1/2 Pulp Density	1/5 Pulp Density
Iron (Fe_2O_3)	0.53	0.63	0.46
Potassium (K_2O)	5.88	17.65	5.88
Phosphorous (P_2O_5)	11.76	23.53	5.88

In a previous study by Williams and Cloete (2008) (Chapter 6), chemical leaching using a 1M solution of citric acid at 30ºC achieved a maximum P and K removal of 17.65% after a 5 day leaching period, while a 0.75M solution of citric acid at 60ºC achieved a P removal of 35.29% and K removal of 17.65% after a 5 day leaching period. The abovementioned results are similar to that reported in this study using a pulp density of 1/2 at 30ºC for 10 days. This indicates that by adding *A. niger* to create a bioleaching process, it is not necessary to increase the leaching temperature to 60ºC as required by chemical leaching, and also that similar results to what was reported by Williams and Cloete (2008) (Chapter 6) at 30ºC was possible at a much lower concentration of citric acid (~0.25M vs 1M).

7.5 Conclusions

Compared to chemical leaching, which requires high concentrations of citric acid and/or high leaching temperatures (Williams and Cloete, 2008) (Chapter 6), this bioleaching process offers a more economical method with similar efficiency for the removal of P and

K from the iron ore concentrate of the Sishen Iron Ore Mine. This bioleaching process using *A. niger* NRRL 567 requires a pulp density [bioleaching solution (ml)/iron ore (g)] of 1/2 for the most efficient removal of P and K from the iron ore concentrate at 30ºC, as the most P (23.53±5.88%) and K (17.65±5.88%) was removed from the iron ore concentrate with only a negligible amount of iron lost due to acid corrosion. Therefore, it is suggested that bioleaching using *A. niger* NRRL 567 is a feasible process for the removal of P and K from the iron ore concentrate of the Sishen Iron Ore Mine.

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CHAPTER 8

CONCLUSIONS

As the progressive depletion of high-grade iron ore deposits, due to the advancing global technologies and civilisation, have been observed throughout the world, it has become necessary to process lower-grade iron ore bodies (Torma, 1986; Ehrlich, 1999; Costa *et al*., 2003; Jian and Sharma, 2004). Impurities, such as phosphorous (P) and potassium (K), contained within the lower-grade iron ore have a detrimental effect on the steel-making process by damaging the refractory linings of blast furnaces (Yusfin *et al*., 1999). Steel-making companies in the leading industrial nations have, therefore, established limits on the concentration of alkali's (such as K_2O) in the charge material without changing the smelting practice (Yusfin *et al*., 1999). Therefore, it has become important to develop an economically viable and environmentally friendly process to reduce the high P and K levels contained in the ore to improve the quality of iron ore that is being exported from the Sishen Iron Ore Mine. Currently such an economically viable biotechnological process for the reduction of P and K present in iron ore does not exist, and would therefore give Sishen Iron Ore Mine a competitive advantage in the international iron ore arena.

Chapter 3

The first step in developing a process for the removal of P and K from the iron ore concentrate was to conduct a population study of the process- and ground water, as well as the iron ore of the mine that would give insight into the microorganisms present in the mine environment. These microorganisms could prove to be useful in the removal of the P and K from the iron ore, as they may have novel metabolic properties, which could enable them to produce acids that may prove invaluable when applied in industrial practice (Gupta and Sharma, 2002; Lesniak *et al*., 2002). It is hypothesised that indigenous microorganisms already living in the Sishen Iron Ore Mine environment are capable of using the P and K in the iron ore as structural components for their cell walls

and membranes, as well as many other metabolic processes, such as organic acid production, since the environment selects for them to do so. A community study of the process- and ground water of the Sishen Iron Ore Mine showed the presence of various bacterial species, including *Aeromonas hydrophila*, *Alcaligenes faecalis*, *Brevundimonas vesicularis*, *Acinetobacter junii*, *Pantoea* spp., *Flavobacterium meningosepticum*, *Chryseomonas luteola*, *Enterobacter sakazakii*, *Pseudomonas aeruginosa* and *Herbaspirillum* spp. All of the abovementioned microorganisms have previously been associated with aquatic systems (Pellett *et al*., 1983; Aspinall and Graham, 1989; Havelaar *et al*., 1990; Morais and da Costa, 1990; Davis *et al*., 1994; Römling *et al*., 1994; Nishio *et al*., 1998; Squier *et al*., 2000; Chauret *et al*., 2001; Lynch *et al*., 2002; Bomo *et al*., 2004; Kim *et al*., 2004; Ozdemir and Baysal, 2004; Connon *et al*., 2005; Vacca *et al*., 2005; Canals *et al*., 2006; Chale-Matsau and Snyman, 2006; Joo *et al*., 2007). Except for *C. luteola* which may be used for the biosorption of chromium from waste water, there is no indication of bioleaching properties for any of the microorganisms isolated in the process- and ground water systems, and therefore, they should be excluded as bioleaching candidates for the removal of undesirable substances from the iron ore of the Sishen Iron Ore Mine.

Chapter 4

The community study of the iron ore concentrate of the Sishen Iron Ore Mine revealed several bacterial and fungal species present in the Sishen Iron Ore Mine environment. According to phylogeny, the bacterial isolates were closely related to *Herbaspirillum* species, as well as *Acidithiobacillus ferrooxidans*, while the fungal isolates were closely related to *Aureobasidium pullulans*, *Phaeosphaeria nodorum*, *Aspergillus fumigatus*, and *Candida parapsilosis*. The most likely candidate for the removal of P and K from the iron ore concentrate would seem to be *Acidithiobacillus ferrooxidans*, however, this bacterium produces sulphuric acid, which is undesired in the iron ore industry due its corrosive properties. Furthermore, the P and K contained in the iron ore is in a non-sulphidic phase, and therefore, the use of organic acids, such as citric acid, for the removal of these contaminants may have potential (Rawlings, 2005). These organic acids are typically produced by certain types of fungi, such as *Aspergillus niger* (Jianlong,

2000; Vandenberghe *et al*., 2000; Rawlings, 2005). Although *A. niger* was not isolated from the iron ore/soil of the Sishen Iron Ore Mine, isolating *A. fumigatus* may indicate that *A. niger* may have the potential to adapt to the environment that prevails at the mine. This would allow the application of *A. niger* for the production of citric acid, which in turn may be used for the removal of P and K from the iron ore concentrate.

Chapter 5

Conventional bioleaching involves the oxidation of insoluble metal sulphides to form corresponding soluble metal sulphates, enabling their removal from complex ore bodies (Rohwerder *et al*., 2002; Rawlings, 2005). The P and K contained within the iron ore concentrate are, however, in non-sulphidic phases, and therefore, conventional bioleaching processes are not viable for their removal from the ore. Metals in certain non-sulphide minerals, such as the iron ore concentrate of the Sishen Iron Ore Mine may be solubilised by a process of complexation using microbially produced inorganic or organic acids (Rawlings, 2005). Citric acid seemed to be the best leaching candidate for the removal of P and K from the iron ore of the Sishen Iron Ore Mine. Although no P was removed by citric acid during this investigation, further analyses are needed to determine whether higher leaching temperatures would play a role in the removal of P from the iron ore. Organic acids, such as citric acid produced by *Aspergillus niger*, possess several carboxyl groups, and therefore can act as chelating agents (Sayer and Gadd, 2001). These carboxyl groups tend to donate protons (H^+), resulting in negatively charged carboxyl groups that are capable of forming stable complexes with several metal cations, such as copper and zinc (Sayer and Gadd, 2001). Therefore, it is possible that these negatively charged carboxyl groups are able to form stable complexes with the K^+ cations present, while the H^+ cations are able to react with the negatively charged phosphorous, resulting in their removal from the iron ore concentrate.

Chapter 6

Solid substrate- and submerged fermentation using *Aspergillus niger* were compared for the production of citric acid, which is to be used for the chemical leaching of P and K from the iron ore concentrate. During solid substrate- and submerged fermentation using

A. niger a high concentration of glucose was required to ensure that citric was excreted from the fungal mycelia. The maximum citric acid yield of 54.2 $g.kg^{-1}$ DPM by solid substrate fermentation and 102.3 $g.l^{-1}$ by submerged fermentation was attained after a period of 96 h in both processes. The fermentation media of both solid substrate- and submerged fermentation contained similar constituents, with the difference that the submerged fermentation medium contained less of each constituent. In addition, solid substrate fermentation required peat moss as a solid substrate, which increases the costs incurred by using this fermentation process. Therefore, taking all the variable factors into account it is suggested that submerged fermentation be used for citric acid production by *A. niger*, as this fermentation process was found to be more economical and produced more citric acid in the same time.

Chemical leaching of the iron ore concentrate was compared to "heap leaching" of the iron ore, whereby iron ore concentrate was mixed with fermentation medium inoculated with *A. niger* with a v/m ratio of 1/10. Chemical leaching of the iron ore concentrate proved to be more efficient than "heap leaching", as more P and K was removed from the iron ore concentrate, as well as in a shorter time frame. In addition, using "heap leaching" technology proved to be uneconomical in this case, as growth medium will have to be added every 5 days to enhance the fungal growth. It is suggested that a 1M citric acid leaching solution be used at 60ºC for the chemical leaching process, as the most P and K is removed from the iron ore concentrate using these leaching conditions. Therefore, the most economical and efficient chemical leaching process for the removal of P and K from the iron ore concentrate of the Sishen Iron Ore Mine, involves a two-stage process whereby citric acid is produced by *A. niger*, followed by the chemical leaching of the P and K from the iron ore concentrate using a 1M citric acid leaching solution at 60ºC.

Chapter 7

Various technologies developed in the last couple of decades include microbial use for the extraction of different valuable metals, which is commonly known as bioleaching (Gilbertson, 2000; Rohwerder *et al.*, 2002). Conventional bioleaching, also known as

biooxidation, refers to the microbial conversion of insoluble metals into soluble forms (Sand, 2001; Rawlings, 2002, Roberto, 2002). The P and K contained within the iron ore concentrate are, however, in non-sulphidic phases, and therefore, conventional bioleaching processes are not viable for their removal from the ore. Therefore, the use of an alternative bioleaching microorganism was investigated to remove the undesirable P and K from the iron ore concentrate.

The possibility to use *A. niger* as a bioleaching microorganism was investigated, due to its ability to produce organic acids, such as citric acid, which has the ability to remove P and K from the iron ore concentrate by chemical leaching (Chapter 6). Compared to chemical leaching, which requires high concentrations of citric acid and/or high leaching temperatures (Williams and Cloete, 2008) (Chapter 6), bioleaching using *A. niger* offers a more economical method with similar efficiency for the removal of P and K from the iron ore concentrate of the Sishen Iron Ore Mine. This bioleaching process using *A. niger* NRRL 567 requires a pulp density [bioleaching solution (ml)/iron ore (g)] of 1/2 for the most efficient removal of P and K from the iron ore concentrate at 30ºC, as the most P (23.53%) and K (17.65%) was removed from the iron ore concentrate with only a negligible amount of iron lost due to acid corrosion. Therefore, it is suggested that bioleaching using *A. niger* NRRL 567 is a feasible process for the removal of P and K from the iron ore concentrate of the Sishen Iron Ore Mine.

With all results taken into account, the best results in terms of the removal of P and K from the iron ore concentrate of the Sishen Iron Ore Mine was obtained by the chemical leaching of P and K from the iron ore concentrate using a 1M solution of citric acid, which was produced by *A. niger*, at 60ºC for 5 days. This method, however, may prove to be uneconomical due to the energy costs involved to maintain the 60ºC temperature required for this process. The most economically viable process, although not as efficient as the abovementioned process for the removal of P and K from the iron ore concentrate, proved to be the bioleaching process using *A. niger* NRRL 567 with a bioleaching pulp density of 1/2 at 30ºC for 10 days. This is the first report of the use of *A. niger* for use in any bioleaching process.

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APPENDIX A

A1 Preparation of culture media and reagents used during the bacterial community study of the process- and ground water of the Sishen Iron Ore Mine

A1.1 Standard Nutrient Agar Medium (Merck, Darmstadt, Germany)

Composition

Special Peptone	15.6 g.ℓ^{-1}
Yeast Extract	2.8 g.ℓ^{-1}
Sodium Chloride	5.6 g.ℓ^{-1}
D (+) Glucose	1.0 g.ℓ^{-1}
Agar	12.0 g.ℓ^{-1}

Suspend 37 g in 1 ℓ of distilled water (dH_2O)
Boil to dissolve completely
Autoclave at 121°C for 15 min

A1.2 Oxidation-Fermentation (OF) Basal Medium (Merck, Darmstadt, Germany)

Composition

Peptone from Casein	2.0 g.ℓ^{-1}
Yeast Extract	1.0 g.ℓ^{-1}
Sodium Chloride	5.0 g.ℓ^{-1}
di-Potassium Hydrogen Phosphate	0.2 g.ℓ^{-1}
Bromothymol Blue	0.08 g.ℓ^{-1}
Agar	2.5 g.ℓ^{-1}

Suspend 11 g in 1 ℓ of dH_2O
Heat in a boiling water bath to dissolve completely

Autoclave at 121ºC for 15 min

A2 Preparation of culture media and reagents used during the microbial community study of the iron ore concentrate of the Sishen Iron Ore Mine

A2.1 Nutrient Broth (Sigma-Aldrich Chemie, Buchs, Switzerland)

Composition

Peptone	15.0 g.ℓ^{-1}
Yeast Extract	3.0 g.ℓ^{-1}
Sodium Chloride	6.0 g.ℓ^{-1}
D(+)-Glucose	1.0 g.ℓ^{-1}

Suspend 25 g in 1 ℓ of dH_2O
Heat in a boiling water bath to dissolve completely
Autoclave at 121ºC for 15 min

A2.2 Half-Strength Potato Dextrose Agar (PDA) Medium (Merck, Darmstadt, Germany)

Composition

Potato Extract	2.0 g.ℓ^{-1}
Dextrose	10.0 g.ℓ^{-1}
Agar	15.0 g.ℓ^{-1}

Suspend 27 g in 1 ℓ of dH_2O
Biol while stirring to dissolve completely
Autoclave at 121ºC for 15 min

Appendix A

A2.3 1 M Tris, pH 8.0 (Merck, Darmstadt, Germany)

Dissolve 121.1 g of Tris in 700 mℓ of dH_2O
Adjust the volume to 900 mℓ using dH_2O
Adjust the pH to 8.0 using 1 M Hydrochloric Acid
Adjust the volume to 1 ℓ using dH_2O

A2.4 0.5 M Ethylene Diamine Tetracetic Acid (EDTA) (Merck, Darmstadt, Germany)

Dissolve 186.12 g of EDTA in 750 mℓ dH_2O
Add 20 g of Sodium Hydroxide pellets and allow to dissolve
Adjust the pH to 8.0 using Sodium Hydroxide
Adjust the volume to 1 ℓ using dH_2O

A2.5 5 M Sodium Chloride (Merck, Darmstadt, Germany)

Dissolve 292.2 g of Sodium Chloride in 1 ℓ of dH_2O

A2.6 Tris-EDTA (TE) Buffer

Add 10 mℓ of 1 M Tris, pH 8.0 (A2.3) and 2 mℓ of 0.5 M EDTA (A2.4) to 988 mℓ of dH_2O

A2.7 Cetyltrimethyl Ammonium Bromide (CTAB)/NaCl Buffer

Add the following to 580 mℓ of dH_2O:

100 mℓ of 1 M Tris, pH 8.0 (A2.3)
280 mℓ of 5 M Sodium Chloride (A2.5)
40 mℓ of 0.5 M EDTA (A2.4)
20 g of CTAB (Merck, Darmstadt, Germany)

Appendix A

A3 Preparation of culture media and reagents used during the production and use of citric acid for the removal of phosphorous and potassium from the iron ore concentrate of the Sishen Iron Ore Mine

A3.1 Potato Dextrose Agar (PDA) Medium (Merck, Darmstadt, Germany)

Composition

Potato Extract	4.0 g.ℓ^{-1}
Dextrose	20.0 g.ℓ^{-1}
Agar	15.0 g.ℓ^{-1}

Suspend 39 g in 1 ℓ of dH_2O
Biol while stirring to dissolve completely
Autoclave at 121ºC for 15 min

A3.2 Basal Salt Solution for Solid Substrate Fermentation

Composition

$(NH_4)_2SO_4$	3.84 g.ℓ^{-1}
KH_2PO_4	10.98 g.ℓ^{-1}
NaCl	1.01 g.ℓ^{-1}
$MgSO_4.7H_2O$	1.01 g.ℓ^{-1}
$FeSO_4.7H_2O$	1.01 g.ℓ^{-1}

Add all the above to 1 ℓ of dH_2O
Biol while stirring to dissolve completely
Autoclave at 121ºC for 15 min

Appendix A

A3.3 Fermentation Medium for Submerged Fermentation

Composition	
D-glucose	15.0 g.ℓ^{-1}
$(NH_4)_2SO_4$	2.5 g.ℓ^{-1}
KH_2PO_4	2.0 g.ℓ^{-1}
$MgSO_4.7H_2O$	0.5 g.ℓ^{-1}
$Fe_2(SO)_4.24H_2O$	0.1×10^{-3} g.l^{-1}
$ZnSO_4.7H_2O$	0.1×10^{-3} g.l^{-1}
$CuSO_4.5H_2O$	0.06×10^{-3} g.l^{-1}

Add all the above to 1 ℓ of dH_2O

Biol while stirring to dissolve completely

Autoclave at 121ºC for 15 min

APPENDIX B

B1 16S Nucleotide Sequences Obtained from the Pure Bacterial Cultures Isolated from the Ground Water of the Sishen Iron Ore Mine

B1.1 KUMBA WATER SEQ 1

```
1    AACAGCAAGG TATTAACTTA CTGCCCTTCC TCCCAACTTA AAGTGCTTTA
51   CAATCCGAAG ACCTTCTTCA CACACGCGGC ATGGCTGGAT CAGGCTTTCG
101  CCCATTGTCC AATATTCCCC ACTGCTGCCT CCCGTAGGAG TCTGGACCGT
151  GTCTCAGTTC CAGTGTGACT GATCATCCTC TCAGACCAGT TACGGATCGT
201  CGCCTTGGTA GGCCTTTACC CCACCAACTA GCTAATCCGA CCTAGGCTCA
251  TCTGATAGCG TGAGGTCCGA AGATCCCCCA CTTTCTCCCT CAGGACGTAT
301  GCGGTATTAG CGCCCGTTTC CGGACGTTAT CCCCCACTAC CAGGCAGATT
351  CCTAGGCATT ACTCACCCGT CCGCCGCTGA ATCCAGGAGC AAGCTCCCTT
401  CATCCGCTCG ACTTGCATGT GTTAGGCCTG CCGCCAGCGT TCAATCTGAG
451  CCAGGATCAA ACTCTCCCCC CGTGCCCCCG CCCCGCCCGC CGCGCG
```

B1.2 KUMBA WATER SEQ 2

```
1    AACAGCAAGG TATTAACTTA CTGCCCTTCC TCCCAACTTA AAGTGTTTTA
51   CAATCCGAAG ACCTTCTTCA CACACGCGGC ATGGCTGGCT CAGGCTTTCG
101  CCCATTGTCC AATATTCCCC ACTGCTGCCT CCCGTAGGAG TCTGGACCGT
151  GTCTCAGTTC CAGTGTGACT GATCATCCTC TCAGACCAGT TACGGATCGT
201  CGCCTTGGTA GGCCTTTACC CCACCAACTA GCTAATCCGA CCTAGGCTCA
251  TCTGATAGCG TGAGGTCCGA AGATCCCCCA CTTTCTCCCT CAGGACGTAT
301  GCGGTATTAG CGCCCGTTTC CGGACGTTAT CCCCCACTAC CAGGCAGATT
351  CCTAGGCATT ACTCACCCGT CCGCCGCTGA ATCCAGGAGC AAGCTCCCTT
401  CATCCGCTCG ACTTGCATGT GTTAGGCCTG CCGCCAGCGT TCAATCTGAG
451  CCAGGATCAA ACTCTCCCCC CGTGCCCCCG CCCCGCCCGC CGCG
```

B1.3 KUMBA WATER SEQ 3

```
1    AACAGCAAGG TATTAACTTA CTGCCCTTCC TCCCAACTTA AAGTGCTTTA
51   CAATCCGAAG ACCTTCTTCA CACACGCGGC ATGGCTGGAT CAGGCTTTCG
101  CCCATTGTCC AATATTCCCC ACTGCTGCCT CCCGTAGGAG TCTGGACCGT
```

```
151   GTCTCAGTTC CAGTGTGACT GATCATCCTC TCAGACCAGT TACGGATCGT
201   CGCCTTGGTA GGCCTTTACC CCACCAACTA GCTAATCCGA CCTAGGCTCA
251   TCTGATAGCG TGAGGTCCGA AGATCCCCCA CTTTCTCCCT CAGGACGTAT
301   CGCCCGTTTC CGGACGTTAT CCCCCACTAC CAGGCAGATT CCTAGGCATT
351   ACTCACCCGT CCGCCGCTGA ATCCAGGAGC AAGCTCCCTT CATCCGCTCG
401   ACTTGCATGT GTTAGGCNNN CCGCCAGCGT TCAATCTGAG CCAGGATCAA
451   ACTCTCCCCC CGTGCCCCCG CCCCGCCCGC CGCGCGCGGG CGGGCGA
```

B1.4 KUMBA WATER SEQ 4

```
1     TACGTCAAAC AGCAAGGTAT TAACTTACTG CCCTTCCTCC CAACTTAAAG
51    TGCTTTACAA TCCGAAGACC TTCTTCACAC ACGCGGCATG GCTGGATCAG
101   GCTTTCGCCC ATTGTCCAAT ATTCCCCACT GCTGCCTCCC GTAGGAGTCT
151   GGACCGTGTC TCAGTTCCAG TGTGACTGAT CATCCTCTCA GACCAGTTAC
201   GGATCGTCGC CTTGGTAGGC CTTTACCCCA CCAACTAGCT AATCCGACCT
251   AGGCTCATCT GATAGCGTGA GGTCCGAAGA TCCCCCACTT TCTCCCTCAG
301   GACGTATGCG GTATTAGCGC CCGTTTCCGG ACGTTATCCC CCACTACCAG
351   GCAGATTCCT AGGCATTACT CACCCGTCCG CCGCTGAATC CAGGAGCAAG
401   CTCCCTTCAT CCGCTCGACT TGCATGTGTT AGGCCTGCCG CCAGCGTTCA
451   ATCTGAGCCA GGATCAAACT CTCCCCCCGT GCCCCCGCCC CGCCCGCCGC
501   GCG
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B1.5 KUMBA WATER SEQ 5

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1     GGTACGTCAA ACAGCAAGGT ATTAACTTAC TGCCCTTCCT CCCAACTTAA
51    AGTGCTTTAC AATCCGAAGA CCTTCTTCAC ACACGCGGCA TGGCTGGATC
101   CCATTGTCCA ATATTCCCCA CTGCTGCCTC CCGTAGGAGT CTGGACCGTG
151   TCTCAGTTCC AGTGTGACTG ATCATCCTCT CAGACCAGTT ACGGATCGTC
201   GCCTTGGTAG GCCTTTACCC CACCAACTAG CTAATCCGAC CTAGGCTCAT
251   CTGATAGCGT GAGGTCCGAA GATCCCCCAC TTTCTCCCTC AGGACGTATG
301   CGGTATTAGC GCCCGTTTCC GGACGTTATC CCCCACTACC AGGCAGATTC
351   CTAGGCATTA CTCACCCGTC CGCCGCTGAA TCCAGGAGCA AGCTCCCTTC
401   ATCCGCTCGA CTTGCATGTG TTAGGCCTGC CGCCAGCGTT CAATCTGAGC
451   CAGGATCAAA CTCTCCCCCC GTGCCCCCGC CCCGCCCGCC GCGCG
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B1.6 KUMBA WATER SEQ 6

```
1    CTTTCCGGAC  AAAAGAGCTT  TACAACCCGA  AGGCCTTCTT  CACATCACGC
51   GATCAGGGTT  GCCCCCATTG  TCCAAAATTC  CCCACTGCTG  CCTCCCGTAG
101  GAGTCTGGGC  CGTGTCTCAG  TCCCAGTGTG  GCTGGTCGTC  CTCTCAGACC
151  AGCTACTGAT  CGTCGCCTTG  GTGGGCCTTT  ACCCCACCAA  CTAGNTAATC
201  AGATATCGGC  CGCTCCAAAA  GCATGAGGTC  TTGCGGTCCC  CCACTTTCAT
251  CCGTAGATCG  TATGCGGTAT  TAGCTAGTCT  TTCGACTAGT  TATCCCCCAC
301  TCCAGGGGAC  GTTCCGATAT  GTTACTCACC  CGTTCGCCAC  TCGCCGCCAG
351  GATGGATCCC  GCGCTGGCGT  TAGACTTGTA  TGTGTAAGGT  ATGCCGCCAG
401  CGTTCAATCT  GAGACAGGAT  CAAACT
```

B1.7 KUMBA WATER SEQ 7

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1    GCTTTACAAC  CCGAAGGCCT  TCTTCACACA  CGCGGCATTG  CTGGATCAGG
51   GTTGCCCCCA  TTGTCCAAAA  TTCCCCACTG  CTGCCTCCCG  TAGGAGTCTG
101  GGCCGTGTCT  CAGTCCCAGT  GTGGCTGGTC  GTCCTCTCAG  ACCAGCTACT
151  GATCGTCGCC  TTGGTGGGCC  TTTACCCCAC  CAACTAGCTA  ATCAGATATC
201  GGCCGCTCCA  CGAGCATGAG  GTCTTGCGGT  CCCCCACTTT  CATCCGTAGA
251  TCGTATGCGG  TATTAGCTAG  TCTTTCGACT  AGTTATCCCC  CACTCCAGGG
301  CACGTTCCGA  TATGTTACTC  ACCCGTTCGC  CACTCGCCGC  CAGGATTGCT
351  CCCGCGCTGC  CGTTCGACTT  GCATGTGTAA  GGCATGCCGC  CAGCGTTCAA
401  TCTGAGCCAG  GATCAAACTC  TCCCCCCGTG
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B1.8 KUMBA WATER SEQ 8

```
1    TCTTTCCGGA  CAAAAGTGCT  TTACAACCCG  AAGGCCTTCT  TCACACACGC
51   GGCATTGCTG  GATCAGGGTT  GCCCCCATTG  TCCAAAATTC  CCCACTGCTG
101  CCTCCCGTAG  GAGTCTGGGC  CGTGTCTCAG  TCCCAGTGTG  GCTGGTCGTC
151  CTCTCAGACC  AGCTACTGAT  CGTCGCCTTG  GTGGGCCTTT  ACCCCACCAA
201  CTAGCTAATC  AGATATCGGC  CGCTCCACGA  GCATGAGGTC  TTGCGATCCC
251  CCACTTTCAT  CCGTAGATCG  TATGCGGTAT  TAGCTAGTCT  TTCGACTAGT
301  TATCCCCCAC  TCCAGGGCAC  GTTCCGATAT  GTTACTCACC  CGTTCGCCAC
351  TCGCCGCCAG  GATTNATCCC  GCGCTGCCGT  TCGACTTGCA  TGTGTAAGGC
401  ATGCCGCCAG  CGTTCAATCT  GAGCCAGGAT  CAAACTCTC
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B1.9 KUMBA WATER SEQ 9

1 ACAAAAGTGC TTTACAACCC GAAGGCCTTC TTCACATCAC GCGGCATTGC
51 TGGATCAGGG TTGCCCCCAT TGTCCAAAAT TCCCCACTGC TGCCTCCCGT
101 AGGAGTCTGG GCCGTGTCTC AGTCCCAGTG TGGCTGGTCG TCCTCTCAGA
151 CCAGCTAAAG ATCGTCGCCT TGGTGGGCCT TTACCCCACC AACTAGCTAA
201 TCAGATATCG GCCGCTCCAC GAGCATGAGG TCTTGCGGTC CCCCACTTTC
251 ATCCGTAGAT CGTATGCGGT ATTAGCTAGT CTTTCGACTA GTTATCCCCC
301 ACTTCAGGGC ACGTTCCGAT ATGTTACTCA CCCGTTCGCC ACTCGCCGCC
351 AGGATGGCTC CCGCGCTGCC GTTCGACTTG CATGTGTAAG GCATGCCGCC
401 AGCGTTCAAT CTGAGCCAGG ATCAAACTCT C

B1.10 KUMBA WATER SEQ 10

1 CTTCCCTGAC AAAAGAGCTT TACAACCCGA AGGCCTTCTT CACTCACGCG
51 GCATTGCTGG ATCAGGCTTG CGCCCATTGT CCAAAATTCC CCACTGCTGC
101 CTCCCGTAGG AGTCTGGGCC GTGTCTCAGT CCCAGTGTGG CTGGTCGTCC
151 TCTCAGACCA GCTACTGATC GTCGCCTTGG TGGGCCTTTA CCTCACCAAC
201 TAGCTAATCA GATATCGGCC GCTCCACGAG CATGAGGTCT TGAGGTCCCC
251 CACTTTCATC CGTAGATCGT ATGCGGTATT AGCTAATCTT TCGATTNGTT
301 ATCCCCCACT TCTGGGTACG TTCCGATATA TTACTCACCC GTTCGCCACT
351 CGCCGCCAGG ATGGCTCGCG CTGTGGCGCT AGA

B1.11 KUMBA WATER SEQ 11

1 TCCGAACAAA AGTGCTTTAC AACCCGAAGG CCTTCTTCAC ACACGCGGCA
51 TGCTGGATCA GGGTTGCCCC CATTGTCCAA AATTCCCCAC TGCTGCCTCC
101 CGTAGGAGTC TGGGCCGTGT CTCAGTCCCA GTGTGGCTGG TCGTCCTCTC
151 AGACCAGCTA CTGATCGTCG CCTTGGTGGG CCTTTACCCC ACCAACTAGC
201 TAATCAGATA TCGGCCGCTC CACGAGCATG AGGTCTTGCG ATCCCCCACT
251 TTCATCCGTA GATCGTATGC GGTATTAGCT AGTCTTTCGA CTAGTTATCC
301 CCCACTCCAG GGCACGTTCC GATATGTTAC TCACCCGTTC GCCACTCGCC
351 GCCAGGATTG CTCCCGCGCT GCCGTTCGAC TTGCATGTGT AAGGCATGCC
401 GCCAGCGTTC AATCTGAGCC AGGATCAAAC TCTC

B1.12 KUMBA WATER SEQ 12

```
1     TCCACCCAGG  GATATTAACC  CAGGCGATTT  CTTTCCGAAC AAAAGTGCTT
51    TACAACCCGA  AGGCCTTCTT  CACACACGCG  GCATTGCTGG  ATCAGGGTTG
101   CCCCCATTGT  CCAAAATTCC  CCACTGCTGC  CTCCCGTAGG  AGTCTGGGCC
151   GTGTCTCAGT  CCCAGTGTGG  CTGGTCGTCC  TCTCAGACCA  GCTACTGATC
201   GTCGCCTTGG  TGGGCCTTTA  CCCCACCAAC  TAGCTAATCA  GATATCGGCC
251   GCTCCACGAG  CATGAGGTCT  TGCGATCCCC  CACTTTCATC  CGTAGATCGT
301   ATGCGGTATT  AGCTAGTCTT  TCGACTAGTT  ATCCCCCACT  CCAGGGCACG
351   TTCCGATATG  TTACTCACCC  GTTCGCCACT  CGCCGCCAGG  ATTGCTCCCG
401   CGCTGCCGTT  CGACTTGCAT  GTGTAAGGCA  TGCCGCCAGC  GTTCAATCTG
451   AGCCAGGATC AAACTCTC
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B1.13 KUMBA WATER SEQ 13

```
1     TATCCTCCCT GATAAAAGAG CTTTACATCC AAAAGGGCCT TCATCACTCA
51    CGCGATATTG CTGGATCAGG CTTGCGCCCA TTGTCCAAGA TTCCCCACTG
101   CTGCCTCCCG TAGGAGTCTG GGCCGTGTCT CAGTCCCAGT GTGGCTGATC
151   ATCCTCTAAG ACCAGCTAAG GATCATCGGC TTGGTAGGCC ATTACCCTAC
201   CAACTACCTA ATCCTACGCA GGCTCATCTT TTAGCGGATT ACTCCTTTCG
251   ATATATACGG TATTCTATTT TCAGTTTCCC GAAAATATTA TCCCATGCTA
301   AAAGGCAGAT TCCTACGCAT TACTCACCCG TGTGCCATGG AAAATAAATT
351   TCCCATACGA CTTGCATGTG TTAAGCATAT CGCTAGCGTT CATTCTGAGC
401   CAGGATCAAA CTCTCCCCCC GTG
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B2 16S Nucleotide Sequences Obtained from the Enriched Iron Ore Concentrate of the Sishen Iron Ore Mine

B2.1 KS1

```
1     CTTTCCGGAC AAAAGAGCTT TACAACCCGA AGGCCTTCTT CACATCACGC
51    GGCATTGCTG GATCAGGGTT GCCCCCATTG TCCAAAATTC CCCACTGCTG
101   CCTCCCGTAG GAGTCTGGGC CGTGTCTCAG TCCCAGTGTG GCTGGTCGTC
151   CTCTCAGACC AGCTACTGAT CGTCGCCTTG GTGGGCCTTT ACCCCACCAA
201   CTAGNTAATC AGATATCGGC CGCTCCAAAA GCATGAGGTC TTGCGGTCCC
251   CCACTTTCAT CCGTAGATCG TATGCGGTAT TAGCTAGTCT TTCGACTAGT
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301   TATCCCCCAC TCCAGGGGAC GTTCCGATAT GTTACTCACC CGTTCGCCAC
351   TCGCCGCCAG GATGGATCCC GCGCTGGCGT TAGACTTGTA TGTGTAAGGT
401   ATGCCGCCAG CGTTCAATCT GAGACAGGAT CAAACT
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B2.2 KS2

```
1     TATCCTCCCT GATAAAAGAG CTTTACATCC AAAAGGGCCT TCATCACTCA
51    CGCGATATTG CTGGATCAGG CTTGCGCCCA TTGTCCAAGA TTCCCCACTG
101   CTGCCTCCCG TAGGAGTCTG GGCCGTGTCT AGTCCCAGTC GTGGCTGATC
151   ATCCTCTAAG ACCAGCTAAG GATCATCGGC TTGGTAGGCC ATTACCCTAC
201   CAACTACCTA ATCCTACGCA GGCTCATCTT TTAGCGGATT ACTCCTTTCG
251   ATATATACGG TATTCTATTT TCAGTTTCCC GAAAATATTA TCCCATGCTA
301   AAAGGCAGAT TCCTACGCAT TACTCACCCG TGTGCCATGG AAAATAAATT
351   TCCCATACGA CTTGCATGTG TTAAGCATAT CGCTAGCGTT CATTCTGAGC
401   CAGGATCAAA CTCTCCCCCC GTG
```

B2.3 KS3

```
1     TCTTTCCGGA CAAAAGTGCT TTACAACCCG AAGGCCTTCT TCACACACGC
51    GGCATTGCTG GATCAGGGTT GCCCCCATTG TCCAAAATTC CCCACTGCTG
101   CCTCCCGTAG GAGTCTGGGC CGTGTCTCAG TCCCAGTGTG GCTGGTCGTC
151   CTCTCAGACC AGCTACTGAT CGTCGCCTTG GTGGGCCTTT ACCCCACCAA
201   CTAGCTAATC AGATATCGGC CGCTCCACGA GCATGAGGTC TTGCGATCCC
251   CCACTTTCAT CCGTAGATCG TATGCGGTAT TAGCTAGTCT TTCGACTAGT
301   TATCCCCCAC TCCAGGGCAC GTTCCGATAT GTTACTCACC CGTTCGCCAC
351   TCGCCGCCAG GATTNATCCC GCGCTGCCGT TCGACTTGCA TGTGTAAGGC
401   ATGCCGCCAG CGTTCAATCT GAGCCAGGAT CAAACTCTC
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B2.4 KS5

```
1     ACAAAAGTGC TTTACAACCC GAAGGCCTTC TTCACATCAC GCGGCATTGC
51    TGGATCAGGG TTGCCCCCAT TGTCCAAAAT TCCCCACTGC TGCCTCCCGT
101   AGGAGTCTGG GCCGTGTCTC AGTCCCAGTG TGGCTGGTCG TCCTCTCAGA
151   CCAGCTAAAG ATCGTCGCCT TGGTGGGCCT TTACCCCACC AACTAGCTAA
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201   TCAGATATCG GCCGCTCCAC GAGCATGAGG TCTTGCGGTC CCCCACTTTC
251   ATCCGTAGAT CGTATGCGGT ATTAGCTAGT CTTTCGACTA GTTATCCCCC
301   ACTTCAGGGC ACGTTCCGAT ATGTTACTCA CCCGTTCGCC ACTCGCCGCC
351   AGGATGGCTC CCGCGCTGCC GTTCGACTTG CATGTGTAAG GCATGCCGCC
401   AGCGTTCAAT CTGAGCCAGG ATCAAACTCT C
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B2.5 KS6

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1     CTTCCCTGAC AAAAGAGCTT TACAACCCGA AGGCCTTCTT CACTCACGCG
51    GCATTGCTGG ATCAGGCTTG CGCCCATTGT CCAAAATTCC CCACTGCTGC
101   CTCCCGTAGG AGTCTGGGCC GTGTCTCAGT CCCAGTGTGG CTGGTCGTCC
151   TCTCAGACCA GCTACTGATC GTCGCCTTGG TGGGCCTTTA CCTCACCAAC
201   TAGCTAATCA GATATCGGCC GCTCCACGAG CATGAGGTCT TGAGGTCCCC
251   CACTTTCATC CGTAGATCGT ATGCGGTATT AGCTAATCTT TCGATTNGTT
301   ATCCCCCACT TCTGGGTACG TTCCGATATA TTACTCACCC GTTCGCCACT
351   CGCCGCCAGG ATGGCTCGCG CTGTGGCGCT AGA
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B2.6 KS7

```
1     TCCACCCAGG GATATTAACC CAGGCGATTT CTTTCCGAAC AAAAGTGCTT
51    TACAACCCGA AGGCCTTCTT CACACACGCG GCATTGCTGG ATCAGGGTTG
101   CCCCCATTGT CCAAAATTCC CCACTGCTGC CTCCCGTAGG AGTCTGGGCC
151   GTGTCTCAGT CCCAGTGTGG CTGGTCGTCC TCTCAGACCA GCTACTGATC
201   GTCGCCTTGG TGGGCCTTTA CCCCACCAAC TAGCTAATCA GATATCGGCC
251   GCTCCACGAG CATGAGGTCT TGCGATCCCC CACTTTCATC CGTAGATCGT
301   ATGCGGTATT AGCTAGTCTT TCGACTAGTT ATCCCCCACT CCAGGGCACG
351   TTCCGATATG TTACTCACCC GTTCGCCACT CGCCGCCAGG ATTGCTCCCG
401   CGCTGCCGTT CGACTTGCAT GTGTAAGGCA TGCCGCCAGC GTTCAATCTG
451   AGCCAGGATC AAACTCTC
```

B2.7 KS8

```
1     TCCGAACAAA AGTGCTTTAC AACCCGAAGG CCTTCTTCAC ACACGCGGCA
51    TTGCTGGATC AGGGTTGCCC CCATTGTCCA AAATTCCCCA CTGCTGCCTC
101   CCGTAGGAGT CTGGGCCGTG TCTCAGTCCC AGTGTGGCTG GTCGTCCTCT
151   CAGACCAGCT ACTGATCGTC GCCTTGGTGG GCCTTTACCC CACCAACTAG
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201   CTAATCAGAT ATCGGCCGCT CCACGAGCAT GAGGTCTTGC GATCCCCCAC
251   TTTCATCCGT AGATCGTATG CGGTATTAGC TAGTCTTTCG ACTAGTTATC
301   CCCCACTCCA GGGCACGTTC CGATATGTTA CTCACCCGTT CGCCACTCGC
351   CGCCAGGATT GCTCCCGCGC TGCCGTTCGA CTTGCATGTG TAAGGCATGC
401   CGCCAGCGTT CAATCTGAGC CAGGATCAAA CTCTC
```

B2.8 KS10

```
1     GCTTTACAAC CCGAAGGCCT TCTTCACACA CGCGGCATTG CTGGATCAGG
51    GTTGCCCCCA TTGTCCAAAA TTCCCCACTG CTGCCTCCCG TAGGAGTCTG
101   GGCCGTGTCT CAGTCCCAGT GTGGCTGGTC GTCCTCTCAG ACCAGCTACT
151   GATCGTCGCC TTGGTGGGCC TTTACCCCAC CAACTAGCTA ATCAGATATC
201   GGCCGCTCCA CGAGCATGAG GTCTTGCGGT CCCCCACTTT CATCCGTAGA
251   TCGTATGCGG TATTAGCTAG TCTTTCGACT AGTTATCCCC CACTCCAGGG
301   CACGTTCCGA TATGTTACTC ACCCGTTCGC CACTCGCCGC CAGGATTGCT
351   CCCGCGCTGC CGTTCGACTT GCATGTGTAA GGCATGCCGC CAGCGTTCAA
401   TCTGAGCCAG GATCAAACTC TCCCCCCGTG
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B3 ITS Region Nucleotide Sequences Obtained from the Iron Ore Concentrate/Soil of the Sishen Iron Ore Mine

B3.1 KIOL1

```
1     TAGTAGAGGG ATAGCTCAGC GCCCGACCTC TCAACCCTTT GTTGTTAAAA
51    CTACCTTGTT GCTTTGGCGG GACCGCTCGG TCTCGAGCCG CTGGGGATTC
101   GTCCCAGGCG AGCGCCCGCC AGAGTTAAAC CAAACTCTTG TTATTAAACC
151   GGTCGTCTGA GTTAAAATTT TGAATAAATC AAAACTTTCA ACAACGGATC
201   TCTTGGTTCT CGCATCGATG AAGAACGCAG CGAAATGCGA TAAGTAATGT
251   GAATTGCAGA ATTCAGTGAA TCATCGAATC TTTGAACGCA CATTGCGCCC
301   CTTGGTATTC CGAGGGGCAT GCCTGTTCGA GCGTCATTAC ACCACTCAAG
351   CTAAGCTTGG ATTGGGTGCC GTCCTTAGTT GGGCGCGCCT TAAAGACCTC
401   GGCGAGGCCT CACCGGCTTT AGGCGAGTAG AATTTATTCG AACGTCTGTC
451   AAAGGAGAGG ACTTCTGCCG ACTGAAACCT TTATTTTTCT AGGTTGACCT
501   CGGATCAGGT AGGGATACCC GCTGAACTTA AGCATATCAT AAAAGCGGA
```

B3.2 KIOL2

```
1     CTCTACATTA CACTACCGTG ATCCGAGCTC TTACCTAGGA AAAATGGCGG
51    TTTCAGTCGG GAGGAAGTCC TCTCCTTTGA CAGACGTTCG AATAAATTCT
101   ACTACGCCTA AAGCCGGTGA GGCCTCGCCG AGGTCTTTAA GGGGCGCCCA
151   ACTAAGGACG GCACCCAATA CCAAGCTTAG CTTGAGTGGT GTAATGACGC
201   TCGAACAGGC ATGCCCCTCG GAATACCAAG GGGCGCAATG TGCGTTCAAA
251   GATTCGATGA TTCACTGAAT TCTGCAATTC ACATTACTTA TCGCATTTCG
301   CTGCGTTCTT CATCGATGCG AGAACCAAGA GATCCGTTGT TGAAAGTTTT
351   GATTTATTCA AAATTTTAAC TCACGACGAC CGGTTTAATA ACAAGAGTTT
401   GGTTTAACTC TGGCGGGCGC TCGCCTGGGA CGAATCCCCA ACGGCTCGAG
451   ACCGAGCGGT CCCGCCAAAG CAACAAGGTA GTTTTAACAA CAAAAGGGTT
501   GGAGGTCGGG CGCTGAGCAC CCTTACTCTT TAATGATCCT TCCGCAGGTT
551   CACCTACAA
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B3.3 KIOL3

```
1     GATGAAAAGG CTTAACTGCA TTTCTTTCTA CACATGTGTT TTTCTTTTTT
51    TGAAAACTTT GCTTTGGTAG GCCTTCTATA TGGGGCCTGC CAGAGATTAA
101   ACTCAACCAA ATTTTATTTA ATGTCAACCG ATTATTTAAT AGTCAAAACT
151   TTCAACAACG GATCTCTTGG TTCTCGCATC GATGAAGAAC GCAGCGAAAT
201   GCGATAAGTA ATATGAATTG CAGATATTCG TGAATCATCG AATCTTTGAA
251   CGCACATTGC GCCCTTTGGT ATTCCAAAGG GCATGCCTGT TTGAGCGTCA
301   TTTCTCCCTC AAACCCTCGG GTTTGGTGTT GAGCGATACG CTGGGTTTGC
351   TTGAAAGAAA GGCGGAGTAT AAACTAATGG ATAGGTTTTT TCCACTCATT
401   GGTACAAACT CCAAAACTTC TTCCAAATTC GACCTCAAAT CAGGGTAGGA
451   CTACCCGCTG AACTTAAGCA TATCAATAAA ACGCGGAA
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B3.4 KIOL4

```
1     TATGCTACGA TCCATACCCT GATTTGAGTC TGAATTTGGA AGAAGTTTTG
51    GAGTTTGTAC CAGATGAGTG GAAAAAACCT ATCCATTAGT TTATACTCCG
101   CCTTTCTTTC AAGCAAACCC ACGCGTATCG CTCAACACCA AACCCGAGGG
151   TTTGAGGGAG AAATGACGCT CAAACAGGCA TGCCCTTTGG AATACCAAAG
201   GGCGCAATGT GCGTTCAAAG ATTCGATGAT TCACGAATAT CTGCAATTCA
251   TATTACTTAT CGCATTTCGC TGCGTTCTTC ATCGATGCGA GAACCAAGAG
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301   ATCCGTTGTT GAAAGTTTTG ACTATTAAAT AATCGGTTGA CATTAAATAA
351   AATTTGGTTG AGTTTAATCT CTGGCAGGCC CCATATAGAA GGCCTACCAA
401   AGCAAAGTTT TCAAAAAAAG AAAAACACAT GTGTAAGAAA AAATGCAGTT
451   AAGCACTTTT CATTCTGTAA TGATCCTTCC GCAGGTTCAC CTACA
```

B3.5 KIOL5

```
1     AAAAGTGCTT AACTGCATTC TTTCTTACAC ATGTGTTTTT CTTTTTTTGA
51    AAACTTTGCT TTGGTAGGCC TTCTATATGG GGCCTGCCAG AGATTAAACT
101   CAACCAAATT TTATTTAATG TCAACCGATT ATTTAATAGT CAAAACTTTC
151   AACAACGGAT CTCTTGGTTC TCGCATCGAT GAAGAACGCA GCGAAATGCG
201   ATAAGTAATA TGAATTGCAG ATATTCGTGA ATCATCGAAT CTTTGAACGC
251   ACATTGCGCC CTTTGTATTC CAAAGGGCAT GCCTGTTTGA GCGTCATTTC
301   TCCCTCAAAC CCTCGGGTTT GGTGTTGAGC GATACGCTGG GTTTGCTTGA
351   AAGAAAGGCG GAGTATAAAC TAATGGATAG GTTTTTTCCA CTCATTGGTA
401   CAAACTCCAA AACTTCTTCC AAATTCGACC TCAAATCAGG TAGGGACTAC
451   CCGCTGAACT TAAGCATATC AATAAAGGCG GAA
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B3.6 KIOL6

```
1     CAGACAGATA CACAATACCT GATTTAGACG CTTAATTTGG AAGAAGTCTT
51    TGGAGTTTGA CCAATGAGTG GAAAAAACCT ATCCATTAGT TTATACTCCG
101   CCTTTCTTTC AAGCAAACCC GGCGTATCGC TCAACACCAT AACCCGAGGG
151   TTTGAGGGAG AAATGACGCT CAAACAAGGC ATGCCCTTTG GAATACCAAA
201   GGGCGCAATG TGCGTTCAAA GATTCGATGA TTCACGAATA TCTGCAATTC
251   ATATTACTTA TCGCATTTCG CTGCGTTCTT CATCGATGCG AGAACCAAGA
301   GATCCGTTGT TGAAAGTTTT GACTATTAAA TAATCGGTTG ACATTAAATA
351   AAATTTGGTT GAGTTTAATC TCTGGCAGGC CCCATATAGA AGGCCTACCA
401   AAGCAAAGTT TTCAAAAAAA GAAAAACACA TGTGTAAGAA AAAATGAGTT
451   AAGCACTTTT CATTCTGTAA TGATCCTTCC GCAGGTTCAC CTACAA
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