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Biobenefication of Sishen Hematite Iron Ore, using bacterial cultures to remove potassium (Muscovite) and phosphorous (Apatite)

By

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

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

Signed:

Date:

“Bacteria represent the world's greatest success story. They are today and have always been the modal organisms on earth; they cannot be nuked to oblivion and will outlive us all. This time is their time, not the "age of mammals" as our textbooks chauvinistically proclaim. But their price for such success is permanent relegation to a micro-world, and they cannot know the joy and pain of consciousness. We live in a universe of trade-offs; complexity and persistence do not work well as partners.”

Stephen Jay Gould (Eight Little Piggies, 1993)

Dedication

This thesis is dedicated to my grandparents and mother for their life of dedication, support and overwhelming love that inspired me to overcome the perils in life and reach my dreams.

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List of abbreviations

Al – aluminum

AMD – Acid mine drainage

ATP – Adenosine triphosphate

Ca – Calcium

Cl – Chlorine

DGGE – denaturing gradient gel electrophoresis

DNA – Deoxyribonucleic acid

Fe – Iron

g – gram

h – hour

HiPIP – High redox potential iron oxidase

K – potassium

KGT – Conglomerate ore

Li – Lithium

Mg – Magnesium

mg – Milligram

min – minute

Mtpa – million ton per annum

n/d – not determined

Ni – Nickel

P – Phosphate

PCR – polymerase chain reaction

RNA – Ribonucleic acid

rpm – revolution per minute

Rus –rusticyanin

Si – silicon

Summary

Kumba Iron Ore, Ltd. is the world's fourth largest supplier of sea-borne iron ore and currently operates two mines in South Africa namely: the Sishen mine in the Northern Cape and Thabazimbi mine in Limpopo.

The Sishen mine, located at the northern end of the Maremane anticline where the bulk of the hematite ore is buried beneath younger cover lithologies, was our focus area. Here the iron resources are made up by laminated and massive ore bodies that belong to the Asbestos Hills Subgroup. These ore bodies are overlain by conglomerates, shales, flagstone and quartzite. The alkalis, potassium and phosphorous, are common constituents of iron ore, which is known to have a deleterious effect on the manufacturing of iron and steel. Therefore steel making companies charge penalties when purchasing iron ore concentrates with alkali concentrations above predetermined levels.

To ensure that the export batches at the Sishen mine stay within set limits, the ores from different batches (with alkali concentration greater and below set limits) are mixed to produce a batch which meet requirements. However this solution will soon become ineffective as the low alkali ore is progressively depleted. Conventional methods used to treat high alkali ores include pyro- and hydrometallurgical methods. These approaches have several limitations such as poor product recovery, involvement of high process and energy cost and an increase in pollution load of water resources. Therefore necessitating research and development of alternative cheap and environment friendly procedures, which could supplement or replace conventional methods to ensure that mining stays economically feasible at the Sishen Iron Ore mine.

The application of microorganisms to minng practices is collectively referred to as biohydrometallurgy and includes bioleaching and biooxidation processes. The phrase bioleaching refers to the conversion of an insoluble metal (typically a metal sulfide) into a soluble form (typically a metal sulfate), *via* microbial activity. When metals are extracted into solution, the process is referred to as bioleaching, whereas if the metal remains in the mineral, it is referred to as biooxidation. The latter term biobeneficiation refers to the selective dissolution of undesired minerals from the ores by direct or indirect action of microbes, thereby enriching the desirable mineral content. Therefore the objective of this study was to determine whether

bacteria (naturally occurring on the ore or introduced species) could be used to selectively remove the alkalis from the iron ore mined at Sishen. The species evaluated were able to change the solution pH and/or form biofilms, which is assumed to have affected mineral mobilization. Data obtained during this study suggests that the composition of the ore plays a significant role in its susceptibility to bioleaching. Furthermore we also found that the indigenous cultures were more effective than the introduced species to mobilize the alkalis, which could possibly be ascribed to an adaptation of the microbes present.

These preliminary results suggest that bioleaching is an effective alternative cost effective approach to treat iron ore and could possibly be implemented in future into the mining schedule at Sishen.

CHAPTER 1

GENERAL INTRODUCTION

1.1 BACKGROUND

Kumba Iron Ore, Ltd. is the world's fourth largest supplier of sea-borne iron ore. The company exports 73% of its 32Mtpa production to 30 international customers, mainly in Europe and Asia. It currently operates two mines in South Africa namely the Sishen Mine in the Northern Cape and Thabazimbi Mine in Limpopo. Our focus area was the Sishen Iron Ore Mine, situated 30 kilometers from Kathu in South Africa (Figure 1). It was established in 1953 after extensive exploitation revealed the potential of the iron resource. The mine has since grown to a major supplier of iron ore to both local and international markets. Today it is one of the largest open cast mines in the world with an open pit of approximately 11km long, 1.5km wide and 400 deep according to D. Krige (Personal communication, 2006). The Sishen mine is located at the northern end of the Maremane anticline where the bulk of the hematite ore is buried beneath younger cover lithologies. Most of the iron ore resource of the mine is made up by laminated and massive ore bodies that belong to the Asbestos Hills Subgroup. These ore bodies are overlain by conglomerates, shales, flagstone and quartzite (Carney and Mienie 2003).

Figure 1. The Sishen Iron Ore Mine located in the Northern Cape, South Africa (http://www.kumba.co.za/media_gallery_sishen.php).

The mining industry is constantly confronted with several difficulties such as depletion of high grade minerals, worsening metal prices and mounting operation costs (Jain and Sharma 2004). In the recent past, iron ore was a low priced commodity which discouraged industrial adoption of hydrometallurgical beneficiation of these ores. At present an increase in global steel production has increased the requirement for iron ore, with a consequent increase in the price for this commodity, making hydrometallurgical approaches viable (Delvasto *et al*., 2008).

The iron ore mined at the Sishen Iron Ore Mine contains several different mineral phases (Table 1) which includes potassium and phosphorous. These alkalis are known to have deleterious effects on the manufacturing of iron and steel (Delvasto *et al*., 2008), therefore, steel-making companies charge penalties when purchasing iron ore concentrates with alkali concentrations above predetermined levels. The limits are determined by the steel making companies and ranges from 0.25% mass in Japan to 0.55% mass in Switzerland for potassium allowed in the iron ore concentrates. Kumba Iron Ore, Ltd., has an industrial set limit of 0.24% potassium allowed in their export ore according to C. Taljaard (Personal communication, 2006). The ore mined at different sits at the mine have varying composition and therefore different alkali concentrations. To ensure that their export batches stay within this set limit, the ores from different batches (with potassium >0.24% and <0.24%) are mixed to produce an average potassium value of below 0.24% according to D. Krige (Personal communication, 2006). However this is only a temporary solution as the low potassium ore (< 0.24%) is progressively depleted according to D. Krige (Personal communication, 2006). Certain pyro – and hydrometallurgical methods can be applied to decrease the alkali concentration (Cheng *et al*., 1999; Kokal *et al*., 2003), however there are several drawbacks when using these methods such as: poor product recovery, involvement of high process and energy cost and an increase in pollution load of water resources (Jain and Sharma 2004). Thus an alternative, natural and economical feasible process is required to aid conventional methods (such as pyro- and hydrometallurgy processes) to remove unwanted alkalis from the ores concentrates mined at Sishen.

Biohydrometallurgy[1] is an option for the removal of the deleterious phosphate and potassium, as it is well established that many microorganisms are capable of mobilizing these minerals, especially in nutrient limited environments (Banfield *et al*., 1999; Nautiyal 1999).

1.2 ALKALI BEARING MINERALS

The Sishen Iron Ore Mine is located at the Northern end of the Maremane anticline, where the bulk of the hematite iron ore is buried beneath younger cover lithologies. The ore bodies are overlain with conglomerates, shales, flagstones and quartzite (Carney and Mienie 2003). Kumba Iron Ore, Ltd. supplied some of these samples for the bioleaching experiments. The samples were labeled and characterized (Table 1) as follow: SK (shale with high potassium concentrations), KGT (conglomerates with high phosphorous concentration), SPHP (sample with high phosphorous concentration) and Export Ore (alkali concentrations at accepted limits)[2]. Mineralogy of the samples was determined by Kumba Iron Ore Ltd. according to B. Ntsoelengoe (Personal communication, 2006) and as part of the thesis objectives.

Following is a brief description of the mineral phases detected in the different iron ore samples, as to familiarize the reader with the terms used in subsequent chapters. Emphasis was placed on minerals containing the alkali's, potassium and phosphorous as well as hematite which was found to be a mayor constituent of all the iron ore samples.

1.2.1 Hematite

Hematite is the mineral form of iron (III) oxide, which serves as a major supply of iron to industry (Jorgenson and Kirk 2003). It crystallizes in the rhombohedral[3] system and has the same crystal arrangement as ilmenite ($FeTiO_3$) and corundum (Al_2O_3) (Harrison *et al*., 2006). Various forms of the mineral exist namely kidney ore, martite, iron rose and specularite

[1] Biohydrometallurgical processes include bioleaching and biobenefication (Ehrlich 1991).

[3] The term rhombohedral is used in crystallography. This crystal system is one of the seven lattice point groups, named after the two dimensional rhombus (http://en.wikipedia.org/wiki/Rhombohedral).

(Chapman *et al*., 2006). Deposits of hematite are regularly found in banded iron formation[4] (Klein 2005), but can also occur as a secondary mineral formed during weathering processes in soil (Hersman *et al*., 1995). The mineral can be colored black, silver-gray, brown or red (Figure 2-C).

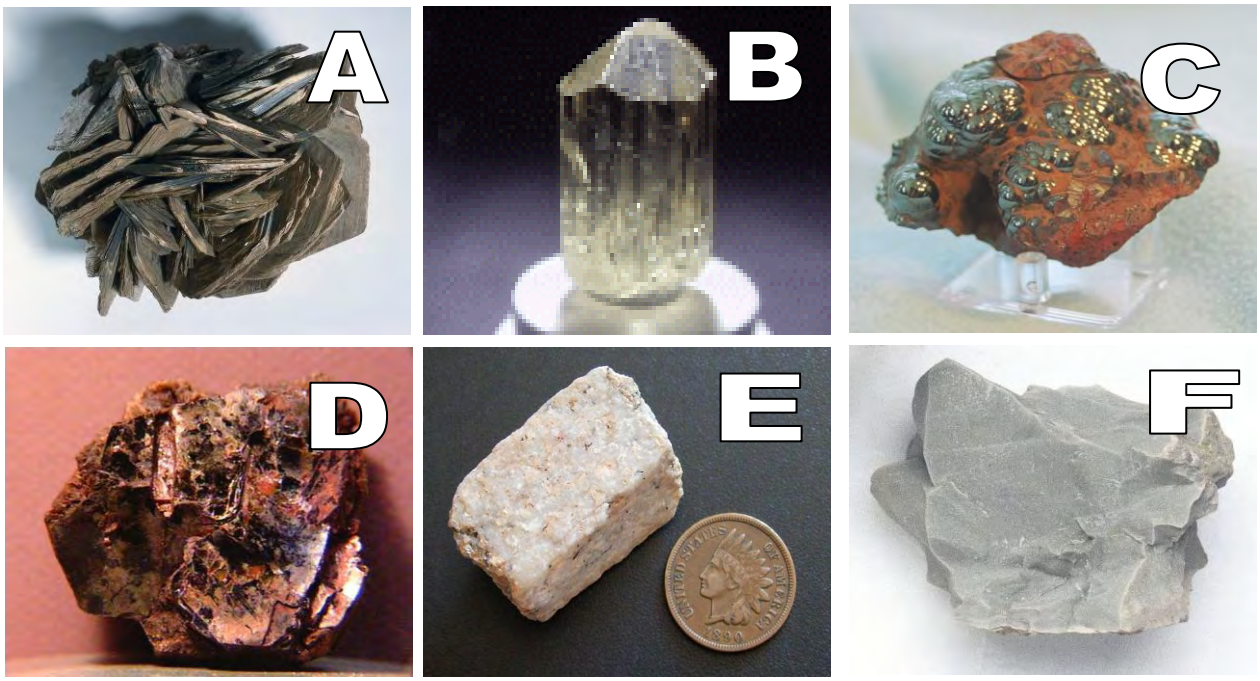

Figure 2. Illustration of the different mineral phases present in the Sishen iron ore. A – Muscovite (www.dkimages.com); B – Apatite (www.galleries.com); C – hematite (http://en.wikipedia.org/wiki/Image:Hematite.jpg); D – biotite (www.cs.cmu.edu); E – K-Feldspar (geology.about.com); F – Illite (www.dkimages.com).

1.2.2 Muscovite

Muscovite is a dioctahedral mica, which consists of tetrahedral sheets, bound by interlayer potassium and octahedral aluminum (Deer *et al*., 2001; Kalinowski and Schweda 1996; Mazzucato *et al*., 1998; McKeown *et al*., 1999). It is frequently found in igneous metamorphic[5] and detrital sedimentary rocks (Figure 2-A). Muscovite has several properties such as perfect cleavage, elasticity, high dielectric constant and low thermal conductivity, which made it ideal

[4] Banded iron formation is a typical rock type, often found in primordial sedimentary rocks. Its structure consists of repeated thin layers of iron oxides [magnetite (Fe_3O_4) or hematite (Fe_2O_3)], alternating with bands of iron-poor shale and chert (Kappler *et al*., 2005).
[5] Igneous rocks are formed by solidification of cooled magma [http://en.wikipedia.org/wiki/Igneous_rock]

for many electrical, electronic and thermal insulation processes (Deer *et al*., 2001). Rausell and coworkers (1965) determined the sensitivity of muscovite to potassium concentration in solution. They discovered that muscovite would not release interlayer potassium when it is placed in a dilute electrolyte solution. Moreover, Pal and coworkers (2001) demonstrated that if biotite and muscovite were both present in soil, no potassium dissolution from muscovite would occur. A 0.1mg/l potassium concentration in solution was found to inhibit the exchange of potassium in muscovite with other ions in solution (vermiculation) (Wilson 2004).

1.2.3 Apatite

Apatite is the most common phosphate mineral in nature (Figure 2-B). It occurs as an accessory phase in igneous, metamorphic[6] and sedimentary rocks (Welch *et al*., 2002). It is also produced by biological systems, where it is a major constituent of tooth enamel and bone material. Apatite is a group of phosphate minerals than can be categorized as three different minerals, namely hydroxylapatite, fluoroapatite and chlorapatite. The fluorine, chlorine and hydroxyl ions are able to substitute each other freely, as they are commonly found in the same specimen (Amethyst Galleries' Mineral Gallery, 1996). Apatite is somewhat insoluble at near neutral pH (Welch *et al*., 2002), however its reactivity and solubility is determined by its composition (Cazalbou *et al*., 2004). It is known that fluoroapatite is less soluble than hydroxyapatite, however the solubility and reactivity of apatite can be increase if there is a carbonate substitution into the phosphate site (Welch *et al*., 2002).

1.2.4 Biotite

Biotite is a common phyllosilicate (or layered alumino-silicate) mineral within the mica group (Douche 1993). It occurs as an essential, accessory or secondary mineral in most plutonic and volcanic igneous rocks of crystal origin (Deer *et al*., 2001). Like other mica minerals, it has a

[6] Metamorphic rock is the result of the transformation of an existing rock type (http://en.wikipedia.org/wiki/Metamorphic_rocks).

highly perfect basal cleavage and consists of flexible sheets or lamellae[7], which can easily flake off. The weathering of micas such as feldspar and biotite has been intensively studied due to their importance as potassium source for plants (Wilson 2004). Weathering of biotite can progress in one of the following ways: congruent (destruction of the mineral surface) and incongruent (transformation into vermiculite by release of interlayer potassium) (Calvaruso *et al*., 2006). The dissolution of biotite is thought to be diffusion-controlled and therefore depends on the potassium concentration in the bulk solution (Wilson 2004). The sheets in the crystal structure of biotite are made up of iron, magnesium, aluminum, silicon, oxygen and hydrogen ions that are weakly bound together by potassium. The interlayer potassium can be substituted in part by sodium, calcium, barium. Magnesium can be completely replaced by ferrous iron and ferric iron and in part by titanium and manganese (Deer *et al*., 2001). Biotite is often referred to as the iron mica as it contains more iron than phlogopite (Hashemi-Nezhad 2005). It has a monoclinic crystal system[8], with tabular to prismatic crystals with a pinacoid termination. The structure contains four prism faces and two pinacoid faces to form a pseudohexagonal crystal. The mineral can appear greenish to brown or black and even yellow when it is weathered (Figure 2-D).

1.2.5 K-feldspar

Feldspars crystallize from magma in both intrusive and extrusive igneous rocks. It also occurs as compact minerals, veins, and may also be present in many types of metamorphic rock. Feldspars ubiquity and varying composition has led to its use as the primary tool in classifying igneous rocks. It is however absent in certain rocks such as ultrabasic and rare alkaline rocks. Compositions between $NaAlSi_3O_8$ and $KAlSi_3O_8$ are referred to as alkali feldspar and those between $NaAlSi_3O_8$ and $CaAl_2Si_2O_8$ as plagioclase. Alkali feldspar is white or colorless when pure but appears pink when contaminated with iron (Figure 2-E) (Deer *at al*., 2001). The structure consists of cross-linked, 'double-crankshaft' chains of Si^{4+} and Al^{3+} tetrahedral with charge compensating such as sodium, potassium and calcium occupying small cavities in the

[7] A lamella is a gill-shaped structure: fine sheets of material held adjacent one another, with fluid in-between-(or simply 'welded'-plates)[http://en.wikipedia.org/wiki/Lamellae_(materials)]
[8] In crystallography, the monoclinic crystal system is one of the 7 lattice point groups. (http://en.wikipedia.org/wiki/Monoclinic_crystal_system)

framework (Han and Lee 2005). Alkali feldspar can be weathered into a secondary mineral kaolinite [$Al_2Si_2O_5(OH)_4$] under the influence of H^+ ions. The low solubility of aluminum at pH above 4.5 is a major reason for the slow dissolution rate of feldspar (Landeweert *et al.*, 2001). Experimental data has shown that dissolution of feldspar can either be surface-controlled or leached-layer control. In the latter, incongruent[9] dissolution occurs, thus certain ions will go preferentially into solution, whereas others will remain in the solid crystalline phase. According to this mechanism, a cation-depleted coating of silicon and aluminum (leached layer) builds up around the feldspar and controls dissolution (Wilson 2004). The possible existence of a surface controlled mechanism was illustrated by Holdren and Stillings during feldspar dissolution experiments, where no leached layer occured (Wilson 2004).

1.2.6 Illite

Illite (hydromuscovite or hydromica) is a non-expanding, clay-sized phyllosilicate or layer alumino-silicate that commonly occurs in sediments, soils and argillaceous sedimentary rocks (Figure 2-F). Its structure consists of repeated tetrahedron – octahedron – tetrahedron layers. The interlayer space is largely occupied by hydrated potassium cations which is possibly responsible for the absence of swelling during reactions. Illite is structurally similar to muscovite (Section 1.2.2) or sericite [$KAl_2(OH)_2(AlSi_3O_{10})$], with more silicon, magnesium, iron and water, and less tetrahedral aluminum and interlayer potassium in its structure. Illite occurs as an alternation product of muscovite and feldspar during weathering (Mengel and Uhlenbecker 1993).

1.2.7 Mineral dissolution

Mineral dissolution is thought to progress *via* two hypothetical mechanisms namely transport- and surface-controlled, which is influenced by different rate-limiting steps. With transport-controlled dissolution, the diffusion of the reactants or weathering products progresses through a diffusion layer, towards the mineral surface or the bulk solution (Scheckel *et al.*, 2005). Here the

[9] Incongruent dissolution – Ions in the mineral lattice do not dissolve according to their stochiometric ration.

reaction is controlled by the rate of diffusion or advection (Brantley 2004). Surface controlled (interface-controlled) dissolution is limited by chemical reactions at the surface of the mineral (Scheckel and Impellitteri 2005). As the reaction is controlled by the dissolution of the mineral, an increase in diffusion would have no effect on the reaction rate due to an absence of a concentration gradient at the mineral-water interface (Brantley 2004). Laboratory and field experiments suggest that silicate dissolution under environmental conditions is surface-controlled; however the discovery of a leached layer in many dissolving silicates has again raised the question whether dissolution is either transport or surface-controlled (Brantley 2004; Wilson 2004).

Table 1 Mineral phases present in the Sishen Hematite Iron Ore.

Iron ore sample	Mineral composition	Chemical group	Chemical formula	Mayor	Minor	Trace
Export Ore	Apatite		$Ca_5(PO_4)_3(F, Cl, OH)$	n/d		
	Hematite	Oxide	Fe_2O_3	n/d		
	K-Feldspar	Silicate	$K(Mg, Fe^{2+})_3(Al, Fe^{3+}) Si_3O_{10} (OHF)_2$	n/d		
	Muscovite	Silicate (Mica)	$KAI_2(Si_3Al)O_{10}(OH,F)_2$	n/d		
	Quartz	Silicate	SiO_2	n/d		
KGT (Conglomerate)	Greenalite	Silicate	$(Fe^{2+}Fe^{3+})_{2-3}Si_20_5(OH)_4$		X	
	Hematite	Oxide	Fe_2O_3	X		
	Illite	Phyllosilicate	$(K,H_3O)(Al, Mg, Fe)_2(Si, Al)_4O_{10}[(OH)_2,H_2O]$			X
	Muscovite	Silicate (Mica)	$KAI_2(Si_3Al)O_{10}(OH,F)_2$			X
	Nacrite	Phyllosilicate	$Al_2Si_20_5(OH)_4$			X

*n/d – not determined

Table 1 (Continue)

Iron ore sample	Mineral composition	Chemical group	Chemical formula	Mayor	Minor	Trace
SPHP (High phosphorous)	Apatite		$Ca_5(PO_4)_3(F, Cl, OH)$	n/d		
	Hematite	Oxide	Fe_2O_3	n/d		
	Muscovite	Silicate (Mica)	$KAI_2(Si_3Al)O_{10}(OH,F)_2$	n/d		
	Quartz	Silicate	SiO_2	n/d		
SK (shale)	Biotite	Silicate	$KAl_2(Si_3Al)O_{10}(OH,F)_2$		X	
	Greenalite		$(Fe^{2+}Fe^{3+})_{2\text{-}3}Si_20_5(OH)_4$			X
	Hematite	Oxide	Fe_2O_3	X		
	Illite	Phyllosilicate	$(K,H_3O)(Al, Mg, Fe)_2(Si, Al)_4O_{10}[(OH)_2,H_2O]$			X
	Magnetite	Oxide	Fe_3O_4			X
	Muscovite	Silicate (Mica)	$KAl_2(Si_3Al)O_{10}(OH,F)_2$			X
	Nacrite	Phyllosilicate	$Al_2Si_20_5(OH)_4$			X
	Quartz	Silicate	SiO_2			X
	Siderite	Calcite	$FeCO_3$			X

*n/d – not determined

1.3 IRON ORE PRODUCTION

Pyrometallurgical processes have been used for decades for the recovery of base metal values such as copper, lead, zinc, etc. from scrap and secondary materials. The traditional process includes: reverberatory -, rotary kiln (short rotary furnace), electric-, slag fuming – and blast furnaces. In addition to these methods new, more intense pyrometallurgical processes have been designed and include the following: Flash smelting, Bath smelting and Top blown rotary converter (Hancock and Peacey 1994). Following is a brief overview of iron production using a blast furnce, as well as the importance of alkalis in iron manufacturing and living cells. The importance of the alkalis to living organisms is covered in this section, as the main objective of this study is to remove the unwanted alkalis from the ores *via* microbial processes.

The iron ore is smelted in a blast furnace at 1500°C (Figure 3). Ore, coke (carbonaceous material) and limestone flux are continuously supplied through the top of the furnace (Figure 3-6), while air (or pure oxygen) is blown into the bottom of the chamber (Figure 3-1). The main chemical reaction producing molten iron is: $Fe_2O_3 + 3CO \rightarrow 2Fe + 3CO_2$. The air (or pure oxygen) blown into the furnace reacts with the coke (carbon source) to produce carbon monoxide and heat. The carbon monoxide then reacts with the iron oxide (found in the iron ore) to produce molten iron and carbon dioxide. The carbon dioxide, unreacted carbon monoxide and nitrogen from the air passes up through the furnace as fresh feed material travels down into the reaction zone (Figure 3-2). As the material travels downward, the counter-current gases preheat the feed, decompose the limestone to calcium oxide and begin to reduce the iron oxides (Figure 3-5). "Four uptakes" allow the gases to exit the furnace (Figure 3-7 and Figure 3-11). The gases are passed through a "dust catcher", to allow small particles to be removed, and a gas cooling unit, before the gas is released into the atmosphere. The end products, molten metal and slag phases are tapped from the bottom of the furnace (Figure 3-10).

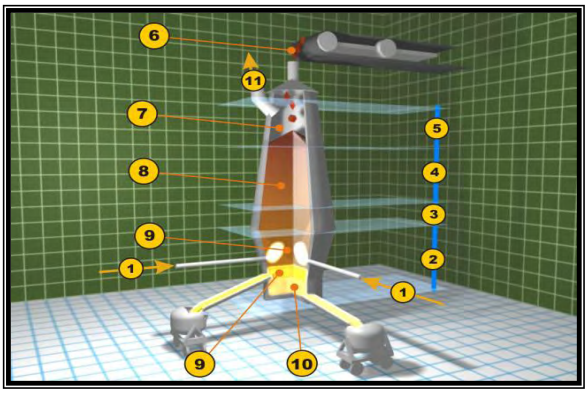

Figure 3. Blast furnace diagram. (1) Hot blast from Cowper stoves; (2) Melting zone (bosh); (3) Reduction zone of ferrous oxide (barrel); (4) Reduction zone of ferric oxide (stack); (5) Pre-heating zone (throat); (6) Feed of ore, limestone and coke; (7) Exhaust gases; (8) Column of ore, coke and limestone; (9) Removal of slag; (10) Tapping of molten pig iron; (11) Collection of waste gases (http://en.wikipedia.org/wiki/Blast_furnace#Chemistry).

1.3.1 Effect of alkali

The alkalis that commonly circulate through the blast furnace interior *via* gaseous phases and their effect on the blast furnace are listed in Table 2. The alkali, potassium in the iron ore concentrates, poses the greatest problem for metal production at the Sishen Iron Ore Mine according to D. Krige (Personal communication, 2006), and will therefore be the main focus of the current study.

Potassium effect the smelting process as follows: Alkali metals deposited on the surface of the coke (carbonaceous mineral) during iron production act as a catalyst which intensifies the gasification of carbon in the presence of carbon dioxide. This shifts the gasification reaction toward lower temperatures and reduces the strength of the coke. In addition to speeding up the gasification reaction, potassium present in the pores and cracks of coke leads to the formation of compounds such as $K_2O.SiO_2$ and $K_2O.Al_2O_3.2SiO_2$, which increases the volume of the coke and leads to its subsequent fracture. Furthermore potassium also affects the lining of the blast furnace. Once it condensates on the surface of the lining; it actively penetrates the monolithic alumosilicate structure and alters it; forming minerals such as silicide ($K_2O.Al_2O_3.6SiO_2$) or leucite ($K_2O.Al_2O_3.4SiO_2$) (Yusfin *et al.*, 1999). This alteration causes the lining to become soft and eventually leads to the mechanical weakening of the furnace (Yusfin *et al.*, 1999).

Table 2 Alkalis and their role in the blast furnace ("Blast furnace phenomena and modeling" 1987).

Alkali	Effect on blast furnace
Sodium	Forms Ansatz Behavior of softening-melting of ores Changes coke properties
Potassium	Forms Ansatz Behavior of softening-melting of ores Changes coke properties
Zinc	Forms Ansatz
Sulphur	Behavior of softening-melting of ores Changes composition of the hot metal
Silicon	Increase of last pressure due to overheating at the tuyere front Changes composition of the hot metal

1.3.2 Importance of phosphorous and potassium to living cells

Phosphorous and potassium are important nutrients for all living organisms. Potassium is a major intracellular cation, which plays an essential role in the metabolism of heterotrophic and chemoautotrophic bacteria (Barreto *et al*., 2003). Phosphorous forms a major component of DNA, RNA, ATP, ribosomes, microbial cell surfaces and other cell materials (Madigan *et al*., 2002). It is a common growth limiting nutrient for organisms in many environments and can therefore affect their ability to leach minerals (Martin 1991; Seeger and Jerez 1993; Tuovinen 1990). Consequently organisms have devised several strategies to survive in low nutrient conditions and to obtain these limiting nutrients from their environment, for example *Escherichia coli* has an emergency system known as the Pho regulon, consisting of a number of genes coding for proteins that allow the bacteria to scavenge traces of usable phosphate sources from the environment (Makino *et al.*, 1994; Wanner 1996). Furthermore, Seeger and Jerez (1993) found that the iron oxidizing bacterium *Acidithiobacillus ferrooxidans* is able to cope with phosphate limiting conditions by: Firstly decreasing its growth rate to increase ferrous iron oxidation and carbon dioxide fixation; and secondly by increasing the expression of certain proteins which could possibly play a role in scavenging for available phosphorous. Hinsinger and Gilkes (1997) identified a phosphatase enzyme, which enabled microorganisms to

cleave phosphates from organophosphates, thereby enabling them to utilize it. However, when orthophosphate and organophosphates are limited in the environment, microorganisms are forced to scavenge for other nutrient sources (Wilson 2004). Rogers *et al*. (1998) found that if feldspar contained trace amounts of phosphorous as apatite inclusions, it would be highly colonized by microorganisms and caused the mineral surface to become etched. They further discovered that the feldspar samples which, did not contain apatite inclusions but had a similar bulk composition, were not colonized by microorganisms. Therefore they concluded that it is possible that the microorganism could identify the phosphatase in the mineral and actively remove and incorporate the phosphates into their metabolism. Several reports have demonstrated that microorganisms are able to accelerate the release of dissolved phosphate to solution from rock phosphate by producing inorganic and organic acids (Drever and Vance 1994; Hinsinger and Gilkes 1997; Jennings 1995; Margolis and Moreno 1992). Therefore we hypothesize that when microorganisms are grown under potassium and phosphorous limiting conditions, they could possibly remove these limiting nutrients from the Sishen hematite iron ore.

1.4 EXPERIMENTAL APPROACH

The mining industry constantly faces various challenges such as depletion of high grade minerals, worsening metal prices and mounting operation costs (Jain and Sharma 2004). In the past, iron ore was a low priced product which discouraged industrial adoption of hydrometallurgical beneficiation of these ores. However due to an increase in global steel production, the requirement of iron ore increased, with a consequent increase in the commodities value, making research and application of alternative processing and treatment processes viable (Delvasto *et al*., 2008).

The iron ore mined at the Sishen Iron Ore Mine contains several phases of minerals (Apatite, biotite, illite and muscovite) which contains the alkali's potassium and phosphorous. Alkali's have a deleterious effect on the manufacturing of iron and steel (Delvasto *et al*., 2008), forcing companies to charge penalties when purchasing iron ore concentrates with alkali concentrations above certain levels. This urged preliminary research into an alternative, cheap and environmentally friendly approach to treat high alkali ore bodies. Kumba Iron Ore, Ltd. faces a severe crisis within the next 3-4 years, as the low alkali ore becomes more depleted according to

R. Grunewaltd (Personal communication, 2006). This spurred additional research into optimizing the current approaches reported in this thesis. Our preliminary experiments were to test the ability of iron oxidizing, heterotrophic (Figure 4) and indigenous bacteria to remove the unwanted substances (biobenefication[10]). The applicability of biohydrometallurgy to remove alkali from iron ore was tested during this study. Several reports supported the hypothesis that bacteria might be able to mobilize the alkalis.

Microbes and minerals are closely linked, such that one often cannot exist without the other in nature (Lower *et al*., 2001). Several researchers have commented on the role that microorganisms play in the cycling of elements and sorption of metals (Langley and Beveridge 1999), the dissolution of minerals (Banfield and Hamers 1997; Barker *et al*., 1998; Bennett *et al*., 2001; Edwards *et al*., 1998; Stone 1997) and mineral crystallization (Fortin and Beveridge 1997; Fortin *et al*., 1997; Warren and Ferris 1998). Experiments done by Bennett (2001) provided evidence that silicate weathering by bacteria is sometimes driven by the nutrient requirements of the microbial consortium. This suggested that the weathering of a mineral may be influenced by its nutritional potential, with the microorganisms destroying only the beneficial minerals. The researchers further commented that the microorganisms could thus directly benefit from the weathering of specific minerals. Other research supporting the findings showed that microorganisms would colonize various different minerals such as feldspar (Bennett *et al*., 2001; Rogers *et al*., 1998), when nutrient became limiting in the environment. More recently Rogers and Bennett (2004), further supported the idea that microbes would preferentially leach minerals with beneficial nutrients. They found that in petroleum contaminated aquifers; silicate glasses that contained phosphorous and iron were preferentially colonized compared to glasses that did not contain these nutrients.

Krebs *et al*. (1997) listed three principles that enable microorganism to leach and mobilize metals from solid material, namely: (i) redox potential; (ii) the formation of organic and inorganic acids; and (iii) the excretion of complexing agents.

In 1997 Bosecker commented that the dissolution of non-sulfidic ores can be achieved by exploiting the metabolic capabilities of heterotrophic microorganisms (Table 3). These

[10] Biobeneficiation refers to the selective dissolution of undesired minerals from the ores by direct or indirect action of microbes, thereby enriching the desirable mineral content (Deo *et al*., 2001; Vasan *et al*., 2001)

organisms produce metabolites that are possibly able to solubilize and/or aid in solubilizing oxide, silicate, carbonate and hydroxide minerals (Ivarson 1981; Jain and Sharma 2004).

Therefore we chose bacteria with different nutritional requirement (chemolithotrophic and heterotrophic) to conduct our experiments. The specific strains for this study will be discussed in subsequent chapters. Furthermore the ability of indigenous bacteria to remove the alkali from the ore was tested, as it is hypothesized that these organisms might be adapted to scavenge for limiting nutrients. The most important parameter included in the heterotrophic (Chapter 5) and indigenous (Chapter 6) leaching experiments, is to starve the cells for potassium, as this could possibly increase the expression of genes that might enable the organisms to remove the nutrient from the ore.

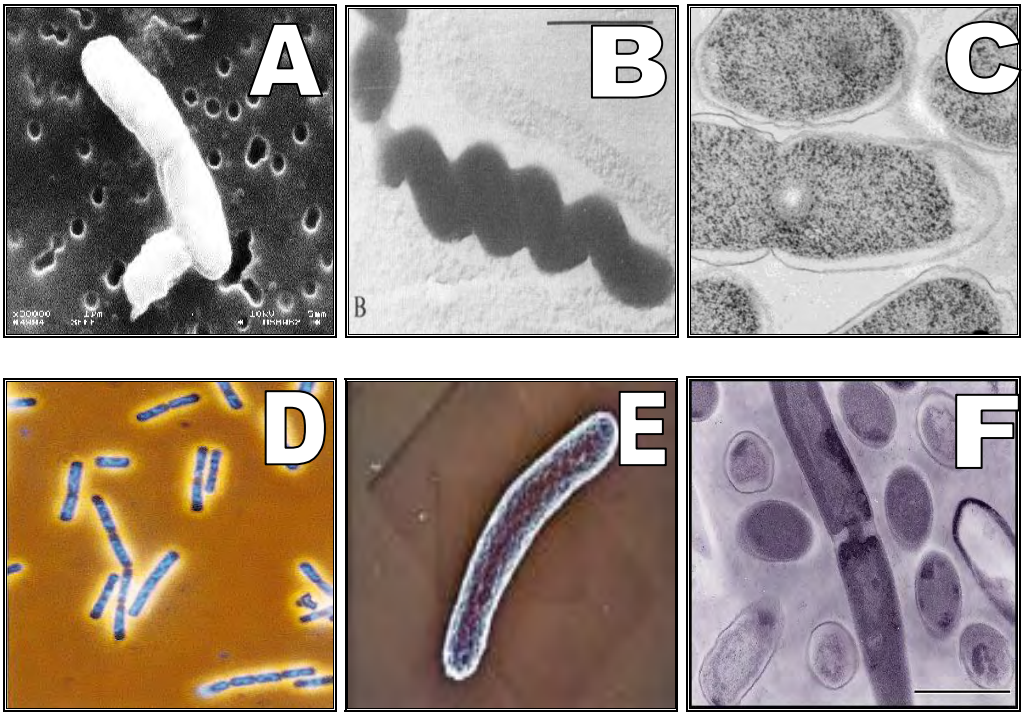

Figure 4. Illustration of bacteria selected from literature. A – *Acidithiobacillus ferrooxidans* (www.geosfreiberg.de); B – *Leptospirillum ferrooxidans* (www.microbewiki.kenyon.edu); C – *Pseudomonas putida* (www.micro.iastate.edu); D – *Bacillus cereus* (www.uio.no); E – *Bacillus megaterium* (www. db2.photoresearchers.com); F – *Bacillus subtilis* (www. sci.agr.ca).

Table 3 Patents on biohydrometallurgical processing of ores. Adopted from Krebs *et al*. (1997).

Type of ore	Metals recovered	Microorganisms	Reference
Iron ore	Fe	*Pseudomonas* species	Hoffmann *et al*., 1988
Gold ore	Au	*Chromobacterium violoceum* *Chlorella vulgaris*	Kleid *et al*., 1990
Carbon containing gold ore	Au	*Thiobacillus* species heterotrophic fungal and bacterial strains	Portier 1991
Sulfidic gold ore	Au, Ag, Cu	*Thiobacillus ferrooxidans*	Reid and Young 1991
Manganiferous silver ore	Ag	*Bacillus* species *Bacillus polymyxa*	Rusin 1992
Sulfidic ore	Au, Ag, Cu	*Thiobacillus* species *Leptospirillum ferrooxidans*	Hill and Brierley 1992
Sulfidic ore	Au, Ag, Pt	Sulfate and hydrogen reducing bacteria	Hunter *et al*., 1996

The objectives for this study were as follow:

- Quantitative and qualitative mineralogy survey on the different iron ore samples (KGT, SK, SPHP and Export ore).
- Determine effectiveness of organic and inorganic acids for leaching potassium and phosphorous from the export, KGT, SK and SPHP iron ore mined at Sishen.
- Test effectiveness of iron oxidizing bacteria for removing potassium and phosphorous from the Export ore.
- Test effectiveness of heterotrophic bacteria for removing potassium and phosphorous from the Export ore.

- Survey the effectiveness of indigenous bacteria to remove potassium and phosphorous from KGT, Export and SK samples.
- Determine bacterial community of indigenous bacteria enriched for during the biobenefication experiment.

CHAPTER 2

LITERATURE REVIEW

IRON OXIDIZING BACTERIA AND THEIR IMPORTANCE IN THE BIOMINING INDUSTRY

2.1 INTRODUCTION

Biohydrometallurgical processes include bioleaching and biobenefication (Ehrlich 1991). The phrase bioleaching refers to the conversion of an insoluble metal (typically a metal sulfide) into a soluble form (typically a metal sulfate), *via* microbial activity (Rawlings 2002). When metals are extracted into solution, the process is typically referred to as bioleaching, whereas if the metal remains in the mineral, it is referred to as biooxidation (Rawlings 2005). The latter term biobenefication refers to a process in which microorganisms are used to selectively remove undesirable mineral components from ores (Ehrlich 1991). The solubilization process is considered to be largely a chemical process, with the microorganisms providing the chemicals (Table 4) and the space where the mineral dissolution reaction occurs (Gehrke *et al*., 1998; Rawlings 2005). These processes utilize biogeochemical activities of which various bacteria and fungi are capable. The function of bacteria is to act as a catalyst of the dissolution reaction or generator of metabolic products, which causes chemical dissolution. Whereas fungi exclusively act as a generator of metabolic products that causes chemical dissolution of metal values (Ehrlich 1991).

Bioleaching is a technology that is applicable to metal extractions from samples such as: low-grade ores, ore benefication, coal benefication, metal detoxification and recovery of metals from waste materials (Bosecker 2001; Jain and Sharma 2004). Except for its industrial application to raw materials, microbial leaching has several other potential uses such as remediation of mining sites, treatment of metal containing waste products and detoxification of sewage sludge (Bosecker 2001).

Table 4 Examples of bacterial and fungal metabolic products with a potential for leaching metals from ores non-enzymatically.

Compound	Organism source	Mechanism of action	Reference
Fe^{3+} + H_2SO_4	Acidophilic iron oxidizing bacteria	Oxidation of metal sulfides	Ehrlich 1991
Nitric acid	Nitrifying bacteria	Acidulation	Ehrlich 1991
Sulfuric acid	Thiobacilli and Acidithiobacilli	Acidulation	Ehrlich 1991
Formic acid	*Arthrobacter agilis*	Reduction of oxides	Liermann *et al*., 2000
Oxalic acid	Fungi *Arthrobacter* species *Paenibacillus stellifer*	Acidulation; Complexation	Ehrlich 1991 Liermann *et al*., 2000
Acetic acid	*Acetobacter* species *Achromobacter* species *Arthrobacter* species *Clostridium* species *Paenibacillus stellifer*	Acidulation Complexation	De Faveri *et al*., 2003 Ehrlich 1991 Liermann *et al*., 2000 Ma *et al*., 2008 Tabak *et al*., 2005 Welch *et al*., 2002
Lactic acid	Fermenting bacteria	Acidulation	Ehrlich 1991
Pyruvic acid	*Bacillus megaterium* *Micrococcus luteus*	Complexation	Ehrlich 1991 Urzi *et al*., 1991
Succinic acid	*Micrococcus luteus*	Acidulation	Ehrlich 1991 Urzi *et al*., 1991
Citric acid	Fungi *Arthrobacter* species	Acidulation	Ehrlich 1991 Liermann *et al*., 2000
2-ketogluconic acid	*Erwinia herbicola* *Pseudomonas* species	Complexation	Ehrlich 1991 Krebs *et al*., 1997
Siderophores	Various bacteria and fungi	Complexation	Ehrlich 1991
Exopolysaccharides	Various bacteria	Reaction with silicates	Ehrlich 1991

Conventional methods used to process and remove impurities from ores include pyrometallurgical and hydrometallurgical methods. The application of these process poses several difficulties such as: emission of sulfur – and carbon dioxide, disposal of arsenic wastes, consumption of fresh water (Scaife 1994), poor product recovery and high process and energy cost (Jain and Sharma 2004). In contrast biohydrometallurgical processes have several advantages over conventional mining procedures, such as: it does not require high amounts of

energy as with roasting and smelting; the process does not produce harmful gaseous emissions such as sulfur dioxide (Rawlings 2002), the technology is relatively inexpensive and safe (Jain and Sharma 2004). However acid mine drainage can be generated by certain microorganisms, which in turn can harm the environment if it is not properly controlled (Olson *et al*., 2003; Rawlings 2005). Bioleaching will however not completely replace conventional methods, because: firstly the bioleaching process does not recover precious metals from the ores which are often an essential component in the profitability of the operation; and secondly when ore bodies do not contain sufficient acid consuming minerals, the residual acids generated have to be neutralized during the leaching process, thus increasing the operational cost (Dreshner 2004).

Leaching of sulfidic minerals using chemolithotrophic bacteria are the best studied and commercially exploited biotechnology today (Jain and Sharma 2004). Iron oxidizing bacteria have been applied in laboratory and/or large scale heap processes to solubilize insoluble metal sulfates of copper (Brierley and Brierley 2001; Pinches *et al*., 1997; Qiu et a. 2005), cobalt, gold (Aswegen 1993; Olson 1994), nickel (Dew and Miller 1997), zinc (Kai *et al*., 2000) and uranium (Khalid *et al*., 1993) have been solubilized in the laboratory or in large-scale heap or tank aeration processes (Amankwah 2005; Rawlings 2002; Rawlings *et al*., 2003). The metabolic capability of iron oxidizing bacteria has been exploited in several designed industrial scale processes such as the BIOX® process (Van Aswegen *et al*., 2006), GEOCOAT^{TM} (Harvey *et al*., 2002), BacTech^{TM} process (Olson *et al*., 2003) and the BioCOP^{TM} process (Batty and Rorke 2006). These microorganisms produce chemicals such as ferric iron and sulfuric acid from the ferrous iron and sulfur contained in the mineral or solution (Rawlings *et al*., 2003). The ferric ion serves as an oxidation attack on the mineral (Takai *et al*., 2001), whereas sulfuric acid is responsible for a proton attack (Rawlings 2005). Furthermore bacteria are able to leach minerals possibly *via* two proposed systems namely ‘contact leaching’ and ‘non-contact leaching’. With contact leaching biofilms are produced. Here the extracellular polymeric substance can function in the following way: it can mediate attachment to a (metal) sulfide surface and it may concentrate the ferric ions by complexation through uronic acids or other residues at the mineral surface, thereby allowing an oxidative attack on the sulfide (Sand and Gehrke 2006).

The focus of this review is the iron oxidizing bacteria currently used in industry. Reference will be made to: general characteristic of biomining organisms; factors affecting leaching (both microbial and physiochemical); microbial oxidation and leaching mechanisms; iron oxidizing bacteria currently used in industry and candidates with future prospects; and acid mine drainage formation and treatment strategies.

2.2 IRON OXIDIZING BACTERIA

2.2.1 General characteristics

The microorganisms currently employed in biomining operations have several characteristics in common (Table 5). These abilities enable them to leach the minerals or impurities from the ores and enable them to survive in the biomining process environment. For example with a passive process such as heap leaching (Section 2.6.1), all the requirements of the leaching organisms are met. The air in the heap provides the carbon source in the form of carbon dioxide and the preferred electron acceptor (O_2). The mineral leached supplies the electron donor (ferrous iron and/or inorganic sulfur) (Section 2.2.2) and the water used to irrigate the heap, functions as the growth medium (Rawlings 2002). Some species of biomining organisms are able to fix nitrogen from the air (Section 2.2.3); however, they may not be able to do so in a highly aerated environment (Rawlings 2002). In commercial processes, small quantities of inexpensive, fertilizer-grade, ammonium sulfate and potassium phosphate may be added to ensure that nutrient limitation does not occur (Rawlings 2002). Due to the few requirements of these organisms, it has become economical feasible to leach low-grade ore.

Table 5 General characteristics of biomining organisms.

Ability	Comment	Reference
Able to produce ferric iron and sulfuric acid.	Compounds play an essential role in solubilizing metals from metal-bearing mineral.	Rawlings 2005
Autotrophic organisms	Organisms have a high requirement for compounds such as NAD(P)H to reduce their carbon source to produce sugars, nucleotides, amino acids and other molecules from which new biomass is formed.	Rawlings 2005
Chemolithotrophic	Able to use minerals from the leached ores.	Rawlings 2002
Acid tolerant	Although the external pH is low, the internal pH of the organism is close to neutral. These organisms have to maintain a high proton gradient, implying a high ATP (adeonosine triphosphate) cost. White (1995) has suggested that iron oxidizing bacteria regulate their internal pH by consuming cytoplasmic protons during respiration rather than by proton pumping across the membrane as with other aerobic microorganisms. A low external pH is required for metabolic activity, because at a pH greater than 5, ferrous iron will rapidly be oxidized to ferric iron, thus the cells struggle to compete with chemical oxidation. However organisms have been characterized that are able to oxidize iron at different pH than the norm. This includes organism that can oxidize iron near neutral pH, or archae that can oxidize iron at pH 0.5.	Emerson and Moyer 1997 Fortin *et al*., 2005 Mignane *et al*., 2004 Migone and Donati 2004 White 1995
Variable electron acceptors	Organisms more adaptable to environment.	Rawlings 2002
Resistant to a range of metals such as arsenic, copper, zinc cadmium and nickel.	Certain species poses a Pho regulon, which is unregulated during phosphate starvation. This mechanism plays a crucial role in metal resistance. Metals such as copper stimulate polyphosphate hydrolysis, which eventually forms metal-phosphate complexes, which is then transported, out of the cell.	Rawlings 2005 Valenzuela *et al*., 2005 Vera *et al*., 2003
Oxidize under anoxic conditions.	Organism more adaptable to environment	Sand 2003

2.2.2 Leaching mechanisms

Bioleaching is the use of microorganisms to solubilize minerals from ores (Rawlings 2002). The solubilization is thought to be mainly a chemical process, with the organisms supplying the chemicals and the space in which the reaction occurs (Gehrke *et al.*, 1998; Rawlings 2005). The iron oxidizing bacteria supplies ferric iron and/or acids, typically sulfuric acid (proton hydrolysis attack), which acts upon the minerals (Rawlings 2005; Takai *et al*., 2001). Following is a general description of the ferrous (production of ferric iron) and sulfur oxidation (production of sulfuric acid) mechanisms of iron oxidizing bacteria.

2.2.2.1 Ferrous oxidation

Under aerobic conditions, ferrous iron (Fe^{2+}) is spontaneously oxidized to ferric iron (Fe^{3+}), unless the pH is low (White 1995). Lacey and Lawson (1970) found that if the solution pH was below 2, the oxidation kinetics of ferrous ion to ferric iron is low; however when *A. ferrooxidans* was inoculated into the solution the reaction increased 5 to 6 times. The ferrous/ferric iron redox couple has a positive standard electrode potential and therefore only oxygen can act as a natural electron acceptor (Reaction 1) and iron as an electron donor under aerobic conditions (Rawlings 2005).

Reaction 1: $Fe^{2+} + H^{+} + ½O_2 \longrightarrow Fe^{3+} + ½H_2O$

Acidophilic bacteria are able to use ferrous iron as an electron acceptor. The difference between the ferrous/ferric iron and oxygen/water redox couples are small, and only one mole of electrons are released per mole of iron (Reaction 1), therefore a lot of ferrous iron is needed to sustain bacterial life. The large quantities of ferrous iron required are not transported into the cell, but only deliver its electron to a carrier situated in the cell envelope. The iron oxidation mechanism of *Acidithiobacillus ferrooxidans* has been extensively studied (Rawlings 2005). The proposed model is illustrated in figure 5. *A. ferrooxidans* contains a *rus* operon, which is suspected to encode for the electron transport chain that is used during oxidation (Appia-Ayme *et al*., 1999). This operon consists of genes for an aa_3-type cytochrome oxidase (Kai *et al.*, 1992; Yarzábal *et al*., 2002), an outermembrane located cytochrome-c (Cyc2) (Sugio *et al*., 1998), a c_4 type cytochrome (Cavazza *et al.*, 1996) , a copper-containing protein rysticyanin and an open

reading frame proposed to encode a periplasmic protein of unknown function (Rawlings 2005). Yarzábal and coworkers (2004) found that the expression of the *rus* operon was 5 to 25 times higher when *A. ferrooxidans* was grown on iron, compared to when it was grown on only sulfur. Sand *et al*. (1993) proposed that the rusticyanin serves as an electron reservoir that readily takes up electrons available at the outer membrane and channels them down the respiratory pathway. Furthermore rusticyanin also possibly functions as a redox buffer which ensures the cytochrome-*c* (electron acceptor) remains in a fully oxidized state, to readily receive electrons from ferrous iron. Kusano *et al*. (1992) commented that rusticyanin is clearly involved in iron oxidation however it is not yet known whether the components of the operon are sufficient for iron oxidation, electron transport, or whether other components such as the *iro* gene for a high redox potential iron oxidase (HiPIP) might also play a role. HiPIP is not present in all *A. ferrooxidans* strains, and might therefore play a bigger role in sulfur oxidation (Rawlings 2005). Research indicated that the iron oxidation mechanisms between iron oxidation species such as *Acidithiobacillus ferrooxidans, Leptospirillum ferrooxidans, Sulfobacillus metallicus* and *Metallosphaera sedula* all differ (Rawlings 2005). *L. ferrooxidans* is known to lack a rusticyanin gene, but has a soluble acid stable cytochrome *a* gene. The cytochrome-*a* is known to be reduced directly by sulfato-iron (II) (Takai *et al*., 2001).

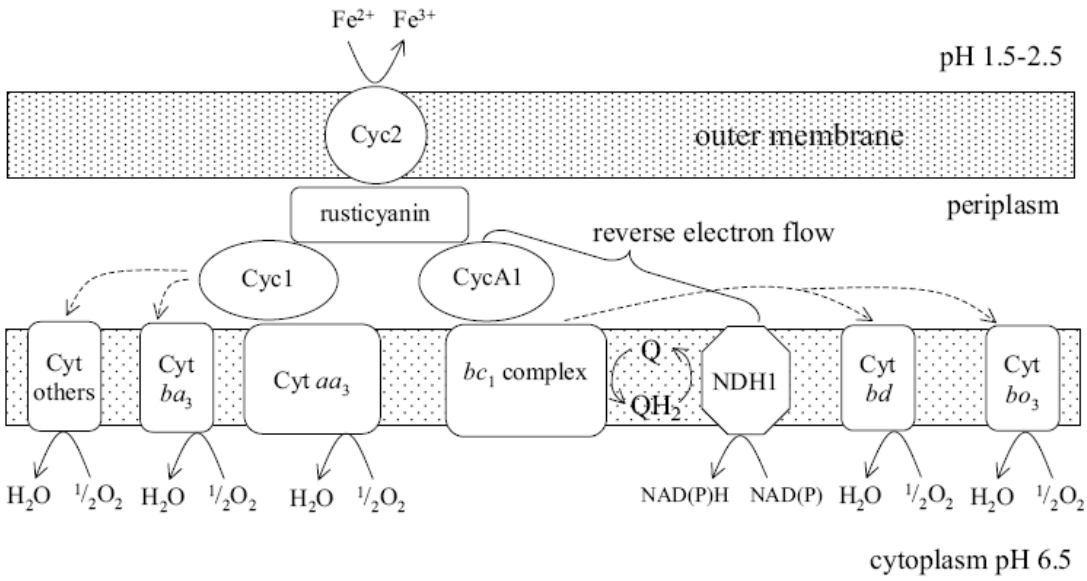

Figure 5. Model of the iron oxidation electron transport pathway of *A. ferrooxidans* (Rawlings 2005).

2.2.2.2 Sulfur as an energy source

Acidophilic bacteria grow in environments with a low pH. These organisms produce several acids, with sulfuric acid being the most important (Rawlings *et al.*, 2003). Sulfuric acid is produced by the oxidation of reduced inorganic sulfur compounds. For biological oxidation to occur, the reduced inorganic sulfur compounds serve as an electron donor with oxygen serving as the electron acceptor. Pronk and coworkers (1990) determined that more metabolic energy was made when bacteria oxidizes sulfur, than when they oxidize iron.

Rohwere and Sand (2003) proposed the following mechanism for sulfur oxidation (Figure 6). Firstly extracellular elemental sulfur is mobilized by the thiol groups of a specific outer membrane proteins and transported into the cytoplasm as persulphide sulphane sulfur. The persulphide sulfur is oxidized to sulfate by a sulphite:acceptor oxidoreductase with the electrons most likely being transferred to cytochromes. Glutathione plays a catalytic role in sulfur activation, but is not consumed during eynzymic sulphane sulfur oxidation. Sulphide oxidation requires the disulpide of glutathione persulphide prior to enzymaticc oxidation. The free sulphide is oxidized to elemental sulfur in the periplasm by a separate sulphide:quinine oxidoreductase. Reaction with the thiol groups of the outer membrane proteins keeps the zero valence sulfur from precipitating. Several other proteins involved in sulfur oxidation have also been identified including a sulfur dioxygenase, a rhodanase and an outer membrane protein. There are indications that there may be more uniformity in the pathways used by the gram-negative sulfur oxidizing bacteria, than there is in iron oxidation pathways (Rawlings 2005).

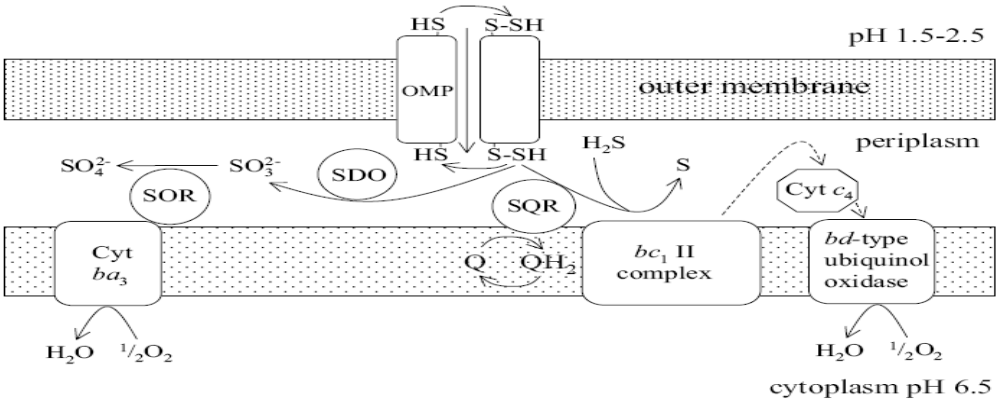

Figure 6. Model of the sulfur oxidation electron transport pathway of *A. ferrooxidans* (Rawlings 2005).

2.2.3 Iron oxidizing bacteria currently used in the biomining industry

Iron oxidizing bacteria have been used in different commercial processes such as the BIOX®, BACTECH™, GEOCOAT™ and BACFOX™ process. These organisms have been isolated from various locations such as: sulfur springs (Maeda *et al.*, 1999), hot-spring biomats (Belkova *et al.*, 2004), acid mine drainage sites (Fowler *et al.*, 1999; Maeda *et al.*, 1999; Rawlings 2005), corroded concrete (Maeda *et al.*, 1999; Terunobu *et al.*, 1999), roots from Wetland plants (Emerson *et al*., 1999) and water systems (Coetser and Cloete 2005), etc. The following section will assess iron oxidizing bacteria found in non-sterile commercial or pilot scale processes.

2.2.3.1 *Acidithiobacillus*

These organisms were previously placed in the genus *Thiobacillus*, but due to 16S rRNA sequence results, it became clear that this genus contained sulfur oxidizing bacteria that belonged to α-, β and γ-division of the *Proteobacteria* (Lane *et al*., 1992). To solve this problem, the genus was subdivided (Kelly and Wood 2002) and a new genus *Acidithiobacillus* was created, which includes all the highly acidophilic organisms. This new genus includes the organisms *Acidithiobacillus ferrooxidans*, *Acidithiobacillus thiooxidans* and *Acidithiobacillus caldus*. These bacteria appear to be ubiquitous and have been isolated from various sites such as: sulfur springs (Maeda *et al.*, 1999), hot-spring biomats (Belkova *et al.*, 2004), acid mine drainage sites (Fowler *et al.*, 1999; Maeda *et al.*, 1999; Rawlings 2005), corroded concrete (Maeda *et al.*, 1999; Terunobu *et al.*, 1999), roots from Wetland plants (Emerson *et al*., 1999) and water systems (Coetser and Cloete 2005).

2.2.3.2 *Acidithiobacillus ferrooxidans*

Acidithiobacillus ferrooxidans was first isolated from an acid mine in 1947 (Colmer and Hinkle 1947). It was the first bacterium discovered that was able to oxidize minerals (Rawlings 2002) and is to date the most studied (Battaglia *et al*., 1997). It is a mesophilic, rod-shaped, gram-negative, motile (Ohmura *et al.*, 1996) and non-sporeforming bacterium. It has an optimal pH range of 1.5-2.5 and temperature range of 28-37°C (Niemelä *et al*., 1994; Razzell *et al*., 1962; Solisio *et al*., 2002), with a maximum of 46°C (Nemati *et al*., 1989). The low pH makes the environment inhospitable to other bacteria

(Mason and Rice 2002). Although the external pH is low, the internal pH of the organism is close to neutral. Therefore these organisms have to maintain a high proton gradient implying a high ATP (adenosine triphosphate) cost. White (1995) suggested that iron oxidizing bacteria regulate their internal pH by consuming cytoplasmic protons during respiration rather than by proton pumping across the membrane as with other aerobic microorganism. A low external pH is required for metabolic activity, because at a pH greater than 5, ferrous iron will rapidly be oxidized to ferric iron, thus the cells struggle to compete with chemical oxidation. Kupka and Kupsăvo (1999) demonstrated that the oxidation of ferrous ions by *A. ferrooxidans* decreased when the pH exceeded 2.5.

A. ferrooxidans obtains energy by oxidizing ferrous iron and sulfur, and does not favor environments in which the redox potential is high (high ferric ion to ferrous ion ratio) (Rawlings *et al*., 1999). *A. ferrooxidans* is able to obtain energy from alternative sources such as: i) hydrogen, with oxygen as terminal electron acceptor under aerobic conditions (Barreto *et al*., 2003; Drobner *et al.,* 1990) or under anaerobic conditions by dissimilatory reduction of ferric iron (Ohmura *et al*., 1999; Pronk *et al*., 1991**)** and ii) formate with oxygen or ferric iron as terminal electron acceptor (Pronk *et al*., 1991). The enzyme, hydrogenase, enables *A. ferrooxidans* to use hydrogen as an energy source. When hydrogen is utilized, the optimal pH range shifts to 3.0-5.8. *A. ferrooxidans* assimilates carbon dioxide (its main carbon source) *via* the Calvin Benson cycle (Levicán *et al*., 2002), which is catalyzed by the ribulose-biphosphate carboxylase enzyme. The nitrogen requirement of the organism is met through ammonium compounds present in the biomining mediums (Ramírez *et al.,* 2004). The G+C mole ratio of *A. ferrooxidans* isolates are between 57%-59% and belong to possibly 4 different DNA-DNA hybridization groups (Rawlings 2002).

The composition of its extracellular polymeric substance (EPS) changes depending on whether ferric iron or sulfur is present. *A. ferrooxidans* is sensitive to sodium (Lavalle *et al.,* 2005) and organic matter (Fournier *et al.,* 1998; Gu *et al*., 2007; Manning 1975; Rawlings 2002; Sand *et al*., 1992). Strains have been isolated that showed low tolerance to sodium, however its growth is completely inhibited by the presence of 10 000 mg/l glucose, regardless of the initial cell density and in spite of favorable pH and aeration conditions (Marchand 2003). Its sensitivity to organic matter, makes it hard to grow in the laboratory, however several different medias has

been developed: 9k media (Ishigaki *et al*., 2005; Kai *et al*., 2000; Park *et al.*, 2005; Tyagi *et al.*, 1993), ISP media (Manning 1975), Sodium thiosulfate media (Colmer *et al*., 1950) and Leathen media (Chan *et al*., 2003). A two layer technique has also been developed, where the iron oxidizing bacterium is grown along with heterotrophic bacteria, which removes the compounds that are toxic to the iron oxidizer (Johnson 1995; Rawlings 2002).

A. ferrooxidans is resistant to a wide range of metals such as copper (Alvarez and Jerez 2004), zinc, silver, mercury (Barreto *et al*., 2003), arsenic (Nies and Silver 1995) and uranium (Pronk *et al*., 1991). Solisio *et al*. (2002) applied this ability to leach zinc and aluminum from industrial waste sludge. Several researchers have commented that the polyphosphate degradation of *A. ferrooxidans* might play a role in metal detoxification. Furthermore research has determined that *Acidithiobacillus* resistance to arsenate greatly depends on its arsenic resistance *ars* operon (Nies and Silver 1995) and also on the phosphate concentration present in the growth media, as arsenate is a structural analog of phosphate and will therefore be readily taken up by the cell during starvation. Also copper ions stimulate polyphosphate degradation and phosphate efflux, again supporting a model for metal detoxification in which heavy metals stimulate polyphosphate hydrolysis and the metal-phosphate complexes formed would be transported out of the cell as part of a possibly functional heavy metal tolerance mechanism (Alvarez and Jerez 2004).

Researchers have demonstrated that the existence and importance of a hydrogen sulphide:ferric ion oxidoreductase and sulfate:ferric iron oxidoreductase in sulfur oxidation. These enzymes use ferric iron as an electron acceptor in the oxidation of elemental sulfur and sulfite (Sugio 1992). Sugio *et al*. (1996) found that these enzymes were responsible for the solubilization of copper from copper concentrates (Sugio *et al.*, 1996). Research by Kai *et al.* (2000) further supported the existence of these enzymes. They demonstrated that *A. ferrooxidans* was able to leach zinc sulfide and manganese dioxide simultaneously. The ferric iron oxidized the zinc sulfide and the ferrous iron reduced the manganese oxide. This allowed the consumption of the oxidizing and reducing agents to decrease and the possibility that these two minerals could be simultaneously electrowon. The researchers commented that during simultaneous leaching of metal oxides and metal sulfides, the consumption of oxidizing and reducing agents decreases.

2.2.3.3 *Acidithiobacillus thiooxidans*

A. thiooxidans is nutritionally very similar to *A. ferrooxidans*; however it is not able to oxidize ferrous iron and is therefore limited to using reduced sulfur compounds as an electron donor. *A. thiooxidans* is a mesophilic (Rawlings 2002), motile by flagella (Ohmura *et al*., 1996) and grows in a pH range of 0.5-5.5. The organism has a G + C ratio of 53% with a DNA-DNA similarity with *A. ferrooxidans* of about 20% or less (Rawlings 2002).

2.2.3.4 *Acidithiobacillus caldus*

Acidithiobacillus caldus is able to oxidize reduces sulfur compounds by not ferric iron (Hallberg and Lindstrőm 1994). Dopson and Lindstrőm (2004) isolated this organism from a bioleaching community that grew at 45°C on pyrite, arsenical pyrite and chalcopyrite. This is a moderate thermophilic organism which has an optimum temperature of about 45°C. Some strains are able to grow mixotrophically using yeast extract or glucose (Rawlings 2002).

2.2.3.5 *Thiobacillus*

Thiobacillus species were found to be satellite organisms, which grew with *A. ferrooxidans*. Furthermore, researchers demonstrated that *Thiobacilli* was able to grow mixotrophically on tetrathionate with glucose, which could therefore aid *A. ferrooxidans* growth in the presence of glucose (Ehrlich 1996). Tuffin and coworkers (2005) isolated a tronsposon *TnAtcArs* that carries a set of arsenic resistance genes from *Acidithiobacillus caldus* which was islated from a commercial biooxidation treatment plant of arsenopyrite. Research found that the conditions in the tank made this organism highly resistant. Other species of acidophilic *Thiobacilli* have been described by Huber and Stetter (1990) which includes *Thiobacillus prosperus* and *T. cuprinus*. *Thiobacillus prosperus* is a new group of halotolerant metal mobilizing bacteria. *T. cuprinus* is a facultative chemolithotrophic bacterium which can oxidize metal sulfides but cannot oxidize ferrous iron. Bosecker (1997) demonstrated that these organisms can preferentially mobilize copper from chalcopyrite, which makes them ideal candidates for future bioleaching technology.

2.2.3.6 *Leptospirillum*

Leptospirillum was first isolated in 1972 from a copper leach dump and coal spoil heap in Armendia (Inoue *et al.*, 2000). The bacteria of this genus are quite similar to that of *Acidithiobacillus*. They are also acid tolerant, with the ability to grow in a pH range of between 1.5-1.8 (Rawlings 2002); gram negative, autotrophic (Johnson *et al*., 2006), spiral shaped, mesophilic, non-sporeforming (Johnson *et al.*, 1992), motile by means of flagella (Ohmura *et al*., 1996) and chemolithoautrophic (Rawlings 2002). Based on 16S rRNA sequence analysis, this genus does not form part of the division *Proteobacteria*, but rather *Nitrospora* (Lane *et al*., 1992). All *Leptospirilli* are only able to use ferrous iron as an electron donor, resulting in a higher affinity for ferrous iron compared to *Acidithiobacilli* (Rawlings 2002). The growth of *Acidithiobacillus ferrooxidans* is inhibited above 36 mM ferric iron concentration whereas *L. ferrooxidans* is resistant even to 500 mM ferric iron concentration (Dave 2008; Mignane *et al.*, 2004). *Leptospirilli* have not been reported to grow on sulfur-salt medium, however they are able to efficiently degrade pyrite (FeS_2) (Sugio *et al*., 1994). This organism also poses the hydrogen sulphide:ferric ion oxidoreductase, as *A. ferrooxidans*, which catalyzes the oxidation of elemental sulfur with ferric ion as an electron acceptor to give ferrous ion and the bisulfite ion. However research showed that during sulfur oxidation, the organism produces bisulfate as an intermediate in low quantities, which inhibits the hydrogen sulphide:ferric ion oxidoreductase, thereby inhibiting sulfur oxidation in *Leptospirilli* (Sugio *et al*., 1994).

Leptospirilli have a G+C content of 49-51%, with four known species (Rawlings 2002). DNA-DNA hybridization between the groups is 11% or less (Coram and Rawlings 2002). *L. ferrooxidans* belongs to the 49-51% G +C group, with *L. ferriphilum* belonging to the higher G + C group (Rawlings 2002). The fourth specie was isolated from an abandoned pyrite mine by Bond and coworkers (2000). *Leptospirilli* have been found widely in biooxidation processes, usually in combination with a sulfur oxidizing bacterium such as *Acidithiobacillus thiooxidans* or *A. caldus* (Rawlings 2002). Moreover, Tuffin *et al*. (2006) also isolated a set of arsenic resistance genes from a highly arsenic resistant strain of *L. ferriphilum*, as with *Acidithiobacillus caldus*.

2.2.3.7 *Acidiphilium*

These are gram-negative, acid tolerant heterotrophs. These organisms are not primarily involved in mineral decomposition (Hallberg and Johnson 2001), but they are included in this section because they have been detected in batch reactors (Goebel and Stackebrandt 1994) and have frequently been found growing near *A. ferrooxidans*, where they are believed to feed on the organic waste products produced by the iron and sulfur oxidizing bacteria (Rawlings 2002). These organisms have been used in the laboratory to cultivate *A. ferrooxidans*, in the overlay plating technique (Section 2.2.3.2), to remove presumably organic toxins form the upper layer, allowing the organic matter intolerant iron and sulfur oxidizing bacteria to grow (Johnson 1995**)**. These organisms are also able to use ferric iron as an electron acceptor, at low oxygen tension, thus regenerating ferrous iron (Rawlings 2002; Romero *et al.*, 2003).

2.2.4 Iron oxidizing bacteria with potential use in the biomining industry

Mineral oxidation is a mixture of chemistry and biology. Current biomining operations run at temperatures ranging from ambient to 40°C. Certain minerals such as chalcopyrite can only be economically leached at elevated temperature. Thus organisms able grow and leach at these elevated temperatures will be valuable in future bioleaching processes (Rawlings 2002). At temperatures exceeding 65°C, the biomining consortia are dominated by Archaea rather than bacteria, with species of *Sulfolobus* and *Metallosphaera* being most prominent (Norris *et al*., 2000). The following organisms discussed are likely to contribute to the functioning of these new bioleaching process (Rawlings 2002).

2.2.4.1 *Sulfobacillus*

Sulfobacilli are gram positive, endospore-forming, non-motile, moderate thermophilic acidophiles, which can grow in a temperature range of 28-60°C, with an optimum around 50°C. It grows in a pH range from 1.9 – 3.0 with an optimum range of 1.9 - 2.4 (Rawlings 2002; Watling *et al*., 2008). These organisms have been isolated from heaps of mineral waste and biomining operations (Rawlings 2002). They are able to grow autotrophically or

heterotrophically. When they grow autotrophically they use ferrous iron, reduced inorganic sulfur compounds or sulfide minerals as electron donors (Norris *et al*., 1996). *S. thermosulfidooxidans* cannot use sulfate as a source of sulfur and therefore must be provided with reduced sulfur. *Sulfobacilli* are not able to efficiently fix carbon dioxide, and therefore need elevated CO_2 in the atmosphere (yeast extract can be added to the media) (Rawlings 2002). Glucose can serve as a carbon and energy source for *Sulfobacilli* when growing heterotrophically. These microorganisms are also able to grow in the absence of oxygen, using ferric iron as an electron acceptor and either organic or inorganic sulfur compounds as electron donor. Four species have been distinguished using phylogenetic and physiological characteristics: *Sulfobacillus thermosulfidooxidans* (DSM 9293), *S. acidophilus* (DSM 10332), *S. sibiricus* (DSM 17363) and *S. thermotolerance* (DSM 17362). *S. acidophilus* (Norris *et al*., 1996) can be differentiated from the other species by being capable of autotrophic growth on sulfur with addition of carbon dioxide enriched air. *S. sibiricus* (Melamud *et al*., 2003), can be differentiated from the other species by its: higher optimal growth temperature, ability to grow on thiosulfate or tetrathionate as sole energy source and inability to grow heterotrophically. *S. thermotolerance* (Bogdanova *et al*., 2006) has larger cells and a lower optimal temperature. A possible fifth specie of the genus *Sulfobacillus* has been isolated from a continuous bioleaching tank, charged with cobaltiferous pyrite (Johnson *et al*., 2007).

2.2.4.2 Ferroplasma

Ferroplasma are archaea. These organisms are mesophilic, pleomorphic in shape, lack cell walls and grows optimally at a temperature of 33°C and pH of 1.7 (Rawlings 2002). *Ferroplasma acidiphilum* was isolated from a pilot plant bioreactor in Kazakstan, which treated arsenopyrite/pyrite (Golyshina *et al.*, 2000). This organism is able to oxidize ferrous iron but not sulfur and appears to be obligatory aerobic (Rawlings 2002).

2.2.4.3 *Sulfolobus*

The first thermophilic acidophile isolated and characterized were *Sulfolobus acidocaldarius*. These organisms are obligated thermophilic autotrophic archaea that obtains energy by oxidizing ferrous iron, reduced inorganic sulfur compounds or sulfide ore (Das *et al*., 1999 as cited in Brandl 2001; Rawlings 2002). They have an optimal growth temperature in the range of 65-70°C. From a biomining perspective, there is particular interest in thermoacidophiles, such as *Sulfolobus metallicus*, *Acidianus* spp. and *Metallosphaera* spp, that mobilize metals from sulfidic minerals (Johnson 2006). The bioleaching kinetics of thermophiles is known to be higher than that of the mesophiles (Das *et al*., 1999 as cited in Brandl 2001; Rawlings 2002).

2.2.4.4 *Gallionella ferruginea*

This is an aerobic, neutralophilic iron oxidizer. It that has been obtained in purified enrichment and pure culture in the laboratory and it grows at very low oxygen tensions in opposing gradients of oxygen and ferrous iron (Hanert 1992). Hallbeck *et al*. (1993) have found that this organism was able to grow lithotrophically on iron, however there is also proof that this organism can grow mixotrophically.

2.2.4.5 *Metallosphaera*

Metallosphaera are aerobic iron and sulfur oxidizing chemolithotrophs archaea that are able to grow on complex organics such as casamino acids or yeast extract, but not on sugars. Researchers demonstrated that *Metallosphaera sedula* is able to grow in a pH range of 1.0-4.5 and oxidize a variety of minerals at temperature 80-85°C (Rawlings 2002). Norris (1997) found *Metallosphaera* like organisms to be the most efficient organisms to leach chalcopyrite at high temperatures. However, 16s rRNA sequence analysis placed some disagreement on these finding as to which are more *Sulfolobus* or *Metallosphaera* like (Norris *et al*., 2000).

2.2.4.6 *Acidianus*

Several species from this group oxidize minerals; however their industrial potential is thought to be less promising than that of *Sulfolobus* and *Metallosphaera*. *Acidianus brierley* can grow autotrophically by oxidizing ferrous iron or sulfur; or it can grow heterotrophically on complex organic substrate. Its optimum pH range is 1.5-2.0 and optimum temperature is 70°C (Rawlings 2002). Other species includes *Acidianus infernus, A. ambivalens* and *A. infernus*, with the latter able to grow optimally at 90°C (Rawlings 2002).

Robert and coworkers (1994) engineered a device, which can increase the growth of iron oxidizing bacteria. Following is a brief description of the design and theory of their device. The device contains a anode, cathode, power supply and a perfluorosulfonic acid membrane (Figure 7) Bacteria are responsible for transferring electrons from soluble ferrous ions to molecular oxygen, thus producing ferric ions and consuming protons (equation 1) (Figure 7). The ferric iron produced is then electrochemically reduced back to ferrous ion at the cathode of the device. The electrons for reduction are derived from the oxidation of water at the anode. The perfluorosulfonic acid membrane between the anode and cathode acts as a semi permeable cation-exchange barrier, permitting the passage of cations between the chambers but prohibiting the passage of anions and bacterial cells. Protons that crossed the semi-permeable barrier concomitantly with current flow to equalize the charge balance in the two chambers replace protons consumed by the bacterium-dependent reduction of oxygen. The apparatus thus replaces the chemical species consumed by the bacterial metabolism. The bacteria therefore should experiences an endless supply of growth substrate and in principle, would continue to prosper until some other factor in the growth medium becomes limiting.

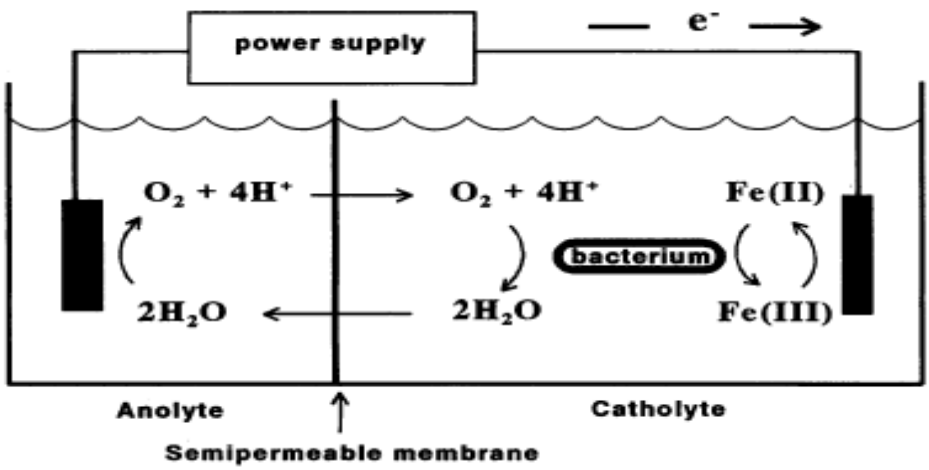

Figure 7. Schematic representation of the chemistry during *in situ* electrolytic cultivation of iron-oxidizing bacteria (Robert *et al.*, 1994).

2.3 ACID MINE DRAINAGE (AMD)

The metabolic capability of iron oxidizing bacteria are exploited in biohydrometallurgical processes, however the metabolic ability of these organisms can have a detrimental effect on the environment. Acid mine drainage (AMD) or acid rock drainage, which is acidic iron/sulfate rich water, formed when sulfide minerals (Table 6) in rocks are exposed to oxidizing conditions (equation 2 to 5), in the absence of alkaline materials (Stumm and Morgan 1996). AMD is found regularly at coalmines and any other practice in which sulfides from geological materials are exposed such as metal mining practices, highway construction or any other deep excavations.

Equation 2: $FeS_2 + 7/2O_2 + H_2O \rightarrow Fe^{2+} + 2SO_4^{2-} + 2H^+$

Equation 3: $Fe_2^+ + 1/4O_2 + H^+ \rightarrow Fe^{3+} + ½\ H_2O$

Equation 4: $Fe^{3+} + 3H_2O \rightarrow Fe(OH)_3 + 3H^+$

Equation 5: $FeS_2 + 14Fe^{3+} + 8H_2O \rightarrow 15Fe^{2+}\ 2SO_4^{2-} + 16H^+$

In equation 2, iron sulfide (FeS_2) is oxidized, thereby releasing ferrous iron (Fe^{2+}), sulfate (SO_4^{2-}) and acid. Ferrous iron is then oxidized (Equation 3) to form ferric iron (Fe^{3+}). The ferric iron can then either be hydrolyzed to form ferric hydroxide, $Fe(OH)_3$, and H^+ acidity (equation 4), or

it can directly attack pyrite, acting as a catalyst in generating much greater amounts of ferrous iron, sulfate, and acidity (equation 5).

Table 6 Typical sulfides occurring in nature (Adopted from Skousen *et al*., 1998)

Chemical formula	Name
FeS_2	Pyrite
FeS_2	Marcasite
Fe_xS_x	Pyrrhotite
Cu_2S	Chalcocite
CuS	Covellite
$CuFeS_2$	Chalcopyrite
MoS_2	Moybdenite
NiS	Millerite
PbS	Galena
ZnS	Sphalerite
FeAsS	Arsenopyrite

If any of the processes represented by equations 2 to 5 were slowed or altogether stopped, the generation of AMD would also slow or cease. Furthermore the removal of air and/or water from the system would stop the mineral oxidation. In nature, pyrite occurs in locations with low oxygen concentrations, thus the pyrite stays relatively unreactive. When pyrite is enclosed within massive rock bodies, only minimal amounts of pyrite are oxidized through natural weathering, thereby generating only small amounts of acid which is sometimes naturally diluted or neutralized by surrounding alkaline rocks. However, when large volumes of pyritic material

are fractured and exposed to oxidizing conditions, which can occur in mining or other major land disturbances, the pyrite reacts, and water dissolves and moves the reaction products (Equation 2 to 5) into ground and surface water sources.

Under many conditions' equation 2 is the rate-limiting step in pyrite oxidation because the conversion of ferrous iron to ferric iron is slow at pH values below 5 under abiotic conditions (Skousen *et al*., 1998). However, iron oxidizing and sulfur oxidizing bacteria can greatly accelerate this reaction, which aids the formation of AMD. Acidophiles commonly found in AMD include *Leptospirillum ferrooxidans* and other *Leptospirilli*, *Acidithiobacillus ferrooxidans*, *A. thiooxidans* and *A. caldus*. Heterotrophs such as *Acidiphilium* species are also found in acid mine drainage, in close association with chemolithotrophs (de Wolf-Durand *et al*., 1997; Hallberg and Johnson 2001). The importance of bacteria in accelerating AMD formation, spurred research into bactericides to control the microbial population and hopefully prevent or treat AMD (Table 8).

The availability of oxygen also limits the formation of acid mine drainage. Spoils with low porosity and permeability, as with those composed of soft shales, often only form AMD in the upper part where enough oxygen is present. In porous and permeable spoil composed of coarse sandstone, air convection driven by the heat generated by pyrite oxidation may provide high amounts of oxygen deep into the spoil (Guo *et al*., 1994, Guo and Cravotta 1996).

Alkaline mine drainage can also be formed. Here water has a pH above 6, with dissolved metals that can create acids *via* reaction 2 and 3 (mentioned before). Skousen and Ziemkiewicz (1996) grouped mine drainage into several basic types according to their various different properties or stages of formation (Table 7).

Table 7 Classification of acid mine drainage (Adopted from Skousen *et al*., 1998).

Mine drainage	Description
Type 1	▪ Drainage has little or no alkalinity present (pH below 4.5). ▪ Contains high concentrations of iron, aluminum, manganese and other metals.
Type 2	▪ Drainage has a pH above 6, with high total dissolved solids (containing high ferrous iron and manganese with a low oxygen content). ▪ Upon oxidation, the pH of this type will drease and will become a Type 1 AMD.
Type 3	▪ Drainage has a pH above 6, with moderate to high total dissolved solids (containing low to moderate ferrous iron and manganese with low oxygen content). ▪ The alkalinity present is greater than the acidity formed. ▪ Upon oxidation, the acid generated from metal hydrolysis and precipitation reactions are neutralized by alkalinity present in the water.
Type 4	▪ This type of AMD is neutralized, with pH above 6 and high total suspended solid content. ▪ Settling of metal hydroxides in the water has not occurred.
Type 5	▪ This type is a neutralized AMD, with pH above 6.0 and high total dissolved solids.

2.3.1 Forms of sulfur in rocks

Sulfur in coal and rocks associated with coalmines can occur as organic sulfur, sulfate sulfur, and pyritic sulfur. Some sulfur in coal appears to have been introduced after the peat had been converted to coal, as is evidenced by pyrite coatings on vertical fracture surfaces, called cleat, in the seam. Much of the pyrite present in rocks and overburdens of coal mines occurs as very small crystalline grains intimately mixed in sandstones and shales (Skousen *et al*., 1998). The origin of sulfur in large concretions, nodules, lenses, bands, and fillings in porous layers of coal is less well understood (Skousen *et al*., 1998). Organic sulfur is thought to be complexed - and combined with organic constituents of coal. This form of sulfur is only found in appreciable quantities in coal beds and in other carbonaceous rocks. Generally, the organic sulfur component is not chemically reactive and has little or no effect on acid producing potential. Sulfate sulfur is usually only found in minor quantities in fresh coal and other undisturbed pyrite-containing rocks, and is commonly the result of weathering and recent oxidation of sulfide sulfur. Some sulfate minerals like jarosite [$KFe_3(SO_4)_2(OH)_6$] can dissolve and form acid solutions in near

surface environments. Pyritic or sulfide sulfur is the dominant form of sulfur in the majority of coal and associated rocks. It is the sulfur form of greatest concern. Of all the sulfide minerals that may be present, iron disulfides predominate and are the major acid producers. Accordingly, the maximum potential acidity (MPA) of a fresh overburden sample correlates closely with the pyritic sulfur content. Studies indicated that variations in total sulfur contents of overburden samples reflect similar variations in pyritic sulfur content. Several types of pyritic sulfur are known based on physical appearance, and are classed into six groups: 1) primary massive, 2) plant replacement pyrite, 3) primary euhedral pyrite, 4) secondary cleat (joint) coats, 5) mossy pitted pyrite, and 6) framboidal pyrite. Caruccio *et al*. (1988) provide an extensive review of the different forms, morphologies, and reactivity of pyritic materials. The equations for pyrite oxidation show that a material containing 1% sulfur, all as pyrite, would yield upon complete reaction an amount of sulfuric acid that requires 31.25 mg of $CaCO_3$ to neutralize the material (Sobec *et al*., 1978). When sulfur in the rock is exclusively pyrite, the total sulfur content of the rock accurately quantifies the acid-producing potential, if it were all to react. When organic or sulfate sulfur are present in significant amounts in partially weathered rocks, total sulfur measurements overestimate the amount of acid that will be formed upon oxidation. Therefore, correction for sulfates and organic sulfur naturally present in some overburdens or resulting from partial weathering of pyritic materials may be necessary to increase accuracy in predicting the acid-producing potential of materials containing mixed sulfur species (Skousen *et al*., 1998).

The rate of pyrite oxidation depends on numerous variables (Skousen *et al*., 1998) such as:

- reactive surface area of pyrite
- form of pyritic sulfur
- oxygen concentrations
- solution pH
- catalytic agents
- the presence of *Thiobacillus* bacteria. The possibility of identifying and quantifying the effects of these and other controlling factors with all the various rock types in a field setting is unlikely.

2.3.2 Alkalinity in rocks

The natural base content of overburden materials (alkali and alkaline earth cations, commonly present as carbonates or exchangeable cations on clays) is important in evaluating the future neutralization potential (NP) of the materials. The amount of alkaline material in unweathered overburden may be sufficient to equal or overwhelm the acid-producing potential of the material. Of the many types of alkaline compounds present in rocks, carbonates (specifically calcite and dolomite) are the primary alkaline compounds, which occur, in sufficient quantities to be considered as effective deterrents to AMD generation. In overburden containing both alkaline and pyritic material, the alkaline material may be sufficient to reduce oxidation from occurring or to neutralize the acid formed from pyrite. Higher alkalinities also help control bacteria and restrict solubility of ferric iron, which are both known to accelerate acid generation. Although a number of factors must be considered, a balance of the acid-producing potential and neutralizing capacity of an overburden sample will indicate whether acidity or alkalinity is expected in the material upon complete weathering (Skousen *et al*., 1998).

2.3.3 Treatment strategies

There are various options available to remediate AMD, which may be divided into those that use either chemicals or biological mechanisms to neutralize acidity and remove metals from solution. Both abiotic and biotic systems include those that are classified as active (Table 8) and passive (Table 9). Active treatment systems require continuous inputs of resources to sustain the process, whereas passive systems require relative little resource input once in operation (Johnson and Hallberg 2005). Active treatment systems are very effective; however the treatments are expensive when cost of equipment, chemicals and manpower are considered (Skousen *et al*., 1998). Due to the high cost of active systems, passive treatment systems have been developed that do not require continuous chemical inputs and take advantage of naturally occurring chemical and biological processes to clean contaminated mine waters, however these systems also suffer from several drawback as discussed below. These systems include constructed wetlands, anoxic limestone drains and vertical flow systems such as successive alkalinity producing systems, limestone ponds and open limestone channels. Compared to active

treatment systems, passive treatment require longer retention times and greater space, but a lower long term cost (Skousen *et al*., 1998).

Table 8 Active treatment processes for acid mine drainage (Adopted and modified from Skousen *et al*., 1998).

System	Description
Aeration/oxidation	Aeration is the process of introducing oxygen into water. Oxygen combines with the metals in water, thereby oxidizing them and resulting in their precipitation at lower pH. Oxidation is limited naturally because oxygen has a low solubility in water. Research found that by increasing oxidation, chemical treatment of AMD became more efficient.
Neutralizers	Six primary chemicals have been used to treat AMD, namely: Calcium carbonate, calcium hydroxide, calcium oxide, sodium carbonate, sodium hydroxide and anhydrous ammonia. Enough of these alkaline materials have to be added to neutralize acids and raise water pH to a level that will cause the metals to precipitate.
• Calcium carbonate	Used to increase the pH and precipitate metals in AMD. It has several advantages such as low material cost, very safe, easy to handle and it produces the most compact sludge material. However its application has been limited by its low solubility especially in cold water.
• Calcium oxide	Calcium oxide has been used in conjunction with a waterwheel application system. The amount of calcium oxide to be applied is determined by the movement of the waterwheel. This system has mainly been used to treat small and/or periodic flows of high acidity because calcium oxide is very reactive.
• Trapzene (calcium oxide)	This is a trade name for a specially formulated compound of calcium peroxide. This compound can be used as an oxidant and neutralizer. It appears to be most effect at oxidizing and removing manganese from AMD.
• Calcium hydroxide	This is the most common used chemical for treating acid mine drainage. It is a cost effective compound to use, however initial capital expenditure is high with the topography of the construction site playing an important role.
• Magna lime (Magnesium oxide)	This is a mixture of calcium and magnesium oxide. It has also been dispensed with a waterwheel as with calcium oxide. The dissolution of this compound is slower than calcium oxide.
• Caustic soda (Sodium hydroxide)	It is often used in secluded locations and in low flow, high acidity situations. Caustic soda is frequently used when manganese concentrations in the AMD are high. Using liquid caustic soda has several drawbacks: high cost, dangers in handling the chemical and high sludge volumes.
• Soda ash briquettes	System frequently used in remote locations with low flow and low amounts of acidity and metals.

Table 8 (Continued).

System	Description
• Ammonia	It is present as a gas at ambient temperatures, which is then compressed and stored as a liquid. Ammonia is highly soluble in water and act as a strong base in treating AMD. There are several disadvantages when using this compound: dangerous to handle; potential increase in nitrate and acid downstream attributable by microbial action; and consequences of excessive application rates.
Flocculants/coagulants	Flocculants of coagulants are used to increase particle settling efficiency. These compounds are normally limited to cases where unique metal compositions require a specialized treatment system or where residence time and aeration is inadequate for complete metal precipitation. The most common coagulants used in water treatment are aluminum sulfate and ferric sulfate.
Reverse osmosis	Osmosis takes place when two solutions of different concentrations are placed in a common solvent and separated by a semi permeable membrane (permeable to the solvent and not the solute). This will cause the solvent to flow from the more dilute solution to the more concentrated solution until an equilibrium concentration is reached. With reverse osmosis, the direction of solvent flow reversed by applying pressure to the more concentrated solution. This produces a brine which is high in acid, iron and sulfate, and high quality water which is suitable for potable and industrial use.
Ion exchange resins	The process is defined as the reversible interchange of ions between a solid medium and the aqueous solution. An example of ion exchange is the removal of calcium and magnesium from 'hard' water by passing it through a bed of ion exchange material which is charged with monovalent cations. The divalent calcium and magnesium cations are exchanged for the sodium ions. Current ion exchange technology uses the following resins: strong-acid cation, weak acid cation, strong-base ion and weak-base anion.
Electrodialysis	This device consists of a number of narrow compartments separated by closely spaced membranes. The compartments are separated by both cation and anion membranes. Positive and negative electrodes are placed at opposite ends of the device. The solution fills the channels between the membranes, and when a current is place through the electrodes, the ions in solution migrates towards the positive or negative poles and are collected on the membranes.
Natural zeolites	These are hydrous aluminosilicates that can be used to exchange ions for treatment of AMD. Zeolites contain sodium ions which are preferentially exchanged for metal cations. Once the zeolite is fully loaded with metals, the material must be regenerated using a sodium chloride solution to remove the metal cations from the aluminosilicate matrix.

Table 9 Passive treatment systems for Acid mine drainage (Adopted and modified from Skousen *et al*., 1998).

System	Description
Natural wetlands	Researchers have noted that wetlands are able to change AMD effluent. Natural occurring wetland plants show long term adaptation to low pH and high metal concentration; however AMD eventually degrades the quality of natural wetlands. Wetlands are protected by federal law, thus wetlands cannot be used to treat AMD. This spurred the research into construction artificial wetlands.
Constructed wetlands	Constructed wetlands are able to remove heavy metals such as iron, manganese and aluminum from AMD effluents. Mechanisms that contribute to this are as follow: formation and precipitation of metal hydroxides; microbial sulfate reduction forming metal sulfide and organic complexation reactions. The way in which a wetland is constructed affects how water treatment occurs. There are currently two construction styles namely; 'aerobic wetlands' and anaerobic wetlands.
• Aerobic wetlands	These wetlands are used to collect water and provide enough residence time and aeration to precipitate the metals present. The water in this case typically has a n*et al*kalinity. Iron and manganese present in the effluent are oxidized, which causes their precipitation. The precipitates are retained in the wetland or downstream. Plants in the wetland promote uniform flow and therefore also more effective wetland area for water contact.
• Anaerobic wetlands	Anaerobic wetlands encourage interaction of water with organic-rich substrates, which contribute significantly to treatment. The substrate may contain a layer of limestone in the bottom of the wetland or the limestone may be mixed among the organic matter. Wetland plants are transplanted into the organic substrate. Anaerobic wetlands are used when the water to be treated has a net acidity. Thus alkalinity must be generated in the wetland and introduced to the net acid water in order to accomplish significant precipitation of dissolved metals. Alkalinity in anaerobic systems can be generated in two ways. Firstly certain bacteria such as Desulfovibrio and Desulfotomaculum can utilize reactions between organic substrates and sulfate as a source of energy for their metabolism. These bacteria convert sulfate to hydrogen sulfate, and in this reaction bicarbonate is produced. Secondly alkalinity can be generated as the limestone reacts with the acidic water. The surface water in anaerobic wetlands is oxidizing, thus oxidation and precipitation of metals will occur.

Table 9 (Continued).

System	Description
Anoxic limestone drains	Anoxic limestone drains are buried cells or trenches of limestone into which anoxic water is introduced. Alkalinity is provided by the limestone which is dissolved in the mine water. The main concern with this system is its durability. When high amounts of ferric ion and aluminum are present, clogging of the limestone pores with these metals hydroxides can occur, which could decrease efficiency or stop AMD treatment.
Vertical flow systems	With this system, water flows downwards, typically from a pond, through organic matter and limestone, before it flows out of the system into a drainage system. In contrast with horizontal flow wetlands, vertical flow systems greatly enhance the interaction of water with organic matter and limestone. The aim is to optimize sulfate reduction in the organic layer by causing water to flow through the organic matter. The limestone may be present as a supplementary reactant to promote optimum pH.
Limestone ponds	Here a pond is constructed on the upwelling of an acid mine drainage seep or underground discharge point. Limestone is positioned at the bottom of the pond and the water flows upward through the pond. This system is recommended for systems that contains no ferric iron ore aluminum, because these metals can precipitate as with the anoxic limestone drain system and cause problems. However this system has the advantage that it is not buried thus an operator can observe whether the limestone is being coated (by ferric ion or aluminum), and periodically disturb the limestone to uncover it.
Open limestone channels	This system introduces alkalinity to acid water in open channels or ditches lined with limestone. The acid water is introduced to the channel and the AMD is treated by limestone dissolution. This system is usually employed as a long term strategy.
Bioremediation	Bioremediation is the application of the metabolic capability of microorganisms to convert contaminants to less harmful substances. Metal oxidation reactions can be accelerated by microorganisms and thus their eventual precipitation. Certain microorganisms are able to reduce metals, thus aiding in the formation and eventual precipitation of metal sulfides. These reduction processes can raise pH, generate alkalinity (as previously mentioned), and remove metals from AMD solutions. Bioremediation has been applied in anaerobic systems.
Diversion wells	This system consists of a cylinder or vertical tank of metal or concrete, which is filled with sand-sized limestone. AMD gets diverted into these wells, where limestone dissolution will increase the pH.
Limestone sand treatment	Limestone can also be added directly to AMD streams at different locations. The limestone is sand-sized which allow it to be distributed through the system.

2.4 DIRECT VS INDIRECT BIOLEACHING

In 1964 Silverman and Ehrlich introduced the phrase "direct leaching" which described a hypothesized enzymatic reaction taking place between an attached cell and an underlying mineral surface (Edwards *et al*., 2001; Sand *et al*., 1995). However, it is now generally accepted that this mechanism does not exist (Sand and Gehrke 2006). The "indirect leaching" mechanism now consist of two sub-mechanisms namely the "contact" and "non-contact" mechanism (Rawlings 2002; Sand and Gehrke 2006). Non-contact leaching describes the oxidation of ferrous iron to ferric iron by planktonic bacteria. The resulting ferric iron acts on the mineral surface where it is reduced and then re-enters the cycle to be oxidized to ferrous iron by the planktonic bacteria (Sand and Gehrke 2006).

Contact leaching takes into account that most of the microbial cells are attached to the surface of sulfide minerals and by means of ferric iron ions, which are complexed in their slime/glycocalyx or extracellur polymeric substances (EPS), they begin to degrade the sulfide minerals (Schippers and Sand 1999). Figure 8 illustrates the proposed contact mechanism of the indirect leaching approach. The model depicts that the dissolution of the mineral takes place at an interface between the bacterial cell wall and the mineral surface. This interfacial process is controlled by electrochemical parameters. The microorganisms contributes to the electrochemical parameters by generating oxidizing agents such as ferric iron ions, and also by subsequently oxidizing the sulfur compounds resulting from the dissolution of the mineral (Rawlings 2002).

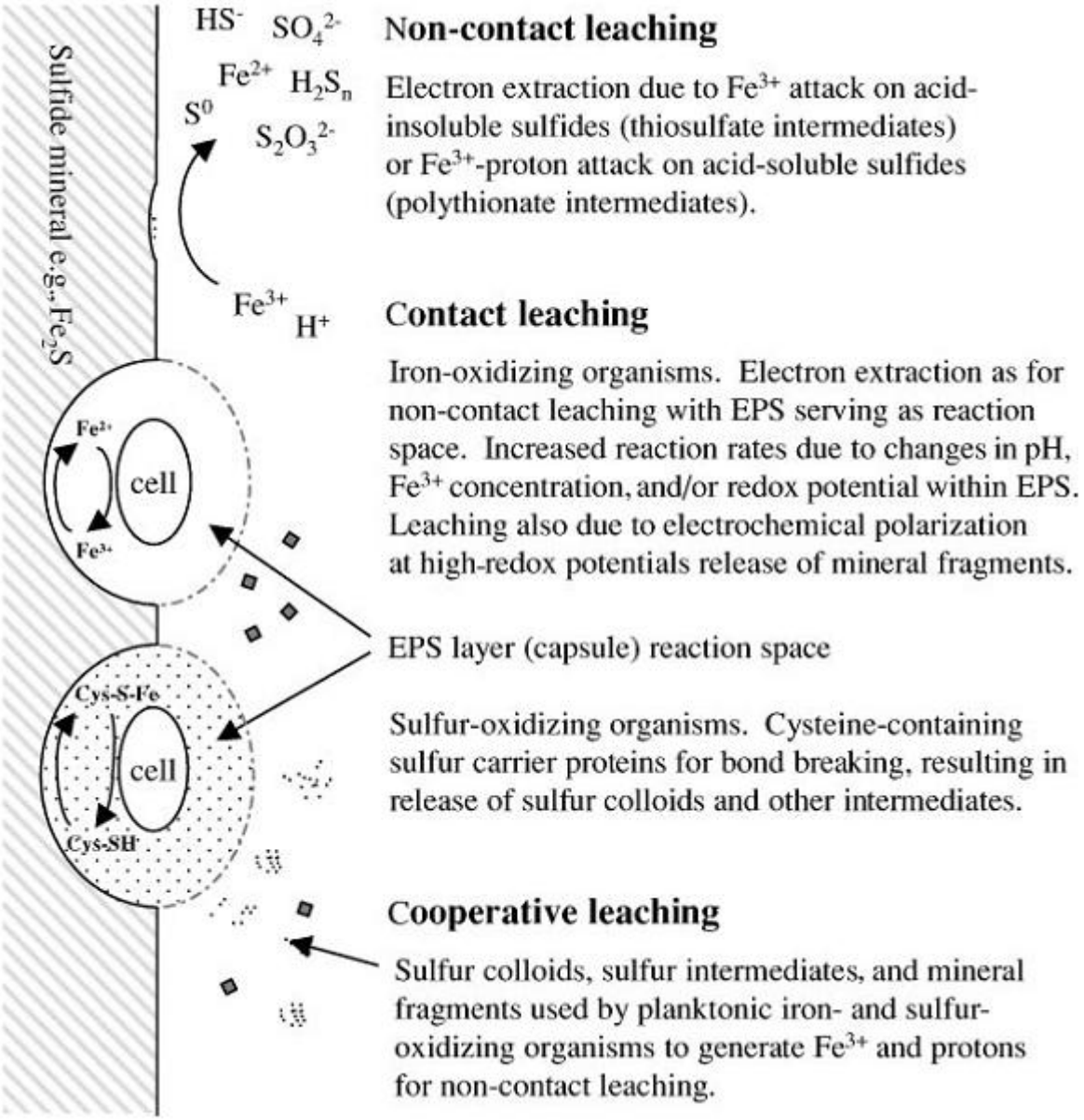

Figure 8. Illustration of the contact leaching mechanism of the indirect leaching attack as catalyzed by a pyrite attached *A. ferrooxidans* cell (Rawlings 2002).

The dissolution rate of a mineral can be affected by galvanic interactions. Metal sulfides have different electrode potentials, thus polarization can occur if there is direct electrical contact. The sulfide which has a low potential, will function as the anode, while the metal sulfide with the high potential will be the cathode. At the anode, the metal sulfide is dissolved by electron transfers to the cathode, where ferric iron ions are reduced without oxidation by the cathode itself. Thus the metal sulfide with the high electrode potential will be protected against oxidative

attack as long as there is an electrical contact and will thus be dissolved at a slower rate than the galvanically unprotected sulfide (Rossi 1990).

2.4.1 Reaction pathway

Several experimental results have indicated that the reaction pathway for metal sulfide dissolution is not determined by the crystal structure of the mineral (Sand *et al*., 2001; Schippers and Sand 1999). The only decisive factor is the reactivity of the mineral with protons. For example, research has found that the acid non-soluble monosulfides such as molybdenite and tungstenite are degraded by the same mechanism as the acid non-soluble disulfide pyrite, but structurally similar disulfides pyrite and the acid soluble hauerite are dissolved by different mechanisms (Sand *et al*., 2001; Schippers and Sand 1999).

2.4.1.1 Thiosulfate pathway

The thiosulfate pathway describes the degradation of acid non-soluble metal sulfides such as pyrite, molybdenite and tungstenite (Schippers and Sand 1999, Schippers *et al*., 1996). Pyrite is dissolved as a result of electron extraction by hydrated ferric ions from the crystal lattice (Equation 6).

Equation 6: $FeS_2 + 6[Fe(H_2O)6]^{3+} + 9H_2O = S_2O_2^{3-} + 7[Fe(H_2O_6]^{2+} + 6H^+$

Thiosulfate, by means of a series of reactions, will subsequently form either sulfate and protons or higher polythionates and elemental sulfur (Figure 9). A number of reactions of the thiosulfate degradation pathway have been found to be mediated by enzymes. Researchers have isolated an enzyme from *A. ferrooxidans* which is responsible for the oxidation of thiosulfate to tetrathionate (thiosulfate dehydrogenase) (Sand and Gehrke 2006). The enzyme responsible for the hydrolysis of tetrathionate (tetrathionate hydrolyses) has also been isolated from *A. ferrooxidans* (de Jong *et al*., 1997; Sugio *et al*., 1996; Tano *et al*., 1996). However the degree to which enzymatic catalysis contributes to thiosulfate degradation remains to be determined. Schippers and coworkers (1999) determined that the amount of biomass produced during pyrite dissolution, indicates that some of the reactions must release electrons at a level sufficient for cytochrome β-flavine reduction. This would permit the two oxidation phosphorylation reactions, whereas

electrons at the cytochrome c level (provided by ferrous ion), would only proved one ATP per mol of oxidized substrate. The increased growth yields found by Schippers *et al*. (1999) are only explainable with this assumption. It is important to determine whether the tetrathionate degradation is dominated by abiotic or enzymatic catalyzed reaction, as the reaction determines the end product (either elemental sulfur or sulfate and protons). If the reactions produces sulfur as an end product, acidification in acid mine drainage could be reduced. When sulfate and eventual sulfuric acid is produced, the acid production would enhance acid mine drainage problems. Furthermore, the control of these reactions is important to the industrial bioleaching of pyrite prior to gold extraction and cyanidation (Schippers *et al*., 1999).

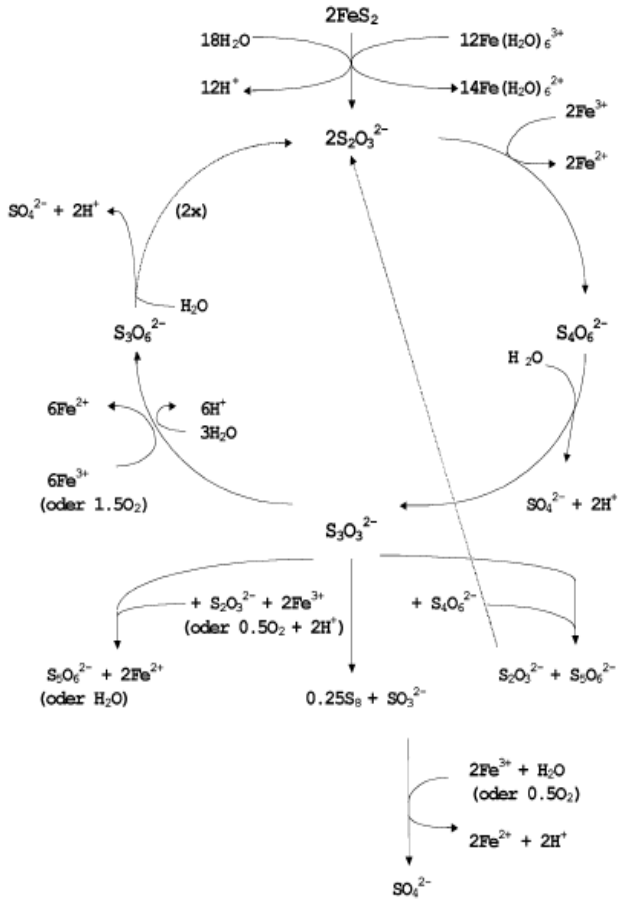

Figure 9. Cycle of oxidative pyrite degradation by bacterial and/or chemical leaching (adapted from (Steudel *et al*., 1987).

2.4.1.2 Polysulfide pathway

The polysulfide pathway, describes the metal sulfide degradation of acid soluble sulfide minerals such as hauerite, sphalerite, galena, chalcopyrite and arsenopyrite. The degradation of these minerals yields a polysulfide intermediate (Schippers and Sand 1999). The crystal lattice is attacked by a combined action of protons and ferric ions. Polarization of a surface sulfides are induced by the protons, which enables the release of the sulfide from the crystal lattice concomitantly with electron transfer to a ferric ion (Schippers and Sand 1999). The first free intermediate of the proton attack is an H_2S^+ cation radical, which after subsequent reactions forms a polysulfide molecule. The polysulfide compound decomposes to H_2S^+ and elemental sulfur. Bacteria regenerate the protons by further oxidizing the elemental sulfur to sulfuric acid (Steudel 1996).

2.4.1.3 Hydrogen sulfide pathway

When ferric ions are absent, readily acid-soluble metal sulfides such as sphalerite can be dissolved by simple acid attack liberating hydrogen sulfide. Hydrogen sulfide is further oxidized by sulfur-oxidizing bacteria to sulfate. This reaction releases a proton, which can enter the cycle again to dissolve more metal sulfides. For example, Chan and Suzuki (1993) found that *A. ferrooxidans* is able to solubilize hydrogen sulfide to elemental sulfur. Moreover, Porro *et al*. (1997) reported that *A. ferrooxidans* was able to leach covellite in the absence of iron, supporting the hydrogen sulfide pathway.

2.4.1.4 Attachment to mineral surfaces

Bacteria are able to form biofilms on the surface of minerals. Biofilm formation is a multistep process which is influenced by many factors, including the specific mineralogy of the rock, solution chemistry (pH, ionic strength) and the characteristics of the microorganism (hydrophobicity, surface charge) (Characklis 1989, Banfield and Hamers 1997; Little *et al*., 1997). The attachment of the cells to the surface can occur *via* random processes such as diffusion and convection or more specifically *via* chemotaxis. Chemotaxis is the active

orientation of a bacterium to a chemical gradient (Jerez 2001). Several researchers studied the chemotatic response of bacteria towards metal ions and other compounds, for example: research by Chakraborty and Roy (1992) and Acuňa *et al.* (1992). The organisms can detect the site of metal sulfide dissolution by sensing a dissolution gradient of dissolved ferrous ions and/or sulfate. This allows the bacterium to respond to the attractant/repellent by swimming away or towards the increased concentration. When the bacterium reaches the surface, the cell can attach by mostly an unknown process (Sand and Gehrke 2006). Blake and coworkers (2001) concluded from their research that aporusticyanin could possibly play an important role in the early attachment of *A. ferrooxidans* to mineral surfaces. Moreover, exopolysaccharides produced by the microbes also, play an important role in attachment of the leaching bacterium.

The exopolysaccharides formed by iron oxidizing bacteria has a net positive charge and at acidic pH, pyrite has a negative charge (Devasia *et al*., 1993; Ohmura *et al*., 1993; Hallman 1992). Thus, attachment of a positively charged bacterium to a negatively charged surface may take place due to electrostatic interaction between the two surfaces. Lazar (2004) found that both electrostatic and hydrophobic interactions could be responsible for the tight adhesion of cells to mineral surfaces (as cited in Sand and Gehrke 2006). The specificity of exopolysaccharides for various metal sulfides has not been termed. Gehrke *et al*. (1998) has however indicated that the composition of the EPS of *A. ferrooxidans* changed when the cells were grown in different substrates. Sulfur-grown cells had more lipids, less neutral sugars and only trace glucuronic acid as compared to those of pyrite grown cells.

2.4.1.5 Importance of exopolysaccharides in bioleaching

The attachment of bacterial cells to the surface of metals is thought to be predominately mediated by the exopolysaccharides surrounding the cells (Gehrke *et al*., 1998; Rojas *et al*., 1995; Tributsch 2001). Research by Gehrke *et al.* (2001) indicated that the attachment does not occur randomly (Gehrke *et al*., 2001). Moreover, Sand *et al.* (2001) demonstrated using atomic force microscopy that the cells of *A. ferrooxidans* preferentially attached to mineral sites with visible surface defects and that mineral dissolution possibly occurs in the exopolysaccharides. Malinovskaya *et al*. (1990) found that acid polysaccharide bacterial

slimes promoted silicate dissolution; however Welch and Van Devivere (1994) found that EPS could also inhibit the dissolution of feldspar under various conditions. The EPS layer formed is thought to breach the gap between the outer membrane of the cell and the surface of the metal surface. Currently there is a proposed electrochemical mechanism for bioleaching of metal sulfides, which is supported by two plausible hypotheses and illustrates the importance of the EPS layer (Medvedev and Stuchebrukhov 2001). The first assumption is that there is an electron tunneling effect present in the EPS. It is known that electrons can bridge distances up to 2nm by tunneling from one electron hole to another. Therefore the distance of ferric ions from the pyrite (sulfide mineral) surface cannot exceed this distance, if the assumption is to be correct. When we consider the distance between the cell membrane and the substrate surface, this assumption seems to be reasonably sound and could explain the reduction of the ferric ion. The second hypothesis is that ferrous ion-glucuronic acid complexes are not stable and this allows the ferrous ions to migrate in the EPS space. If the ferrous ion diffuses towards the outer membrane it will be reoxidized by the enzymatic system of the cells. Thus they serve as a substrate and can enter the oxidation/reduction cycle again (Medvedev and Stuchebrukhov 2001).

2.4.1.6 Role of ferric/ferrous iron

Researchers have found that the amount of EPS-bound ferric ions varies with the metabolic activity of the leaching bacteria. Cells with ample amounts of iron ions and glucuronic acid in their exopolymers exhibit higher oxidation activity than those with low amounts of these constituents. This observation has also been made for microbial strains such as *A. ferrooxidans, L. ferrooxidans* and *L. ferriphilum*. The ferrous ions oxidation mechanism between *L. ferrooxidans* and *A. ferrooxidans* seems to be completely different (Blake *et al*., 1993), however the attachment of the cells to the surface of metal sulfides and EPS formation prior to the onset of leaching is achieved by similar mechanisms (Gehrke *et al*., 1995; Gehrke *et al*., 1998). Moreover, glucuronic acids and ferric ions are key components of the exopolymers of both species. Research by Edwards and coworkers (2001) has indicated that the ferric ions within the EPS-layers are connected to the metabolism of the bacteria, due to its complex formation on the surface of the cell.

2.5 FACTORS AFFECTING BIOLEACHING

Bioleaching processes need to be optimized with regard to the rate of the leaching reactions and the rate of microbial growth. To be able to optimize the bioleaching process, it is necessary to understand the nature of the biotic and abiotic reactions of the system. The factors that influence these reactions are listed in Table 10 (Brandl 2001) and discussed in the following sections.

Table 10 Factors affecting the rate of bioleaching.

Factor	Parameter	Reference
Microbiological	-Microbial diversity -Population density -Microbial activities -Spatial distribution of microorganisms -Metal tolerance adaptation of microorganisms	Das *et al*., 1999 as cited in Brandl 2001 Jain and Sharma 2004 Kawai *et al*., 2000 Tzeferis *et al*., 1994 Valix *et al*., 2001
Physiochemical	-Temperature -pH -Redox potential -Water potential -Oxygen content and availability -Carbon dioxide content -Nutrient availability -Ferric iron concentration	Burgstaller *et al*., 1992 Castro *et al*., 2000 Hallberg *et al*., 1996 Jain and Sharma 2004 Lan *et al*., 1994 Welch and Ullman 1999
Processing	-Leaching mode (heap, dump or tank) -Pulp density -Stirring rate -Heap geometry	Baldi *et al*., 1992
Properties of minerals to be leached	-Mineral type -Mineral composition -Mineral dissemination -Grain size -Surface area -Porosity -Hydrophobicity -Galvanic interaction	Ballester *et al*., 1989 Das *et al*., 1999 as cited in Brandl 2001

2.5.1 Microbial, physiochemical and process parameters

From a microbial aspect, parameters such as their diversity, density, activity, etc. can influence the rate of bioleaching (Jain and Sharma 2004). Das and coworkers (1999) found that the iron and sulfur oxidation rate from different microbial species and strains differ. The researchers contemplated that the observed difference in oxidation rates could be due to several factors such as change in morphology, difference in the chemical composition of the lipopolysaccharides, difference in nutrient metabolism and the microorganisms DNA base ratio. Moreover they stated that the concentration of the microbial cells also influences the oxidation rate. An increase in bacterial numbers should hypothetically increase the oxidation rate. Therefore, various different approaches have been developed to increase the bacterial numbers during a bioleaching process, such as: membrane filtration or centrifugation of bacterial solution, followed by inoculation in a smaller volume; use of a biofilm type reactor called BACFOX (bacterial film oxidation) or using the organisms' affinity for a mineral. As an example of the latter, *Acidithiobacillus* and other acidophilic microorganisms have a special affinity towards jarosite. Therefore, in a reactor, if a thin film of jarosite can be developed under suitable conditions, the microorganism would get the necessary site for surplus growth (Das *et al*., 1999 as cited in Brandl 2001).

The type of ore leached can also affect the bacterial number and therefore the oxidation rate. When metals are leached from ores and wastes, the toxicity of certain metals may influence the rate of leaching. Thus the use of metal tolerant species could enhance the leaching process (Valix *et al*., 2001b). Research has suggested that high concentration of heavy metals act as a broad protoplasmic poison which also denatures proteins and nucleic acids (Avakyan 1994). Avakyan (1994) showed the ability of microorganisms to survive in a heavy metal environment by selecting mutants at high concentrations of heavy metals. Several other researchers have also shown the possibility of isolating microbial strains resistant to heavy metals, for example: Nickel tolerant strains (Tzeferis *et al*., 1994), nickel and cobalt tolerant strains (Valix *et al*., 2001) and aluminum tolerant strains (Kawai *et al*., 2000).

Oxygen concentration is another important parameter for bioleaching. In oxidation process it serves as the electron acceptor (Rawlings 2002). In a heap leaching operation (Section 2.6.1), steps can be taken to ensure adequate oxygen supply, by constructing the heap to ensure optimum aeration and/or by installing an aeration system. With stirred tank bioleaching

(Section 2.6.2), the design of the reactor, impeller, oxygen distribution and even bubble size can determine the amount of oxygen that is available to the cells. If oxygen becomes limiting in leaching reactions, it imposes a limit on the reaction kinetics (Das *et al*., 1999 as cited in Brandl 2001). Oxygen supply has been found to be important in the production of citric acid, which affects the leaching rate of zinc from filter dust by *P. simplicissimum* (Burgstaller *et al*., 1992; Franz *et al.*, 1991). Burgstaller *et al*. (1992) found that if the supply of oxygen was interrupted, citric acid production stopped immediately, production could be reinitiated by supplying oxygen again.

2.5.2 Properties of minerals

When chemical species such as ferric iron or sulfuric acid leach ores, it can form a product on the mineral surface, which can affect the solubilization of metals. This product can be impermeable to the chemical species, and thus no further leaching would be possible. However if the product layer that is formed is porous, the leaching reaction can go on independent of the product layer (Das *et al*., 1999 as cited in Brandl 2001). In bioleaching processes, there are three common product layers that could be formed, namely: gypsum, precipitated iron compounds and sulfur. Ores can contain acid consuming gangue minerals such as carbonates and silicates which are neutralized by the acid forming gypsum during leaching. The organisms commonly used in biomining operations are acidophilic organisms, thus a low pH is required. Therefore the precipitation of gypsum on the surface of the mineral will hinder the activity of the acidophiles (Das *et al*., 1999 as cited in Brandl 2001). Ores containing iron can precipitate products such as hydroxide, goethite, jarosite (Wary *et al.*, 2005 as cited in Brandl 2001) or hematite (Das *et al*., 1999 as cited in Brandl 2001). The formation of precipitated iron compounds depends on pH, Eh and the concentration of ferric iron. Therefore to ensure that these products do not precipitate certain parameter has to be kept stable (Das *et al*., 1999 as cited in Brandl 2001). Sulfur is formed during the dissolution of metal sulphides and forms a dense, protective and insulating layer over the sulfide matrix. This layer limits the transport of species to and from the reaction site, thereby limiting the dissolution of the mineral. Researchers have developed a strategy in which this impermeable layer can be made porous. This is accomplished by using catalysts like Ag, which reacts with the iron and sulfur within the ore, to ensure that the sulfur will not be precipitated (Das *et al*., 1999 as cited in Brandl 2001).

Galvanic interactions can also affect the rate of biooxidation. Das and coworkers (1999) found that the dissolution of pyrite (FeS_2) is faster than similar minerals such as sphalerite (ZnS), galena (PbS) or chalcopyrite ($CuFeS_2$). Moreover when pyrite is leached along with one of the before mentioned minerals, the rate of dissolution decreases. Das and coworkers (1999) ascribed the results to galvanic interaction which is explained as follow: the sulfide minerals are considered semiconductors and can act as electron carriers. Due to the electronic nature of the minerals, the sulfide dissolution reaction can be considered as two half cells (anode and cathode). Each of the sulphide minerals has its own rest potential depending on various factors such as crystallographic structure, solution composition and ionic concentration. If pyrite is in intimate contact with sphalerite, then pyrite, having the higher rest potential will act as cathode and sphalerite as the anode. Therefore sphalerite would be anodically dissolved whereas pyrite would be cathodically protected. So galvanic interactions not only increase the dissolution of anodic sulphide minerals but preferentially the leach of a particular mineral (Das *et al*., 1999 as cited in Brandl 2001; Mason and Rice 2002).

2.6 INDUSTRIAL BIOLEACHING

There are two different leaching approaches used in the biomining industry, namely heap and tank leaching. The approaches selected, depends on the type of mineral to be leached (Rawlings 2002). Stirred tanks (continuous flow) are distinguished from heap leaching, by homogeneous and constant growth conditions, which selects for rapidly growing microbes and are therefore usually dominated by two or three species. Heap leaching is characterized by a heterogeneous growth environment which can change with the age of the heap and therefore the microbial community tends to be more diverse. Here the microorganisms grow as a biofilm which is not subject to washout as with tank leaching (Rawlings and Johnson 2007). Following is a general description of these processes, as well as examples where they have been used.

2.6.1 Heap and dump leaching

Bioleaching has been practiced for centuries. Initially, leaching was initiated by simply irrigating a waste ore dump. This was seen as a low technology process, with low metal recovery efficiency. However, since the early 1960s, metal recovery processes were made more efficient by constructing and irrigating specially designed heaps (Rawlings *et al*., 2003; Rawlings 2005; Schnell 1997). To construct a heap, ore is piled onto an impermeable base and supplied with an efficient leach liquor, distribution and collection system. Acidic leaching solution is percolated through the heaped ore and microbes growing on the surface of the mineral produce ferric iron and acids that result in mineral dissolution and metal solubilization. Aeration can be active or passive, with the former pipes have to be installed that blows air from the bottom of the heap (Figure 10). In the latter case air is drawn into the heap due to the flow of liquid (Rawlings *et al*., 2003; Rawlings 2005). Oxygen is a very important component for effective leaching (Section 2.5), due to its use as an electron acceptor. Thus the leaching kinetics can become limiting if this component is lacking. Moreover, the supply of air also supplies the organism with the necessary carbon source. The metal-containing leach solutions that drain from the heap are collected and sent for metal recovery (Figure 11). The heaps are cheaper to construct than tank reactors, and are thus suited for the leaching of low-grade ores (Bennett *et al*., 2004; Rawlings *et al*., 2003; Rawlings 2005). However the heap leaching process is more difficult to control. It is difficult to aerate the process and gradients of pH and nutrients from inside the heaps which affect bacterial growth and eventually the leaching kinetics (Rawlings 2005). If the pH rises above 2.5, ferric iron precipitation might occur; that will hinder bioleaching (Rawlings *et al*., 2003) by forming an impermeable product layer (Section 2.5.2). Furthermore, due to the low-grade of the ore, it might also contain compounds such as silicates, which can limit the leaching rate.

Heap leaching has been applied mainly to the treatment of copper ores (Figure 11) (Rawlings *et al*., 2003). However it can also be applied to the pretreatment of gold bearing ores (Rawlings *et al*., 2003). Several advances have been made to the heap leaching process, but here the target has mainly been from an engineering perspective and not microbiological (Rawlings 2002).

Figure 10. Heap-leaching operation at Cerro Colorado in Chile. (a) Heaps with aeration blowers, (b) insulating thermofilm and (c) buried irrigation drippers (Rawlings *et al*., 2003).

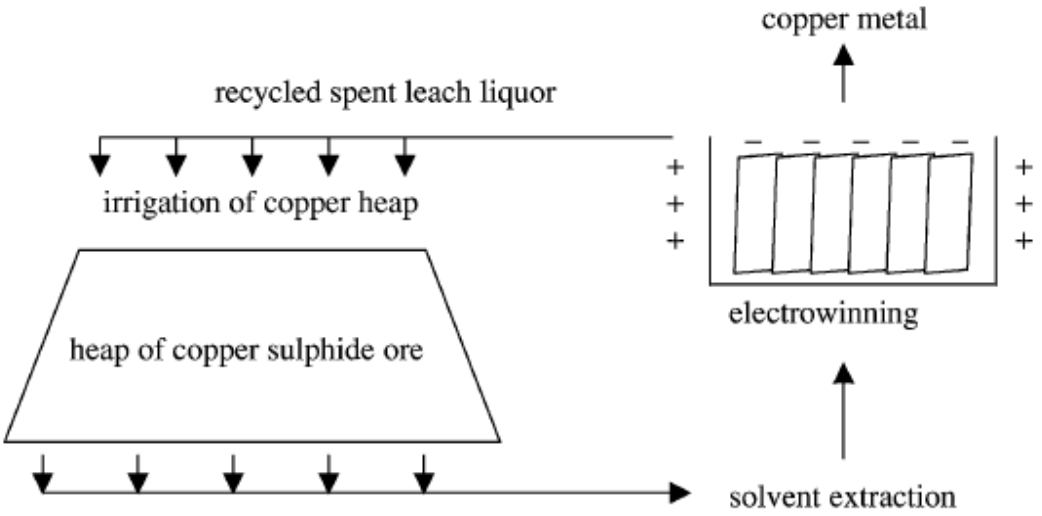

Figure 11. Illustration of copper leaching. The ore is crushed and stacked onto plastic-lined pads and irrigated with recycled leach liquor. The bacteria growing on the ore, oxidizes ferrous iron to ferric iron, which solubilizes the copper, and the pregnant copper-containing solution is recovered from the heap. The copper is concentrated through a solvent extraction process, followed by recovery though electrowinning and the spent leach liquor is recycled to the heap (Rawlings 2002).

2.6.2 Tank leaching

The tank leaching process is reserved due to its high installation and running cost for high value minerals such as gold. The conditions within a stirred reactor are homogenous, with a constant aeration, pH, temperature, nutrient concentration and microbial growth rate. With the stirred tank process, continuous flow reactors are placed in series to treat the mineral (Figure 12). The

ore or finely milled mineral concentrate is added to the first tank together with inorganic nutrient in the form of ammonia and phosphate containing fertilizer (Rawlings *et al*., 2003). The mineral suspension flows through a series of highly aerated tanks that are temperature and pH controlled (Rawlings 1995; Rawlings 2002; Van Aswegen *et al*., 1991). Mineral dissolution takes days in a stirred tank reactor, compared to months with a heap reactor (Rawlings 1997). The major constraint with tank leaching is the quantity of solids that can be maintained in suspension. The liquid can become too thick in the reactor, thereby decreasing gas transfer, which will decrease the oxygen concentration and finally affect the leaching kinetics (Rawlings *et al*., 2003). Examples of industrial use of the tank leaching process includes the leaching of gold containing arsenopyrite at the Fairview mine (Barberton, South Africa) and Ashanti goldfields in Ghana; and treatment of cobalt-containing pyrite at Kasese (Uganda) (Rawlings 2005). Rawlings *et al*. (1999b) found that in a continuous-flow, stirred tank reactor the ferric iron concentration remains high, thus it appears that *A. ferrooxidans* appears to be less important than a combination of Leptospirillum and *A. ferrooxidans* or *A. caldus*.

2.7 INDUSTRIAL BIOLEACING PROCESSES

The metabolic capabilities of microorganisms have been exploited commercially to treat various types of ores (Table 11). Following is a brief description of processes that utilizes a consortium of iron oxidizing bacteria. These processes include the BIOX®, GEOCOAT™, GEOLEACH™, BIOCOP™ and the BacTech/Mintek process.

Table 11 Patents on biohydrometallurgical processing of ores (Adopted from Krebs *et al*., 1997).

Type of ore	Metals recovered	Microorganisms
Iron ore	Iron	*Pseudomonas* species
Gold	Gold	*Chromobacterium violoceum* *Chlorella vulgaris*
Carbon containing gold ore	Gold	*Thiobacillus* species heterotrophic fungi and bacterial strains
Sulfidic gold ore	Gold, silver, copper	*Thiobacillus ferrooxidans*
Manganiferous silver ore	Silver	*Bacillus* species *Bacillus polymyxa*
Sulfidic ore	Gold, silver, copper	*Thiobacillus* species *Leptospirillum ferrooxidans*
Sulfidic ore	Gold, silver, platinum	Sulfate and hydrogen reducing bacteria

2.7.1 BIOX ®

Gold ores can be classified as "free milling" or "refractory". "Free milling" ores only require physical pre-treatments, such as grinding and crushing, to liberate the gold for cyanidation. Refractory ores however require a pre-treatment stage such as roasting, pressure oxidation or fine grinding, to render the ore modifiable to cyanide extraction (Climo *et al*., 2000). The BIOX® process was developed by the Gencor Process Research (now BHP-Billiton, Johannesburg Technology Centre) in the 1970's for the pre-treatment of refractory gold ores and concentrates ahead of conventional cyanide leach for gold recovery. In 1986 the first commercial bioleach plant was commissioned for the pre-treatment of sulfidic gold concentrate to enhance gold recovery (Brierley and Briggs 2002; Van Aswegen *et al*., 1988) and since numerous plant have been build across the world (Table 12) (Olson *et al*., 2003).

Table 12 BIOX® plants commissioned across the world (Olson *et al*., 2003).

Plant and location	Technology	Years in operation
Fairview, South Africa	BIOX®	1986-present
Sao Bento, Brazil	BIOX®	1990-present
Harbour lights, Australia	BIOX®	1992-1994
Wiluna, Australia	BIOX®	1993-present
Sansu, Ghana	BIOX®	1994-present
Youanmi, Australia	BacTech	1994-1998
Tamboraque, Peru	BIOX®	1990-present
Beaconsfield, Australia	BacTech	2000-present
Laizhou, China	BacTech	2001-present

When gold is encapsulated in sulphide minerals such as pyrite, arsenopyrite or pyrrhotite, it hinders the gold from being leached by cyanide. The BIOX® process exploits the metabolic capability of microorganisms to break down the sulphide mineral matrix, thereby exposing the gold minerals for succeeding cyanidation steps. Figure 12 illustrates a typical process flow sheet for the BIOX® process. Sulphide concentrates from the flotation section of the plant is pumped to the BIOX® tank. A sulphide-S concentration of roughly 6% is required to ensure sufficient bacterial activity. When flash flotation is used, a regrind circuit is included in the circuit before the stock tank. The plant normally consists of six reactors that are configured as three primary reactors running in parallel, followed by three secondary reactors running in series (Figure 12). The residence time of the pulp in the biooxidation reactors is usually 4-6 days, depending on the oxidation rates achieved. Approximately have half of the residence time is spent in the primary reactors to allow a stable bacterial population. A shorter time in the secondary reactors can be

tolerated, where the sulphide oxidation is completed. Nutrients such as potassium, phosphorous and nitrogen are added to the primary reactors to increase bacterial growth. The microbial community usually includes the following species, namely: *Acidithiobacillus ferrooxidans*, *Acidithiobacillus thiooxidans* and *Leptospirillum ferrooxidans*; however the cultures are not controlled in the process, but rather left to adapt to the concentrate and operating conditions (Olson *et al*., 2003). Rawlings *et al*. (1999) found that *Acidithiobacillus caldus* and *L. ferriphilum* dominated the microbial community when arsenopyrite was biooxidized at 40°C. Tuffin *et al*. (2005) isolated *A. caldus* from a biooxidation plant, where arsenopyrite was processed, that was highly resistant to arsenic. Air is injected into the BIOX® process to supply oxygen to the biooxidation process. When sulphide minerals such as pyrite are oxidized, acids are produced. However biooxidation of arsenopyrite and pyrrhotite consumes acids, thus the pH (optimum range 1.2-1.8) in the BIOX® process has to be regulated using lime and sulfuric acid (van Aswegen *et al*., 2006). The product of the BIOX® process contains high concentrations of dissolved ions and must be washed in a three-stage countercurrent decantation circuit before cyanide leaching (Figure 12).

Research conducted by Amankwah and coworkers (2005) enabled more gold to be extracted. They tested the effect of a two stage bacterial pretreatment process for double refractory gold ores (ores that contain both sulfides and carbonaceous material). The gold ores were first treated with chemolithotrophic bacteria to oxidize the sulfides and in a second stage carbonaceous matter was removed using *Streptomyces setonii*. It is well known that carbonaceous material is not oxidized by the pretreatment steps and continue to serve as a preg-robber during cyanidation. Using this additional step, 13.6% more gold could be extracted from the ores.

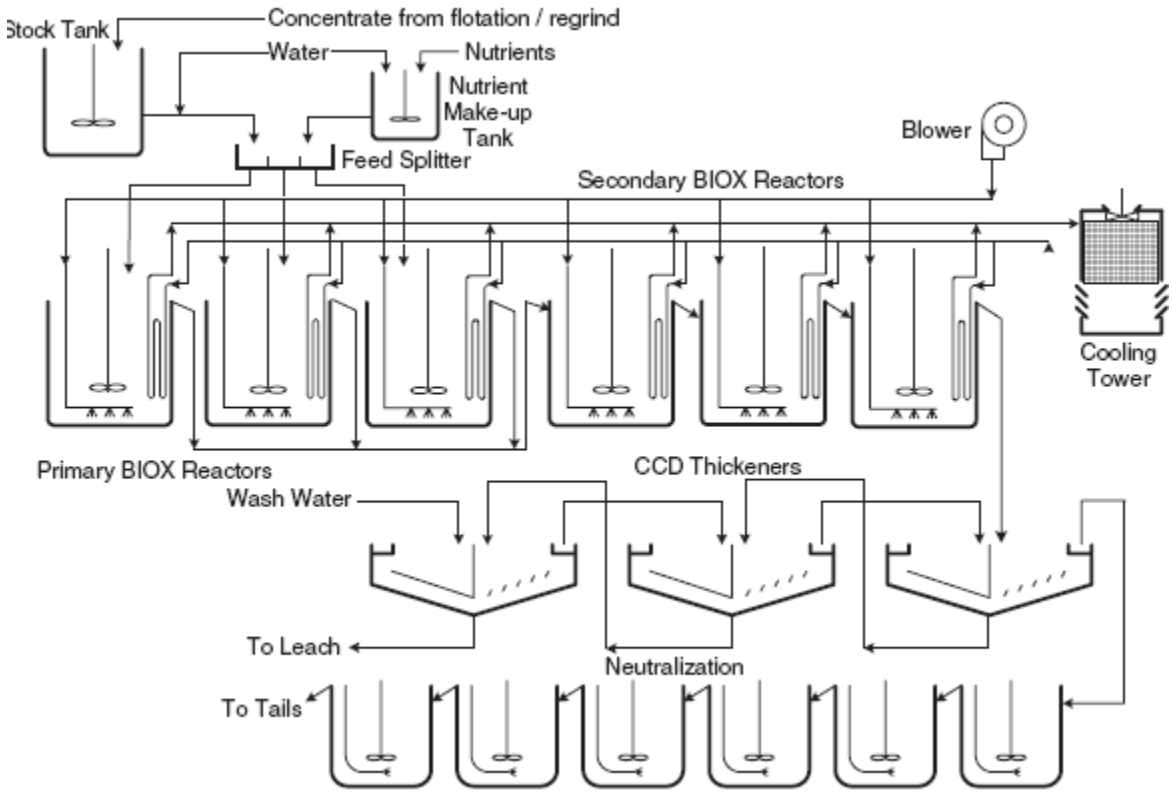

Figure 12. A typical BIOX process flow sheet (van Aswegen *et al*., 2006).

The BIOX® process has numerous advantages compared to conventional refractory processes such as roasting, pressure oxidation and nitric acid leach. These include: improved gold recovery rates; low capital cost; low running cost; low operational skills required and the BIOX® process is an environment friendly technology.

2.7.2 GEOCOAT™

The GEOCOAT® system was originally developed for the treatment of refractory gold deposits by GeoBiotics, LLC. Its use has since been expanded for the treatment of copper, nickel and cobalt (Harvey *et al.*, 2002). A collaboration between Geobiotics and Kumba Iron Ore, has further extended the technologies application, by testing its applicability for extracting zinc from sphalerite concentrates (Harvey *et al.*, 2002). The GEOCOAT® system makes use of two proven technologies namely heap leaching and biooxidation. The process for zinc extraction is as follow: zinc bearing sulphide minerals are concentrated by flotation. The slurry produced during flotation is coated onto a support rock and stacked onto a line pad and left to biooxidize.

Low-pressure blowers are used to supply sufficient air to the system and to remove excess heat. The concentrated slurry adheres strongly to the support rock and does not wash out during irrigation of the heap. The cultures inoculated into the heap, depends upon the desired operating temperature. Sulphide oxidizing bacteria such as *Acidithiobacillus caldus* (DSMZ strain 8584), *Sulfobacillus thermosulfidooxidans* (DSMZ strain 9293 and 1192), *Acidianus brierleyi* (DSMZ strains 1651 and 6334), *Acidianus infernus* (DSMZ strain 3191), *Metallosphaera sedula* (ATCC strain 3390) *Sulfolobus acidocaldarius* (ATCC strain 49426) *Sulfolobus shibatae* (DSMZ strain 5389) and *Sulfolobus metallicus* (DSMZ strain 6482) have been successfully applied in the GEOCOAT® process (Harvey *et al.*, 2002). Nutrients for the microorganisms are added to the heap by recirculating solutions. During the biooxidation process, the sulphides in the concentrated slurry are oxidized and the solubilized zinc, iron, arsenic and sulfates are removed from the heap *via* the recirculating solution. A portion of this recirculated solution is used for metal recovery. Once the biooxidation process is complete, the coated rocks may be unloaded from the pad and recycled or replaced with fresh support rock. The concentrates can then be removed and screened for precious metals (Harvey *et al.*, 2002).

The GEOCOAT® process has a number of advantages over conventional refractory processes and hydrometallurgical base metal recovery systems, such as: low operating cost due to its simplicity; lower construction cost, because it does not require exotic materials; ability to economically process low grade ores and the GEOCOAT® process is safe, because there is no necessity for high pressures (Harvey *et al.*, 2002).

2.7.3 GEOLEACH^{TM}

The GEOLEACH^{TM} process was developed by GeoBiotics, LLC, for treatment of base metal sulphide ores (www.geobiotics.com/GeoL_Process.cfm). The process utilizes iron and sulfur oxidizing microorganisms to assist in oxidizing and leaching sulfide minerals in a constructed whole ore heap environment. The microbial community includes mesophilic microorganisms such as *Thiobacillus ferrooxidans*, *Thiobacillus thiooxidans*, and *Leptospirillum ferrooxidans*, and thermophilic microorganisms such as the Archaea *Sulfolobus* and *Acidianus* (www.geobiotics.com/GeoL_Process.cfm). The GEOLEACH^{TM} process utilizes the HotHeap^{TM}

technology in which the process retains their chemical reaction heat so that the heap can operate at high temperatures. As with copper leaching, high temperatures are necessary to leach this ore economically. Aeration of the heap is controlled according to the oxidation rate within the heap and irrigation of the heap is controlled according to the temperature inside the heap. GeoBiotics, LLC commented that the GEOLEACH^{TM} process can be used to economically recover zinc, cobalt and nickel from sulfide ore (www.geobiotics.com/GeoL_Process.cfm).

2.7.4 BioCOP^{TM}

BioCOP™ Process is owned by BHP Billiton. The BioCOP™ Process has been specifically developed for treatment of concentrates not suitable for commercial smelting due to the content of deleterious elements, such as arsenic, which is harmful to the environment. The process utilizes thermophilic microorganisms operating at temperatures up to 80 °C to leach copper sulphide mineral concentrates. The copper is subsequently recovered by conventional solvent extraction and electrowinning, producing a high value copper metal product (Batty and Rorke 2006).

2.7.5 BacTech/Mintek process

An alternative to the BIOX® process is the Bacox process developed by BacTech (Australia) (Miller 1997). The BacTech process is similar to the BIOX® process, with the major difference being that the BacTech process is operated at 50°C. Moderately thermophilic bacteria therefore carry out biooxidation of concentrates; however the exact composition of the microbial consortium is unclear (Franzmann and Williams 1997**)**. The BacTech process has the added advantage over the BIOX® process in that less cooling is required however the solubility of oxygen and carbon dioxide is less soluble at these higher temperatures (Rawlings 2004).

2.8 CONCLUSION

Biomining exploits the metabolic capabilities of microorganisms for industrial scale mineral processing operations. Conventional treatment methods which include pyro - and hydrometallurgical methods have several limitations that encouraged research into alternative methods. Here biohydrometallurgical processes have proved to be useful; with several industrial scale plants such as the BIOX®, BacTech, BioCOP™, GEOLEACH™ and GEOCOAT™ currently in operation. These systems employ iron oxidizing bacteria such as *A. ferrooxidans*, *L. ferrooxidans*, *L. ferriphilum*, etc. which can leach minerals *via* a 'contact' or 'non-contact mechanism'. Several factors however affect the efficiency of leaching such as: physiochemical parameters, mineral characteristics and microbial strain variations. Except for its industrial bioleaching application, microbial leaching capabilities have also been exploited in bioremediation operations. Apart from their beneficial application in bioleaching, the metabolic capability of iron oxidizing bacteria, can also negatively affect nature, in the form of acid mine drainage. Here the microorganism accelerates the certain reactions which cause acid mine drainage to form more easily. Numerous active and passive strategies have been developed to control or prevent the formation of acid water.

Biomining is a safe, low energy and inexpensive technology. It may not completely replace conventional processing methods, but its application has made mining more economical feasible and environmentally friendly.

CHAPTER 3

REMOVAL OF POTASSIUM AND PHOSPHOROUS FROM SISHEN HEMATITE IRON ORE USING VARIOUS (IN)ORGANIC ACIDS

Abstract

The Sishen Iron Ore Mine is situated in the Northern Cape, South Africa, and forms part of the northern end of the Maremane anticline where the bulk of the hematite is buried beneath younger cover lithologies. The iron ore bodies mined are overlain by conglomerates, shales, flagstone and quartzite. Potassium and phosphorous are common constituents of the iron ore bodies and are known to have deleterious effect on iron and steel manufacturing. Therefore steel making companies charge penalties when purchasing iron ore with alkali concentrations above predetermined limits. Conventional methods used to treat high alkali ore have several drawbacks such as poor product recovery, high running cost and increase pollution of water thus an alternative approach is needed to supplement/replace these methods. Biohydrometallurgy is a natural alternative approach which has been applied commercially to extract or treat various ore bodies. Here we aim to assess the ability of (in)organic acids, known to be produced by microorganisms, to solubilize potassium and phosphorous from different iron ore samples mined at Sishen. We found that the composition of the ore particle plays a crucial role in the ability of each acid to solubilize potassium and phosphorous. Moreover, iron ore particles that contained different combinations of the potassium bearing mineral phase's muscovite, illite, K-feldspar and biotite were solubilized differently by the acids tested. The organic acids tested were able to remove more alkalis than the inorganic acids, which is ascribed to their ability to bind minerals, thereby decreasing its saturation state in solution.

Keywords: Iron ore, Acid leaching, Muscovite, Illite, K-feldspar, Biotite, Apatite

3.1 INTRODUCTION

The Sishen Iron Ore Mine is one of the major suppliers of iron ore to both local and international markets. It is situated in the Northern Cape (South Africa) and forms part of the northern end of the Maremane anticline where the bulk of the hematite ore is buried beneath younger cover lithologies. The majority of the iron ore resource of the mine is made up of laminated and massive ore bodies that belong to the Asbestos Hills Subgroup. These ore bodies are overlain by conglomerates, shales, flagstone and quartzite (Carney and Mienie 2003). The alkalis, potassium and phosphorous, are common constituents of iron ore and have deleterious effects on the manufacturing of iron and steel (Delvasto *et al*., 2008). Therefore, steel making companies charge penalties when purchasing iron ore concentrates with alkali concentrations above predetermined levels according to D. Krige (Personal communication, 2006).

Kumba Iron Ore, Ltd. has an industrial set limit of 0.24% for potassium and phosphorous allowed in their export ore according to D. Krige (Personal communication, 2006). To ensure that their batches stay within acceptable levels; the ores from different excavation sites (with alkalis' >0.24% and <0.24%) are mixed to produce a batch with an average potassium/phosphorous level below 0.24% (Dukino *et al*., 2000). This approach is however a temporary solution as the low potassium/phosphorous ore (< 0.24%) is becoming progressively depleted according to R. Grunewaldt (Personal communication, 2006). Alternative methods that can be employed to treat high impurity ore include pyro- and hydrometallurgical processes (Cheng *et al*., 1999; Kokal *et al*., 2003). However, the application of these methods poses several problems such as poor product recovery, involvement of high process and energy cost and an increase in pollution load of water (Jain and Sharma 2004). Therefore a low maintenance, environment friendly and efficient approach is needed, that can supplement conventional approaches and be incorporated into the mining schedule at minimal cost.

Primary rock-forming minerals can be chemically weathered by water, acids, complexing agents and oxygen into dissolved substances and secondary residual minerals. These reactions can be mediated by living organisms (Landeweert *et al*., 2001). It is well established that many microorganisms are capable of mobilizing minerals from various sources in nature especially in

nutrient limited environments (Banfield *et al*., 1999; Nautiyal 1999; Paris *et al*., 1996). Plant roots and their associated rhizosphere microorganisms produce low molecular weight organic compounds such as aliphatic acids and aldehydes, phenols, sugar acids and amino acids, all of which may accelerate mineral weathering (Drever and Vance 1994). Several reports have demonstrated that microorganisms or their metabolites could solubilize minerals from ores. Leyval and Berthelin demonstrated in 1991 that rhizosphere microorganism, associated with pine roots, could leach potassium from phlogopite by possibly producing organic acids. Jennings (1995) commented on the importance of organic acids in fungal nutrition and physiology. He stated that apart from their possible utilization as carbon and energy source, they also contribute to intracellur osmotic potential, charge balance and pH homeostasis. Margolis and Moreno (1992) found that apatite dissolution was enhanced in the presence of organic acids.

Schnitzer and Kodama (1976) showed that fulvic acid was able to solubilize iron, magnesium, potassium, aluminum and silicon from biotite, phlogopite and muscovite, by forming complexes between the carboxyl/phenolic group of the acid and the mineral. Moreover, they also commented that the mineral with the highest iron concentration was the most susceptible to solubilization by fulvic acid. According to Schnitzer and Kodama (1976), iron ions are transition metal ions and contain low crystal field stabilization energy; therefore fulvic acid can readily remove it. The complexing of iron with fulvic acid affected the structural stability of the mineral and thus other mineral constituents could also be readily released. Growing evidence has suggested that microbial metabolites, especially acids, can be applied to leach various metals from different ore bodies (Ehrlich 1991; Jain and Sharma 2004). However, there is still controversy in literature regarding this (Drever and Stillings 1997; Jones *et al*., 2003). Drever and Stillings (1997) listed three different ways in which organic acids and their anions might affect mineral weathering rates, namely: the organic acids can change the dissolution rate of the mineral far from equilibrium by decreasing the solution pH or by forming complexes at the mineral surface; secondly they can affect the saturation state of the solution with respect to the mineral and lastly the organic acid can affect the speciation in solution of ions such as aluminum that affect minerals dissolution themselves (Table 13). For example, citric acid contains several carboxyl groups which tends to donate protons (H^+), resulting in a negatively charged carboxyl

group. The protons attack the mineral surface (acidulation/hydrolysis reaction), while the negatively charged carboxyl group can form stable complexes with cations thus removing it from solution (Sayer and Gadd 2001). If the organic acid, for example citric or oxalic acid, contains two or more electron donor groups so that one or more rings are formed, during the reaction with a metal, the organic acid can be termed a chelating agent and the resulting complexes termed metal chelates (Gadd 1999; Martell and Calvin 1952).

Here we aim to asses the aptitude of known microbially produced (in)organic acids to solubilize potassium and phosphorous from the various ore bodies mined at Sishen. Abiotic tests are known to be more reproducible than biotic tests, therefore these preliminary results will aid us in determining the effectiveness of microbial leaching in subsequent steps.

Table 13 Examples of bacterial and fungal products of metabolism with a potential for leaching metals from ores non-enzymatically

Acid	Chemical formula	Organism producing	Mode of action	Reference
Acetic acid	CH_3CO_2H	*Acetobacter* species *Achromobacter* species *Arthrobacter* species *Clostridium* species *Paenibacillus stellifer* *Bacillus cereus* *Bacillus subtilis*	Acidulation[11] Complexation	De Faveri *et al*., 2003 Fry *et al*., 2000 Jain and Sharma 2004 Ma *et al*., 2008 Tabak *et al*., 2005 Welch *et al*., 2005 Zigova *et al*., 2000 Wong *et al*., 1998
Ascorbic acid	$C_6H_8O_6$	*Chlorella pyrenoidosa*	Complexation[12]	Ehrlich 1991 United States Patent 5001059
Boric acid (Control)	H_3BO_3	Chemical process	Acidulation	Ehrlich 1991
Citric acid	$HOC[CH_2CO_2H]_2CO_2H$	Fungi *Arthrobacter* species *Bacillus megaterium Pseudomonas putida*	Complexation Acidulation	Ehrlich 1991 Jain and Sharma 2004 Neaman *et al*., 2005 Sayer and Gadd 2001
Gluconic acid	$HO_2CC[O](CH[OH])_3CH_2OH$	*Erwinia herbicola* *Pseudomonas* species *Burkholderia* species *Micrococcus luteus*	Complexation Acidulation	Ehrlich 1991 Krebs *et al*., 1997 Lin *et al*., 2006 Urzi *et al*., 1991
Hydrochloric acid (Control)	HCl	Chemical process	Acidulation	Ehrlich 1991
Malic acid	$HO_2CCH_2CH[OH]CO_2OH$	Plant roots and associated microbial community	Complexation	Burford *et al*., 2003 Little *et al*., 2005
Oxalic acid	HO_2CCO_2H	Fungi *Arthrobacter* species *Paenibacillus stellifer*	Complexation Acidulation	Ehrlich 1991 Gadd 1999 Lierman *et al*., 2000
Sulfuric acid	H_2SO_4	*Thiobacilli*	Acidulation	Ehrlich 1991

[11] Acids able to donate a proton which can act on minerals (Gadd 1999).
[12] Organic acid anions are capable of forming soluble complexes with metal cations thereby increasing its mobility (Gadd 1999).

3.2 MATERIALS AND METHODS

3.2.1 Iron Ore

The majority of ore resources at the Sishen Iron ore Mine, Northern Cape, South Africa is made up of laminated and massive ore bodies that belong to the Asbestos Hills Subgroup. These ore bodies are overlain by conglomerates, shales, flagstone and quartzite (Carney and Mienie 2003). Kumba Iron Ore, Ltd. supplied various different types of iron ore samples from the Sishen Mine for leaching, and labeled them as follow according to B. Ntsoelengoe (Personal communication, 2006): export (alkali levels within set limits); SK (shale - high potassium), SPHP (high phosphorous) and KGT (conglomerate – high potassium). Prior to leaching, the samples were rinsed with distilled water in a sieve (< 1mm) to remove small debris which could hamper subsequent analysis, followed by sterilization at 121°C for 15 min.

3.2.2 Leaching

The ability of different organic acids to solubilize potassium/phosphorous from the export iron ore sample was evaluated by adding 50g of sterilized ore to 100ml of each acid (1M) in 500 ml Erlenmeyer flasks, followed by incubation at 30°C and a duplicate at 60°C for 5 days (Table 15).

The ability of different (in)organic acid to solubilize potassium/phosphorous from the SK, KGT or SPHP samples were assessed by adding 50g of sterilized ore to 100ml of each acid (1M) in 500ml Erlenmeyer flasks, followed by incubation at 30°C and a duplicate at 60°C for 5 days (Table 16, 17 and 18).

After 5 days the iron ores were removed from the various acids and washed three times with distilled water. The samples were then placed in glass petridishes and dried in an oven at 100°C overnight.

3.2.3 Quantitative and Qualitative mineralogy

Treated samples were rinsed with distilled water to remove residual acids. Quantitative and qualitative mineralogy of iron ore samples were preformed as described by Loubser and Verryn (2008). 50 g of each iron ore samples was ground to <75µm in a tungsten carbide milling vessel, followed by roasting at 1000°C to determine the loss of ignition. 1g of the milled sample was then added to 6g $Li_2B_4O_7$ and fused into glass beads. The remaining sample was pressed (2 tons) into a powder briquette for minor element analysis. Major element analyses were done on the fused bead using an ARL9400XP+ spectrometer (wavelength dispersive). Mineral phases were determined using the PANalytical X'Pert PRO X-Ray Powder diffractometer.

Quantitative and qualitative mineralogy of untreated iron ore samples were determined with X-ray powder diffraction (XRD) and X-ray fluorescence (XRF) at the X-ray analytical facility of the University of Pretoria, Pretoria, South Africa. Alkali removal from the ore was determined by measuring the residual alkali contained in the ore after treatment using XRF.

3.2.4 Scanning electron microscopy (SEM)

Acid treated KGT samples were rinsed with distilled water to remove residual acids, followed by incubation at 50°C overnight to dry the samples.

KGT samples were scatter-coated with gold using, a Polaron Equipment LTD SEM Autocoating unit E5200, to make the samples conductive. Samples were observed with a JEOL 5800LV scanning electron microscope at an accelerating voltage of 5kV.

3.3 RESULTS AND DISCUSSION

3.3.1 Quantitative and Qualitative mineralogy

Export iron ore samples were treated with various acids, as several mineral weathering studies have demonstrated their ability to increase the rate of the mineral dissolution reaction and affect its stoichiometry, over a wide pH range (Drever and Stillings 1997; Jones *et al*., 2003; Stillings *et al*., 1996; Wogelius and Walther 1991). Drever and Stillings (1997) listed three different ways in which organic acids and their anions can affect mineral weathering rates, namely: the organic acids can change the dissolution rate of the mineral far from equilibrium by decreasing the solution pH or by forming complexes at the mineral surface; secondly they can affect the saturation state of the solution with respect to the mineral and lastly the organic acid can affect the speciation in solution of ions such as aluminum that affect minerals dissolution themselves (Table 13). Two models have been proposed to describe mineral dissolution *via* protonation and complexation. Firstly a surface complexation model, where protonation of oxide minerals takes place on the mineral surface. Protonation of oxygen and hydroxyl groups on the mineral surface changes the electrostatic interactions between the metal and oxygen subsequently leading to polarization (Brantley 2004). Thus, electrons are shifted towards the more electronegative oxygen, consequently weakening the bonding that holds the metal in the mineral lattice. The formation of surface proton complexes is therefore a requirement for surface controlled dissolution of minerals (Scheckel *et al*., 2005). This model has been applied to silicate dissolution and researchers have proposed that protonation of the terminal aluminum hydroxyl sites controls the dissolution of feldspar (Brantley 2004). The second model is the ligand promoted dissolution, but due to disagreement among scientist in interpreting data for silicate dissolution, no model has been well established (Brantley 2004). One model that possibly explains the mechanism of organic ligand-promoted dissolution is related to surface complexation reactions. Here the attachment of a ligand on the hydroxyl-binding site of a mineral oxide is thought to polarize the metal, which consequently shifts electrons towards the metal, weakening the bonding between the metal and the mineral lattice (Brantley, 2004). The detachment of the metal-ligand complex is thought to be the rate-limiting step in ligand-promoted dissolution of oxide minerals (Furrer and Stumm1986).

Untreated export iron ore (control) samples were subjected to X-ray powder diffraction (XRD) and X-Ray fluorescence (XRF) to determine the quantitative and qualitative mineralogy of the iron ore samples before treatment. Acid treated iron ore samples were subjected to XRF analysis to determine the residual mineral concentration after leaching. The "mineral phases" detected in the untreated sample included hematite (Section 1.2.1), quartz and muscovite (Section 1.2.2) (Figure 13), however two additional phases, namely apatite (Section 1.2.3) and K-feldspar (Table 1) were reported by Kumba Iron Ore, Ltd.according to M. Reyneke (Personal communication, 2007).

X-ray fluorescence is known to be a reasonably sensitive detection method, with detection limits[13] for most elements in the low ppm range (Jenkins 1988). Jenkins (1988) determined the detection limits of a wavelength dispersive spectrometer (Table 14). According to this data, the measurements obtained for magnesium and sodium (Table 15) are not reliable and will therefore be excluded.

Table 14 Detection limits for a wavelength dispersive spectrometry major elements in whole rocks (Jenkins 1988).

Element	Detection limit
Sodium	0.16
Magnesium	0.08
Aluminum	0.032
Silicon	0.050
Phosphate	0.016
Potassium	0.008
Calcium	0.004
Titanium	0.006
Manganese	0.014

[13] Lower limit of detection is defined as that concentration equivalent to a certain number of standard deviations of the background count rate.

Four untreated export iron ore samples (control) were analyzed with XRF, to give a more accurate estimate of the batch sample (Table 15). Focus will be placed mainly on the residual alkali, as these minerals have a deleterious effect in steel manufacturing (Delvasto *et al*., 2008). Results of treated samples were expected to exceed the control sample due to sample variation; therefore a range was determined which included the minimum and maximum amount of alkali measured in the batch untreated sample. From this data set we can possibly determine the amount of alkali removed due to acid leaching and excluded data sets possibly influenced by sample variation. Moreover, the effect of acid leaching on silicon and aluminum will be investigated due to a report by Mojallali and Weed (1978) which demonstrated that a loss of aluminum and silicon caused structural rearrangement of a mineral. Wilson and Jones (1983) found that a decrease in magnesium also shows a rearrangement of a mineral. From XRD analysis (Table 1), the minerals in which potassium occurs also includes aluminum, therefore it is likely that potassium or aluminum/silicon might be solubilized indirectly due to the action of the acid on the other mineral as discovered by Scott and Amonette (1988).

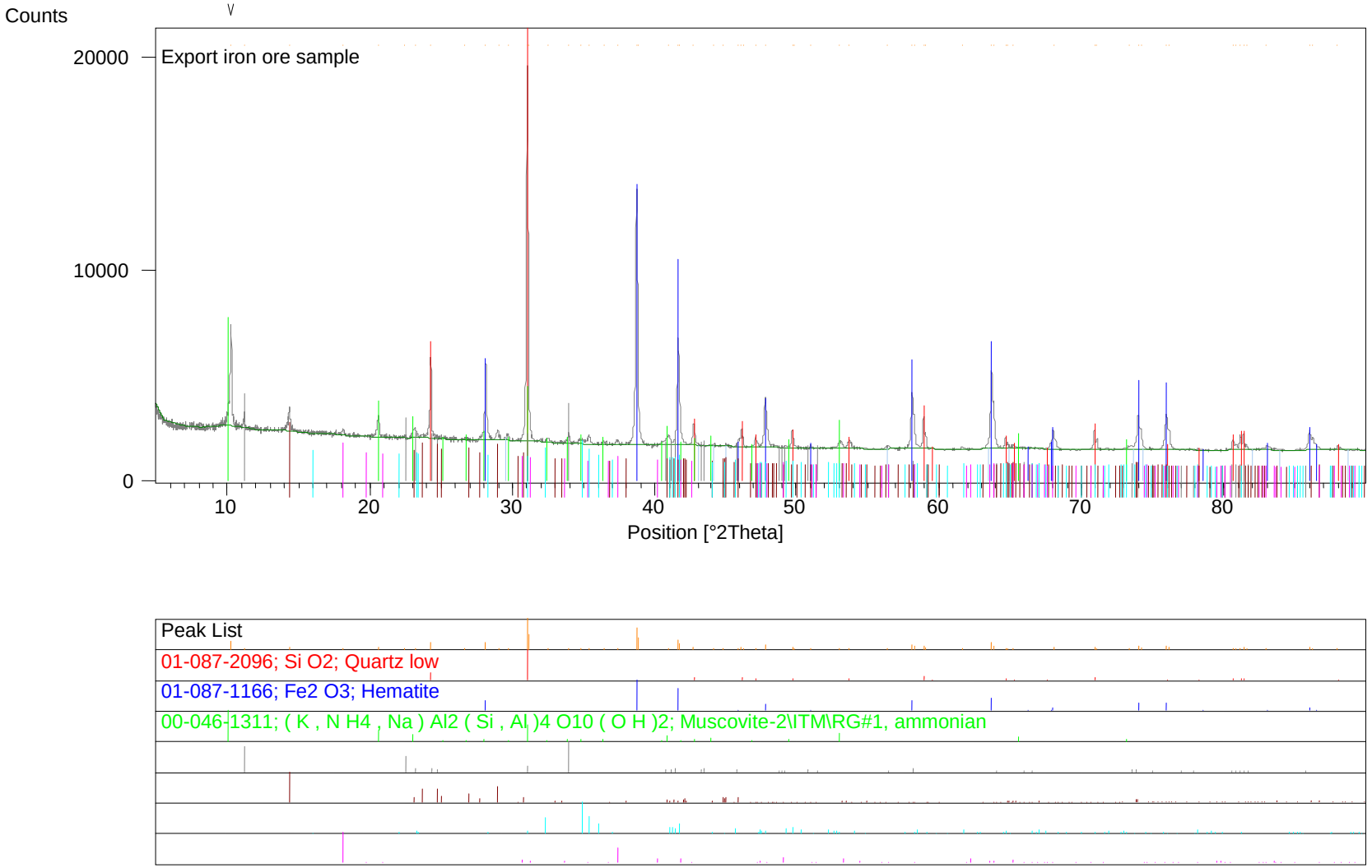

Figure 13. X-ray powder diffraction analyses of untreated export ore.

They demonstrated that the micas biotite and muscovite undergo structural changes and possible dissolution under low pH conditions or in the presence of an organic chelating agent. Moreover, they also found that other cations are ejected along with potassium from the interlayer of the mica. Landeweert *et al*. (2001) commented that oxalate and citrate are able to form stable complexes with cations such as aluminum, thus decreasing the solution concentration and thereby increasing the weathering rate of minerals containing aluminum.

From the above, we examined the following minerals and used the calculated parameters during this discussion: Al_2O_3 (1.15% - 1.56%), SiO_2 (1.92% - 2.80%), K_2O (0.16% - 0.18%) and P_2O_5 (0.13% - 0.17%). Concentrations of each mineral removed below these parameters will be reported as "large quantities[14]" removed. Silicon was present in quartz, muscovite, biotite, etc. in all of the samples analyzed (Table 1). Quartz is known to dissolve slowly, due to the high activation energy needed to break the silicon-oxygen bond (Brehm *et al*., 2005 as cited in Wilson 2004). We did not determine the source of silicon dissolution, as it was outside the scope of this study; however it is assumed that the silicon released was from the less resistant mineral. Hereon we accept the average value of the minerals present in the untreated export sample analysed with XRF as an acceptable representative of the batch sample.

Muscovite and K-feldspar were identified as the major potassium bearing mineral present in the export sample. Muscovite is an aluminosilicate mineral with compositional affinity to feldspar, feldspathoids and quartz (Deer *et al*., 2001; Kalinowski and Schweda 1996; McKeown *et al*., 1999). The structure of feldspar consists of cross-linked, 'double-crankshaft' chains of Si^{4+} and Al^{3+} tetrahedral with charge compensating such as sodium, potassium and calcium occupying small cavities in the framework (Han and Lee 2005). Alkali feldspar can be weathered into a secondary mineral kaolinite [$Al_2Si_2O_5(OH)_4$] under the influence of H^+ ions. The acids assayed during this section of the study are able to aid the solubilization process *via* protonation and/or complexation mechanisms. All of the acids tested, except gluconic acid (60°C), were able to remove potassium so that the residual was below 0.16%. Its inability to

[14] A maximum and minum concentration for each mineral present in the untreated sample was determined. Once the ore was treated with the specific assay, we analyzed it again with XRF. If the residual amount of a specific mineral was below the minimum concentration, we ascribe it to the assay tested.

efficiently remove potassium (below 0.16%) is ascribed to sample variation[15] and/or mineral dissemination[16], as it was able to remove potassium from the other ore bodies analyzed.

XRF analysis indicated that the selected minerals were solubilized from the export iron ore (Table 15). The amount of silicon and aluminum removed, illustrates a rearrangement of the minerals as discussed by Mojallali and Weed (1978). Acetic (30°C), ascorbic (60°C) and boric acid (30°C and 60°C) were all able to remove aluminum, silicon, potassium and phosphorous below the set parameters, as indicated previously. Huang and Longo (1992) tested the dissolution of K-feldspar in oxalic and acetic acid. They found that dissolution of Si and K from the mineral was increased with decreasing pH. They further discovered that the presence of Ca and Mg decreased the dissolution of Si and K. The dissolution of feldspar is known to be affected by various different factors such as twinning lamellae, dislocation, surface roughness and surface area (Deer *et al*., 2001). The mechanism suggested by some researchers for mineral dissolution by organic acids refers to the complexation of aluminum, which can be seen as an indirect mechanism or ligand-promoted dissolution (Wilson 2004). Preliminary results obtained during this study and work done as part of a Phd thesis, at the University of Pretoria, justified repeating the citric and acetic acid experiments. Here XRF data confirmed that acetic and citric acid were both able to efficiently remove potassium and phosphorous from the export iron ore sample.

It was thought that the acids that were able to form complexes and attack the minerals with protons would be able to remove the most alkali as Drever and Stillings (1997) stated that bidentate acids are more effect than monodentate acids at solubilization minerals, due to stronger complex formation (Drever and Stillings 1997), however from our data we found that acetic and citric acid were able to remove comparable amounts. The data suggested that acetic and gluconic acid, which is known to have protonation and complexation mechanisms (Ehrlich 1991), were able to remove large concentrations of the alkalis (Table 15). However boric acid, which is not able to form complexes with the solubilized minerals were able to remove comparable amounts of alkali. This observation is ascribed to saple variation, as acids

[15] Sample contains an alkali concentration higher than the set parameter and therefore no apparent removal will be reported.
[16] Alkali is located in the core of the particle and therefore inaccessible to the acid

able to form complexes with solubilized minerals showed greater potential with the other samples.

Most literature reports focused on leaching experiments conducted on almost pure minerals and some on heterogonous samples such as iron ore. We ascribe the difference in results obtained, compared to literature, to the varying composition of the ore and/or the accessibility of the minerals leached as the alkali could be located in the core of the particle. This observation spurred additional research into determining the effect of particle size on mineral leaching, as Bosecker (1997) found that a particle size of 42 µm was optimum for leaching. However, Mortland and Lawton found that potassium released from biotite in a NaCl solution was slower from fine particles than from large ones (as cited in Wilson 2004). Reichenbach and Rich (1969) found similar results to Mortland and Lawton when they experimented with different particle sizes of muscovite and its exchange with $BaCl_2$ at elevated temperatures. Reichenbach and Rich (1969) proposed that the decreased leaching efficiency with smaller particles was due to a difference in the exchange mechanism brought about by the splitting of the particles. The splitting caused a release in stress brought about by the expansion and bending of the flakes following potassium depletion form mineral, thus resulting in inhibition of further potassium exchange.

During this study, the effect of extended leaching times were also assayed. Optimum leaching was obtained after 5 days for most acids tested and were therefore selected as a standard. There are several possible reasons that only a limited number of minerals were solubilized. Firstly it is possible that after 5 days all the accessible minerals were removed and only the inaccessible (inside the core of the particle) remains. Moreover the available protons could also be exhausted and therefore no further solubilization of the minerals could occur as described by Welch *et al*. (2002). Lastly, a saturation state could have been reached in solution and therefore no further minerals would be solubilized. Scott and Smith (1966) conducted experiments in which they determined the susceptibility of interlayer potassium in micas to exchange with sodium. They found that essentially, all the potassium in muscovite, biotite, phlogopite and vermiculite were exchangeable when the concentration of the potassium in solution was kept low.

Table 15 XRF analysis of acid treated export iron ore.

Element	Untreated Export ore*	Ace 30°C ***	%	Ace ** 60°C	%	Asc ** 30°C	%	Asc 60°C	%	Bor ** 30°C	%	Bor 60°C	%	Gluc ** 30°C	%	Gluc 60°C	%	Cit 30°C ***	%
SiO_2	**2.36**	1.92	19	1.72	27	1.50	36	1.86	21	1.48	37	1.83	22	1.89	20	1.90	20	1.93	19
TiO_2	**0.08**	0.06	25	0.07	13	0.06	25	0.07	13	0.06	25	0.06	25	0.06	25	0.06	25	0.06	25
Al_2O_3	**1.32**	0.78	41	1.02	23	0.93	30	1.00	23	0.87	34	0.93	30	0.85	36	1.18	11	0.93	30
Fe_2O_3	**95.68**	94.77	1	96.86	n/a	96.85	n/a	96.44	n/a	97.06	n/a	96.98	n/a	96.96	n/a	96.23	n/a	95.87	n/a
MnO	**0.03**	0.03	0	0.04	n/a	0.03	0	0.03	0	0.04	n/a	0.04	n/a	0.04	n/a	0.04	n/a	0.04	n/a
MgO	**0.03**	0.00	100	0.00	100	0.00	100	0.00	100	0.00	100	0.00	100	0.00	100	0.00	100	0.00	100
CaO	**0.04**	0.00	100	0.00	100	0.00	100	0.00	100	0.00	100	0.00	100	0.00	100	0.00	100	0.00	100
Na_2O	**0.01**	0.01	0	0.01	0	0.01	0	0.01	0	0.01	0	0.01	0	0.01	0	0.01	0	0.01	0
K_2O	**0.17**	0.12	29	0.14	18	0.14	18	0.15	12	0.14	18	0.13	24	0.13	24	0.16	6	0.13	24
P_2O_5	**0.15**	0.11	27	0.14	7	0.17	n/a	0.10	33	0.13	13	0.11	27	0.11	27	0.13	13	0.13	13
Cr_2O_3	**0.02**	0.02	0	0.02	0	0.02	0	0.02	0	0.02	0	0.02	0	0.02	0	0.02	0	0.02	0
NiO	**0.01**	0.00	100	0.00	100	0.00	100	0.00	100	0.00	100	0.00	100	0.00	100	0.00	100	0.00	100
V_2O_5	**0.01**	0.01	0	0.01	0	0.01	0	0.02	n/a	0.02	n/a	0.02	n/a	0.02	n/a	0.02	n/a	0.02	n/a
ZrO_2	**0.00**	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0

*Average (Triplicate analysis)

**Ace – Acetic acid; Asc – Ascorbic acid; Bor – Boric acid; Gluc – Gluconic acid; Cit – Citric acid

*** Average (Triplicate analysis)

Phosphorous was detected as apatite in the export ore (Table 1). All the acids tested, except acetic acid at 60°C and ascorbic acid at 30°C were able to remove "large quantities" of potassium and phosphorous. This can again be attributed to sample variation or mineral distribution inside the particle, as Welch and coworkers (2002) demonstrated that acetic and oxalic acid were able to increase apatite dissolution. They attributed the ability of these acids to solubilize the phosphorous from the mineral to their ability to form complexes with either the calcium on the mineral surface or in the solution. Moreover, they further commented that the acids were also effective at near neutral pH, thereby supporting the complexing mechanism as fewer protons would be present for protonation. Welch and coworkers (2002) further demonstrated that calcium was preferentially released from apatite than phosphate. They proposed that the increased release could be due to an ore reactive calcium rich phase such as carbonate, calcium carbonate or calcium fluoride. Denêvre *et al.* (1996) found that complexation is dependent on the relative concentration of the anions and metals in solution, the pH and stability constants of the various complexes; therefore it is probable that these factors played a role in our leaching experiments. We found that boric (60°C), gluconic (30°C) and ascorbic acid (60°C) were able to remove more than 25% of the phosphorous from the export ore. Gluconic acid is one of the strongest naturally occurring organic acids and has been associated with bacteria that are able to solubilize high levels of phosphates (Goldstein and Krishnaraj 2007). Furthermore, boric acid is able to release a proton, which can attack the mineral surface and aid in solubilization (Sayer and Gadd 2001). Research has demonstrated that gluconic and ascorbic acid were able to form complexes with the minerals released, thus affecting the saturation state and the amount of alkali that could be removed (Drever and Stillings 1997; Goldstein 1995). Moreover, when these acids form complexes, protons are released which can aid further solubilization of the minerals (Drever and Stillings 1997; Ehrlich 1991).

We found during this section of the study that the organic acids were more effective at removing the alkali from the ore than the inorganic acids. This is in accordance with other research that found mineral weathering by organic acids to be 3-5 times higher compared to inorganic acids at the same pH. This observation was ascribed partly due to the complex forming ability of organic acids, which lowers the saturation state of cations in solution (Banfield *et al.*, 1999). Moreover,

researchers indicated that increased temperatures favor the release of phosphorous from apatite (Kim *et al*., 2003), which is in accordance with some of the XRF data obtained during this study.

From the XRF data (Table 15), it was observed that none of the manganese, sodium, chromium, vanadium or zirconium was removed. These minerals do not form a component of the mineral phases detected in the ore by XRD (Table 1). The inability of the acids to remove these minerals could be due to the minerals intrinsic characteristics or possible position inside the ore particle. For example, vanadium is a type of transition metal, which is known to have corrosion resistance to alkali, sulfuric-, hydrochloric acid and saline solution (Moskalyk and Alfantazi 2003). Furthermore, all of the magnesium and calcium were removed from the ore (Table 15), which is ascribed to the leaching mechanisms of the acids tested.

KGT (conglomerate) samples were supplied by Kumba Iron Ore, Ltd. and categorized as a high potassium/low phosphorous iron ore samples according to B. Ntsoelengoe (Personal communication, 2006). The susceptibility of this sample to acid leaching was tested at two different temperatures (Table 16). Four untreated KGT samples (control) were analyzed with XRF, to give a more accurate estimate of the mineral composition of the batch sample (Table 15). Focus will be placed mainly on the residual alkali, as these minerals have a deleterious effect on steel manufacturing (Delvasto *et al*., 2008). Results of treated samples are expected to exceed the control sample due to sample variation; therefore a range was determined which included the minimum and maximum amount of alkali measured in the replicate untreated sample. Therefore the following minerals and parameters for the export iron ore samples were used during this study: Al_2O_3 (1.90 - 2.14); SiO_2 (3.90 – 4.90), K_2O (0.48 – 0.56) and P_2O_5 (0.06 – 0.07). Concentrations of each mineral removed below these parameters will be reported as “large quantities” removed. From this data set we can possibly determine the amount of alkali removed due to acid leaching and exclude the data sets possibly influenced by sample variation. Moreover, the effect on silicon and aluminum will also be investigated as these might be solubilized with the alkalis as stated previously. Scott and Amonette (1988) found that the micas biotite and muscovite undergo structural changes and possible dissolution under low pH conditions or in the presence of an organic chelating agent. Moreover, they also found that other cations are ejected along with potassium from the interlayer of the mica.

Landeweert *et al*. (2001) commented that oxalate and citrate are able to form stable complexes with cations such as aluminum, thus decreasing the solution concentration and thereby increasing the weathering rate of minerals containing aluminum. Mojallali and Weed (1978) stated that a loss of aluminum and/or silicon shows a more structural rearrangement of the mineral. Later Wilson and Jones (1983) found that a decrease in magnesium and iron shows an even more rearrangement of a mineral.

Untreated KGT samples (control) were subjected to X-ray powder diffraction (XRD) and X-Ray fluorescence (XRF) to determine the quantitative and qualitative mineralogy of the iron ore samples initially. Acid treated KGT samples were subjected to XRF analysis to determine the residual mineral concentration after leaching (Table 16). The "mineral phases" detected in the untreated sample included hematite, quartz and muscovite (Figure 14), however additional phases, namely greenalite, illite, etc (Table 1) were reported by Kumba Iron Ore, Ltd.. Silicon was present in quartz, muscovite, biotite, etc. in all of the samples analyzed (Table 1). Quartz is known dissolved slowly, due to the high activation energy needed to break the silicon-oxygen bond (Brehm *et al*., 2005 as cited in Wilson 2004). We did not determine the source of silicon dissolution, as it was outside the scope of this study; however it is assumed that the silicon released was from the less resistant mineral. Jenkins (1988) reported the lower detection limits of a wavelength dispersive spectrometer (Table 14), therefore sodium and magnesium will be excluded from further analysis as it is below the limits for the machine.

Potassium was detected as illite and muscovite in the KGT sample (Table 1). Illite is a non-expanding, clay-sized phyllosilicate or layer alumino-silicate that commonly occurs in sediments, soils and argillaceous sedimentary rocks. It is structurally similar to muscovite or sericite [$KAl_2(OH)_2(AlSi_3O_{10})$], with more silicon, magnesium, iron and water, with less tetrahedral aluminum and interlayer potassium. Illite occurs as an alternation product of muscovite and feldspar during weathering (Mengel and Uhlenbecker 1993).

Acetic (30°C), citric (60°C), sulfuric (30°C) and malic acid (30°C) were able to remove "large quantities" of potassium and aluminum. The amount of aluminum removed, illustrates a rearrangement of the minerals (Mojallali and Weed 1978). Han and Lee (2005) found that *Bacillus mucilaginosus*, a known potassium solubilizing bacterium, was able solubilize

potassium from mica, illite and orthoclase by producing organic acids. Acetic, malic and citric acid are able to form complexes with the minerals released from the ore (Table 16). During complex formation, protons are released which can aid solubilization of other minerals. Sulfuric acid and malic acid are able to donate a proton, thus solubilization *via* protonation (Drever and Stillings 1997). The inability of the other acids tested to solubilize alkali from the ore is ascribed to mineral dissemination and sample variation, as certain of the data reported quantities are outside of the set parameters. Illite and muscovite have similar structures. Muscovite is known to have considerable resistance toward leaching of interlayer potassium, compared to trioctahedral micas (such as biotite). It was proposed by Kalinowski and Schweda (1996) that an inclined orientation of hydroxyl ions in the dioctahedral micas results in stronger binding of potassium.

XRD analysis of the KGT sample did not detect a phosphorous phase. This is in accordance with results obtained from Kumba Iron Ore, Ltd. (Table 1). Manganese, sodium, chromium and vanadium were not leached from the ore by the acids tested. It is assumed that this is due to mineral dissemination or mineral characteristics for example. Vanadium is a type of transition metal, which is known to have corrosion resistance to alkali, sulfuric-, hydrochloric acid and saline solution (Moskalyk and Alfantazi 2003) and therefore we expect it not to be affected, as with the export iron ore analysis. Magnesium, calcium, nickel and zirconium were not detected in the KGT sample which is probable that the concentrations of these minerals were below the detection limit of XRF (Jenkins 1988).

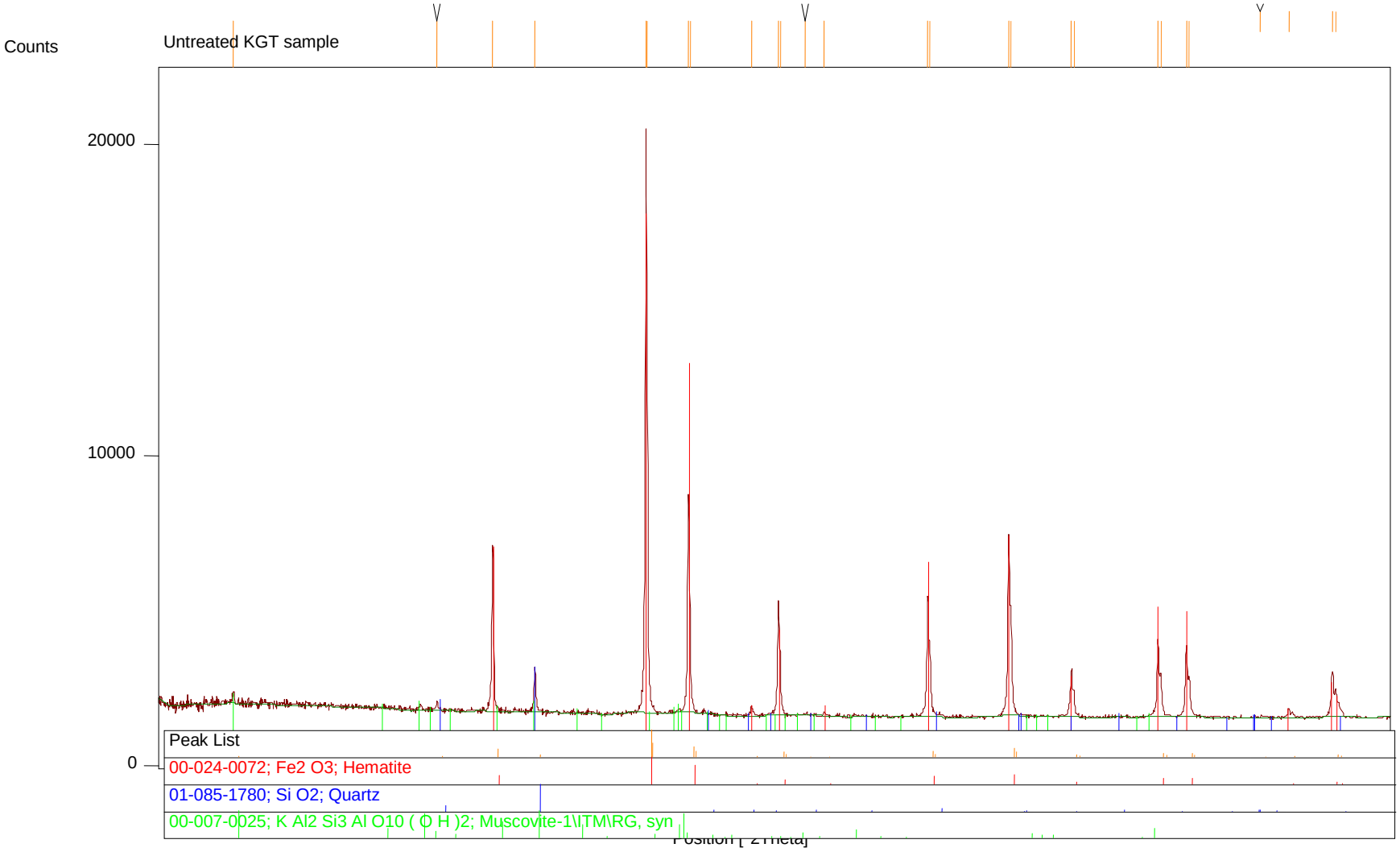

Figure 14. X-ray powder diffraction analyses of untreated KGT (conglomerate) iron ore

Table 16 XRF analysis acid treated KGT samples

Element	Untreated KGT sample*	Ace 30°C**	% removed	Ace 60°C**	% removed	Asc 30°C**	% removed	Asc 60°C**	% removed	Cit 30°C**	% removed	Cit 60°C**	% removed
SiO_2	**4.41**	4.86	n/a	4.49	n/a	5.08	n/a	4.57	n/a	4.60	n/a	4.56	n/a
TiO_2	**0.10**	0.10	0	0.09	10	0.11	n/a	0.11	n/a	0.10	0	0.10	0
Al_2O_3	**2.02**	1.79	11	1.95	3	2.45	n/a	2.22	n/a	1.90	6	2.46	n/a
Fe_2O_3	**92.68**	90.40	2	90.98	2	88.88	4	90.18	3	90.70	2	92.32	0
MnO	**0.03**	0.03	0	0.03	0	0.03	0	0.03	0	0.03	0	0.03	0
MgO	**0.00**	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0
CaO	**0.00**	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0
Na_2O	**0.01**	0.01	0	0.01	0	0.01	0	0.01	0	0.01	0	0.01	0
K_2O	**0.52**	0.47	10	0.50	4	0.64	n/a	0.60	n/a	0.50	4	0.48	7
P_2O_5	**0.07**	0.06	14	0.06	14	0.07	0	0.07	0	0.07	0	0.07	0
Cr_2O_3	**0.02**	0.02	0	0.02	0	0.02	0	0.02	0	0.02	0	0.02	0
NiO	**0.00**	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0
V_2O_5	**0.02**	0.02	0	0.02	0	0.02	0	0.02	0	0.02	0	0.02	0
ZrO_2	**0.00**	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0

* Average (Triplicate analysis)

**Ace – Acetic acid; Asc – Ascorbic acid; Cit – Citric acid

Table 16 (Continue)

Element	Untreated KGT sample*	Oxa 30°C**	% removed	Oxa 60°C**	% removed	H_2SO_4 30°C**	% removed	HCl 30°C**	% removed	HCl 60°C**	% removed	Mal 30°C**	% removed
SiO_2	**4.41**	4.97	n/a	4.48	n/a	4.63	n/a	5.54	n/a	4.30	2	4.07	8
TiO_2	**0.10**	0.10	0	0.10	0	0.09	10	0.11	n/a	0.12	n/a	0.10	0
Al_2O_3	**2.02**	2.44	n/a	1.90	6	1.89	6	2.24	n/a	2.04	n/a	1.78	12
Fe_2O_3	**92.68**	91.49	1	90.24	3	93.16	n/a	90.72	2	92.56	0	93.50	n/a
MnO	**0.03**	0.03	0	0.03	0	0.04	n/a	0.03	0	0.03	0	0.03	0
MgO	**0.00**	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0
CaO	**0.00**	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0
Na_2O	**0.01**	0.01	0	0.01	0	0.02	n/a	0.01	0	0.01	0	0.01	0
K_2O	**0.52**	0.60	n/a	0.49	6	0.46	12	0.54	n/a	0.52	0	0.47	10
P_2O_5	**0.07**	0.07	0	0.07	0	0.06	14	0.07	0	0.07	0	0.07	0
Cr_2O_3	**0.02**	0.02	0	0.02	0	0.02	0	0.02	0	0.02	0	0.02	0
NiO	**0.00**	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0
V_2O_5	**0.02**	0.02	0	0.02	0	0.02	0	0.02	0	0.02	0	0.02	0
ZrO_2	**0.00**	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0

**Ace – Acetic acid; Asc – Ascorbic acid; Cit – Citric acid

SK iron ore samples (shale) are known to be high in potassium/low phosphorous. The aptitude of various acids to remove the alkalis from the SK sample was tested (Table 17). Mineral phases detected with XRD included muscovite, hematite and quartz (Figure 15). Additional phases were reported by Kumba staff (Table 1). The alkali potassium was present as biotite, muscovite and illite. Biotite is a ferromagnesium mineral with compositional affinity to olivine [$(Mg,Fe)_2SiO_4$], pyroxene [$XY(Si,Al)_2O_6$][17] and amphiboles[18] (Deer *et al*., 2001). As with other mica minerals, it has a highly perfect basal cleavage and consists of flexible sheets or lamellae[19], which can easily flake off. Calvaruso *et al*. (2006) stated that biotite can undergo two types of weathering namely: congruent (destruction of the mineral surface) and incongruent (transformation into vermiculite by release of interlayer potassium). The sheets in the crystal structure of biotite are made up of iron, magnesium, aluminum, silicon, oxygen and hydrogen ions that are weakly bound together by potassium. The potassium can be substituted in part by sodium, calcium, barium. Magnesium can be completely replaced by ferrous iron and ferric iron and in part by titanium and manganese. Biotite is often referred to as the iron mica because it contains more iron than phlogopite (Hashemi-Nezhad 2005). No phosphorous phases were detected in the SK sample.

The following parameters for the alkalis and mineral described by Mojallali and Weed (1978) and Wilson and Jones (1983) were calculated from XRF data of untreated SK sample: Al_2O_3 (9.69 – 10.17); SiO_2 (11.56 – 12.26); K_2O (2.31 – 2.43) and P_2O_5 (0.03 – 0.04). These results are in accordance with classification by Kumba Iron Ore, Ltd. staff. Treated samples analyzed did contain quantities outside the parameters set with the untreated samples (Table 17), which is attributed to sample variation. Concentrations of each mineral removed below these parameters will be reported as "large quantities" removed as this will be used to determine which acids were able to solubilize the most alkali. Alkali removal from the ore was determined by measuring the residual contained in the ore after the acid treatment and comparing it to the

[17] X represents calcium, sodium, ferrous iron, magnesium, zinc, manganese and lithium. Y represents ions such as chromium, aluminium, ferric iron, magnesium, manganese, scandium, titanium, vanadium. (http://en.wikipedia.org/wiki/Pyroxene)
[18] The main differences between amphiboles and pyroxenes are that amphiboles contain essential hydroxyl (OH) or halogene (F, Cl) and secondly the basic structure is a double chain of tetrahedra (as opposed to the single chain structure of pyroxene). (http://en.wikipedia.org/wiki/Amphiboles)
[19] A lamella is a gill-shaped structure: fine sheets of material held adjacent one another, with fluid in-between-(or simply 'welded'-plates)[http://en.wikipedia.org/wiki/Lamellae_(materials)]

untreated sample (control). Jenkins (1988) reported the lower detection limits of a wavelength dispersive spectrometer (Table 14), therefore sodium will be excluded from further analysis. We accept the average value of the untreated SK sample as an acceptable representative (Table 17).

Acetic (30°C), ascorbic (60°C), sulfuric (30°C and 60°C) and malic acid (30°C) were able to remove large quantities of potassium from the ore. Only acetic and sulfuric acid were able to remove = aluminum, potassium and silicon from the sample. The amount of silicon and aluminum removed, illustrates a rearrangement of the minerals as stated by Mojallali and Weed (1978). These acids aid solubilization *via* protonation in the (Ehrlich 1991). It was expected that citric acid would be able to remove some of the potassium, as it was effective at removing the alkali from the KGT sample. It is assumed that the difference in mineral composition of the KGT, SK and export iron ore samples played a role in the amount of potassium release (Das *et al*., 1999 as cited in Brandl 2001).

The low concentration of potassium solubilized was expected as the sample contained illite, biotite and muscovite which are all known to be effected by the potassium concentration in solution. Rausell and coworkers (1965) determined the sensitivity of muscovite to potassium concentration in solution. Later Pal and coworkers (2001) demonstrated that if biotite and muscovite were both present in soil, no potassium dissolution from muscovite would occur, further supporting findings by Rausell *et al*. (1965). Also biotite weathering is known to be diffusion-controlled and depends on the potassium concentration in the solution (Wilson 2004) and therefore we expected low potassium solubilization to occur. Scott and Smith (1966) determined the susceptibility of various different micas to exchange their interlayer potassium with sodium from the surrounding environment. They found that almost all of the interlayer potassium of muscovite and biotite were exchangeable, as long as the solution potassium concentration was kept below certain limits. It is therefore possible that the inability of the acids to remove greater amounts of potassium from the SK (high potassium) sample is due to a saturation state of potassium reached in solution.

Manganese was removed by oxalic (30°C and 60°C), hydrochloric (30°C), ascorbic (30°C) and citric acid (30°C and 60°C). It susceptibility to leaching could be due to its possible position on the surface of the particle. Phosphorous, vanadium and chromium were not leached, possibly

due to their position closer to the core of the particle (Table 17). Ballester *et al*. (1989) and Das *et al*. (1999) listed several properties of minerals which could influence leaching. These include: mineral type, gain size, mineral dissemination, etc. Therefore the position of the mineral in the particle could determine its susceptibility to leaching.

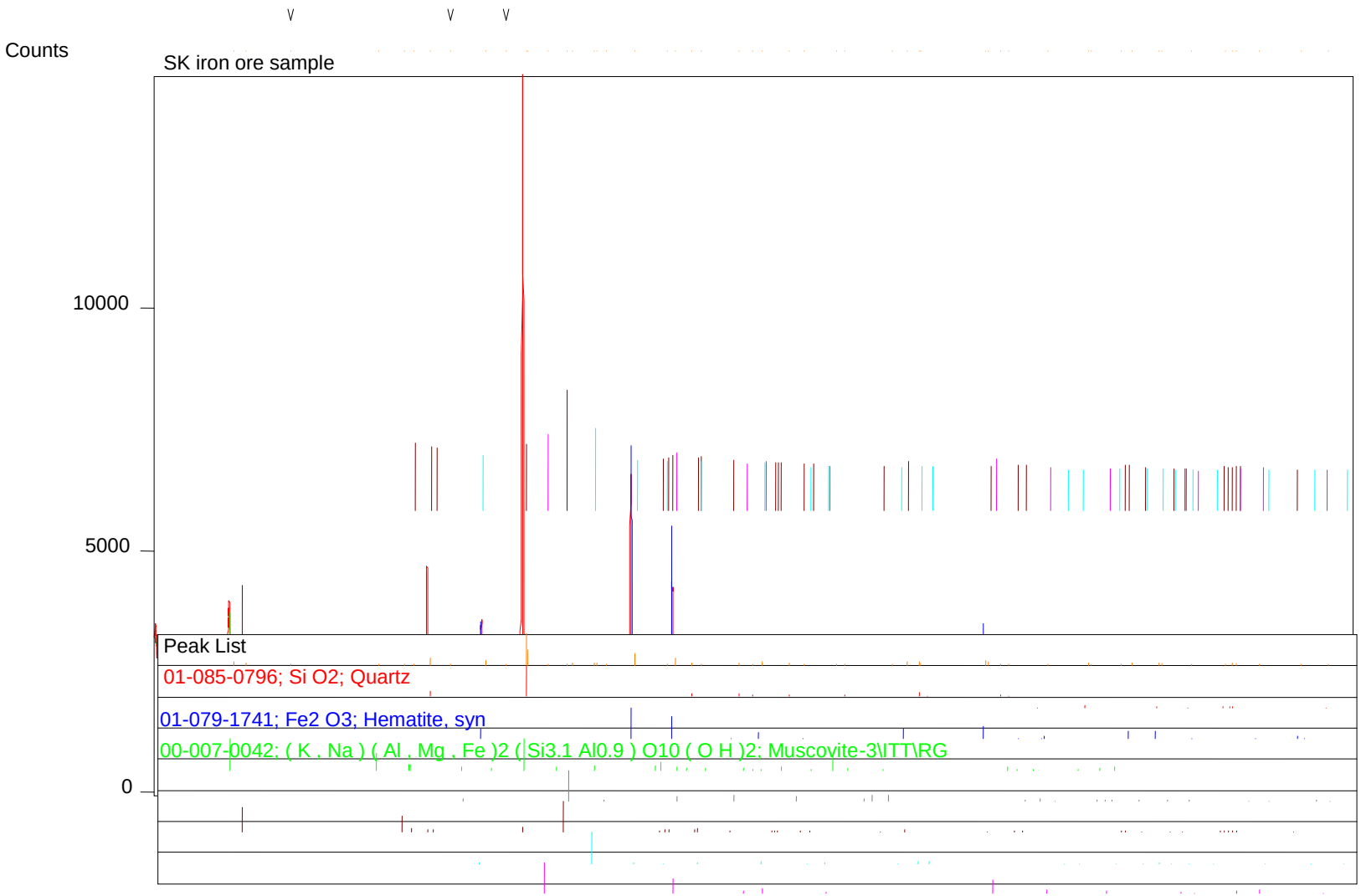

Figure 15. X-ray powder diffraction analyses of untreated SK (shale) iron ore.

Table 17 XRF analysis of acid treated SK iron ore.

Element	Untreated SK Sample*	Ace 30°C**	% removed	Asc 30°C**	% removed	Asc 60°C**	% removed	Cit 30°C**	% removed	Cit 60°C**	% removed
SiO_2	**11.91**	11.57	3	12.08	n/a	13.27	n/a	12.68	n/a	12.24	n/a
TiO_2	**0.61**	0.57	7	0.59	3	0.60	2	0.61	0	0.62	n/a
Al_2O_3	**9.93**	9.57	4	9.91	0	10.13	n/a	10.30	n/a	10.07	n/a
Fe_2O_3	**74.12**	74.62	n/a	73.32	1	72.04	3	72.19	3	73.54	1
MnO	**0.03**	0.03	0	0.02	33	0.02	33	0.02	33	0.02	33
MgO	**0.00**	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0
CaO	**0.00**	0.00	0	0.03	0	0.00	0	0.00	0	0.00	0
Na_2O	**0.13**	0.10	23	0.12	8	0.22	n/a	0.10	23	0.18	n/a
K_2O	**2.37**	2.28	4	2.37	0	2.30	3	2.45	n/a	2.38	n/a
P_2O_5	**0.04**	0.04	0	0.05	n/a	0.04	0	0.04	0	0.04	0
Cr_2O_3	**0.03**	0.03	0	0.04	n/a	0.04	n/a	0.04	0	0.04	n/a
NiO	**0.00**	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0
V_2O_5	**0.03**	0.03	0	0.03	0	0.03	0	0.03	0	0.03	0
ZrO_2	**0.00**	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0

* Average (Triplicate analysis)

**Ace – Acetic acid; Asc – Ascorbic acid; Cit – citric acid; Oxa – Oxalic acid

Table 17 (Continue)

Element	Untreated SK sample	Oxa 30°C**	% removed	Oxa 60°C**	% removed	H_2SO_4 30°C**	% removed	H_2SO_4 60°C**	% removed	HCl 30°C**	% removed	Mal 30°C**	% removed
SiO_2	**11.91**	11.95	n/a	11.56	3	11.35	5	11.85	0	12.41	n/a	11.94	n/a
TiO_2	**0.61**	0.59	3	0.59	3	0.61	0	0.59	3	0.64	n/a	0.58	5
Al_2O_3	**9.93**	10.17	n/a	9.78	2	9.59	3	9.92	0	10.47	n/a	9.83	1
Fe_2O_3	**74.12**	74.15	n/a	75.01	n/a	75.00	n/a	72.66	2	72.35	2	74.05	0
MnO	**0.03**	0.02	33	0.02	33	0.03	0	0.03	0	0.02	33	0.03	0
MgO	**0.00**	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0
CaO	**0.00**	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0
Na_2O	**0.13**	0.12	8	0.12	8	0.12	8	0.14	n/a	0.12	8	0.14	n/a
K_2O	**2.37**	2.35	1	2.31	3	2.29	3	2.31	3	2.41	n/a	2.26	5
P_2O_5	**0.04**	0.04	0	0.04	0	0.04	0	0.04	0	0.05	0	0.05	0
Cr_2O_3	**0.03**	0.04	n/a	0.03	0	0.04	n/a	0.04	n/a	0.04	n/a	0.10	n/a
NiO	**0.00**	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.01	n/a
V_2O_5	**0.03**	0.03	0	0.03	0	0.03	0	0.03	0	0.03	0	0.03	0
ZrO_2	**0.00**	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0

**Oxa – Oxalic acid; H_2SO_4 – sulfuric acid; HCl – hydrochloric acid; Mal – Malic acid

SPHP iron ore was categorized by Kumba Iron Ore, Ltd., as a high phosphorous/low potassium sample. This is in accordance with our results (Table 18). The mineral phases detected were (Figure 16): apatite, hematite, muscovite and quartz (Table 1). XRF analysis of the untreated SPHP iron ore gave the following parameters, as selected for the other samples analyzed: Al_2O_3 (0.47 - 0.51); SiO_2 (0.57 - 0.83); K_2O (0.03 - 0.04) and P_2O_5 (0.34 - 0.40). Samples analyzed did contain minerals with quantities outside the parameters (Table 18), which is attributed to sample variation. Concentrations of each mineral removed below these parameters will be reported as "large quantities" removed. Alkali removal from the ore was determined by measuring the residual contained in the ore after the acid treatment and comparing it to the untreated sample (control). Jenkins (1988) reported the lower detection limits of a wavelength dispersive spectrometer (Table 14), therefore sodium was excluded from further analysis. According to XRF analysis, magnesium, calcium, nickel and zirconium were absent from the sample. The amount of these minerals could possibly be below the detection limit, explaining their absence from the mineral composition. We accept the average value of the untreated SK sample as an acceptable representative (Table 18).

There was no amount of potassium removed from the SPHP sample that could exclude the role of sample variation as none of the samples contained residual amounts of potassium that was below the set parameters stated previously. There are three possible reasons why none of the potassium could be removed. Firstly the alkali could have been present in the core of the particle, therefore inaccessible to leaching; secondly a minute quantity of potassium was released, which could not be detected by XRF; and lastly sample composition might be highly variable between batches. Several acid were however able to remove a "large quantity" of phosphorous. These included the following (Table 18): Citric acid (60°C), oxalic (30°C and 60°C), sulfuric acid (30°C and 60°C), hydrochloric (30°C and 60°C) and malic acid (30°C). The acetic and ascorbic acids were able to solubilize minerals from the export, KGT and SK sample, but not from the SPHP sample. This is ascribed to mineral composition and dissemination within the ore particle. More phosphorous was released from the SPHP sample when the acid leaching experiments were conducted at elevated temperatures. This is in accordance with research by Goldstein and Krishnaraj (2007) and Kim *et al.* (2003). Kim and coworkers (2003) determined the type of phosphorous found in the sediments of lakes and rivers and the effect of

temperature on phosphorous solubilization. They found that more phosphorous was released as the temperature increased. Goldstein and Krishnaraj (2007) discovered that the most common method by which calcium phosphate are solulized, is by acidulation. All of the acids tested are able to donate a proton and thus consequently acidify the solution (Ehrlich 1991), which would therefore influence the mobility of the various minerals in the ore.

Oxalic acid and hydrochloric acid were able to remove a "large quantity" of aluminum. Oxalic acid is able to aid solubilization by forming complexes with minerals released and by protonation. Hydrochloric acid is able to donate a proton and thus assist solubilization by a protonation attack (Table 13). We expected that aluminum and potassium would be leached simultaneously as with the other iron ore samples tested. This was not the case with the SPHP sample. We ascribed the results obtained for the amount of aluminum and potassium released to sample variation or to low concentration of potassium present in the sample, therefore any removal seem insignificant from XRF analysis. Furthermore, all the effective acids for solubilizing phosphorous are able to aid solubilization *via* protonation, which is in accordance with results from Goldstein and Krishnaraj (2007), which found that calcium phosphate is mostly solubilized *via* acidulation.

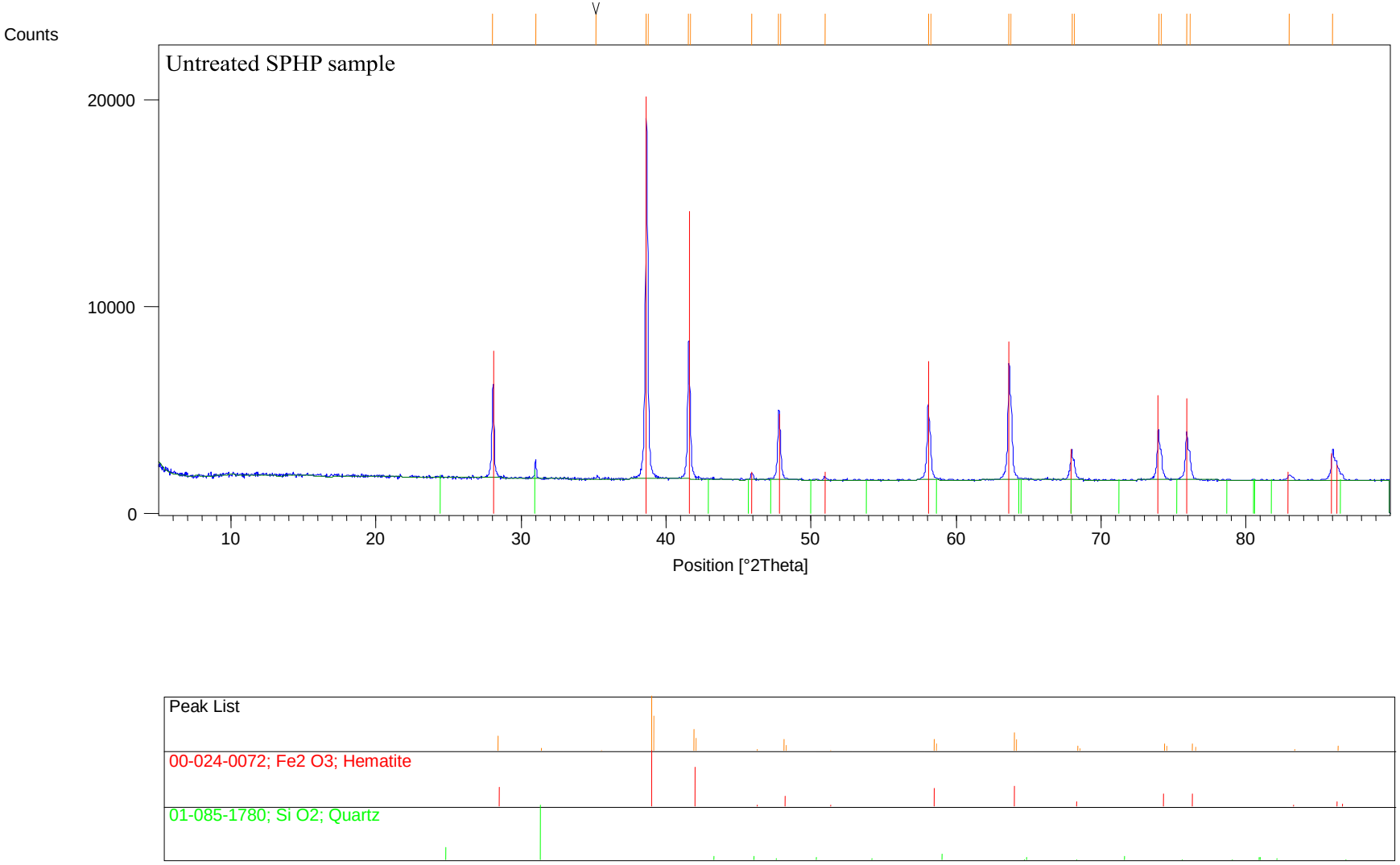

Figure 16. X-ray powder diffraction analyses of untreated SPHP iron ore.

Table 18 XRF analysis of acid treated SPHP iron ore.

Element	Untreated SPHP Sample*	Ace 30°C **	% removed	Ace 60°C **	% removed	Asc 30°C **	% removed	Asc 60°C **	% removed	Cit 30°C **	% removed	Cit 60°C* *	% removed	Oxa 30°C **	% removed
SiO_2	**0.70**	0.54	23	0.77	n/a	0.70	0	0.98	n/a	0.80	n/a	0.96	n/a	0.83	n/a
TiO_2	**0.02**	0.02	0	0.02	0	0.02	0	0.02	0	0.02	0	0.02	0	0.02	0
Al_2O_3	**0.49**	0.54	n/a	0.47	4	0.46	6	0.78	n/a	0.61	n/a	0.83	n/a	0.34	31
Fe_2O_3	**98.48**	98.33	0	96.02	3	97.78	0	95.48	3	98.24	0	97.79	0	98.27	0
MnO	**0.04**	0.04	0	0.04	0	0.04	0	0.04	0	0.04	0	0.04	0	0.04	0
MgO	**0.00**	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0
CaO	**0.00**	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0
Na_2O	**0.01**	0.01	0	0.01	0	0.01	0	0.01	0	0.01	0	0.01	0	0.01	0
K_2O	**0.04**	0.03	25	0.04	0	0.05	n/a	0.04	0	0.03	25	0.04	0	0.03	25
P_2O_5	**0.37**	0.38	n/a	0.38	n/a	0.39	n/a	0.34	8	0.36	3	0.28	24	0.21	43
Cr_2O_3	**0.02**	0.02	0	0.02	0	0.02	0	0.03	n/a	0.02	0	0.03	n/a	0.02	0
NiO	**0.00**	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0
V_2O_5	**0.02**	0.02	0	0.02	0	0.01	50	0.01	50	0.02	0	0.02	0	0.01	50
ZrO_2	**0.00**	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0

* Average (Triplicate analysis)

**Ace – Acetic acid; Asc – Ascorbic acid; Cit – citric acid; Oxa – Oxalic acid

Table 18 (Continue)

Element	Untreated SPHP sample	Oxa 60°C**	% removed	H_2SO_4 30°C**	% removed	H_2SO_4 60°C**	% removed	HCl 30°C**	% removed	HCl 60°C**	% removed	Mal 30°C**	% removed
SiO_2	**0.70**	0.58	17	0.55	21	0.71	n/a	0.61	13	0.56	20	0.62	11
TiO_2	**0.02**	0.02	0	0.02	0	0.02	0	0.02	0	0.02	0	0.02	0
Al_2O_3	**0.49**	0.47	4	0.43	12	0.47	4	0.59	n/a	0.40	18	0.51	n/a
Fe_2O_3	**98.48**	98.65	n/a	97.04	2	97.28	1	98.62	n/a	99.44	n/a	97.84	1
MnO	**0.04**	0.04	0	0.04	0	0.04	0	0.04	0	0.04	0	0.04	0
MgO	**0.00**	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0
CaO	**0.00**	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0
Na_2O	**0.01**	0.01	0	0.01	0	0.01	0	0.01	0	0.01	0	0.01	0
K_2O	**0.04**	0.03	25	0.03	25	0.03	25	0.03	25	0.03	25	0.04	0
P_2O_5	**0.37**	0.18	51	0.16	57	0.22	41	0.25	32	0.30	19	0.29	12
Cr_2O_3	**0.02**	0.02	0	0.02	0	0.03	n/a	0.02	0	0.02	0	0.02	0
NiO	**0.00**	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0
V_2O_5	**0.02**	0.01	50	0.01	50	0.02	0	0.02	0	0.01	50	0.02	0
ZrO_2	**0.00**	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0

**Oxa – Oxalic acid; H_2SO_4 – sulfuric acid; HCl – hydrochloric acid; Mal – Malic acid

From XRF data obtained (Table 13 – Table 18), we observed that the acids were able to remove varying amounts of minerals from each sample. Table 19 serves as a summary of the composition of the various samples. Ballester *et al*. (1989) and Das *et al*. (1999) commented that the intrinsic characteristics of a mineral can affect the rate of leaching. These factors include: mineral type and composition, grain size, surface area, porosity, mineral dissemination, formation of secondary minerals, etc (Table 10). Therefore it was expected that the different samples (Export, KGT, SK and SPHP) obtained from Kumba Iron Ore, Ltd., would have varying susceptibility to acid leaching, due to their intrinsic mineral composition. These preliminary results are therefore important for the design of a pilot scale testing plant, as the type of ore determines the amount of potassium and/or phosphorous that can be leached effectively.

Table 19 Quantitative mineralogy of iron ore sample supplied by Kumba Iron Ore, Ltd.

	Export	KGT	SK	SPHP
Mineral	**Quantity**	**Quantity**	**Quantity**	**Quantity**
SiO_2	**2.36**	**4.41**	**11.91**	**0.70**
TiO_2	**0.08**	**0.10**	**0.61**	**0.02**
Al_2O_3	**1.32**	**2.02**	**9.93**	**0.49**
Fe_2O_3	**95.68**	**92.68**	**74.12**	**98.48**
MnO	**0.03**	**0.03**	**0.03**	**0.04**
MgO	**0.03**	**0.00**	**0.00**	**0.00**
CaO	**0.04**	**0.00**	**0.00**	**0.00**
Na_2O	**0.01**	**0.01**	**0.13**	**0.01**
K_2O	**0.17**	**0.52**	**2.37**	**0.04**
P_2O_5	**0.15**	**0.07**	**0.04**	**0.37**
Cr_2O_3	**0.02**	**0.02**	**0.03**	**0.02**
NiO	**0.01**	**0.00**	**0.00**	**0.00**
V_2O_5	**0.01**	**0.02**	**0.03**	**0.02**
ZrO_2	**0.00**	**0.00**	**0.00**	**0.00**

3.3.2 Scanning electron microscopy

KGT samples (treated and untreated), were observed in a JEOL 5800LV scanning electron microscope (SEM) at an accelerating voltage of 5kV, as supplementary analysis (Figure 17). Results from SEM can be ambiguous and was therefore only used to support the information derived from XRF.

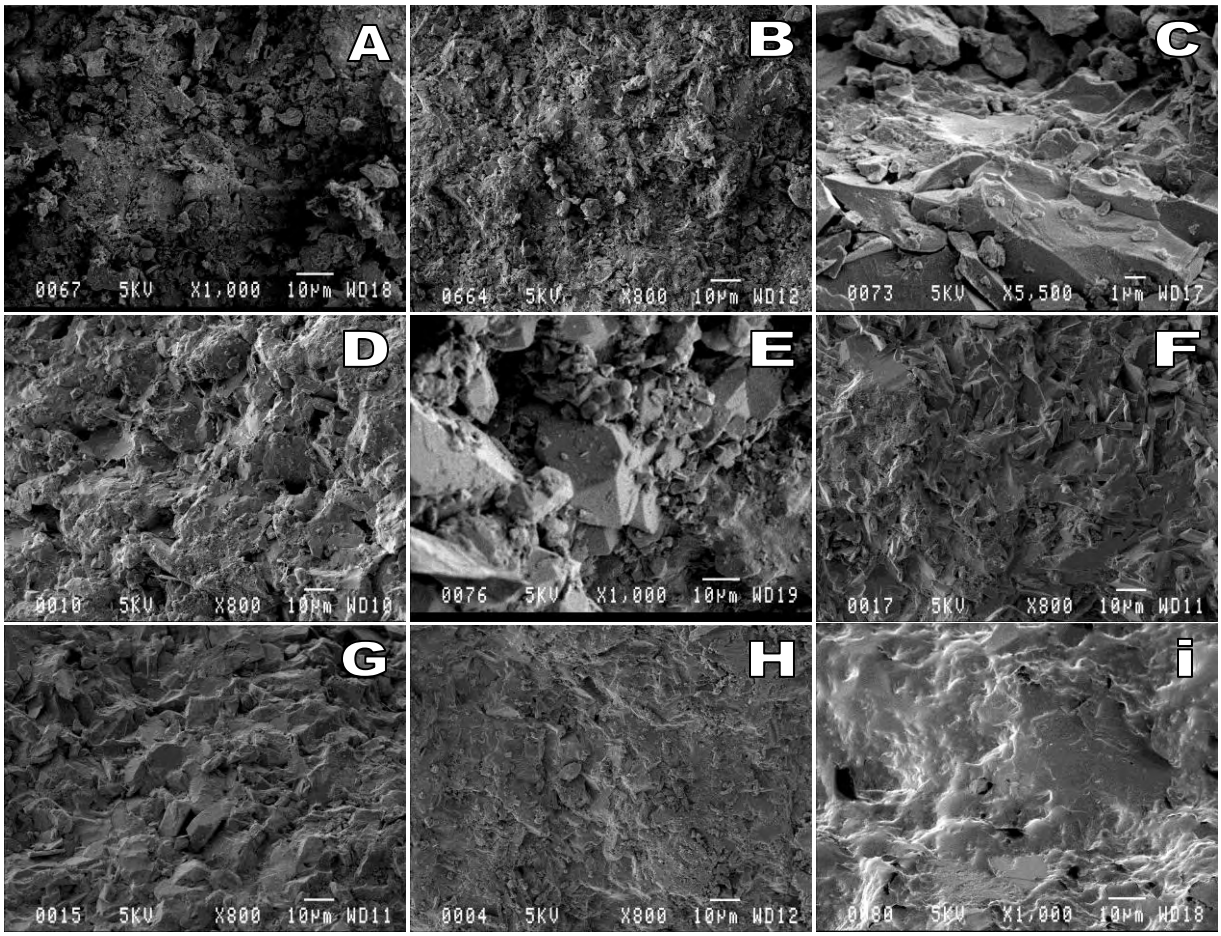

Figure 17. Scanning electron microscopy analysis of acid treated KGT iron ore. A – untreated export ore; B-acetic acid; C – ascorbic acid; D – boric acid; E – citric acid; F – hydrochloric acid; G – oxalic acid; H – malic acid; I – sulfuric acid.

The KGT sample is known to contain the mineral phase's greenalite, hematite, illite, muscovite and nacrite (Table 1). From XRF data (Table 16) we established that acetic, citric, sulfuric and malic acid were able to remove "large quantities" of potassium and aluminum. The inability of

other acids tested to remove these elements was attributed to mineral dissemination within the ore particles. The untreated iron ore particle (Figure 17 A) has an uneven surface due to sample handling and processing at the Sishen mine, which makes surface comparison to treated samples difficult and unreliable. However hydrochloric – and sulfuric acid was able to change the surface of the particle (Figure 17 F and I) as smoothening of the surface was observed. From Table 16 we found that sulfuric acid was able to remove potassium and aluminum, which lets us deduce that the change in surface morphology is due to the removal of these elements (Figure 17 – I). Although hydrochloric acid was unable to remove any of the potassium from the KGT iron ore sample, we still saw a change in surface structure, which is ascribe to the removal of other ore components (Figure 17 - F).

3.4 CONCLUSION

The Sishen Iron Ore Mine is one of the major suppliers of iron ore to both local and international markets. The majority of the iron ore resource of the mine is made up of laminated and massive ore bodies that belong to the Asbestos Hills Subgroup. These ore bodies are overlain by conglomerate, shales, flagstone and quartzite. Potassium and phosphorous are common constituents of iron ore, which have a deleterious effect on iron and steel manufacturing. Therefore steel making companies charge penalties when purchasing iron ore concentrates with an alkali concentration above predetermined limits. Conventional pyro- and hydrometallurgical processes used to treat high alkali ore suffer from various limitations. Therefore the objective of this thesis was to determine whether microorganisms could be applied to supplement conventional methods. This study accessed the ability of microbially produced (in)organic acids to solubilize potassium and phosphorous from different iron ore bodies mined at the Sishen Iron Ore Mine. From the XRF data obtained, we observed that the acids tested were able to remove varying amounts of potassium and phosphorous from the different iron ore samples. This was expected as the mineral composition and distribution of the alkali in the ore particle affects leaching efficiency.

Research has demonstrated that bidentate acids were more efficient at leaching than monodentate acids, as they were able to form stronger complexes with minerals. In contrast, our results

showed that a monodentate acid was as effective as a bidentate acid. This was however attributed to mineral dissemination in the particle.

The SK sample analyzed contained illite, biotite and muscovite as potassium phases. It is known that muscovite is sensitive to minute quantities of potassium in solution, and will therefore not release this element if other phases such as biotite are present. The dissolution of biotite is diffusion-controlled and also depends on the potassium concentration of the bulk solution. Therefore only minute quantities of potassium could be released from this sample.

The KGT sample contained muscovite and illite. Here some of the potassium could be released. Drever and Stillings (1997) stated that organic acids were able to affect the saturation state of a solution by forming complexes with the minerals released. It was therefore expected that the effective acids used in our study to remove potassium/phosphorous would be an organic acids. Oxalic acid was able to leach of potassium from the KGT sample. However, sulfuric acid and malic acid were also able to remove potassium from the KGT sample. These acids aid weathering *via* a protonation mechanism (Table 13). Therefore the results obtained were attributed to sample variation and mineral dissemination in the ore particle.

The SPHP sample analyzed contained a high phosphorous and low potassium concentration compared to the other samples (Table 19). No potassium could be removed from the SPHP sample, which was ascribed to the mineral composition and/or distribution of the alkali in the ore particle. The acids tested were effective at removing phosphorous at higher temperatures, which is in accordance with research done by Kim *et al*. (2003) which demonstrated that a higher temperature favors more phosphorous release from apatite.

From this section of the study we therefore found that the composition of the ore body determines its susceptibility to acid leaching. Furthermore, we found that sample variation played a significant role in our data analysis and therefore more repitions needs to be completed. However due to cost limitations this was not possible. However, these preliminary results can still serve as a guideline for future acid leaching experiments on various iron ore bodies.

CHAPTER 4

THE USE OF IRON OXIDIZING BACTERIA TO LEACH POTASSIUM AND PHOSPHOROUS FROM SISHEN HEMATITE IRON ORE

Abstract

The Sishen Iron Ore Mine is situated in the Northern Cape, South Africa, and forms part of the northern end of the Maremane anticline where the bulk of the hematite is buried beneath younger cover lithologies. The iron ore bodies at the mine are overlain by conglomerates, shales, flagstone and quartzite. Potassium and phosphorous are common constituents of iron ore that have deleterious effect on iron and steel manufacturing, therefore steel making companies charge penalties when purchasing iron ore with alkali concentrations above predetermined limits. Conventional methods used to treat high alkali ore have several drawbacks such as poor product recovery, high running cost and increase pollution of water. Biohydrometallurgy is a natural alternative approach which could be applied to supplement conventional methods. Our aim was to assess the ability of commercially exploited iron oxidizing bacteria to remove potassium and phosphorous from export iron ore samples mined at Sishen. We found that the organisms were able to solubilize varying degrees of potassium and phosphorous, which was attributed to the different metabolic capabilities of each organism tested. The Iron oxidizing bacteria were more effective than the abiotic tests with sulfuric acids, which is ascribed to their ability to form exopolysaccharides which influenced the saturation state of the minerals released in solution. The iron oxidizing bacteria showed a synergistic effect on removing potassium and phosphorous from export iron ore, which decreased the time required to efficiently leach.

Keywords: Iron oxidizing bacteria, Sulfuric acid, Ferric iron, Bioleaching, Iron ore

4.1 INTRODUCTION

Leaching[20] of sulfidic minerals using chemolithoautotrophic bacteria are the best studied and commercially exploited of mineral biotechnology today (Jain and Sharma 2004). The application of biohydrometallurgical processes have increased due to favorable process economics and reduced environmental problems compared to conventional methods such as pyro – and hydrometallurgical methods (Olson *et al*., 2003). The metabolic capabilities of these organisms enable their use in industrial scale processes such as the BIOX® (Van Aswegen *et al*., 1988), GEOCOAT™ (Harvey *et al*., 2002), GEOLEACH™ (www.geobiotics.com/GeoL_Process.cfm), BIOCOP™ (Batty and Rorke 2006) and the BacTech/Mintek (Miller 1997) process (Table 5). The solubilization of the minerals in these processes is considered to be largely a chemical process, with the microorganisms providing the chemicals and the space where mineral dissolution reaction occurs (Gehrke *et al*., 1998, Rawlings 2005) (Table 4). Iron oxidizing bacteria are able to aid mineral solubilization by producing chemicals such as ferric iron and sulfuric acid from the ferrous iron and sulfur contained in the mineral or solution (Rawlings *et al*., 2003). The ferric iron serves as an oxidation attack on the mineral (Takai *et al*., 2001), whereas sulfuric acid is responsible for a proton attack (Rawlings 2005). Bioleaching of non-sulfidic minerals occurs by acid attack and/or complexation, while dissolution of sulfides requires a combined acid and/or oxidative attack (Sand and Gehrke 2006). Bacteria are able to leach the minerals possibly *via* two proposed systems namely 'contact leaching' and 'non-contact leaching' (Figure 8) (Sand and Gehrke 2006). With contact leaching, biofilms are produced; that function in the following way: the extracellular polymeric substances can mediate attachment to a (metal) sulfide surface and it may concentrate the ferric ions by complexation through uronic acids or other residues at the mineral surface, thus allowing an oxidative attack on the sulfide (Corzo *et al*., 1994; Comte *et al*., 2006; Sand and Gehrke 2006). Kinzler *et al*. (2003) reported that *Acidithiobacillus ferrooxidans* was able to accumulate ferric iron in its exopolysaccharides during biofilm formation *via* a complexation mechanism. This feature modulated the colonization of the bacteria on a pyrite surface.

[20] Bioleaching refers to the conversion of an insoluble metal (typically a metal sulfide) into a soluble form (typically a metal sulfate), *via* microbial activity (Rawlings 2002). When metals are extracted into solution, the process is referred to as bioleaching, whereas if the metal remains in the mineral, it is referred to as biooxidation (Rawlings 2005).

Kumba Iron Ore, Ltd. is the world's fourth largest supplier of sea-borne iron ore. The company exports 73% of its 32Mtpa (million ton per annum) production to 30 international customers, mainly in Europe and Asia. It currently operates two mines in South Africa namely the Sishen Mine in the Northern Cape and Thabazimbi Mine in Limpopo. The iron ore mined at Sishen is composed of several different minerals. Alkali (potassium and phosphorous) are a common constituent of iron ore, which is known to have a deleterious effect on the manufacturing of iron and steel (Delvasto *et al*., 2008). Therefore, steel making companies charge penalties when purchasing iron ore concentrates with alkali concentrations above predetermined levels. The limits allowed is determined by the steel making companies and ranges from 0.25% mass in Japan to 0.55% mass in Switzerland for the alkali potassium. Kumba Iron Ore, Ltd., has an industrial set limit of 0.24% potassium allowed in their export ore according to D. Krige (Personal communication, 2006). To ensure that their export batches stay within this set limit, the ores from different batches, at the Sishen Iron Ore Mine (with potassium >0.24% and <0.24%) are mixed to produce an average potassium value of below 0.24%. However this solution will soon become ineffective as the low potassium ore (< 0.24%) is progressively depleted according to R. Grunewaldt (Personal communication, 2006). Certain pyro – and hydrometallurgical methods can be applied to decrease the alkali concentration (Cheng *et al*., 1999; Kokal *et al*., 2003), however there are several disadvantages when using these methods such as: poor product recovery, involvement of high process and energy cost and an increase in pollution load of water resources (Jain and Sharma 2004). Thus an alternative, natural and economical feasible process is required aid conventional methods (such as pyro- and hydrometallurgy processes) to remove unwanted alkalis from the ores. Biohydrometallurgy[21] is an option for the removal of the deleterious phosphate and potassium, as it is well established that many microorganisms are capable of mobilizing these minerals, especially in nutrient limited environments (Banfield *et al*., 1999; Nautiyal 1999),

The aim of our study was to determine whether the commercially exploited iron oxidizing bacteria *Acidithiobacillus ferrooxidans* (ATCC 13598), *Leptospirillum ferrooxidans* (ATCC 49879), *L. ferriphillum* (ATCC 49881) and *Sulfobacillus thermosulfidooxidans* (Kora)

[21] Biohydrometallurgical processes include bioleaching and biobenefication (Ehrlich 1991).

(Figure 4) can be employed in a biobenefication[22] approach to remove potassium/phosphorous from the iron ore mined at Sishen, as iron oxidizing bacteria have been shown to be able to remove phosphorous from ore bodies (He and Wei 2000).

4.2 MATERIALS AND METHODS

4.2.1 Iron Ore

Export iron ore (alkali <0.24%), with a particle size range of 1mm - 5mm, from the Sishen Iron Ore Mine, Northern Cape, South Africa was supplied by Kumba Iron Ore, Ltd., for iron oxidizing bacteria leaching. Prior to leaching, the samples were rinsed with distilled water in a sieve (< 1mm) to remove small debris which could hamper subsequent analysis, followed by sterilization at 121°C for 15 min.

4.2.2 Culture

Acidithiobacillus ferrooxidans (ATCC 13598) was obtained from the American Type Culture Collection (ATCC). *Leptospirillum ferrooxidans* (ATCC 49879) *L. ferriphilum* (ATCC 49881) and *Sulfobacillus thermosulfidooxidans* (Kora) were obtained from the culture collection of the University of Stellenbosch, Stellenbosch, South Africa. Iron oxidizing bacteria were grown in iron-oxidizing medium, as described by Atlas (1993). Media prepared as follow. Solution A contains: $(NH_4)_2SO_4$ - 3.0g; K_2HPO_4 - 0.50g; $MgSO_4.7H_2O$ - 0.50g; KCl - 0.10g; $Ca(NO_3)_2$ - 0.01g. Solution B contained 44.22g of $FeSO_4.7H_2O$. Distilled/deionized water was added to solution A (700ml) and solution B (300ml). The solutions were autoclaved for 15min at 121°C and then left to cool down to 25°C before adding them together. The pH was then adjusted to 2.20 by adding H_2SO_4 (V/V). The cultures were grown in 500ml Erlenmeyer to which 300ml of the iron-oxidizing media was added. *A. ferrooxidans*, *L. ferrooxidans* and *L. ferriphilum* were

[22] Biobenefication refers to a process in which microorganisms are used to selectively remove undesirable mineral components from ores (Ehrlich 1991).

incubated at 30°C and *S. thermosulfidooxidans* was incubated at 37°C, with shaking (150 rpm) for 30 days before subsequent leaching experiments.

4.2.3 Leaching

Bioleaching experiments were carried out in 500ml Erlenmeyer flasks containing 150g export ore, 200ml iron oxidizing media (Atlas 1993) and were each inoculated with 5 ml of a different bacterial culture, followed by incubation at 30°C with agitation (150 rpm). Size of the inoculums was not determined, as subsequent measuring would be inaccurate due to bacterial attachment on the mineral surface (Kinzler *et al*., 2003). Therefore a decrease in solution pH was used to determine bacterial activity.

The bacterial cultures were also added together, in equal volumes (2ml each), for a synergistic effect on ore leaching.

4.2.4 DNA extraction

Total genomic DNA was extracted from the inoculated flasks using the cetyltrimethylammonium bromide (CTAB method), as described by Ausubel *et al*. (2002), to ensure that no contaminants were present in the samples. Cells from 1.5ml of the inoculated flasks were pelleted by centrifugation at 13 400 rpm for 5 min and suspended in 567µl of 1 × TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8). The cells were lysed by adding sodium dodecyl phosphate (SDS) to a final concentration of 0.5% (v/v) and the proteins were digested by adding Proteinase K to a final concentration of 100 µg/ml in total volume of 600 µl. The suspension was then incubated overnight at 37°C. Following incubation, 100 µl of 5M NaCl and 80µl of CTAB/NaCl solution (10% [w/v] CTAB in 0.7 M NaCl) was added, mixed thoroughly and incubated at 65°C for 10 min. An equal volume of chloroform:isoamyl alcohol (24:1) was then added and followed by centrifugation at 13 400 rpm for 5 min. The supernatant was then transferred to a clean microfuge tube to which an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added and centrifuged at 13 400 rpm for 5 min. The supernatant was then transferred to a new microfuge tube, to which 0.6 volume isopropanol was added to precipitate the DNA. The

precipitated DNA was pelleted by centrifugation at 13 400 rpm for 20 min, washed with 70% ethanol, dried under vacuum and suspended in 20 µl of 1 × TE buffer. The suspension was then incubated overnight at 37°C before analyzing an aliquot of the extracted genomic DNA was analyzed by electrophoresis on a 1% (w/v) agarose gel.

4.2.5 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) was used to amplify a section of the 16S rRNA of the DNA extracted. The genes were amplified using the oligonucleotides PRUN518r (5'-ATT-ACC-GCG-GCT-GCT-GG-3') (Siciliano *et al.,* 2003) and pA8f-GC (5'-CGC-CCG-CCG-CGC-GCG-GCG-GGC-GGG-GCG-GGG-GCACGG-GGG-GAG-AGT-TTG-ATC-CTG-GCT-CAG-3') (Fjellbirkeland *et al.,* 2001). Each PCR reaction mixture (20 µl) contained ~27ng/µl of genomic DNA as template, 10.3 µl sterile distilled MilliQ water, 2.5µl PCR buffer with $MgCl_2$ (10×), 2µl dNTPs (2.5µM), 1µl primer PRUN518r (50µM), 1µl primer pA8f-GC (50µM) and 0.2µl Supertherm *Taq* (5U/µl) (Southerm Cross Biotechnology). A negative control (all reagents and no template DNA) was added to rule out contamination. The tubes were placed in a Perkin-Elmer GeneAMP® 2700 thermal cycler. Following incubation at 95 °C for 10 min, the reaction mixture were subjected to 35 cycles of denaturing at 94 °C for 30 s, annealing at 53°C for 30 s and elongation at 72 °C for 1 min. Once the cycles were completed, a final elongation step was performed at 72°C for 10 min to complete DNA synthesis. An aliquot of the amplified DNA was analyzed by electrophoresis on a 1.5% (w/v) agarose gel in the presence of appropriate molecular weight marker. The PCR products were stored at 4°C, until further analysis.

4.2.6 Denaturing gradient gel electrophoresis (DGGE)

PCR products were subjected to denaturing gradient gel electrophoresis (DGGE) according to the method described by Muyzer *et al*. (1993), to ensure that no contamination were present in our bioleaching experiments. 10µl of the each PCR product was loaded onto a 40-50% denaturing gradient gels (Table 20). Gels were run at 70 V for 17 h at a constant temperature of 60 °C. Image analysis was performed using the Gel2K (Norland 2004) program. Selected bands

were picked under blue light from the DGGE gels using a sterile scalpel and forceps. Each band was assigned a number for sequence analysis. The gel fragments were place into 25µl filter-sterilized deionized water and allowed to stand overnight to dissolve. The dissolved fragments were then subjected to PCR as described in the previous section.

Table 20 Denaturing gradient table showing volumes in milliliters of DSSA (denaturing stock solution A: 8% acrylamide in 0.5x TAE (40mM Tris, 20 mM acetic acid, 1nM EDTA (pH 8.3) buffer) and DSSB (denaturing stock solution B: 8% acrylamide, 7M urea, 40% formamide in 0.5x TAE buffer) mixed to form a gradient within the gel.

Denaturing percentage	DSSA(ml)	DSSB (ml)
30	10.2	4.4
35	9.4	5.1
40	8.7	5.8
45	8.0	6.5
50	7.3	7.3
55	6.5	8.0
60	5.8	8.7
65	5.1	9.4

4.2.7 Sequencing

The amplified products from the DGGE bands were cleaned by adding 15ul sterile water, transferring the entire volume to a 0.5ml Eppendorf sequencing tube, adding 2ul of sodium acetate (3M) and 50ul ethanol (95%), and allowing it to stand on ice for 10min. The tubes were then centrifuged at 10 000 rpm for 30 min. The ethanol solution was removed, the pellet rinsed in 150µl ethanol (70%), and the tubes again centrifuged for 5 min at 10 000 rpm. The ethanol was aspirated and the pellet dried under vacuum for approximately 10 min. These samples were then submitted to Macrogen (U.S.A.) for sequencing using the oligonucleotides PRUN518r (Siciliano *et al.*, 2003). Nucleotide sequences were analyzed with BioEdit v.5.0.9.1 (Hall 2001)

and their identity were verified by sequence match searches against the RDP database (available at http://rdp.cme.msu.edu).

4.2.8 Quantitative mineralogy

The composition of iron samples were analyzed with X-ray fluorescence (XRF) at the X-ray analytical facility of the University of Pretoria. Four samples of untreated export iron ore were analyzed as controls. Samples were analyzed after 30 and 60 days of leaching.

The samples were prepared as follow for analysis: Iron ore samples were first ground to <75µm in a tungsten carbide milling vessel. The loss of ignition (LOI) was determined by roasting the milled samples at 1000ºC. 1g of a milled iron ore sample were added to 6g $Li_2B_4O_7$ and fused into a glass beads. The rest of the sample was then used to make a powder briquette for minor element analysis (Loubser and Verryn 2008). Major element analyses were executed on the fused bead using an ARL9400XP+ spectrometer.

4.2.9 Scanning electron microscopy (SEM)

Bacteria cells were fixed by placing the iron ore in a Greiner tube containing 20 ml fixing solution (2.5% gluteraldehyde in 0.0075 M phosphate buffer) for 1 h. The ore was washed three times for 15 min with 0.0375 M $NaPO_4$ buffer, before dehydration by sequential treatment for 15 min in 50%, 70%, 90% and 100% ethanol. The 100% ethanol step was repeated twice more to ensure complete dehydration. The iron ore samples were critical point-dried, scattered with gold with a Polaron Equipment LTD SEM Autocoating unit E5200 and observed with a JEOL 5800LV scanning electron microscope at an accelerating voltage of 5kV.

4.3 RESULTS AND DISCUSSION

4.3.1 pH measurement

Iron oxidizing bacteria were grown in an iron-oxidizing media, as described by Atlas (1993). The pH of the media was adjusted to 2.20 and measured every 5^{th} day as iron oxidizing bacteria

are able produce chemicals such as ferric iron and sulfuric acid from the ferrous iron and sulfur contained in the mineral or solution, which the latter product would influence pH (Rawlings *et al*., 2003). Moreover, Štyriaková *et al.* (2004) stated that these organisms were also able to produce negligible amounts of organic acids. Therefore the acids produced would decrease the pH of the solution and make it inhospitable to other bacteria (Mason and Rice 2002). Research indicated an increase in dissolution rate with increasing acidity, which they ascribed in part to proton adsorption to oxygen sites on the mineral surface. This weakens metal-oxygen bonds and therefore increases dissolution (Welch *et al*., 2002). In the abiotic experiments (Chapter 3) these protons possibly decreased and therefore mineral dissolution stopped/decreased. In contrast bacteria are able to regenerate these protons and would hypothetically be able to solubilize more minerals. Therefore we assumed that a decrease in pH would indirectly indicate bacterial activity. Although the solution pH was low, research has demonstrated that the internal pH of the iron oxidizing bacteria is close to neutral, implying that these organisms have to maintain a high proton gradient which would have a high ATP (adenosine triphosphate) cost (White 1995). White (1995) has suggested that iron oxidizing bacteria regulate their internal pH by consuming cytoplasmic protons during respiration rather than by proton pumping across the membrane as with other aerobic microorganisms. A low external pH is required for metabolic activity, because at a pH greater than 5, ferrous iron will rapidly be oxidized to ferric iron, therefore the cells would struggle to compete with chemical oxidation. However organisms have been characterized that are able to oxidize iron at different pH than the norm. This includes organism that can oxidize iron near neutral pH, or archaea that can oxidize iron at pH 0.5.

The lowest pH measured during this study was for *A. ferrooxidans* (Table 21), and was attributed to sulfuric acid production during microbial oxidation (Rawlings *et al*., 2003). Research demonstrated that this bacterium was able to grow optimally in a pH range of 1.5 – 2.5 (Niemelä *et al*., 1994; Razzell *et al*., 1962; Solisio *et al*., 2002). The low pH is essential for the organism survival as Kupka and Kupsăvo (1999) demonstrated that the oxidation of ferrous ions by *A. ferrooxidans* decreased when the pH exceeded 2.5. The pH measured for *Leptospirilli* was 1.86 – 1.91 (Table 21). These are known acid tolerant organisms, with the ability to grow in a pH range of between 1.5-1.8. These organisms are only able to use ferrous iron as electron

donor (Rawlings 2002), however ferric iron can effectively interact with surface species and promote its dissolution which can results in sulfuric acid production (Schrenk *et al*., 1998). *Sulfobacillus thermosulfidooxidans* had the highest pH measurement compared to the other strains (Table 21). *Sulfobacilli* grow in a pH range from 1.9 – 3.0 with an optimum range of 1.9 – 2.4 (Rawlings 2002; Watling *et al*., 2008). It was assumed that the varying decreases measured (Table 21), was due to varying or different metabolic capabilities of each organism, as research by Blake and coworkers (1993) showed that *L. ferrooxidans* and *A. ferrooxidans* use different ferrous iron oxidation mechanisms. Additionally *A. ferrooxidans* is able to obtain energy from alternative sources such as hydrogen which enable the organism to better adapt to a wider range of different environments (Barreto *et al*., 2003; Drobner *et al.*, 1990). *Leptospirilli* are only able to use ferrous iron as an electron donor (Rawlings 2002). *S. thermosulfidooxidans* is able to use ferrous iron, reduced inorganic sulfur compounds or sulfide minerals as electron donors, but unable to use sulfate as a source of sulfur (Norris *et al*., 1996). The iron oxidizing media (Atlas 1993) used during the experiments contains ammonium-sulfate and iron-sulfate, thus *S. thermosulfidooxidans* was only able to utilize ferrous iron as an energy source, sequentially causing a smaller decrease in pH. Therefore we concluded that due to *A. ferrooxidans* ability to use a broader range of energy sources enable it to decrease the pH more compared to the other iron oxidizing bacteria. The mixed culture experiment had a mean pH of 1.81, which is an optimum pH for most of the cultures tested.

Table 21 Average pH measurement of iron oxidizing leaching experiment

Organisms	pH average
A. ferrooxidans (ATCC 13598)	1.65
L. ferrooxidans (ATCC 49879)	1.86
L. ferriphilum (ATCC 49881)	1.91
S. thermosulfidooxidans (Kora)	1.98
Mixed IOB	1.81

4.3.2 Quantitative mineralogy

Export ore (alkali <0.24%) was treated with different iron oxidizing bacteria (Table 22-25), to determine their ability to remove alkali. Alkali removal was determined by measuring the residual contained in the ore after treatment and comparing it to the untreated sample. The alkali, aluminum and silicon concentrations were the main focus of the current research as Mojallali and Weed (1978) stated that a loss of aluminum and/or silicon showed a structural rearrangement of a mineral. Furthermore, Wilson and Jones (1983) found that a decrease in magnesium and iron shows an even greater rearrangement of a mineral.

The mineral composition of the untreated iron ore sample is depicted in Table 19. X-ray fluorescence is known to be a reasonably sensitive detection method, with detection limits[23] for most elements in the low ppm range (Table 14) (Jenkins 1988). Magnesium and sodium were below these detection limits and will therefore be excluded from further analysis and not used as an indication of structural rearrangement as stated by Wilson and Jones (1983). Results of treated samples were expected to exceed the control sample due to sample variation; therefore a range was determined which included the minimum and maximum amount of alkali measured in the replicate untreated sample. From this data set we can possibly determine the amount of alkali removed due to acid leaching and excluded data sets influenced by sample variation. From XRF analysis the following ranges for the minerals were calculated as described in Chapter 3: aluminum (1.15% - 1.56%), silicon (1.92% - 2.80%), potassium (0.16% - 0.18%) and phosphorous (0.13% - 0.17%), as discussed by Mojallali and Weed (1978). Concentrations of each mineral removed below these parameters will be reported as “large quantities” removed. Silicon was detected as quartz, muscovite, biotite, etc. in all of the samples analyzed (Table 1). Quartz is known dissolved slowly, due to the high activation energy needed to break the silicon-oxygen bond (Brehm *et al*., 2005 as cited in Wilson 2004). We did not determine the source of silicon dissolution, as it was outside the scope of this study, however it is assumed that the silicon released was from less resistant mineral. We accept the average value of the untreated export sample as an acceptable representative. Mineral phases detected in the export iron ore sample include apatite, hematite, muscovite and K-feldspar (Table 1).

[23] Lower limit of detection is defined as that concentration equivalent to a certain number of standard deviations of the background count rate.

Acidithiobacillus ferrooxidans obtains energy by oxidizing ferrous iron and sulfur present in the sample or solution. The iron ore analyzed did not contain sulfur (Table 22), therefore the nutritional requirements of the organism was satisfied by the media (Atlas 1993). *A. ferrooxidans* is able to obtain energy from alternative sources such as: hydrogen, with oxygen as terminal electron acceptor under aerobic conditions (Barreto *et al.*, 2003; Drobner *et al.*, 1990) or under anaerobic conditions by dissimilatory reduction of ferric iron (Ohmura *et al.*, 1999; Pronk *et al.*, 1991**)** and formate with oxygen or ferric iron as terminal electron acceptor (Pronk *et al.*, 1991). The organism is known to produce sulfuric acid and ferric iron (Ehrlich 1991; Rawlings 2005), which is possibly able to aid solubilization of metals. Bioleaching of non-sulfidic minerals occurs by acid attack and/or complexation, while dissolution of sulfides requires a combined acid and/or oxidative attack (Sand and Gehrke 2006).

A. ferrooxidans was able to remove "large quantities" of potassium (35%), phosphorous (27%), aluminum (55%) and silicon (42%) from the export iron ore. Increased solubilization of these minerals (except phosphorous), occurred after 60 days (Table 22). This is ascribed to mineral dissemination in the particle or possibly due to saturation of the alkali in solution. It was demonstrated that a 0.1mg/l potassium concentration in solution would inhibit the exchange of the alkali with other ions in solution (vermiculation) (Wilson 2004). The amount of silicon and aluminum removed, illustrates a rearrangement of the minerals (Mojallali and Weed 1978). It was not determined whether the organisms actively scavenged for the minerals, or whether the solubilization of the minerals was indirectly due to the production of metabolites such as ferric iron and/or sulfuric acid. There is however support for the former as Seeger and Jerez (1993) found that when *A. ferrooxidans* was grown under phosphate-limited conditions, the organism would adapt by reducing its growth rate, to be able to increase its capacity to oxidize ferrous iron and to fix carbon dioxide. The researchers further discovered that the organism increased its expression of certain proteins under nutrient limited conditions and they concluded that these proteins might assist in scavenging for available phosphates. More recently work by Bennett and coworkers (2001) demonstrated that microbial colonization of a mineral can be nutrient driven. They using *in situ* and laboratory microcosms to determine this. The *in situ* (field experiments) were conducted at two groundwater sites, which had similar characteristics: abundant dissolved carbon and anoxic conditions. Here mineral chips were placed in permeable

chambers and suspended in the groundwater for months. In the laboratory, mineral fragments were inoculated with groundwater (from the two sites) and aquifer materials. They found that in the groundwater, which was rich in carbon but contained limited amounts of phosphate, microbial mediated weathering of minerals were sometimes determined by the nutritional requirements of the microbial community. They further found that feldspar could be rapidly dissolved in such nutrient limiting environments. Furthermore the authors argued that microorganisms might be able to select minerals that contain beneficial elements, and leave others intact.

Table 22 XRF analysis of *A. ferrooxidans* (ATCC 13598) treated export iron sample.

Element	Untreated Export Ore	*A. ferrooxidans*			
		30 days	% removed	60 days	% removed
SiO_2	2.36	1.82	23	1.38	42
TiO_2	0.08	0.07	13	0.06	25
Al_2O_3	1.32	1.17	11	0.60	55
Fe_2O_3	95.68	96.89	n/a	97.54	n/a
MnO	0.03	0.04	n/a	0.04	n/a
MgO	0.03	0.00	100	0.00	100
CaO	0.04	0.00	100	0.00	100
Na_2O	0.11	0.03	73	0.01	91
K_2O	0.17	0.14	18	0.11	35
P_2O_5	0.15	0.11	27	0.11	27
Cr_2O_3	0.02	0.02	0	0.02	0
NiO	0.01	0.00	100	0.00	100
V_2O_5	0.01	0.02	n/a	0.02	n/a
ZrO_2	0.00	0.00	0	0.00	0

Potassium was detected as K-feldspar and muscovite in the export iron ore sample (Table 1). Muscovite has considerable resistance toward leaching of interlayer potassium, compared to trioctahedral micas (such as biotite). It was proposed that an inclined orientation of hydroxyl ions in the dioctahedral micas results in stronger binding of potassium (Kalinowski and Schweda 1996) resulting in its resistance. However, Scott and Smith (1966) conducted experiments in which they determined the susceptibility of interlayer potassium in micas to exchange with sodium. They found that essentially, all the potassium in muscovite,

biotite, phlogopite and vermiculite were exchangeable when the concentration of the potassium in the solution was kept low. Leaching was not extended beyond 60 days, as the implementation of this approach into the mining schedule of the Sishen mine would not allow a longer time span.

Phosphorous was detected as apatite. It is relatively insoluble at neutral pH, although its solubility and reactivity varies as a function of its composition (Anderson *et al*., 1985; Jahnke 1984; LeGeros and Tung 1983; Valsami-Jones *et al*., 1998). Therefore the low pH present in the bioleaching solution (Table 21) could possibly have aided the release of phosphorous from the ore (Welch *et al*., 2002). Goldstein and Krishnaraj (2007) commented that the most common mechanism used by microorganisms to solubilize phosphate from apatite, seems to be by acidifying the media when organic acids were produced and released. Moreover, biofilms bound to the surface (Figure 19), would also actively take up phosphate, due to its importance in microbial cell processes (Delvasto *et al*., 2008; Tempest and Neijssel 1992). Phosphate uptake by the biofilms is accomplished by the metals complexing with active moieties (such as carboxyl groups) present in the exopolysaccharides or other cellular materials (Comte *et al*., 2006; Corzo *et al*., 1994). Moreover, studies conducted with microbial extracellular polymers and simple analogs of these compounds showed that acid polysaccharides could increase dissolution of alumino-silicate minerals under mildly acidic conditions, by complexing with the minerals solubilized into solution (Welch and Vandevivere 1994; Welch *et al*., 1999) or inhibit dissolution at near neutral pH when bound on the mineral surface (Poumier *et al*., 1999).

Microorganisms are able to produce other compounds that can affect mineral dissolution. For example, when iron limitation occurs, microbes can produce siderophores, that have a high affinity for iron, but can also bind to other cations. Moreover, organisms can also produce phosphatases to degrade organophosphates and release orthophosphate. This led Welch and coworkers (2002) to deduce that there could possibly be similar compounds that are able to release phosphate from minerals such as apatite.

Sulfuric acid was able to remove 7% potassium and 29% phosphorous from the export ore (Williams 2008), whereas the iron oxidizing bacteria could solubilize more potassium. This could be due to a synergistic effect of all its metabolic capabilities, for example: it is able to

produce sulfuric acid and ferric iron (oxidation and proton attack); able to produce biofilms which can complex with minerals lowering the saturation state; actively take up phosphates as a nutrient (lowers saturation state) and lastly it could produce compounds similar to phosphatases/siderophores which further increase mineral dissolution (Welch *et al.,* 1999). The ability of sulfuric acid to remove more phosphorous was ascribed to sample variation[24], as only a single repetition of the experiment was done.

Leptospirillum was first isolated in 1972 from a copper leach dump and coal spoil heap in Armendia (Inoue *et al.,* 2000). The bacteria of this genus are quite similar to that of *Acidithiobacillus*. They are also acid tolerant, with the ability to grow in a pH range of between 1.5-1.8. However, based on 16S rRNA sequence analysis, the genus does not form part of the division *Proteobacteria* as Acidithiobacilli, but rather *Nitrospora* (Lane *et al*., 1992). All *Leptospirilli* are only able to use ferrous iron as an electron donor, resulting in higher affinity for this compound (Rawlings 2002). This organism produced small quantities of hydrogen sulfide: ferric ion reductase, which aids sulfur oxidation, but is inhibited by an intermediate product (bisulfate) of sulfate oxidation (Sugio *et al*., 1994).

We calculated a range for the following minerals, as mentioned before: aluminum (1.15% - 1.56%), silicon (1.92% - 2.80%), potassium (0.16% - 0.18%) and phosphorous (0.13% - 0.17%). After 30 days leaching, no potassium could be removed using *L. ferrooxidans* (Table 23) or *L. ferriphilum* (Table 24), however extended leaching enabled removal. *L. ferrooxidans* and *L. ferriphilum* were able to remove "large quantities" of phosphorous, silicon and aluminum. The amount of aluminum and silicon demonstrates that mineral rearrangement occurred (Mojallali and Weed 1978). Several factors such as intrinsic mineral characteristics, processing, physiochemical and microbiological factors can influence leaching (Table 10). *A. ferrooxidans* was able to remove 35% of the potassium and 27% of the phosphorous (Table 22); compared to <30% for potassium and above 30% for phosphorous by *Leptospirilli.*

[24] Sample contains an alkali concentration higher than the set parameter and therefore no apparent removal will be reported.

We concluded that sample variation, mineral dissemination[25] or metabolic differences between the strains played an important role in the amount of minerals solubilized by each organism.

Table 23 XRF analysis of *L. ferrooxidans* (ATCC 49879) treated export sample.

Element	Untreated Export Ore	*L. ferrooxidans*			
		30 days	% removed	60 days	% removed
SiO_2	2.36	2.11	11	1.67	29
TiO_2	0.08	0.07	13	0.06	25
Al_2O_3	1.32	1.08	18	0.65	51
Fe_2O_3	95.68	93.92	2	97.50	n/a
MnO	0.03	0.04	n/a	0.04	n/a
MgO	0.03	0.00	100	0.00	100
CaO	0.04	0.00	100	0.00	100
Na_2O	0.11	0.01	91	0.01	91
K_2O	0.17	0.17	0	0.13	24
P_2O_5	0.15	0.12	20	0.10	33
Cr_2O_3	0.02	0.02	0	0.02	0
NiO	0.01	0.00	100	0.00	100
V_2O_5	0.01	0.02	n/a	0.01	0
ZrO_2	0.00	0.00	0	0.00	0

[25] Alkali is located in the core of the particle and therefore inaccessible to the acid

Table 24 XRF analysis of *L. ferriphilum* (ATCC 49881) treated export sample.

Element	Untreated Export Ore	*L. ferriphilum*			
		30 days	% removed	60 days	% removed
SiO_2	2.36	1.56	33	1.66	30
TiO_2	0.08	0.07	13	0.06	25
Al_2O_3	1.32	0.99	25	0.72	46
Fe_2O_3	95.68	97.01	n/a	97.08	n/a
MnO	0.03	0.04	n/a	0.04	n/a
MgO	0.03	0.00	100	0.00	100
CaO	0.04	0.00	100	0.00	100
Na_2O	0.11	0.01	91	0.01	91
K_2O	0.17	0.16	6	0.12	29
P_2O_5	0.15	0.14	7	0.10	33
Cr_2O_3	0.02	0.02	0	0.02	0
NiO	0.01	0.00	100	0.00	100
V_2O_5	0.01	0.02	n/a	0.01	0
ZrO_2	0.00	0.00	0	0.00	0

Sulfobacillus thermosulfidooxidans are able to grow autotrophically or heterotrophically. When growing autotrophically they use ferrous iron, reduced inorganic sulfur compounds or sulfide minerals as electron donors (Norris *et al*., 1996). *Sulfobacilli* are not able to efficiently fix carbon dioxide, and therefore need elevated CO_2 in the atmosphere or yeast extract can be added to the media, furthermore they are not able to use sulfate as a source of sulfur and must therefore be provided reduced sulfur (Rawlings 2002). When growing heterotrophically, glucose can serve as a carbon and energy source. Furthermore these microorganisms are also able to grow in the absence of oxygen, using ferric iron as an electron acceptor and either organic or inorganic sulfur compounds as electron donor. During this study, *S. thermosulfidooxidans* were grow in an iron oxidizing media described by Atlas (1993). Here the organisms could grow autotrophically as the media supplied the sulfur source and the carbon dioxide in the air served as a carbon source.

S. thermosulfidooxidans was able to remove a "large quantity" of potassium after 30 days, but was unable to remove more after extended leaching (Table 25). Furthermore phosphorous, aluminum and silicon were removed in "large quantities" after 30 days. The data obtained is

ascribed to sample variation, due the higher value of residual minerals after extended leaching time (Table 25). However research conducted by Delvasto *et al*. (2008) found that when dense biofilms were formed, under limited air exchange, it resulted in lower phosphate mobilization from the ore than under unlimited air exchange. This was found to be a result of a dynamic process of iron and phosphate re-precipitation within the biofilm formed (Delvasto *et al.*, 2008).

Table 25 XRF analysis of *Sulfobacillus thermosulfidooxidans* treated export sample.

Element	Untreated Export Ore	*S. thermosulfidooxidans*			
		30 days	% removed	60 days	% removed
SiO_2	2.36	1.59	33	1.74	26
TiO_2	0.08	0.05	38	0.06	25
Al_2O_3	1.32	0.90	32	1.00	24
Fe_2O_3	95.68	97.18	n/a	96.82	n/a
MnO	0.03	0.04	n/a	0.04	n/a
MgO	0.03	0.00	100	0.00	100
CaO	0.04	0.00	100	0.00	100
Na_2O	0.11	0.01	91	0.16	n/a
K_2O	0.17	0.15	12	0.17	0
P_2O_5	0.15	0.12	20	0.13	13
Cr_2O_3	0.02	0.02	0	0.02	0
NiO	0.01	0.00	100	0.00	100
V_2O_5	0.01	0.02	n/a	0.02	n/a
ZrO_2	0.00	0.00	0	0.00	0

A synergistic effect was also tested, in which equal volumes of *A. ferrooxidans*, *L. ferrooxidans*, *L. ferriphilum* and *S. thermosulfidooxidans* were added together to the export ore. The mixed culture removed "large quantities" of all the minerals analyzed after 30 days, with the highest removal for potassium and silicon occurring after 60 days residence time (Table 26). The pH of 1.81, was near the optimum pH for most of the organisms (Table 21), therefore the cultures could grow near optimal. The ability of the organisms to remove more alkali in a shorter time period, is assumed to be due to the minerals becoming rapidly depleted and therefore the organisms actively produce compounds to scavenge for potassium and phosphorous as described by Seeger and Jerez (1993). They found that if *A. ferrooxidans* was grown under phosphate limited conditions, the organism would adapt by reducing its growth rate, to be able to increase

its capacity to oxidize ferrous iron and to fix carbon dioxide. The researchers further discovered that the organism increased its expression of certain proteins under the limiting conditions and they concluded that these proteins might assist in scavenging for available phosphates.

Table 26 XRF analysis of mix iron oxidizing bacteria leaching of export sample.

Element	Untreated Export Ore	Mixed Iron oxidizing bacteria			
		30 days	% removed	60 days	% removed
SiO_2	2.36	1.48	37	1.51	36
TiO_2	0.08	0.06	25	0.07	13
Al_2O_3	1.32	0.97	27	0.65	51
Fe_2O_3	95.68	97.00	n/a	96.66	n/a
MnO	0.03	0.04	n/a	0.04	n/a
MgO	0.03	0.00	100	0.00	100
CaO	0.04	0.00	100	0.00	100
Na_2O	0.11	0.01	91	0.01	91
K_2O	0.17	0.14	18	0.12	29
P_2O_5	0.15	0.11	27	0.11	27
Cr_2O_3	0.02	0.02	0	0.02	0
NiO	0.01	0.00	100	0.00	100
V_2O_5	0.01	0.02	n/a	0.02	n/a
ZrO_2	0.00	0.00	0	0.00	0

We found that the iron oxidizing bacterial experiments were more variable than the abiotic tests (Chapter 3), which is in accordance with other reports (Welch *et al.*, 2004). Welch and coworkers (2004) found that bacteria produce varying amounts of acids. We assume that these varying amounts of acids are responsible for the different amounts of alkali removed. *A. ferrooxidans* was able to remove greatest amount of potassium (Table 22) and the *Leptospirillum* cultures were able to remove the largest concentration of phosphorous (Table 22 and 23). *S. thermosulfidooxidans* was the most inefficient at removing potassium and aluminum, which was ascribed to the metabolic capability of this organism. The mixed cultures proved to be the most efficient for removing the minerals, which was ascribed to a synergistic effect.

4.3.3 Sequencing

Total genomic DNA was extracted from the *A. ferrooxidans, L. ferrooxidans, L. ferriphilum* and *S. thermosulfidooxidans* inoculated flasks using the CTAB method, as described by Ausubel *et al*. (2002), and amplified using the oligonucleotides PRUN518r (5'-ATT-ACC-GCG-GCT-GCT-GG-3') (Siciliano *et al.,* 2003) and pA8f-GC (5'-CGC-CCG-CCG-CGC-GCG-GCG-GGC-GGG-GCG-GGG-GCACGG-GGG-GAG-AGT-TTG-ATC-CTG-GCT-CAG-3') (Fjellbirkeland *et al.,* 2001). Øvereås and Torsvik (1998), found that these primers were valuable in molecular ecological and systematic studies which focused on the 16s rRNA gene. Denaturing gradient gel electrophoresis of the iron oxidizing bacterial inoculated samples, showed that a single band was present (Figure 18 – lane 1 and 2) and therefore theoretically no microbial contamination was present. Therefore the leaching data obtained for each iron oxidizing bacterium can be ascribed to each specific specie used and not to a contaminant or indigenous organisms still present on the ore.

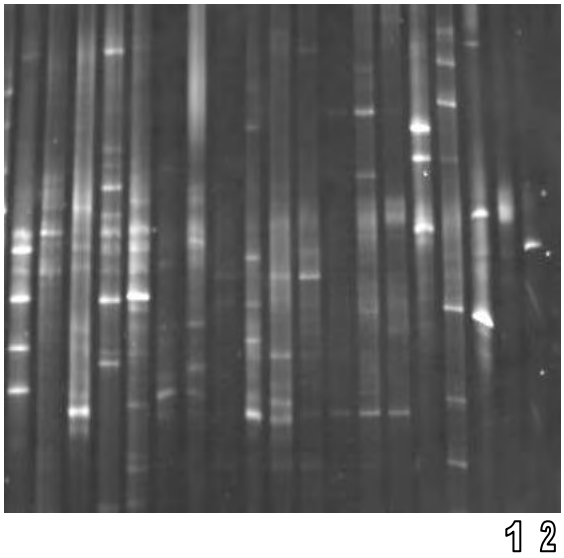

Figure 18. Denaturing gradient gel electrophoresis (DGGE) of iron oxidizing bacterial cultures. 1 and 2 illustrates pure cultures obtained for iron oxidizing bacteria leaching experiments.

The selected bands were subjected to sequencing and verified against the RDP database. Organisms were identified as *Acidithiobacillus ferrooxidans, Leptospirillum ferrooxidans,*

L. ferriphilum and *Sulfobacillus thermosulfidooxidans*, but not the specific strains as depicted in Table 21. Taxonomic resolution of 16S rRNA sequences are known to be insufficient for discriminating between close-related organisms (Janse *et al*., 2003), however it was adequate to identify the selected strains.

4.3.4 Scanning electron microscopy

Bacterial leached iron ore samples were observed with scanning electron microscopy. Data obtained indicated that the bacteria were able to form biofilms on the surface of the mineral (Figure 19). Therefore the bacteria could possibly leach the impurities by a contact leaching mechanism, as described by Sand and Gehrke (2006). Here the extracellular polymeric substance functions as follows: it mediates attachment to the (metal) sulfide surface and concentrates ferric ions by complexation through uronic acids or other residues on the cell surface, thereby allowing an oxidative attack on the mineral (Corzo *et al*., 1994; Comte *et al*., 2006; Sand and Gehrke 2006).

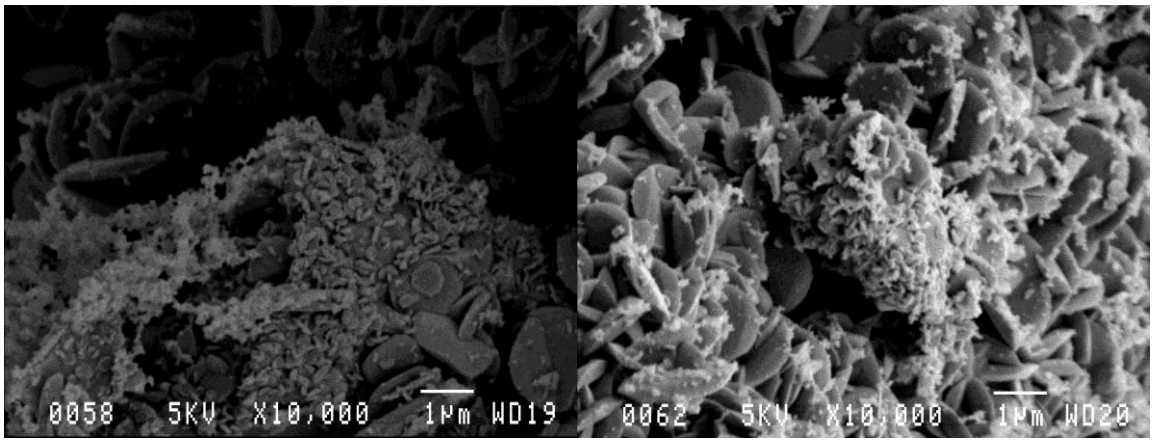

Figure 19. Scanning electron microscopy images portraying biofilm formation on the surface of the iron ore sample.

Biofilm formation is a multistep process which is influenced by many factors, including the specific mineralogy of the rock, solution chemistry (pH, ionic strength) and the characteristics of the microorganism (hydrophobicity, surface charge) (Banfield and Hamers 1997; Characklis 1989; Little *et al*., 1997). The attachment of cells to the surface can occur *via* random processes such as diffusion and convection or more specifically *via* chemotaxis, which is the active orientation of a bacterium to a chemical gradient (Jerez 2001). Several researchers studied the chemotatic response of bacteria towards metal ions and other compounds, for example: chemotaxis of *A. ferrooxidans* (Chakraborty and Roy 1992) and *L. ferrooxidans* (Acuña *et al*., 1986; Acuña *et al*., 1992). The organisms can detect the site of mineral dissolution (possibly potassium and phosphorous) by sensing a dissolution gradient. This allows the bacterium to respond to the attractant/repellent by swimming away or towards the increased concentration. When the bacterium reaches the surface, the cell can attach by mostly an unknown process (Sand and Gehrke 2006). Blake *et al*. (2001) concluded that aporusticyanin could possibly play an important role in the early attachment of *A. ferrooxidans* to mineral surfaces. The exopolysaccharides produced by the cell, plays an important role in attachment of the leaching bacterium. Research indicated that the exopolymers produced by *A. ferrooxidans* and *L. ferrooxidans*, contain besides neutral sugars and lipids, some glucuronic acid residues and complexed ferric ions (Gehrke *et al*., 1995; Gehrke *et al*., 1998, Gehrke *et al*., 2001). The stoichiometry of glucuronic acid to ferric ions amounts to a molar ratio of about 2:1 for both *A. ferrooxidans* and *L. ferrooxidans* tested thus far. Thus regardless of the phylogenetic class of the bacterium, the physicochemical characteristics of the environment enforced a similar adaption between the organisms. The exopolysaccharides formed by iron oxidizing bacteria has a net positive charge and at acidic pH, pyrite has a negative charge (Devasia *et al*., 1993; Hallman 1992; Ohmura *et al*., 1993). Thus, attachment of a positively charged bacterium to a negatively charged surface may take place due to electrostatic interaction between the two surfaces. Lazar (2004) found that both electrostatic and hydrophobic interactions could be responsible for the tight adhesion of cells to mineral surfaces (as cited in Sand and Gehrke 2006). The specificity of exopolysaccharides for various metal sulfides has not been termed. Gehrke *et al*. (1998) has however indicated that the composition of the EPS of *A. ferrooxidans* changed when the cells were grown in different substrates. Sulfur-grown cells had more lipids, less neutral sugars and only trace glucuronic acid as compared to those of pyrite grown cells.

4.4 CONCLUSION

Leaching of sulfidic minerals using chemolithoautotrophic bacteria are the best studied and commercially exploited mineral biotechnology today (Jain and Sharma 2004). The solubilization of the minerals is considered to be largely a chemical process, with the microorganisms providing the chemicals and the space where the mineral dissolution reaction occurs. Bioleaching of non-sulfidic ores, such as the export ore in this study, occurred by acid attack and complexation. Iron oxidizing bacteria are able to provide ferric iron and/or sulfuric acid which can serve as an oxidative attack in the former and a proton attack in the latter on mineral samples. Moreover, EPS produced by the bacteria are able to form complexes with the minerals which will influence the saturation state of the minerals in solution. The objective of this study was to determine, whether the commercially exploited iron oxidizing bacteria, were able to remove the alkali impurities from the export iron ore samples, provided by Kumba Iron Ore, Ltd. We found that the various strains were indeed able to remove different amounts of potassium and phosphorous from the export iron ore samples. Furthermore the organisms also demonstrated a symbiotic/synergistic relationship, which enable mobilization of the minerals from the ore. Scanning electron microscopy analysis demonstrated that biofilms were formed on the surface of the iron ore, supporting a contact leaching mechanism.

The preliminary results suggest that iron oxidizing bacteria leaching could be a viable option for treating the iron ore mined at Sishen. Bioleaching will however never completely replace the conventional methods but rather supplement them, as bioleaching does not recover precious metals from the ores which are often an essential component in the profitability of the operation; also when ore bodies do not contain sufficient acid consuming minerals, the residual acids generated have to be neutralized during the leaching process, thus increasing the operational cost (Dreshner 2004).

CHAPTER 5

LEACHING OF POTASSIUM AND PHOSPHOROUS FROM SISHEN HEMATITE IRON ORE USING HETEROTROPHIC BACTERIA

Abstract

The Sishen Iron Ore Mine is situated in the Northern Cape, South Africa, and forms part of the northern end of the Maremane anticline where the bulk of the hematite is buried beneath younger cover lithologies. The iron ore bodies minedare overlain by conglomerates, shales, flagstone and quartzite. Potassium and phosphorous are common constituents of the iron ore bodies and are known to have a deleterious effect on iron and steel manufacturing. Therefore steel making companies charge penalties when purchasing iron ore with alkali concentrations above predetermined limits. Conventional methods used to treat high alkali ore have several drawbacks such as poor product recovery, high running cost and increase pollution of water thus an alternative approach is needed to supplement or replace these methods. Biohydrometallurgy is a natural alternative approach which has been applied commercially to extract or treat various ore bodies. Here we aim to asses the ability of the heterotrophic bacteria *Bacillus megaterium, B. cereus, B. subtilis* and *Pseudomonas putida* to remove potassium and phosphorous from export grade iron ore mined at Sishen. We found that the organisms were able to solubilize varying degrees of potassium and phosphorous, which was attributed to their ability to produce different organic. Furthermore we found that the biotic test with the heterotrophic organims were more efficient at removing alkali than the abiotic test with with acids (Chapter 3). This ability is ascribed to their ability to form EPS, which is able to form complexes with minerals, thereby affecting the saturation state of the mineral in solution.

Keywords: Heterotrophic bacteria, organic acids, muscovite, exopolysaccharides

5.1 INTRODUCTION

Microbes are able to contribute to the biogeochemical cycles by transforming the metal phases (soluble to volatile) and/or changing the oxidation state of the metal. They play a major role in the: (i) cycling of elements and sorption of metals (Langley and Beveridge 1999); (ii) the dissolution of minerals (Banfield and Hamers 1997; Hersman *et al*., 1996; Roden and Zachara 1996; Schrenk *et al*., 1998), and (iii) mineral crystallization (Fortin *et al*., 1998). Whereas, mineralogical processes affect the distribution, activity and diversity of microbes (Schrenk *et al*., 1998); the expression of their genes (Gehrke *et al*., 1998), the structure and development of communities (Kennedy and Gewin 1997; Torseth *et al*., 1995; Wolfaardt *et al*., 1994) and the transfer of genetic material (Trevors and van Elsas 1997).

Kumba Iron Ore, Ltd. is the world's fourth largest supplier of sea-borne iron ore. The company exports 73% of its 32Mtpa production to 30 international customers, mainly in Europe and Asia. It currently operates two mines in South Africa namely the Sishen Mine in the Northern Cape and Thabazimbi Mine in Limpopo. The iron ore mined at Sishen is composed of several different minerals phases, with potassium and phosphorous as common constituents. These alkalis have a deleterious effect on the manufacturing of iron and steel (Delvasto *et al*., 2008). Therefore, steel making companies charge penalties when purchasing iron ore concentrates with alkali concentrations above predetermined levels. The limits allowed is determined by the steel making companies and range from 0.25% mass in Japan to 0.55% mass in Switzerland for the alkali potassium. Kumba Iron Ore, Ltd., has an industrial set limit of 0.24% potassium allowed in their export ore D. Krige (Personal communication, 2006). To ensure that their export batches stay within this set limit, the ores from different batches, at the Sishen Iron Ore Mine (with potassium >0.24% and <0.24%) are mixed to produce an average potassium value of below 0.24%. However this solution will soon become ineffective as the low potassium ore (<0.24%) is progressively depleted according to D. Krige (Personal communication, 2006). Certain pyro – and hydrometallurgical methods can be applied to decrease the alkali concentration (Cheng *et al*., 1999; Kokal *et al*., 2003), however there are several disadvantages when using these methods such as: poor product recovery, involvement of high process and energy cost and an increase in pollution load of water resources (Jain and Sharma 2004). Thus an alternative, natural and economical feasible process is required

aid conventional methods (such as pyro- and hydrometallurgy processes) in removing unwanted alkalis from the ores. Biohydrometallurgy[26] is an option for the removal of the deleterious phosphate and potassium, as it is established that many microorganisms are capable of mobilizing these minerals, especially in nutrient limited environments (Banfield *et al*., 1999; Nautiyal 1999). Bosecker (1997) found that the dissolution of non-sulfidic ores can be achieved by exploiting the metabolic capabilities of heterotrophic microorganisms. These organisms have the potential to produce metabolites that are able to solubilize and/or aid in solubilizing oxide, silicate, carbonate and hydroxide minerals *via* mechanisms such as reduction, acidolysis and complexation (Burgstaller and Schinner 1993; Gadd and Sayer 2000; Jain and Sharma 2004; Sayer and Gadd 1997). Metabolites include exopolysaccharides (Banfield *et al*., 1999; Malinovskaya *et al*., 1990; Welch *et al*., 1999), amino acids, proteins (Bosecker 1997) and organic acids (Agatzini and Tzeferis 1997; Barker *et al*., 1997; Castro *et al*., 2000; Natarajan and Deo 2001; Štyriaková *et al*., 1999; Valsami-Jones and McEldowney 2000).

Among the heterotrophic bacteria, members of the genus *Bacillus* and *Pseudomonas* have been shown to be effective in leaching non-sulfidic minerals (Karavaiko *et al*., 1988). Welch and Ullman (1999) found that certain heterotrophic microorganisms were able to mobilize elements such as Si, Al, Fe, K, Li, Ni, Zn, and Mg from the rock-forming minerals feldspar, pegmatite, hornblende and spodumene.

Leaching with heterotrophic bacteria can occur at higher pH than with autotrophic microorganisms, thus no equilibration of acids is required which would increase operational costs. However, the higher solution pH makes the environment favorable for many microbes, which again can contaminate the process and outcompete the leaching microbes (Jain and Sharma 2004). The bacteria used in this study are reported to be capable of solubilizing potassium and/or phosphates from various rocks and minerals. These include: *Bacillus megaterium* (Han 2006; Jain and Sharma 2004), *Bacillus cereus* (Štyriaková *et al.*, 2004), *Bacillus subtilis* and *Pseudomonas putida* (Jiang *et al.*, 2007).

Here we aim to asses the ability of the selected heterotrophic microorganisms to remove potassium and phosphorous under nutrient limiting conditions for these minerals. It is

[26] Biohydrometallurgical processes include bioleaching and biobenefication (Ehrlich 1991).

hypothesized that under potassium/phosphorous limiting conditions, certain genes will be activated, that could enable the organism to actively scavenge for these minerals. This hypothesis is supported by research research done by Bennet (2001), which found that silicate weathering by bacteria is driven by nutrient requirement. Due to difficulty in establishing selective growth conditions, heterotrophic microorganisms were not readily applied in industrial scale bioleaching processes (Ehrlich 1991). Heterotrophs require one or more organic nutrient to serve as an energy and carbon source, which increase operational costs. Furthermore the organic nutrient can support growth of a great variety of different organisms, which could contaminate the leaching processes and outcompete the organisms able to leach the ore (Ehrlich 1991).

5.2 MATERIALS AND METHODS

5.2.1 Iron Ore

Export iron ore (alkali <0.24%), with a particle range 1mm - 5mm, from the Sishen Iron Ore Mine, Northern Cape, South Africa was supplied by Kumba Iron Ore, Ltd., for heterotrophic bacterial leaching. Prior to leaching, the samples were rinsed with distilled water in a sieve (< 1mm) to remove small debris which could hamper subsequent analysis, followed by sterilization at 121°C for 15 min.

5.2.2 Cultures

Pseudomonas putida (ATCC 15070) were obtained from the American Type Culture Collection (ATCC). *Bacillus megaterium* (J3-10DQ363436), *B. subtilis* (MG1-DQ228954) and *B. cereus* (AMM202-AB092795) were obtained for the culture collection of the University of Pretoria, Pretoria, South Africa.

5.2.3 Inoculum media

The cultures were grown in a media described by Štyriaková *et al.* (2004) at 30°C with shaking (150rpm). The composition of the media per liter was as follow: 0.42g - NaH_2PO_4; 0.8g – $(NH_4)_2SO_4$; 0.19g NaCl; 19.8g – glucose. The cultures were maintained on Nutrient Agar (Biolab) at 4°C or as glycerol cultures at -70°C.

5.2.4 Leaching

Bioleaching experiments were carried out in 500ml Erlenmeyer flasks containing 150g export ore, 300ml inoculum media (Štyriaková *et al.*, 2004) and were inoculated with 5 ml of each different bacterial culture, followed by incubation at 30°C with agitation (150 rpm) and a duplicate without agitation (static). A decrease in solution pH was used to determine bacterial activity (Kinzler *et al*., 2003).

The different bacterial cultures were also mixed, in equal volumes (2ml each), to evaluate a potential synergistic effect on ore leaching.

5.2.5 pH calibration

pH of the inoculated samples were neutralized with 1M NaOH every 5^{th} day, as described by Štyriaková *et al*. (2004).

5.2.6 DNA extraction

Total genomic DNA was extracted from the heterotrophic bacterial inoculated flasks using the cetyltrimethylammonium bromide (CTAB method), as described by Ausubel *et al*. (2002), to ensure that no contaminants were present in the samples. Cells from 1.5ml of the inoculated flasks were pelleted by centrifugation at 13 400 rpm for 5 min and suspended in 567µl of 1 × TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8). The cells were lysed by adding sodium dodecyl phosphate (SDS) to a final concentration of 0.5% (v/v) and the proteins were digested by

adding Proteinase K to a final concentration of 100 µg/ml in total volume of 600 µl. The suspension was then incubated overnight at 37°C. Following incubation, 100 µl of 5M NaCl and 80µl of CTAB/NaCl solution (10% [w/v] CTAB in 0.7 M NaCl) was added, mixed thoroughly and incubated at 65°C for 10 min. An equal volume of chloroform:isoamyl alcohol (24:1) was then added and followed by centrifugation at 13 400 rpm for 5 min. The supernatant was then transferred to a clean microfuge tube to which an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added and centrifuged at 13 400 rpm for 5 min. The supernatant was then transferred to a new microfuge tube, to which 0.6 volume isopropanol was added to precipitate the DNA. The precipitated DNA was pelleted by centrifugation at 13 400 rpm for 20 min, washed with 70% ethanol, dried under vacuum and suspended in 20 µl of 1 × TE buffer. The suspension was then incubated overnight at 37°C before analyzing an aliquot of the extracted genomic DNA was analyzed by electrophoresis on a 1% (w/v) agarose gel.

5.2.7 Polymerase chain reaction

Polymerase chain reaction (PCR) was used to amplify a section of the 16S rRNA of the DNA extracted. The genes were amplified using the oligonucleotides PRUN518r (5'-ATT-ACC-GCG-GCT-GCT-GG-3') (Siciliano *et al.*, 2003) and pA8f-GC (5'-CGC-CCG-CCG-CGC-GCG-GCG-GGC-GGG-GCG-GGG-GCACGG-GGG-GAG-AGT-TTG-ATC-CTG-GCT-CAG-3') (Fjellbirkeland *et al.*, 2001). Each PCR reaction mixture (20 µl) contained ~27ng/µl of genomic DNA as template, 10.3 µl sterile distilled MilliQ water, 2.5µl PCR buffer with $MgCl_2$ (10×), 2µl dNTPs (2.5µM), 1µl primer PRUN518r (50µM), 1µl primer pA8f-GC (50µM) and 0.2µl Supertherm *Taq* (5U/µl) (Southern Cross Biotechnology). A negative control (all reagents and no template DNA) was added to rule out contamination. The tubes were placed in a Perkin-Elmer GeneAMP® 2700 thermal cycler. Following incubation at 95 °C for 10 min, the reaction mixture were subjected to 35 cycles of denaturing at 94 °C for 30 s, annealing at 53°C for 30 s and elongation at 72 °C for 1 min. Once the cycles were completed, a final elongation step was performed at 72°C for 10 min to complete DNA synthesis. An aliquot of the amplified DNA was analyzed by electrophoresis on a 1.5% (w/v) agarose gel in the presence of appropriate molecular weight marker. The PCR products were stored at 4°C, until further analysis.

5.2.8 Denaturing gradient gel electrophoresis (DGGE)

The PCR products were subjected to denaturing gradient gel electrophoresis (DGGE) according to the method described by Muyzer *et al*. (1993), to ensure that no contamination were present in our bioleaching experiments. 10µl of the each PCR product was loaded onto a 40-50% denaturing gradient gels (Table 20). Gels were run at 70 V for 17 h at a constant temperature of 60 °C. Image analysis was performed using the Gel2K (Norland 2004) program. Selected bands were picked under blue light from the DGGE gels using a sterile scalpel and forceps. Each band was assigned a number for sequence analysis. The gel fragments were place into 25µl filter-sterilized deionized water and allowed to stand overnight to dissolve. The dissolved fragments were then subjected to PCR as described in the previous section.

5.2.9 Sequencing

The amplified products from the DGGE bands were cleaned by adding 15µl sterile water, transferring the entire volume to a 0.5ml Eppendorf sequencing tube, adding 2µl of sodium acetate (3M) and 50ul ethanol (95%), and allowing it to stand on ice for 10min. The tubes were then centrifuged at 10 000 rpm for 30 min. The ethanol solution was removed, the pellet rinsed in 150µl ethanol (70%), and the tubes again centrifuged for 5 min at 10 000 rpm. The ethanol was aspirated and the pellet dried under vacuum for approximately 10 min. These samples were then submitted to Macrogen (U.S.A.) for sequencing using the oligonucleotides PRUN518r (Siciliano *et al.*, 2003). Nucleotide sequences were analyzed with BioEdit v.5.0.9.1 (Hall 2001) and their identity were verified by sequence match searches against the RDP database (available at http://rdp.cme.msu.edu).

5.2.10 Qualitative mineralogy

The composition of iron samples were analyzed with X-ray fluorescence (XRF) at the X-ray analytical facility of the University of Pretoria. Four samples of untreated export iron ore were analyzed as controls. Samples were analyzed after 30 and 60 days of bioleaching.

The samples were prepared as follows for analysis: Iron ore samples were first ground to <75µm in a tungsten carbide milling vessel. The loss of ignition (LOI) was determined by roasting the milled samples at 1000ºC. 1g of a milled iron ore sample were added to 6g $Li_2B_4O_7$ and fused into a glass beads. The rest of the sample was then used to make a powder briquette for minor element analysis. Major element analyses were executed on the fused bead using an ARL9400XP+ spectrometer (Loubser and Verryn 2008).

5.2.11 Scanning electron microscopy

Bacteria cells were fixed by placing the iron ore in a Greiner tube containing 20 ml fixing solution (2.5% gluteraldehyde in 0.0075 M phosphate buffer) for 1 h. The ore was washed three times for 15 min with 0.0375 M $NaPO_4$ buffer, before dehydration by sequential treatment for 15 min in 50%, 70%, 90% and 100% ethanol. The 100% ethanol step was repeated twice more to ensure complete dehydration. The iron ore samples were critical point-dried, scattered with gold with a Polaron Equipment LTD SEM Autocoating unit E5200 and observed with a JEOL 5800LV scanning electron microscope at an accelerating voltage of 5kV.

5.3 RESULTS AND DISCUSSION

5.3.1 pH measurement

Heterotrophic bacteria were grown in a media described by Štyriaková *et al.* (2004), with an initial pH under shaking (150 rpm) and static conditions. The media pH was neutralized every 5^{th} day, to ensure optimum conditions and to prohibit endospore formation as described by Štyriaková *et al.* (2004). The selected organisms produce metabolites such as exopolysaccharides (Banfield *et al.*, 1999; Fortin 2004; Malinovskaya *et al.*, 1990; Welch *et al.*, 1999), amino acids, proteins (Bosecker 1997) and organic acids (Agatzini and Tzeferis 1997; Barker *et al.*, 1997; Castro *et al.*, 2000; Natarajan and Deo 2001; Valsami-Jones and McEldowney 2000; Štyriaková *et al.*, 1999). It is known that organic acid production constitutes an adaption strategy by which bacteria and other microorganisms can extract limiting nutrients such as potassium, phosphorous

and calcium from insoluble mineral matrices, by chemical attack on the crystal structure of the nutrient containing minerals (Banfield *et al*., 1999). The mineral dissolution rate as well as the effectiveness of low/high molecular weight organic ligands on dissolution varies as a function of pH (Stillings *et al*., 1996; Ullman and Welch 1998; Welch and Ullman 1996). Optimum mineral dissolution should occur near the pH of the pKa of the acids produced. At the pKa of the acid there will be both protons available to dissociate from the ligand and react with the mineral surface and free ligands available to complex with metal ions (Ullman *et al*., 1997; Ullman and Welch 1998). Welch (1999) found that at near neutral pH, the dissolution rate of silicate minerals were relatively slow and independent of small changes in pH, however as the acidity increased to below pH 4 to 5, the dissolution became more dependent on changes in pH. With organic acid solutions, the rate of mineral dissolution increased with increasing acidity due to proton-promoted dissolution and metal-ligand complexation. The average pH measured for each culture used in this study is reported in Table 27. The decrease in pH may have been due to: (i) the release of organic acid by the bacteria into the surrounding media (Krebs *et al*., 1997; Štyriaková *et al*., 2004); (ii) hydrolysis of carbon dioxide to carbonic acid produced during respiration and/or due to assimilation of ammonium sulfate present in the media (Illmer and Schinner 1995; Illmer *et al*., 1995). Although the organic acids were not identified and measured, we know from literature which acids these organisms might produce (Table 4) and therefore we can compare these results to the finding in Chapter 3.

We found that the cultures that were grown under static conditions decreased the solution pH more. This is in accordance with research from Štyriaková *et al*. (2004), which ascribed the lower pH measured for static grown cultures to low dissolved oxygen present in solution.

Table 27 pH measurement of heterotrophic bacterial leaching experiments.

Organisms	pH average before neutralization	Adjusted pH
Bacillus megaterium (static)	4.10	6.95
Bacillus subtilis (static)	3.94	7.20
Bacillus cereus (static)	4.18	7.10
Heterotrophic *mixture* (static)	3.81	6.90
Pseudomonas putida (static)	4.25	6.90
B. megaterium (shaking)	4.49	6.95
B. subtilis (shaking)	4.50	6.90
B. cereus (shaking)	4.46	6.95
Heterotrophic *mixture* (shaking)	4.46	7.10
P. putida (shaking)	4.40	6.95

5.3.2 Quantitative mineralogy

Export ore (alkali <0.24%) were treated with different heterotrophic bacteria (Table 28-32), to assess their ability to solubilize the alkalis present. Numerous heterotrophic organisms, both bacteria and fungul species, are known for their leaching abilities on minerals, in particular oxidic, siliceous or carbonaceous materials. Examples of applied heterotrophic leaching include: copper oxides and carbonates (Agatzini-Leonardou and Tzeferis 1992; Rusin *et al*., 1992; Sharma *et al*., 1994); manganese oxide (Abbruzzese *et al*., 1990; Moy and Madgwick 1996); refractory gold ores (Groudev and Groudeva 1994; Torma and Oolman 1992); oxidic nickel ores (Tzeferis 1995); quartz sands and silicates (Strasser *et al*., 1993); cobalt ores (Tzeferis 1995) and spodumene (Ilgar *et al*., 1993). These organisms produce metabolites that are able to solubilize and/or aid in solubilizing oxide, silicate, carbonate and hydroxide minerals *via* mechanisms such as reduction, acidolysis, complexation and alkalinization (Burgstaller and Schinner 1993; Gadd and Sayer 2000; Jain and Sharma 2004; Sayer and Gadd 1997).

The amount of alkali removed was determined by measuring the residual retained in the ore after treatment and comparing it to the untreated sample (control) (Table 28-32). The alkali, aluminum and silicon concentrations in the ore will be the main focus of the current research, as Mojallali and Weed (1978) stated that a loss of aluminum and/or silicon shows a structural rearrangement of the mineral. Furthermore we will also focus on iron and magnesium as Wilson and Jones (1983) found that a decrease in these minerals also show a rearrangement of the mineral.

Mineral composition of the untreated iron ore sample is depicted in Table 19. X-ray fluorescence was used to determine the mineral composition of the export iron ore sample. This method is reasonably sensitive, with detection limits[27] for most elements in the low ppm range as stated by Jenkins (1988) (Table 14). The magnesium and sodium concentrations in the ore, were below the detection limit listed by Jenkins (1988) and will therefore be excluded from further analysis. Results of treated samples were expected to exceed the control sample due to sample variation; therefore a range was determined which included the minimum and maximum amount of alkali measured in the quadruple analyzed untreated sample. From this data set we can possibly determine the amount of alkali removed due to acid leaching and excluded data influenced by sample variation. From XRF analysis of the untreated export iron ore, the following ranges for the minerals were calculated: aluminum (1.15% - 1.56%), silicon (1.92% - 2.80%), potassium (0.16% - 0.18%) and phosphorous (0.13% - 0.17). Concentrations of each mineral removed below these parameters will be reported as "large quantities[28]" removed. We accept the average value of the untreated export sample as an acceptable representative. The amount of magnesium present in the sample was excluded from analysis (Table 14); therefore it could not be used as an indication of structural rearrangement of the mineral (Wilson and Jones 1983).

Silicon was detected as quartz, muscovite, biotite, etc. in all of the samples analyzed (Table 1). Quartz is known to dissolve slowly, due to the high activation energy required to break the

[27] Lower limit of detection is defined as that concentration equivalent to a certain number of standard deviations of the background count rate.
[28] A maximum and minum concentration for each mineral present in the untreated sample was determined. Once the ore was treated with the specific assay, we analyzed it again with XRF. If the residual amount of a specific mineral was below the minimum concentration, we ascribe it to the assay tested.

silicon-oxygen bond (Brehm *et al*., 2005 as cited in Wilson 2004). We did not determine the source of silicon dissolution, as it was outside the scope of this study; however it is assumed that the silicon released was from less resistant mineral phases present in the export iron ore. Mineral phases identified in the export sample using XRD, was apatite, hematite, muscovite and K-feldspar (Table 1). Muscovite has considerable resistance toward leaching of interlayer potassium, compared to trioctahedral micas (such as biotite). It has been proposed that an inclined orientation of hydroxyl ions in the dioctahedral micas results in stronger binding of potassium (Kalinowski and Schweda 1996) and therefore it has considerable resistance toward leaching. However, Scott and Smith (1966) found that essentially all the potassium in muscovite, biotite, phlogopite and vermiculite were exchangeable when the concentration of the potassium in solution was kept low.

Bioleaching during this study was not extended beyond 60 days, as the implementation of this approach into the mining schedule at Sishen would not allow such a time span.

Controversy exists in literature on the role of organic acids leaching in nature, due to its relative low concentrations. However, recent experiments have demonstrated that naturally occurring concentrations of organic acids, could increase mineral dissolution. The organic ligands can directly or indirectly catalyze the reaction by forming complexes with either the metal ions in solution (lowering the solution saturation state), or the metal ions on the mineral surface, thereby weakening the metal oxygen bonds (Banfield *et al*., 1999; Welch *et al*., 1999). The effect of organic ligands on mineral weathering reactions is affected by: (i) the ligand composition and structure; (ii) metal-ligand stability and (iii) solution composition (pH, ligand concentration, concentration of other metals) (Welch *et al.*, 1999). Van Schöll and coworkers (2006) found that mineral composition can also affect the efficiency of ligand weathering.

The bacteria selected for this section of the study includes: *Bacillus megaterium*, *B. cereus* and *B. subtilis*. *Bacillus megaterium* is a gram positive, aerobic, spore-forming bacterium that naturally occurs in soil, and has shown to effectively increase the mineral uptake of eggplants, pepper and cucumber (Han *et al*., 2005). It is known to produce citric acid, which is a relatively strong organic acid, and can assist mineral mobilization *via* acidulation and/or complexation (Jain and Sharma 2004) (Table 13). From the acid leaching experiments (Chapter 3) we found

that citric acid was able to remove "large quantities" of potassium (24%) and phosphorous (13%) (Table 15). We assume that the inability of the organisms to remove as much potassium as the acid experiment is due to sample variation and/or mineral dissemination, as the iron oxidizing bacterial results (Chapter 4) has an increased ability of the biotic experiments to remove these alkalis.

Static grown cultures were able to remove silicon, aluminum and phosphorous after 60 days. Shaken cultures were able to remove of silicon, aluminum, phosphorous and potassium after 60 days (Table 28). pH measured suggested that the static grown cultures were able to produce more acids and was therefore thought to be more affective at solubilizing the alkalis (Table 27). It is however likely that mineral dissemination[29] and/or sample variation[30] influenced these XRF results and change the expected outcome.

Phosphorous was detected as apatite, which is known to be relatively insoluble at neutral pH, although its solubility and reactivity varies as a function of its composition (Anderson *et al*., 1985; Jahnke 1984; LeGeros and Tung 1983; Valsami-Jones *et al*., 1998). Therefore the low pH present in the bioleaching solution (Table 28) could possibly have aided the release of phosphorous from the ore (Welch *et al*., 2002). Recently, Goldstein and Krishnaraj (2007) showed that the most common mechanism used by microorganisms to solubilize phosphate from apatite, seems to be by acidifying the media when organic acids are produced and released. Moreover, biofilms bound to the surface (Figure 22), would also actively take up phosphate, because it is a key nutrient for cell processes (Delvasto *et al*., 2008; Tempest and Neijssel 1992). This is accomplished by metals complexing with active moieties (such as carboxyl groups) present in the exopolysaccharides or other cellular materials (Comte *et al*., 2006; Corzo *et al*., 1994). Studies conducted with microbial extracellular polymers and simple analogs of these compounds showed that acid polysaccharides could increase dissolution of alumino-silicate minerals under mildly acidic conditions, by complexing with the minerals solubilized into solution (Welch and Vandevivere 1994; Welch *et al*., 1999) or inhibit dissolution at near neutral pH when bound on the minerals surface (Poumier *et al*., 1999).

[29] Alkali is located in the core of the particle and therefore inaccessible to the acid
[30] Sample contains an alkali concentration higher than the set parameter and therefore no apparent removal will be reported.

The amount of silicon and aluminum removed, illustrates a structural rearrangement of the minerals (Mojallali and Weed 1978). It was not determined whether the organisms actively scavenged for the minerals, or whether the solubilization of the minerals was indirectly due to the production of metabolites such as organic acid. There are however support for the former by Bennett and coworkers (2001). They used *in situ* and laboratory microcosms to determine whether colonization of minerals can be nutrient driven. The *in situ* (field experiments) were conducted at two groundwater sites, which had similar characteristics: abundant dissolved carbon and anoxic conditions. Here mineral chips were placed in permeable chambers and suspended in the groundwater for months. In the laboratory, mineral fragments were inoculated with groundwater (from the two sites) and aquifer materials. They found that in the groundwater, which was rich in carbon but contained limited amounts of phosphate, microbial mediated weathering of minerals were sometimes determined by the nutritional requirement of the microbial community. They further found that feldspar could be rapidly dissolved in such nutrient limiting environments. Furthermore the authors argued that microorganisms might be able to select minerals that contain beneficial elements, and leave others intact. Previously, Ullman *et al*. (1996) showed that bacteria are able to produce organic acids when carbon sources are abundant but other nutrients scarce, therefore the organisms would still be able to scavenge for nutrients**.** Furthermore, they showed that the excretion of organic acids can also be achieved by fermentation under anoxic conditions. Therefore, the main conclusion of their research study was that the dissolution rates of minerals should increase in organic-rich and nutrient-poor environments, as well under anoxic conditions.

Table 28 XRF analysis of *Bacillus megaterium* leached export iron ore.

Element	Untreated Export Ore	*B. megaterium* (static)				*B. megaterium* (shaking)			
		30 days	% removed	60 days	% removed	30 days	% removed	60 days	% removed
SiO_2	2.36	1.77	25	1.88	20	2.33	1	1.40	41
TiO_2	0.08	0.08	0	0.07	13	0.08	0	0.06	25
Al_2O_3	1.32	1.14	14	0.97	27	1.31	077	0.67	50
Fe_2O_3	95.68	96.76	n/a	96.09	n/a	95.41	0.3	97.59	n/a
MnO	0.03	0.05	n/a	0.04	n/a	0.03	0	0.04	n/a
MgO	0.03	0.00	100	0.00	100	0.00	100	0.00	100
CaO	0.04	0.00	100	0.00	100	0.00	100	0.00	100
Na_2O	0.11	0.01	91	0.01	91	0.01	91	0.01	91
K_2O	0.17	0.17	0	0.23	n/a	0.20	n/a	0.14	18
P_2O_5	0.15	0.14	7	0.13	13	0.16	n/a	0.13	13
Cr_2O_3	0.02	0.02	0	0.02	0	0.02	0	0.02	0
NiO	0.01	0.00	100	0.00	100	0.00	100	0.00	100
V_2O_5	0.01	0.02	n/a	0.02	n/a	0.02	n/a	0.02	n/a
ZrO_2	0.00	0.00	0	0.00	0	0.00	0	0.00	0

Bacillus cereus is a gram positive, aerobic, spore-forming bacterium that naturally occurs in soil, and has shown to be able to effectively change the structure of the trioctahedral mica phlogopite (Štyriaková *et al.,* 2004). This organism is known to produce lactic and acetic acid (Wong *et al,*. 1998) (Table 13) which we assume to be responsible for the decrease in solution pH (Table 27). From our acid leaching experiments (Chapter 3) we found that acetic acid was able to remove "large quantities" of potassium (29%) and phosphorous (27%) (Table 15). Static grown *B. cereus* culture was able to remove potassium (24%) and phosphorous (13%) after 30 days, compared to 12% potassium and 20% phosphorous after 60 days for aerated cultures (Table 29). It is assumed that mineral dissemination and/or mineral distribution influenced the results obtained. Also it is hypothesized that the more concentrated acid tested had more available protons and the bacterial experiment and were therefore able to remove more of the alkali. *Bacillus cereus* was able to remove more potassium and phosphorous than *B. megaterium*. *B. megaterium* produces a bidentate acid which is thought to be more effective in enhancing dissolution than the monodenate ligand produces by *B. cereus* (Welch and Ullman 1993). Therefore it is possible that other factors such as EPS, enzyme, etc. produced by each organism could also have affected the XRF results. It has experimentally been

demonstrated that high molecular weight organic ligands (phospholipids, peptidoglycan, carbohydrates, peptides and teichoic acids) can affect mineral weathering by acting as a binding site for metals derived from minerals (Daughney *et al*., 1998; Fein *et al*., 1997). Welch (1999) found that certain polysaccharides that had multiple functional groups could form bidentate complexes with mineral ions which then enabled them to enhance the solubilization reaction more. Moreover, they found that there is a strong dependence on pH for dissolution to occur with high molecular weight organic ligands. At near neutral pH, feldspar dissolution was inhibited by the acid polysaccharides, whiles at mildly acid pH all the polymers tested enhanced weathering. Drever (1997) showed that the release of aluminum from feldspar is also pH dependent (as the pH increases, less of the aluminum were removed from the feldspar).

Table 29 XRF analysis of *Bacillus cereus* leached export iron ore.

Element	Untreated Export Ore	*B. cereus* (static)				*B. cereus* (shaking)			
		30 days	% removed	60 days	% removed	30 days	% removed	60 days	% removed
SiO_2	2.36	1.73	27	2.16	8	2.03	14	1.52	36
TiO_2	0.08	0.06	25	0.07	13	0.07	13	0.06	25
Al_2O_3	1.32	1.03	22	1.18	11	1.11	16	0.70	47
Fe_2O_3	95.68	96.64	n/a	96.67	n/a	95.98	n/a	97.63	n/a
MnO	0.03	0.04	n/a	0.04	n/a	0.04	n/a	0.04	n/a
MgO	0.03	0.00	100	0.00	100	0.00	100	0.00	100
CaO	0.04	0.00	100	0.00	100	0.00	100	0.00	100
Na_2O	0.11	0.01	91	0.01	91	0.01	91	0.01	91
K_2O	0.17	0.13	24	0.17	0	0.18	n/a	0.15	12
P_2O_5	0.15	0.13	13	0.13	13	0.14	7	0.12	20
Cr_2O_3	0.02	0.03	n/a	0.02	0	0.02	0	0.03	n/a
NiO	0.01	0.00	100	0.00	100	0.00	100	0.00	100
V_2O_5	0.01	0.02	n/a	0.02	n/a	0.02	n/a	0.01	0
ZrO_2	0.00	0.00	0	0.00	0	0.00	0	0.00	0

Bacillus subtilis is a known mycorrhiza-helping bacterium and has been implicated as responsible for mobilizing inaccessible phosphorous (Toro *et al*., 1997). Fry and coworkers (2000) determined that this organism was able to produce lactic and acetic acid. Furthermore, gluconic acid can also be produced by this organism when glucose is supplied (Štyriaková *et al*., 2004; Welch *et al*., 1999). Therefore, the decrease in solution pH

was assumed to be due to acid production (Table 27). Leaching experiments with 1M acetic acid, showed that it was able to solubilize 29% potassium and 27% phosphorous from the export iron ore. 1M gluconic acid was able to solubilize 24% of the potassium and 27% of the phosphorous present in the export iron ore (Table 15). Static grown cultures were able to remove 6% potassium and 13% phosphorous after 60 days, however static grown cultures were able to remove 29% potassium and 27% phosphorous. Moreover, the cultures were also able to remove "large quantities" of aluminum and silicon. Experiments conducted by Delvasto *et al*. (2008) found that removal of phosphorous from iron ore appears not to be a continuous process, which they attributed to the re-immobilization of the mineral due to the high phosphate-binding capacity of iron oxides, and the ability of biofilms to readily take up this mineral. Consequently, we conclude that the XRF data obtained for phosphorous (aerated cultures) is possibly due to sample variation or re-immobilization of the mineral on the surface. Therefore, *Bacillus subtilis* is able to remove a "large quantity" of the alkalis compared to acetic acid. The static grown cultures were able to decrease the solution pH more than the aerated (shaken) cultures, which is in accordance Štyriaková *et al*. (2004). It was expected that the lower pH present in the static grown cultures would favor more phosphorous release as Welch *et al*. (2002) stated that a low solution pH increased apatite dissolution; however it is known that its solubility and reactivity varies as a function of its composition (LeGeros and Tung 1983; Jahnke 1984; Anderson *et al*., 1985; Valsami-Jones *et al*., 1998). We found that the aerated cultures were able to solubilize more alkali, compared to the static cultures. We presume that this is due to mineral dissemination, sample variation and/or the difference in mineral composition. Furthermore it is also probable that the static grown cultures produced more lactic acid, which might be less efficient than acetic acid, however due to insufficient data we are unable to draw this conclusion.

Table 30 XRF analysis of *Bacillus subtilis* leached export samples.

Element	Untreated Export Ore	*B. subtilis* (static)				*B. subtilis* (shaking)			
		30 days	% removed	60 days	% removed	30 days	% removed	60 days	% removed
SiO_2	2.36	2.18	8	1.52	36	2.36	0	1.67	29
TiO_2	0.08	0.06	25	0.07	12.5	0.07	12.5	0.06	25
Al_2O_3	1.32	1.04	21	0.83	37	1.12	15	0.74	44
Fe_2O_3	95.68	96.64	n/a	95.95	n/a	96.01	n/a	96.70	n/a 1
MnO	0.03	0.05	n/a	0.04	n/a	0.04	n/a	0.03	0
MgO	0.03	0.00	100	0.00	100	0.00	100	0.00	100
CaO	0.04	0.00	100	0.00	100	0.00	100	0.00	100
Na_2O	0.11	0.01	91	0.01	91	0.01	91	0.01	91
K_2O	0.17	0.16	6	0.16	6	0.20	n/a	0.12	29
P_2O_5	0.15	0.19	n/a	0.13	13	0.11	27	0.13	13
Cr_2O_3	0.02	0.02	0	0.02	0	0.02	0	0.04	n/a
NiO	0.01	0.00	100	0.00	100	0.00	100	0.00	100
V_2O_5	0.01	0.02	n/a	0.02	n/a	0.02	n/a	0.02	n/a
ZrO_2	0.00	0.00	0	0.00	0	0.00	0	0.00	0

Pseudomonas putida is a gram negative, rod shaped, saprophytic soil bacterium that has been found to effectively leach zinc, cadmium and copper from filter dust and fly ash from municipal waste incineration (Jain and Sharma 2004). It is known to produce citric and gluconic acid (Jain and Sharma 2004) which was included in our preliminary acid leaching survey (Chapter 3). 1M gluconic acid was able to solubilize 24% of the potassium and 27% of the phosphorous present in the export iron ore sample (Table 15). 1M citric acid was able to remove 24% potassium and 13% phosphorous. Static grown *Pseudomonas putida* cultures were able to remove "large quantities" of silicon, aluminum and potassium after 30 days, and phosphorous after 60 days. Aerated cultures were able to remove a "large quantity" of silicon, aluminum, potassium and phosphorous after 60 days (Table 31). Leaching with aerated cultures showed a decrease in the amount of potassium leached after extended leaching time. This is ascribed again to either sample variation, mineral distribution inside the particle or possible due to re-immobilization of the mineral, as discussed by Delvasto *et al*. (2008).

Table 31 XRF analysis of *Pseudomonas putida* leached export sample.

Element	Untreated Sishen Iron Ore	*P. putida* (static)				*P. putida* (shaking)			
		30 days	% removed	60 days	% removed	30 days	% removed	60 days	% removed
SiO_2	2.36	1.86	21	1.87	21	1.77	25	1.68	29
TiO_2	0.08	0.07	13	0.08	0	0.07	13	0.07	13
Al_2O_3	1.32	1.13	15	1.04	21	0.94	29	0.96	28
Fe_2O_3	95.68	96.85	n/a	96.05	n/a	96.72	n/a	97.05	n/a
MnO	0.03	0.04	n/a	0.04	n/a	0.04	n/a	0.03	0
MgO	0.03	0.00	100	0.00	100	0.00	100	0.00	100
CaO	0.04	0.00	100	0.00	100	0.00	100	0.00	100
Na_2O	0.11	0.01	91	0.01	91	0.01	91	0.01	91
K_2O	0.17	0.15	12	0.17	0	0.14	17	0.15	12
P_2O_5	0.15	0.16	n/a	0.12	20	0.14	7	0.12	20
Cr_2O_3	0.02	0.02	0	0.02	0	0.02	0	0.04	n/a
NiO	0.01	0.00	100	0.00	100	0.00	100	0.00	100
V_2O_5	0.01	0.02	n/a	0.02	n/a	0.02	-100	0.02	n/a
ZrO_2	0.00	0.00	0	0.00	0	0.00	0	0.00	0

A synergistic effect was also determined, by adding equal volumes of the different bacterial strains tested during this study. Several factors influence leaching such as microbial numbers, activity of the strains, metal tolerance, etc. (Table 10). Therefore it was thought an increase in bacterial numbers and different acids would increase the amount of alkali mobilized from the ores. Static grown mixed cultures were able to remove 29 % potassium and 33% phosphorous, compared to 18% potassium and 27% phosphorous for aerated cultures (Table 32). Here the static grown cultures had a solution pH of 3.81 compared to 4.46 for the aerated cultures (Table 27), which can possibly be ascribed to increased acid production due to a low oxygen concentration in the media as discussed by Štyriaková *et al*. (2004). However, data from the other heterotrophic leaching suggests that acid production alone cannot be responsible for the amount of minerals leached therefore other compounds such as EPS, enzymes, etc. could also play an important role.

The significance of sample variation and/or mineral dissemination cannot be fully determined for any of the experiments as more repetitions are needed, to determine its significance, but analysis

was limited due to funding. However, these preliminary results are still valuable, as they justify further exploration into bacterial treatment of iron ore samples.

Table 32 XRF analysis of heterotrophic leached export sample.

Element	Untreated Export Ore	Mixed heterotrophs (static)				Mixed heterotrophs (shaking)			
		30 days	% removed	60 days	% removed	30 days	% removed	60 days	% removed
SiO_2	2.36	1.36	42	2.17	8	1.70	28	1.55	34
TiO_2	0.08	0.06	25	0.06	25	0.08	0	0.06	25
Al_2O_3	1.32	0.76	43	0.74	44	1.15	13	0.78	41
Fe_2O_3	95.68	96.38	n/a	97.08	n/a	97.14	n/a	97.32	n/a
MnO	0.03	0.04	n/a	0.04	n/a	0.04	n/a	0.04	n/a
MgO	0.03	0.00	100	0.00	100	0.00	100	0.00	100
CaO	0.04	0.00	100	0.00	100	0.00	100	0.00	100
Na_2O	0.11	0.01	91	0.01	91	0.01	91	0.01	91
K_2O	0.17	0.15	12	0.12	29	0.16	6	0.14	18
P_2O_5	0.15	0.10	33	0.12	20	0.11	27	0.11	27
Cr_2O_3	0.02	0.02	0	0.02	0	0.02	0	0.02	0
NiO	0.01	0.00	100	0.00	100	0.00	100	0.00	100
V_2O_5	0.01	0.02	n/a	0.02	n/a	0.02	n/a	0.02	n/a
ZrO_2	0.00	0.00	0	0.00	0	0.00	0	0.00	0

5.3.3 Denaturing gradient electrophoresis (DGGE)

Ehrlich (1991) commented that several challenges, such as establishing selective growth conditions (thus controlling contamination), will have to be solved before leaching with heterotrophic organisms will become a viable approach. During this study, some of the flasks became contaminated (Figure 20) with unknown cultures, which was excluded from further analysis and discussion and therefore necessitated the use of DGGE to ensure that the XRF results obtained was de to the selected microorganisms and not a contaminant. Therefore total genomic DNA was extracted from the inoculated flasks using the cetyltrimethylammonium bromide (CTAB method), as described by Ausubel *et al*. (2002), amplified using the oligonucleotides PRUN518r (5'-ATT-ACC-GCG-GCT-GCT-GG-3') (Siciliano *et al.,* 2003) and pA8f-GC (5'-CGC-CCG-CCG-CGC-GCG-GCG-GGC-GGG-GCG-GGG-GCACGG-GGG-

GAG-AGT-TTG-ATC-CTG-GCT-CAG-3') (Fjellbirkeland *et al.*, 2001), and subjected it DGGE, as described by Muyzer *et al*. (1993), to ensure that no contaminants were present in the samples.

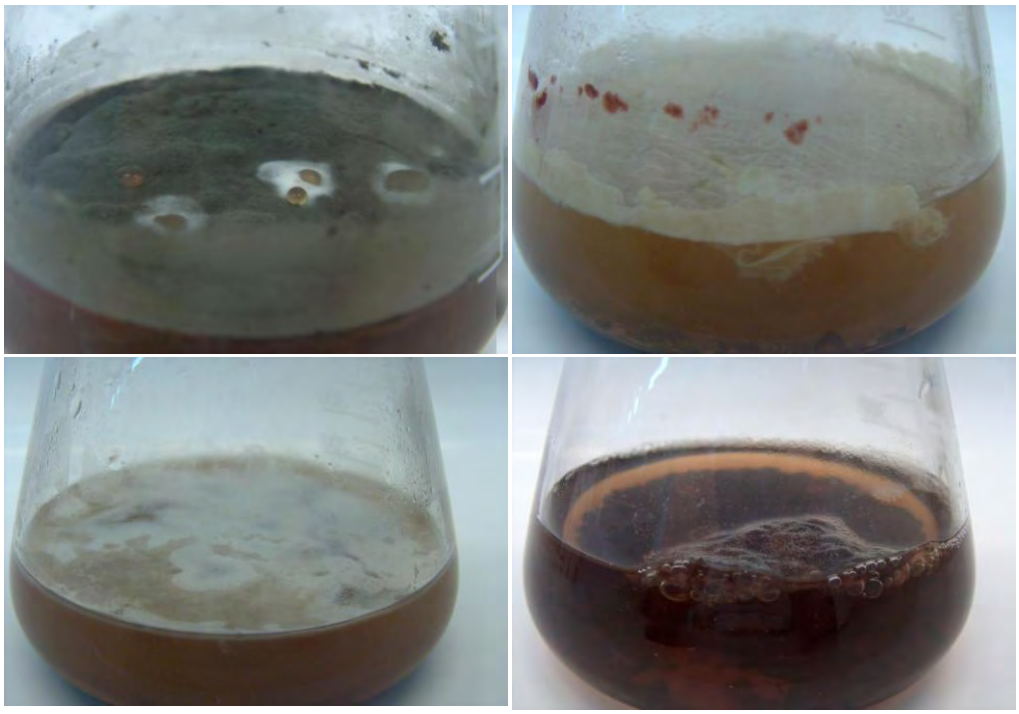

Figure 20. Contamination of heterotrophic leaching experiment with unknown cultures.

Sequencing of the excised bands identified the cultures as follow: *Bacillus megaterium* (J3-10DQ363436), *B. cereus* (MG1-DQ228954), *B. subtilis* (AMM202-AB092795) and *Pseudomonas putida* (ATCC 15070). Therefore the XRF data obtained can be ascribed to the action of each culture tested, and not to an unidentified contaminant. These preliminary results allow future research into optimizing conditions or modifying the organism for optimal leaching efficiency.

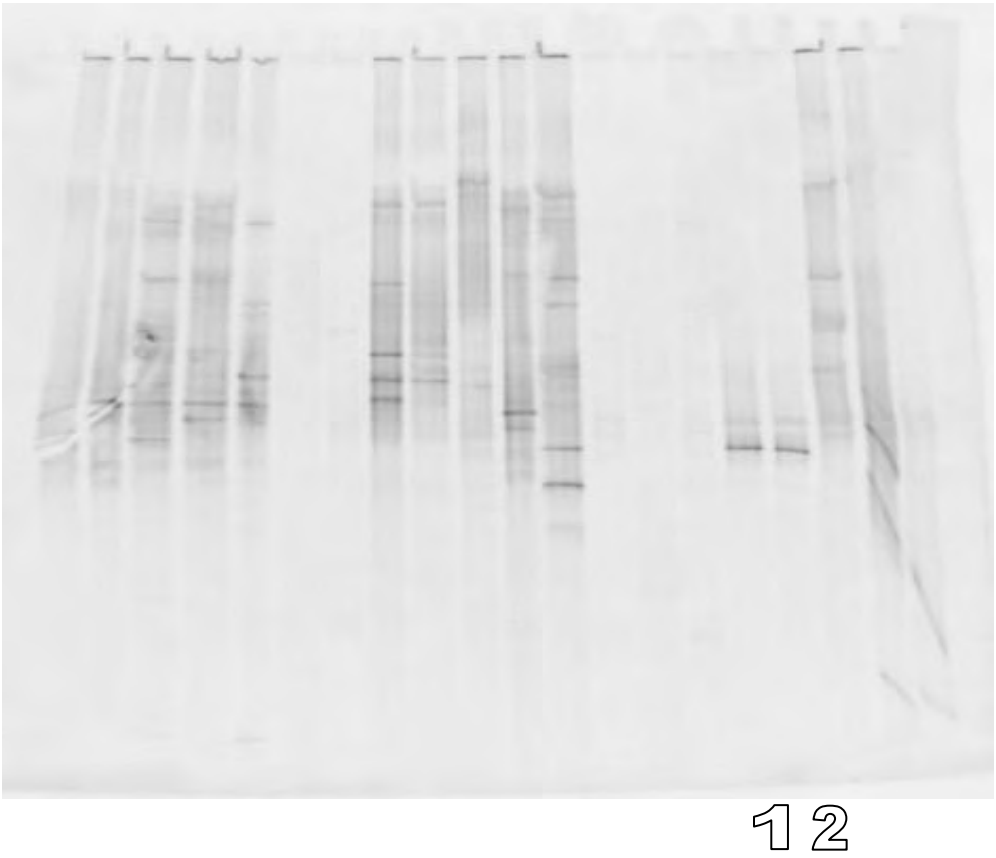

Figure 21. Denaturing gradient gel of amplified genomic DNA from heterotrophic leaching experiments showing dominant strains present in leaching solution (1 and 2).

5.3.4 Scanning electron microscopy

Bacterial leached iron ore samples were observed with scanning electron microscopy. Data obtained suggests that the bacteria were able to form biofilms on the surface of the mineral (Figure 22), which would actively take up phosphate, due to its importance in cell processes (Delvasto *et al*., 2008; Tempest and Neijssel 1992). This is accomplished by metals complexing with active moieties (such as carboxyl groups) present in the exopolysaccharides or other cellular materials (Comte *et al*., 2006; Corzo *et al*., 1994). Biofilm formation is a multistep process which is influenced by many factors, including the specific mineralogy of the rock, solution chemistry (pH, ionic strength) and the characteristics of the microorganism (hydrophobicity, surface charge) (Banfield and Hamers 1997; Characklis 1989; Little *et al*., 1997).

The attachment of cells to the surface can occur *via* random processes such as diffusion and convection or more specifically *via* chemotaxis (Jerez 2001).

Studies conducted with microbial extracellular polymers and simple analogs of these compounds showed that acid polysaccharides could increase dissolution of alumino-silicate minerals under mildly acidic conditions, by complexing with the minerals solubilized into solution (Welch *et al*., 1999; Welch and Vandevivere 1994) or inhibit dissolution at near neutral pH when bound on the minerals surface (Poumier *et al*., 1999).

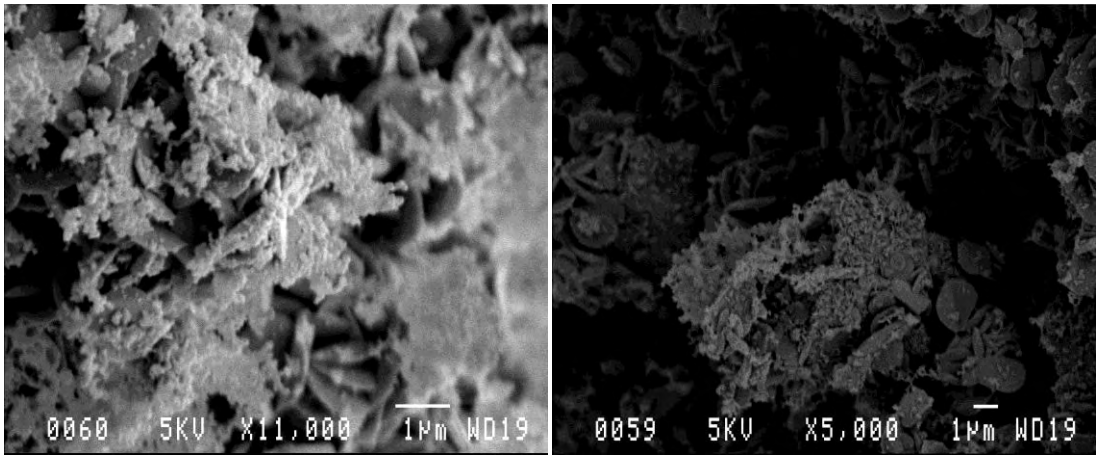

Figure 22. Scanning electron microscopy images of heterotrophic leached export iron ore.

During our study we found several instances in which extended leaching (60 days) removed less alkali from the ore. This was ascribed to sample variation and/or mineral dissemination in the particle; however biofilms could also influence the XRF data. Experiments conducted by Delvasto *et al*. (2008) found that removal of phosphorous from iron ore appeared not to be a continuous process, which they attributed to the re-immobilization of the mineral due to the high phosphate-binding capacity of iron oxides, and the ability of biofilms to readily take up this mineral.

Moreover, the presence of biofilms shows that the mobilization of the minerals from the export sample can be ascribed to other compounds, than just to the action of organic acids.

5.4 CONCLUSION

Heterotrophic bacteria posses several mechanisms, which enable them to aid mineral mobilization. These include exopolysaccharides (Banfield *et al*., 1999; Malinovskaya *et al*., 1990; Welch *et al*., 1999), amino acids, proteins (Bosecker 1997) and organic acids (Agatzini and Tzeferis 1997; Barker *et al*., 1997; Castro *et al*., 2000; Natarajan and Deo 2001; Štyriaková *et al*., 1999; Valsami-Jones and McEldowney 2000). Heterotrophic organisms have been applied to leach compounds such as: copper oxides and carbonates (Agatzini-Leonardo and Tzeferis 1992; Rusin *et al*., 1992; Sharma *et al*., 1994); manganese oxide (Abbruzzese *et al*., 1990; Moy and Madgwick 1996); refractory gold ores (Groudev and Groudeva 1994; Torma and Oolman 1992); oxidic nickel ores (Tzeferis 1995); quartz sands and silicates (Strasser *et al*., 1993); cobalt ores (Tzeferis 1995) and spodumene (Ilgar *et al*., 1993). However due to difficulty in establishing selective growth conditions, heterotrophic leaching is not preferred for industrial scale leaching as autotrophic leaching (Ehrlich 1991; Jain and Sharma 2004). The objective of this study was to determine, whether known potassium/phosphorous solubilizing heterotrophic bacteria, are able to remove the alkali impurities from the export iron ore samples, provided by Kumba Iron Ore, Ltd. We found that the different strains were able to remove varying amounts of potassium and phosphorous. Moreover, the organisms demonstrated a synergistic relationship, which enable mobilization of the mineral. Scanning electron microscopy analysis of the leached ore, demonstrated the formation of biofilms, which could also have affected the leaching ability of the organisms.

The preliminary results suggest that heterotrophic bacteria leaching could possibly be applied to remove some of the impurities from the ore. However due to difficulty in establishing selective growth conditions, will make its introduction into the mining schedule more difficult than the autotrophic bioleaching approach (Chapter 4). Furthermore bioleaching will never completely replace the conventional methods but rather supplement them, as bioleaching does not recover precious metals from the ores which are often an essential component in the profitability of the operation; also when ore bodies do not contain sufficient acid consuming minerals, the residual acids generated has to be neutralized during the leaching process, thereby increasing the operational cost (Dreshner 2004).

CHAPTER 6

MICROBIAL COMMUNITY STRUCTURE OF INDIGENOUS BACTERIA PRESENT ON THE SISHEN HEMATITE IRON ORE DURING BIOBENEFICATION

Abstract

The Sishen Iron Ore Mine is situated in the Northern Cape, South Africa, and forms part of the northern end of the Maremane anticline where the bulk of the hematite is buried beneath younger cover lithologies. The iron ore bodies at the mine are overlain by conglomerates, shales, flagstone and quartzite. Potassium and phosphorous are common constituents of iron ore that have deleterious effect on iron and steel manufacturing, therefore steel making companies charge penalties when purchasing iron ore with alkali concentrations above predetermined limits. Conventional methods used to treat high alkali ore have several drawbacks such as poor product recovery, high running cost and increase pollution of water. Biohydrometallurgy is a natural alternative approach which could be applied to supplement conventional methods. Our aim was to determine the microbial community structure of the indigenous bacteria present on the iron ore and assess their ability to remove potassium and phosphorous from the iron ore. We found that the organisms were able to solubilize varying degrees of potassium and phosphorous from the different iron ore samples, which is attributed to different metabolic capabilities of the organisms and the composition of each ore body. Furthermore, from this section of the study, we concluded that indigenous bacter are beter adapted than introduced species to remove the alkalis from the ore.

Keywords: Indigenous bacteria, Iron ore, muscovite, exopolysaccharide, organic acid, muscovite

6.1 INTRODUCTION

Microorganisms play an important role in: (i) the cycling of elements and sorption of metals (Langley and Beveridge 1999); (ii) the dissolution of minerals (Banfield and Hamers 1997; Hersman *et al.*, 1996; Roden and Zachara 1996; Schrenk *et al.*, 1998) and (iii) mineral crystallization (Fortin *et al.*, 1997; Warren and Ferris 1998). They are able to achieve this *via*: (1) assimilation/adsorption and mineralization; (2) precipitation and dissolution; (3) oxidation and reduction and (4) methylation and dealkylation. For a full review on these mechanisms please refer to Johnson (2006). Research has found that mineralogical process in turn play a significant role in the distribution, activity and diversity of microbes (Schrenk *et al.*, 1998), the expression of their genes (Gehrke *et al.*, 1998), development and structure of communities (Kennedy and Gewin 1997; Thorseth *et al.*, 1995; Wolfaardt *et al.*, 1994), and transfer of genetic material (Holben 1997; Trevors and van Elsas 1997).

Research by Johnson (1998) and Hallberg and Johnson (2001) indicated that there is a rich diversity of microorganisms present in the mining environments. Several researchers have reported that microorganisms are able to solubilize potassium from various minerals, for example: Berthelin and Belgy (1979) demonstrated the complete removal of potassium and titanium from biotite by a microbial consortium in a granitic sand, which resulted in brittle, white micaceous particles. Leyval and Berthelin (1991) showed that rhizosphere microorganisms associated with pine roots extracted potassium from phlogopite and this was attributed to organic acid mediated solution. Buis (1995) and Parks *et al*. (1990) conducted work in which they used fungal strains with high phosphate-solubilizing activity to treat phosphatic iron ore. The main drawback of their investigation was that they used strains that were not associated with the ore being treated. When artificially inoculated in an environment, the indigenous microorganisms as a general rule compete better in terms of adaptation and cause fewer ecological distortions than exogenous microorganisms (Delvasto *et al*., 2008b).

Iron ore mined at the Sishen, Northern Cape, South Africa contains several different mineral phases (Table 1). Alkalis (potassium and phosphorous) are frequently found as constituents of iron ore, which has a deleterious effect on the manufacturing of iron and steel (Delvasto *et al*., 2008). Therefore, steel making companies charge penalties when purchasing iron ore concentrates with alkali concentrations above certain levels. The limits allowed is

determined by the steel making companies and ranges from 0.25% mass in Japan to 0.55% mass in Switzerland for the alkali potassium. Kumba Iron Ore, Ltd., has an industrial set limit of 0.24% potassium allowed in their export ore according to D. Krige (Personal communication, 2006). To ensure that their export batches stay within this set limit, the ores from different batches, at the Sishen Iron Ore Mine (with potassium >0.24% and <0.24%) are mixed to produce an average potassium value of below 0.24%. However this solution will soon become ineffective as the low potassium ore (< 0.24%) is progressively depleted according to D. Krige (Personal communication, 2006). Certain pyro – and hydrometallurgical methods can be applied to decrease the alkali concentration (Cheng *et al*., 1999; Kokal *et al*., 2003), however there are several disadvantages when using these methods such as: poor product recovery, involvement of high process and energy cost and an increase in pollution load of water resources (Jain and Sharma 2004). Thus an alternative, natural and economical feasible process is required aid conventional methods (such as pyro- and hydrometallurgy processes) to remove unwanted alkalis from the ores. Biohydrometallurgy[31] is an option for the removal of the deleterious phosphate and potassium, as it is well established that many microorganisms are capable of mobilizing these minerals, especially in nutrient limited environments (Banfield *et al*., 1999; Nautiyal 1999),

It is thought that indigenous microorganisms occurring on the iron ore mined at Sishen might possess the ability to leach the potassium and phosphorous from the iron, as these minerals might have become limiting previously in their immediate environment. Experiments by Bennett *et al*. (2001) provided evidence that silicate weathering by bacteria is sometimes driven by the nutrient requirements of the microbial consortium. They used *in situ* and laboratory microcosms to determine whether colonization of minerals can be nutrient driven. The *in situ* (field experiments) were conducted at two groundwater sites, which had similar characteristics: abundant dissolved carbon and anoxic conditions. Here mineral chips were placed in permeable chambers and suspended in the groundwater for months. In the laboratory, mineral fragments were inoculated with groundwater (from the two sites) and aquifer materials. They found that in the groundwater, which was rich in carbon but contained limited amounts of phosphate, microbial mediated weathering of minerals were sometimes determined by the nutritional

[31] Biohydrometallurgical processes include bioleaching and biobenefication (Ehrlich 1991).

requirement of the microbial community. They further found that feldspar could be rapidly dissolved in such nutrient limiting environments. Furthermore the authors argued that microorganisms might be able to select minerals that contain beneficial elements, and leave others intact.

Therefore, the purpose of the study was to amplify the indigenous bacteria, using Aleksandrov and Mikhauloskaya media previously described as able to select for potassium/phosphorous solubilizing bacteria and assessing their ability to mobilize the detrimental minerals from the iron ore mined at Sishen.

6.2 MATERIALS AND METHODS

6.2.1 Iron ore

Export (alkali <0.24%), KGT (conglomerate) and SK (shale) iron ore, as defined by supplier[32], with a particle size range of 1mm - 5mm, from the Sishen Iron Ore Mine, Northern Cape, South Africa was supplied by Kumba Iron Ore, Ltd., for indigenous bacterial leaching experiment. Samples were not kept under sterile conditions; therefore an array of contaminant would be present. However, due to the experimental setup, only those organisms with potassium/phosphate-solubilizing abilities would survive.

6.2.2 Enrichment media

Bioleaching experiments were carried out in 500ml Erlenmeyer flasks containing 150g unsterilized ore and 200ml media, followed by incubation at 30°C with agitation (150 rpm). Indigenous bacteria were amplified from the export iron ore using Aleksandrov media (Hu *et al.*, 2006), Nutrient broth (Biolab) and a media described by Štyriaková *et al.* (2004), which will hence be referred to as Štyriaková media (Table 33). Indigenous bacteria from the KGT sample (Conglomerate) were amplified using Aleksandrov media (Hu *et al.*, 2006), Nutrient broth (Biolab) and a media described by Mikhailouskaya *et al.*, 2005, which will consequently be referred to as Mikhailouskaya media. The SK sample (Shale) was treated with Nutrient broth (Biolab), Aleksandrov-, Mikhailouskaya- and Štyriaková media. The various iron ore samples

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were treated with different combinations of media, due to limited sample. The iron ore sample served as the sole phosphorous and potassium source. Prior results obtained with the SPHP samples were inconclusive and is therefore excluded from further bacterial leaching experiments (data not shown). Cultures were not enumerated during this study as results would be inaccurate due to microbial attachment on mineral surface (Kinzler *et al.*, 2003), therefore changes in solution pH was used to determine bacterial activity.

Table 33 Enrichment media for amplifying indigenous bacterial cultures.

Media	Constituent	g/l	Reference
Mikhailouskaya media	Sucrose	0.75	Mikhailouskaya *et al.*, 2005
	$(NH_4)_2SO_4$	0.15	
	Na_2HPO_4	0.30	
	$MgSO_4$	0.075	
	$FeCl_3$	1	
Aleksandrov media	Glucose	0.5	Hu *et al.*, 2006
	$MgSO_4.7H_2O$	0.05	
	$FeCl_3$	0.05	
	$CaCO_3$	0.01	
Štyriaková media	NaH_2PO_4	0.42	Štyriaková *et al.* (2004)
	$(NH_4)_2SO_4$	0.8	
	NaCl	0.19	
	Glucose	19.8	

6.2.3 pH measurements

pH of the inoculated samples were neutralized with 1M NaOH every 5th day, as described by Štyriaková *et al*. (2004).

6.2.4 DNA extraction

Total genomic DNA was extracted from the flasks using the cetyltrimethylammonium bromide (CTAB method), as described by Ausubel *et al*. (2002), to ensure that no contaminants were present in the samples. Cells from 1.5ml of the inoculated flasks were pelleted by centrifugation at 13 400 rpm for 5 min and suspended in 567µl of 1 × TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8). The cells were lysed by adding sodium dodecyl phosphate (SDS) to a final concentration of 0.5% (v/v) and the proteins were digested by adding Proteinase K to a final concentration of 100 µg/ml in total volume of 600 µl. The suspension was then incubated overnight at 37°C. Following incubation, 100 µl of 5M NaCl and 80µl of CTAB/NaCl solution (10% [w/v] CTAB in 0.7 M NaCl) was added, mixed thoroughly and incubated at 65°C for 10 min. An equal volume of chloroform:isoamyl alcohol (24:1) was then added and followed by centrifugation at 13 400 rpm for 5 min. The supernatant was then transferred to a clean microfuge tube to which an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added and centrifuged at 13 400 rpm for 5 min. The supernatant was then transferred to a new microfuge tube, to which 0.6 volume isopropanol was added to precipitate the DNA. The precipitated DNA was pelleted by centrifugation at 13 400 rpm for 20 min, washed with 70% ethanol, dried under vacuum and suspended in 20 µl of 1 × TE buffer. The suspension was then incubated overnight at 37°C before analyzing an aliquot of the extracted genomic DNA was analyzed by electrophoresis on a 1% (w/v) agarose gel.

6.2.5 Polymerase chain reaction

Polymerase chain reaction (PCR) was used to amplify a section of the 16S rRNA of the DNA extracted. The genes were amplified using the oligonucleotides PRUN518r (5'-ATT-ACC-GCG-GCT-GCT-GG-3') (Siciliano *et al.,* 2003) and pA8f-GC (5'-CGC-CCG-CCG-CGC-GCG-GCG-

GGC-GGG-GCG-GGG-GCACGG-GGG-GAG-AGT-TTG-ATC-CTG-GCT-CAG-3') (Fjellbirkeland *et al.*, 2001). Each PCR reaction mixture (20 µl) contained ~27ng/µl of genomic DNA as template, 10.3 µl sterile distilled MilliQ water, 2.5µl PCR buffer with $MgCl_2$ (10×), 2µl dNTPs (2.5µM), 1µl primer PRUN518r (50µM), 1µl primer pA8f-GC (50µM) and 0.2µl Supertherm *Taq* (5U/µl) (Southerm Cross Biotechnology). A negative control (all reagents and no template DNA) was added to rule out contamination. The tubes were placed in a Perkin-Elmer GeneAMP® 2700 thermal cycler. Following incubation at 95 °C for 10 min, the reaction mixture were subjected to 35 cycles of denaturing at 94 °C for 30 s, annealing at 53°C for 30 s and elongation at 72 °C for 1 min. Once the cycles were completed, a final elongation step was performed at 72°C for 10 min to complete DNA synthesis. An aliquot of the amplified DNA was analyzed by electrophoresis on a 1.5% (w/v) agarose gel in the presence of appropriate molecular weight marker. The PCR products were stored at 4°C, until further analysis.

6.2.6 Denaturing gradient gel electrophoresis (DGGE)

PCR products were subjected to denaturing gradient gel electrophoresis (DGGE) according to the method described by Muyzer *et al*. (1993). 10µl of the each PCR product was loaded onto a 40-50% denaturing gradient gels (Table 20). Gels were run at 70 V for 17 h at a constant temperature of 60 °C. Image analysis was performed using the Gel2K (Norland 2004) program. Selected bands were picked under blue light from the DGGE gels using a sterile scalpel and forceps. Each band was assigned a number for sequence analysis. The gel fragments were place into 25µl filter-sterilized deionized water and allowed to stand overnight to dissolve. The dissolved fragments were then subjected to PCR as described in the previous section.

6.2.7 Sequencing

The amplified products from the DGGE bands were cleaned by adding 15ul sterile water, transferring the entire volume to a 0.5ml Eppendorf sequencing tube, adding 2ul of sodium acetate (3M) and 50ul ethanol (95%), and allowing it to stand on ice for 10min. The tubes were then centrifuged at 10 000 rpm for 30 min. The ethanol solution was removed, the pellet rinsed in 150µl ethanol (70%), and the tubes again centrifuged for 5 min at 10 000 rpm. The ethanol

was aspirated and the pellet dried under vacuum for approximately 10 min. These samples were then submitted to Macrogen (U.S.A.) for sequencing using the oligonucleotides PRUN518r (Siciliano *et al.*, 2003). Nucleotide sequences were analyzed with BioEdit v.5.0.9.1 (Hall 2001) and their identity were verified by sequence match searches against the RDP database (available at http://rdp.cme.msu.edu).

6.2.8 Qualitative mineralogy

The composition of iron samples were analyzed with X-ray fluorescence (XRF) at the X-ray analytical facility of the University of Pretoria. Four samples of untreated iron ore were analyzed as controls

The samples were prepared as follow for analysis: Iron ore samples were first ground to <75µm in a tungsten carbide milling vessel. The loss of ignition (LOI) was determined by roasting the milled samples at 1000ºC. 1g of a milled iron ore sample were added to 6g $Li_2B_4O_7$ and fused into a glass beads. The rest of the sample was then used to make a powder briquette for minor element analysis. Major element analyses were executed on the fused bead using an ARL9400XP+ spectrometer (Loubser and Verryn 2008).

6.2.9 Scanning electron microscopy

Bacteria cells were fixed by placing the iron ore in a Greiner tube containing 20 ml fixing solution (2.5% gluteraldehyde in 0.0075 M phosphate buffer) for 1 h. The ore was washed three times for 15 min each with 0.0375 M $NaPO_4$ buffer, before being dehydrated by sequential treatment for 15 min each in 50%, 70%, 90% and 100% ethanol. The 100% ethanol step was repeated twice more to ensure complete dehydration. The iron ore samples were critical point-dried, scatter with gold with a Polaron Equipment LTD SEM Autocoating unit E5200 and observed with a JEOL 5800LV scanning electron microscope at an accelerating voltage of 5kV.

6.3 RESULTS AND DISCUSSION

6.3.1 pH measurement

The initial pH of the different medias tested, were as follow: Aleksandrov media: 6.25; Mikhailouskaya: 6.67; Nutrient broth: 6.9 and Štyriaková media: 6.58. The pH of the solution were neutralized every 5th day to ensure that pH conditions remained optimal (Štyriaková *et al*., 2004). Table 34 reports the average pH measured during this study.

Table 34 Average pH measurement for indigenous bacterial leaching experiments.

Description	Measurement
Export ore Aleksandrov media	5.72
Export ore Nutrient broth	7.48
Export ore Štyriaková media	4.09
KGT* sample Aleksandrov media	5.90
KGT sample Mikhailouskaya media	4.87
KGT sample Nutrient broth	8.95
SK** sample Aleksandrov media	5.91
SK sample Mikhailouskaya media	2.38
SK sample Nutrient broth	9.14
SK sample Štyriaková media	3.14

*Conglomerate iron ore sample

** Shale iron ore sample

Microorganisms produce an array of metabolites which might assist in the mobilization of minerals from the ore. These include exopolysaccharides (Banfield *et al*., 1999; Malinovskaya *et al*., 1990; Welch *et al*., 1999), amino acids, proteins (Bosecker 1997) and organic acids (Agatzini and Tzeferis 1997; Barker *et al*., 1997; Castro *et al*., 2000; Natarajan and Deo 2001; Valsami-Jones and McEldowney 2000; Štyriaková *et al*., 1999). Mineral dissolution and the effectiveness of low/high molecular weight organic ligands are known to be influenced by the solution pH (Stillings *et al*., 1996; Welch and Ullman 1996; Ullman and Welch 1998). Most metals are cationic and are therefore more soluble in acidic than in neutral pH and alkaline solutions, however the reverse is true for oxyanionic metal species (Johnson 2006). For example, silicate dissolution is reasonably slow at near neutral pH and independent on small pH changes, whereas an increase in acidity to below pH 4 to 5, the dissolution becomes more dependent on the changes in pH. With organic acid solutions, the rate of mineral dissolution increases with increasing acidity due to proton-promoted dissolution and metal-ligand complexation (Welch *et al.,* 1999). The ore bodies used during this study were composed of an array of minerals which included apatite and biotite (Table 1). Welch and coworkers (2002) demonstrated that microorganisms are able to influence apatite and biotite dissolution by producing organic acids, primarily pyruvate, fermentation products and oxalate. They found that by lowering the pH between 3 to 5, the microorganisms were able to solubilize more of the minerals. Moreover, they further found that some microorganisms were able to solubilize phosphorous from the mineral bodies without lowering the solution pH by producing pyruvate. Therefore we assumed that some of the potassium and phosphorous solubilized where from the ore, were due to the low pH and complex forming ability of the acids.

We found that the microbes enriched for, using the Aleksandrov, Mikhailouskaya and Štyriaková media, increased solution acidity, whereas the microbes amplified with Nutrient broth (Biolab) increased alkalinity. The changes in pH measured during this study, was ascribed to the following (Table 34). A decrease in pH may have been due to: the release of organic acid by the bacteria into the surrounding media (Krebs *et al*., 1997); production of gluconic acid from the carbon source (Štyriaková *et al*., 2004; Welch *et al*., 1999); hydrolysis of carbon dioxide to carbonic acid produced during respiration and/or due to assimilation of ammonium sulfate present in the media (Illmer and Schinner 1995; Illmer *et al*., 1995). Although the organic acids

were not identified and measured, it is known that organic acid production constitutes an adaption strategy by which bacteria and other microorganisms can extract limiting nutrients such as potassium, phosphorous and calcium from insoluble mineral matrices, by chemical attack on the crystal structure of the nutrient containing minerals (Banfield *et al*., 1999). Microbial processed, such fermentation, nitrification and sulfur-oxidation generate acidity, whilst others such as ammonification, denitrification, sulfate-reduction and methanogenesis generate alkalinity, therefore it is assumed that the cultures enriched with nutrient broth possess such as mechanism(s) (Johnson 2006). In addition, microorganisms are also able to produce other compounds such as siderophores, which can actively scavenge for ferric iron and other trivalent metals (such as aluminum and gallium), at neutral pH (Johnson 2006; White *et al*., 1995), which could have aided mineral solubilization at near neutral pH (Table 34).

6.3.2 Quantitative mineralogy

Indigenous bacteria with possible potassium/phosphorous leaching abilities were enriched for by inoculating Aleksandrov - (Hu *et al*., 2006), Mikhailouskaya – (Mikhailouskaya *et al.*, 2005), Štyriaková media and Nutrient broth (Biolab) with export iron ore. Bacteria with phosphate solubilizing ability has been isolated from various environments such as eutrophic lakes (Gen-Fu and Xue-Ping 2005), mangrove tree roots (Vazquez *et al*., 2000), the cactus rhizoplane (Puente *et al*., 2004) and tree rhizospheres (Leyval and Berthelin 1991). Mikhailouskaya *et al*. (2005) assessed the ability of two local strains to mobilize potassium from biotite, muscovite-2, glauconite and hydromuscovite. They found that the mobility of potassium in soil minerals and its availability for bacteria depend on factors such as the chemical structure of the mineral, the degree of potassium dispersion, activity of the bacterial strain and the ecological conditions. Hu *et al*. (2006) used a modified Aleksandrov media to isolate two phosphate- and potassium-solubilizing bacteria from the Tianmu Mountain, Zhejang, China. Preliminary results obtained with Aleksandrov media, justified repeating the experiment. Alkali removal was determined by measuring the residual contained in the ore after treatment and comparing it to the untreated sample (control). The alkali, aluminum and silicon concentrations will be the main focus of the current research as Mojallali and Weed (1978) stated that a loss of aluminum and/or silicon shows a structural rearrangement of the mineral structure. Furthermore,

Wilson and Jones (1983) found that a decrease in magnesium and iron also shows a rearrangement of the mineral structure.

The mineral composition of the untreated iron ore sample is depicted in Table 19. X-ray fluorescence is known to be a reasonably sensitive detection method, with detection limits[33] for most elements in the low ppm range (Table 14) (Jenkins 1988). Therefore magnesium and sodium will be excluded from further analysis. Focus is placed mainly on the residual alkali, as these minerals have a deleterious effect in steel manufacturing (Delvasto *et al*., 2008). Results of treated samples are expected to exceed the control sample due to sample variation; therefore parameters were calculated to be able to determine the amount of alkali/mineral removed due to microbial leaching and not sample variation. From XRF analysis the following ranges for the minerals were calculated: aluminum (1.15% - 1.56%), silicon (1.92% - 2.80%), potassium (0.16% - 0.18%) and phosphorous (0.13% - 0.17%), as discussed by Mojallali and Weed (1978). The amount of magnesium present in the sample was excluded from analysis (Table 14), therefore it could not be used as an indication of structural rearrangement of the mineral (Wilson and Jones 1983). Silicon was detected as quartz, muscovite, biotite, etc. in all of the samples analyzed (Table 1). Quartz is known dissolved slowly, due to the high activation energy needed to break the silicon-oxygen bond (Brehm *et al*., 2005 as cited in Wilson 2004). We did not determine the source of silicon dissolution, as it was outside the scope of this study, however it is assumed that the silicon released was from less resistant mineral. Concentrations of each mineral removed below these parameters will be reported as "large quantities[34]" removed. We accept the average value of the untreated export sample as an acceptable representative. Mineral phases detected in the sample include apatite, hematite, muscovite and K-feldspar (Table 1). We accept the average value of the untreated export sample as an acceptable representative.

Potassium was detected as K-feldspar and muscovite (Table 1). Muscovite has considerable resistance toward leaching of interlayer potassium, compared to trioctahedral micas (such as biotite). It was proposed that an inclined orientation of hydroxyl ions in the dioctahedral micas results in stronger binding of potassium (Kalinowski and Schweda 1996). Leaching was not

[33] Lower limit of detection is defined as that concentration equivalent to a certain number of standard deviations of the background count rate.
[34] A maximum and minum concentration for each mineral present in the untreated sample was determined. Once the ore was treated with the specific assay, we analyzed it again with XRF. If the residual amount of a specific mineral was below the minimum concentration, we ascribe it to the assay tested.

extended beyond 30 days, as the implementation of this approach into the mining schedule of the Sishen mine would not allow such a time span. Scott and Smith (1966) conducted experiments in which they determined the susceptibility of interlayer potassium in micas to exchange with sodium. They found that essentially, all the potassium in muscovite, biotite, phlogopite and vermiculite were exchangeable when concentration of the potassium in solution was kept low.

Phosphorous was detected as apatite. It is relatively insoluble at neutral pH, although its solubility and reactivity varies as a function of its composition (Anderson *et al*., 1985; Jahnke 1984; LeGeros and Tung 1983; Valsami-Jones *et al*., 1998). Therefore the low pH present in some of the solution (Table 34) could possibly have aided the release of phosphorous from the ore (Welch *et al*., 2002). Goldstein and Krishnaraj (2007) commented that the most common mechanism used by microorganisms to solubilize phosphate from apatite, seems to be by acidifying the media when organic acids are produced and released. Moreover, biofilms bound to the surface (Figure 24), would also actively take up phosphate, because it is a key nutrient for cell processes (Delvasto *et al*., 2008; Tempest and Neijssel 1992). This is accomplished by metals complexing with active moieties (such as carboxyl groups) present in the exopolysaccharides or other cellular materials (Comte *et al*., 2006; Corzo *et al*., 1994). Studies conducted with microbial extracellular polymers and simple analogs of these compounds showed that acid polysaccharides could increase dissolution of alumino-silicate minerals under mildly acidic conditions, by complexing with the minerals solubilized into solution (Welch and Vandevivere 1994; Welch *et al*., 1999) or inhibit dissolution at near neutral pH when bound on the minerals surface (Poumier *et al*., 1999).

The indigenous bacteria amplified using the Aleksandrov media (Hu *et al*., 2006) were able to solubilize "large quantities" of aluminum, silicon, potassium and phosphorous. The range of potassium solubilized was 0.13 to 0.15 and phosphorous was 0.11 to 0.15 (Table 35). The amount of silicon and aluminum solubilized indicates a rearrangement of the minerals inside the ore particle (Mojallali and Weed 1978). Here we observed a relationship between the amount of aluminum and potassium leached. Greater amounts of potassium were released when large an amount of aluminum was solubilized. We assume that this is due to the presence of aluminum and potassium in the mineral phases muscovite and K-feldspar (Table 1). Cultures amplified using Nutrient broth (Biolab) was unable to solubilize potassium but able to mobilize

"large quantities" of phosphorous, aluminum and silicon (Table 35). Štyriaková media selected for cultures, that were able to solubilize "large quantities" of aluminum, silicon, potassium and phosphorous. Štyriaková media had the lowest measured pH for the export iron ore inoculated experiments, which could have aided in phosphorous solubilization (Welch *et al*., 2002). The high pH measured for Nutrient broth is assumed to be due to the microbial action of the cultures present in solution. The increase in pH could have affected the amount of alkali solubilized from the export iron ore.

Table 35 Indigenous bacterial leaching of export iron ore.

Element	Untreated Export Ore	Aleksandrov	% removed	Aleksandrov	% removed	Aleksandrov	% removed	Nutrient broth	% removed	Štyriaková media	% removed
SiO_2	2.36	1.60	32	1.77	25	1.50	36	1.92	19	1.72	27
TiO_2	0.08	0.06	25	0.07	13	0.06	25	0.08	0	0.06	25
Al_2O_3	1.32	0.65	51	0.91	31	0.81	39	1.09	18	0.82	38
Fe_2O_3	95.68	97.32	n/a	96.81	n/a	96.55	n/a	96.02	n/a	96.58	n/a
MnO	0.03	0.04	n/a	0.04	n/a	0.04	n/a	0.04	n/a	0.04	n/a
MgO	0.03	0.00	100	0.00	100	0.00	100	0.00	100	0.00	100
CaO	0.04	0.00	100	0.00	100	0.00	100	0.00	100	0.00	100
Na_2O	0.11	0.01	91	0.01	91	0.01	91	0.01	91	0.01	91
K_2O	0.17	0.13	24	0.15	12	0.15	12	0.19	n/a	0.16	6
P_2O_5	0.15	0.11	27	0.14	7	0.15	0	0.14	7	0.13	13
Cr_2O_3	0.02	0.02	0	0.02	0	0.02	0	0.02	0	0.02	0
NiO	0.01	0.00	100	0.00	100	0.00	100	0.00	100	0.00	100
V_2O_5	0.01	0.02	n/a	0.02	n/a	0.02	n/a	0.02	n/a	0.01	0
ZrO_2	0.00	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0

KGT (conglomerate) samples were categorized as a high potassium/low phosphorous iron ore samples according to B. Ntsoelengoe (Personal communication, 2006). Indigenous cultures from the sample were enriched for by inoculating an unsterilized sample into Aleksandrov - (Hu *et al*., 2006), Mikhailouskaya media and Nutrient broth (Biolab) (Table 36). Untreated KGT samples (control) were analyzed in triplicate with XRF, to give a more accurate estimate of batch sample (Table 19). Focus is placed mainly on the residual alkali, as these minerals have a deleterious effect in steel manufacturing (Delvasto *et al*., 2008). Results of treated samples are expected to exceed the control sample due to sample variation; therefore parameters were calculated to be able to determine the amount of alkali/mineral removed due to microbial leaching and not sample variation. Moreover, the effect on silicon and aluminum will also be investigated. Scott and Amonette (1988) found that the micas biotite and muscovite undergo structural changes and possible dissolution under low pH conditions or in the presence of an organic chelating agent. Moreover, they also found that other cations are ejected along with potassium from the interlayer of the mica.

From XRD analysis (Table 1), the minerals in which potassium occurs also includes aluminum, therefore it is hypothesized that potassium or aluminum/silicon might be solubilized indirectly due to the action of the acid on the other mineral, as discussed by Wilson (2004). Mojallali and Weed (1978) stated that a loss of aluminum and/or silicon shows a structural rearrangement of the mineral. Wilson and Jones (1983) found that a decrease in magnesium and iron also shows a rearrangement of the mineral structure.

Therefore the following minerals and parameters for the export iron ore samples will be used during this study: Al_2O_3 (1.90 - 2.14); SiO_2 (3.90 – 4.90), K_2O (0.48 – 0.56) and P_2O_5 (0.06 – 0.07). Silicon was present in quartz, muscovite, biotite, etc. in all of the samples analyzed (Table 1). Muscovite was detected as the main potassium bearing mineral, while no phosphorous bearing mineral was identified. Concentrations of each mineral removed below these parameters will be reported as “large quantities” removed.

Untreated KGT samples (control) were subjected to X-ray powder diffraction (XRD) and X-Ray fluorescence (XRF) to determine the quantitative and qualitative mineralogy of the iron ore samples initially. Acid treated KGT samples were subjected to XRF analysis to determine the residual mineral concentration after leaching (Table 16). The “mineral phases” detected in the

untreated sample included hematite, quartz and muscovite (Figure 14), however additional phases, namely greenalite, illite, etc (Table 1) were reported by Kumba Iron Ore, Ltd. Jenkins (1988) reported the lower detection limits of a wavelength dispersive spectrometer (Table 14), thus sodium and magnesium will be excluded from further analysis. We accept the average value of the untreated KGT sample as an acceptable representative (Table 16).

Potassium was detected as illite and muscovite in the KGT sample (Table 1). Illite is a non-expanding, clay-sized phyllosilicate or layer alumino-silicate that commonly occurs in sediments, soils and argillaceous sedimentary rocks. It is structurally similar to muscovite or sericite [$KAl_2(OH)_2(AlSi_3O_{10})$], with more silicon, magnesium, iron and water, with less tetrahedral aluminum and interlayer potassium. Illite occurs as an alternation product of muscovite and feldspar during weathering (Mengel and Uhlenbecker 1993).

XRF data obtained from the Aleksandrov media (Hu *et al*., 2006) were inconclusive, as some of data suggests a removal of potassium and others not (Table 36). Rausell and coworkers (1965) determined the sensitivity of muscovite to potassium concentration in solution. They discovered that muscovite will not release potassium when it is placed in a dilute electrolyte solution. Furthermore, Pal and coworkers (2001) demonstrated that if biotite and muscovite were both present in soil, no potassium dissolution from muscovite would occur. It has been determined that a 0.1mg/l potassium concentration in solution would inhibit the exchange of the alkali with other ions in solution (vermiculation) (Wilson 2004). Therefore it is assumed that the presence of illite and muscovite as potassium bearing minerals, inhibited/retarded the solubilization of the alkali. Data obtained can also be ascribed to sample variation and/or mineral dissemination as alkali was solubilized by the other experiments. With the former, there are varying amounts of minerals in the sample which differs from the batch and therefore solubilization of some metals from the sample would not apparent. In the latter, the minerals potassium/phosphorous might be located mostly on the inside of the particle and therefore inaccessible for leaching. XRF analysis of Mikhailouskaya media and Nutrient broth (Biolab), illustrated an inability of the microbial cultures to remove potassium.

The KGT sample is a known low phosphorous ore, which is in accordance with our results. No phosphorous bearing mineral could be determined. XRF data of the Nutrient broth (Biolab)

experiment, reported that the cultures were able to remove a “large quantity” of phosphorous. Here the pH was more alkaline compared to the other media (Table 34). Microorganisms possess mechanisms such as ammonification, denitrification, sulfate-reduction and methanogenesis which can generate alkalinity (Johnson 2006), and therefore it is assumed that the cultures enrich possess such a mechanism(s).

Table 36 Indigenous bacterial leaching of KGT sample.

Element	Untreated KGT Iron Ore	Aleksandrov	% removed	Aleksandrov	% removed	Aleksandrov	% removed	Nutrient broth	% removed	Mik* media	% removed
SiO_2	4.41	4.09	7	4.61	n/a	3.93	11	3.97	10	4.80	n/a
TiO_2	0.10	0.10	0	0.11	n/a	0.11	n/a	0.10	0	0.12	n/a
Al_2O_3	2.02	1.73	15	2.17	n/a	1.90	6	1.75	14	2.15	n/a
Fe_2O_3	92.68	92.81	n/a	91.96	0.77	92.91	n/a	92.51	0.18	91.22	2
MnO	0.03	0.03	0	0.03	0	0.09	n/a	0.04	n/a	0.05	n/a
MgO	0.00	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0
CaO	0.00	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0
Na_2O	0.01	0.01	0	0.01	0	0.01	0	0.01	0	0.01	0
K_2O	0.52	0.48	8	0.60	n/a	0.52	0	0.51	2	0.61	n/a
P_2O_5	0.07	0.07	0	0.08	n/a	0.08	n/a	0.06	14	0.07	0
Cr_2O_3	0.02	0.02	0	0.02	0	0.02	0	0.02	0	0.16	n/a
NiO	0.00	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0
V_2O_5	0.02	0.02	0	0.02	0	0.02	0	0.02	0	0.02	0
ZrO_2	0.00	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0

SK samples (shale) are high potassium/low phosphorous iron ore. Indigenous bacteria with were amplified by inoculating Aleksandrov -, Mikhailouskaya-, Štyriaková media and Nutrient broth (Biolab) with unsterilized SK sample.

Mineral phases detected with XRD included muscovite, hematite and quartz (Figure 15). Additional phases were reported by Kumba staff (Table 1). Potassium was present as biotite, muscovite and illite. Biotite is a ferromagnesium mineral with compositional affinity to olivine [$(Mg,Fe)_2SiO_4$], pyroxene [$XY(Si,Al)_2O_6$][35] and amphiboles[36] (Deer *et al*., 2001). As with other mica minerals, it has a highly perfect basal cleavage and consists of flexible sheets or lamellae[37], which can easily flake off. Calvaruso *et al*. (2006) stated that biotite can undergo two types of weathering namely: congruent (destruction of the mineral surface) and incongruent (transformation into vermiculite by release of interlayer potassium). The sheets in the crystal structure of biotite are made up of iron, magnesium, aluminum, silicon, oxygen and hydrogen ions that are weakly bound together by potassium. The potassium can be substituted in part by sodium, calcium, barium. Magnesium can be completely replaced by ferrous iron and ferric iron and in part by titanium and manganese. Biotite is often referred to as the iron mica because it contains more iron than phlogopite (Hashemi-Nezhad 2005). No phosphorous phases were detected.

The following parameters for the alkalis and mineral described by Mojallali and Weed (1978) and Wilson and Jones (1983) were calculated from XRF data of untreated SK sample: Al_2O_3 (9.69 – 10.17); SiO_2 (11.56 – 12.26); K_2O (2.31 – 2.43) and P_2O_5 (0.03 – 0.04). These results are in accordance with classification by Kumba Iron Ore, Ltd. staff. Concentrations of each mineral removed below these parameters will be reported as "large quantities" removed. Alkali removal from the ore was determined by measuring the residual contained in the ore after treatment and comparing it to the untreated sample (control). Jenkins (1988) reported the lower detection limits of a wavelength dispersive spectrometer (Table 14), therefore sodium will be

[35] X represents calcium, sodium, ferrous iron, magnesium, zinc, manganese and lithium. Y represents ions such as chromium, aluminium, ferric iron, magnesium, manganese, scandium, titanium, vanadium. (http://en.wikipedia.org/wiki/Pyroxene).
[36] The main differences between amphiboles and pyroxenes are that amphiboles contain essential hydroxyl (OH) or halogene (F, Cl) and secondly the basic structure is a double chain of tetrahedra (as opposed to the single chain structure of pyroxene). (http://en.wikipedia.org/wiki/Amphiboles).
[37] A lamella is a gill-shaped structure: fine sheets of material held adjacent one another, with fluid in-between-(or simply 'welded'-plates)[http://en.wikipedia.org/wiki/Lamellae_(materials)].

excluded from further analysis. We accept the average value of the untreated SK sample as an acceptable representative (Table 37).

None of the potassium and phosphorous could be solubilized from the SK sample by the strains amplified using the various media. This is ascribed to the intrinsic characteristic of the mineral (Ballester *et al*., 1989; Das *et al*., 1999 as cited in Brandl 2001). The SK sample contains illite, biotite and muscovite as potassium bearing minerals. Rausell and coworkers (1965) demonstrated that the dissolution of muscovite is controlled by the concentration of potassium in solution. Wilson (2004) determined that a 0.1mg/l potassium concentration in solution would inhibit the exchange of the alkali with other cations in solution. The diffusion of biotite is thought to be diffusion-controlled and therefore also depends on the potassium concentration in the bulk solution (Wilson 2004). Pal *et al*. (2001) found that if biotite and muscovite were both present in soil, no potassium dissolution from muscovite would occur. We assume that a saturation state for potassium was reached in the solutions, and therefore none could be mobilized.

Table 37 Indigenous bacterial leaching of SK sample.

Element	Untreated SK Iron Ore	Aleksandrov	% removed	Nutrient broth	% removed	Mik media	% removed	Štyriaková media	% removed
SiO_2	11.91	12.32	n/a	11.51	3	11.73	2	12.26	n/a
TiO_2	0.61	0.65	n/a	0.59	3	0.60	2	0.62	n/a
Al_2O_3	9.93	10.11	n/a	9.51	4	9.63	3	10.20	n/a
Fe_2O_3	74.12	74.28	n/a	74.70	n/a	74.57	n/a	72.50	2
MnO	0.03	0.02	33	0.02	33	0.02	33	0.02	33
MgO	0.00	0.00	0	0.00	0	0.00	0	0.00	0
CaO	0.00	0.00	0	0.00	0	0.00	0	0.00	0
Na_2O	0.13	0.20	n/a	0.17	n/a	0.15	n/a	0.21	n/a
K_2O	2.37	2.43	n/a	2.33	2	2.33	2	2.49	n/a
P_2O_5	0.04	0.05	n/a	0.05	n/a	0.06	n/a	0.05	n/a
Cr_2O_3	0.03	0.03	0	0.03	0	0.03	0	0.03	0
NiO	0.00	0.00	0	0.00	0	0.00	0	0.00	0
V_2O_5	0.03	0.03	0	0.03	0	0.03	0	0.03	0

6.3.3 Indigenous bacterial cultures

The different iron ore samples (Export, KGT and SK) supplied by Kumba Iron Ore, Ltd. was inoculated into different media, to determine the selection of indigenous bacteria cultures. Total genomic DNA was extracted from these media, using the cetyltrimethylammonium bromide (CTAB method), as described by Ausubel *et al*. (2002), followed by PCR amplification using the oligonucleotides PRUN518r (5'-ATT-ACC-GCG-GCT-GCT-GG-3') (Siciliano *et al.*, 2003) and pA8f-GC (5'-CGC-CCG-CCG-CGC-GCG-GCG-GGC-GGG-GCG-GGG-GCACGG-GGG-GAG-AGT-TTG-ATC-CTG-GCT-CAG-3') (Fjellbirkeland *et al.*, 2001). The amplicons were then subjected to DGGE as described by Muyzer *et al*. (1993) (Figure 23). Selected bands were sequenced using the oligonucleotides PRUN518r (Siciliano *et al.*, 2003), and the nucleotide sequences were analyzed with BioEdit v.5.0.9.1 (Hall 2001). The identity of the sequences was verified by sequence match searches against the RDP database (available at http://rdp.cme.msu.edu).

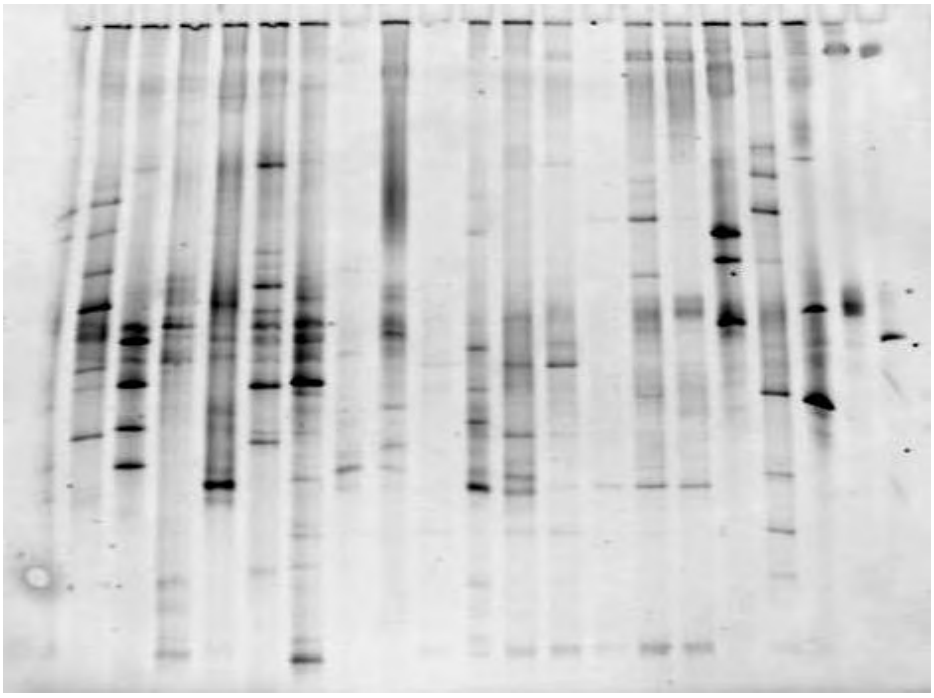

Figure 23. Denaturing gradient gel electrophoresis of amplified genomic DNA from indigenous cultures.

Cultures identified from each media are listed in Table 38, with a literature survey of each organism identified in Table 39, which includes bioleaching/biooxidation/bioremediation studies, environments isolated from and possible leaching mechanisms which might be responsible for the potassium and phosphorous leached from the iron ore. Organisms enriched during this study have been isolated from various environments such as soil, ditch water, oil polluted samples, constructed wetlands for treating AMD, humans, sewage, etc. The occurrence of organisms previously isolated from oil polluted samples, is assumed to be due to pollution by mining machinery, however these organisms do form a part of the natural environment. Furthermore, organisms from human origin are ascribed to the handling of the ore bodies. Some of the organisms identified have been isolated from mineral such as muscovite and K-feldspar, for example Cyanobacteria and *Pseudomonas putida* (Gleeson *et al*., 2006) which is assumed to form part of the natural flora on the iron ore. The literature survey on previous bioleaching/biooxidation/bioremediation reports showed that the identified species have been exploited commercially to treat various types of ores. Furthermore, from the survey we found that some of the species could produce an array of compounds that possibly aided alkali solubilization.

The survey of the organisms identified, also includes bioleaching/biooxidation/bioremediation studies. Most of the organisms have been used in multiple experiments and have proved to have unique capabilities which industry exploited, for example United States patent 5244493 describes the use of *Achromobacter* specie to treat precious metal ores that contain carbon.

Table 38 Indigenous bacteria identified from study.

Sample	Media	Culture
Export Iron Ore	Aleksandrov media (Hu *et al*,. 2006)	*Acetobacter* specie *Achromobacter* specie *Achromobacter xylosoxidans* *Arthrobacter agilis* *Citrobacter* specie *Paenibacillus stellifer*
	Nutrient broth (Biolab)	*Acinetobacter junii* *Comamonas* specie *Pantoea agglomerans*
	Štyriaková media (Štyriaková *et al.*, 2004)	*Bacillus* specie *Burkholderia* specie Cyanobacterium *Pseudomonas putida*
KGT sample	Aleksandrov media (Hu *et al*., 2006)	*Acetobacter* species *Burkholderia* species *Paenibacillus stellifer*
	Nutrient broth (Biolab)	*Acinetobacter jejunni* *Micrococcus luteus* *Sphingomonas* specie
	Mikhailouskaya media (Mikhailouskaya *et al.* 2005)	*Alcaligenes faecalis* *Citrobacter* specie *Sphingomonas* specie
SK sample	Aleksandrov media (Hu *et al*., 2006)	*Arthrobacter* specie *Citrobacter* specie *Paenibacillus* specie
	Nutrient broth (Biolab)	*Acinetobacter junii* *Burkholderia* specie Cyanobacterium *Stenotrophomonas* specie
	Mikhailouskaya media (Mikhailouskaya *et al.* 2005)	*Bacillus* specie *Brevundi diminuta* *Citrobacter* specie *Paenibacillus* specie
	Štyriaková media (Štyriaková *et al.*, 2004)	*Bacillus* specie *Clostridium* species *Stenotrophomonas*

Table 39 Survey of each identified bacterial culture.

Organism	Isolated from	Biooxidation/Bioleaching/Bioremediation Studies	Possible leaching mechanisms
***Acetobacter* specie**	Sugar cane, ditch water, sewage, soil and fruits (Cleenwerck *et al*., 2002).	Used in a bioleaching experiment with rich-in-carbonates copper ore conducted at alkaline pH (Groudeva *et al*., 2007).	Able to produce acetic acid (De Faveri *et al*., 2003) and gluconate (Krebs *et al*., 1997).
Achromobacter xylosoxidans	Soil (McGuirl 1998; Rodriguez and Fraga 1999)	Tesar *et al*. (2002) found that this organism is able to degrade hydrocarbons. Babenko (1987) established that this organism is able to solubilize manganese from ores, by producing organic acids. Patent registered in which this organism is used in a process to treat precious metal ores which contains refractory carbon (United States Patent 5244493). Patent registered in which this organism is used, in a process, to immobilize arsenic waste (United States Patent 6656722). Rodriguez and Fraga (1999) and Ma *et al*. (2008) found that *Achromobacter* species are plant growth promoting bacteria which are resistant to copper.	Able to produce acetic acid (Ma *et al*., 2008).

Table 39 (Continued)

Organism	Isolated from	Biooxidation/Bioleaching/Bioremediation Studies	Possible leaching mechanisms
Acinetobacter junii	Sludge treating industries (Sharifi-Yazdi*et al*., 2001) and oil polluted samples (Nkwelang *et al*., 2008)	Able to degrade organometallic compounds from metallic waste (Farbiszewska-Kiczma and Farbiszewska 2005). Lante *et al*. (2000) established that a *Acintobacter* species was able to degrade phenol. *Acinetobacter calcoaceticus* is able to take up excess phosphates from solution (Kargia *et al*., 2005). Boswell *et al*. (1999) isolated a *Acinetobacter* strain from a wastewater-treatment plant operating a biological phosphate removal process. They found that polyphosphates were degraded under anaerobic conditions in the presence of cadmium and uranium-oxide. The latter stimulates the reaction which produces free phosphates, while cadmium is associated with phosphate containing intracellular granules.	• Known ammonifying bacterium (Groudeva *et al*., 2007). • Organism able to produce acinetobactin (catechol siderophores) which is related to the iron chelator anguibactin (Dorsey *et al*., 2004).
Alcaligenes faecalis	Near neutral pH soil and sediment environments (Anderson *et al*., 1993; Smejkal *et al*., 2001); constructed wetland system for treating acid coal drainage (Nicomrata *et al*., 2008) and from activated sludge (Sharifi-Yazdi*et al*., 2001).	*Alcaligene* is able to oxidize arsenite (Rinderle 1984). Patent register in which *Alcaligene* is used to immobilize arsenic waste (United States Patent 6656722). Tesar *et al*. (2002) listed *Alcaligenes* as an organism able to degrade hydrocarbons.	Sayyed and Chincholkar (2005) established that this organism is able to produce two siderophores namely hydroxamate and catecholate.
Arthrobacter agilis	Untreated and oily sludge treated soil (Koch *et al*., 1995; Nkwelang *et al*., 2008)	Patent registered for the treatment of precious metal ores with refractory carbon content (United States Patent 5244493). Organism found to be able to leach lithium, aluminum and silicon from the mineral spodumene (Karavaiko *et al*., 1980). Tesar *et al*. (2002) listed *Arthrobacter* as an organism able to degrade hydrocarbons . Cardone *et al*. (1999) found an *Arthrobacter* specie able to solubilize manganese from pyrolusite. Organism able to grow in the presence heavy metals such as Cd, Cr, Zn, Cu, Pb, Fe, Ni and Co in water and also able to actively remove them (El-Bestawy *et al*., 1998).	• Organism able to produce a range of different acids which includes: formic acid, acetic acid, oxalic acid, malonic acid, citric acid, phthalic acid (Liermann *et al*., 2000). • Organism is known to produce exopolysaccharides (Liermann *et al*., 2000).

Table 39 (Continued)

Organism	Isolated from	Biooxidation/Bioleaching/Bioremediation Studies	Possible leaching mechanisms
***Bacillus* specie**	Has been isolated from numerous environments (Sharifi-Yazdi*et al*., 2001).	*Bacillus* species are able to solubilize silicate (Ehrlich 1991; Malinovskaya *et al*., 1990). Able to degrade hydrocarbons (Tesar *et al*., 2002; Widada *et al*., 2002). Štyriaková *et al*. (2003) successfully applied a Bacillus strain to remove iron bearing minerals from quartz sand. Toro *et al*. (1997) found *B. subtilis* to have a beneficial effect on the growth of onion (*Allium cepa L*.) mycorrhized with *Glomus intraradices via* the release of phosphate from rock phosphate.	• Several *Bacillus* species produce exopolysaccharides (Mohanty *et al*., 1990; Malinovskaya *et al*., 1990) • Organisms are able to produce acids such as pyruvic acid (Urzi *et al*., 1991).
Brevundimonas diminuta	Crude oil samples (Yoshida *et al*., 2005).	Trifluralin degrading bacterium (Bellinaso *et al*., 2006).	This organism is able to produce phosphotriesterases (Horne *et al*., 2002).
***Burkholderia* specie**	Soil (LiPuma *et al*., 2002), crude-oil samples from Japanese oil stockpiles (Yoshida *et al*., 2005); human patients suffering from cystic fibrosis (LiPuma *et al*.,2002) and iron ore (Valverde *et al*., 2006)	Widada *et al*. (2002) established that this organism is able to degrade polyaromatic hydrocarbons. Calvaruso *et al*. (2006) found *Burkholderia glathei* PML1 able to significantly increased biotite weathering by a factor of 1.4 for magnesium and 1.5 for potassium compared to pine plant treatment alone. O'Sullivan and Mahenthiralingam (2005) found *Burkholderia* to be the predominant genus isolated from polyaromatic hydrocarbon polluted soil. Valverde *et al*. (2006) isolated a strain from high phosphorous iron ore that was able to solubilize highly insoluble phosphate minerals. Calvaruso *et al*. (2007) found a *Burkholderia* strain to be capable of weathering biotite. Lower *et al*. (2001) found that when *Burkholderia* showed strong affinity for muscovite under limiting conditions.	• Able to solubilize mineral phosphate by producing organic acids such as acetic (Kim *et al*., 2005), citric (Delvasto *et al*., 2008b) and gluconic acid (Lin *et al*., 2006) • Known to produce biofilms (Delvasto *et al*., 2008)

Table 39 (Continued)

Organism	Isolated from	Biooxidation/Bioleaching/Bioremediation Studies	Possible leaching mechanisms
***Citrobacter* specie**	Soil, water, and sewages (Patel *et al*., 2007).	*Citrobacter* is able to accumulate uranium (basic mineral for nuclear technology) by forming phosphate-uranium complexes (Puchenkova 1996). Basnakova and Macaskie (1999) found that a *Citrobacter* specie was able to mineralize zirconium to a mixture of $Zr(HPO_4)_2$ and hydrated zirconia (ZrO_2). The biomineralization of uranium as HUO_2PO_4 is repressed by zirconium in the presence of excess phosphates. Roig *et al*. (1997) established that *Citrobacter* can be used to recover uranium from the acid drainage waters. Patel *et al*. (2007) identified *Citrobacter* as a phosphate solubilizing bacterium. The *Citrobacter* acid phosphatase has been characterized and found to exist in two isoforms (CPI and CPII) with differing stabilities, which would relate to their suitability for metal phosphate precipitation (Jeong and Macaskie 1999).	• Produces phosphatases which makes it resistant to some diagnostic reagents (Hillel 1998) and also helps to accumulate heavy metals (Roig *et al*., 1997). • Excretes acetic acid when grown on sucrose and fructose (Patel *et al*., 2007). • Excretes gluconic acid when grown on glucose (Patel *et al*., 2007).
Clostridium bifermentas, C.celerecrenscens, C.subterminale	Soil, decomposing biological material, lower gut of mammals (Suresh *et al*., 2007) and waste water sludge(Wang *et al*., 2003)	Francis and Dodge (1994) isolated a *Clostridium* specie from co-precipitated metals. The organism was able to recover metals from solution. The metabolic capability of *Clostridium bifermentas* has been exploited in wastewater sludge. Here the organism can convert the wastes to hydrogen gas (Wang *et al*., 2003). *Clostridium bifermentas* has been used in bioremediation approaches to treat nitroaromotic contaminants (Sembries and Crawford 1997).	• Able to reduce ferric oxide enzymatically (Munch *et al*., 1980). • Known to produce acetic and lactic acid (Tabak *et al*., 2005).

Table 39 (Continued)

Organism	Isolated from	Biooxidation/Bioleaching/Bioremediation Studies	Possible leaching mechanisms
***Comamonas* specie**	Soil (Lévy-Schil *et al*., 1995)	Able to degrade polyaromatic hydrocarbons (Widada *et al*., 2002). Boon *et al*. (2000) conducted bioaugmentation experiments on activated sludge. They found that an indigenous bacterium, *Comamonas testosteroni*, was able to degrade 3-chloroaniline that was present in the sludge. Produces aliphatic nitrilase which is active on adiponitrile and cyanovaleric acid (Lévy-Schil *et al*., 1995).	Able to produce exopolysaccharides (Bossier *et al*., 1997).
Cyanobacterium	Weathered muscovite (Gleeson *et al*., 2006), water (Bender *et al*., 1994)	Ferris *et al*. (1994) found that cyanobacteria serve as nucleation sites for carbonate minerals during the weathering of basalt. A process has been designed which uses floating cyanobacterial mats to remove metals from waters. The removal of the metals is due to binding to the polysaccharides produced by the organism (Bender *et al*., 1994).	• Organism is able to produce formate, acetate, oxalate, and lactate (Heyer and Krumbein 1991; Moezelaar *et al*., 1996; Stal and Moezelaar 1997). • Able to produce siderophores of hydroxamate and catechol (Straus 1994).

Table 39 (Continued)

Organism	Isolated from	Biooxidation/Bioleaching/Bioremediation Studies	Possible leaching mechanisms
Micrococcus luteus	Has been isolated from soil (Costa *et al*., 2006) and activated sludge (Sharifi-Yazdi*et al*., 2001)	Tesar *et al*. (2002) included *Micrococcus luteus* in a list of organisms able to degrade hydrocarbons. Padoley *et al*. (2006) found *Micrococcus luteus* able to degrade pyridine. Rahman *et al*. (2002) included Micrococcus along with *Bacillus* and *Pseudomonas* in a list of organisms able to increase degradation of petrol, diesel and crude oil. Able to solubilize poorly soluble calcium phosphates such as hydroxyapatite (Calvaruso *et al*., 2007).	Organism is able to produce gluconic, lactic, pyruvic and succinic acid (Urzi *et al*., 1991).
Paenibacillus stellifer	Soil (Costa *et al*., 2006)	Research conducted by Deo and Natarajan (1997) showed that *Paenibacillus stellifer* is able to leach iron, aluminum and calcium from minerals such as calcite, hematite and corundum. Organism is able to remove calcium and iron impurities from low grade bauxite (Vasan *et al*., 2001). Two phosphate- and potassium-solubilizing strains (KNP413 and KNP414) were isolated which showed close relationship to Paenibacillus. These strains were able to effectively remove alkali from phosphorite, montmorillonite, kaolinite and potassium feldspar (Hu *et al*., 2006). Daane *et al*. (2002) found a number of *Paenibacillus* species to have agricultural importance due to their ability to degrade several polyaromatic hydrocarbons.	Organism is able to produce acetic and oxalic acid (Deo and Natarajan, 1997).
Pantoea agglomerans	Soil (Amellal *et al*., 1998)	Organism able to reduce chromium anaerobically (Francis *et al*., 2000). Son *et al*. (2006) established that this organism is able to solubilize phosphates from hydroxyapatite. It has been shown for the first time that a mesophilic facultatively anaerobic Fe(III)-reducing bacterium, closely related to *Pantoea* (*Enterobacter*) *agglomerans*, can couple the oxidation of acetate or H_2 to dissimilatory reduction of Fe(III), Mr(IV) and Cr(VI) (Francis *et al*., 2000)	• Able to produce exopolysaccharides (Amellal *et al*., 1998). • Possibly produces organic acids (Son *et al*., 2006).

Table 39 (Continued)

Organism	Isolated from	Biooxidation/Bioleaching/Bioremediation Studies	Possible leaching mechanisms
Pseudomonas putida	Weathered K-feldspar (Gleeson *et al.*, 2006) and activated sludge (Sharifi-Yazdiet *et al.*, 2001).	Patent has been registered in which this organism is used to treat precious metals which contains refractory carbon (United States Patent 5244493). Research conducted by Muller and coworkers (1995) showed that this organism is able to leach zinc from filter dust. Organism able to degrade phenol (Boaventura 2001). Citrate-utilizing strains of *Pseudomonas aeruginosa* and *Pseudomonas putida* have been isolated that can remove Cd, Zn, Cu, Fe, Co and Ni from solution with incorporation of inorganic phosphate as a precipitant. Such organisms may have potential in the treatment of metal-citrate wastes: citrate is used as a complexing agent in certain decontamination processes (Thomas *et al*., 2000). Calvaruso *et al*. (2007) found that a *Pseudomonas* strain was able to weather biotite.	• Able to reduce ferric iron enzymatically (Ehrlich 1991) • Organism able to produce citric acid and gluconic acid (Krebs *et al*. 1997) • Able to produce phosphatase (Horne *et al*., 2002) • Produces siderophore which can increase dissolution of hematite (Hersman 1995)
Sphingomonas specie	Soil from reclaimed land (Fukuda *et al*., 2002).	Sphingomonas is able to utilize dibensofuran as a sole carbon source (Fukuda *et al*., 2002). Organism able to degrade the phenyl-urea herbicide, isoproturon (Shi and Bending 2007) Calvaruso *et al*. (2007) found that a *Sphingomonas* was able to weather biotite.	• Organism able to produce catechol, salicylic acid, 4H-1-benzopyran-4-one (Fukuda *et al*., 2002). • Able to produce biofilms (Venugopalan *et al*., 2005).
***Stenotrophomonas* specie**	Constructed wetland (Nicomrat *et al*., 2008), crude-oil samples (Yoshida *et al*., 2005), anaerobic reactor (Assih *et al*., 2002), medical devices (De Rossi *et al*., 2007) , soil (Emerson and Moyer 1997)	Able to degrade high molecular weight polycyclic aromatic hydrocarbons (Boonchan *et al*., 1998). Organism is able to degrade ethylene glycol, which is a toxic byproduct from polyester hydrolysis (Kim *et al*., 2001).	Able to produce biofilm (De Rossi *et al*., 2007).

For each of the enrichment medias different organims were identified. From the literature survey we identified several species, which is known to possess an alkali-solubilizing mechanism or have shown to be effective in mineral solubilization, for example: Organisms from export iron ore inoculated Aleksandrov media (Hu *et al*., 2006) included *Acetobacter*, *Achromobacter*, *Arthrobacter* and *Paenibacillus* which could produce an array of organic acids such as acetic -, oxalic -, formic -, malonic -, citric – and phthalic acid, which could leach the minerals *via* protons and/or complexation mechanism. Moreover, *Arthrobacter* is able to produce exopolysaccharides (Liermann *et al*., 2000), which could further aid solubilization of metals by binding with them and therefore decrease the saturation state (Delvasto *et al*., 2008; Tempest and Neijssel 1992). *Citrobacter*, a known phosphate solubilizing bacterium, was also identified in the Aleksandrov media which could produce gluconic acid from the carbon source supplied by the media (Patel *et al*., 2007). This organism was also isolated from Mikhailouskaya media, where it was possibly able to produce acetic acid due to the carbon source present (Patel *et al*., 2007). Furthermore, this organism could aid solubilization of phosphorous by excreting phosphatases which can accumulate heavy metals (Roig *et al*., 1997). *Paenibacillus* was also identified in the Aleksandrov media. Hu and coworkers (2006) identified two potassium/phosphorous solubilizing bacteria which showed close relationship to *Paenibacillus.* They further demonstrated that the organisms were able to remove alkali from phosphorite, montmorillonite, kaolinite and K-feldspar. Therefore it is possible that this organism played a vital role in the solubilization of alkali from the ore samples.

Acinetobacter junii were identified in the KGT and export iron ore inoculated nutrient broth. This is an ammonifying bacterium. We found that the pH of the nutrient broth increased during our study, therefore we assume that this is due to the presence of *Acinetobacter junii* (Groudeva *et al*., 2007). The cocktail of organisms present in the Nutrient broth (Biolab) could mobilize of phosphorous from export (Table 35) and KGT (Table 36) samples, possibly due to *Acinetobacter junii* ability to take up excess phosphates in solution (Kargi *et al*., 2005), thereby affecting the saturation state in solution.

Bacillus species were identified from export and SK inoculated Štyriaková media. Research has shown that these organisms were able to solubilize silicates (Ehrlich 1991; Malinovskaya *et al*., 1990). From research (Chapter 5), we found that these organisms are able

to solubilize alkalis from the export ore (Table 28), but not from the SK sample (Table 37). This is ascribed to the composition of the sample, as it contains illite, muscovite and biotite as previously discussed. *Clostridium* and *Stenotrophomonas* species were also isolated from the SK sample (Table 38). *Clostridium* is known to be a obligative anaerobe, and therefore we assume that the organism produced biofilms to protect itself. Furthermore Tobak *et al.* (2005) reported that these organisms are able to produce acetic – and lactic acid. Therefore the measured decrease in pH and alkali solubilization is ascribed to the metabolic products of the organism.

Burkholderia species were identified from Export iron ore inoculated Štyriaková media (Table 35); KGT inoculated Aleksandrov media (Table 36) and SK inoculated Nutrient Broth (Table 37). These organisms produce biofilms which could influence the saturation state in solution (Delvasto *et al*., 2008). Reports indicated that these organisms are able to solubilize phosphorous from biotite (Calvaruso *et al*., 2006) and iron ore (Valverde *et al*., 2006), possibly by producing acids such as acetic - (Kim *et al*., 2005), citric – (Delvasto *et al*., 2008b) and gluconic acid (Lin *et al*,. 2006). Therefore it is thought that this organism played an important role in alkali solubilization for the export and KGT ore samples. Its inability to remove alkali from the SK sample is ascribed to the intrinsic characteristics of the sample and to the presence of *Acinetobacter junii* which increased the solution pH, which would allow the acids to only form complexes with the metals.

Micrococcus luteus was isolated from KGT inoculated Nutrient broth (Table 38). Calvaruso *et al*. (2007) demonstrated that this organism is able to solubilize poorly soluble calcium phosphate such as hydroxylapatite. The pH of the nutrient broth was alkaline (Table 34), possibly due to the presence of *Acinetobacter junii*, however some of the alkali could be solubilized from the ore sample possibly due to the metabolic action of the organisms present (Table 36).

6.3.4 Scanning electron microscopy

Bacterial leached iron ore samples were observed with scanning electron microscopy. Data obtained suggests that the bacteria were able to form biofilms on the surface of the mineral (Figure 24; A and B), which would actively take up phosphate, due to its importance in cell processes (Tempest and Neijssel 1992; Delvasto *et al*., 2008). This is accomplished by metals complexing with active moieties (such as carboxyl groups) present in the exopolysaccharides or other cellular materials (Comte *et al*., 2006; Corzo *et al*., 1994). Biofilm formation is a multistep process which is influenced by many factors, including the specific mineralogy of the rock, solution chemistry (pH, ionic strength) and the characteristics of the microorganism (hydrophobicity, surface charge) (Banfield and Hamers 1997; Characklis 1989; Little *et al*., 1997). The attachment of cells to the surface can occur *via* random processes such as diffusion and convection or more specifically *via* chemotaxis (Jerez 2001).

Studies conducted with microbial extracellular polymers and simple analogs of these compounds showed that acid polysaccharides could increase dissolution of alumino-silicate minerals under mildly acidic conditions, by complexing with the minerals solubilized into solution (Welch and Vandevivere 1994; Welch *et al*., 1999) or inhibit dissolution at near neutral pH when bound on the minerals surface (Poumier *et al*., 1999).

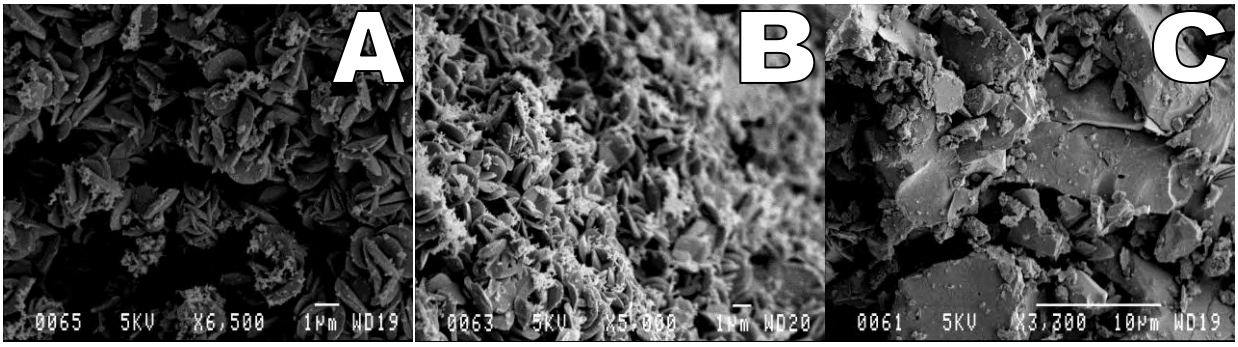

Figure 24. Scanning electron microscopy images of indigenous bacterial leached samples. A and B - biofilm formation; C – no biofilm.

Scanning electron microscopy of Nutrient broth (Biolab) samples revealed that no biofilms were formed (Figure 24 C). Chan and Ting (1995) found that a decrease in pH leads to an increase in negative charges on bacterial surfaces with subsequent attraction to the positively charged metal ions. Therefore we assume that the opposite is true for the Nutrient broth (Biolab) cultures, which would hinder attachment of cells on the mineral surface.

6.4 CONCLUSION

Microorganisms play an important role: in the cycling of elements and sorption of metals, the dissolution of minerals and mineral crystallization. They are able to achieve this *via* mechanisms such as: assimilation/adsorption and mineralization; precipitation and dissolution; oxidation and reduction and methylation and dealkylation. On the other hand, research has found that mineralogical process in turn play a significant role in the distribution, activity and diversity of microbes, the expression of their genes, development and structure of communities, and transfer of genetic material. The iron ore mined at Sishen, Northern Cape, South Africa contains several different minerals, with potassium and phosphorous as common constituents. The latter minerals have a deleterious effect on the manufacturing of steel. Experiments by Bennett *et al*. (2001) provided evidence that silicate weathering by bacteria is sometimes driven by the nutrient requirements of the microbial consortium. Therefore we hypothesized that natural occurring bacteria on the surface of the ore, would have mineral solubilization capabilities, as these two minerals are essential nutrients and might have become limiting in the environment.

From our research we found a rich consortium of indigenous microorganisms, with possible leaching mechanisms which enabled them to remove mobilize some of the alkali from certain ore bodies. Here we observed that the composition of the ore plays a important role in its leachability and therefore the eventual application of indigenous bacterial leaching in the mining schedule of Sishen. We found that the indigenous bacteria were beter adapted than the introduced species (Chapter 4 and 5) to leach alkali. Furthermore, the indigenous organisms were also able to solubilize more alkali than the abiotic tests (Chapter 3), which is ascribed to the organisms ability to effect the saturation state of the minerals in solution.

Therefore our preliminary results justifies additional research into indigenous bacterial leaching for the removal of alkali from the ore mined at Sishen.

CHAPTER 7

GENERAL CONCLUSION

- The alkalis potassium and phosphorous are common constituents of the various different iron ore mined at Sishen, according to XRD analysis.
- Effectivity of alkali removal from the different iron ore samples were possibly influenced by mineral composition and – dissemination.
- The presence of different potassium bearing minerals in each iron ore sample influenced the amount of potassium that could be solubilized.
- Low solution pH favored the release of phosphorous from the different iron ore samples.
- Organic and inorganic acids were able to remove alkali from the export ore, but not from KGT and SK sample.
- Commercially used iron oxidizing bacteria were able to mobilize alkali from the export iron ore sample. The iron oxidizing bacteria tested differed in their ability to mobilize alkali from the export iron ore sample, which is ascribed to the metabolic capability of each organism. However the effect of sample variation could not be disregarded.
- Iron oxidizing bacteria were able to mobilize more alkali than 1M sulfur acid. This is ascribed to biofilms formation which can bind metals and thereby decrease the solution saturation state.
- Heterotrophic bacteria were able to solubilize alkali, by decreasing the solution pH and possibly by forming biofilms. The microorganisms tested produce several organic acids, which were tested separately for their mobilization ability. We found that the acids (abiotic) were less effective at solubilizing alkalis than the microorganisms (biotic) tested, which is ascribed to the diverse metabolic capability of the organisms.

- Bacteria with possible potassium/phosphorous solubilizing ability were enriched for from the different iron ore samples, using different medias. These bacteria were more effective in mobilizing alkali from the ore than the introduced species. This is ascribed to possible adaptation of the organisms to scavenge for limiting nutrients.
- Acid production by the microorganisms do not account for all the alkali mobilized, therefore it is assumed that the organisms possess alternative mechanisms which aids in the solubilization process.
- Aluminum and potassium were leached simultaneously from the ore particle, which is ascribed to the presence of aluminum in the potassium bearing minerals.
- Preliminary results suggest that a biotic approach could be used to remove the alkali from the export iron ore mined at Sishen.

CHAPTER 8

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