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Biobeneficiation development for the reduction of potassium and phosphorus from Sishen iron ore

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RASHEED ADEGBOLA ADELEKE

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

Signature _____

Date _____

ABSTRACT

High levels of elements such as sodium (Na), potassium (K) and phosphorus (P) in iron ore minerals are known to reduce the quality and price of these minerals. South Africa, as one of the world largest exporter of iron ore, is affected by this problem. Both potassium (K) and phosphorus (P) are peculiar to South African iron ore. The present study has therefore focussed on developing an environmentally friendly biological method for lowering the levels of K and P in iron ore minerals. Short and long term experiments were set up to isolate, identify, screen and test potential bioleaching bacteria and fungi from different environmental samples.

The study started by investigating the possible relationship that exists between weathering and bioleaching processes. The investigation was intended to provide relevant information on the natural role of microorganisms such as ectomycorrhizal (ECM) fungi in the mining environment. The experiments involved the use of both mycorrhizal and non-mycorrhizal *Pinus patula* seedlings for the weathering of iron ore minerals. Four types of ECM fungi were used, namely *Pisolithus tinctorius* (PT), *Paxillus involutus* (PI), *Laccaria bicolor* (LB) and *Suillus tomentosus* (ST). From the results, ectomycorrhizal weathering can be said to be species-specific and significantly influenced by fungal type and particle size. In addition, it was also discovered that both mycorrhizal and non-mycorrhizal roots can participate in weathering processes.

Further investigations of ECM fungi when not in symbiosis, were carried out to know how or if they can be potential candidates to mobilise K and P from iron ore minerals. The experimental set up involved *in vitro* pure cultures of four different ECM fungi, namely *Pisolithus tinctorius* (PT), *Paxillus involutus* (PI), *Phialocephala fortini* (PFR), and *Suillus tomentosus* (ST). In addition, the treatments involved the use of five different particle sizes of each ore type. The results obtained indicated the potential of the ECM fungi to mobilise P and K from the two iron ore types though at different levels. Factors such as ore type, particle size, organic acid production and attachment of the fungi to the iron ore were all found to influence the mobilisation of nutrients from these ores.

Another experiment that addressed some of the limitations encountered with the use of pure cultures of ECM fungi was conducted. Isolated indigenous fungal pure cultures from the surfaces of iron ore minerals were screened for their abilities to solubilise minerals by lowering the levels of K and P. These isolates were identified molecularly as close relatives of three genera that included *Penicillium*, *Alternaria* (2 isolates) and *Epicoccum* for isolates FO, SFC2/KFC1 and SFC2B respectively. The identified *Penicillium* sp. turned out to be the only phosphate solubiliser among these isolates. Direct bioleaching capability of the fungus was compared to that of its metabolite. At the end, the metabolite showed better K removal than the direct use of the fungi. Interpretation of these results indicates possible relationship between K and P removal, and the organic acids production by this fungus. Other factors such as particle size and mineral type were also found to significantly influence the leaching process.

Additional experiment was conducted to investigate the indigeous bacteria and their potentials in reducing the K and P contents of iron ore minerals. A total of 23 bacterial strains that belong to *Proteobacteria*, *Firmicutes*, *Bacteroidetes* and *Actinobateria* were isolated from the iron ore minerals and identified with molecular methods. All the bacterial isolates were screened for their potential as mineral solubilisers. Only eight of the isolates were selected and used in shake flask experiments that contained both KGT and SK mineral types as their sources of K and P. The experiment showed that all the eight isolates have potentials to produce organic acids especially high levels of gluconic acid but lower quantities of acetic, citric and propanoic acid. Scanning electron microscopy (SEM) and fourier transform infrared (FITR) analyses also helped to uncover the role that biofilm and extracellular polymeric substances could play in mineral solubilisation.

Finally, an investigation of a new method for reduction of K and P levels of iron ore minerals was carried out, focussing on the use of cheap resources as well as septic conditions. The study involved the use of fermented spoilt grape fruits (*Vitis* sp.) and the solution from the product utilised in shake-flask experiments. Treatments involved two types of iron ore minerals (KGT and SK) and two different particle sizes. The result suggests the significant effect of particle size, time and organic acids on the reduction of K and P from the iron ore minerals. The important part of this finding is the discovery of a cheap microbial energy source (spoilt grape) that can be

further exploited for full biobeneficiation of iron ore minerals. Another advantage of this method is the fact that the experiment can be conducted under non–sterile conditions, making it a system that can be operated outdoor.

LIST OF PUBLICATIONS, SUBMITTED MANUSCRIPTS AND CONFERENCE CONTRIBUTIONS

- Adeleke, R.A., Cloete, E., Bertrand, A., and Khasa, D.P. (2010). Mobilisation of potassium and phosphorus from iron ore by ectomycorrhizal fungi. World Journal of Microbiology and Biotechnology. doi: 10.1007/s11274-010-0372-0 - Accepted

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- Adeleke, R.A., Cloete, E., Bertrand, A., and Khasa, D.P. (2008). "Nutrient absorption from iron ore by ectomycorrhizal seedlings.", 13th Congress of the African Association for Biological Nitrogen Fixation, Sol Azur Beach Hotel, Hammamet, Hammamet, Tunisia, December 15-18, 2008.

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List of Symbols and Abbreviations

<	Less than
>	Greater than
µ	Micron (10^{-6})
ANOVA	analysis of variance
BLAST	Basic local alignment search tool
bp	base pairs
d	Day(s)
df	degrees of freedom
DM	Dry mass
DNA	Deoxyribonucleic acid
ECM	Ectomycorrhizal fungi
EMC	Ectomycorrhizal colonised
EPS	Extracellular polymeric substances
F	*F*-statistic
Fig.	Figure
FTIR	Fourier transform infrared
g	Gram
GS	Grape solution
h	Hour
i.e.	That is
ITS	Internal transcribed spacer
KGT	Sishen iron ore type - conglomerates
L or l	Liter
LB	*Laccaria bicolor*
m	Meter
M	Molar
mg	Milligram
min	Minute(s)
mm	Millimeter
MMN	Modified Melin-Norkrans
MP	Maximum parsimony
n	(not *N*) sample size (number)
NA	Nutrient agar

NB	Nutrient broth
NCBI	National center for biotechnology information
NJ	Neighbour joining
NJ	Neighbour joining
NMC	non-mycorrhizal colonised root
P	probability value
P	Phosphorus
PCR	polymerase chain reaction
PDA	Potato dextrose agar
PFR	*Phialocephala fortini*
pH	Potential hydrogen
PI	*Paxillus involutus*
ppm	Part per million
ppm	parts per million
PSM	Phosphate solubilising medium
PT	*Pisolithus tinctorius*
RDM	root dry mass
rpm	rotation per min
SD	standard deviation
SDM	shoot dry mass
SE	standard error
SEM	Scanning electron microscope
SK	Sishen iron ore type - Shale
sp.	Species (singular)
spp.	Species (plural)
ST	*Suillus tomentosus*
T_m	Melting temperature
vs	versus
W1	week one
W2	week two
W3	week three

Dedication

Dedicated to the memory of my mother, who taught me how to read and write at home, as well as my class one.

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CHAPTER ONE

LITERATURE REVIEW

1.1 Introduction – Biohydrometallurgy

Production, trading and utilisation of minerals have become a very important part of human lives. Existence of life without these minerals is almost unimaginable due to their vital contributions to industrial developments such as construction and manufacturing industries. Few minerals such as talc, asbestos and sulfur can be used just after mining without any processing. However, most of the minerals, especially the more important ones such as iron ore and gold, need to be refined and reprocessed to produce utilisable materials (Klein, 2002).

The current pace of development has led to increases in the demand for minerals and their by-products. This has subsequently resulted in rapid depletion and exhaustion of quality mineral ores, which means most mineral ores are no longer found in their pure forms (Dale, 1984). Over the last century, scientists have queried and investigated reasons for this disparity in existence of minerals. Long-term natural events such as mineralisation, deposition, solubilisation and weathering were suggested as possible causes of changes in the chemical constitution of minerals (Rawlings, 2002; Rawlings, *et al*., 2003; Astrup and Hammerbeck, 1998). Geologists, microbiologist or biotechnologists may have different definitions for these processes; but the inter-relationship between these processes cannot be disputed. For example, mineralisation and solubilisation have been described as possible stages in weathering. Meanwhile, weathering itself can be a natural way by which mineral ores can be purified or leached through solubilisation by some microbes or could lead to formation of more complex types of minerals such as the deposition or precipitation by anaerobic sulphate-reducing bacteria (Banfield *et al*., 1999; Rawlings, 2002). In any of these scenarios, microorganisms play active roles through their interaction with the mineral environment.

Different microbial functionalities have been exploited by scientists to develop processes that can mobilise and solubilise the elemental composition of mineral ore. These have been achieved through laboratory simulation and repetition of microbial activities to yield a technology known as biohydrometallurgy (Rawlings, 2002; Jain and Sharma, 2004; Rawlings, 2005). The word "biohydrometallurgy" has been used widely and interchangeably with "bioleaching" to describe

microbial processes utilised in the mineral industry such as metal extraction from low grade ores, metal detoxification, ore beneficiation, coal beneficiation and recovery of metals from waste materials (Olson *et al*., 2003; Jain and Sharma, 2004).

1.2 Relationship between biohydrometallurgy and weathering

Weathering is described as the breakdown of materials on the earth's crust, which leads to changes in the composition of the original materials yielding more stable products (Banfield *et al*., 1999; White *et al*., 1992; Burford *et al*., 2003). Weathering could be biological, physical or chemical (Banfield *et al*., 1999; White *et al*., 1992), but weathering described in this study is biological. This is in some situations referred to as bioweathering, defined by Sollas (1880) as a process that involves the erosion, decay and decomposition of rocks and minerals caused by living organisms. There are numerous advantages associated with weathering in nature. The most discussed topic pertaining to weathering is its natural effects, which ensure nutrient cycling and availability to both plants and microbes in the soil (Banfield *et al*., 1999; White *et al*., 1992; Burford *et al*., 2003; van Scholl *et al*., 2006b). Putting the pieces of this puzzle together, weathering could therefore be described as a natural bioleaching process that involves slow and gradual degradation or purification or solubilisation of some important minerals which can occur over a long period of time. In addition, it is also a process that could remove toxic metals from the environment (Willscher and Bosecker, 2003).

Apart from the fact that both biological weathering and bioleaching are natural processes, there are many other features that are also shared between them (Rawlings, 2002; Rawlings, 2005; Willscher and Bosecker, 2003). These include production of organic acid, mobilisation, binding and solubilisation of metals. Therefore, it may be true that some natural microbial weathering agents could also be bioleaching agents. In some situations, weathering roles of some microorganisms have been translated as bioleaching (Styriakova *et al*., 2003; Jain and Sharma, 2004). However, further studies on both processes could provide more information on how bioleaching occurs in nature. In addition, potential bioleaching agents may also be identified by investigating microorganisms that are known to participate in weathering.

1.3 Biohydrometallurgy as a technology

Biohydrometallurgy is a word that evolved from hydrometallurgy, which means biological hydrometallurgy. In hydrometallurgy, the system relies on dissolution of metallic artifacts by acids or alkalis to produce solubilisation effects that leach metallic ions from minerals into solutions (Bodsworth, 1994). Meanwhile, leaching conditions are normally adjusted to ensure the desired part of the mineral remains insoluble. If soluble, additional techniques are introduced to separate the desired portion from other compounds in the solution (Bodsworth, 1994). Processes involved in biohydrometallurgy are valued for being more environmentally friendly and cheaper compared to most physical and chemical methods of mineral extraction (Rawlings, 2002). Generally in biohydrometallurgy, minerals can be categorised into two types, i.e. the sulfidic and non-sulfidic minerals (Jain and Sharma, 2004). Chemolithoauthotrophic bacteria are in most cases used for bioleaching of sulfidic minerals, while heterotrophs, which could be bacteria or fungi, are used for the bioleaching of non-sulfidic minerals (Rawlings, 2005; Jain and Sharma, 2004). Currently, there is a growing distinction in terms of mechanisms involved between bioleaching and another related process known as biobeneficiation. Biobeneficiation involves the use of microbes to dissolve only unwanted parts of a mineral ore (Vasan *et al*., 2001; Jain and Sharma, 2004). However, both processes can be referred to as biohydrometallurgy technologies (Rawlings, 2002; Jain and Sharma, 2004).

1.4 Bioleaching methods

Processes involved in bioleaching are broadly divided into two types, namely irrigation- and stirred tank–type processes (Brierley and Brierley, 2001; Rawlings, 2002). Irrigation type processes involve infiltration of ores arranged in heaps, columns or dumps with the leaching solution (Schnell, 1997; Brierley and Brierley; 2001). The irrigation method has been successfully commercialised in different countries (e.g. Chile) for different minerals such as copper and cobalt (Brierley and Brierley, 2001). For the stirred tank process, tanks containing ores and leaching solution are exposed to aeration by continuous stirring. An example is the use

of this technology at the Youanmi plant, Australia, for bioleaching of gold-containing ores (Brierley and Brierley, 2001).

1.5 Bioleaching of sulfidic minerals

It is generally believed that activities of microbes for mining began a long time ago, around 1556 at the Rio Tinto mine, Spain, where copper and silver were said to have been mined in this ancient period (Rawlings, 2002). However, it was not until around half a century ago that proper documentation of biohydrometallurgical processes began (Zimmerley *et al*., 1958). After this period, most of the studies and technological developments in biohydrometallurgy focussed on sulfidic minerals. These are minerals that could provide sources of energy for bioleaching microorganisms through the sulfur and iron cycles (Jain and Sharma, 2004). Various groups of bacteria have been confirmed as having potential in the bioleaching industry. This diverse group of bacteria can be classified into two groups depending on their oxidation mechanisms. These comprise thiosulfate and polysulfide mechanisms. The thiosulphate mechanism is a type of oxidation mechanism proposed for solubilisation of acid insoluble metal sulphides such as pyrite and molybdenite, while the polysulfide mechanism is proposed for oxidation of acid- soluble metal sulphides (Rawlings, 2005). Examples of common sulfidic microbes are *Acidithiobacillus thiooxidans*, *Leptospirillum ferroxidans*, *Acidiphilium acidophilum*, *Acidianus infernus*, *Sulfobacillus thermosulfodooxidaans*, *Ferroplasma acidarmanus*, *Sulfolobus* sp. and *Metallosphaera sedula* (Rawlings *et al*., 1999; Rawlings, 2001; Rawlings, 2005).

1.6 Bioleaching of non-sulfidic minerals

Heterotrophs consist of a wide group of organisms, including fungi and some bacteria that use naturally occurring organic substrates for generation of energy (Jain and Sharma, 2004). Heterotrophs have been highlighted as having the best potential in bioleaching of non-sulfidic minerals (Jain and Sharma, 2004; Rawlings, 2005). This group of microorganisms can be used for the recovery of valuable metals from low-grade mineral ores, beneficiation of mineral raw materials, recovery of metals from wastes and detoxification of heavy metal-contaminated soils

and solid residues (Bosecker, 1997; Jain and Sharma, 2004; Rawlings, 2005). Heterotrophic microorganisms have been successfully used in mobilisation of elements such as Si, Al, Fe, K, Li, Ni, Zn and Mg from mineral ores (Table 1.1). Functional roles of heterotrophic microbes used in bioleaching strongly depends on their ability to produce low-molecular-weight organic acids, exopolysaccharides, amino acids and proteins that can be used to solubilise mineral ores through a variety of mechanisms (Burgstaller and Schinner, 1993; Jain and Sharma, 2004).

1.6.1 Mechanisms of biobeneficiation of non-sulfidic minerals

Jain and Sharma (2004) classified mechanisms involved in the solubilisation of non-sulfidic minerals into four different groups. These include: bioreduction, acidification, ligand production or complexolysis and alkalinisation. Bioreduction has been described as the process through which microbes could reduce some minerals through their activities such as the biological reduction of iron with *Pseudomonas* (Hoffman *et al*., 1989). Acidification is directly related to the production of organic acids such as citric, oxalic, acetic, propionic and tartaric acids by heterotrophic leaching microbes. This mechanism could also be due to selective utilisation of alkaline food substrates by these microbes (Jain and Sharma, 2004).

Another important mechanism of leaching non-sulfidic minerals by heterotrophs is complexolysis. This is the reaction that involves the organic ligands produced by microbes and some mineral constituents such as Fe, Al, Cu, Zn, Ni, Mn, Ca and Mg. The ability of these ligands to form stable ligand-metal complexes helps to increase solubilisation of non-sulfidic minerals (Amrhein and Surez, 1988; Bennett *et al*., 1988; Wieland *et al*., 1988; Jain and Sharma, 2004). Microbial extracellular polysaccharides that are produced by some microorganisms also help to increase the solubility of non-sulfidic minerals, because of their ability to complex with ions in solutions (Welch and Ullman, 1999; Welch and Vandevivere, 1994; Welch *et al*., 1999; Jain and Sharma, 2004).

1.6.2 Importance of organic acid to biobeneficiation of non-sulfidic minerals

Organic acids are low-molecular-weight carbon compounds such as oxalic, citric, acetic, lactic, tartaric, malic and malonic acids. The action of these acids could be explained in two different ways. This involves direct mineral attack by the metal complexing organic acid anion and protons (Gadd, 1999). Due to the high chelation constants of $[Al(C_2O_4)_3]^{3-}$; 2.0×10^{16} and $[Fe(C_2O_4)_3]^{3-}$; 3.9×10^{16}, mineral sites containing Al^{3+} and Fe^{3+} are easy targets for attack by organic acid anions. For instance, oxalate chelation of these cations results in structural imbalance and therefore, may lead to the dissolution and release of different elements and nutrients contained in minerals such as Si, K and P (Yuan *et al*., 2004; Delvasto *et al*., 2009). Secondly, there is similarity (monovalent structures) of both protons from organic acids, and K from minerals such as muscovite, but the size of the protons (0.32×10^{-10} m) is much smaller than that of K (2.03×10^{-10} m) (Lapeyrie *et al*., 1987; Yuan *et al*., 2004). The size of the proton is therefore an advantage, as it enables them to replace interlayer K contained in layer minerals associated with iron ores investigated in this study.

Therefore, organic acids production has been highlighted as one of the most important factors that determines leaching ability of heterotrophs (Castro *et al*., 2000, Jain and Sharma, 2004). Sheng *et al*. (2008) reported an example of this phenomenon where high gluconic and acetic acid production were suggested to be responsible for the dissolution of feldspar by *Bacillus globisporus* Q12. In addition, Delvasto *et al*. (2008) also reported the possible effects of gluconic acid released by *Burkhoderia caribensis* FeGL03 on the mobilisation of P from iron ore. Furthermore, Williams (2008) utilised citric acid obtained from *Aspergillus niger* for the biobenefication of Sishen iron ore.

1.6.3 Factors affecting bioleaching of non-sulfidic minerals

There are various groups of heterotrophs that are involved in the biohydrometallurgy of non-sulfidic minerals. The diversity of these microbes has a great influence on their mode of action, because of the associated differences with regards to their requirements for growth and metabolism. Below are some of the previously investigated factors:

Mineralogy: Mineral composition has been found to significantly affect the type of microorganisms that can solubilise the minerals (Schippers and Sand, 1999). An important example here is the grouping of minerals into sulfidic and non-sulfidic minerals (Jain and Sharma, 2004). Presence of sulfur as an energy source in sufuldic minerals is a "privilege" to organisms such as *Thiobacillus ferrooxidans* that can utilise sulfur as their source of energy (Rawlings, 2002; Jain and Sharma, 2004). On the other hand, absence of sulfur in non-sufuldic minerals also provided a competitive advantage to heterotrophs that can utilise other forms of mineral constituents as their energy source (Rawlings, 2002; Jain and Sharma, 2004). It is however important to mention that ores can exist in different forms containing different minerals. For instance, iron ore can be categorised as sulfudic (Biswas, 1981) when it contains sulfur or non-sulfudic (Williams, 2008) when it contains no sulfur (e.g Sishen iron ore with no sulfur).

Nutrient limitation: Whether in a biohydrometallurgy process or in a normal growth condition, there is need for microorganisms to survive in order to grow and multiply. Whenever there is shortage or lack of essential nutrients, microbes reengineer their metabolic process in order to ensure availability of such nutrients (Willey *et al*., 2007). In the process of doing this, mechanisms for solubilisation of unavailable nutrients are triggered through processes such as increased production of organic acids and scavenging (Banfield *et al*., 1999; Sheng *et al*., 2008).

This method can therefore, be used to isolate potential bioleaching organisms. For example, K-limited media were used by Hutchens *et al*. (2003) for the isolation of *Serratia marcescens* that could solubilise feldspar. The isolate was then grown in this same medium for feldspar solubilising experiment. Furthermore, Sheng *et al*. (2008), also utilised K-limited medium to isolate the silicate-solubilising bacterium *Bacillus globisporus* Q12. They eventually discovered that using these silicate minerals as the sole K source of the bacterium increases the number of cells in the medium and subsequently an increase in the solubilsation rate was recorded. In another study by van Scholl *et al*. (2006a), K deficiencies significantly increased the oxalate production by tree seedlings colonised by the fungus *Paxillus involutus*, while Mg deficiencies increased the oxalate production in both mycorrhizal and non-mycorrhizal tree seedlings in the same experiment.

Microbial type: Due to differences in their metabolic activities, there is a diverse range of mechanisms that microbes adopt in bioleaching processes and this invariably affects their rate, mode and manner of bioleaching. These differences can be intra- or inter-species, depending on other factors such as exposure to high levels of heavy metals. For instance, some species of *Aspergillus* and *Penicillium* have mutants that can withstand heavy metals and are essentially different from other strains in their group because of the genetic adaptation (mutation) they developed to heavy metals during exposure to high levels of heavy metal concentration. Rezza *et al*. (2001) also pointed out the differences in organic acid production among some hetrotrophs. In this situation, a decrease in concentration of organic acids was recorded for *Rhodotorula rubra* after 15 d of incubation during the dissolution of aluminosilicate mineral. However, an opposite result was obtained for *Penicillium purpurogenum* under the same conditions, where an increase in citric acid production was recorded after 15 d.

Physicochemical factors:

pH (acidification or alkalinisation): The acidity or alkalinity of the growth medium of bioleaching microbes plays an important role in the bioleaching potential of these microorganisms (Jain and Sharma, 2004). This becomes a vital advantage when bioleaching organisms can only grow at very high or very low pH. This restricts the number of microbes that can grow under these conditions and eliminates the task and cost of carrying out the leaching experiment under sterile conditions (Vasan *et al*., 2001; Jain and Sharma, 2004). In a study conducted by Vasan *et al*. (2001), it was discovered that the increase in pH to near neutral levels of metabolite produced by *Paenibacillus polymyxa* reduces the Ca solubisation from bauxite. In another example, at pH 3 of the medium, Welch *et al*. (1999), reported increased feldspar dissolution compared to pH 4.

Cultural composition and carbon source: This factor is also related to nutrient limitation and mineralogy. Chemical constitution of the growth medium determines the substrate availability for utilisation by microbes for metabolic activities (Willey *et al*., 2007). For example, the source, type or quantity of carbon in the culture may determine the nature and quantity of organic acids

to be produced. This invariably affects the mineral dissolution, because mineral solubilisation is in most cases "organic acid specific". Various sources of carbon have been used in previous studies to grow the heterotrophs in bioleaching experiments. For instance, Hutchens *et al*. (2003) used 0.2 g of glucose in 100 ml of medium to leach potassium from feldspar. In their own study, Sheng *et al*. (2008) used 1% sucrose as carbon source in an experiment that resulted in dissolution of feldspar by *Bacillus globisporus* Q12. Meanwhile, the ambition of developing a good biohydrometallurgical method for non-sulfidic minerals may only be realised if a cheap form of carbon source utilizable by the microbes is integrated into the process (Jain and Sharma, 2004).

Temperature: High temperature exposure of minerals before and during bioleaching processes has been found to enhance bioleaching of these minerals. For example, in the study conducted by Groudev and Groudeva, (1986), it was discovered that initial heat treatment of clay mineral prior to the bioleaching process improved their vulnerability to leaching. A decrease of 5 °C in temperature also led to improved feldspar weathering by a factor of 20 in a study conducted by Welch *et al*. (1999).

Other physical factors include shaking, aeration and time. Most of these remaining factors are interconnected. Franz *et al*. (1991) recorded increased solubilisation of zinc from an industrial filter with increasing shaker speed. The speed of the shaker was related to the aeration (oxygen supply) by these investigators as a cause of increased dissolution of zinc. Time (period of incubation) is another important factor. Rezza *et al*. (2001) reported a sharp decrease in the concentration of organic acid produced by some heterotrophs after 15 d of incubation. This decrease directly corresponded to a decrease in the dissolution of aluminosilicate by these microbes.

Pulp density: This factor has been found to be indirectly related to the rate of mineral solubilisation. For instance, Vasan *et al*. (2001) compared the bioleaching rates at two different pulp densities of 5% and 10% and concluded that the former produced a better leaching of Ca

from bauxite. Pradhan *et al*. (2008) also recorded good bioleaching of copper at a low pulp density.

Particle size: The size of the mineral particles has a great influence on the weathering (solubilisation) rate of minerals (White and Brantley, 1995; Rosling *et al*., 2004). This importance was indicated in the study conducted by Leake *et al*. (2008) where weathering was found to be dependent on particle size in *Pinus sylvestris* colonised by the root microbe *Paxillus involutus*. In another study by Modak *et al*. (2001), bioleaching of bauxite by *Paenibacillus polymyxa* was found to be better with finer particle size of bauxite compared to when the particle size is coarser. In contrast, Srihari *et al*. (1994) discovered that "grinding effect" on minerals can change this trend. Better leaching were obtained from coarser particle size pyrite minerals compared to finer particle size.

1.6.4 Bacterial leaching of non-sulfidic minerals

Bacteria were the first type of microorganisms to be commercially developed in biohydrometallurgy and they are still the most widely used (Rawlings, 2002; Rawlings, 2005). The high level of bacterial technology development in biohydrometallurgy has eluded those involved in biohydrometallurgy of non-sulfidic minerals. Only recently have investigators began in-depth studies on bioleaching of non-sulfidic minerals through adoption and adaptation of some natural processes such as weathering. For instance, 27 fast-growing aerobic heterotrophs were isolated by Hutchens *et al*. (2003) from feldspar-rich soil to investigate their potential in the dissolution of feldspar. *Serratia marcescens* emerged as the best isolate in the dissolution of this mineral and factors such as growth conditions, bacterial type, growth phase, metabolites production and subculturing were all highlighted to significantly affect the dissolution rate.

Furthermore, He and Sheng (2006) reported the ability of *Bacillus edaphicus* in the solubilisation of some K-bearing minerals (feldspar and Illite) and the subsequent release of K in the rhizosphere for utilization for plant growth. Such bacterial-solubilising abilities have been transformed into their bioleaching potentials in the laboratory by testing for their ability to dissolve artificial insoluble chemical compounds for final selection as a potential bioleaching

candidate. Delvasto *et al*. (2008) utilised this method to isolate and characterise different phosphate-solubilising bacteria that included *Leifsonia xyli* Fe G1 02, *Burkholdera cenocepacia* FeSu 01, *Burkholdera caribensis* FeG1 03 and *Burkholdera ferrariae* FeG1 01. Among the potential bioleaching microbes obtained from their study, *Burkholderia caribensis* FeGL03 was able to show high potential in mobilising P contained in iron ore (Delvasto *et al*., 2009). A few other examples of bacteria that have been used in bioleaching are listed below in Table 1.1.

Table 1.1: Examples of bacteria that have been used for heterotrophic leaching of non-sulfidic minerals (adapted from Jain and Sharma, 2004).

Bacteria	Mineral/Mineral ore	Bioleached metal	Reference
Bacillus sp.	Manganiferous ore	Ag	Rusin, 1992
Paenibacillus polymyxa	Calcite, hematite, corundum	Fe, Al, Ca	Deo and Natarajan (1998)
Arthrobacter sp., *Norcadia* sp., *Pseudomonas* sp	Spodumene	Li, Al, Si	Karavaiko *et al*., 1980
Mixed culture of *Agrobacter radiobacter*, *Spaphilococcus* sp., *Candida* sp.	Manganiferous ore	Mn	Veglio *et al*., 1997
Bacillus circulans	Iron ore	Al	Pradhan *et al*., 2006
Bacillus globisporus Q12	Feldspar	K and Si	Sheng *et al*., 2008
Burkholdera caribensis FeGL03	Iron ore	P	Delvasto *et al*., 2008

1.6.5 Fungal leaching of non-sulfidic minerals

Not many studies have attempted to discuss or investigate fungal leaching (Burgstaller and Schinner, 1993; Jain and Sharma, 2004). Fungi generally have the capability of being used in separation of metals from low-grade ores, mine wastes or in the removal or reduction of contaminations from ores (Groudev, 1987; Burgstaller and Schinner, 1993; Valix *et al*., 2001). Burgstaller and Schinner (1993), listed four factors that had probably discouraged intensive research into the use of fungi as bioleaching agents. These included the high carbon demand of

fungi needed for growth, lack of thorough knowledge about fungi, slower rate of leaching by the fungi and an inability to use refined genetic methods for improvement of the fungi. The most important of these factors is the provision of a carbon source for these microbes that can serve as their source of energy during the leaching process (Jain and Sharma, 2004).

Generally, in fungal bioleaching, only *Aspergillus* and *Penicillium* spp. have received much attention (Table 1.2). In a study conducted on *Aspergillus* and *Penicillium* by Valix *et al*. (2001), cobalt and nickel were effectively leached by direct microbial activities and the effects of organic acids produced by these microbes. In another study, Castro *et al*. (2000) confirmed the better potential of *A. niger* to bacteria (*Bacillus* sp. and *Pseudomonas* sp.) in the leaching of zinc and nickel.

Although great progress has been made in the use of fungi such as *Aspergillus* and *Penicillium* spp. for bioleaching processes; however the health implication of the aerosols formed by the spores of these fungi (Gorny, 2004) has raised a serious question about their safety. The focus on the use of fungi for bioleaching should therefore extend beyond their leaching potential, but should also include the cost implication, as well as their short and long time impacts on the environment.

One of the most studied groups of microorganisms involved in weathering is the ectomycorrhiza (ECM) fungi. The capacity of ectomycorhizae to participate in weathering through the production of organic acid has been proven in many studies (Jongman *et al*., 1997; Hoffland, 2003; Gadd, 1999). Both the ectomycorrhizal root tips and the ectomycorrhizal hyphae in the soil are capable of releasing organic acids into the soil environment (Landeweert *et al*., 2001). In addition, other important metabolites of ECM fungi produced during weathering are siderophores. These are low-molecular-weight iron-chelating compounds synthesised under conditions of low Fe availability. Siderophores are Fe-loving compounds that help in solubilisation of insoluble Fe compounds or minerals, thereby contributing significantly to weathering of minerals that contain such compounds. Several studies have confirmed the increased uptake of siderophores by mycorrhizal plants (Haselwandter *et al*., 1992; Haselwandter, 1995). A combination of siderophores and organic acids was said to be very effective in dissolution of iron-containing compounds such as goethite (Cheah *et al*., 2003; Haselwandter, 2008). Meanwhile, organic acids

could sometimes function like siderophores in the mobilisation of Fe (Guerinot *et al*., 1990; Carson *et al*., 1992 in Machuca *et al.,* 2007).

In one of the earliest studies on ECM weathering, Jongmans *et al. (*1997) suggested that tubular pores found in feldspars and hornblende in podzol E horizons and granitic bedrocks at some places in Europe were formed by ECM fungi. This is one of the strategies used by these fungi to search for K^+, Mg^{2+} and Ca^{2+} on behalf of their host plants that grow under severe nutrient conditions, thereby contributing to the weathering of these minerals. Wallander and Wickman (1999) attempted a study that focussed on the mobilisation of potassium from biotite and microcline using two ECM fungi, namely *Paxillus involutus* and *Suillus variegatus*. Absence of K in their control experiments in *P. involutus*-colonised soils resulted in a decreased biomass of the ECM fungi in the soil compared to the situation where biotite was added as the source of K. In addition, they suggested that the relationship that existed between the foliar K and the citric acid (in *S. variegatus*-colonised seedlings) was an indication that K was released from biotite by weathering and later transferred to the host plant of the fungus.

Hoffland *et al*. (2003) also established the direct relationship between feldspar tunneling and ECM root density as a proof of participatory role of these fungi in weathering of feldspar. In addition, the study also provided the correlation between high weathering and low nitrogen availability. The ability of ECM fungi to repeatedly gain assess to nutrient trapped inside different minerals such as Phlogopite, feldspar and biotite (Paris *et al*., 1995), leaves no doubt that the weathering ability of these fungi could possibly indicate their high bioleaching and metal binding potentials.

Assuming time is not a factor (such as the case in mine wastes) and a cheap carbon source could be provided, then fungi could compete with bacteria as candidates in bioleaching. For example, fungi can grow in higher pH environments (created by materials contained in metals to be leached) (Willscher and Bosecker, 2003) compared to some bacteria that are used in bioleaching and thrives best in acidic environments. In addition, the production of organic acids, amino acids, proteins, protons and peptides by fungi increases the solubility of metals in solution, thereby aiding in the bioleaching process.

Nevertheless, prior to the full development of a technology where ECM fungi could be used as bioleaching agents, the immediate focus should be on screening of potential ECM fungi that could function in this area of biotechnology. Indeed, if there are ECM fungi that are bioleaching agents, then improvements could be made in their culturability whether in the presence or absence of a host plant, such as those developed in the mushroom industries where some mushrooms that are ectomycorrhizal could be cultivated without the host plant (Debaud and Gay, 1987; Hall *et al*., 2003). Furthermore, there should also be investigations into different methods in which minerals could be leached with these fungi, bearing in mind the ease of separation of the fungi and mineral products after leaching, as well as the cost implication of the method should be low. For instance, the consequence of direct leaching with the fungi should be compared to the use of fungal filtrates for the leaching process. The use of fungal metabolite for this purpose was demonstrated by Williams (2008), that utilised citric acid produced by *A. niger* was utilised to reduce K of iron ore by 17.65%.

A difficult challenge regarding the use of ECM fungi in bioleaching will be the growth of the fungi under non-sterile conditions. The pH and temperature ranges at which fungi grow normally supports the growth of many contaminants (Jain and Sharma, 2004). In conclusion, use of fungi such as ECM in bioleaching could be feasible if there is thorough understanding of the metabolic pathways and genetics of these fungi (Burgstaller and Schinner, 1993; Jain and Sharma, 2004).

Since it has been established that performance of ECM fungi in pure culture does not necessarily translate to their role when in symbiosis (Smith and Read, 2008), separate investigations of the two scenarios should be encouraged. This would increase the knowledge about different potentials of the ECM fungi with or without the host plants. Therefore, there is a high prospect in the ability of ECM fungi to solubilise varieties of minerals, as proven in their weathering capabilities (Paris *et al*., 1995; Landeweert *et al*., 2001; van Scholl *et al*., 2006b).

Table 1.2: Examples of fungi that have been used for heterotrophic leaching of non-sulfidic minerals (adapted from Jain and Sharma, 2004).

Fungi	Mineral/Mineral ore	Bioleached metal	Reference
Aspergillus sp.	Manganese ore	Mn	Ghiorse, 1988
	Laterite ore	Ni, Co, Mn	Tzeferis, 1994
A. clavatus	Mercury compounds	Hg	Puerner and Siegel, 1976
A. niger	Laterite ore	Ni, Co	Tzeferis *et al*. 1994
A. niger	Manganese nodule	Cu, Ni	Ehrlich, 1980
	Coal fly ash	Al	Singer *et al*., 1982; Torma and Singh, 1993
	Clay	Al	Groudev and Groudeva, 1986
	Nepheline	Al	King and Dudeney, 1987
	β-spodumene	Li	Ilger and Tormer, 1989
		Li, Al	Rezza *et al*., 1997
	Copper converter slag	Cu, Ni, Co	Sukla *et al*., 1992
	Silicate ore	Zn, Ni	Castro *et al*., 2000
	Iron ore	Al	Pradhan *et al*., 2006
A. ochraceous	Rocks	U	Munier-Lamy and Berthelin, 1987
Penicillium sp.	Manganese ore	Mn	Ghiorse, 1988
	Gold dust	Au	Groudev and Groudeva, 1988
	Silver ore	Mn, Ag	Gupta and Erlich, 1989
	Iron ore	Fe	Hoffman *et al*., 1989
	Laterite ore	Ni, Co	Agatzini and Tzeferis, 1997
P. funiculosum	Rocks	U	Munier-Lamy and Berthelin, 1987
P. notatum	Pagmetite rock	Li, Si, Al, Fe	Avakyan *et al*., 1981
P. simplicissimum	Rocks	Ti	Silverman and Munoz, 1971
	Basalt rock	Al	Mehta *et al*., 1978, 1979
	Red Mud	Al	Vachon *et al*., 1994
P. purporogenum	Spodumene	Al, Li	Rezza *et al*., 1997
P. variotti	Lead zinc ore	Zn	Dave *et al*., 1981
Trichoderma ligneruom	Pagmetite ore	Li, Si, Al, Fe	Avakyan *et al*., 1981
Yarrowia lipolytica	Used catalyst	Cu, Pb, Sn	Hahn *et al*., 1993
Candida sp.	Gold dust	Au	Groudev and Groudeva, 1988

1.7 Iron ore

Iron ore is one of the oldest metals and an essential source of primary iron for the global iron and steel industries. Iron is one of the most common elements on earth, traded and consumed in different forms in many countries (Astrup and Hammerbeck, 1998). There are different types of iron-bearing minerals, but the highly exploited ones include: Magnetite – $FeO.Fe_2O_3$ (72% Fe), Haematite - Fe_2O_3 (70% Fe), Goethite - FeO.OH (61% Fe), Lepidocrocite - FeO.OH (61% Fe), Siderite – $FeO.CO_2$ (48% Fe) and Chamosite – $3FeO.Al_2O_3.2SiO_2.6H_2O$ (35% Fe). In addition, there are associated gangue minerals found in iron ores and these include feldspar, quartz, calcite, dolomite, clays and carbonaceous matter (Astrup and Hammerbeck, 1998). Iron ore could also contain some deleterious elements such as phosphorus, silica, potassium, zinc, sulfur and sodium (Astrup and Hammerbeck, 1998; Yusfin *et al*., 1999). Iron is desirable because of its physical properties such as hardness, strength, malleability, ductility, durability and the ease with which it can form alloys with other elements to form different types of steel (Klemic *et al*., 1973; Astrup and Hammerbeck, 1998).

Iron ore deposits in South Africa can be categorised into four types of deposits, namely: Banded iron formations (BIF), Magnetic deposits, Gossan and residual properties and Lode, vein and replacement deposits (Astrup and Hammerbeck, 1998). However, most of the world's iron ore currently being mined are from the high-grade haematite iron hosted by the Precambrian branded iron formations (BIF) (Gutzmer *et al*., 2001; Beukes *et al*., 2003). The BIF is generally divided into two groups, i.e. martite-goethite ores and martite hematite ores. The martite-goethite ores consist of a range of strongly indurated, brown goethite-rich material to friable yellow ochre, consisting of martite and early formed prismatic hematite. These deposits are characterised by variable preservation of BIF features such as banding and pseudomorphing of gangue minerals by goethite. This group is generally considered to result from supergene enrichment of BIF during Mesozoicto Tertiary lateritic weathering (Morris *et al*., 1980; Morris, 1985; Barley *et al*., 1999). The second group, martite-hematite (or microplaty hematite) ores, is characterised by well-preserved primary lamination, but with variable loss of internal texture and the growth of abundant secondary prismatic and microplaty hematite. Though the origin of these ore deposits is

not certain, it is generally believed that they represent metamorphosis of martite-goethite ore bodies that occurred during Palaeoproterozoic burial, where goethite is susbstituted for hematite at <100 °C (Morris, 1985). These types of deposits are found in South Africa and Brazil (Morris, 1985; Barley *et al*., 1999). In South Africa, these BIF consist of distinctive hills, stretching a distance of 400 km from the south in Prieska to Pomfret in the north (Carney and Mieney, 2003).

1.7.1 Iron ore in South Africa

Records have shown that mining must have started in South Africa during the middle stone age when black people obtained minerals either by picking them at the surface or digging holes to get these minerals (Mason, 1982). Iron and copper were the first two minerals mined by the early farmers in South Africa (Miller, 1995). Beumont (1973) estimated the exploitation of iron ore minerals in Southern Africa to be 40000 years old when specularite and haematite were extracted by ancient miners from Ngwenya, Swaziland. There are also documented traces of early mining of specularite in the Nothern Cape province of the country, whereas historical evidence of early smelting and fabrication were recorded from Tzaneen and Broederstroom, near Hartbeespoort dam (Astrup and Hammerbeck, 1998). However, proper documentation of mining in South Africa is only available for the last two centuries. South African iron ore mines include Sishen, Beeshoek, Thabazimbi, Palabora and Mapochs, having combined reserves of 2879 million tonnes of iron ore. Up to 80% of these reserves belong to the Sishen iron ore mine located in the Northern Cape (Fig. 1.1) (Astrup and Hammerbeck, 1998). This huge deposit means South Africa has the largest iron ore deposit in Africa.

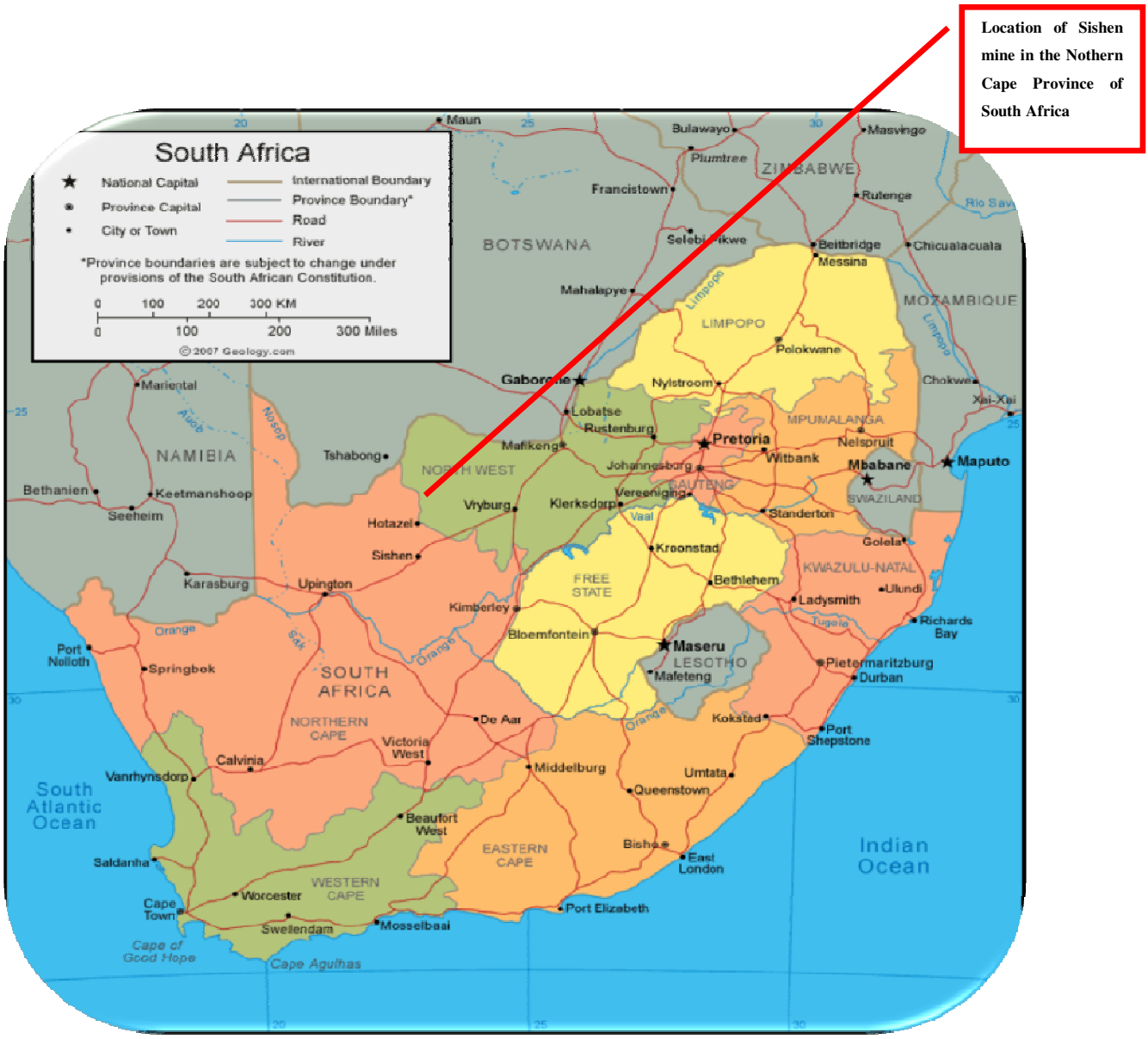

Figure 1.1: Map of South Africa showing the location of Sishen mine in the Northern Cape Province (adapted from http://geology.com/world/south-africa-satellite image.shtml).

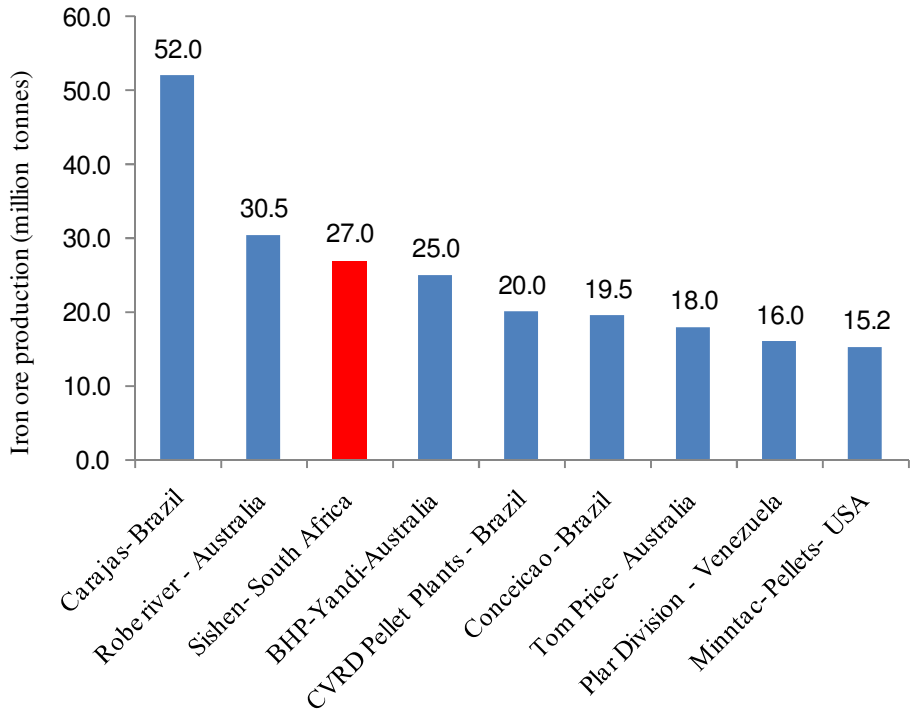

Figure 1.2: Top iron ore mining sites in the world, Sishen is 3rd on the list (adapted from (http://www.saldanhaportoperations.com/operations_io_sishen_largest.php?sid=)

South African iron ore mining is dominated by KUMBA resources that controls the two biggest iron ore mining sites in South Africa, i.e. Sishen in the Northern Cape and Thabazimbi in the Northern Province. Sishen was founded in 1953, and presently has the capacity to produce up to an average of 27 million tonnes of iron ore a year. This corresponds to 78% of the total iron ore in South Africa and it is the third largest iron ore company in the world (Fig. 1.2) (Internet 1). At Sishen, South Africa, the iron ore company faces the problem of increasing mining volume of impure iron ore minerals, which contain potassium and phosphorus that affect the commercial value of the ore.

1.7.2 Biobeneficiation of iron ore

Despite the popularity of iron ore, the biobeneficiation of this ore has not received much attention like the other mineral ores. This is mostly due to the cheap cost of this mineral, regardless of its importance (Williams, 2008; Delvasto *et al*., 2009). However, fast depletion and difficulty in finding the mineral in the pure form has increased awareness about the extraction and biobeneficiation processes of iron ore. Problematic elements such as P, K and Na have been identified to reduce the commercial value or render the mineral valueless. These elements have various ways of interfering with the processing of iron ore. Phosphorus, which is an element needed by both plants and animals, is, however, one of the most deleterious elements that can associate with iron ore mineral. High phosphorus levels in steel cause a reduction or loss of strength, hardness and ductility, as well as increasing the chances of corrosion (Marshall, 1984). High levels of alkaline - K and Na, can also affect iron ore processing, especially the smelting of iron ore in the blast furnace. The problem with alkalis occurs when they are volatilised as elements. Some parts of the vapour normally react with the refractory lining or burden material to generate compounds in relation to their relative stability. This mainly happens in the cooler regions where oxygen potential is high. The compounds formed progress to the hotter regions where they are reduced and rise again to form recirculating load of alkalis (Biswas, 1981; Elkasabgy, 1984; Yusfin *et al*., 1999). In summary, the adverse effects of alkalis on the blast furnace include an increase in coke rate, poor quality of hot metal and mechanical weakening of the furnace lining. They also cause a decrease in production; conditions that are more pronounced when there is low stability of coke and the iron ore (Davies *et al*., 1978; Elkasabgy, 1984; Yusfin *et al*., 1999). Due to these problems, iron ore with high contents of both P (>0.03%) and K (>0.24%) attracts penalties (low cost) in the international market. It has therefore become imperative for iron ore industries to find an economical and environmentally friendly method that could solve these problems.

To solve these problems, several methods have been proposed and these normally rely on the principle of dilution (with better quality ore) and adjustment of temperature, basicity and acidity inside the blast furnace. The method that was adopted by Sishen iron ore mine, South Africa, to deal with this problem prior to this time is the mixing of standard (low levels of K and P) and low

grade (high levels of K and P) iron ore to avoid penalty charges and meet international standards (Williams, 2008). However, with increasing volumes of iron ore that have high levels of K and P, this method has become unsustainable. Traditionally, different experiments have been conducted using chemical and physical methods to get rid of these contaminants found in iron ore (Delvasto *et al*., 2005). The choice of method in this regard strongly depends on the basic characteristics of the iron ore and the type or degree of association that exist between the iron ore and the P content (Delvasto *et al*., 2005). For example, Cheng *et al*. (1999) utilised sulfuric acid for the biobeneficiation of Australian iron ore with a phosphorus level of 0.126%. In this situation, more than 67% of P was leached within 5 h at 60 °C by a 0.1 M concentration of sulfuric acid. In addition, Changde iron ore with a high P content (1.125%) was successfully leached using an alkali and acid solution that contained sodium hydroxide, sulfuric, hydrochloric and nitric acids. Another hydrometallurgical method was proposed by Muhammed and Zhang (1989), where isoamyl alcohol, phosphoric acid and nitric acid were used in an integrated leaching process to remove P from the iron ore.

Unfortunately, further developments of these technologies have been hindered by cost implications and their potential negative impacts on the environment (Delvasto *et al*., 2005). For this reason, this last decade has seen the gradual development of affordable and environmentally friendly methods of leaching iron ore. Focus is now more on the use of microorganisms for the leaching of this mineral, a process known as biobeneficiation (Jain and Sharma, 2004). Development in this area has been slow due to the non-sulfidic nature of some iron ore minerals (Williams, 2008), which has made it difficult to apply already detected sulfidic bioleaching microorganisms for leaching of the iron ore mineral. However, two decades ago, the use of microorganisms to leach iron ore was patented by Hoffman *et al*. (1989). In their work, domestic wastewater containing *Pseudomonas* 200 was used under anaerobic conditions to reduce the ferric iron in iron ore to ferrous iron and which was later precipitated from the solution with the help of a base to recover the iron mineral. Another study was carried out by Parks *et al*. (1995) where metabolites containing itaconic and oxalic acid produced by a *Penicillium* sp. significantly reduced the phosphorus contents of the iron ore. Further reduction of the P content was obtained by addition of a low concentration of hydrochloric acid. The only report regarding the use of

ECM fungi for the solubilisation of P from iron ore was reported by Buis (1995) in Delvasto *et al*. (2005), where *Paxillus involutus, Hebeloma crustiniforme, Thelopora terrestris* and *Laccaria bicolour* failed to solubilise P from iron ore, despite their ability to solubilise P from hidroxylapatite.

Recently, Delvasto *et al*. (2005) investigated the biobeneficiation of iron ore in greater detail. In this case, after bioactivation of the iron ore samples, one of the phosphate-solubilising fungi that was isolated from the iron ore, *Aspergillus niger*, was tested for its ability to solubilise the P content of the iron ore. Up to 30% desphosphorisation was attained, signifying high potential in the use of microorganisms for the bioleaching of iron ore. This was followed by another study by Delvasto *et al*. (2008), where four different phosphate-solubilising bacteria were isolated from the high phosphorus Brazilian ore using tricalcium phosphate [$Ca_3(PO_4)_2$] as the insoluble form of P. The bacteria isolated included *Leifsonia xyli* FeGl 02, *Burkholderia cenocepacia* FeSu 01, *Burkholderia caribensis* FeGl 03 and *Burkholderia ferrariae* FeGl 01. Further studies were then carried out on *Burkholderia caribensis* FeGl 03 to investigate its potential in desphophorisation of the iron ore. The results indicated that this bacterium was able to mobilise between 5% and 20% of the initial P value of the iron ore within a period of 21 d. Particle size, organic acid and exopolymeric substances production were all listed as possible factors that affected the leaching process (Delvasto *et al*., 2009).

The problem associated with this method is mostly related to the growth conditions of the bacteria. Heterotrophs are known to grow at temperature and pH ranges near neutral (around pH 7, ±2), a situation that encourages easy contamination of their growth medium (Jain and Sharma, 2004). Iron ore being an inexpensive mineral, it is very difficult to develop this processes into heap leaching that can be used in outdoor leaching. It has therefore been suggested that focus should shift to finding a better source of carbon such as organic wastes from food or agricultural industrial wastes. Another problem is the strong attachment of fungi used in biobeneficiation to the mineral to be leached. Separation in such scenario may be quite difficult and in this case, the use of metabolites produced by the fungi could be the solution. In addition, separating techniques could also be introduced into the fungal biobenefication process from the initial stage to prevent the fungal attachment to the surfaces of the mineral.

From the examples above, it can be said that technologies in the biobeneficiation of iron ore is gradually gaining momentum. However, what is not clear to investigators in this area of research is the exact type of microorganisms that can perform this task. Both fungi and bacteria have been used for the bioleaching process. A knowledge gap here is the absence of such studies on other contaminants of iron ore such as K. From the available literature, there has not been development or investigation of biobeneficiation for other impurities (such as K) found in iron ore. Information about P removal from previous studies provided direction on how to approach this study. This means technologies already developed for P-solubilising bacteria could provide a platform for studies concerning solubilisation of other P-containing mineral. The current study therefore, provides an elaborate investigation of both uninvestigated fungi and bacteria that have the potential to participate in the bioleaching of iron ore. The study started by investigating the bioleaching roles of some soil microbes. This was expected to provide insight into the relationship between bioleaching and weathering, an investigation that can lead to detection of potential bioleaching agents of iron ore. Furthermore, potential influences of iron ore-associated microorganisms (fungi and bacteria) were also investigated to establish the possibility of using indigenous microbes for the biobeneficiation of iron ore. Finally, a novel method was designed to initiate a future development of an economical and environmental friendly method of leaching iron ore. Based on the above-mentioned points, the aims of this study are as listed under the objectives.

1.8 Objectives of the study:

a. To investigate the relationship between natural weathering and biohydrometallurgy potentials of ectomycorrhizal fungi

b. To investigate the *in vitro* capabilities of pure cultures of different ectomycorrhizal fungi in mobilisation of potassium and phosphorus from iron ore

c. To isolate and identify potential organic acid-producing fungi that can mobilise nutrients from iron ore minerals

d. To investigate indigenous bacterial flora of two types of Sishen iron ore and the potential of the bacterial isolates in mobilisation of nutrients from the ore

e. To initiate an economically viable method for biobeneficiation of iron ore using a cheap energy source and septic conditions.

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CHAPTER TWO

IMPLICATION OF ECTOMYCORRHIZAL WEATHERING OF IRON ORE MINERALS: THE BIOLEACHING CONNECTION

Abstract

Ectomycorrhizal bioleaching as a stage in mineral weathering was investigated in order to establish the relationship between the effects of ectomycorrhizal (ECM) fungal and chemical constitution of minerals. Such relationships can have a great impact on mining as it can either improve or reduce the quality of the minerals. Low grade quality iron ore minerals from Sishen iron ore mine were utilised in this study in an experiment that involved both mycorrhizal and non-mycorrhizal *Pinus patula*. Four types of ECM fungi were used, namely *Pisolithus tinctorius* (PT), *Paxillus involutus* (PI), *Laccaria bicolor* (LB) and *Suillus tomentosus* (ST). The results suggest that different roles of ectomycorrhizal fungi in mineral weathering (such as nutrient absorption and transfer, improving the health of plants and ensuring nutrient circulation in the ecosystem) are species specific, and both mycorrhizal roots and non-mycorrhizal roots can influence the chemical constitution of the iron ore minerals.

2.1 Introduction

Mineral weathering can be described as a combination of different physical, chemical and biological processes that can improve and boost the nutrients supply to plants (Smith and Read, 1908; van Breemen *et al*., 1900a; van Breemen *et al*., 1900b; Schoenholtz *et al*., 1900). Soil biological systems play a major role in mineral weathering (Arocena and Glowa, 2000). In the process of establishing some survival strategies, soil organisms such as plants, saprophytic and symbiotic microbes are able to interact in different ways to produce varieties of metabolites that are involved in weathering (Banfield *et al*., 1999; Schoenholtz *et al*., 1900). At the symbiotic level, ectomycorrhizal (ECM) fungi are actively involved in mineral weathering (Smith and Read, 2008). Ectomycorrhizal fungi are beneficial and like other types of mycorrhizal fungi, they form mutualistic associations with suitable plants (Smith and Read, 2008). These fungi colonise the roots of compatible plants by forming an association that is intercellular, just outside the root cells and the hyphae never penetrate the root cells (Smith and Read, 2008). They are able to absorb nutrients on behalf of their host plants, especially when nutrients are in short supply or unavailable to plants. Such nutrients are subsequently passed onto their host in return for carbon supply by the plants (Bolan, 1991; Smith and Read, 2008).

Ectomycorrhizal fungi have special capability to absorb nutrients from rocky and hard mineral materials (Smith and Read, 1908; Jongmans *et al*., 1997; Arocena and Glowa, 2000; van Scholl *et al*., 2006b). The link between weathering and ECM fungi was first noted by Jongmans *et al*., (1997) while investigating the tunnels found inside mineral grains obtained from podzolic soil. These authors suggested that the tunnels were created by ECM fungi over a long period of time. Their findings were further corroborated by another study that investigated feldspar tunneling. In that study, it was discovered that ECM root density was constantly proportional to the feldspar tunneling, which was also related to the release of nutrients such as K and Ca from the mineral (Hoffland *et al*., 2003). In another study by Leake *et al*., (2008), the ectomycorrhiza formed between *Paxillus involutus* and *Pinus sylvestris* was suggested to be actively involved in the

weathering of apatite, quartz and biotite, and also altered the potassium content of these minerals. Their study also confirmed that weathering rate is dependent on mineral type and proportional to the surface area-to-volume ratio of the mineral. Although other soil microorganisms contribute to mineral weathering (Banfield *et al*., 1999), ECM fungi play a major role through high production of low-molecular-weight HOC=O compounds known as organic acids (Paris *et al*., 1995; Jones, 1998; Smith and Read, 1908). Many studies had proposed that factors such as pH, nutrient limitation, grain size, organic acid production, and mechanical penetration of the mineral have different influences on mineral weathering (Burford *et al*., 1903; Jain and Sharma, 1904; Balogh-Brunstad *et al*., 1908). Of all these factors, production of organic acid is probably the most acknowledged (Burford *et al*., 1903; Jain and Sharma, 1904).

Investigation of mineral weathering in the mining industry has been mostly in two ways – bioleaching and bioremediation (bioremediation is outside the scope of present study) - both processes rely primarily on production of organic acids by microorganisms (Burford *et al*., 1903; Jain and Sharma, 1904). The word bioleaching is mostly used to describe the microbial solubilisation of metals for extraction purpose (Bosecker, 1997) and can also be used to estimate the weathering budget (Balogh-Brunstad *et al*., 1908; Calvaruso *et al*., 1909). Most biological weathering investigations that involved ECM fungi have focused on agricultural benefits (Balogh-Brunstad, *et al*., 1908; Calvaruso *et al*., 1909), leaving a gap about the knowledge of how this process can change the chemical constitution of minerals. It was therefore hypothesised in this study that when ECM fungi are present, their weathering capabilities can contribute to the quality of naturally occurring minerals through bioleaching. In this situation, non–exportable Sishen iron ore minerals, with high levels of K and P (Williams, 1909), were used. High levels of K (>0.24%) and P (>0.03%) contents diminish the market values of iron ore minerals and could render them non-exportable (Parks *et al*., 1990; Yusfin *et al*., 1999; Williams and Cloete, 1908; Delvasto *et al*., 1909). The purpose of this study was therefore to investigate potential mobilisation effects of ectomycorrhizal colonised plants on chemical constitution of minerals such as iron ore, through weathering.

2.2 Materials and Methods

2.2.1 Origin of ectomycorrhizal fungi

Ectomycorrhizal isolates used and their origins are *Pisolithus tinctorius* - # PT 7 (Plant Health Care Inc., Pittsburgh, USA), *Paxillus involutus* - NOF 2340 (Canada), *Laccaria bicolor* - LB (Canada) and *Suillus tomentosus* - UAMH 6252 (Canada).

2.2.2 Iron ore preparation

The iron ore sample used, KGT (conglomerates), was supplied by KUMBA iron ore resources Ltd. The KGT sample, originally contained an average of 0.995% of K_2O and 0.152% of P. KGT also contains SiO_2 (4.87%), Al_2O_3 (3.26%) and Fe_2O_3 (90.70%). Trace values of TiO_2, CaO, MgO, Na_2O, MnO, Cr_2O_3, NiO, V_2O_5 and ZrO_2 were present in the ore sample. The ore materials were milled and separated into two particle sizes of 3.36 mm - 1.68 mm and 1.68 mm - 0.84 mm with mesh of different sizes. Henceforth, these would be referred to as particle sizes A and B, respectively. The iron ore materials were treated with 0.1M HCl by soaking overnight to reduce the effects of high-energy surface sites created by grinding (van Scholl *et al*., 2006b). After 24 h, the samples were thoroughly washed under distilled water to remove fine particles and the pH adjusted to 4. This was followed by the addition of distilled water to the iron ore mineral and shaking for 7 d at 100 rpm. The iron ore materials were finally washed with distilled water and dried overnight in an oven at 60 °C.

2.2.3 Preparation of seeds

Seeds of *Pinus patula* obtained from Komatiland Forest (KLF), SABIE, South Africa, were surfaced-sterilised in 30% H_2O_2 for 15 min, washed continuously under distilled water for 3 min and soaked overnight in autoclaved distilled water with a drop of Tween-20. After 24 h, the seeds were re-sterilised in 10 % sodium hypochlorite (NaOCl) for 60 s. This was followed by washing 3-5 times under distilled water before inoculation onto

15% water agar plates where they were pre-germinated for 4 weeks. Germinants were considered ready for mycorrhizal synthesis experiment when radicles were 1 to 2cm long.

2.2.4 Mycorrhizal synthesis experiment

Autoclavable Magenta boxes (Magenta. Corp., Chicago, Ill., USA) were used for the experiment. A 50 ml aliquots of Modified Melin Norkrans (MMN) medium (Marx, 1969) containing Malt extract (3g/l), $(NH_4)_2HPO_4$ (0.25g/l), $MgSO_4.7H_2O$ (0.075g/l), $CaCl_2.H_2O$ (0.067g/l), NaCl (0.025 g/l), $FeCl_3$ (1%), thiamine (100µg/l) and Agar (10g) was used for the experiment. Glucose was omitted from the medium composition in order to starve the fungi of carbon source, which they could get from their host plant by forming the mycorrhizal association. After the autoclaved medium was poured into the boxes, they were covered with a layer of autoclaved cellophane paper, which was meant to prevent the ectomycorrhizal root from penetrating the medium.

A 6-mm cork-borer was used to cut plugs of the mycelial culture of the ECM fungi from the actively growing edge of 7-d old cultures. Three plugs of ECM mycelia were inoculated on the cellophane paper at equal distances from one another. This was incubated at a temperature of 28 °C. After 5 d, germinated seedlings of *Pinus patula* were carefully introduced into the centre of the flasks containing growing mycelia of the fungi, and the boxes were incubated under sterile conditions at 23 °C/16 °C (d/night), 16 h photoperiod and 80% humidity for a period of 12 weeks. All the control plant seedlings (non-mycorrhizal) were also transferred to the magenta boxes containing the same type of MMN medium but no ECM fungi. After 12 weeks, the root samples were selected at random and carefully examined for signs of mycorrhizal association. The root samples were observed under the dissecting microscope Leica S4E microscope (Leica Microsystems Imaging Solutions, Cambridge, UK) for hyphal sheath structural development as an indication of mantle formation. Representative roots that could not be confirmed as colonised under dissecting microscopes were further examined under a light microscope after staining using the method described by Smith and Dickson (1997) (Appendix I).

2.2.5 Soil Treatments

The growth medium was 100% sand soil obtained from Sable Marco Inc., Pont-Rouge, Québec Province, Canada. Soil was soaked in 0.1 M HCl overnight in order to remove fine dust and exchangeable bases. After 24 h, the soil was washed continuously for several hours under distilled water and later dried in the oven for 3 d. Soils were sieved with an electroformed sieve to particle sizes of 0.25 – 0.59 mm which allows an easy separation of soil from the iron ore minerals of particle sizes A and B (bigger) at the end of the experiment. The sieved soil was sterilised in the autoclave at 121 °C for 30 min and allowed to cool overnight before the sterilisation was repeated at the same temperature and time. The soil was kept sterile until the beginning of the weathering experiment.

2.2.6 Weathering experiment

Plastic pots of size (80 X 80 X 70 mm) were sterilised by soaking in 3% NaOCl overnight and washed several times under distilled water. These pots were then filled to the brim with the autoclaved/treated soil mentioned above. Planting holes, big enough to hold seedlings were created in the soil and were partially filled with 4 g of the prepared iron ore samples. In the same hole, ectomycorrhizal colonised (EMC) and non-mycorrhizal colonised root (NMC) sections - radicle of all the healthy seedlings - were introduced and covered with soil. The incubation was at 23 °C/16 °C (d/night), 16 h photoperiod and 80% humidity. This experiment lasted for 24 weeks. All treatments were performed with 4 replicates.

2.2.7 Watering and nutrient supply

Watering of the seedlings was done every other day and the nutrient supply was twice a week. The nutrient solution used in this experiment was a Hoagland solution adjusted to reduce the sources of K. In addition, sources of Mg were also halved to limit the alternative K source (Mg) available to the fungi during the weathering process (Van

Scholl *et al*., 1906b). The final solution of the Hoagland contained the following – $Ca(NO_3)_2$ -7 ml, $NH_4(PO_4)$ – 2 ml, $MgSO_4$ – 1 ml, trace elements-1 ml (H_3BO_3 - 2.8 g/L, $MnCl_2.4H_2O$ - 1.8 g/L, $ZnSO_4.7H_2O$ - 0.2g/L, $CuSO_4.5H_2O$ - 0.1 g/L and $Na_2MO_4.2H_2O$ – 0.025 g/L) and 1 ml FeEDTA (15 g/L). For the controls that included 1.) no iron ore samples (CT) and 2.) no ECM fungi and no iron samples (CTR), the nutrient solution contained - $Ca(NO_3)_2$ - 7 ml, $KH_2(PO_4)$ – 2 ml, KNO_3 - 5 ml, $MgSO_4$ – 2 ml, Trace elements - 1 ml (H_3BO_3 - 2.8 g/L, $MnCl_2.4H_2O$ - 1.8 g/L, $ZnSO_4.7H_2O$ - 0.2 g/L, $CuSO_4.5H_2O$ - 0.1 g/L and $Na_2MO_4.2H_2O$ – 0.025 g/L) and 1 ml FeEDTA (15 g/L).

2.2.8 Harvesting

All plants were harvested at the end of 24th week. The first step in harvesting included the careful separation of the seedling from the soil and iron ore samples through sieving. The roots were thereafter severed from the shoot system, washed free of the soil and transported to the laboratory for further processing. In the laboratory, the root samples were thoroughly washed again with distilled water to remove any trace of the soil and then prepared for light microscopy examination where percentage root colonisation was determined. The plant shoots and roots were separately dried in the oven and weighed to obtain root dry mass (RDM) and shoot dry mass (SDM). The sieved soil samples free of the iron ore samples were divided into two for dry mass calculation, pH determination and organic acid analysis.

2.2.9 Organic acid analyses

Four g of the harvested soil was put into 25 ml centrifuge tubes and 20 ml of 10 mM NaH_2PO_4 were added (Mimmo *et al*., 2008). The mixture was shaken for 4 hrs at room temperature and then centrifuged at a speed of 9300 rpm for 8 min at 20 °C. Ten ml of the supernatant were collected and kept at -20 °C until further analysis. A 1-ml subsample was collected and evaporated to dryness on a SAVANT Speed Vac Plus evaporator (SC210A) system (Fisher Scientific, ON, Canada) and then re-suspended in 200 µl of demineralised water, vortexed and left at room temperature for 15 min. The

resuspended samples were vortexed, transferred into 1.5 ml tubes and centrifuged at 13,000 rpm. Fifty µl of each sample were analysed by high pressure liquid chomatography (HPLC). The chromatographic conditions were a modification of the separation method described in Schneider *et al*. (1987). The HPLC analytic system was controlled by WATERS Empower software (WATERS, Milford , MA , USA) and was composed of Model 1525 pump, a Model 717 ^{plus} autosampler, and a Model 2487 dual absorbance detector. Organic acids were separated on a Bio-Rad HPX-87H column (Bio-Rad, Hercules, Ca, USA), eluted isocratically at 25 °C at a flow rate of 0.6 mL min^{-1} with 0.008 N sulfuric acid and detected on dual absorbance detector set at 210 nm. Peak identity and organic acid quantity were determined by comparison with standards. The organic acid standard included oxalic acid, citric acid, malonic acid and maleic acid that were well separated under the described chromatographic conditions.

2.2.10 Plant root and shoot analyses

Roots and shoots of the harvested plants were dried at 65 °C for 48 h. Dry weights were recorded and the root and shoot samples were further analysed for potassium and phosphorus content by inductively coupled plasma-atomic emission spectrometer (ICP-OES Optima 4300 DV, Perkin Elmer, Waltham, MA, USA).

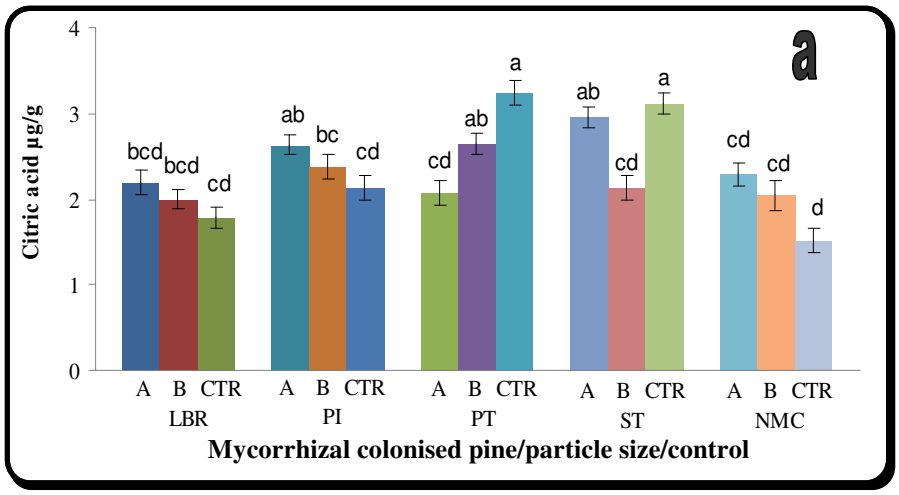
2.2.11 Statistical analyses

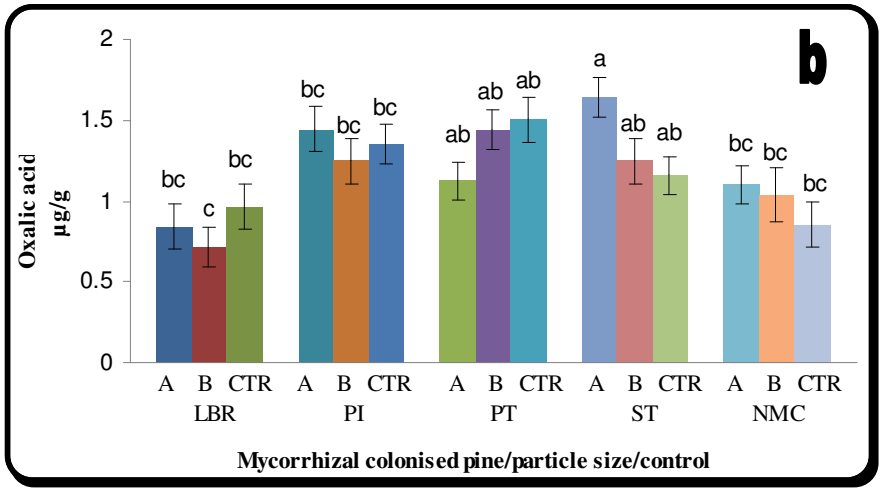
The weathering experiment was designed as a factorial involving the following treatments: 1.) Ectomycorrhizal-colonised plants with iron ore samples (EMC); 2.) Non-mycorrhizal plants (NMC) with iron ore samples; and 3.) Controls (CTR) for both EMC (EMC with no iron ore) and NMC (NMC with no iron ore). The statistical analyses for the weathering experiment therefore involved comparison of these treatments i.e. EMC, NMC and CTR. For organic acid analysis (oxalic, citric, maleic and malonic acids) and other dependant variables, two-way Anova model was used that included the plants colonised by the four fungal types for EMC and the NMC (i.e. PT, PI, ST, LBR and NMC), 3 levels of particle size (A, B and CTR). Similarly, for %K and %P loss, a two-

way Anova model was used with one group consisting of EMC and NMC (as described earlier) and the other consisting 2 levels of particle sizes (A and B). Following significant effects, multiple comparisons with the step down Bonferroni method were conducted to identify the differences between the treatments.

2.3 Results

The mycorrhizal synthesis experiment was successfully carried out with colonisation of between 40 and 100% recorded for all the four types of fungi used and more than half the entire population (EMC) having more than 60% colonisation rates. These levels of mycorrhization were enough to assess the effects of mycorrhization on the weathering process. The statistical analyses revealed different significant effects of both dependent and independent factors. For the organic acid analysis, there was a statistically significant interaction (P< 0.0001) between the fungal type, particle size and organic acids produced during the experiment.





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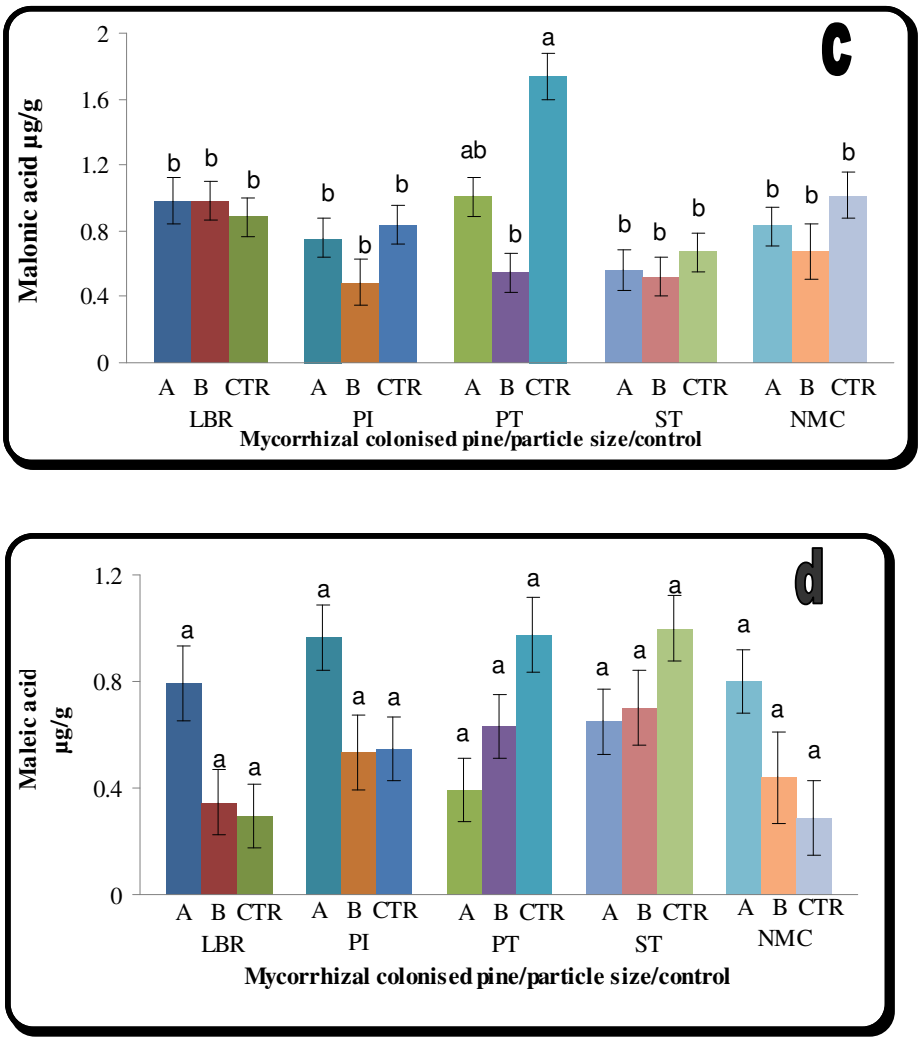


Figure 2.1: Amount of Citric acid (a), Oxalic acid (b), Malonic acid (c) and Maleic acid (d) released by the four EMC (LBR, PI, PT, ST) and the NMC roots grown in the presence of KGT iron ore (particle sizes A and B; CTR - control without iron ore). Bars with the same number are not significantly different (P<0.0001).

In general, citric acid was the highest produced organic acid detected in the soil samples of all the treatments (Fig. 2.1 a). Control and EMC (particle size A for ST and B for PT) treatments of both PT and ST produced the highest amount of citric acid. However, for

oxalic acid, there was no significant difference in the quantities produced among the EMC and CTR treatments, except for LBR and NMC/CTR treatments with statistically significant lower values (Fig. 2.1 b). There was no significant difference between the quantities of maleic and malonic acid detected from all the EMC and CTR treatments (Fig. 2.1 c and d).

Table 2.1: Two-way analysis of variance (ANOVA) models with F and P values that showed the effects of fungal type, particle size and their interactions on K and P reduction from iron ore as well as the effects on K and P of the soil, shoot and root. The effects of these factors on shoot and root DM were also shown. P values <0.05 are considered significant

Factors	df		Sources of Variation		
			Fungi	Particle size	Fungi vs particle size
		df	4	1	4
%K	**134**	*F*	98.36	193.32	46.95
		P	<.0001	<.0001	<.0001
%P	**134**	*F*	11.13	64.34	6.63
		P	<.0001	<.0001	<.0001
		df	4	2	8
Soil K	**201**	*F*	24.09	1135.82	16.9
		P	<.0001	<.0001	<.0001
Soil P	**201**	*F*	35.63	14.31	5.35
		P	<.0001	<.0001	<.0001
		df	4	2	8
Shoot K	**189**	*F*	15.22	1868.44	8.74
		P	<.0001	<.0001	<.0001
Shoot P	**189**	*F*	11.81	35.43	5.39
		P	<.0001	<.0001	<.0001
		df	4	2	8
Root K	**197**	*F*	52.5	2030.39	35.82
		P	<.0001	<.0001	<.0001
Root P	**197**	*F*	29.44	3.44	13.98
		P	<.0001	0.034	<.0001
		df	4	2	8
Shoot DM	**197**	*F*	138.99	3.56	40.23
		P	<0.0001	0.0301	<0.0001
Root DM	**197**	*F*	265.09	1.84	37.41
		P	<0.0001	0.1616	<0.0001

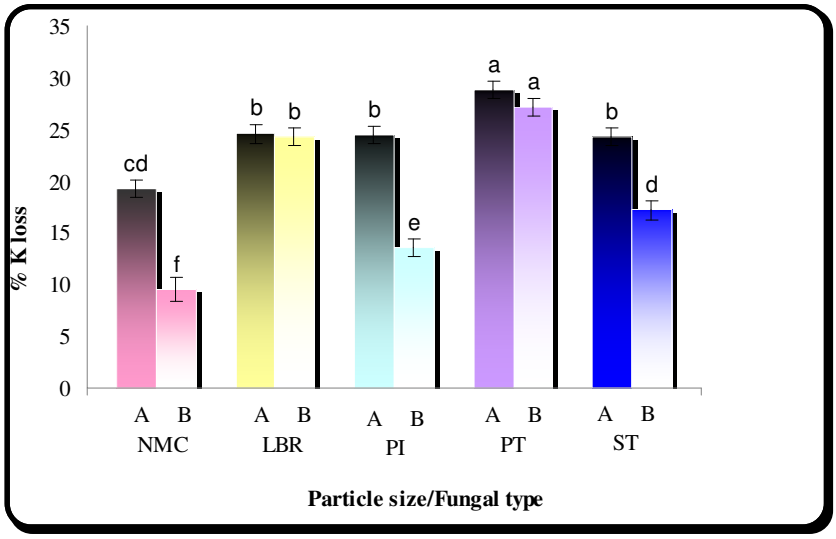


Figure 2.2: Percentage of K reduction from iron ore mineral in response to the four different fungal treatments (PFR, PI, PT and ST) and NMC using two particle sizes (A and B) of the iron ore materials. Bars represent standard errors (P<0.0001).

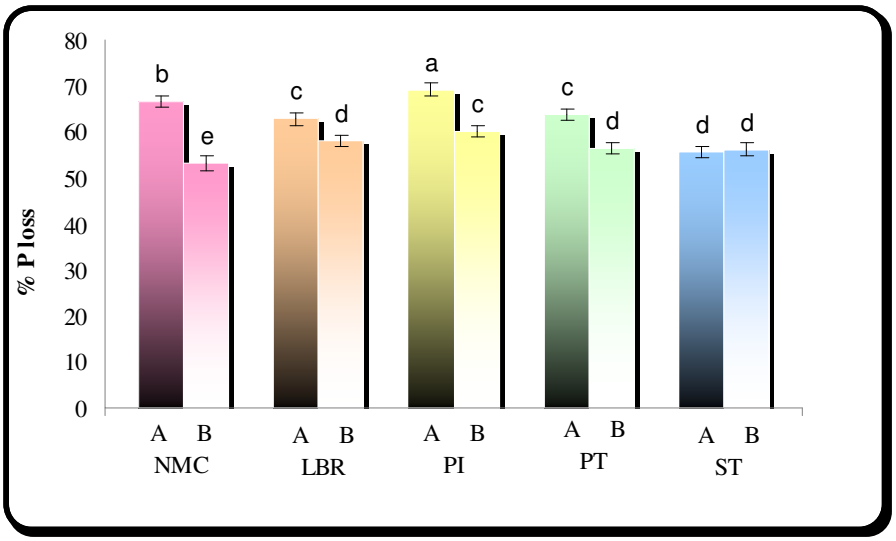
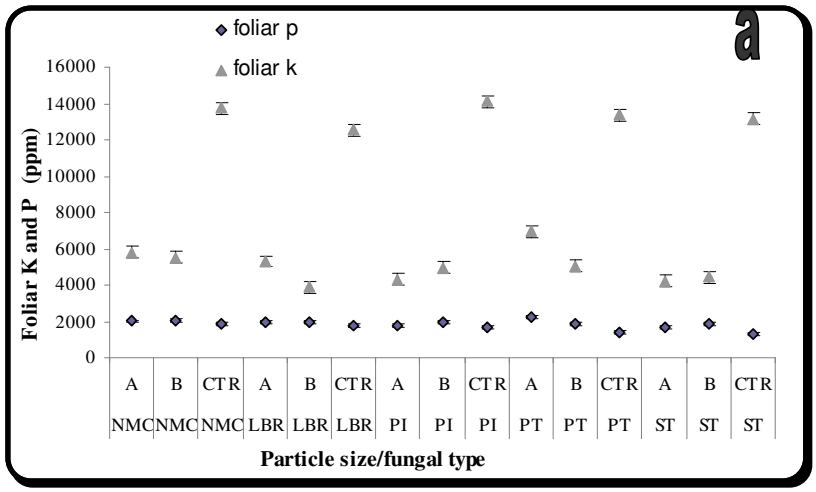
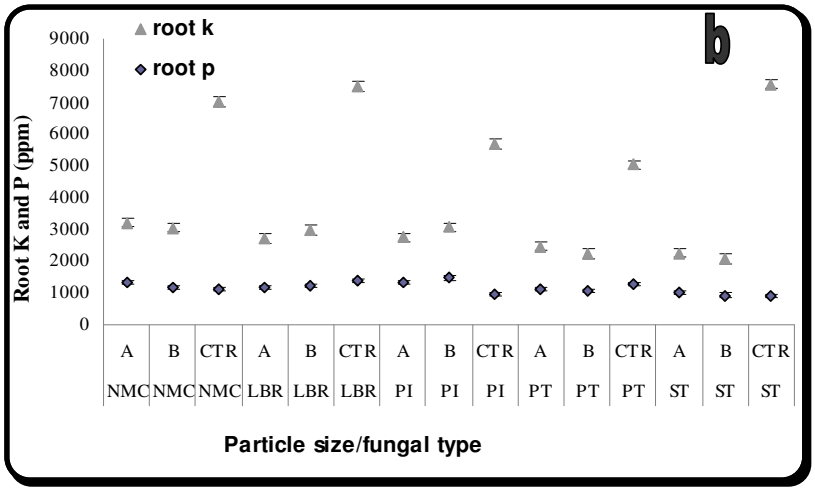


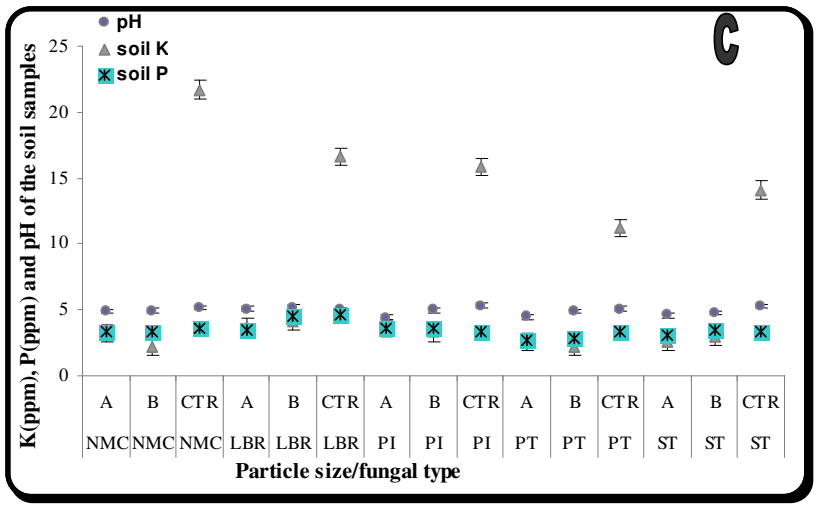
Figure 2.3: Percentage of P reduction from iron ore mineral in response to the four different fungal treatments (PFR, PI, PT and ST) and NMC using two particle sizes (A and B) of the iron ore materials. Bars represent standard errors (P<0.0001).

Fungal type, particle size and the interaction between the two factors significantly affected the mobilisation of K and P from iron ore minerals (Table 2.1). More K and P quantities were removed from treatments involving particle size A compared to B (Fig. 2.2 and 2.3). Highest amounts of % K loss (28.8%) from the iron ore was from treatments containing fungal type PT with particle size A (Fig. 2.2). In addition, the lowest K reduction was from the NMC treatment (particle size B). Similarly, more %P loss was recorded from treatments involving particle size A compared to B (Fig. 2.3). Meanwhile, the highest P reduction from the iron ore was recorded from PI treatment of particle size A (69%), while the lowest (53.16%) was from the CTR treatment with particle size B (Fig. 2.3). In addition, the soil pH was significantly affected by interaction between the fungal type and particle size (Table 2.1). All EMC (except LBR) and NMC plant treatments have a slightly lower pH compared to CTR treatments (Fig. 2.4 c). Therefore, mycorrhizal status seemed not to directly affect the pH status of the soil. Particle size was observed to have statistically significant effect on the soil pH. The pH was lower in presence of particle size A compared to particle size B.

All the interactions between the fungal type and particle size have effects on foliar, root and soil P and K (Table 2.1). Meanwhile, due to the full Hoagland nutrient solution used to fertilise CTR treatments, they had significantly higher values of foliar K compared to EMC and NMC treatments (Fig. 2.4 a). The highest value of foliar K for the EMC treatment was recorded in PT treatment with particle size A, while the lowest was from LBR treatment with particle size B (Fig. 2.4 a). Comparable trend was observed for foliar P where the treatment involving PT (particle size A) had the highest shoot P, while the lowest was ST, particle size A (Fig. 2.4 a). However, there were higher values of foliar P in both the EMC and NMC treatments compared to CTR treatments (Fig. 2.4 a). This is due to the presence of two different phosphate sources for these treatments during the experiment; one in the Hoagland solution and the other from iron ore minerals.


a
foliar p
foliar k
Foliar K and P (ppm)
16000
14000
12000
10000
8000
6000
4000
2000
0
A B CTR A B CTR A B CTR A B CTR A B CTR
NMC NMC NMC LBR LBR LBR PI PI PI PT PT PT ST ST ST
Particle size/fungal type


b
root k
root p
Root K and P (ppm)
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
A B CTR A B CTR A B CTR A B CTR A B CTR
NMC NMC NMC LBR LBR LBR PI PI PI PT PT PT ST ST ST
Particle size/fungal type


c
pH
soil K
soil P
K(ppm), P(ppm) and pH of the soil samples
25
20
15
10
5
0
A B CTR A B CTR A B CTR A B CTR A B CTR
NMC NMC NMC LBR LBR LBR PI PI PI PT PT PT ST ST ST
Particle size/fungal type

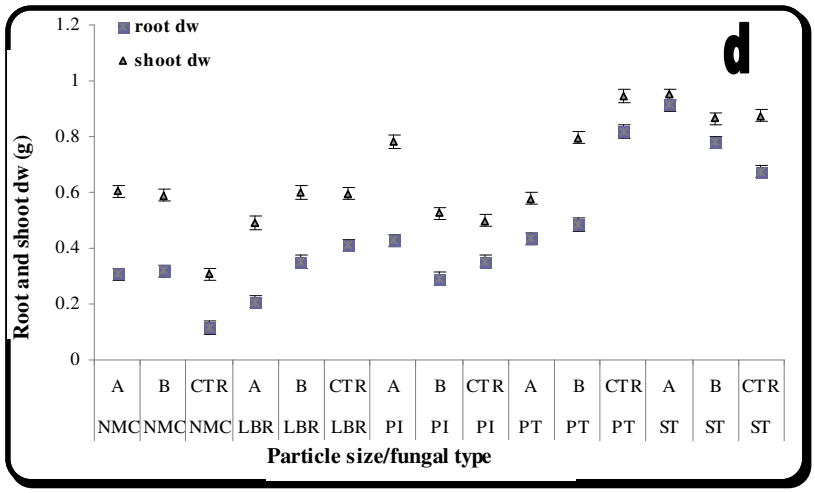


Figure 2.4: Amounts of measured (a) foliar K and P, (b) root K and P, (c) soil K and P, as well as pH, and (d) values of root and shoot DM for EMC (LBR, PI, PT and ST), NMC, as well as the controls (CTR). A and B are the two particle sizes. Bars represent standard errors (P<0.0001).

The NMC plant treatments with particle size A and B had statistically significantly higher root K values compared to EMC plant treatments (Fig. 2.4 b). For the EMC treatments, highest root K was recorded in LBR treatment with particle size B while the lowest was recorded from ST treatment with particle size B (Fig. 2.4 b). Plants with PI treatments retained more P than all other EMC treatments in the roots, while the lowest P root retention was obtained in ST with both particle size A and B (Fig. 2.4 b).

Statistical analyses of the soil nutrient status after the experiment revealed that there was no significant difference between the amount of K in soil with particle size A and B for all the mycorrhizal plant treatments (Fig. 2.4 c). However, as expected, CTR treatments (full hoagland nutrient solution supply), had higher amounts of soil K. Highest value of soil K recorded was from LBR treatment with particle size B, while the lowest was from PT treatment (Fig. 2.4 c). Similarly, values for soil P in both treatments with particle size A and B were not significantly different. The NMC treatments had slightly higher values of P than the EMC plants except, for PI where the values from the CTR treatment was slightly lower than those with iron ore of the two particle sizes (Fig. 2.4 c).

There were significant effects of fungal type, particle size and their interaction on both SDM and RDM (Table 2.1). The highest values of both shoot and root DM were recorded in ST treatments, while the lowest was in the CTR treatment of NMC (Fig. 2.4 d).

2.4 Discussion

Mineral weathering is a process that has been interpreted in different ways to enable its applicability in various industrial applications. Land rehabilitation and remediation, biohydrometallurgy, agroforestry, among others, are some of the technological advancements that have benefited from underlying mechanisms of mineral weathering (Ahonen *et al*, 2000; Vasan *et al*., 2001; Jain and Sharma, 2004; van Scholl *et al*., 2006b; van Scholl *et al*., 2008; Khosla and Reddy, 2008). Traditionally, mineral weathering is believed to largely depend on organic acid availability that affects the mineral dissolution through three major mechanisms. These mechanisms are complexation of cations from the mineral surface, lowering of the pH towards acidic range and selective binding of Fe and Al ions (Drever and Stillings, 1997). Both plant roots and their associated microorganisms have been implicated in organic acid production in the rhizosphere. Of the most important soil microbes that produce organic acids, ectomycorrhizal fungi have been well acknowledged (van Scholl *et al*., 2008). However, because of the complex microbial population mix in the rhizosphere, it is difficult to estimate the organic acid released into rhizosphere by ECM fungi (van Scholl *et al*., 2008). Nevertheless, investigators have managed to study this process under sterile and semi-sterile conditions, thereby reducing the risk of over- or under-estimation of the organic acids released by ECM fungi (van Scholl *et al*., 2006b; van Scholl *et al*., 2008).

This study has revealed that pine roots, whether mycorrhial or not, were able to mobilise between 9 and 29% K and more than 50% P from the iron ore sample, thereby confirming their participation in the weathering process. Higher values of P mobilisation indicate that roots are more efficient in P than in K mobilisation. This is in agreement with previous studies that identified both mycorrhizal roots and non-mycorrhizal roots as capable of participating in mineral weathering (Wallander and Wickman, 1999; Calvaruso *et al*., 2006; Calvaruso *et al*, 2009). For instance, Calvaruso *et al*. (2009) reported the participation of both mycorrhizal and non-mycorrhizal pine root in biotite weathering. Furthermore, this study shows that all the mycorrhizal plants had little

significant increase in K mobilisation from iron ore compared to non-mycorrhizal plants, while in contrast, non-mycorrhizal plant showed a better mobilisation of P than most mycorrhizal plant treatments. These contradictory results make it difficult to directly correlate the mobilisation of nutrients from the iron ore to the presence of ECM fungi. On the other hand, the present study clearly demonstrates the efficiency of PT to pass the absorbed K and P onto the shoot system, compared to the other mycorrhizal and non-mycorrhizal treatments. This suggests that PT participated in the absorption and transfer of dissolved mineral nutrients to the plant and the possibility of little drainage loss during the process. As explained by Calvaruso *et al*. (2009), it is possible to have similar efficiency in mineral weathering process between non-mycorrhizal roots and mycorrhizal roots. However, when it comes to the absorption of the dissolved mineral nutrients, mycorrhizal plants have been shown to be more effective (Smith and Read, 2008). This is due to the nature of ECM hyphal network, which usually spread to cover large surface area and to the ability of the fungus to penetrate hard mineral surfaces, while scavenging for nutrients. On the other hand, effective absorption of dissolved mineral nutrients through ECM fungi has also been identified to indirectly facilitate mineral weathering processes (Paris *et al*., 1995; Glowa *et al*., 2003). This can happen when there is a shortage of a particular nutrient in the growth medium. For instance, in the present study, the shortage of K in the nutrient solution available to mycorrhizal plants can favour scavenging as a feeding mechanism by the ectomycorrhizal hyphae (Banfield *et al*., 1999). The mineral constitution of the iron ore is probably another factor that affected the rate of K and P release from the iron ore. Sheng *et al*. (2008) showed in an experiment, involving three silicate minerals, that mineral type could determine the rate of nutrient release from minerals. The iron ore used in this study contained different types of minerals from which the K-bearing mineral was different from P-bearing mineral.

Among the four organic acids analysed from the soil samples in this study, citric acid was the highest produced, followed by oxalic acid. Several studies have highlighted the importance of these two organic acids in mineral weathering due to their high capability to chelate metals (Wallander and Wickman, 1999; Ahonen-Jonnarth, 2000; Smith and Read,

2008; van Scholl *et al*., 2008). Akin to the result obtained in this study, Wickman and Wallander (1999) reported large quantities of citric acid in weathering experiment involving *Pinus sylvestris* seedlings inoculated with *S. variegatus* using biotite mineral as source of K. Similarly, Ahonen-Jonnarth (2000) reported the increased oxalic acid production by *S. variegatus* and *Rhizopogon roseolus* colonised plants when Al concentrations were elevated.

Neither the nutrient (K) limitation nor the addition of iron ore minerals increased organic acid production by both mycorrhizal and non-mycorrhizal plants. For instance, the largest quantity of citric acid was produced by mycorrhizal (PT and ST) treatments that were fertilised with complete Hoagland's solution without iron ore minerals. This is similar to the result obtained by van Scholl *et al*. (2006a), where the P limitation did not increase the organic acid production by ECM plants compared to non-mycorrhizal plants. In that study, ECM colonisation did not increase total organic acid compared to non-mycorrhizal fungi, but affected the total organic acid production depending on the fungal species. With no statistically significant difference between the amount of citric acid detected in treatments with highest (PT) and lowest (NMC/CTR) K mobilisation, the quantity of organic acid produced in the present study can therefore, not be directly correlated to mobilisation of either P or K from the iron ore minerals. This suggests that mineral weathering occurred as a result of combination of factors; not only in response to organic acid. This view was shared in the review by Banfield *et al*. (1999) where it was explained that other mechanisms such as scavenging by microbes can lead to mineral weathering. This is contrary to the findings of Wallander and Wickman (1999), where weathering by *Suillus variegatus* was linked to the production of citric and oxalic acid. However, caution must be exercised in the interpretation of organic acid result because the response could vary with soil type, treatments and storage (Mimmo *et al*., 2008).

Another factor that significantly affected the weathering process in this study was the particle size. Generally, there were more nutrients release from larger particle size A than B. This is in contrast to the result obtained by Modak *et al*. (2001) that showed better mobilisation of calcium from finer particle size mineral and concluded that exposure of

larger surface area (finer particle size) of mineral particle to leaching agent increases the possibility of solubilisation. However, the opposite result obtained in the present study may be due to aeration of the growth medium. Larger particle size ores may allow more aeration of the growth medium, a factor which has been mentioned to affect mineral weathering by microorganisms (Calvaruso *et al*., 2006). Similar effects of aeration were also suggested by Kazantseva *et al*. (2009), especially for mycorrhizal plants. In addition, the importance of aeration to the functionality of mycorhizal and non-mycorrhizal roots was highlighted in the study by Wallander and Wickman (1999), where improper aeration was suggested as a factor affecting the weathering process.

In contrast to previous studies where pH of soil was shown to be lowered by mycorrhizal plants as compared to non-mycorrhizal plants (Cromack *et al*., 1979; Berthelin, 1983; Arocena and Glowa, 2009), the result in the present study showed that non-mycorrizal plants can also lower the pH of the growth medium. This suggests the capability of non-mycorrhizal roots to also participate in weathering processes. Furthermore, statistical analyses of the pH values indicated that mycorrhizal colonisation had little effects on K and P mobilisation, a situation which may be connected to significant effect of interaction between fungal type and particle size. Another reason could be the causal effects of pH, which may lead to the oxidation of inorganics such as sulfur, organic acid production and high rate of NH_4^+ uptake by ECM plant roots (Berthelin, 1983; Marschner *et al*., 1987; Arocena and Glowa, 2009). The lower pH in the presence of particle size A compared to B can be connected to positive effects of proper aeration (Calvaruso *et al*., 2006) on soils with bigger particle size of iron ore. This may allow better metabolic activities in the soil, thereby lowering the pH to favour mineral weathering. Statistical results of pH from non-mycorrhizal, PI, PT and ST treatments with iron ore minerals compared to their respective CT, suggest that the presence of iron ore minerals and probably the omission of K from the Hoagland solution, lowered the pH of the soil. The only exception was the LBR where the presence of iron ore minerals raised the pH of the soil. This is similar to the result of Scholl *et al*. (2006b) that reported how hornblende addition affected the pH, and concluded that the effect of pH can be species-specific.

The mycorrhizal inoculation seems to have positive effects on the health of the plants (especially for ST treatment) when RDM and SDM of both mycorrhizal and non-mycorrhizal plants were compared. This may be due to functionalities of the mycorrhizal hyphal network that penetrates and covers more area as compared to ordinary roots when in search of nutrients (Smith and Read, 2008). However, potassium and phosphorus released / absorbed from the iron ore do not seem to have much effect on the health of the plants, as indicated in the result. ST mycorrhizal treatments did not absorb much K and P compared to other mycorrhiza treatments, but had higher SDM and RDM than PT treatment with the highest K mobilisation. This may be due to the fact that both mycorrhizal and non-mycorrhizal plants need not only K and P for optimal growth but also additional nutrients such as N (Smith and Read, 2008). In addition, effects of other microorganisms cannot be excluded because the experiment was carried out under non-sterile conditions.

In a previous study, Wallander and Wickman (1999) suggested that weathering and release of K from biotite by *Suillus variegatus* occurred because of the fungal production of oxalate and citrate, which promoted plant growth as well as an increase in foliar K. In contrast, Calvaruso *et al*. (2009), observed that colonisation by *Lacarria bicolor* S238N did not cause any increase in biotite weathering when compared to non-mycorrhizal pine, but significantly contributed to plant health through absorption of weathered nutrients. These two findings support the observation in the present study of different rates of weathering and absorption exhibited by the four mycorrhizal fungi investigated. This indicates that weathering roles of ECM fungi is species-specific. Therefore, the results obtained in the present study provided additional information about the differences that exist between weathering, absorption and transfer of nutrient to plants. There are many implications of weathering and other subsequent events that follow the process.

This study has shown that whether mycorrhizal or not, pine roots are able to mobilise nutrients from iron ore minerals. However, depending on the species, mycorrhizal plants can be more effective in nutrient mobilisation compared to non-mycorrhizal plants. If ECM fungi are truly involved in mineral tunnelling then it could be suggested that they

are part of the biotic factors that determine the chemical constitution of minerals. Despite the positive results obtained in this study regarding this hypothesis, there are still more investigations that need to be carried out. This is very necessary in a natural environment where presence of ECM fungi could be directly linked to changes in chemical constitution of minerals. Long term studies are needed and these could be conducted by comparing minerals in ECM infested environments to those in non-ECM environments. Other factors such as biotic and abiotic factors should also be considered.

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CHAPTER THREE

MOBILISATION OF POTASSIUM AND PHOSPHORUS FROM IRON ORE BY ECTOMYCORRHIZAL FUNGI

Abstract

Mutualistic roles of ectomycorrhizal (ECM) fungi have been linked to their ability to produce organic acids that aid in the dissolution of insoluble minerals in the rhizosphere. This ability of ECM fungi was utilised to investigate their potential participation in the mobilisation of nutrients such as phosphorus (P) and potassium (K) from a typical insoluble ore - iron ore. *In vitro* pure cultures of four different ECM fungi; *Pisolithus tinctorius* (PT), *Paxillus involutus* (PI), *Phialocephala fortini* (PFR) and *Suillus tomentosus* (ST), were screened for their ability to mobilise P and K from two types of non-exportable Sishen iron ore. When present in iron ore, these elements are deleterious and reduce the commercial values of the ore. Experiment was set up with different treatments that included two ore types (KGT and SK) and five particle sizes of each ore type. Results indicated the potential of the four fungi to mobilise P and K from the two iron ore types though at different levels. Ore type, particle size, organic acid production and attachment of the fungi to the iron ore were all found to play important roles in the mobilisation of nutrients from these ores.

3.1 Introduction

Different kinds of interactions exist between microorganisms and minerals in the environment (Erlich, 1997; Gadd, 1999). These interactions have been extensively studied in the mining industries to produce technologies that are beneficial such as mineralisation, bioremediation and biohydrometallurgy (Ehrlich, 1997; Gadd, 1999; Rawlings, 2002). Though many studies have been conducted in most of these areas, there are still some gaps in the understanding of the full potential and applicability of microorganisms such as ectomycorrhizal (ECM) fungi.

On the basis of available literature, Rinaldi *et al.* (2008) conservatively estimated the number of ECM fungi to be 7750 but went further to state that the actual number could be between 20000 and 25000, based on known and unknown species of this group of fungi. These fungi can penetrate hard mineral materials for the purpose of nutrient absorption (van Breemen *et al.*, 2000; Smith and Read, 2008). Their primary role is to search for and absorb nutrients under severe soil conditions on behalf of their host plants (Smith and Read, 1997). Both ecological and weathering roles of ECM fungi have been investigated and linked to their ability to produce metabolites such as organic acids (van Breemen *et al.*, 2000). These acids are low-molecular weight compounds and confirmed to have great potential in the solubilisation of complex or hard mineral materials (van Breemen *et al.*, 2000; Paris *et al.*, 1995; Gadd, 1999). These acids are naturally produced by different types of microorganisms in the environment but production seems to depend on the environmental conditions (Burgstaller and Schinner, 1993; Erlich, 1997). For instance, ECM fungi exude organic acids in the rhizosphere in order to initiate a chain of chemical reactions that lead to the breaking down of hard mineral materials. Shortage of base cations in the growth medium and the presence of some minerals in the growth medium can increase the production of organic acid by ECM fungi (van Scholl *et al.*, 2006a). These attributes are the theoretical base for the consideration of ECM fungi as

potential candidates for mobilisation of nutrients from some mineral ores such as the low-grade iron ore.

In South Africa, there is a huge deposit of iron ore that is hosted by the Palaeo-Proterozoic Transvaal Supergroup in the Northern Cape Province of the country; it is currently being exploited by Sishen mine (Carney and Mienie, 2003). The Transvaal Supergroup consists of different subgroups such as Campbell rand, Asbestos Hills and Gamagaga subgroups. High grades iron ore are usually associated with the Asbestos Hills subgroup, while the medium and lower grade iron ore are found in the Campbell Rand and Gamagara subgroups respectively (Carney and Mienie, 2003). The low grade iron ore of Sishen mine has high K (>0.24%) and P (>0.03%) contents that diminishes their market value and could render them non-exportable because of their negative impact on the smelting process in the blast furnace (Parks *et al*., 1990; Yusfin *et al*., 1999; Williams and Cloete, 2008; Delvasto *et al*., 2009).

Most studies in this area have adopted bacteria to clean up such low grade ores especially in biohydrometallurgy of sulfide minerals (Jain and Sharma, 2004; Rawlings, 2005). However, in the leaching of non-sulfidic minerals such as silicate, carbonate and oxide minerals that cannot be directly attacked by sulfur-oxidising bacteria, fungi are the more suitable candidates (Burgstaller and Schinner, 1993; Jain and Sharma, 2004; Rawlings, 2005; Williams and Cloete, 2008). In the leaching of non-sulfide ores, only two fungal types; *Aspergillus* spp. and *Penicillium* spp., have received considerable attention (Burgstaller and Schinner, 1993). In the study conducted on *Aspergillus* and *Penicillium* by Valix *et al*. (2001) it was discovered that a combination of both microbial activities and production of organic acids produced effective leaching of cobalt and nickel. In another study, Castro *et al*. (2000) confirmed the better potential of *A. niger* compared to bacteria (*Bacillus* and *Pseudomonas*) in the leaching of zinc and nickel. *Aspergillus niger* HNA-1 isolated from the surface of iron ore samples was also used by Delvasto *et al*. (2005) for the desphosphorisation of iron ore, while Williams (2008) was able to use metabolites produced by *Aspergillus* sp. for the reduction of P and K content of iron ore.

Despite the remarkable progress that has been made in the use of *Aspergillus* and *Penicillium* spp. for biohydrometallurgical processes, the health implication of their aerosols and the mycotoxins they produce to both human health and agricultural products are of serious concern (Desjardins and Hohn, 1997; Gorny, 2004) . The focus on the use of fungi in biohydrometallurgy should therefore extend beyond their leaching potentials, but should also include their immediate and long-term impact on the environment. Having established the positive effects of ECM fungi in the areas of weathering and nutrient cycling (Smith and Read, 2008), it is interesting to investigate the ability of these fungi in the mobilisation of P and K from a typical mineral ore - Sishen iron ore. The objectives of this study were therefore to investigate the possible biobeneficiation potentials of four ECM fungi and establish the link between the production of organic acids, particle size, ore type and mobilisation of nutrients from ores by ECM fungi.

3.2 Materials and Methods

3.2.1 Origin of fungal isolates and iron ore preparation

Four different isolates were used in this study. These included *Pisolithus tinctorius* (PT)- # PT 7 (Plant Health Care Inc., Pittsburgh, USA), *Paxillus involutus* (PI) - NOF 2340 (Canada), *Phialocephala fortini* (PFR)- PB6B (South Africa) and *Suillus tomentosus* (ST) - UAMH 6252 (Canada).

Two different types of iron ore samples were supplied by Kumba Iron Ore, Ltd. and were originally characterised by the company as KGT (conglomerates) and SK (shale). Particle size effects were also investigated by using different particle sizes of the iron for the experiment. The ore materials (original size of 120 mm to 450 mm - as supplied by the company) were milled and sieved into different particle sizes of <3.36 mm to >1.68 mm, <1.68 mm to >0.84 mm, <0.84 mm to >0.21 mm, <0.21 mm to >0.1 mm, and <0.1 mm. Henceforth, these would be referred to as particle sizes A, B, C, D and E, respectively. Pretreatments of the iron ore samples are as stated in chapter two (section 2.2.2).

3.2.2 Media preparation

Cultures of the ECM fungi were prepared and maintained on Modified Melin Norkrans (MMN) medium (Marx, 1969). Liquid MMN medium was used for this experiment with the source of K omitted and that of magnessium halved. Quantity of P was also reduced because of the omission of KH_2PO_4. The final concentration of the culture medium contained: Malt extract (3 g/l), Glucose (10 g/l), $(NH_4)_2HPO_4$ (0.25 g/l), $MgSO_4.7H_2O$ (0.075 g/l), $CaCl_2.H_2O$ (0.067 g/l), NaCl (0.025 g/l), $FeCl_3.6H_2O$ (0.001 mg/l) and thiamine (100 µg/l). Each of the 100 ml flasks used contained 2.4 g of iron ore per 40 ml of the adjusted concentration of MMN medium while the controls had no iron ore but complete composition of the MMN with 0.5g/l (0.02g/40 ml) of KH_2PO_4 and 0.15g/l (0.006g/40 ml) of $MgSO_4.7H_2O$. Another control was set up to test the media effects

using only iron ore of different particle sizes with the MMN. There were four replicates for each treatment. The flasks used for the experiment were incubated at 28ºC for 28 d and shaken at 100 rev/min.

3.2.3 Organic acid analyses by High Performance Liquid Chromatography (HPLC)

At the end of the experiment, the concentration of the organic acids secreted by the fungi in the growth media was assessed. The growth medium was passed through filter paper (0.45 µm) to separate the fungi and iron ore from the medium of growth. Collected media were homogenised by vortexing, then centrifuged for 180 s at 16060 rpm and the supernatant frozen at –40^{o}C prior to analysis by HPLC.

A volume of 50 µl of the sample was used for HPLC analysis. The chromatographic conditions were a modification of the separation method described in Schneider *et al*. (1987). The HPLC analytic system was controlled by WATERS Empower software (WATERS, Milford, MA, USA) and was composed of a Model 515 pump and a Model 717 ^{plus} autosampler, and a Model 2487 dual absorbance detector. Organic acids were separated on a Bio-Rad HPX-87H column (Bio-Rad, Hercules, Ca, USA) eluted isocratically at 40 °C at a flow rate of 0.6 mL min^{-1} with 0.008 N sulfuric acid and detected on a dual absorbance detector set at 210 nm (Waters, Model 2487). Peak identity and organic acid quantity were determined by comparison with standards. The organic acid standard included oxalic acid, citric acid, malonic acid, maleic acid, lactic acid, acetic acid and formic acid that were well separated under the described chromatographic conditions.

3.2.4 Chemical analyses for nutrients absorbed by the ECM fungi

Based on the result from the mobilisation of P and K from the iron ore samples, further processing of the dried fungal mycelia (65 ^{o}C) and the remaining growth medium were

carried out to analyse the nutrients absorbed by the ECM fungi. This assessment was only done for particle size E treatment. For the digestion of the dried ECM mycelia, samples were dried in the oven at 70 °C, ground in a Wiley mill and passed through a 2-mm sieve screen. A 0.5 g quantity of the each sample was then digested in a sulfuric acid and hydrogen peroxide mix (Parkinson and Allen, 1975). Determinations of P and K were made by Induction Coupled Plasma Optical Emission Spectrometer ICP-OES Optima 4300 DV (Perkin Elmer, Waltham, MA, USA).

3.2.5 Microscopy

Scanning electron microscopy (SEM) was used to view the physical interaction of the ECM fungi with the ore samples. There was random selection of samples from replicates of different treatments for microscopic analysis. The samples were fixed with a solution that contained 2.5% glutaraldehyde, 4% paraformaldehyde in sodium cacodylate buffer, 0.1 M pH 7.3, for 24 h. Fixation was followed by washing with cacodylate buffer three times for 10 min. Post-fixation was carried out with osmium tetroxide (1% in cacodylate buffer), for 90 min, followed by another round of washing with cacodylate buffer, three times for 10 min. The samples were then dehydrated at different alcohol concentrations (50, 70, 95 and 100%) for 10 min each. The dehydrated samples were then soaked in 100% alcohol for an initial period of 40 min and then for another 10 min. This stage was followed by soaking the samples in hexaméthyldisilazan two times for 30 min. Finally, specimens were then air-dried and later coated with gold/palladium. They were then assembled for observation under the microscope at 30 kV on a JEOL 6360LV scanning electron microscope (Tokyo, Japan).

3.2.6 Elemental analyses

X-ray fluorescence (XRF) analyses of iron ore was used to detect K, P and total Fe of the ore samples at the beginning and the end of the experiment. The iron ore samples were collected from the flasks after the treatment. The samples were dried at 110 ^{o}C. Tungsten

carbide milling vessel was used to pulverize the Iron ore samples to a particle size of <75 µm. This was followed by the determination of Lost of Ignition (LOI) which involved roasting of the milled samples at 1000 ºC. Samples (1 g) of the milled iron ore were added to 6 g $Li_2B_4O_7$ and fused into glass beads. Minor elemental analysis was carried out from the powder briquette made from the remaining part of the sample while the major elemental analyses were performed on the fused bead using an ARL 9400XP+ spectrometer at University of Pretoria.

3.2.7 Statistical analyses and experimental design

The statistical analyses were done using SAS software, version 9.2 (SAS Institute 2008, Cary, NC, USA). There were three fixed factors for these analyses and these include fungal isolate (PFR, PI, ST, and PT), iron type (KGT, SK) and particle size (A, B, C, D, E). In addition to these 40 (4X2X5) combination of treatments, there was one control for each fungal isolate consisting of four replicates of isolates PT, PI, PFR and ST and complete MMN medium without iron ore to give a total of 44 treatments. Each of these treatments was replicated four times to give a total of 176 observations.

Dependent variables were in three groups including aqueous P (P_{aq}) and K (K_{aq}) analysed from growth medium as well as P and K from the dried mycelia (P_{dw} and K_{dw}) of the fungi making up the first group while the second group included percentage K and P loss by the iron ore (K_{Loss}, P_{Loss}). K_{Loss} and P_{Loss} were calculated by subtracting the final value of K or P from the initial value prior to the treatments. The total was divided by the initial value of K or P and multiplied by 100. The third group includes organic acids released into the growth medium by the fungi. These were oxalic, citric, maliec, malonic, lactic, fornic and acetic acids. For the first group of dependant variables (P_{aq}, K_{aq}, P_{dw} and K_{dw}), the two iron types and the four fungal isolates were used. All observations in this group had a particle size E and the controls were excluded because of the very high values of P and K in the controls. This was as expected because of the complete MMN medium used in the controls, and the values obtained from the controls interfered with the proper

analysis and comparison of P and K in the different fungal treatments for this group of dependent variable.

The statistical analysis was designed to compare all levels of fixed factors for each dependant variable. The analyses carried out therefore included a 2-way ANOVA (4 fungal type X 2 iron type) for P_{aq}, K_{aq}, P_{dw} and K_{dw}, and a 3-way ANOVA(4 fungal type X 2 iron type) for K_{Loss} and P_{Loss}. For all other dependant variables, an ANOVA was conducted with a factorial 4X2X5 treatment structure to which 4 controls were added. The ANOVA table for this treatment structure was constructed by the way of contrasts to study the main effects of fungal type, Iron type and Particle size, their interactions and to test the difference between treatments and controls. Following a significant effect, multiple comparisons with the step down Bonferrori method were conducted to identify the differences between the treatments. This method was used to control for the type I error rate.

All the assumptions of any Anova model were verified. The homogeneity of variances was verified using the Bartlett's test and the normality assumption was tested with Shapiro-Wilk's statistic. For all the organic acid models, a logarithmic transformation was applied to the data in order to attain normality and homogeneity of variances.

3.3 Results

3.3.1 Mobilisation of K and P in relation to particle size and organic acid production

The XRF analyses of the two Sishen iron ores revealed their chemical composition to contain the following (average of six samples) - SiO_2 (31.6%), Al_2O_3 (2.8%) and Fe_2O_3 (61.30%) and trace values of TiO_2, CaO, MgO, Na_2O, MnO, Cr_2O_3, NiO, V_2O_5 and ZrO_2 for SK. Furthermore, KGT had SiO_2 (4.87%), Al_2O_3 (3.26%) and Fe_2O_3 (90.70%). Trace values of TiO_2, CaO, MgO, Na_2O, MnO, $Cr2O_3$, NiO, V_2O_5 and ZrO_2 were also present in KGT ore type. The SK ore type contains average values of 0.56% and 0.0685 % for K_2O and P_2O_5 respectively while KGT has average values of 0.995% and 0.152% for K_2O and P_2O_5 respectively. The major K_2O bearing mineral contained in both SK and KGT ores was characterised as muscovite by Sishen iron ore company (Richards, 1990 - 1992). The P-bearing minerals are apatite, woodhouseite, goyasite and gorceixite for KGT ore type, while those for SK has not been characterised because of low levels of P contained in this ore (Richards, 1990 -1992; Ogilvie, 2002).

However, full mineralogical characterisation of the Sishen iron ore is not available but from past data obtained through unpublished work, the following were listed: KGT contains minerals such as haematite, greenalite, muscovite, nacrite, apatite, woodhouseite, goyasite and gorceixite. For SK, minerals such as haematite, biotite, greenalite, illite, muscovite, nacrite, magnetite, quartz and siderite were listed. Fractions of these minerals contained in the mineral ores can differ from one site to another but haematite is the major mineral contained in both KGT and SK, while biotite is present as a minor mineral for SK ore type. All other minerals are in trace quantities and they may not be present in each of these minerals at all times.

The XRF results were calculated in terms of percentage quantity of K and P removed from the iron ore. The mobilisation was found to be significantly affected by fungal type,

ore type and particle as well as some of their interactions (Table 3.1) but could not be linked directly to the organic acid production (Fig. 3.1, Fig. 3.2 and Fig. 3.3). In both ore types, particle size was discovered to have "restricted effect" on the mobilisation of K and P. This effect was most observed in treatments involving particle size E where all the fungi used produced significant removal of K from the iron ore (Fig. 3.1A and 3.1B).

Table 3.1: Three-way analysis of variance (ANOVA) with F and P values that show the effects of the fixed factors – fungal type, mineral type, particle size and their interactions on %K and % P reduction from the iron ore materials.

Sources of Variation	**df**	**% K loss**		**%P loss**	
		df= 109		**df= 97**	
		F	*P*	*F*	*P*
Fungal type	3	32.63	<.0001	25.24	<.0001
Iron type	1	45.07	<.0001	475.85	<.0001
Fungal type vs Iron type	3	54.46	<.0001	37.87	<.0001
Particle size	4	209.11	<.0001	28.13	<.0001
Fungal type vs Particle size	12	8.72	<.0001	8.36	<.0001
Iron type vs Particle size	4	51.99	<.0001	3.27	0.0147
Fungal vs Iron type vs Particle size	12	11.63	<.0001	6.2	<.0001
Fungal type	3	32.63	<.0001	25.24	<.0001

P values <0.001 are considered significant.

Mobilisation of K from KGT treatment was best in particle size E (Fig. 3.1A) in all the ECM fungi with no significant difference among the fungal treatments. Values recorded here are far from the value needed to meet the commercial standard value of 75.88%. Meanwhile, for SK ore type, only PFR (Fig. 3.1B) was able to mobilise enough K (65.40%) from the iron ore, which is significantly above the commercial standard of 57.14%. There is however, no significant difference in the highest K leached from ST, PT and PI from particle size E treatment of SK ore type (Fig. 3.1B).

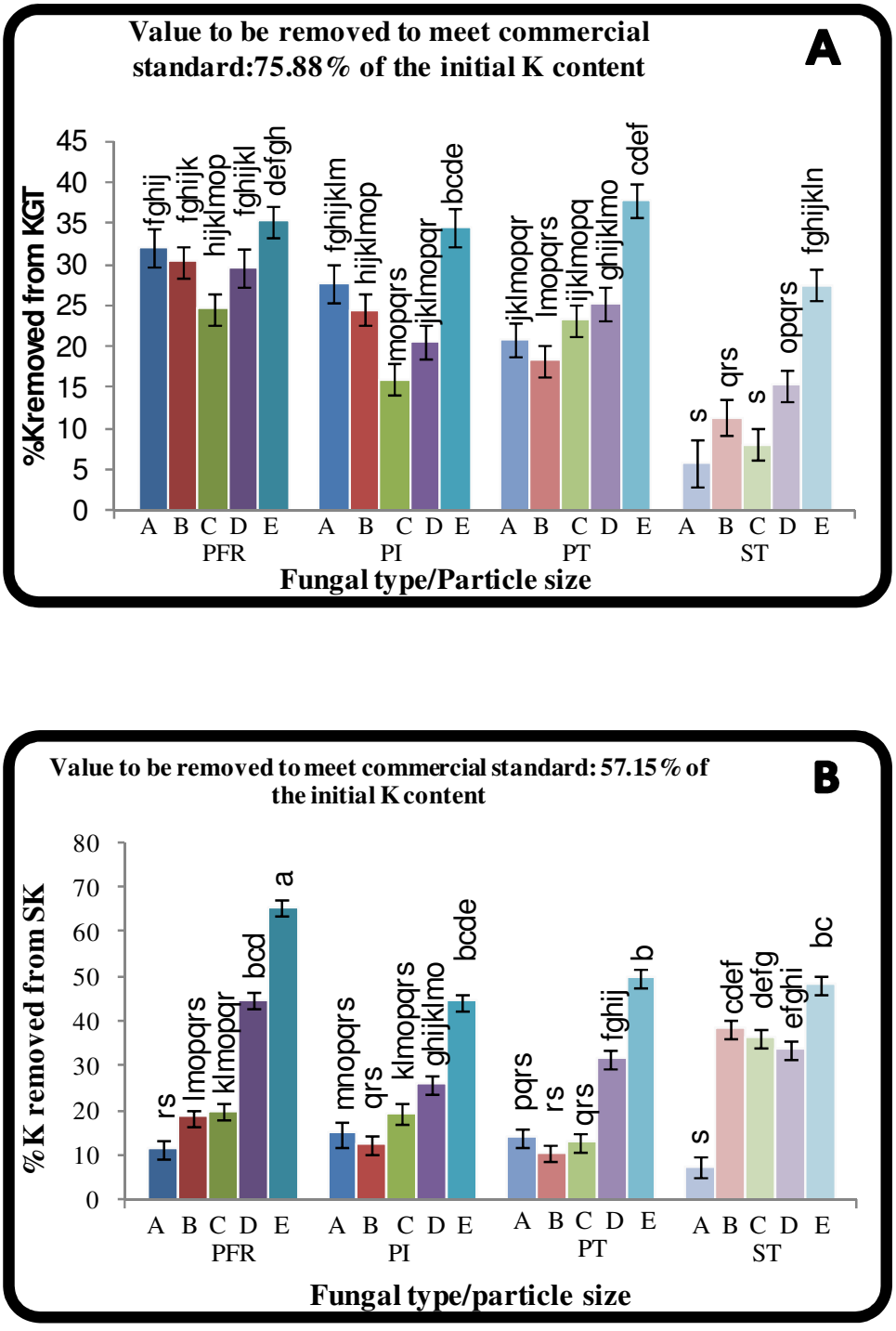
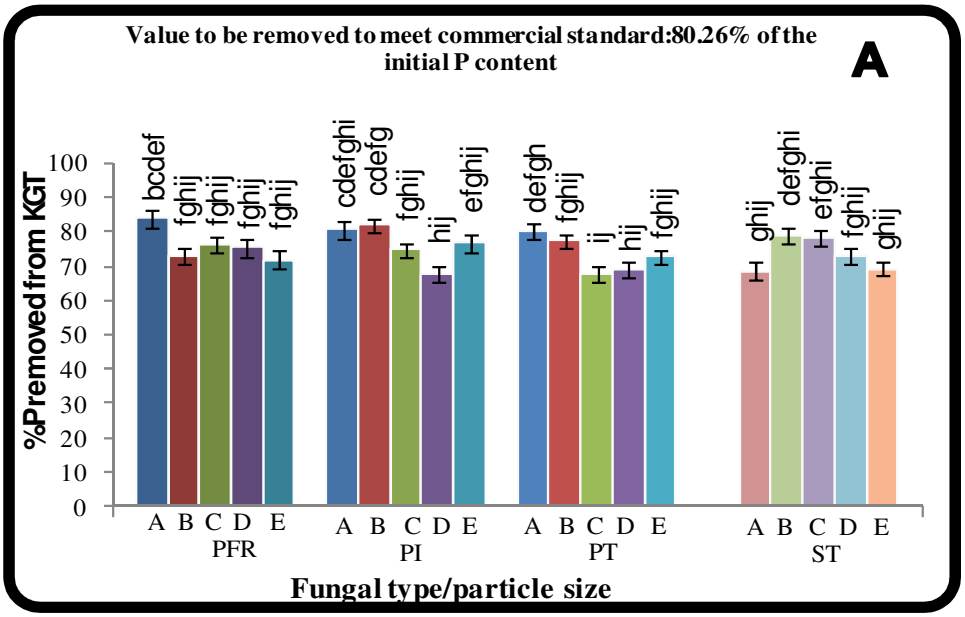


Figure 3.1: Percentage of K reduction from mineral type KGT (**A**) and mineral type SK (**B**) in response to the four different fungal treatments (PFR, PI, PT and ST) using five particle sizes (A,B,C,D and E) of the iron ore materials. Bars with the same letter are not significantly different. (P<0.0001).

The mobilisation of P seemed not to be affected much by particle size (Fig. 3.2 A and 3.2B) except in SK treatment where PI and ST produced less mobilisation from the smaller particle size C, D and E (Fig. 3.2 B). In general, better P removal in the iron ore was recorded in larger particle sizes of the iron ore (Fig. 3.2 A and 3.2 B). Only PFR, PI and PT (particle size A) were able to reduce the P level beyond the commercial standard (80.26%) in KGT treatment (Fig. 3.2 A). In addition, all the fungi reduced the P levels in SK beyond the commercial standard of 56.21% (Fig. 3.2 B). It is also important to mention that the least value of total Fe recorded in both ore types after the leaching experiment was >60%.



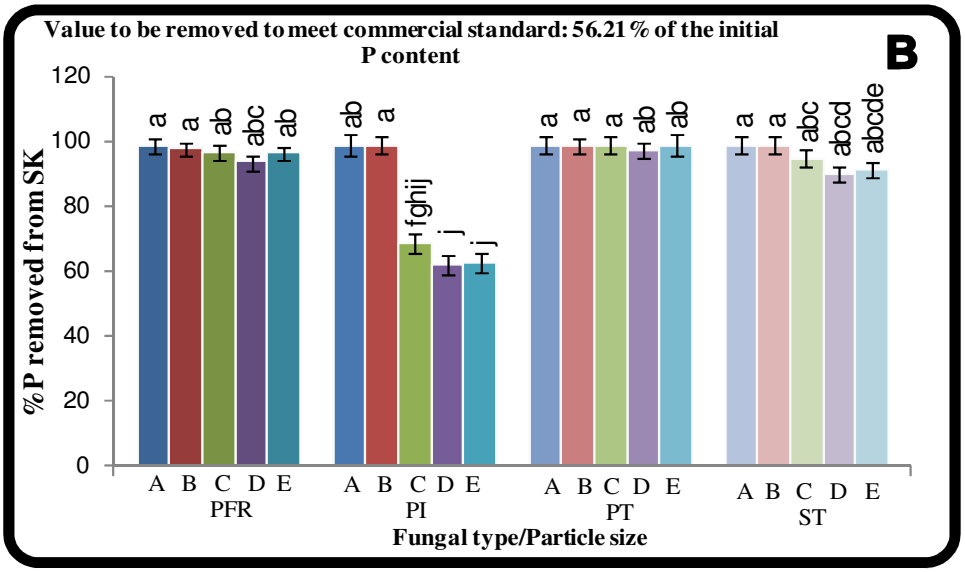


Figure 3.2: Percentage of P reduction from mineral type KGT (A) and mineral type SK (B) in response to the four different fungal treatments (PFR, PI, PT and ST) using five particle sizes (A,B,C,D and E) of the iron ore materials. Bars with the same letter are not significantly different. (P<0.0001).

3.3.2 Organic acid production

All the seven organic acids tested were detected in the medium of growth for all the four ECM fungi studied. As shown in Table 3.2, the release of organic acid was found to be significantly affected by the interactions among the factors: particle size, ore type and fungal type.

Table 3.2: One-way analysis of variance (ANOVA) with F and P values that show the effects of the fixed factors – fungal type, mineral type, particle size and their interactions on the release of seven different organic acids.

Sources of Variation		**Oxalic acid**		**Citric acid**		**Maleic acid**		**Malonic acid**		**Lactic acid**		**Formic acid**		**Acetic acid**	
		df= 124		**df= 125**		**df= 122**		**df= 122**		**df= 125**		**df= 128**		**df=116**	
		F	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Fungal type	3	710.38	<.0001	655.73	<.0001	279.64	<.0001	570.95	<.0001	1034.5	<.0001	79.35	<.0001	2.83	<.0001
Iron ore type	1	0.96	0.3285	21.83	<.0001	55.87	<.0001	9.02	0.0032	5.45	.021	8.31	<.0001	07.5	<.0001
Particle size	4	273.46	<.0001	450.94	<.0001	171.77	<.0001	37.77	<.0001	190.9	<.0001	9.78	<.0001	64.79	<.0001
Fungal type vs Iron type	3	68.94	<.0001	35.18	<.0001	100.23	<.0001	86.59	<.0001	31.63	<.0001	101.33	<.0001	79.67	<.0001
Fungal type vs Particle size	2	33.63	<.0001	16.8	<.0001	26.06	<.0001	28.3	<.0001	80.33	<.0001	2.25	<.0001	37.06	<.0001
Iron type vs Particle size	4	13.08	<.0001	5.21	<.0001	5.19	0.0007	25.38	<.0001	43.02	<.0001	9.46	<.0001	12.01	<.0001
Fungal type vs Iron type vs Particle size	2	14.02	<.0001	46.23	<.0001	6.23	<.0001	21.6	<.0001	13.2	<.0001	21.68	<.0001	56.82	<.0001
Iron vs Control	4	70.18	<.0001	2.37	<.0001	32.94	<.0001	9.1	<.0001	123.53	<.0001	34.29	<.0001	5.73	<.0001

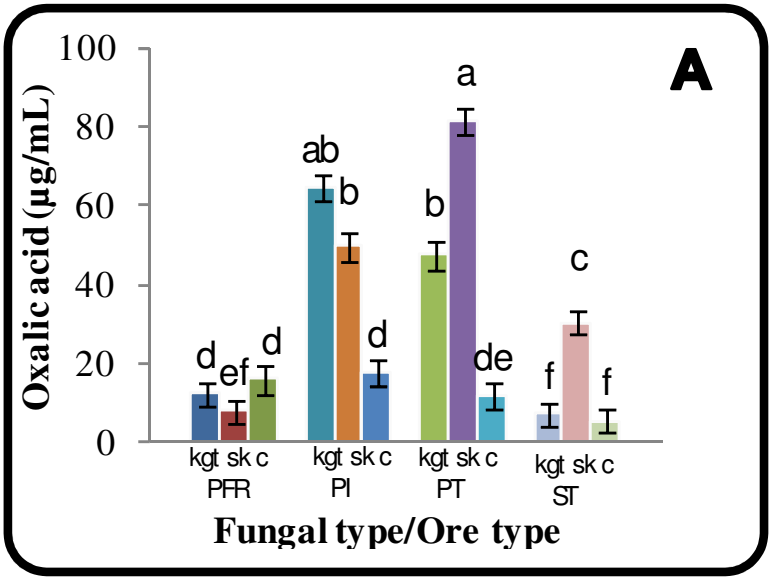
P values <0.0001 are considered significant.

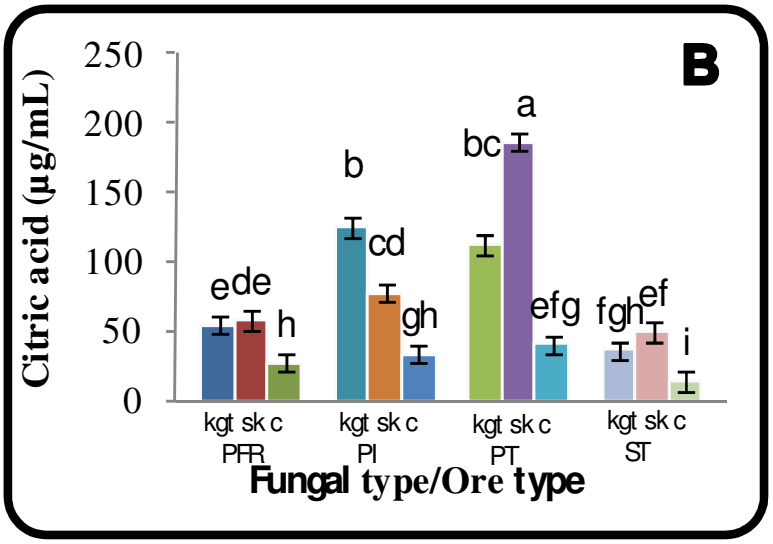
Table 3.3: Two-way analysis of variance (ANOVA) with F and P values that show the effects of the fixed factors – fungal type and mineral type as well as their interactions on the K and P accumulated in the spent broth and fungal dried mycelia.

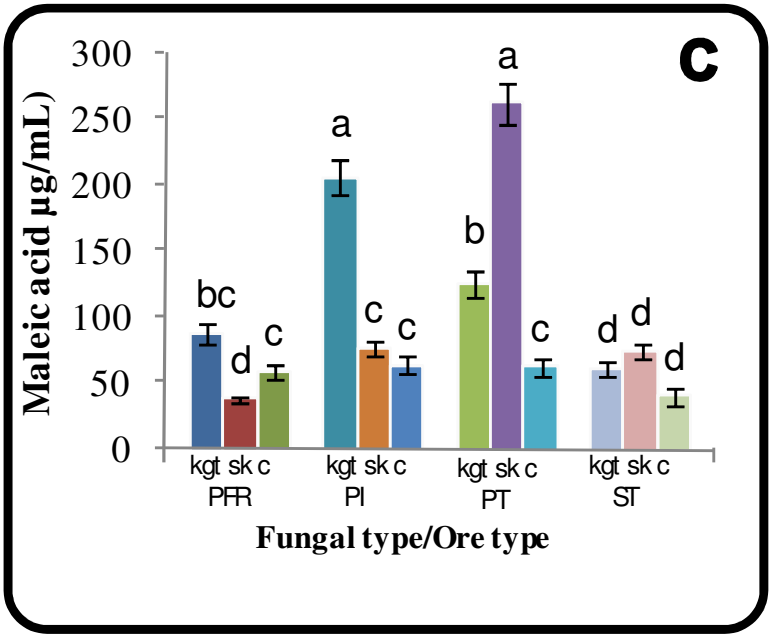
Sources of Variation	**df**	**Paq**		**Kaq**		**Pdw**		**Kdw**	
		df= 24		**df= 24**		**df= 24**		**df= 24**	
		F	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Fungal type	3	49.46	<.0001	102.82	<.0001	32.31	<.0001	20.26	<.0001
Iron ore type	1	12.92	0.0015	3.07	0.0927	0.64	0.4326	26.13	<.0001
Fungal type vs Iron ore type	3	6.94	0.0016	7.1	0.0014	5.74	0.0042	17.84	<.0001

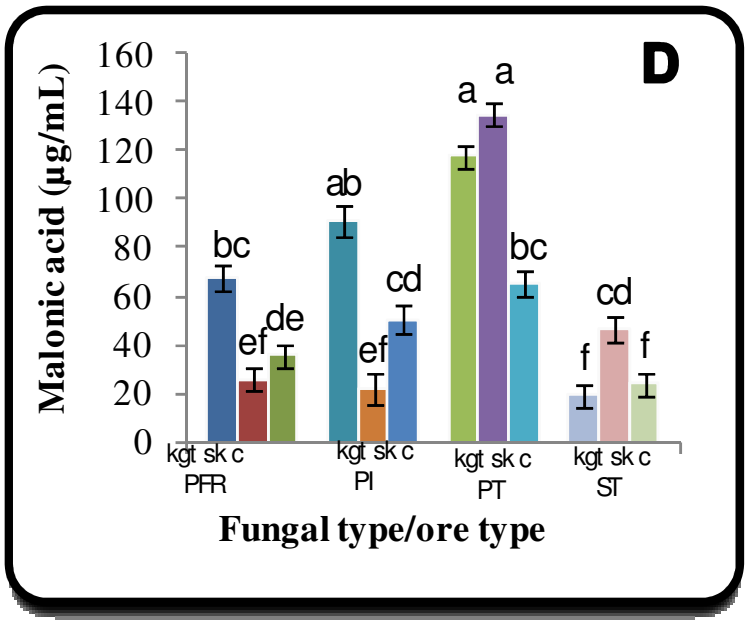
P values <0.0001 are considered significant.

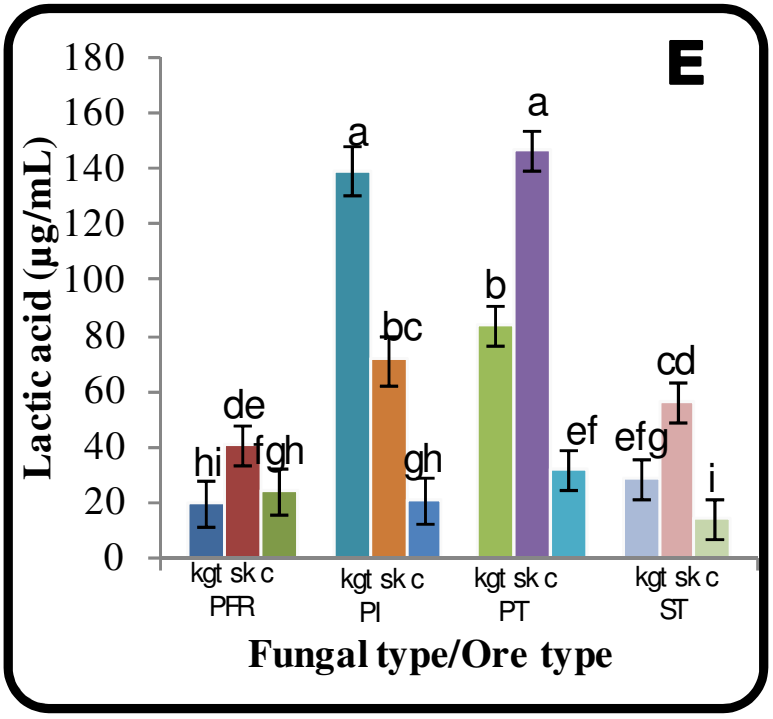
In general, largest quantities of all the acids were recorded in the experiments involving the lowest particle sizes D, E and C of the two iron ore types (Fig. 3.3). The quantity was significantly higher in particle size D than E and C for oxalic, citric, malic, malonic and lactic acids, while the highest was recorded in particle size E in formic and acetic acids (Fig.3.3).






C
Maleic acid µg/mL)
300
250
200
150
100
50
0
bc
d
c
a
c
c
b
c
d
d
d
kgt sk c
PFR
kgt sk c
PI
kgt sk c
PT
kgt sk c
ST
Fungal type/Ore type


D
Malonic acid (µg/mL)
160
140
120
100
80
60
40
20
0
bc
ef
de
ab
ef
cd
a
a
bc
f
cd
f
kgt sk c
PFR
kgt sk c
PI
kgt sk c
PT
kgt sk c
ST
Fungal type/ore type


E
Lactic acid (µg/mL)
180
160
140
120
100
80
60
40
20
0
hi
de
f
gh
a
bc
gh
b
a
ef
efg
cd
i
kgt sk c
PFR
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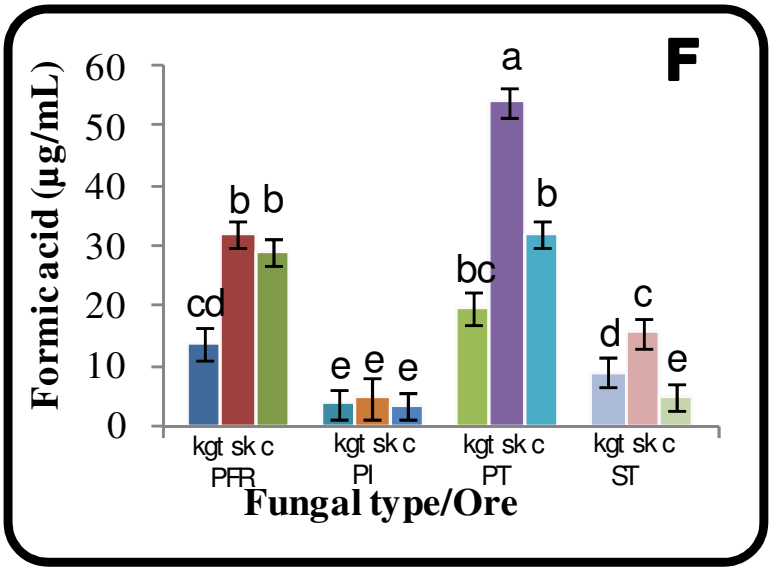
PI
kgt sk c
PT
kgt sk c
ST
Fungal type/Ore type


F
Formic acid (µg/mL)
60
50
40
30
20
10
0
cd
b b
e e e
bc
a
b
d
c
e
kgt sk c
PFR
kgt sk c
PI
kgt sk c
PT
kgt sk c
ST
Fungal type/Ore

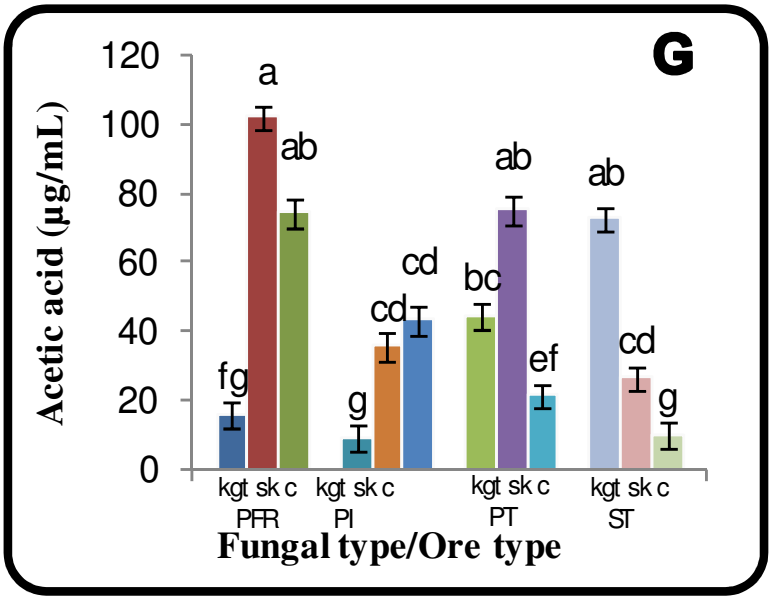


Figure 3.3: Amount of Oxalic acid (A), Citric acid (B), Maleic acid (C), Malonic acid (D), Lactic acid (E), Formic acid (F) and Acetic acid (G) released by the four ECM fungi (PFR, PI, PT and ST) grown under either KGT or SK treatments, or control – c (without fungus), with particle size E. Bars with the same number are not significantlydifferent ($P<0.0001$).

By comparing the organic acid released in particle size E treatment where the highest mobilisation of K occurred, it was revealed that PT released the highest quantity of oxalic acids in both iron types. The same trend was repeated in all other types of organic acids studied, except citric acid where the highest quantity was produced by PFR in SK treatment (Fig. 3.3). Contrary to expectation, the mobilisation of the two types of the iron ore does not correspond to organic acid detected in their respective medium of growth. The highest K reduction in particle size E treatment was from PFR, but this does not correlate with the quantity of organic acid released by the same fungus (Fig. 3.3).

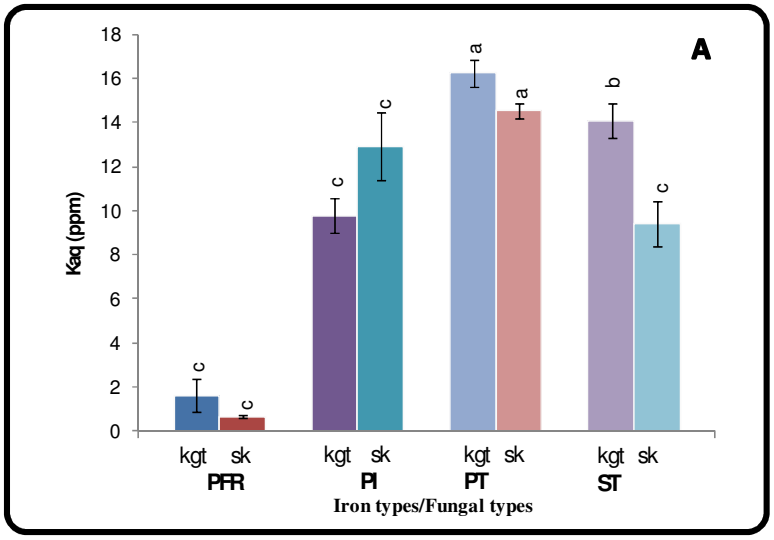
High quantity of acetic acid produced by PFR in particle size E corresponds to the highest leaching percentage of this fungus (Fig. 3.1, Fig. 3.2 and Fig. 3.3G). However, a claim that this acid could be solely responsible for the mobilisation of K could not be verified because of lack of similar evidence in the other ECM fungi that produced high

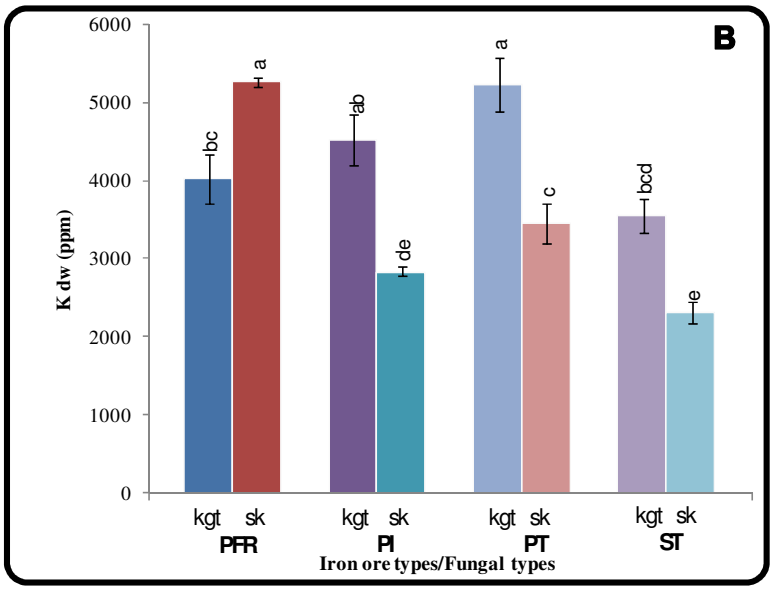
acetic acid Fig. 3.1, Fig. 3.2 and Fig. 3.3G). For example, high acetic acid production by PT and ST (Fig. 3G) did not translate into better mobilisation of K as shown in Fig. 3.1A and 3.1B.

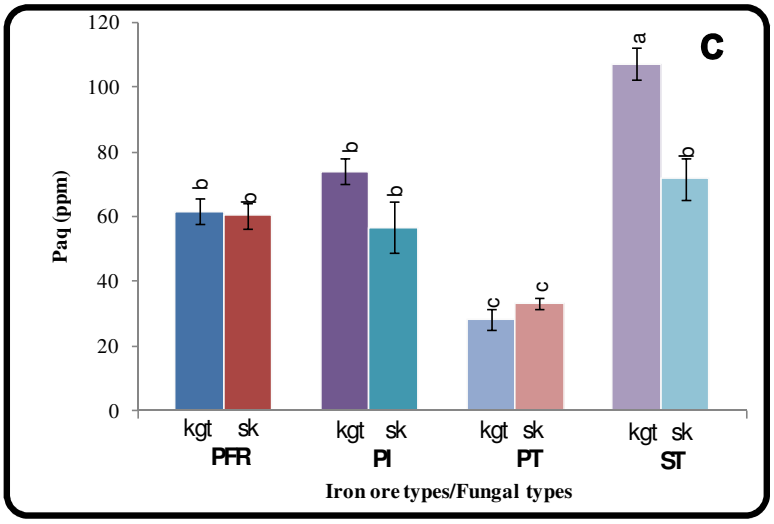
3.3.3 Aqueous K /P and Dry mycelia K/P

The inability to properly link production of organic acid to the mobilisation of K and P from the two iron ore types led to further investigation where K and P contents of both the growth medium and fungal mycelia were analysed. The analysis was carried out only for treatment with particle size E where the best leaching was produced. There were significant effects of fungal type on the amount of K and P detected in aqueous and dried mycelia culture of all the four fungi for treatments involving the two ore types (Table 3.3).

In iron ore type 2 (SK), high quantity of aqueous K was discovered in PT growth medium while the lowest was discovered in PFR growth medium (Fig. 3.4 A).




B
K dw (ppm)
6000
5000
4000
3000
2000
1000
0
bc
a
ab
de
a
c
bcd
e
kgt sk
PFR
kgt sk
PI
kgt sk
PT
kgt sk
ST
Iron ore types/Fungal types


C
Paq (ppm)
120
100
80
60
40
20
0
b
b
b
b
c
c
a
b
kgt sk
PFR
kgt sk
PI
kgt sk
PT
kgt sk
ST
Iron ore types/Fungal types

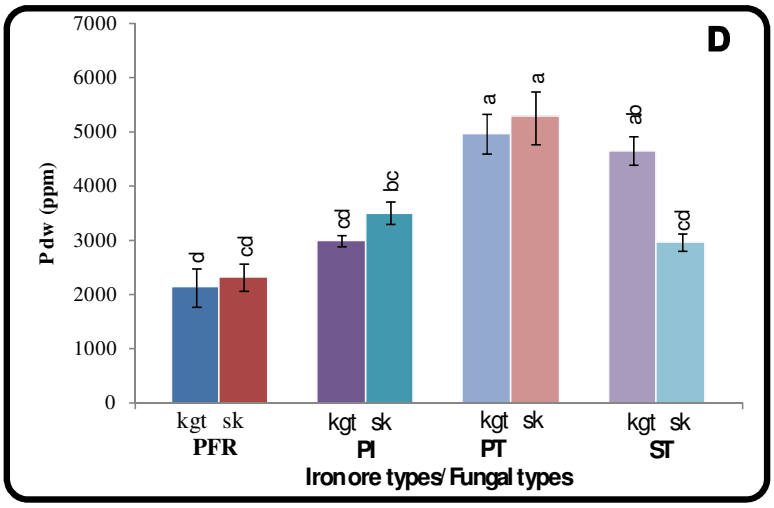


Figure 3.4: Amount of K measured in the growth medium (A) and in the dried mycelium (B) of each of the four fungi with either KGT or SK mineral type. Amount of K measured in the growth medium (C) and in the dried mycelium (D) of each of the four fungi (PFR, PI, PT and ST) with either KGT or SK mineral type. Bars with the same number are not significantly different (P<0.0001).

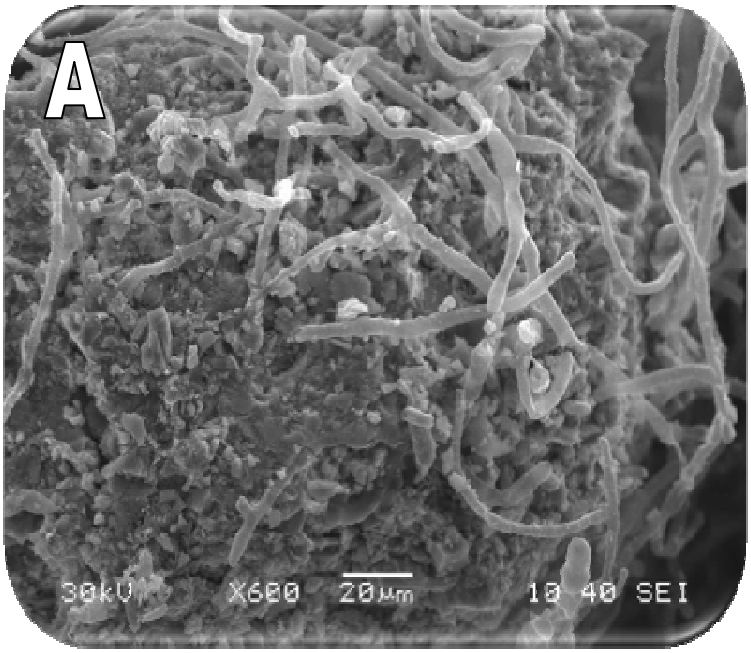
Contrary to this, dried mycelia culture of PFR had the highest concentration of K, whereas the K content of PT mycelia was lower (not significantly different) (Fig. 3.4 B). In addition, PT had the lowest aqueous P and highest mycelial concentration of P in both SK and KGT (Fig. 3.4 C and D) ore types, a reflection of the quantity of P that was mobilised from both ore types by PT. As expected, the controls had high K and P (aqueous and mycelial) contents and are not reported.

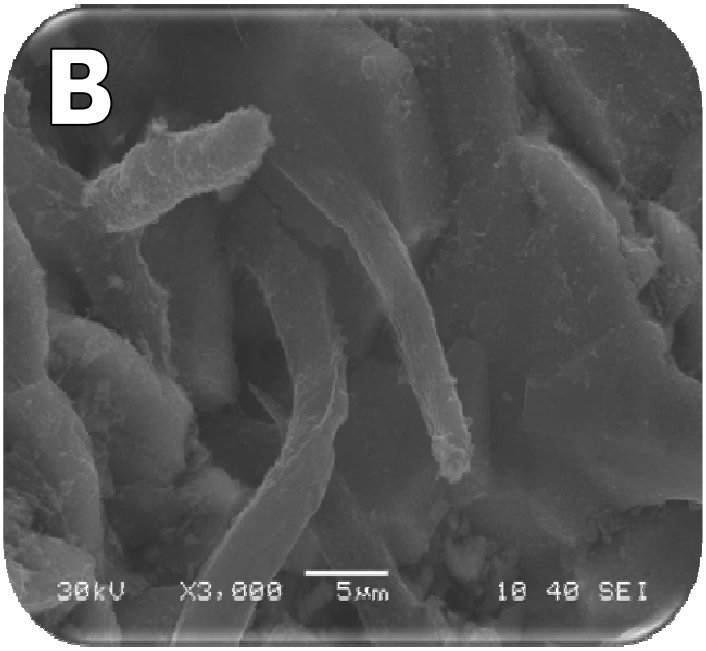
3.3.4 Microscopy

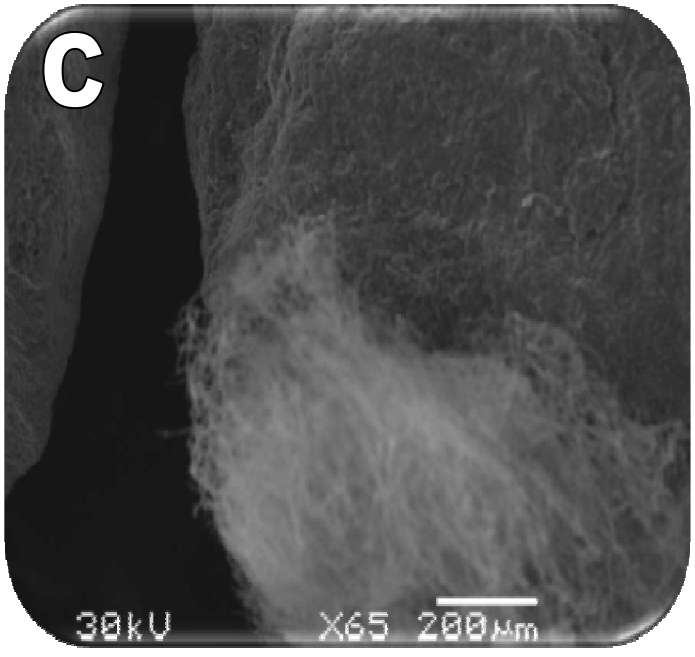
Physical contacts between iron ore and fungi were observed for all types of fungi, though at various levels. There was not much difference between the attachments of the fungi to the two ore types but there were differences among the fungi. The strongest attachment to the iron ore surface was recorded in PFR where mycelia of the fungus seemed to have the

capability of producing sticky materials that helped in proper binding of the mycelial to the iron ore (Fig. 3.5 A and 3.5B). Furthermore, these PFR mycelial structures looked as if they penetrated the ore (Fig. 3.5 B) but this could not be confirmed. In addition, preferential attachment of PT to phosphate rich mineral (Eudral goyasite crystals – as identified by Richards (1990 -1992) was observed on SK ore type (Fig. 3.5 C).

Attachment of ST was only to the surface of the iron ore and never showed any penetration of the ores by the mycelial structures (Fig. 3.5 D). Meanwhile, PI was observed to attach moderately to the iron ore materials (Fig. 3.5 E).




B
30kV X3,000 5µm 10 40 SEI


C
30kV X65 200µm

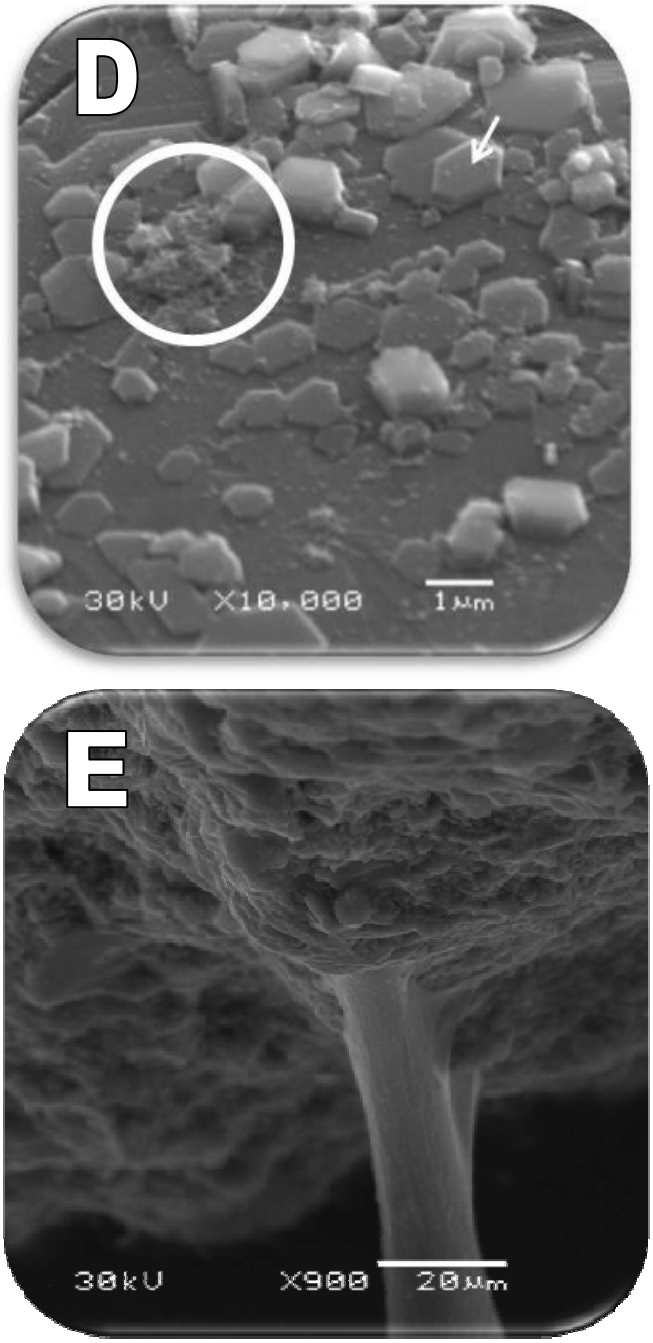


Figure 3.5: A and B represent the SEM images of PT mycelia with scattered attachments to the surface of the iron ore, no sign of penetration of the ore was visible. C and D represent the SEM images of PFR with strong attachment to the iron ore and possibility of penetrating the iron ore (D). E represents the SEM images showing slight attachment of ST mycelia to the iron ore surface. F represents the PI attachment to the surface of the iron ore.

3.4 Discussion

Studies have been able to show the ability of ECM fungi to mobilise nutrients from different minerals under different conditions (Paris *et al*., 1996; Yuan *et al*., 2004; van Scholl *et al*., 2006b). Most of these studies involved the use of mycorrhizal plants as well as single type of K and P bearing minerals such as feldspar, biotite, muscovite and apatite (Wallander and Wickman, 1999; Yuan *et al*., 2004; van Scholl *et al*., 2006b; Balogh-Brunstad *et al*., 2008; Calvaruso *et al*., 2009). However, this study has investigated the mobilisation of nutrients from mineral ore that consists of one or more of such minerals using ECM fungi.

Omission of some key nutrients in the medium of growth has probably helped in the leaching process by triggering the production of organic acids and creating a need for the fungi to source for the missing nutrients from the ores. Similar methods that involved the use of nutrients poor solution in the presence of insoluble minerals have also been adopted in previous related studies (Paris *et al*., 1996; Leake *et al*., 2008; Calvaruso *et al*., 2009). Although biomass was not taken into consideration in this study, visual observation suggested higher biomass of ECM fungal mycelia in the presence of iron ore samples compared to the controls with no addition of iron ore. Such observation has been initially reported by Wallander and Thelin (2008) that addition of minerals such as apatite stimulates the growth of ECM fungi.

Particle size of the ore, production of organic acids by the fungi, and ore types were all observed to significantly affect the nutrient mobilisation. Reduction of particle size increases the total particle surface area of minerals that is exposed to the leaching process and therefore is expected to increase the solubilisation rate (Bosecker, 1997; Vasan *et al*., 2001; Jain and Sharma, 2004). This fact was partially confirmed in this study with leaching of K from both ore types. Very fine particle size of the iron ore (<0.1 mm) led to more leaching than the coarser particle sizes. This was attributed to the ease of releasing

nutrients from such particle size, because of larger particle surface exposed. As discussed by Modak *et al*. (2001) and Jain and Sharma (2004), the use of small particle size minerals could save time and resources during commercial bioleaching processes.

However, data on particle size should be interpreted with caution as it is difficult to identify a general trend through which this factor affects mobilisation of nutrients from minerals. Although time effect was not investigated in this study, it is plausible that particle size is only relevant when time required for leaching is considered. In the study conducted by Modak *et al*. (2001), leaching of calcium from minerals with coarser particle size was similar to those from finer particle size but differ on the basis of time required for the leaching process. Results obtained from this study only indicate consistent "particle size E effect" on K mobilisation and not for other particle sizes investigated. This is in agreement with the suggestion of Modak *et al*. (2001) that recommended >0.1 mm size as optimum for bioleaching process. In spite of this fact, the deviation of P solubilisation from such a trend during this study, suggests that finer particle size may not be applicable to all mineral/ore leaching processes. Delvasto *et al*. (2009) reported a similar scenario where more P was leached from coarser ore particle (2 mm) as compared to those with smaller particle size (0.2 mm).

Fungal leaching largely occurs through four main mechanisms namely acidolysis, complexolysis, redoxolysis/reduction and metal accumulation (Burgstaller and Schinner, 1993). Most of these mechanisms are directly and indirectly related to the ability of fungi to produce organic acids and ligands (Burgstaller and Schinner, 1993; Jain and Sharma, 2004). For example, acidolysis results from a decrease in pH that can be caused by fungal exudation of organic acids as mentioned in a study conducted by Badr *et al*. (2006). In their investigation, they suggested that production of organic acids by silicate-solubilising bacteria lowered the pH to 3.5, which consequently improved the dissolution rate of K- and P-bearing minerals. In another study, reduction of Fe^{3+} to Fe^{2+} was credited to fungal production of oxalate by Ghiorse (1988).

Therefore, on the basis of such theories, the influence of organic acids on nutrient mobilisation was well acknowledged and investigated in this study. The only acid that was produced in higher quantity by PFR (removed highest quantity of K) was acetic acid. However, this tendency could not be linked to other isolates. Numerous studies (Paris *et al*., 1995; Lapeyrie *et al*., 1987; Parks, 1990; Wallander and Wickman, 1999; Rosling, 2004; Williams, 2008) had successfully linked biological weathering and leaching of minerals to the effects of organic acids. These acids act in two major ways that involve the contribution of metal complexing organic acid anion and protons (Gadd, 1999). High chelation constants of $[Al\ (C_2O_4)_3]^{3-}$; 2.0×10^{16} and $[Fe\ (C_2O_4)_3]^{3-}$; 3.9×10^{16} make mineral sites containing Al^{3+} and Fe^{3+} easy target for attack by organic acid anion. Oxalate chelation of these cations results in structural imbalance and therefore may lead to the dissolution and release of other elements embedded in minerals such as K and P (Yuan *et al*., 2004; Delvasto *et al*., 2009). Secondly, both protons (from organic acids) and K (from minerals such as muscovite) are monovalent, but the size of the protons (0.32×10^{-10} m) is much smaller than that of K (2.03×10^{-10} m) (Lapeyrie *et al*., 1987; Yuan *et al*., 2004). The size therefore gives an advantage to protons to replace interlayer K contained in layer minerals such as muscovite associated with iron ores investigated in this study.

Although this study did not specifically investigate solubilisation of any particular mineral type, as earlier mentioned, iron ore samples investigated contained minerals such as muscovite, biotite, goyasite and apatite. Mobilisation of nutrients from these minerals has been previously linked to effects of organic acid produced by microoganisms (Badr *et al*., 2006; Delvasto, 2009; van Scholl *et al*., 2006a; Sheng *et al*., 2008; Williams, 2008). Sheng *et al*. (2008) suggested that gluconic and acetic acid production by *Bacillus globisporus* influenced the release of K from feldspar mineral. Similar idea was pointed out by Delvasto *et al*. (2009) having utilised *Burkholderia caribensis* FeGL03 for beneficiation of iron ore materials. In their study, gluconic acid was noted to play a central role during the leaching process. Williams (2008) went a step further by utilising

Aspergillus niger to produce citric acid for leaching of iron ore materials. About 17.65% K and no P were removed by this process. Organic acids detected in the medium of growth in this study have therefore provided an important relationship to the aforementioned studies. However, results of statistical analyses obtained indicated that organic acids may not be the only reason for the dissolution of iron ore samples. These results could be an indication that though organic acid is important, it may not be the only reason for mineral solubilisation by ECM fungi.

The physical presence, as well as attachment of the fungus to mineral could be equally important for leaching process. In the initial physical observation of the growth inside the conical flask and during the microscopic observation, PFR was observed to strongly attach to both ore types. Despite the fact that penetration of the ore types by PFR could not be confirmed, such attachments are very important and are enhanced by slime production by ECM fungi (Denny and Wilkins, 1987; Gadd, 2000). This is one of the mechanisms that fungi generally used to solubilise minerals (Burgstaller and Schinner, 1993). Apart from the direct binding of elements from the minerals, attachment to mineral surfaces also enhance a particular feeding mechanism in fungi known as scavenging (Banfield *et al*., 1999; Delvasto, 2009). Closeness of the fungal mycelia to the mineral surface during scavenging promotes direct absorption of nutrients contained inside the mineral. In addition, the propinquity also allows faster action of organic acids on the minerals. Any or all these factors may contribute to increased solubilisation of iron ore through scavenging by the ECM fungi (Smith and Read, 2008).

Slime produced by microorganisms largely consists of polysaccharides that can bind metals from solution (Banfield *et al*., 1992). Such a process can possibly create a concentration gradient that can increase movement of ions from mineral into solutions (Delvasto *et al*., 2009). Likely evidence of this was confirmed by the K and P analyses of the aqueous growth medium and the dried mycelium of these fungi. For instance, high levels of K detected in the dried mycelium of PFR may indicate PFR was very effective

in utilising K released into the medium. This may be the reason for the low concentration of K in the growth medium and the high concentration of K in the mycelium. In the presence of organic acid exudation, such process can create a concentration gradient that could allow more K to be released into the solution in response to the low levels of K in solution. In addition, this may further explain the reason behind the lower K reduction for PT fungal type in SK ore type despite high organic acid production. Slow utilisation of K in the medium by PT must have slowed down the process of K release from the medium because of lack of "sucking pressure" that could create a concentration gradient for more K to be released.

Finally, attachments of fungi to specific sites on mineral surfaces, e.g. PT attaching to goyasite, is an indication that complete mineralogical characterisation of both KGT and SK iron ore types will provide more information for development of a reliable biobeneficiation method for these ores. In addition, the higher levels of both K and P contained in KGT iron type must have made it difficult for the fungi to reduce the concentration of both elements from this ore type. Repeated leaching or longer exposure could eventually reduce these elements beyond the commercial standards. It is important to mention that results obtained from this study could differ when the fungi are in symbiosis. However, the study has laid a foundation for future consideration of ECM fungi in biohydrometallurgical processes.

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CHAPTER FOUR

COMPARISON BETWEEN DIRECT SOLUBILISING EFFECTS OF IRON ORE-ASSOCIATED FUNGUS AND ITS METABOLITE

Abstract

Attempts were made to isolate potential mineral-solubilising fungi from the surfaces of iron ore minerals. Four isolates were obtained and identified with molecular and phylogenetic methods as close relatives of three genera that included *Penicillium*, *Alternaria* (2 isolates) and *Epicoccum* for isolates FO, SFC2/KFC1 and SFC2B, respectively. The use of $Ca_3(PO_4)_2$ in the phosphate solubilsing experiment confirmed isolate FO as the only Phosphate solubiliser among the isolated fungi. Two types of iron ore materials (KGT and SK) were used as sources of potassium (K) and phosphorus (P) in this study. Bioleaching capabilities of both the fungus and its metabolites were tested. Direct bioleaching capability of the fungus was compared to that of its metabolites. The result showed a better K removal by the metabolite than the direct use of the fungi, removing up to 32.94% of total K_2O content of the SK ore type. However, for P removal, the direct use of the fungus was better with a maximum removal of 58.33% of the total P content from KGT ore type. The results indicate a potential relationship between K/P removal and the organic acids production by this fungus. High production of gluconic acid by the fungus could be related to the ability of the fungus to remove K and P. Acetic, citric and maleic were the other organic acids produced by the fungus, but in lower quantities. The importance of particle size and ore type was also highlighted in the study. It is therefore concluded that there is a potential prospect in the use of metabolite from this type of fungus for biobeneficiation of iron ore minerals.

4.1 Introduction

One of the consequences of the global technological advancement is the fast depletion rate of valuable minerals, which are also becoming increasingly difficult to find in their pure forms. This has spurred more interest in technologies that investigate the ability of different microorganisms that could mobilise unwanted nutrients from such minerals. Such technologies, popularly referred to as biohydrometallurgy, are positively acknowledged for their environmental and economic advantages (Jain and Sharma, 2004; Rawlings, 2005) and could be utilised in extraction and purification of different minerals during and after actual mining operations. In iron ore materials, the presence of both potassium and phosphorus that are naturally beneficial to living organisms could be a menace when in high concentration (K_2O>0.24% and P>0.03%) (Parks *et al*., 1990; Yusfin *et al*., 1999; Williams and Cloete, 2008; Delvasto *et al*., 2009). This is because of the interference they cause in the operation of the blast furnace, which could eventually reduce the strength and ductility of the iron ore materials. Therefore, iron ore minerals are priced for their low contents of these elements (Davies *et al*., 1978; Elkasabgy, 1984; Yusfin *et al*., 1999).

A common characteristic of potential microbial agents for the mobilization of nutrients from minerals is the production of metabolites that contain organic acids, which could aid the solubilisation of hard and complex mineral materials (Gadd, 1999; Lin *et al*., 2006; Xiao *et al*., 2009). These organic acids are low molecular weight carbon compounds that are capable of forming complexes with various minerals under certain conditions (Paris *et al*., 1995; Gadd, 1999; Goldstein *et al*., 2003). Therefore, an important screening process for bioleaching microorganisms involves the direct or indirect evaluation of the ability of microbes to produce organic acids. Investigators are beginning to acknowledge the importance of the utilisation of indigenous microorganisms to leach elements from minerals. Delvasto *et al*. (2005) utilised *Aspergillus* isolated from iron ore samples to reduce the phosphorus content of the iron ore by 10%. In their investigation, high production of some organic acids by the fungus was directly correlated to the phosphate-solubilising ability of this fungus. In another related study, Williams

(2008) utilised citric acid produced by *Aspergillus niger* to reduce K of Sishen iron ore by 17.65% but there was no P reduction.

In this study, indigenous fungi associated with the surfaces of iron ore were isolated and tested for their phosphate solubilising activities as indicators of their organic acid production (Lin *et al*., 2006; Xiao *et al*., 2009). The potential of both the fungus and its metabolite to mobilise K and P from iron ore samples were evaluated and compared.

4.2 Materials and Methods

4.2.1 Origin and preparation of iron ore samples

Two different types of iron ore samples namely: KGT (conglomerates) and SK (shale) were obtained from Sishen iron ore mine, Northern Cape Province of South Africa. The Induction Coupled Plasma (ICP) analyses of these iron ore types revealed that KGT originally contains an average of 0.805 % K_2O and 0.14 % P whereas SK has an average of 0.423 % K_2O and 0.09 % P. In addition, the chemical composition of the two ore types used for this study as confirmed by the ICP analyses are (average of four samples),: - SiO_2 (32.7 %), Al_2O_3 (3.84 %) and Fe_2O_3 (63.1 %) with trace values of TiO_2, CaO, MgO, Na_2O, MnO, $Cr2O_3$, NiO, V_2O_5 and ZrO_2 for SK. For KGT, the ore contains had SiO_2 (5.01 %), Al_2O_3 (3.61 %) and Fe_2O_3 (90.20 %) with trace values of TiO_2, CaO, MgO, Na_2O, MnO, $Cr2O_3$, NiO, V_2O_5 and ZrO_2.

The iron ore materials were milled and separated into two different particle sizes of <0.84 mm to >0.21 mm and <0.21 mm to >0.1 mm by sieving. Henceforth, these would be referred to as particle sizes A and B, respectively. Pretreatments of iron ore samples are as stated in chapter two (section 2.2.2).

4.2.2 Preparation of media and isolation of fungi from iron ore samples

Two popular fungal growth media were used for the initial isolation of the fungi, and these were Potato Dextrose Agar (PDA) (Biolab) and Modified Melin Norkrans (MMN) medium (Marx, 1969). The fungal isolation process was carried out under sterile conditions, which involved addition of 250 ml of de-ionised water to 100 g of iron ore materials. The mixture was shaken for 24 h at 60 rpm under room temperature. After this, a 10-ml homogenised part of the mixture was vortexed and inoculated onto already prepared plates of PDA and MMN. All the plates were incubated at 37ºC for 5 d.

To get a pure culture of the isolated fungi, mycelia fragments were scrapped off the surface of growth medium and suspended in 1 ml deionised water inside 1.5-ml tubes. The suspension was vortexed to separate the clustered mycelia. A 50-µl aliquot of each suspension was spread onto new plates of MMN and PDA medium with the aid of an autoclaved glass spreader. After 5 d, distinct growing mycelia of the fungi were sub-inoculated onto new plates to obtain pure culture of the fungi. This method enhanced the purity of the isolates by encouraging growth from individual hyphae. Pure cultures obtained were then transferred onto Phosphate Solubilising Medium (PSM) by inoculation at the centre of the agar medium plate. The composition of the PSM was $(NH_4)_2SO_4$ 0.10 g/L; $MgSO_4 \cdot 7H_2O$, 0.25 g/L; $MgCl_2 \cdot 6H_2O$ 5.00 g/L; KCl, , 0.20 g/L; $Ca_3(PO_4)_2$, 2.5 g/L; 10 g/L of glucose and agar, 20 g/L (Mehta and Nautiyal, 2001). After 10 d of incubation at 37 °C, halos formed around the areas of growth of the fungi were taken as indicators of phosphate-solubilising ability of these fungi. Fungi that tested positive were identified with molecular methods.

4.2.3 Molecular identification of the isolates

Genomic DNA extraction was carried out using the Zymo Research Fungal/Bacterial DNA Kit™ (Cat. # 6001) according to the manufacurer's instructions. This was followed by polymerase chain reaction (PCR) to amplify the ITS regions of the isolated fungi. The PCR was carried in a BIO-RAD MJ Mini Personal Thermal Cycler using a 50-µl reaction that consisted of 0.5 µM each of both forward (ITS1F- 5'-CTTGGTCATTTAGAGGAAGTAA-3'; Tm- 49.7 °C) and reverse (ITS4 - 5'-CCTCCGCTTATTGATATGC-3'; Tm- 52.1 °C) primers (White *et al*., 1990; Gardes and Bruns, 1993), 2 µl of the DNA template and 25 µl of Fermentas Master mix (2X), (Cat. # K0171) that contained the Taq, buffer and magnesium chloride. The cycling conditions included an initial denaturing cycle of 3 min at 94 °C followed by 30 cycles of 1 min at 94 °C for DNA denaturing, annealing temperature of 50 °C at 30s and 2 min DNA elongation at 72 °C. There was a final elongation period of 72 °C that lasted for 8 min. The PCR products were separated electrophoretically on a 1% agarose gel and visualised by ethidium bromide-UV fluorescence to determine the size of the amplified bands. Cleaning of the PCR products obtained

was done using the PROMEGA Wizard SV Gel and PCR purification kit (Cat.# A9280) and resuspended in 30 µl of nuclease-free double distilled water. Cleaned PCR product was sent to the Inqaba Biotechnical Industries (Pty) Ltd Sequencing Facility. Forward and reverse sequences of the ITS regions obtained were aligned using BioEdit software prior to BLASTing (Hall, 1999). Homology sequences were thereafter compared on the NCBI website to confirm the nearest identical organisms based on % homology. Four out of many identical ones for each fungus were selected from GenBank for phylogenetic analysis.

4.2.4 Phylogenetic analysis

Phylogenetic analyses were carried out using Mega 4 software (Tamura *et al*., 2007). Using *Aspergillus niger* as an outgroup, neighbour joining (NJ) method (Saitou and Nei, 1987) was used to infer the evolutionary history of the isolates and the bootstrap consensus tree inferred from 1000 replicates. There were 463 positions in the final dataset.

4.2.5 Fungal leaching experiment

Fungi that produced visible halos on PSM were selected for the leaching experiment. The experiment involved the direct use of fungi, as well as the use of fungal metabolites. Three plugs of Phosphate Solubilising Fungi (PSF) taken from the edge of growing medium were inoculated onto PSM that consisted of iron ore materials used to substitute the $Ca_3(PO_4)_2$. Five g of the iron ore materials were added to 50 ml of the medium and incubated at 37 °C for 10 d and shaken at 100 rpm. Two different controls were used, one involving the use of water and the iron ore samples with no fungus (CT), and the other involving the use of fungus growing on Phospahte Solubilising Broth but no iron ore materials (CTR).

4.2.6 The use of fungal metabolites

PSF were grown on PS broth by inoculating three plugs of the fungal culture onto the broth. The incubation period lasted for 10 d at 37 °C. This was also used as the control experiment involving

no fungus. This was followed by separation of fungus and the metabolites through filtration (0.22 µm). The liquid part was then used for leaching process by the addition of autoclaved 5 g of iron ore materials to 50 ml of the metabolite. Incubation was done without shaking (to reduce the number of processes needed to achieve the objective) and lasted for 10 d at 45 °C to discourage the growth of fungal spores.

4.2.7 Harvesting

For both fungal and metabolite treatments, harvesting was done using filteration through filter paper of size 0.45 µm. Iron ore samples collected were then washed with HCl and later rinsed with deionised water. Liquid parts from both treatments were preserved at -40 °C for HPLC analysis.

4.2.8 Organic acids detection

High Performance Liquid Chromatography (HPLC) was used to identify organic acids released for both fungal and metabolites treatments. The method described by Sheng *et al*. (2008) was used to analyse four different organic acids, namely: gluconic, acetic, citric acid and maleic acid.

4.2.9 Statistical analyses

For the statistical analyses, SAS software, version 9.2 (SAS Institute, 2009, Cary, NC, USA) was used. A 3-way Anova model was adjusted to the data with three sets of factors which included 1.) Fungi/metabolite/control - 3 levels: F, M or CTR, 2.) iron ore - 2 levels: KB or SB, and 3.) Particle size (2 levels: A or B). For all other variables, the 3-way Anova model was also adjusted to the data but with the factors 1.) Fungi/metabolite 2.) Iron ore - KB or SB, and 3.) Particle size - A or B. All variables were log-transformed in order to satisfy the assumptions of the model (normality and homogeneity of variances). The normality assumption was verified by the Shapiro-Wilk's statistic, while the homogeneity of variance was verified visually with the

residual plots. Following a significant effect of any source of variation, multiple comparisons were done to point out where the difference occurred.

4.3 Results

The isolates were obtained and identified through sequence homology and phylogenetic analyses (Fig. 4.1). Sequences obtained (Appendix III) have already been deposited to the GenBank and accession numbers allocated (Fig. 4.1). The ITS phylogenetic analyses of the four isolates and their closest relatives obtained from the GenBank supported three major lineages in NJ analysis. The genera identified with this process were *Penicillium* (FO), *Alternaria* (SFC2 and KFC1) and *Epicoccum* (SFC2B) (Fig. 4.1).

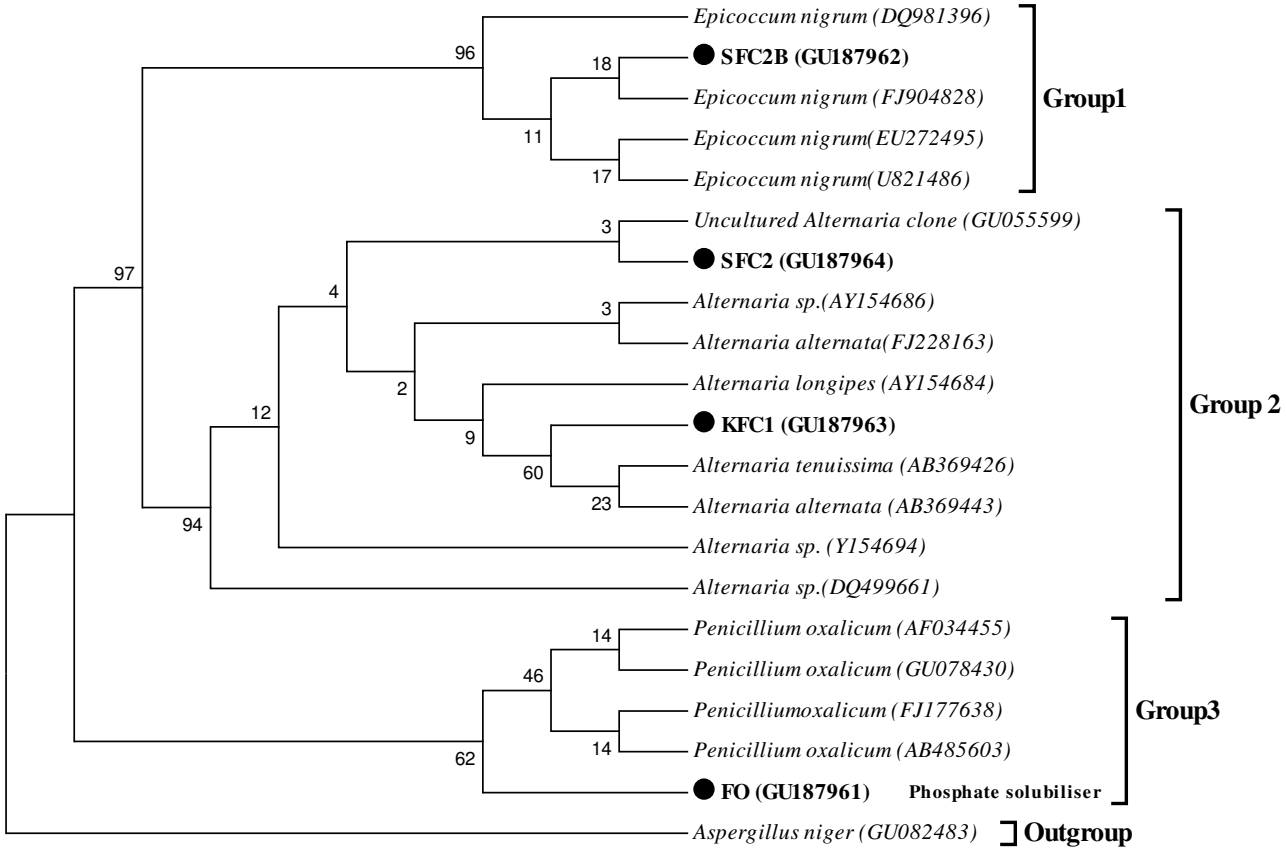

Figure 4.1: Neighbour joining tree constructed from ITS sequences of isolates obtained from iron ore minerals (bold letters) and other sequences obtained from GenBank. The NJ tree was rooted with *Aspergillus niger* as outgroup.

The phosphorus solubilisation experiment confirmed that isolate FO (GU187961) was the only isolate capable of solubilising $Ca_3(PO_4)_2$, therefore, it was the only fungus among the four, used in the biobeneficiation experiment.

Table 4.1: Three-way analysis of variance (ANOVA) with F and P values that show the effects of fungal/metabolite, mineral type, particle size and their interactions on % K_2O and % P reduction from the iron ore materials.

Sources of Variation	**df**	**% K_2O loss df= 23**		**%P loss df= 23**	
		F	*P*	*F*	*P*
Fungal/Metabolite	2	50.4	<0.00001	113.06	<0.0001
Iron type	1	2.33	0.1402	36.07	<0.0001
Particle size	1	72.03	<0.0001	9.91	0.0045

P values <0.005 are considered significant.

Fungal/metabolite usage and particle size had significant effects on % K loss, but not the iron type (Table 1). However, these three factors proved significant for the reduction of P from the iron ore samples (Table 4.1). For both ore types, highest quantities of K_2O (32.94%) was removed in treatments involving fungal metabolite and particle size A of KGT ore type (Fig. 4.2), while highest P removal (58.33%) was recorded in fungal treatment with particle size B of SB ore type (Fig. 4.3).

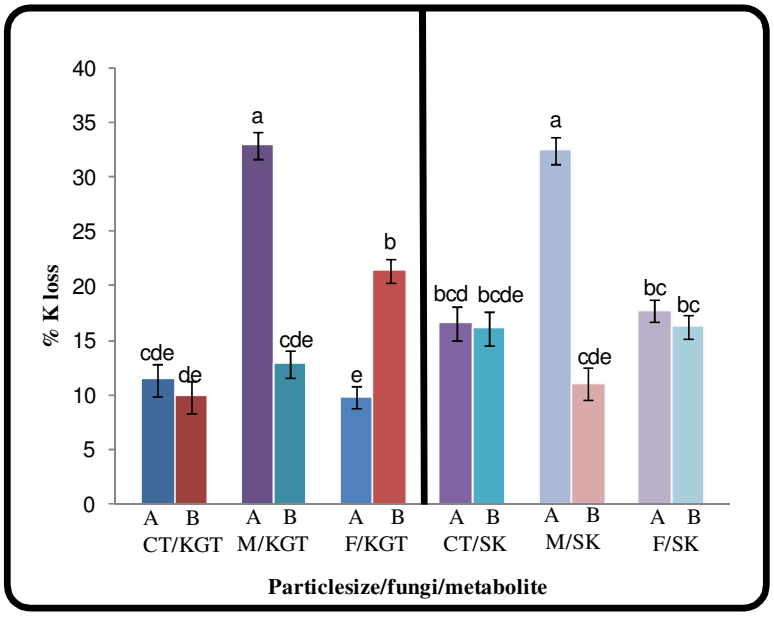

Figure 4.2: Percentage of K reduction from mineral type KGT and mineral type SK in response to fungal (F), metabolite (M) and water – control (CT) treatments two different particle sizes (A and B) of the iron ore materials. Bars with the same letter are not significantly different (P<0.0001).

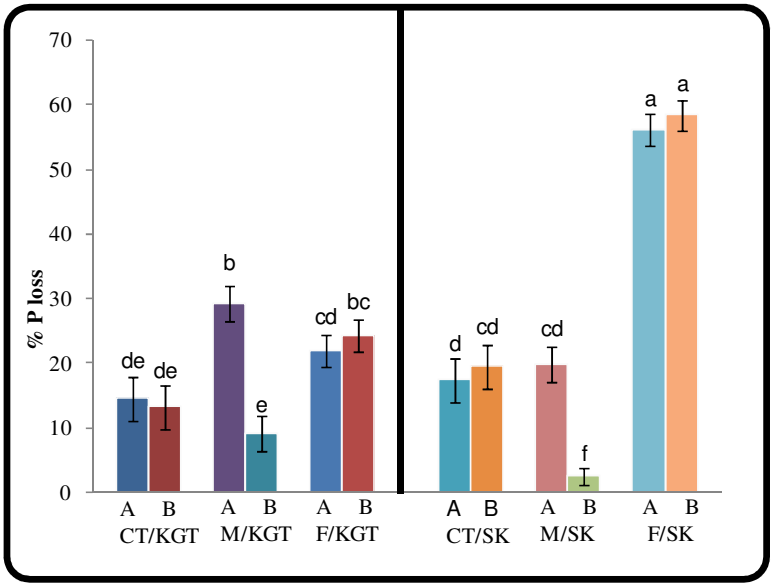

Figure 4.3: Percentage of P reduction from mineral type KGT and mineral type SK in response to fungal (F), metabolite (M) and water – control (CT) treatments two different particle sizes (A and B) of the iron ore materials. Bars with the same letter are not significantly different. (*P*<0.0001)

The fungal culture was able to remove more K from smaller particle size B than A treatment, but there was no significant difference between P removal for both particle size A and B treatments. However, for the metabolite treatments, more K and P removal were recorded from particle size A (Fig. 4.2 and Fig. 4.3).

Table 4.2: Organic acid produced (µg/ml) by the fungus in the presence of the two mineral types KGT and SK as well as two different particle sizes A and B.

Treatments	**Particle**	**Gluconic acid**		**Acetic acid**		**Citric acid**		**Malic acid**	
	size	KGT	SK	KGT	SK	KGT	SK	KGT	SK
Fungus	A	1563.81	1105.09	6.41	3.12	18.49	14.36	440.65	478.57
	SD	±130.57	±92.27	±0.41	±0.2	±1.68	±1.13	±38.19	±35.92
	B	2450.38	2196.25	19.72	9.15	43.88	9.07	404.61	154.10
	SD	±204.6	±158.12	±1.54	±0.58	±4.0	±0.72	±35.07	±11.57
Control	A	1549.21	2649.33	8.34	5.63	7.92	16.40	155.69	91.13
	SD	±125.86	±221.21	±0.53	±0.36	±0.72	±1.50	±13.49	±7.90
	B	1751.66	2463.35	14.35	13.32	10.46	21.261	109.57	33.74
	SD	±146.26	±251.91	±0.91	±1.04	±0.95	±2.37	±9.50	±33.74

*The spent medium from the control experiment was used for metabolite treatment; therefore values presented above (control) represent the organic acid contained in the metabolite.*SD *represents standard deviation from 4 replicates, P<0.0005.*

Among the organic acids, gluconic acid seemed to be the most important as the quantity produced by this fungus is approximately 4 to 200 times greater than other organic acids. The second highest was maleic acid, while quantities of both acetic and citric acids released by this fungus were generally low (Table 4.2).

4.4 Discussion

There are two commonly used methods for the isolation, characterisation and utilisation of mineral-associated microbes in biohydrometallurgical processes. The first method is the direct enrichment culture method where the mineral is added to a defined medium for the purpose of isolation and leaching of the mineral (Goebel and Stackebrandt, 1994). In this situation, the associated microbes are expected to multiply and participate in the leaching of the mineral (Rezza *et al*., 1997; Rezza *et al*., 2001). The problem with this method is the inability to immediately identify the specific organism responsible for the leaching if or when it occurs. The other method involves the direct isolation from the surface of the mineral and subsequent utilisation of the isolates for leaching processes (Delvasto *et al*., 2008). This allows the immediate identification of the organism responsible for the leaching process. The disadvantage here is the inability to obtain information about possible heterotrophic leaching that can occur from more than one microbe. However, both methods have been successfully used to investigate biohydrometallurgical processes (Goebel and Stackebrandt, 1994; Delvasto *et al*., 2008). This study has adopted the second method that allowed initial isolation of four different fungal isolates, namely FO, SFC2, SFC2B and KFC1. In addition, the present study has been able to further show differences that can exist between the direct use of fungi and the use of fungal metabolites. The three fungal genera identified in this study have been previously isolated from different minerals and mining sites (Burford *et al*., 2003; Qui *et al*., 2005; Sabat and Gupta, 2009). For example, Rezza *et al*. (1997) were able to isolate three different fungi from spodumene of which *Penicllium purpurogenum* was one of them.

The production of organic acids has been established in both agro- and biomining industries as essential for natural dissolution of complex mineral materials by microorganisms (Rezza *et al*., 1997; van Scholl *et al*., 2006, Sheng *et al*., 2008). Therefore, an indirect screening approach involving *in vitro* P solubilisation by the fungi was used as indicator of organic acid production. Delvasto *et al*. (2005) screened isolated microbes for their abilities to dissolve insoluble forms of phosphate in an *in vitro* experiment. *Aspergillus niger* obtained from this process was then used

for leaching of iron ore mineral. In another study, Sheng *et al*. (2008) screened isolates obtained from silicate minerals for their solublisation potentials and used one of them, *Bacillus globisporus* Q12 for solubilisation of this mineral.

The influence of particle size on treatment involving fungal treatment was expected. This belief is linked to the fact that larger particle surface of minerals (finer particle size minerals) is exposed to microbial activity when there is reduction in the grain size (Modak *et al*., 2001). However, for the present study, the same mechanism cannot be used to explain leaching with metabolite due to the influence of another factor, non-shaking of the flasks. Franz *et al*. (1991) suggested that shaking of flasks during leaching is essential for production of organic acid and proper aeration. Though more organic acid production was not expected during the metabolite treatment (because fungus was not involved), lack of good aeration due to non-shaking of the flasks probably affected proper mixing of the metabolite and the iron ore minerals during the experiment. Non-shaking of flasks in the metabolite treatment was introduced in order to reduce the number of steps needed to achieve the main goals of the study, K and P reduction of iron ore minerals.

Leaching obtained from the metabolite treatments could probably be linked to the organic acids detected in both the spent and growth media. Some of the organic acids (especially gluconic acid) detected in this study have been previously reported as essential in both weathering and bioleaching experiments. For example, Sheng *et al*. (2008) reported that the ability of *Bacillus globisporus* Q12 for the solubilisation of silicate minerals was due to the production of both gluconic and acetic acid. These acids were suggested to enhance the release of K and Si from these minerals. In addition, Delvasto *et al*. (2009) also reported direct relationship between the quantity of organic acids released and P solubilised from iron ore by *Burkholderia caribensis* FeGL03.

At the beginning of both fungal and metabolite treatments, higher levels of organic acids were contained in the metabolite because it was a spent medium; fungus had already released high amounts of acids into the medium before its usage. Considering these initial organic acid concentrations in the two treatments and the fact that both treatments lasted for the same time, the

high removal of K recorded in metabolite treatment can be attributed to the initial higher quantities of organic acids in this treatment. Such is the influence of organic acid and period of incubation on leaching process. Modak *et al*. (2001) reported that particle size influence is only noticed when period or time of incubation is considered.

For P mobilisation from the iron ore, utilisation of P by the fungus seems to be driving the concentration gradient, which translated into high P removal from the iron ore mineral. This scenario, associated with physical presence of the microbe is sometimes, needed for bioleaching to occur effectively especially in situation where organic acid is not the only factor responsible. It is therefore plausible that in addition to the fungal production of organic acids, scavenging was probably used as a feeding mechanism to get P from the iron ore minerals by the fungus (Banfield, 1999; Delvasto, 2009).

Although values to be removed (KGT-70.19% of K_2O and 78.57% of P, SK – 43.17% of K_2O and 66.67% of P) to meet the commercial standard needed for exportation of these minerals were not attained, this study has highlighted the importance of factors such as ore type and particle size in the bioleaching processes. The result of particle size effect has to be interpreted with caution. Apart from aeration that was earlier mentioned, there could also be grinding effect on mineral solubilisation. In the study conducted by Srihari *et al* (1994), it was discovered that grinding of minerals could help create specific sites for microbial attachment that subsequently affect dissolution of minerals.

Successful development of a biobeneficiation method for iron ore entails many steps. Identification of potential microbes is a major part of the developmental process. Factors such as difficult adaptation of non-indigenous microbes, biofilm formation and lack of cheap carbon sources have slowed down the development of this technology (Jain and Sharma, 2004; Delvasto *et al*., 2009). From the available literature, the present study is probably the first that utilised fungus isolated from Sishen iron ore for bioleaching of this mineral.

Further optimisation of the process developed in this study may give the desired result that can solve the problem of high K and P in iron ore minerals. However, the major problem facing the full development of this process is the use of sterile conditions. Presently, maintenance of such conditions is expensive for the iron ore industry, because of the low price of iron ore. One of the potential solutions to solve such problem is the use of fungal metabolites, as indicated in this study, which may be incorporated into outdoor leaching processes.

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CHAPTER FIVE

CULTURABLE MICROORGANISMS ASSOCIATED WITH SISHEN IRON ORE AND THEIR POTENTIAL ROLES IN BIOBENEFICIATION

Abstract

With one of the largest iron ore deposits in the world, South Africa is recognised to be among the top ten biggest exporters of iron ore mineral. Increasing demand and consumption of this mineral triggered search for processing technologies that can be utilised to "purify" the low-grade iron ore that contain high levels of unwanted potassium (K) and phosphorus (P). The need for a low cost and environmental friendly technology has therefore made biobenefication technology a potential process to solve this problem. This study investigated a potential biological method that can be further developed for a full biobeneficiation of low-grade iron ore minerals. Twenty-three bacterial strains that belong to *Proteobacteria*, *Firmicutes*, *Bacteroidetes* and *Actinobateria* were isolated from the iron ore minerals and identified with sequence homology and phylogenetic methods. Abilities of these isolates to lower the pH of the growth medium, high slime production and solubilisation of tricalcium phosphate were used to screen them as potential mineral solubilisers. Eight isolates were successfully screened with this method and utilised in shake flask experiments using iron ore minerals as sources of K and P. The shake flask experiments revealed that all the eight isolates have potentials to produce organic acids that aided the solubilisation of the iron ore minerals. In addition, all eight isolates produced a high quantity of gluconic acid but lower quantities of acetic, citric and propanoic acid. Scanning electron microscopy (SEM) and Fourier transform infrared (FITR) analyses also helped to uncover the role that biofilm and extracellular polymeric substances could play in mineral solubilisation.

5.1 Introduction

Iron ore is one of the most common minerals on the surface of the earth. The importance of iron is strongly linked to its hardness, durability, strength and ability to form alloys with other metals. These properties have made iron ore special and suitable for different applications in various industrial processes (Gutzmer *et al*., 2001; Beukes *et al*., 2003). Over the past few decades, the surge in the global demand for iron ore has led to increase production and exportation of this mineral by the iron ore-producing countries (Williams and Cloete, 2008; Delvasto *et al*., 2009). With this development, it is becoming increasingly difficult to find this mineral in its pure form. Iron ore mining companies are now faced with the challenges of refining and reprocessing low-grade iron ore minerals. Such poor quality iron ore minerals contain contaminants such as potassium, phosphorus, aluminium and sodium which are deleterious to the processing and products of this mineral (Parks *et al*., 1990; Yusfin *et al*., 1999; Williams and Cloete, 2008; Delvasto *et al*., 2009).

The type of unwanted elements in iron ore samples differs from country to country. For example, iron ore minerals of high P have been reported in Brazil, while Sishen mine in South Africa is having problems of high K (>0.24%) and P (>0.03%) contents of the iron ore minerals. Studies have revealed that these deleterious elements are always embedded in various associative minerals contained in the iron ore materials such as apatite, hematite, muscovite, quartz and goyasite (Parks *et al*., 1990; Williams, 2008; Delvasto, 2008). Both chemical and pyrometallurgical methods developed to solve these problems have not been generally accepted, because of cost and environmental concerns (Brombacher *et al*., 1997; Rawlings and Johnson, 2007). In addition, the development of an acceptable biological method of leaching iron ore has been slow due of the difficulty involved in extrapolating the available biomining procedures into this area of biohydrometallurgy. This is because most of the successfully concluded studies in biohydrometallurgy were based on the use of chemolithoautotrophic bacteria designed for bioleaching of sulfidic minerals. These are bacteria that are able to utilise both sulfur and iron cycles for the generation of their energy during bioleaching processes (Jain and Sharma, 2004;

Rawlings, 2005). Unfortunately, iron ore minerals such as Sishe iron minerals do not belong to this group (Jain and Sharma, 2004; Williams, 2008).

In similarity to bioleaching of sulfidic minerals, the importance of the unwanted elements as nutrients for microorganisms has encouraged scientists to develop methods that enable microbes to utilise them as sources of energy and other metabolic processes. These processes involve the use of bacteria and fungi, usually soil-associated, and some indigenous microflora that are capable of dissolving complex mineral materials. Solubilisation of the minerals is achieved through the production of metabolites that contain organic acids as the active ingredients (Parks *et al*., 1990; Deo and Natarajan, 1998; Pradhan, 2008; Williams, 2008; Delvasto, 2009). Both bacteria and fungi that were previously investigated for biobeneficiation of non-sulfidic minerals have been identified as organic acids producing-microbes. The process occurs through direct oxidation pathway where gram-negative bacteria are mostly involved. In this situation, organic acids produced in the periplasm could easily diffuse into adjacent environment and subsequently dissolve insoluble forms of minerals such as calcium phosphate (Goldstein *et al*., 2003). The production of organic acids by such microbes therefore provides a platform for ion exchange in forms of proton donation and complexation (Gadd, 1999; Jain and Sharma, 2004). A recent example was the solubilisation effects of gluconic acid released by *Burkhoderia caribensis* FeGL03 on the mobilisation of P from iron as reported by Delvasto *et al*. (2009). Furthermore, Williams (2008) also utilised citric acid obtained from *Aspergillus niger* for the biobenefication of Sishen iron ore. In addition, factors such as molecular functionalities of extracellular polymeric substances (EPS) produced by microbes and attachment of the microbes to mineral surfaces have also been indicated as important in iron ore solubilisation (Natarajan and Deo, 2001; Delvasto *et al*., 2009). In their study, Delvasto *et al*. (2009) reported EPS production by *Burkholderia caribensis* FeGL03 as partly responsible for the solubilisation of P from iron ore materials. All these attributes are the theoretical background of the present investigations of bacteria associated with iron ore and their roles in biobenefication of this mineral.

The ability of the isolates to reduce the pH of the growth medium was taken as an indication of medium acidification (Welch and Ullman, 1996) while those that dissolve water-insoluble tricalcium phosphate were assume to have the capability to produce high gluconic acid (Delvasto

et al., 2009). The aims of this study were therefore to i.) isolate and characterise culturable bacterial population inhabiting the iron ore surfaces, ii.) screen the isolates in order to identify potential organic acids-producing bacteria through the use of microbial features - characterisitics such as ability to lower the pH of the growth medium, high slime production and dissolution of insoluble phosphorus were utilised, and iii.) investigate the biobeneficiation (K and P reduction) potential of the organic acids-producing isolates.

5.2 Materials and methods

5.2.1 Origin and preparation of iron ore samples

Two types of iron ore samples were collected from Sishen mine located in the Nothern Cape Province of South Africa. These samples were originally characterised by the company as KGT (conglomerates) and SK (shale). The iron ore materials were milled and sieved into sizes that are between <0.21 mm to >0.1 mm. Pretreatments of iron ore samples are as stated in chapter two (section 2.2.20). ICP was used to check any possible change in the P and K contents of the iron materials after this treatment. Dried samples were used in the leaching experiment as sources of K and P.

5.2.2 Preparation of media

Three different media were used for the isolation of bacteria in this study. This included a phosphate solubilising medium (PSM) (Mehta and Nautiyal, 2001), Nutrient agar (NA) (Biolab) and Typtone soy agar (TSA) (Biolab). The PSM contained $(NH_4)_2SO_4$, 0.10g/L; $MgSO_4 \cdot 7H_2O$, 0.25 g/L; $MgCl_2 \cdot 6H_2O$, 5.00 g/L; KCl, 0.20 g/L; $Ca_3(PO_4)_2$, 2.5 g/L; 10 g/L of glucose and agar, 20 g/L.

5.2.3 Isolation of bacteria from iron ore samples

A 5000-g sample of the iron ore materials was added to 1 L of de-ionised water inside autoclaved 2-litre beakers under sterile conditions and this was replicated three times for each mineral type. The beakers were covered with three-layer sterile foil paper and shaken at 60 rpm at room temperature. After 24 h of shaking, 10 ml of the homogenised liquid part of the mixture was taken from each beaker and replicates pooled together for each mineral type. This was followed by 3 min vortexing and serial dilution (10^{-1}, 10^{-2} and 10^{-3}). Eighty-microliter volumes of the diluted samples was inoculated onto PSM, NA and TSA plates using spread plate technique. Inoculated plates were incubated at 37 °C for 48 h.

Morphologically distinct colonies were identified and obtained from the plates. The colonies were suspended in 1 ml autoclaved double-distilled water. The suspension was serially diluted and the 10^{-2} dilution was plated out onto NA (Biolab) by spread plating of individual colonies. Distinct colonies were obtained after incubation at 37ºC for 24 h. Pure colonies were streaked onto NA as representatives of the individual bacterial isolates.

5.2.4 Screening of phosphorus-solubilising, potassium-solubilising and low pH- isolates

All the isolated bacteria samples were inoculated onto nutrient broth (NB) and incubated at 37 °C. The pH of the growing culture was checked after 24 and 48 h for any change. In addition, 10-µl volume of each isolate was inoculated at the centre of the PSM, containing (g/L): $MgSO_4 \cdot 7H_2O$ - 0.25; $(NH_4)_2SO_4$ - 0.10, $MgCl_2 \cdot 6H_2O$ - 5.00; KCl - 0.20; $Ca_3(PO_4)_2$ - 2.5; glucose-10 and agar – 20, was used for the selection of phosphate-solubilising bacteria. For isolation of potassium-solubilising isolates, a medium containing (g/L): starch - 10, $Na_2HPO_4.12H_2O$ -2, $FeCl_3$ – 0.005, $MgSO_4.7H_2O$ - 0.5, $CaCO_3$ -0.1, Yeast extract – 1 and agar – 20 (at pH 7.4), was used (Lin *et al*., 2001).

5.2.5 Molecular identification of the isolates

Genomic DNA extraction was carried out using the Zymo Rresearch Fungal/Bacterial DNA Kit™ (Cat.# 6001) according to the manufacturer's instructions. The 16S rDNA bacterial genes was the target region for the PCR amplification using a universal pair of bacterial forward and reverse primers; GM5F (5´-CCTACGGGAGGCAGCAG-3´;Tm-58.2ºC) and R907 (5´CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCGCCGTCAATTCCTTTGAGTTT-3´;Tm-1.8ºC) (Muyzer, *et al*., 1995) respectively. PCR was conducted in a 50µl reaction which contained the following: 0.4µM of each of the primers, 1.25 U of *Taq* polymerase, 5 µl of Promega 10X buffer (0.2 mM) promega dNTPs, 1.75 mM of Magnessium chloride and 2µl template DNA. The PCR was performed on a MJ Mini Personal Thermal Cycler (Bio-Rad) using these conditions: initial denaturing at 94 °C for 2 min, followed by 4 cycles of 30 s at 94 °C (denaturing), 45 s at 68 °C (annealing), 2 min at 72 °C (elongation). These steps were repeated

(excluding initial denaturing) with decreasing annealing temperature at 66, 64, 62, 60 and 58 °C running at 4 cycles, except at the annealing temperature of 58 °C that ran at 12 cycles. Final elongation was at 72 °C for 8 min. The different annealing temperatures listed in decreasing order were due to the high variation in the T_m of the two primers. PCR products were separated electrophoretically with ethidium bromide (0.1µg/ml)-stained 1% agarose gel running at 120V for 1 h. DNA was visualised and photographed using a Uviprochem Transilluminator.

Cleaning of the PCR products obtained was done using the PROMEGA Wizard SV Gel and PCR purification kit (Cat.# A9280) and resuspended in 30 µl of nuclease-free double distilled water. Cleaned PCR product was sent to the Inqaba Biotechnical Industries (Pty) Ltd Sequencing Facility. Forward and reverse sequences of the 16s rDNA regions obtained were aligned to obtain consensus sequence using BioEdit software prior to BLASTing. Homology sequences were thereafter compared on the NCBI website (Hall, 1999) to confirm the nearest identical organism.

5.2.6 Phylogenetic analysis

Nucleotide sequences of two bacterial isolates (closest to the isolated strains) for each isolate were obtained from the GenBank. Meanwhile, some of the isolates shared close relatives. All the bacterial sequences obtained were aligned using ClustalX software (Thompson *et al*., 1997). Further alignment was carried out with online version of MAFFT software (Katoh *et al*., 2002). The phylogenetic analyses were carried out using Mega 4 software and the evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2007). Using *Salmonella paratyphi* as an outgroup, neighbour joining (NJ) method (Saitou and Nei, 1987) was performed to infer the evolutionary history of the isolates and the bootstrap consensus tree inferred from 1000 replicates. All positions containing gaps and missing data were eliminated from the dataset.

5.2.7 Leaching experiments

Bacteria with lowest pH values and those that produced visible halos on PSM were selected for the leaching experiment. Isolates were inoculated into Nutrient Broth (NB) (Biolab) and grown at

37 °C overnight. The bacterial cultures were then centrifuged at 13000 rpm after which the supernatant was discarded. The cells were then resuspended in autoclaved deionised water. Concentrations of all the bacterial isolates were adjusted with sterile water using a Beckman spectrophotometer (Du® 530) at OD_{600} to 0.1. Biobeneficiation experiment was conducted in a 100-ml Erlenmeyer flasks containing 5 g of iron ore mineral and 50 ml of modified PSM that contained $(NH_4)_2SO_4$, 0.10 g/L; $MgSO_4 \cdot 7H_2O$, 0.125 g/L; $MgCl_2 \cdot 6H_2O$, 2.5 g/L; KCl, 0.10 g/L; 2.5 g/L; 10 g/L of glucose and agar, 20 g/L. One milliliter of the adjusted concentration of the bacterial culture was inoculated onto the contents of the flask and incubated at 37 ºC for 3 weeks. The experiment was in triplicate and harvesting was done at weekly intervals. There were two control treatments – the first control had the iron ore and the PSM but no bacteria, while the second had the PSM and the bacteria but no iron ore.

5.2.8 pH measurement and high performance liquid chromatography (HPLC)

During harvesting, iron ore samples was separated from the spent medium by decantation. Spent broth from bacterial cultures were homogenised by vortexing, then centrifuged for 180 s at 16060 rpm and the supernatant frozen at –40 ºC prior to analysis by High Performance Liquid Chromatography (HPLC). This was later passed through the filter paper (0.22 µm) to remove any remaining particles from the medium of growth. Prior to the storage, part of the supernatant were used for pH measurement. Organic acids were separated with an Agilent Zorbax SB-Aq (4.6 x 150nm) 5-µm column, eluted isocratically at 1 mL min^{-1} with 20 mM NaH_2PO_4 at pH2 buffer with the column at 25 °C and detected on a diode array detector at 210 nm (Agilent 1100 series). Peak identity and organic acid quantity were determined by comparison with standards. The organic acid standard included gluconic, acetic, citric acid and maleic acid that were well separated under the described chromatographic conditions.

5.2.9 Fourier transform infrared (FTIR) spectroscopy

Exopolymeric substances produced by some of the bacterial isolates were analysed using reflectance infrared (IR) method with a KBr matrix. Measurements were taken with scans using a Perkin Elmer spectrum RX IFT-IR system. The process involved the initial precipitation of the

spent medium with ethanol. A control treatment with the same culture medium and iron ore but with no bacteria was treated the same way and used as a background to set the machine. This was to enable the elimination of any interference from the culture medium and iron ore samples during the reading. The acquisition of the spectra was through the transmission mode. Pellets formed after the ethanol treatments were mixed with KBR and dried for 24 h at room temperature. This was followed by crushing and pressurisation of the mixture to form pellets that were used for the reading. Background was set with the control.

5.2.10 Microscopy

Part of iron ore samples collected during harvesting were fixed by applying 15 ml of fixing solution (2.5% glutaraldehyde in 0.0075 M phosphate buffer) onto the ore inside Greiner tube. Ore samples were washed three times for 15 min each with 0.0375 M phosphate ($Na_3(PO_4)$) buffer. The samples were then dehydrated at different alcohol concentrations (50, 70, 95, 100%), at 10 min each. The dehydrated samples were repeatedly soaked in 100% alcohol twice. This stage was followed by drying of the samples that were later sputter-coated in a Polaron Equipment LimitedSEM Coating Unit E5200 with gold prior to observation under the scanning electron microscope (SEM). They were then assembled for observation under the microscope at 5 kV on a JEOL 5800LV scanning electron microscope (Tokyo, Japan).

5.2.11 Induction Coupled Plasma (ICP)

Iron ore samples collected during harvesting were repeatedly washed with 0.1 M HCl and later left in deionised water for 24 h. The samples were dried at 104 °C before sending them for Induction Coupled Plasma (ICP-OES Optima 4300 DV, Perkin Elmer, Waltham, MA, USA) analysis by UIS Analytical Services, Pretoria, South Africa.

5.2.12 Experimental design and Statistical analyses

The statistical analyses were carried out using SAS software, version 9.2 (SAS Institute, 2008, Cary, NC, USA). The analyses was done as 3-way Anova with two levels of iron type (KB, SB),

nine levels for bacteria including the control with no bacterial sample (KU1, KU7, KC1, KC2, KU6, KU8, SU5, SU7 and CTR-control) and three levels for the time (week)(Week1-W1, Week 2-W2, Week 3 W3). For all the variables, there were two types of control that were included (one with the bacteria and no iron ore and the other with no bacterial sample). The analysis was also done as a 3-way Anova with 3 levels of iron (KB, SB, CT-control), bacteria (9 levels): (KU1, KU7, KC1, KC2, KU6, KU8, SU5 and SU7) and three levels for the time; week (W1, W2, W3). For all these variables, the log transformation was used in order to fulfil the assumptions of the model and each time, an interaction of order 3 was observed, i.e. an interaction between IRON vs Bacterial vs Week. Multiple comparisons were done using the stepdown Bonferroni method in order to protect the type 1 error rate. Normality assumptions were verified with the Shapiro-Wilk's statistic and the homogeneity of variances was verified by the residual plots.

5.3 Results

Phosphorus and potassium contents of the iron ore materials were analysed by Induction Coupled Plasma ICP-OES Optima 4300 DV (Perkin Elmer, Waltham, MA, USA), which showed that KGT originally contains an average of 0.805% K and 0.14% P whereas SK has an average of 0.423% K and 0.09% P. Other major compounds contained in the iron ore samples are SiO_2 (32.48%), Al_2O_3 (4.12%) and Fe_2O_3 (61.51%) for SK, while KGT had SiO_2 (5.32%), Al_2O_3 (2.94%) and Fe_2O_3 (89.75%).

From the three media used, the highest number of isolates was obtained from the MMN medium, followed by TSA and then PSM. A total of 23 morphologically distinct isolates were obtained during the isolation processs (Fig. 5.1). The homology sequence (Appendix III) and phylogenetical analyses of the 16S rDNA of these isolates enabled their division into four different clades which included *Protebacteria*, *Firmicutes*, *Bacteroidetes* and *Actinobacteria* (Fig. 5.1). Most of the isolates belong to the *Protebacteria* clade which was subdivided into Alpha-*Protebacteria* with isolate TKU1, Beta- *Protebacteria* with isolates KC2, KU2, KU13, SC4, SU4 and SU9 and Gamma- *Proteobacteria* with isolates KU6 and SU7. The *Actinobacteria* cluster consists of isolates KU8, SU3, KU4, KU7, KU3, KC4 and SU1, while the *Firmicutes* cluster consists of SU2, KC1, KU5, SU5, KU1 and SC5. Only one isolate (TS4) belongs to the *Bacteroidetes* clade.The isolates, together with their recently allocated accession numbers, as well as their close relatives from the GenBank are presented in Fig. 5.1.

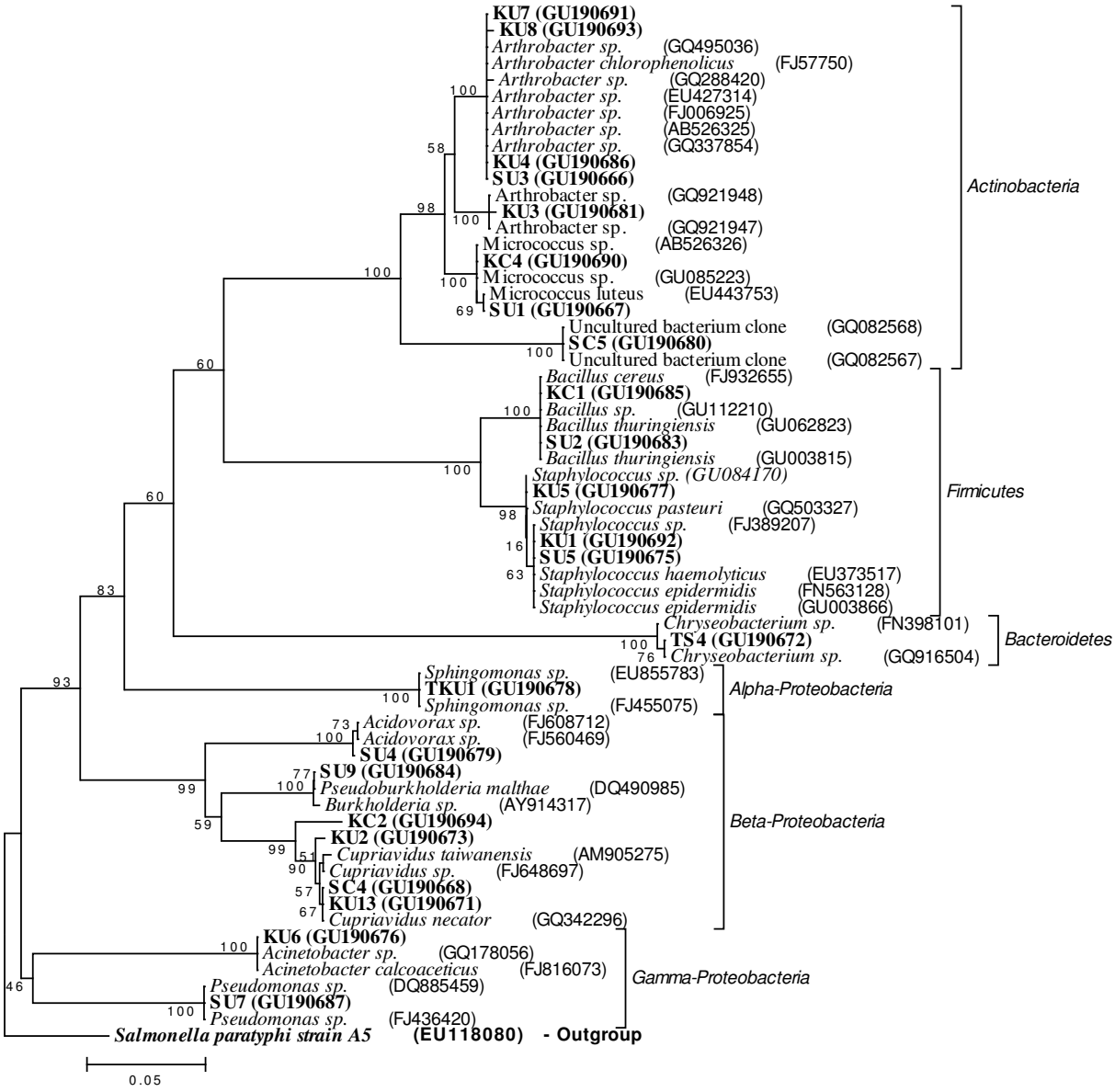


Figure 5.1: Phylogenetic tree of the 16S rDNA of bacterial isolates obtained from KGT and SK mineral types (in bold) and their related species obtained from the GeneBank as established by boostrap neighbor-joining method.

When cultured on PSM, seven of the bacterial isolates tested positive for the P solubilisation (Fig. 5.2). These include isolates KU6, SU5, SU7, KC2, KU8, KU7 and KU1. In addition, only one of the isolates effectively lowered the pH of the medium of growth towards the acidic range -

KC1. For the K solubilisation, four of P solubiliser isolates also showed positive abilities to solubise K by producing high levels of slime. At the end, eight bacterial isolates were positively identified as potential mineral solubilisers with these methods. Molecular and phylogenetic analyses of the nucleotide sequences of these isolates revealed that they are closely related to six genera that included *Staphylococcus* (KU1 and SU5), *Bacillus* (1), *Arthrobacter* (KU8 and KU7), *Acinetobacter* (KU6), *Cupriavidus* (KC2) and *Pseudomonas* (SU7) (Fig. 5.1).

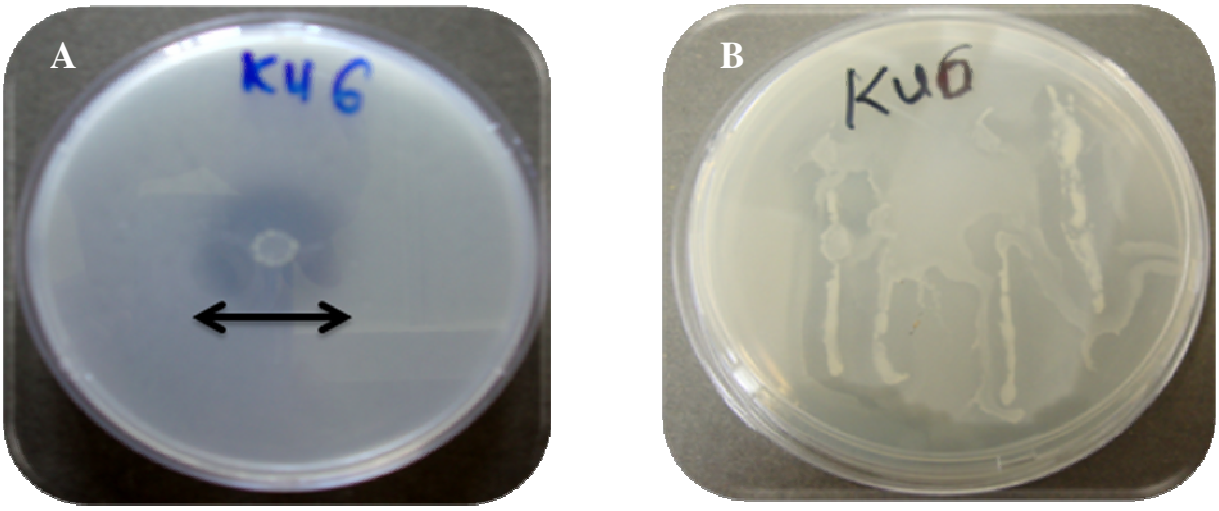


Figure 5.2: Phosphate-solubilising ability of isolate KU6 (A), as indicated by the halos (length of halo showed with the arrow) and high potassium usage, as indicated by high slime production that covered almost the entire plate.

The results of the shake-flask experiment revealed that iron ore type, bacteria type and time, as well as most of the interactions between these factors have significant effects on the rate of K and P removal from the iron ore (Table 5.1).

Table 5.1: Influence of mineral type, iron type, bacterial type and their interactions on the percentage K and P loss.

Sources of Variation	df	% K loss df= 105		%P loss df= 104	
		F	*P*	*F*	*P*
Iron	**1**	1650.2	<.0001	1684.52	<.0001
Bacteria	9	351.5	<.0001	1887.33	<.0001
Iron vs Bacteria	9	156.3	<.0001	555.4	<.0001
Week	2	34.29	<.0001	8.78	0.0003
Iron vs Week	2	3.08	0.0502	34.99	<.0001
Bacteria vs Week	18	14.65	<.0001	15.19	<.0001
Iron vs Bacteria vs Week	18	11.33	<.0001	21.48	<.0001

P-values < 0.005 are considered significant.

Higher percentages of K and P removal were obtained from SK mineral treatments compared to KGT mineral treatments (Fig. 5.4 and 5.6). Treatments involving isolate KU6 turned out to show highest percentage of K removal (up to 52%K removal) from SK mineral type (Fig. 5.4). Other isolates such as KU8, SU5, KU7, KC1 and SU7 also showed significantly higher K removal (>30%) (Fig. 5.4). For KGT mineral type, 29.52% was the highest percentage of K removed by bacterial isolate SU5 and there was no significant difference between this value and those from bacterial isolates KU6, KC2 and SU7 for this mineral type (Fig. 5.3). Potential for P removal (compared to K) seems to be more feasible for some of the isolates, as up to 97.5% of the total P was removed by isolate KU8 from SK mineral type (Fig. 5.6). More than 85% P was also removed from treatments involving isolates KU7, KU6 and KC2, with no significant difference among the values (Fig. 5.6). These isolates (KU8, KU7, KU6 and KC2) are also responsible for the highest P removal from KGT mineral type, removing more than 60% P from this mineral type (Fig. 5.5). In terms of time effect on K and P removal, there was no general trend that is applicable to all the isolates. For instance, isolate KU6 was able to remove a high portion of the K content of SK mineral type for the duration of the experiment (1^{st} to 3^{rd} week), whereas the value of percentage K removed by KU7 dropped from first week through second to third week (Fig. 5.4). Organic acid production by these isolates was significantly affected by mineral type, bacteria type and time, as well as the interactions between these factors (Table 5.2).

Table 5.2: Three-way analysis of variance (ANOVA) with F and P values that that show the effects of bacterial type, mineral type and their interactions on the release of four different organic acids.

Sources of Variation	**df**	**Gluconic acid df= 103**		**Acetic acid df= 102**		**Citric acid df= 106**		**Propanoic acid df= 107**	
		F	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Iron type	2	7438	<.0001	3457.58	<.0001	192.63	<.0001	3153.09	<.0001
Bacterial type	7	2231.6	<.0001	865.68	<.0001	351.2	<.0001	389.48	<.0001
Iron type vs Bacterial type	15	466.49	<.0001	1066.8	<.0001	91.9	<.0001	295.52	<.0001
Time (Week)	2	199.6	<.0001	394.11	<.0001	86.03	<.0001	265.85	<.0001
Iron type vs Time (Week)	4	110.46	<.0001	78.83	<.0001	18.12	<.0001	149.08	<.0001
Iron type vs Bacterial type	16	3.01	4E-04	65.47	<.0001	8.83	<.0001	30.71	<.0001
Iron type vs Bacteria vs Week	32	6.12	<.0001	100.56	<.0001	9.61	<.0001	48.21	<.0001

P-values <0.005 are considered significant.

In general, gluconic acid was constantly detectable and the highest produced organic acid by the isolates. However, there are some discrepancies between the quantity of organic acids produced and leaching of K and P by the isolates. More gluconic acid production was recorded in some of the control treatments (Fig. 5.7). In terms of the two minerals, isolates growing under the treatments involving SK mineral type were able to release more acids, especially gluconic acid, than those growing under the KGT mineral treatments. Generally, there is increase in organic acid production by most of the isolates from 1st to 3rd week, but this does not portend the trend of K and P removal, as the rate of these elements removal in most cases decreases after the 2nd week of the experiment.

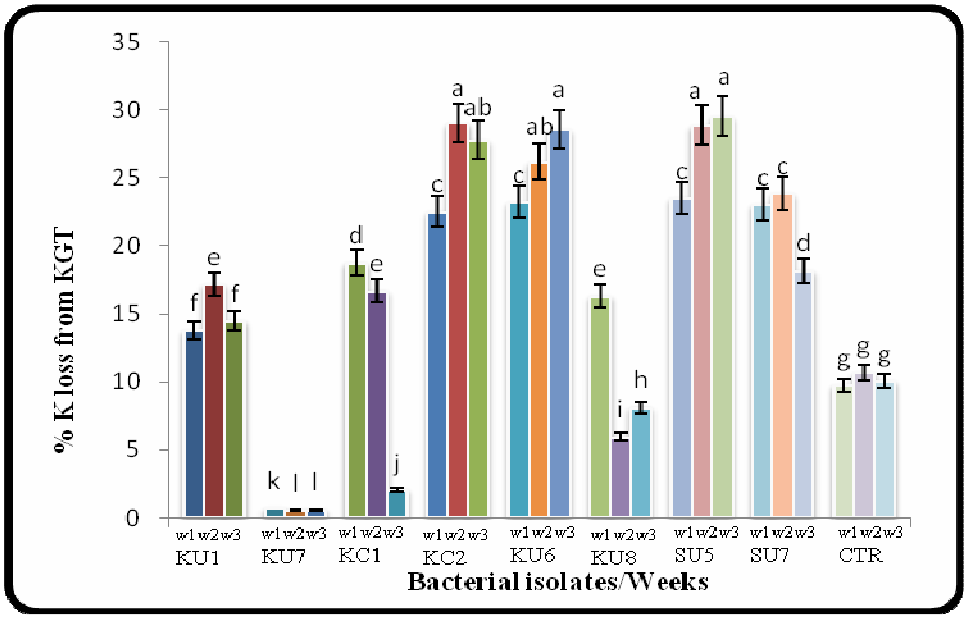


Figure 5.3: Amount of K loss from the KGT mineral type in treatment by all the identified isolates that are "mineral solublisers". Error bars are ±SE (n = 3). CTR represent where iron ore materials were left inside the modified PSM medium with no bacterial inoculation. (P<0.001).

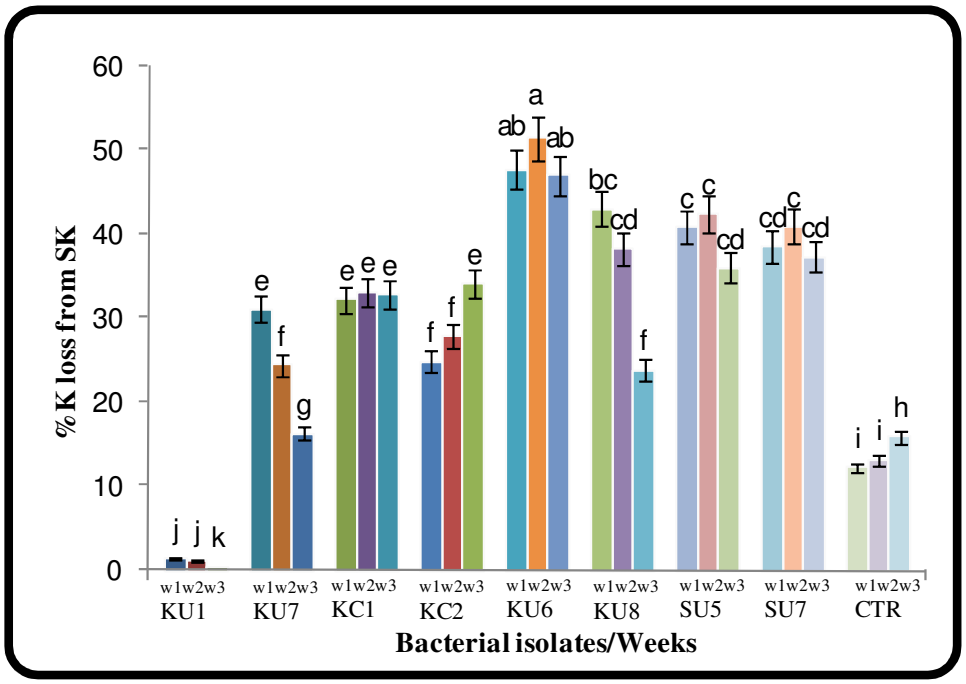


Figure 5.4: Amount of K loss from the SK mineral type in treatment by all the identified isolates that are "mineral solublisers". Error bars are ±SE (n = 3). CTR represent where iron ore materials were left inside the modified PSM medium with no bacterial inoculation. (P<0.001).

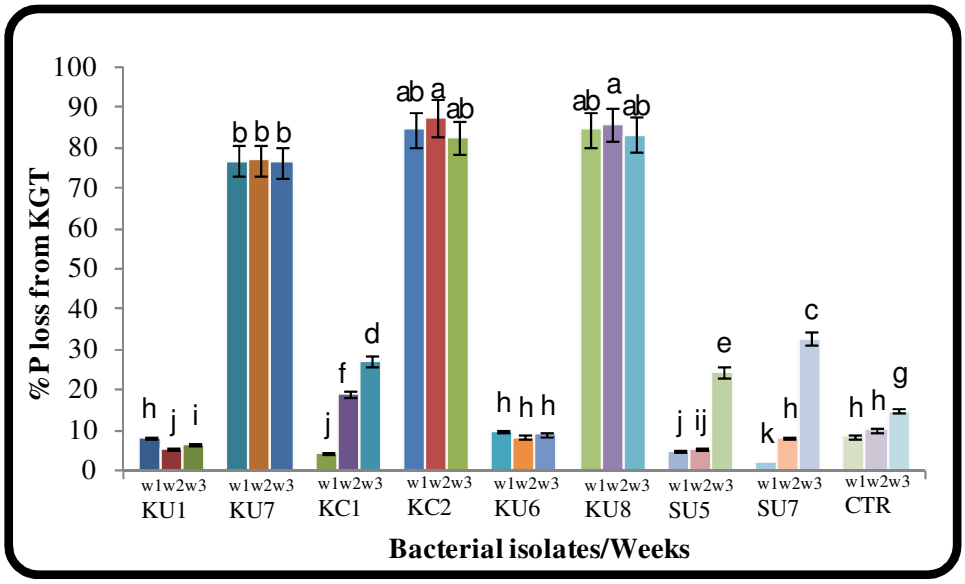


Figure 5.5: Amount of P loss from the KGT mineral type in treatment by all the identified isolates that are "mineral solubilisers". Error bars are ±SE (n = 3). CTR represent where iron ore materials were left inside the modified PSM medium with no bacterial inoculation. (P<0.001).

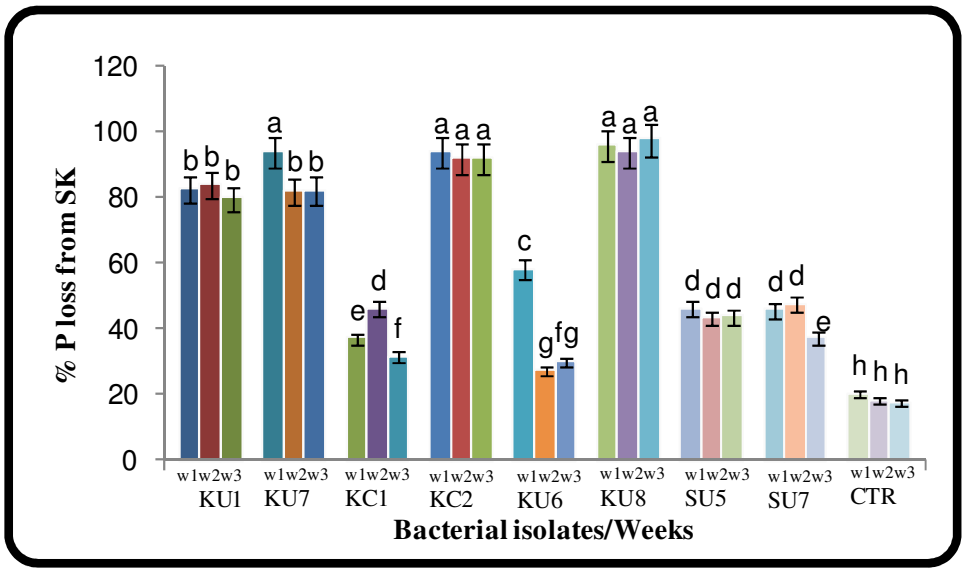


Figure 5.6: Amount of P loss from the SK mineral type in treatment by all the identified isolates that are "mineral solublisers". Error bars are ±SE (n = 3). CTR represent where iron ore materials were left inside the modified PSM medium with no bacterial inoculation. (P<0.001).

Statistical analysis of the pH result indicates a strong influence of the mineral type on the pH (Fig. 5.8). It is apparent that mobilisation of elements from SK mostly occurred at pH above 5 while for KGT it is mostly below pH 5. Few isolates that showed potential to reduce K and P from the ore minerals were selected for microscopic observations. Biofilms of isolates KU6, KU8, SU7, KU7 and SU5 were observable during the SEM analysis (Fig. 5.10A to 5.10E). The biofilm formation was at different intensity with more visible attachments observed for isolates KU6, SU5 and KC2. Isolate KU8 was observed to produce EPS, with very strong binding capacity that influenced the sticking together of a larger quantity of the mineral particles (Fig. 5.10). The FTIR spectra of precipitated substances showed several absorbance bands (Fig. 5.10) from isolates KU6 (best for K removal) and KU8 (best for P removal). As listed in Table 5.3, these bands depict different functional groups of proteins, lipids extracellular polysaccharides and nucleic acids.

Table 5.3: Band assignments of FITR spectra obtained from experiments involving isolates KU6 and KU8.

Functional groups (Reference)	Isolate KU6	Isolate KU8
OH of water (Omoike and Chorover, 2004; Delvasto *et al*., 2009)	3410	
Asymmetric -CH3 stretching vibration, fatty acids (Omoike and Chorover, 2004; Delvasto *et al*., 2009)	2968	2982 2958
Asymmetric -CH2 stretching vibration, fatty acids (Omoike and Chorover, 2004; Delvasto *et al*., 2009)	2908 - 2364	2858
Carboxyl (C=O) stretch; Ester, fatty acids (Jiang *et al*., 2004; Delvasto *et al*., 2009)		1728
Amide I (C=O)	1657	
Amide II, N-H, C-N and structure of proteins (Jiang *et al*., 2004; Delvasto *et al*., 2009)	1537	1530
Carboxylate (Jiang *et al*., 2004; Delvasto *et al*., 2009)	1401	1402
C-O bond from carboxylate ions (Omoike and Chorover, 2004) Vibrations of -COOH and C-O-C in ester (Jiang *et al*., 2004; Delvasto *et al*., 2009)	1314 1234	
P=O bond stretching in phosphate (Jiang *et al*., 2004; Delvasto *et al*., 2009)	1218	1201
C-O-C (Glycosidic linkage), C-O, C-C vibrations (polysaccharides) (Omoike and Chorover, 2004; Jiang *et al*., 2004; Delvasto *et al*, 2009) Phosphate or sulfur functional groups (Comte *et al*., 2006 Delvasto *et al*., 2009)	1111 - 1025 ≤ 903	1141-1070 ≤ 974

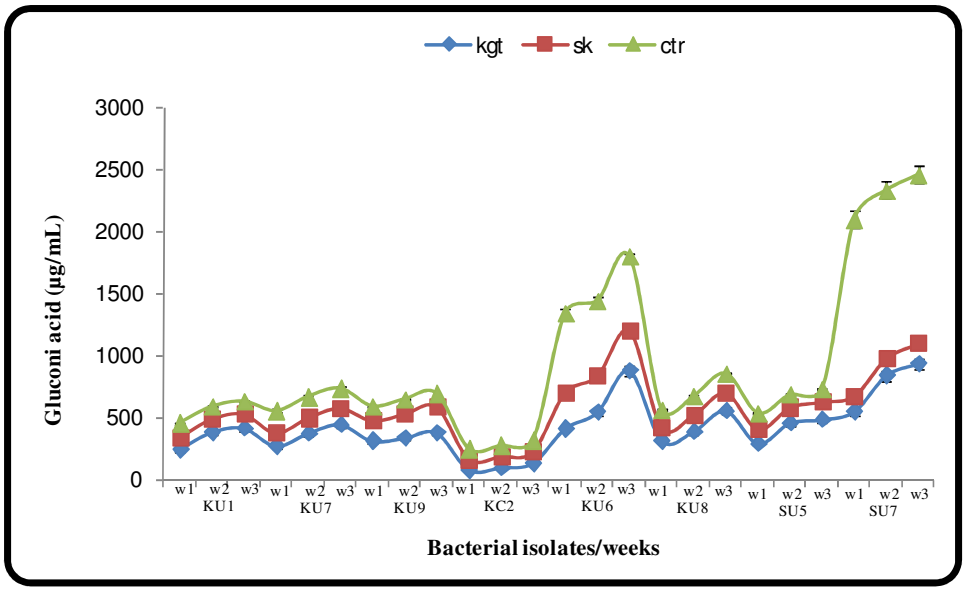


Figure 5.7: Amount of gluconic acid released during the shake flask experiment in the presence of both KGT and SK mineral types. CTR represent where bacterial isolates were cultured with PSM (complete), and no iron ore was present. (P<0.001).

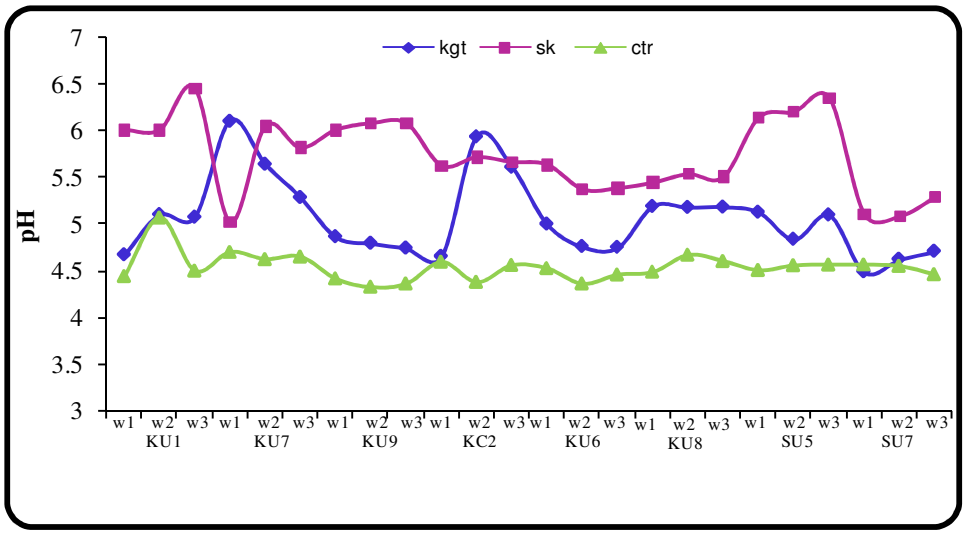
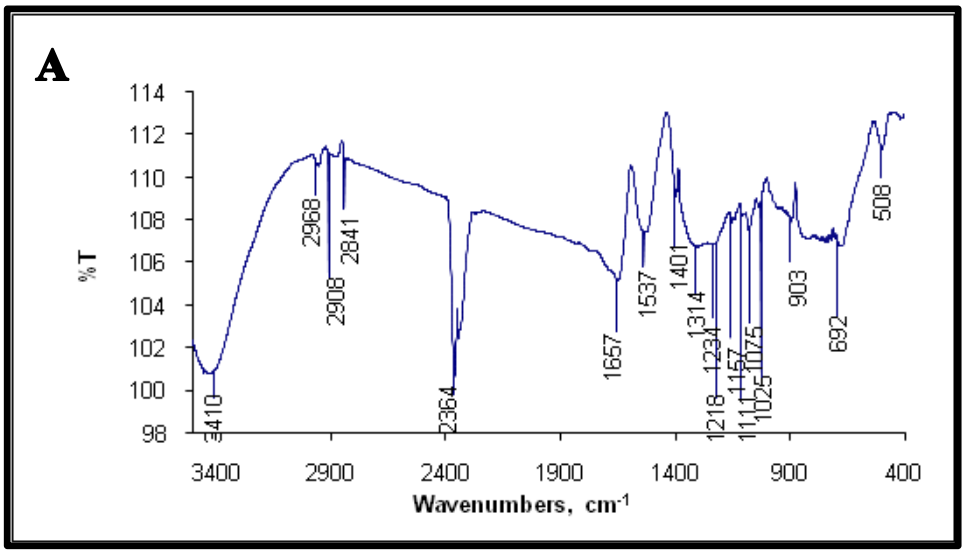


Figure 5.8: pH measurements during the shake flask experiment in the presence of both KGT and SK mineral types. CTR represent where bacterial isolates were cultured with PSM (complte), no iron ore was present. (P<0.001).



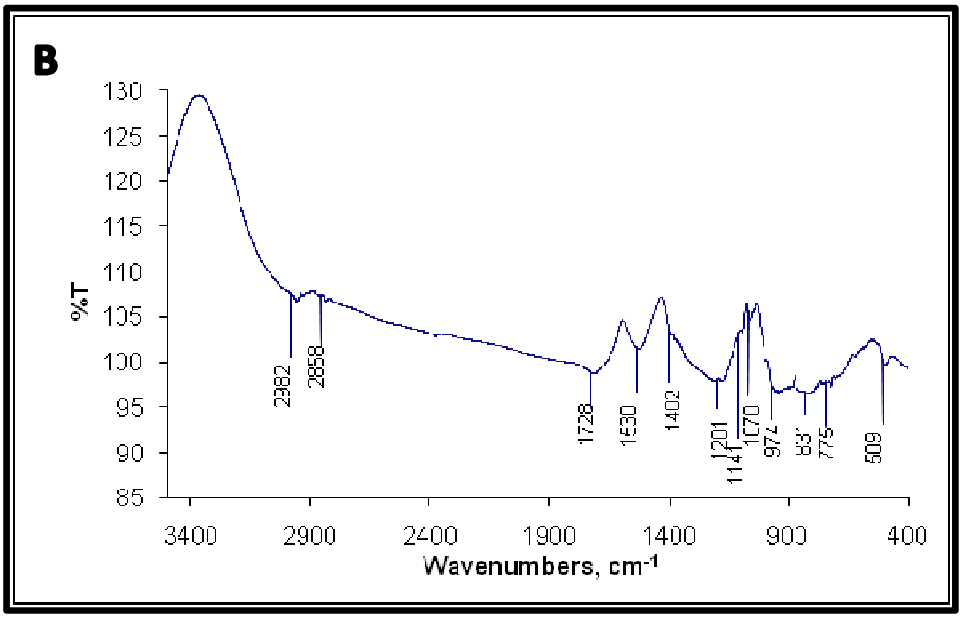
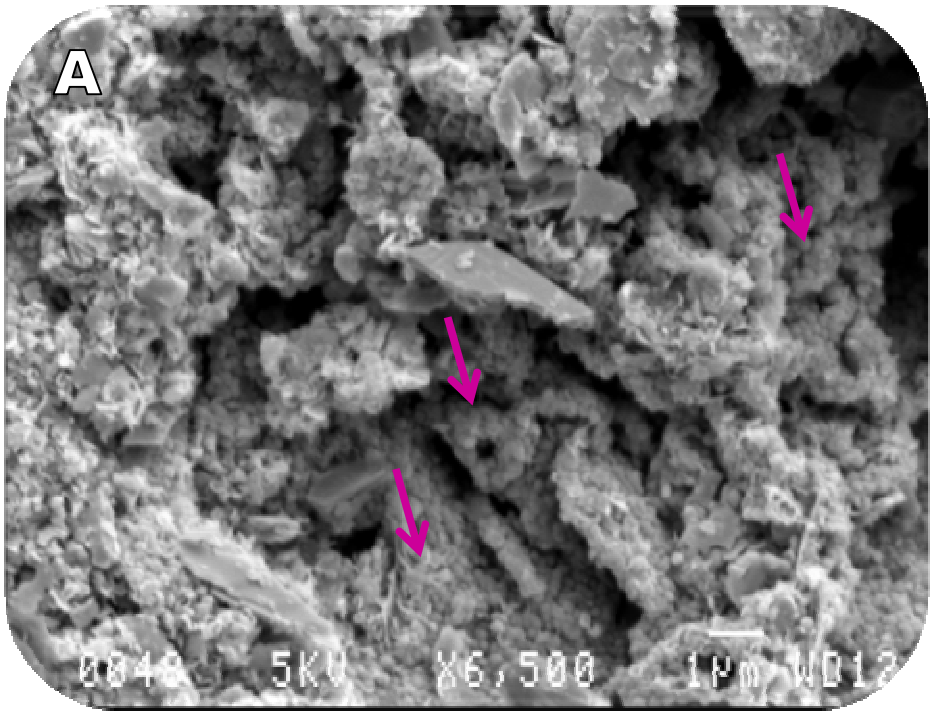
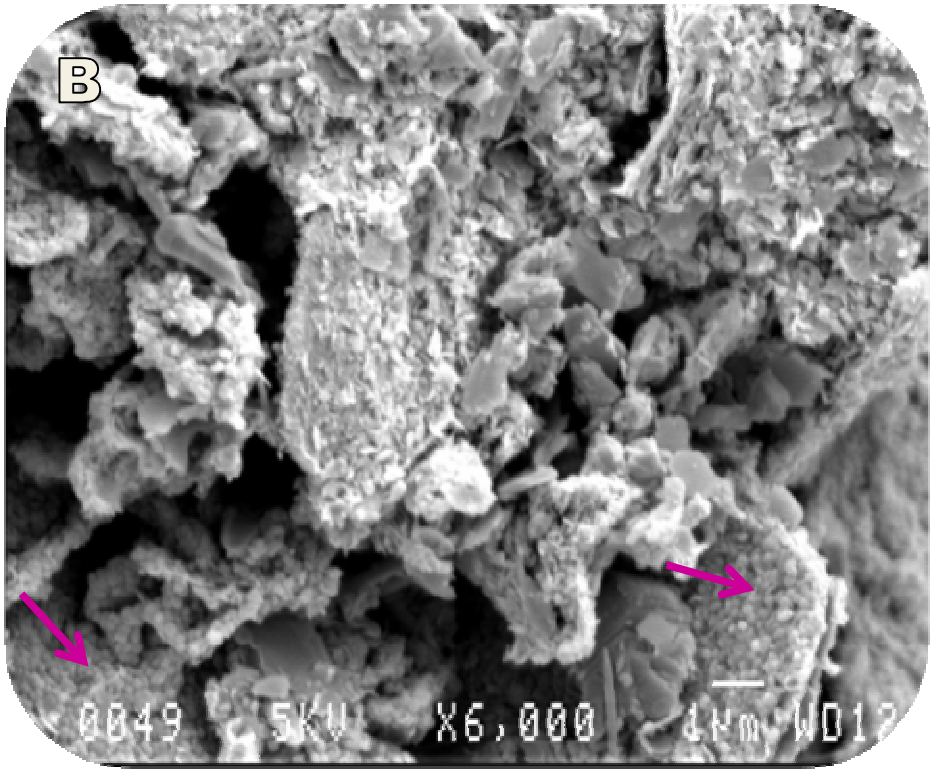
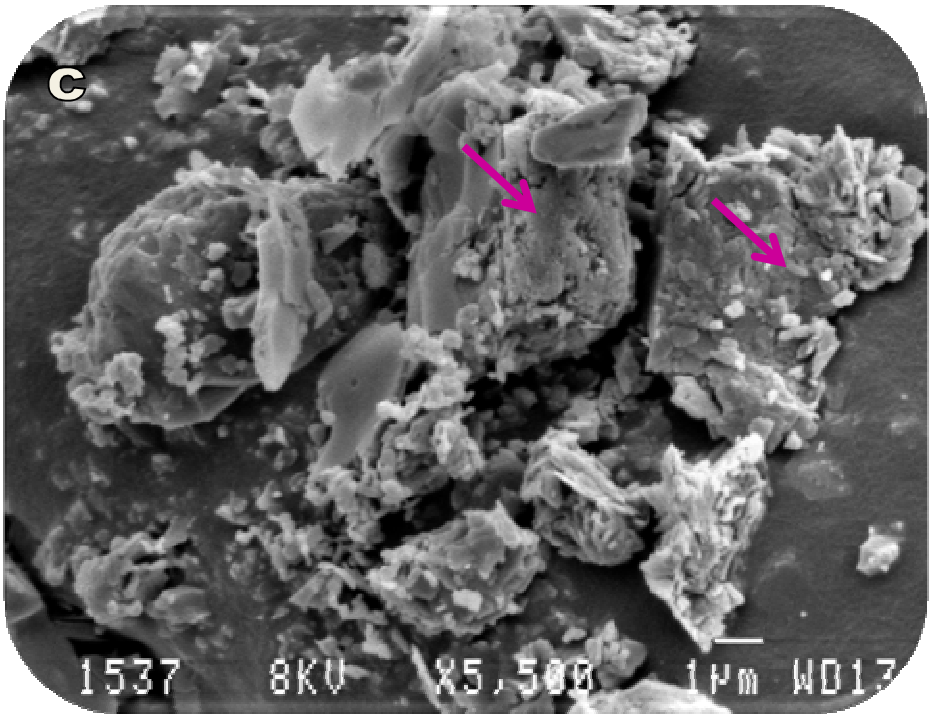
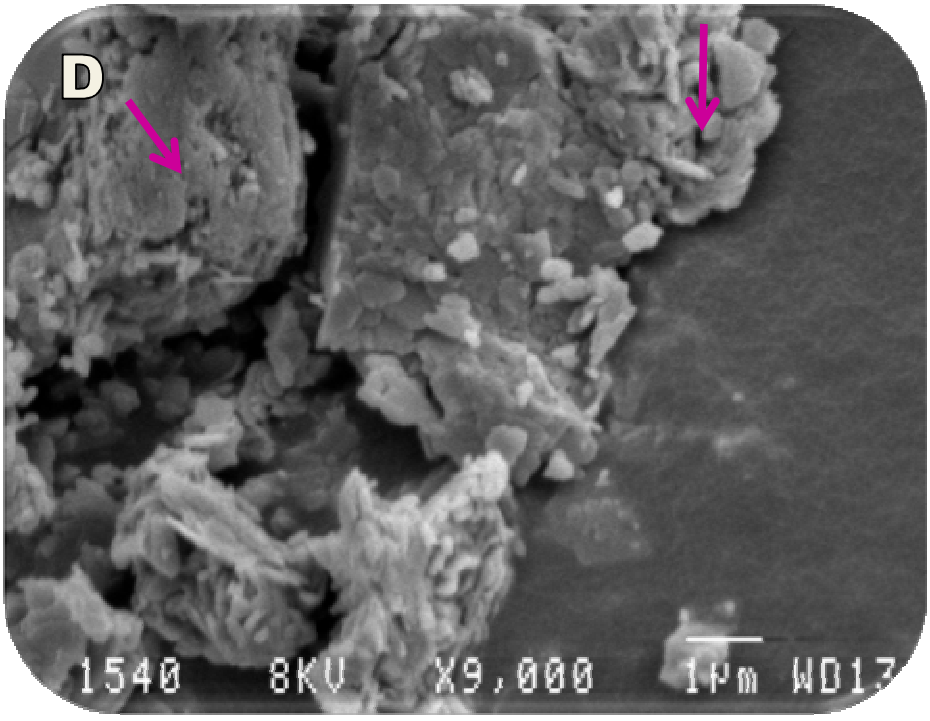


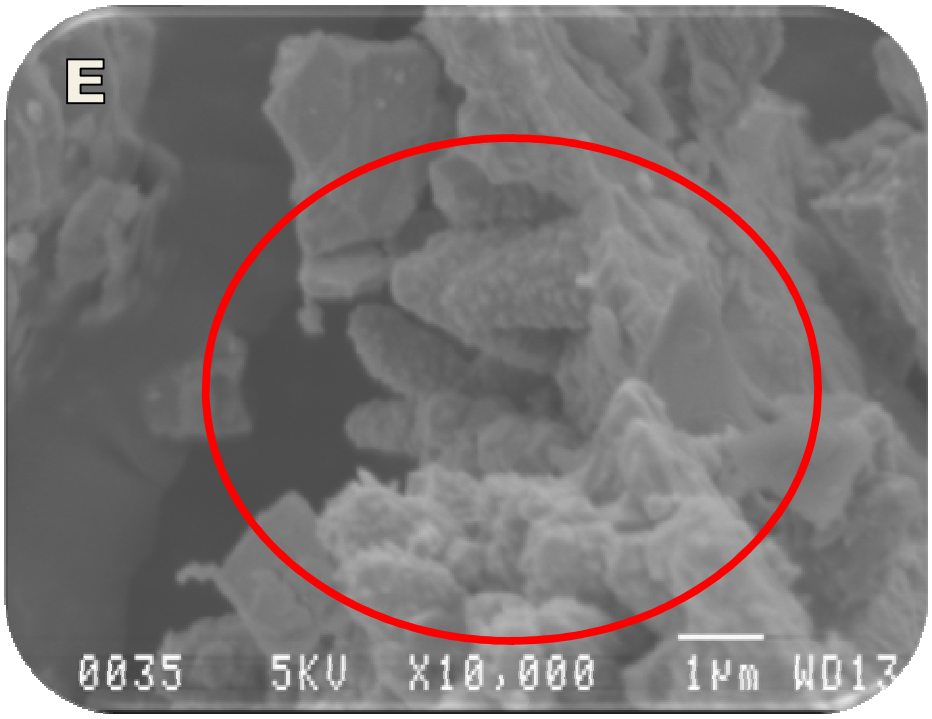
Figure 5.9: FT-IR spectrum of precipitated bacterial EPS from isolate KU6 (A) and isolate KU8 (B) growing in the presence of SK mineral type, week 1.

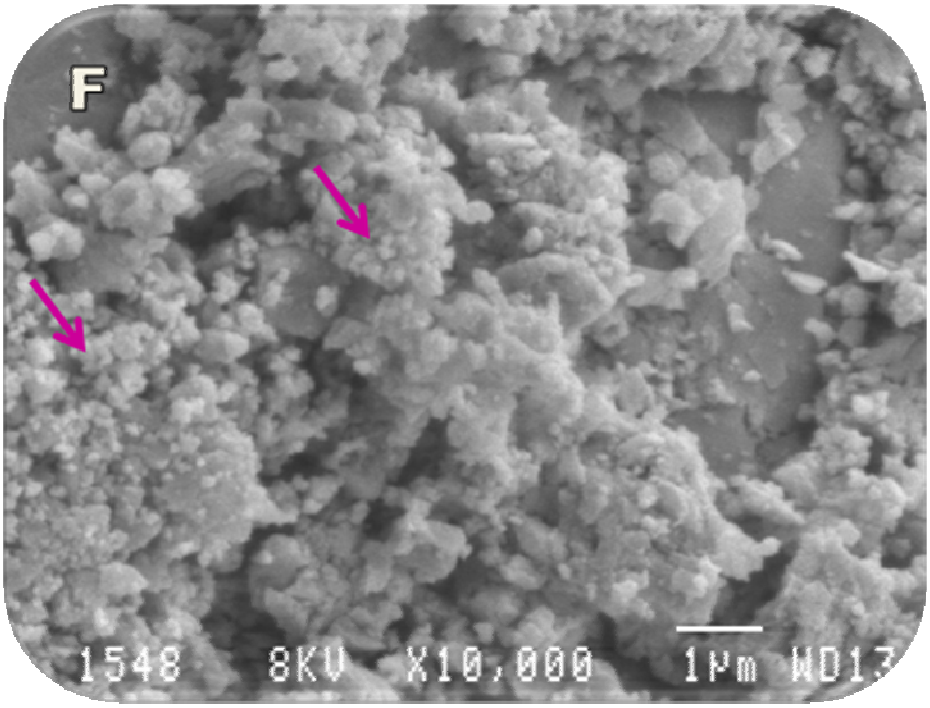

A
5KV
X6,500


B
0049
5KV
X6,000


C
1537 8KV X5,500 1µm WD13


D
1540 8KV X9,000 1µm WD13


E
0035 5KV X10,000 1µm WD13


F
1548 8KV X10,000 1µm WD13

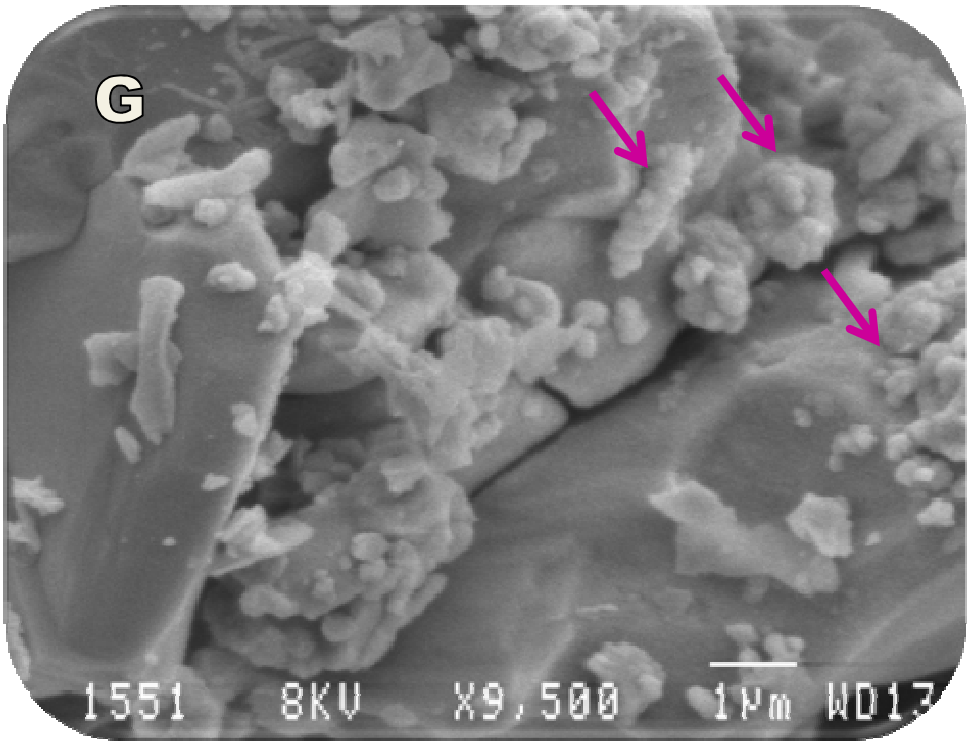


Figure 5.10: A and B represent the SEM images of isolate KU6 treatment of SK and KGT mineral types, respectively, with visible biofilm and strong attachment (arrows) to the iron surface (week 2 of the experiment). C and D represent the SEM images of isolate KU8 with high possibility of EPS secretion that binds the iron ore particles together strongly (arrows). E, F and G represents isolate SU5, KC2 and KU7, respectively, with visible forms of biofilm formation (circles and arrows).

5.4 Discussion

Unlike other metals such as copper and gold, there is no fully developed biohydrometallurgical process for treating iron ore minerals. This is partly due to the low cost of this mineral, as well as the nature of the chemical constitution (Delvasto *et al*., 2009). Biohydrometallurgical processes are mostly developed for sulfidic minerals where microbes can utilise either sulfur and iron or both as sources of energy (Rawlings, 2005). The use of such technology for biobeneficiation of Sishen iron ore may simply defeat the purpose of the technology, as iron is the major element of interest contained in iron ore materials. With all these in mind, this study has focussed on simple methods of isolating potential microbes from the iron materials and subsequently tested their potential in mobilisation of unwanted parts of the ore materials.

The choice of indigenous microbes for this investigation is essentially due to possibility of their better acclimatisation to the biobeneficiation environment. The diversity and number of bacterial isolates obtained from the iron ore surface indicates a high possibility of finding one or more potential bacteria that will be able to mobilise K or P or both from iron ore minerals. All the phylogetically identified groups, *Proteobacteria*, *Firmicutes*, *Bacteroidetes* and *Actinobateria*, isolated in this study have been previously identified and utilised for biohydrometallurgical purposes (Groudeva *et al*., 2007; Qui *et al*., 2007). At the genus level, isolates SU7, KU8, KU4, KU3 and SU3 that are closely related to *Arthrobacter*, are the dominant group. This genus of bacteria was used by Willscher and Bosecker (2003) in the leaching of alkaline siliceous slag.

Although the two iron ore minerals used in the present study are low grade, their chemical constitutions are not the same, with KGT having higher contents of the desired Fe than SK. However, KGT has higher levels of the "unwanted" elements - K and P, than SK. Such difference is also an indication that K and P contents of these minerals may be bonded and exists in different forms inside the ores. Such variation is in most cases discussed under mineralogy and was noted by Sheng *et al*. (2008) in their investigation. In that study, three different silicate minerals, namely feldspar, muscovite and biotite, contained different ratios of different compounds. This

fact can affect microbial leaching because microbes are known to utilise different mechanism to obtain nutrients from different compounds contained inside minerals (Jain and Sharma, 2004).

Bacterial screening methods used in this study were directly and indirectly adapted to acidification and direct nutrient utilisation mechanisms. According to Jain and Sharma (2004), acidification by biobeneficiation microbes can occur through production of acidic metabolites or selective preference for alkaline substrate. The former is evident in the levels of gluconic acid detected in the spent medium of all the isolates used in the shake flask experiment but it is difficult to ascertain the selective utilisation of alkaline substrate. Isolate KC1 that was included in the shake flask experiment, because of its ability to lower the pH of nutrient medium showed no such feature in the presence of the iron ore minerals. This may be due to the differences in the chemical constitution of the two media of growth (nutrient medium and modified PSM). Meanwhile, the direct nutrient utilisation was linked to the slime production by microorganisms that enhance microbial attachment to mineral surfaces and provide nutrient diffusing channels between the microbes and mineral surfaces (Gulevich *et al*; 1968; Willey *et al*., 2007).

The effects of mineral type were noticeable in all the isolates tested. The higher percentage K and P removal from SK treatments compared to KGT treatments may be related to the composition of these minerals, as previously mentioned. Although both are iron ore minerals, compositional differences in their mineral constituents could easily affect their rates of dissolution. In a study conducted on three silicate minerals (feldspar, muscovite and biotite) by Sheng *et al*. (2008), *Bacillus globisporus* Q12 strain exhibited differences in the release of K and Si from the minerals. Such differences are attributable to dissimilarities in sites of attachment for reagents such as protons and ligands involved during the bioleaching process (Huerta *et al*, 1995; Welch and Ullman, 1996). The only isolate that exhibited the ability to remove enough K up to the commercial standard was KU6, identified molecularly to be closely related to *Acinetobacter calcoaceticus*. From the available literature, no study has been able to connect this isolate to biobenefication process. However, studies have emphasized the important role of the biofilms produced by *Acinetobacter calcoaceticus* (Elkeles *et al*., 1994; Rosenberg and Ron, 1997). These are generally referred to as emulsans, highly beneficial for hydrocarbon degradation (Rosenberg

and Ron, 1997). The commercialisation of bioemulsans produced by *Acinetobacter* (Rosenberg and Ron, 1997) is an indication that further investigation of this bacterial isolate and its metabolites can provide direction in the development of a reliable method for solubilisation of iron ore minerals. It was even more interesting to discover that this isolate could reduce the P and K content of the iron ore samples (SK) below commercially approved at the same time. Such characterisitic was exhibited by strains of *Paenibacillus* spp. in a study conducted by Hu *et al*. (2006). In that study, the agricultural importance of the combined solubilising activity of the isolates was acknowledged. For the present study, it means a system that utilise only one organism can be invented for the removal of one or more impurities from iron ore minerals. Moreover, the bacterial isolate with the highest ability to mobilise P from the iron ore mineral is isolate KU8, which is closely related to *Arthrobacter* sp. This genus of bacteria have been isolated and utilised in biohydrometallurgical processes (Cardone *et al*., 1999; Luca *et al*., 2008). For instance, Cardone *et al*. (1999) utilised a species of *Arthrobacter* to remove manganese from chalcopyrite.

The mechanism involved in organic acid production by heterotrophs is essential for mobilisation of nutrients from minerals (Jain and Sharma, 2004). This study seems to have confirmed the same trend. Meanwhile, it is important to mention here that the cross-interactions that exist between the factors studied made it difficult to directly compare the organic acids with percentage K and P removal from the iron ore minerals. However, consistent detection of the organic acid in the growth medium is an indication that they are involved in the leaching process, especially gluconic acid that was detected in higher amounts compared to other organic acids (Sheng *et al*., 2008; Delvasto *et al*., 2009). One of the interesting outcomes of this study is the preferential absorption of elements by the isolates. For instance, it is apparent that isolate KU6 has ability to mobilise more K than P, whereas the reverse is the case with isolate KU8. Although this may simply be explained by the differences in their metabolic pathways, the underlying mechanism involved could be elucidated through the activities of gluconic acid. This acid could have acted in two possible ways during the iron ore solubilisation.The first is the direct activity on the surface of the mineral that involved the complexation with anions from the acid with cations of K^+, Al^{3+} (also contained in the ore) and Fe^{3+}. Secondly, it is also possible for the

protons from the acid to replace interlayer K on the mineral surface due to their similar shapes and smaller size of the protons, thereby creating an "outflow" of K ions from the minerals that subsequently increased the mineral dissolution rate (Gadd, 1999; Yuan *et al*., 2004; Delvasto *et al*., 2009). Apart from the K mobilisation, such activities normally disrupt the structure of the iron ore including the phosphate content. Therefore, the solubilisation of P may be high when P is linked to the iron phase of the mineral that consists ions with high chelation constant such as Al^{3+} and Fe^{3+} contained in Sishen iron ore (Yuan *et al*., 2004; Delvasto *et al*., 2009).

Scavenging is one of the methods through which microbes obtain nutrients from minerals, but this condition is mainly created by need. Nutrient limitation is an essential condition for microbes to "re-engineer" their feeding mechanisms in order to obtain nutrients from complex substrates (Banfield *et al*., 1999; Bennett *et al*., 2001). As observed under the microscope in this study, bacterial isolates attached differently to dissimilar sites on different minerals. Different functional groups found in bacterial cell wall materials such as those detected in the present study through FTIR (carboxyl - COOH, amino - NH_2, and hydroxyl- -OH), are known to greatly influence bacterial attachment to mineral surfaces (Deo and Natarajan, 1998). The attachment is usually preceded by interactions between the bacterial cell wall and the mineral surface, a process that leads to changes in surface chemistry of the mineral (Devasia *et al*., 1993). One or more of electrostatic, hydrophobic and specific protein interactions can make this type of attachment to occur (Sampson *et al*., 2000). Scavenging is made easy for bacteria through the production of special substances that enhance their attachment to surfaces. These substances are high molecular weight compounds known as extracellular polymeric substances (EPS). They are composed of polysaccharides, proteins and nucleic acids (Omoike and Chorover, 2004). The polysaccharides part of EPS are believed to aid the component cells contained in biofilm to dissolve and utilise substrates that are normally inaccessible for utilisation (Sutherland, 2001). During scavenging, due to proximity, there could be direct utilisation of nutrients contained inside the mineral by bacteria cells of the biofilm. On the other hand, there could also be direct action of anions of organic acids from the biofilm on the mineral surface where the anions can react with cations of Fe^{3+} or Al^{3+}.

Any of the above mentioned methods would lead to an increased rate of mineral dissolution. However, as explained by Delvasto (2009), the disadvantage of this process in biobeneficiation is the accumulation of the "unwanted" elements (such as the P and K previously absorbed from the mineral ore) by the biofilm. Furthermore, leached elements could also be re-precipitated from the solution due to saturation. Such scenarios were discovered in this study when percentage quantities of K and P mobilised from the iron ore minerals were reduced between the second and third week. A feasible solution to this problem is to know the time required for the solution to be saturated with leached products, as well as monitoring the accumulation of these elements in the biofilm. This will ensure the benefication process is stopped at the appropriate time, but the idea will depend on the type of bacteria in question. For instance, two weeks could be enough for the beneficiation process involving isolate KU6 with mineral type SK.

For the first time, this study has provided information about bacterial inhabitants of Sishen iron ore. It is, however, pertinent to mention that the number of isolated bacteria from the iron ore may represent a very small fraction of the total population that inhabit the surface of this mineral. A higher number of the representative population could be obtained by utilizing molecular methods such as DGGE (Zwolinski, 2007). In addition, methods adopted in this study to screen the potential mineral solubilisers could have ommited other potential phosphate and mineral solubilisers. According to Pereze *et al*. (2007) not all phosphate-solubilisers can solubilise all forms of phosphate. In their investigation, bacteria that solubilised $Ca_3(PO_4)_2$ were unable to solubilise $FePO_4$ and $AlPO_4$. Such is the reality encountered in this study when some isolates such as KU6 and SU7 cannot solubilise most of the P contained in both minerals. Further investigation is therefore needed to investigate more types of growth media that could be used in the isolation of microbes from the minerals. In addition, from the available literature, there has not been any investigation on the use of indigenous microbes for K removal from iron ore minerals. This study has provided information and the possibilities of using indigenous microbes in this area of biohydrometallurgy. To improve on the results obtained from this study, screening methods of isolated microbes can be expanded to increase the chances of getting more and better mineral solubilisers. With this in mind, this study is therefore expected to provide a guideline for further investigations into use of indigenous microbes for biobeneficiation of iron ore minerals.

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CHAPTER SIX

FERMENTATION IN BIOHYDROMETALLURGY, A NOVEL METHOD FOR K AND P REMOVAL FROM IRON ORE

Abstract

A new method for reduction of potassium (K) and phosphorus (P) levels of iron ore minerals has been developed through the use of fermentation principles. The study was conducted through a process that involved fermentated spoilt grape fruits (*Vitis* sp.) and the solution from the product utilised in shake-flasks experiment. Treatments involved two types of iron ore minerals (KGT and SK) and two different particle sizes. Furthermore, different organic acids contained in the leaching solution were evaluated using High Performance Liquid Chromatography (HPLC). Scanning electron microscopy (SEM) and Fourier transform infrared (FTIR) spectroscopy were the other tools used to achieve study aim. The result linked the solubilisation of the iron ore minerals to high levels of gluconic and acetic acid in the leaching solution, as well as the biofilm and bacterial attachment to ore surfaces.

6.1 Introduction

Iron is an essential source of primary iron for the global iron and steel industries (Meyer, 1985). It is traded and consumed in different forms in many countries (Astrup and Hammerbeck, 1998). Due to high demand, high quality iron ore minerals are being depleted and this has created a situation for accumulation of low-grade iron ore minerals that cannot be exported. Currently, there is a need for the development of a green technology that can be used to treat such poor quality iron ore minerals (Delvasto *et al*., 2009).

Studies have attempted the use of biohydrometallurgical processes to improve the quality of low-grade iron ore minerals (Parks *et al*., 1990; Delvasto *et al*., 2005; Delvasto *et al*., 2009). For instance, Parks *et al*. (1990) utilised metabolites produced by *Penicillium*-like organisms to reduce the phosphorus (P) content of the iron ore mineral. In another study, Delvasto *et al*. (2005) utilised *Aspergillus niger* isolated from the surface of iron ore to mobilise the P content of the iron ore minerals. A similar experiment was also carried out to investigate the potential of *Burkholderia caribensis* FeGL03 in reducing the P content of iron ore minerals (Delvasto *et al*., 2009). Among other reasons cited by these investigators, organic acid production by microbes is probably the most important for leaching of iron ore.

Although microbial leaching has been widely accepted due to friendliness of the technology to the ecosystem, there are still some few factors that have deterred the complete development or acceptance of this technology. Firstly, the source of carbon and energy needed by the microorganisms has always been a cause of concern to investigators (Jain and Sharma, 2004). This is because of the cost implication; the commercially available sources of carbon for microbes such as glucose, sucrose, and fructose are too expensive to be considered for bioleaching processes (Jain and Sharma, 2004). This problem is partially linked to the second factor; the issue of sterility. In heterotrophic leaching, most of the isolated and identified potential bioleaching microbes grow at neutral pH (close to pH 7), which supports growth of many microorganisms, and therefore encourages easy contamination of experiments (Jain and Sharma,

2004). Maintenance of a high level of sterility for biohydrometallurgical processes is probably not a feasible idea, especially for cheap mineral resources such as iron ore minerals.

There is scanty information about studies that have attempted to tackle problems caused by these factors. A good example of an investigation that has focussed in this direction is the study conducted by Vasan *et al*. (2001). They carried out an experiment that involved the formulation of a new medium (Bromfield), using a cheap source of carbon (sugar cane). This special medium was then used to culture *Paenibacillus polymyxa* in the bioleaching of bauxite under septic conditions. Unfortunately, from the available literature, there has not been any such study that utilised septic condition for biobeneficiation of iron ore minerals.

This study was therefore, aimed at investigating the use of organic acid-producing bacteria for biobeneficiation of iron ore under septic conditions. The objective of this study was to utilise spoilt grape fruits - (usually harbouring organic acid-producing bacteria) for the production of a special leaching solution for biobeneficiation process.

6.2 Materials and Methods

6.2.1 Origin and preparation of iron ore samples

Two different types of iron ore samples, namely KGT (conglomerates) and SK (shale), were obtained from Sishen iron ore mine, Northern Cape Province of South Africa. The Induction Coupled Plasma (ICP) analyses of these iron ore types revealed that KGT originally contains an average of 0.805% K_2O and 0.14% P, whereas SK has an average of 0.423% K_2O and 0.09 % P. In addition, the chemical composition of the two ore types used for this study, as confirmed by the ICP analyses are (average of four samples): - SiO_2 (32.7%), Al_2O_3 (3.84%) and Fe_2O_3 (63.1%) with trace values of TiO_2, CaO, MgO, Na_2O, MnO, $Cr2O_3$, NiO, V_2O_5 and ZrO_2 for SK. For KGT, the ore contains SiO_2 (5.01%), Al_2O_3 (3.61%) and Fe_2O_3 (90.20%) with trace values of TiO_2, CaO, MgO, Na_2O, MnO, Cr_2O_3, NiO, V_2O_5 and ZrO_2 .

The iron ore materials were milled and separated into two different particle sizes of >0.21 mm to <0.84 mm and >0.1 mm to<0.21 mm by sieving. Henceforth, these will be referred to as particle sizes A and B, respectively. Pretreatments of the iron ore samples are as stated in chapter two (section 2.2.2).

6.2.2 Preparation of leaching solution

Fermented liquid from *Vitis* sp., common grape, was used for this process. The preparation involved crushing of the grape fruit in a blender under sterile conditions. Three hundred grams of the crushed samples were mixed with 2L of deionised water. The mixture was left for 7 d to allow fermentation and multiplication of the bacteria to take place. This stage was also expected to allow accumulation of bacterial metabolites that can influence the leaching process. After 7 d, the solution obtained was filtered using filter paper (0.45 µm) to remove the carcass of the grape and the filtrate was used for the shake flask experiments. Henceforth, the filtrate will be referred to as grape solution (GS).

6.2.3 Shake flask experiment

The shake flasks experiment was carried out in 100-ml Erlenmeyer flasks containing 5 g of iron ore mineral and 50 ml of GS. The experiment was performed in triplicate and lasted for 3 weeks. Harvesting was done at weekly intervals. There were two control treatments. The first control had the iron ore and water but no bacteria, while the second had the GS but no iron ore.

6.2.4 Cultivation and identification of bacterial isolates from grape solution

Nutrient agar (NA) (Biolab) and acetic acid-ethanol (AE) medium for isolation of acetic acid bacteria were used for this process. Nutrient agar was used because it is a general medium for isolation of different bacteria while AE was chosen in order to isolate the potential organic acidic-producing bacteria. Fifty µl-quantities of the GS were spread on both agar media for the isolation. The AE medium was in a double layer with the first layer (bottom) having 1.5% glucose, 0.2% yeast extract, 0.3% peptone, 4% acetic acid, 25 ethanol and 0.5% agar. The content of the second layer (top) was similar to the first, except it had 1% agar to replace 0.5% agar. Acetic acid and ethanol were added after autoclaving (Entani *et al*., 1985; Sokollek, *et al*., 1998). This method has been identified as ideal for obtaining acetic acid-producing bacteria. Isolates obtained were later identified with molecular and phylogenetical methods.

6.2.5 Harvesting, pH measurement and High Performance Liquid Chromatography (HPLC)

During harvesting, iron ore samples were separated from the spent GS by decantation. The spent GS was homogenised by vortexing and centrifuged for 180 s at 16060 rpm and the supernatant frozen at –40^{o}C prior to analysis by High Performance Liquid Chromatography (HPLC). This was later passed through the filter paper (0.22 µm) to remove any remaining particles from the medium of growth. Prior to the storage, part of the supernatant were used for pH measurement. Organic acids were separated with a Agilent Zorbax SB-Aq (4.6 x 150nm) 5-µm column, eluted isocratically at 1 mL min^{-1} with 20 mM NaH_2PO_4 at pH2 buffer with the column at 25 °C and

detected on a diode array detector at 210 nm (Agilent 1100 series). Peak identity and organic acid quantity were determined by comparison with standards. The organic acid standard included gluconic, lactic, acetic, citric and maleic acid that were well separated under the described chromatographic conditions.

6.2.6 Phylogenetic and molecular identification

Extraction of genomic DNA was done using the Zymo Research bacterial/fungal DNA Kit™ (Cat.# 6001) according to the manufacturers' instructions. The polymerase chain reaction (PCR) involved the amplification of 16S rDNA bacterial genes using a universal pair of bacterial forward and reverse primers; GM5F (5´-CCTACGGGAGGCAGCAG-3´;Tm-58.2ºC) and R907 (5´CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCGCCGTCAATTCCTTTGAGTTT-3´; Tm-1.8ºC) (Muyzer, *et al*., 1995), respectively. The amplification was done in a 50-µl reaction that contained 0.4µM of each of the primers, 1.25 U of *Taq* polymerase, 5 ul of promega 10X buffer (0.2 mM) promega dNTPs, 1.75 mM of Magnessium chloride and 2 µl template DNA. The machine used for the PCR was a MJ Mini Personal Thermal Cycler (Bio-Rad) with the following conditions: initial denaturing at 94 °C for 2 min, followed by 4 cycles of 30 s at 94 °C (denaturing), 45 s at 68 °C (annealing), 2 min at 72 °C (elongation). These steps were repeated (excluding initial denaturing) with decreasing annealing temperature at 66, 64, 62, 60 and 58 °C running at 4 cycles, except at the annealing temperature of 58 °C that ran at 12 cycles. Final elongation was at 72 °C for 8 min. The different annealing temperatures listed in decreasing order were due to the high variation in the T_m of the two primers. PCR products were separated electrophoretically with ethidium bromide (0.1 µg/ml)-stained 1% agarose gel running at 120V for 50 min. Amplified DNA was visualised and photographed using a Uviprochem Transilluminator.

Cleaning of the PCR products obtained was done using the PROMEGA Wizard SV Gel and PCR purification kit (Cat.# A9280) according to manufacturer's instructions. Sequencing of the cleaned PCR product was carried out by Inqaba Biotechnical Industries (Pty) Ltd. Forward and

reverse sequences of the 16S rDNA regions obtained were aligned to obtain consensus sequence using BioEdit software prior to BLASTing. Sequences were thereafter compared on the NCBI website (Hall, 1999) to confirm the nearest identical organisms.

Similar isolates with closest nucleotide sequences to those isolated from GS (three for each) were obtained from GenBank. Fasta format of all the 8 bacterial nucleotide sequences and the outgroup (*Acetobacter* sp.) were aligned using ClustalX software (Thompson *et al*., 1997). Final alignment was carried out with online version of MAFFT software (Katoh *et al*., 2002). The Phylogenetic analyses were carried out using Mega 4 software and the evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2007). Neighbour joining (NJ) method (Saitou and Nei, 1987) was used to infer the evolutionary history of the isolates and the bootstrap consensus tree inferred from 1000 replicates. All positions containing gaps and missing data were eliminated from the dataset.

6.2.7 Leaching with isolates

Identified isolates obtained from GS were tested for their abilities to mobilise nutrients from the iron ore materials. The method used was similar to the one mentioned above but instead of GS, AE medium was used to cultivate the isolates during the leaching process.

6.2.8 Fourier transform infrared (FTIR) spectroscopy

There were visible exopolymeric substances (EPS) produced by some of the bacterial isolates. These were analysed using the reflectance infrared (IR) method with a KBr matrix. The process was started with precipitation of the EPS with ethanol. The acquisition of the spectra was through the transmission mode. Pellets obtained from the ethanol treatments were mixed with KBr powder and dried for 24 h at room temperature. The dried samples were crushed and pressurised to form pellets that was used for the reading.

6.2.9 Microscopy

Fixing of the iron ore samples was done by applying 15 ml of 2.5% glutaraldehyde in 0.0075 M phosphate buffer onto the ore using greiner tubes. The samples were then washed three times for 15 min each with 0.0375 M phosphate (Na_3PO_4) buffer. The washed samples were dehydrated at different alcohol concentrations (50, 70, 95, 100%), for 10 min each. The dehydrated samples were repeatedly soaked in 100% alcohol twice. The treated samples were dried and later sputter-coated in a Polaron Equipment Limited SEM Coating Unit E5200 with gold prior to observation under the scanning electron microscope (SEM). Gold-coated samples were assembled for observation under the microscope at 5 KV on a JEOL 5800LV scanning electron microscope (Tokyo, Japan).

6.2.10 Induction coupled plasma (ICP) analyses

Samples of the iron ore materials collected from the shake flask experiments were repeatedly washed with 0.1 M HCl and left in deionised water for 24 h. The samples were put in the oven and dried at 104 °C before sending them for Induction Coupled Plasma (ICP-OES Optima 4300 DV, Perkin Elmer, Waltham, MA, USA) analysis by UIS Analytical Services, Pretoria, South Africa.

6.2.11 Statistical analyses

Statistical analyses were carried out with SAS software, version 9.2. The data available for analysis are from 4 factors: Culture (2): GS, Control (i.e. water), Iron Ore (2): KGT, SK, Particle Size (2): A, B and Weeks (3): W1, W2, W3. This means, without the factor – culture/GS, there were three replicates for each of the 12 treatment combinations and two replicates with the water culture to give a total of 12*3 + 12*2 = 60 observations. Four-way Anova was thus adjusted to the data.

Analyses of organic acids and pH were carried out using two sets of factors that included Treatments- GCT (control- Grape solution without iron ore), KGT-A, KGT-B, SK-A, SK-B and Time/weeks (W1,W2 and W3). The factor "Trt" is a combined effect of iron ore and particles size with the control without Iron ore. The degree of freedom associated to this factor is df=4. Contrasts were written to test the significance of Iron ore (df=1), particles size (df=1), the interaction of iron ore vs particle size (df=1), and the difference between the control and all other levels (df=1). Two-way Anova was thus adjusted to the data with contrasts to identify the effects of iron ore, particle size, the interaction between those two factors, and the effect of control.

6.3 Results

6.3.1 K and P reduction

Iron ore type, particle size, time and the type of leaching solution (GS or water-control), as well as some of their interactions were all found to significantly affect the reduction of K and P contents of the iron ore minerals (Table 6.1). Reduction of K contents of the iron ore was highest at the second week (52.32% for SK and 38.14% for KGT) for both mineral types, followed by the first and third weeks, respectively (Fig. 6.1). Better K reduction was recorded from particle size B compared to size A (Fig. 6.1).

Table 6.1: Four-way analysis of variance (ANOVA) with F and P values that show the effects of GS, iron ore, particle size, time and their interactions on %K and % P reduction from the iron ore materials.

Sources of Variation	df	% K loss df= 36		%P loss df= 36	
		F	*P*	*F*	*P*
GS	1	5232.46	<.0001	394.16	<.0001
Iron ore	1	1053.06	<.0001	28.1	<.0001
GS vs iron ore	1	42.81	<.0001	8.88	0.0051
Particle size	1	968.43	<.0001	186.98	<.0001
GS vs Particle size	1	586.64	<.0001	236.42	<.0001
Iron ore vs Particle size	1	23.06	<.0001	75.28	<.0001
GS vs Iron ore vs Particle size	1	8.76	0.0054	41.09	<.0001
Time	2	26.54	<.0001	45.25	<.0001
GS vs Time	2	14.13	<.0001	44.2	<.0001
Iron ore vs Time	2	0.21	0.8125	1.71	0.1947
GS vs Iron ore vs Time	2	0.41	0.6698	0.22	0.802
Particle size vs Time	2	0.32	0.7264	5.11	0.0112
GS vs Particle size vs Time	2	10.85	0.0002	1.12	0.3365
Iron ore vs Particle size vs Time	2	17.61	<.0001	16.29	<.0001
GS vs iron ore vs Particle size vs Time	2	2.38	0.1065	3.29	0.0489

P values <0.005 are considered significant.

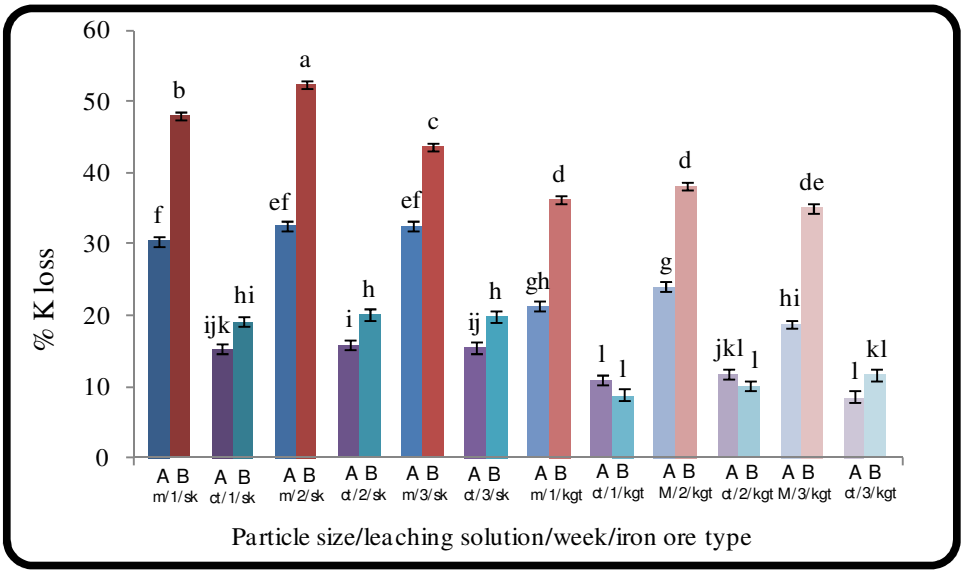


Figure 6.1: Amount of K loss from the KGT and SK mineral types with GS treatments for 3 weeks. Error bars are ±SE. CT represents iron ore with distilled water as leaching solution.($P<0.001$).

In addition, more K reduction was recorded in SK mineral type compared to KGT mineral type (Fig. 6.1). Similar result was obtained for P reduction, with better reduction in SK compared to KGT (Fig. 6.2). However, P reduction was better in bigger particle size A iron ore samples. Additionally, P reduction fell from the first week to third week; at odds to what was observed for K reduction (Fig. 6.1 and 6.2).

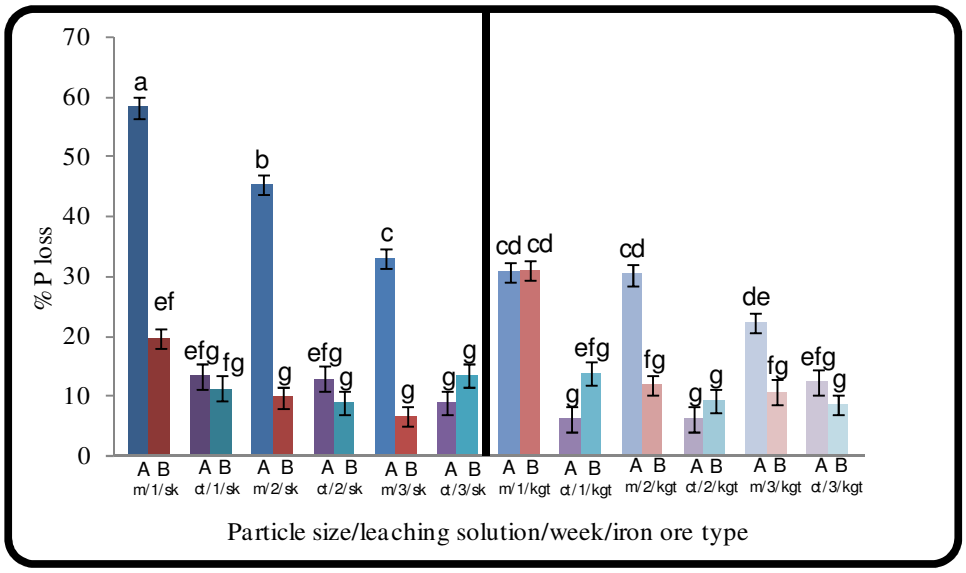


Figure 6.2: Amount of P loss from the KGT mineral type with GS treatments for 3 weeks. Error bars are ±SE. CT represents iron ore with distilled water as leaching solution (P<0.001).

6.3.2 Organic acid in GS and pH medium

Treatments, time and the interactions between these two sets of factors were shown to statistically affect the quantity of organic acid found in GS (Table 6.2). Highest quantity of organic acid produced was gluconic acid with values ranging from approximately 3000 to 8000 µg/ml (Table 6.3). This was followed by acetic acid with values above 2000 µg/ml, but not up to 3000 µg/ml. Lactic and citric acids were also detected but in lower quantities. In general, values of different organic acids detected in the control where no iron ore was added to GS was lower when compared to the situation where iron ore was present (Table 6.3).

Table 6.2: Two-way analysis of variance (ANOVA) with F and P values that that show the effects of iron ore type, particle size, time and their interactions on the release of five different organic acids.

Sources of Variation	df	Gluconic acid df= 29		Lactic acid df= 25		Acetic acid df= 26		Citirc acid df= 23		Malic acid df= 25	
		F	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Iron ore	1	348.33	<.0001	392.92	<.0001	25.33	<.0001	106.39	<.0001	406.58	<.0001
Particle Size	1	49.24	<.0001	98.33	<.0001	49.27	<.0001	37.02	<.0001	2.87	0.1029
Iron ore vs Particle size	1	128.28	<.0001	31.42	<.0001	39.93	<.0001	37.44	<.0001	14.23	0.0009
Ctl	1	1522.78	<.0001	125.7	<.0001	462.19	<.0001	137.19	<.0001	105.06	<.0001
Iron ore vs Time	2	2.44	0.1047	0.04	0.9656	1.75	0.1943	27.58	<.0001	410.03	<.0001
Particle size vs Time	2	8.27	0.0014	1.43	0.2578	2.74	0.0832	6.72	0.005	12.39	0.0002
Iron ore vs Particle size vs Time	2	18.48	<.0001	2.65	0.0905	9.45	0.0008	22.79	<.0001	10.74	0.0004
Ctl vs Time	2	35.14	<.0001	14.29	<.0001	9.09	0.001	29.91	<.0001	128.36	<.0001

Ctl represent the control without any iron ore. P values <0.05 are considered significant.

Table 6.3: Organic acids detected in GS for a period of 3 weeks; K and S represents KGT and SK iron ore types respectively, A and B represent particle size and W1, W2 and W3 represent week1, 2 and 3 respectively.

Iron ore / particle size/Time	**Organic acids**				
	Gluconic	**Lactic**	**Acetic**	**Citric**	**Malic**
K A (W1)	3746.19	126.07	2043.93	85.12	184.71
	±61.51	±4.36	±29.57	±6.76	±4.94
K A (W2)	4768.53	168.24	2389.02	159.66	259.60
	±100.00	±6.20	±23.90	±19.63	±7.47
K A (W3)	6701.19	209.58	2474.56	186.16	827.90
	±146.63	±7.70	±34.12	±6.89	±26.99
K B (W1)	5707.25	167.13	2115.93	89.70	224.49
	±114.09	±6.47	±17.96	±2.20	±11.25
K B (W2)	6597.58	196.77	2314.19	196.75	325.55
	±149.20	±2.20	±13.22	±1.65	±10.34
K B (W3)	7238.63	247.80	2430.52	141.71	724.43
	±165.79	±8.96	±11.77	±7.78	±25.69
S A (W1)	3675.19	42.79	2139.93	72.10	229.28
	±113.47	±3.86	±15.78	±0.30	±4.46
S A (W2)	4342.22	51.99	2360.29	79.81	257.99
	±207.96	±1.74	±25.11	±3.65	±6.23
S A (W3)	5411.79	56.55	2457.62	105.01	285.31
	±127.42	±5.84	±26.10	±2.95	±3.43
S B (W1)	3488.70	58.28	1869.36	75.42	218.25
	±141.92	±18.65	±26.77	±5.31	±1.12
S B (W2)	3786.71	97.22	2120.73	118.99	254.20
	±129.83	±7.17	±8.40	±4.16	±14.78
S B (W3)	5385.91	151.31	2362.87	194.91	273.28
	±94.91	±18.30	±43.26	±10.46	±6.84

Table 6.4: pH of GS for a period of 3 weeks; K and S represents KGT and SK iron ore types respectively, A and B represent particle size and W1, W2 and W3 represent week1, 2 and 3 respectively.

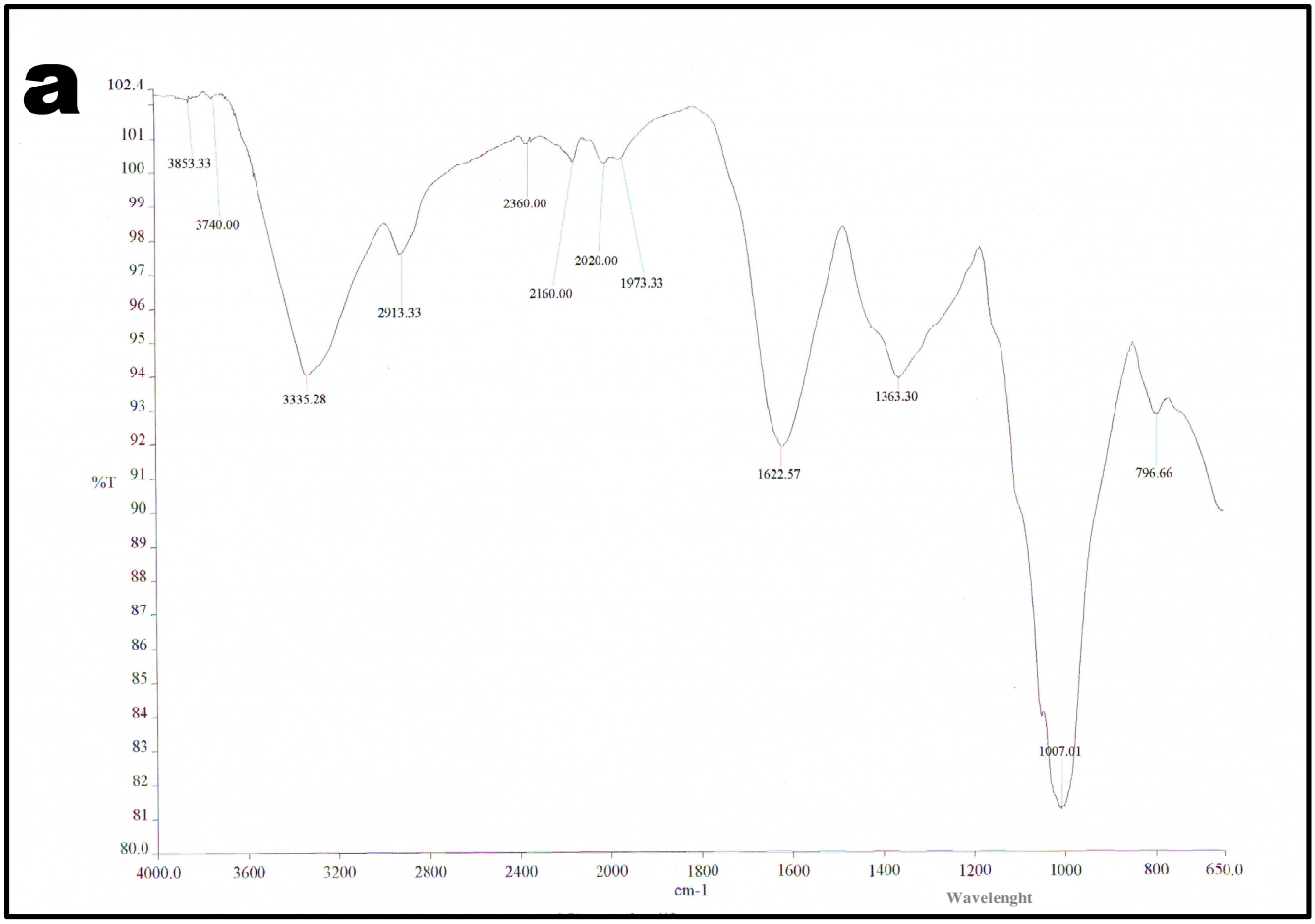
Iron ore / particle size/Time	pH	Iron ore / particle size/Time	pH
K A (W1)	3.06 ±0.01	**SA (W1)**	2.89 ±0.01
K A (W2)	3.09 ±0.05	**SA (W2)**	2.89 ±0.03
K A (W3)	3.06 ±0.01	**SA (W3)**	2.89 ±0.01
K B (W1)	3.15 ±0.03	**SB (W1)**	2.99 ±0.03
K B (W2)	3.23 ±0.01	**SB (W2)**	2.96 ±0.01
K B (W3)	3.31 ±0.01	**SB (W3)**	3.00 ±0.02

There were significant differences in pH of both minerals, with GS containing SK ore type having lower pH than GS with KGT ore type. For both ore types, GS containing particle size B ores had higher pH than those with particle size A (Table 6.3).

6.3.3 FTIR

Several absorbance bands were obtained from the FTIR spectra of EPS produced during the leaching process (Fig. 6.3). For bacterial culture system, most important absorbance bands are usually located in the regions of 1800 – 800 cm^{-1} (Kornmann *et al*., 2004). These bands correspond to various functional groups (Table 6.5) that may have direct and indirect effects on the leaching process.

Fermentation in Biohydrometallurgy, a novel method for K and P removal from iron ore



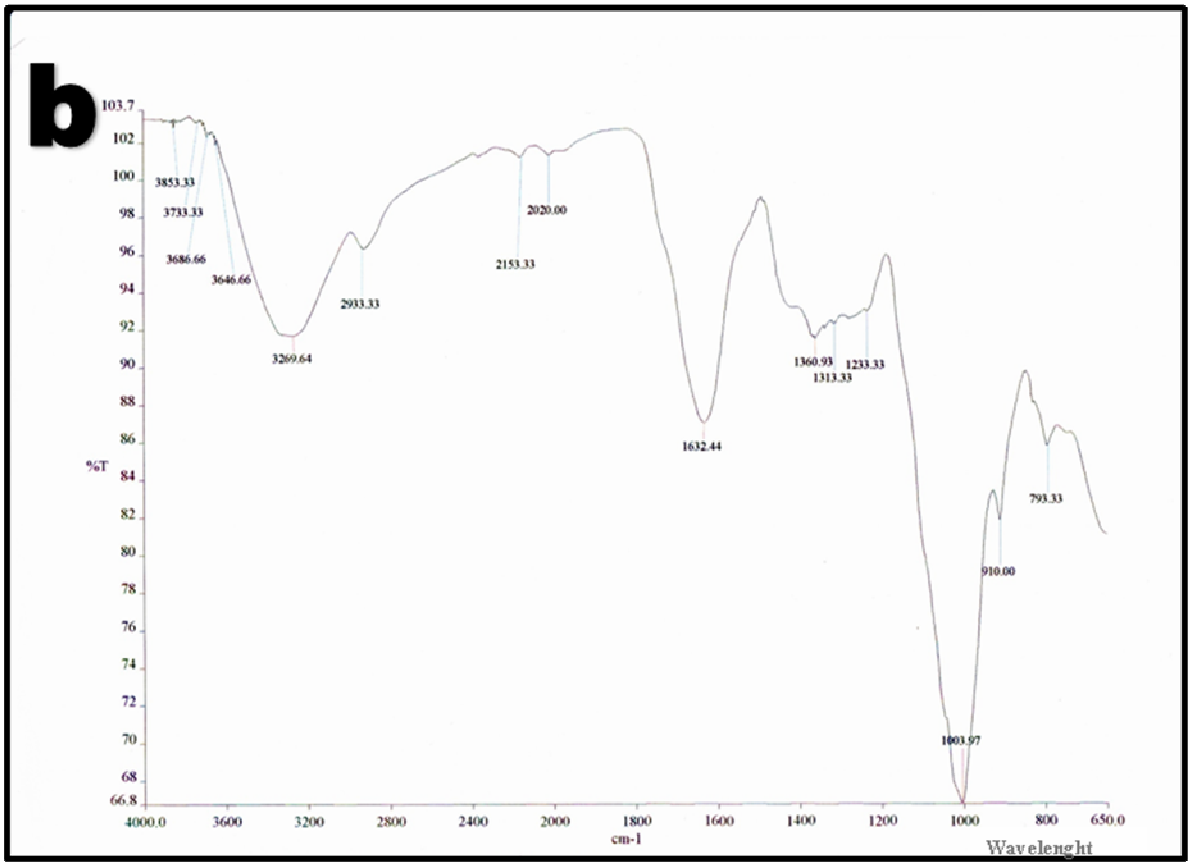


Figure 6.3: FT-IR spectrum of EPS from GS used for shake flask experiment in the presence of KGT (a) and SK (b) mineral types, after 14 d.

Table 6.5: Band assignments of FITR spectra of EPS obtained from treatments involving KGT and SK iron ore types 14th day of the experiment (only bands between 1800 to 790cm^{-1} were listed).

Functional groups (Reference)	KGT	SK
Amide I (C=O)	1622	1632
Amide II, N-H, C-N and structure of proteins (Jiang *et al*., 2004; Delvasto *et al*., 2009)	1363	1361
Carboxylate (Jiang *et al*., 2004; Delvasto *et al*., 2009)		1313
Ester group / DNA phosphate group (-COOH, and C-O-C) (Jiang *et al*., 2004)		1233
Phosphate (PO2- and $P(OH)_2$; Polysaccharides and alcohols (C-OH and C-C) (Jiang *et al*., 2004; Delvasto *et al*., 2009)	1007- 796	1004-793

6.3.4 Bacterial isolates

Two different bacterial isolates named RD1 and RD2 were obtained from GS inoculated on AE medium. Molecular and phylogenetic analyses (Appendix III) showed that both isolates are close relatives of *Gluconacetobacter intermedius* and *Gluconacetobacter* sp., respectively (Fig. 6.4). No microbial growth was observed on NA medium.

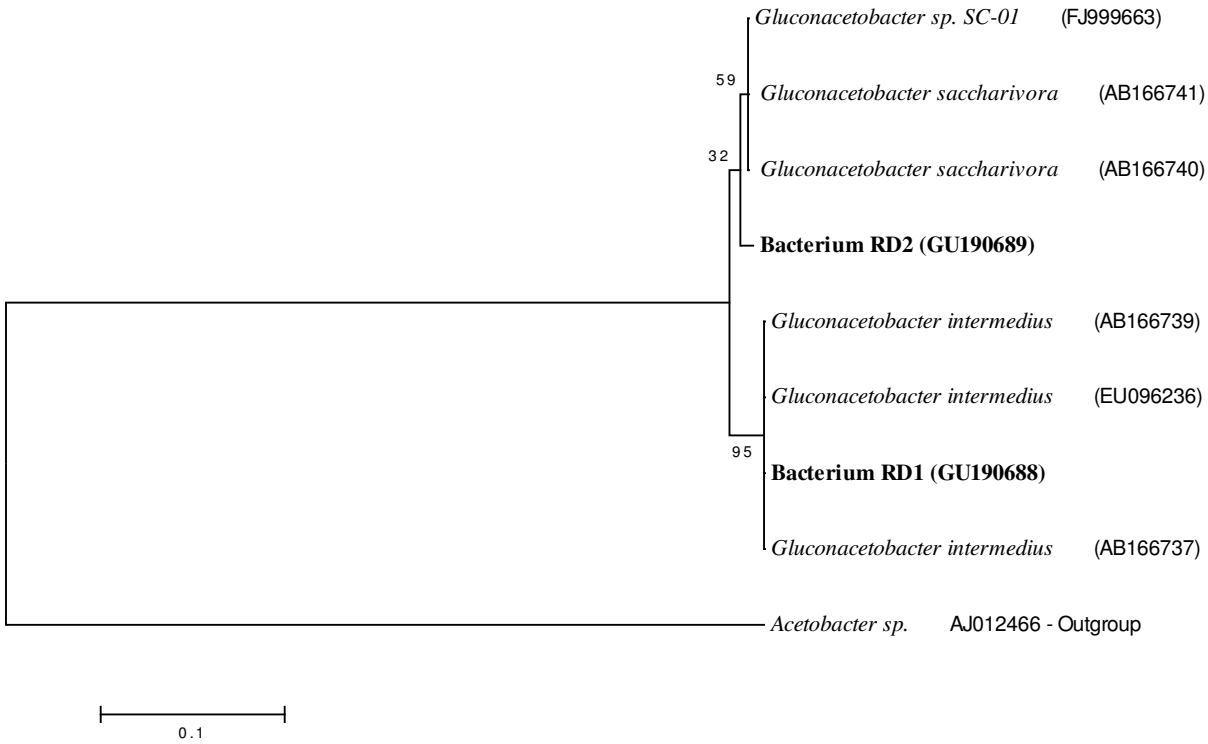


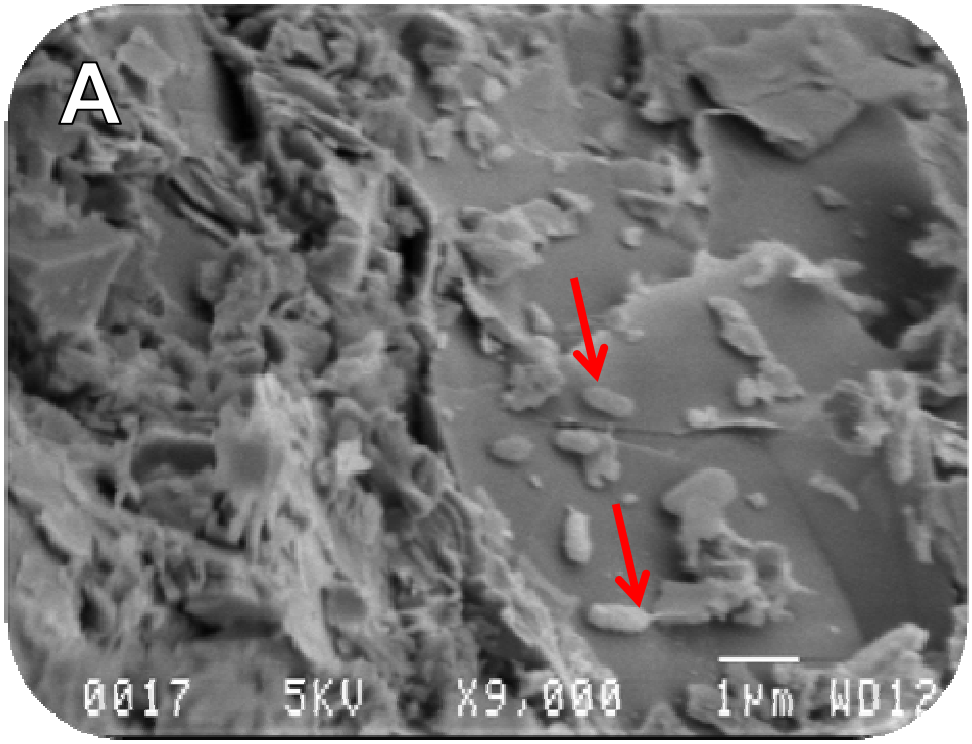
Figure 6.4: Phylogenetic tree of the 16S rDNA of bacterial isolates obtained from GS (in bold) and their related species obtained from the Genebank as established by boostrap neighbor-joining method.

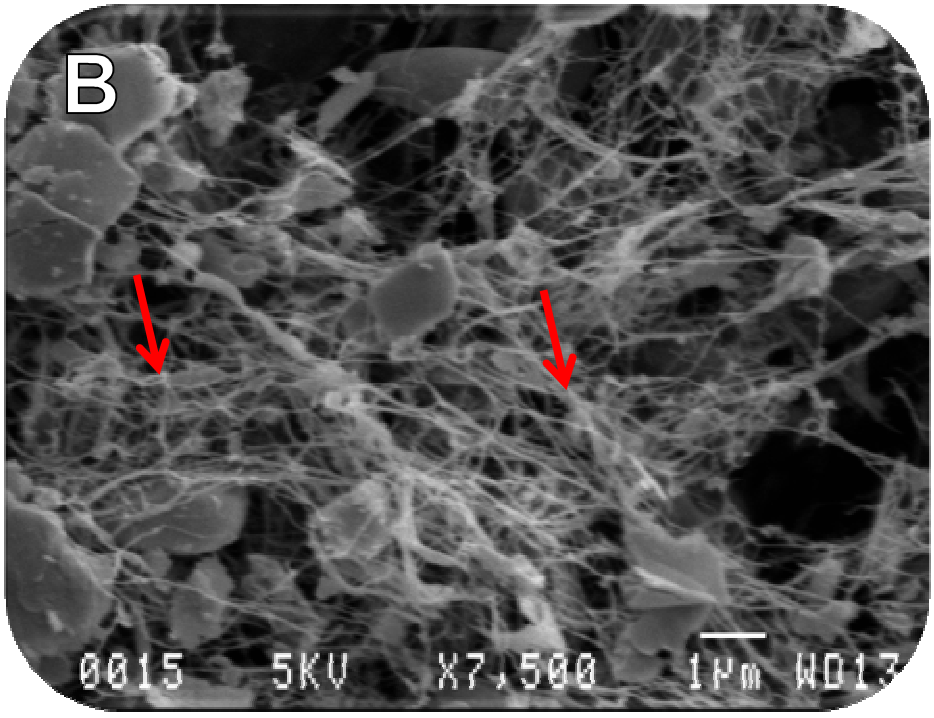
6.3.5 Leaching potentials of bacterial isolates

In order to test the individual effects of the bacterial isolates on the mobilisation of K and P from the iron ore minerals, they were allowed to grow in AE medium in the presence of both types of iron ore types. Unfortunately, the effects of the pH of the AE medium prevented the actual evaluation of the leaching processes involving the isolates. There was no significant difference between the highest leaching obtained from the isolates and the control with no isolate. Therefore, acetic acid contained in the medium of growth participated in the leaching process.

6.3.6 Microscopy

The microscopy observations of biofilms from the leaching experiment revealed the attachment of bacterial cells to the surface of the iron ore particles, as well as the entrapment of iron ore particles by the EPS (Fig. 6.5a – d). This is an indication of the importance of biofilm formation during bioleaching by bacteria involved in this experiment. Observation of the biofilms using SEM showed that they contained bacterial cells interconnected by cords, EPS, ore particles and some other metabolites. For SK ore type, there is an indication of possible preferential attachment of the bacterial cells and their cords to specific sites on the mineral surfaces. Better attachment of the biofilm to smaller particle size B ores was also observed (Fig.6.5a and b).




B
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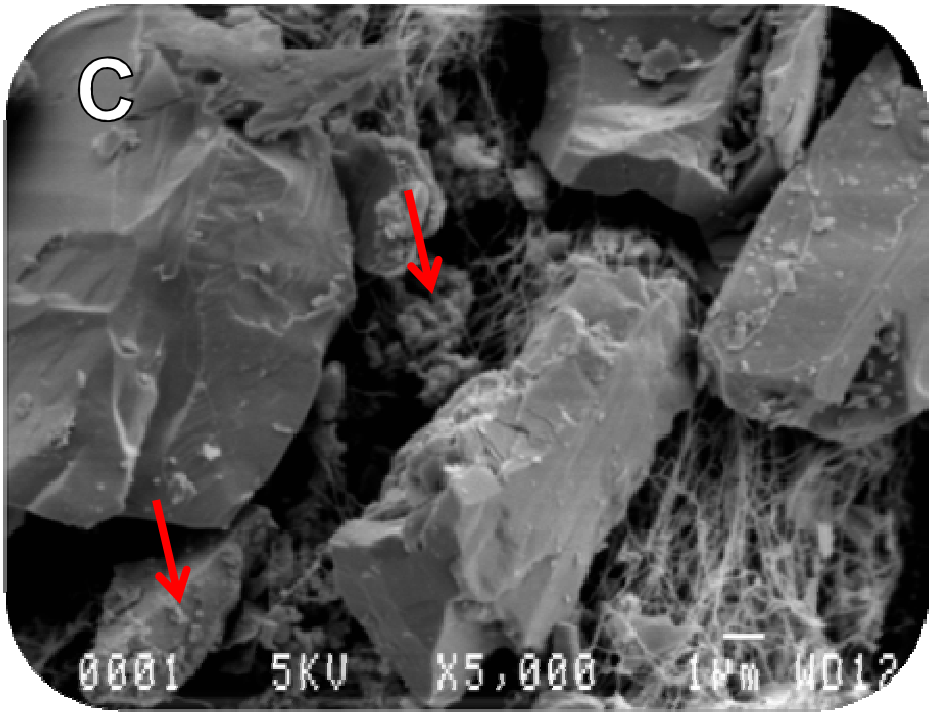
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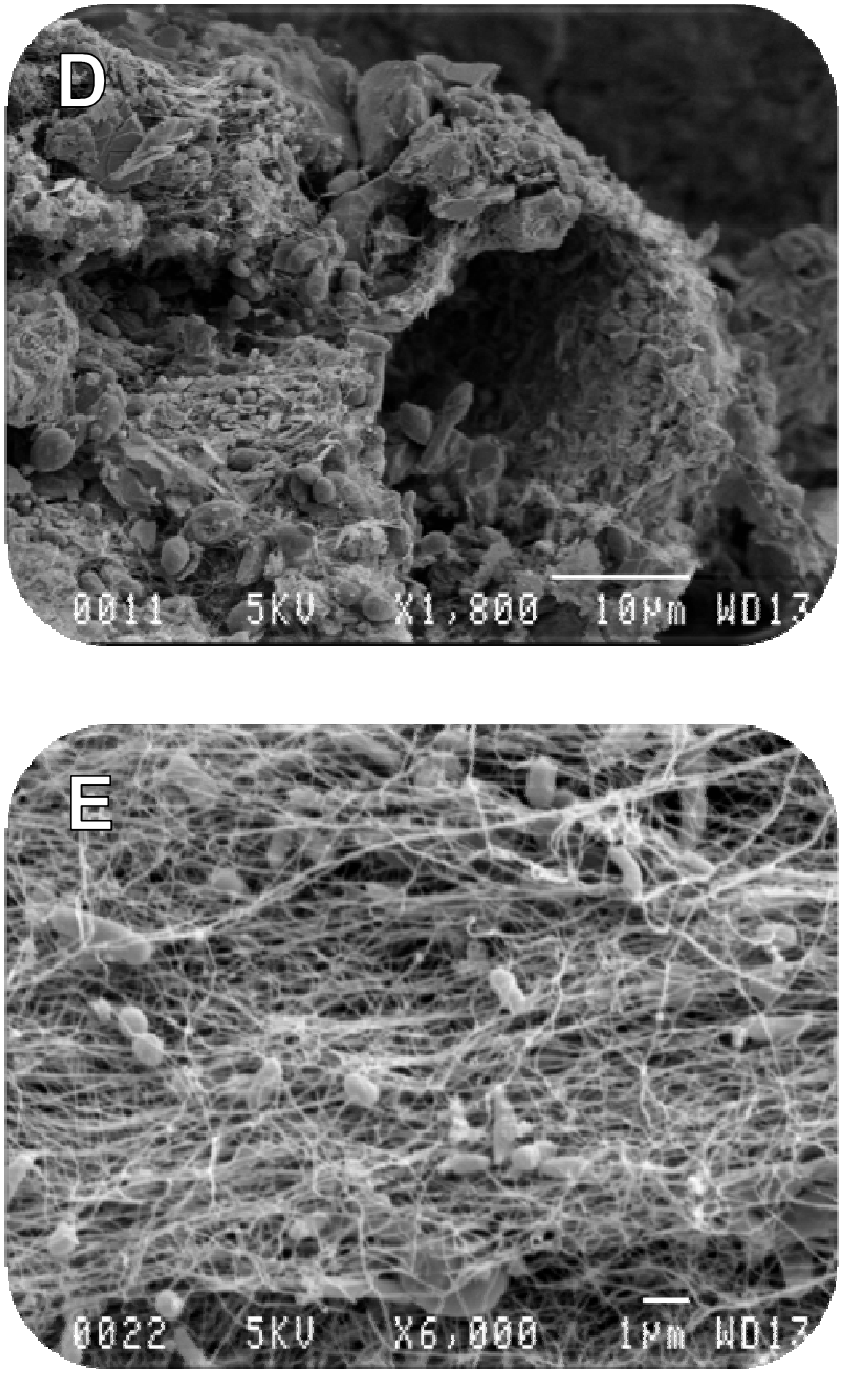

Figure 6.5: A and B represent the SEM images of bacterial attachment and cord formation by bacterial contained in GS in the presence of SK mineral type, while C and D are for SK mineral type with C showing selective adsorption of bacterial cells to mineral surface and d showing biofilm formation. E represents the control showing the cord formation by the bacterial contained in GS in the absence of iron ore mineral.

6.4 Discussion

The search for organic acid-producing bacteria that can leach iron ore minerals has yielded a positive result in this study. One of the most utilised groups of organic acid-producing bacteria are the acetic acid bacteria that includes three major genera, namely *Acetobacter*, *Gluconoacetobacter*, *Gluconobacter* and *Acidomonas* (Yuzo *et al*., 1997; Gupta *et al*., 2001). A usual habitat for some of these bacteria is *Vitis* sp. (grape fruits) that was utilised in this study (Gupta *et al*., 2001). The ability to multiply these bacteria by simple addition of distilled water to spoilt grape fruits provided a good foundation for this investigation. Additionally, the creation of a very low pH by the bacteria contained in GS allowed the promising execution of the experiment under septic conditions.

The significant effect of iron ore type on mobilization of K and P suggests the importance of variation in the chemical compositions of the ores. Although the two iron ore types contain both K and P as impurities, the mineral constitutions of both ores are not the same, with higher values of K and P in KGT ore type. A similar observation was reported by Sheng *et al.* (2008) where *Bacillus globisporus* Q12 mobilised K and Si at different rates from three different silicate minerals, namely: feldspar, muscovite and biotite.

There were also significant effects of particle size - smaller particle size promoted mobilization of K, while the bigger particle size enhanced mobilization of P. The better mobilization of K from smaller particle-sized molecule is due to the exposure of larger particle surface to the activities of the bacteria contained in the GS or the biofilms. Similar situation was reported by Modak *et al*. (2001) where better leaching of calcium was obtained from finer particle size minerals. However, these investigators pointed out the particle size effect was only important when time is considered. As already observed in this study, it is difficult to generalise the issue of particle size. Phosphorus reduction was better in coarser particle size B ores, in contrast to K reduction. Although Delvasto *et al*. (2009) reported similar manner of P mobilization from iron ore where higher % of P was mobilised from coarser iron ore mineral, the underlying factor

responsible for this remains to be investigated. The plausible explanation given by Delvasto *et al*. (2009) was the re-precipitation of phosphate from solution.

Organic acids produced by microbes during bioleaching processes are capable of releasing anions and protons into solution, and can therefore react with mineral surfaces (Gadd, 1999). Due to their high chelation constants of some Al and Fe compounds, sites containing these elements on mineral surfaces are easily attacked, a situation which leads to solubilisation and release of nutrients from such minerals (Yuan *et al*., 2004; Delvasto *et al*., 2009). In addition, the smaller size of protons from organic acids usually enhances easy displacement of similar but bigger sized monovalent ions (Lapeyrie *et al*., 1987; Yuan *et al*., 2004) such as K contained in the iron ore minerals used in this study. These processes can disrupt the structure of minerals such as those contained in iron ore minerals. Therefore, considering the link of organic acid to the leaching process, the result suggests that the mobilisation of K could be direct through the proton attack. However, P mobilisation could be indirect and likely to happen after the disruption of the ore structure caused by anion and proton attacks.

The biofilm formation possibly affects the leaching experiments in three major ways. One is direct ability of the microbes to scavenge nutrients from mineral surfaces (Delvasto *et al*., 2009). Possibility of this feeding mechanism was observed from the SEM images where there was strong attachment of the biofilms to the iron ore surfaces. In addition, the preferential attachment to specific sites on the mineral surfaces is an indication of selective scavenging, as well as preferential utilisation of nutrients from the iron ore surfaces. Furthermore, the attachment of the biofilms to mineral surfaces also provides avenue for close and direct deposition of metabolites such as organic acids onto the mineral surfaces by the microbes (Gadd, 1999; Delvasto *et al*., 2009). Thirdly, the biofilm formation can also lead to enhanced adsorption of some bacterial functional groups associated with EPS to mineral surfaces (Natarajan and Deo, 2001). As shown through the FTIR analyses, functional groups such as carboxylic groups are important for the leaching process to occur especially in the presence of organic acids (Banfield *et al*., 1999).

The isolation and identification of bacteria contained in GS have provided good information on potential utilization and cultivation of these isolates for bioleaching purposes. *Gluconobacter* spp. are usually found on fruits with considerable levels of sugar such as grapes, dates, and apples (Gupta *et al*., 2002). They are obligate aerobic Gram-negative bacteria that can oxidise glucose to produce gluconic and 2-ketogluconic acids (Gupta *et al*., 2002). These acids are very important organic acids for mineral solubilisation (Natarajan and Deo, 2001; Jain and Sharma, 2004). As suggested by Jain and Sharma (2004), the missing link between fermentation and biohydrometallurgy may be an answer to so many questions in this field of study. With the findings in this study, there is every possibility this study could be extrapolated using wastes from the wine industry to develop a potential biobeneficiation method for iron ore minerals.

This is not the first attempt to utilise fermentation ideas in biohydrometallurgy. However, the main importance of the method developed in this study is the use of non-sterile conditions to achieve the aims. Usual setbacks associated with the development of a reliable biobeneficiation technology of iron ore minerals include the inability to develop a cheap source of carbon and energy for the growth of microbes involved during leaching. Secondly, there are problems associated with finding a method that utilises non-sterile conditions for the leaching process. This study was structured to address these two problems by using GS, which can be obtained from spoilt fruits. Furthermore, the high acidity environment created by the bacterial contained in the GS discouraged the growth of any other bacteria, a situation that suggest a good reason for further development of this technology.

6.5 References

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CHAPTER SEVEN

GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATION

The development of biological methods for the removal or reduction of K and P contents of iron ore minerals primarily depends on the direct and indirect approach to find suitable microorganisms that can leach these elements to the required levels. As previously indicated in past studies, commercialisation of biohydrometallurgical processes started through the simulation of natural roles of microorganisms in the laboratory (Rawlings, 2002). This study has therefore been structured around this idea.

7.1 Implication of ectomycorrhizal weathering of iron ore minerals, the bioleaching connection

This investigation was initiated through the study of potential relationship between a natural weathering process and bioleaching. The listing of ECM fungi as rock eating fungi makes this group of fungi a favourite choice for investigation of this idea. The result obtained from the investigation suggests that whether mycorrhizal or not, plants are able to participate in weathering process. Meanwhile in some cases, ECM fungi such as *Pisolithus tinctorius*, were better in terms of nutrient mobilisation from the iron ore minerals. The study further indicated that the rate of ECM weathering can be influenced by fungal type, organic acid production, mineral type and particle sizes of the mineral. It was therefore concluded that when present, ECM fungal colonised plants can contribute to the biomineralisation of iron ore.

7.2 Mobilisation of potassium and phosphorus from iron ore by Ectomycorrhizal fungi

Behaviour of ECM fungi when in symbiosis has been established to be, in most cases, different from their pure cultures (Smith and Read, 2008). This fact led to further investigation of the capability of pure cultures of ECM fungi in mobilisation of nutrients from the iron ore minerals. All the ECM fungi tested were able to mobilise both K and P from the iron ore minerals. Similar to symbiosis experiment, ECM fungal leaching abilities were species-specific and significantly affected by mineral type, particle size and organic acid production. *Phialocephala fortini*, a fungus isolated from the root of a pine tree in South Africa, was able to mobilise more K than the commercial value required for international trade. However, the main setbacks from this method are the slow growth of ECM fungi, strong attachment of the fungi to the mineral surface and the use of sterile conditions to achieve the goals. Such limitations make it difficult to transfer this technology into an outdoor process such as heap leaching.

7.3 Comparison between direct solubilising effects of iron ore associated fungus and its metabolite

Having established that organic acid and particle size can influence mobilisation of nutrients from iron ore minerals, another approach was investigated by isolating indigenous fungi that are associated with iron ore mineral surfaces. This allowed identification of four different fungi, out of which one turned out to be a potential mineral (phosphate) "solubiliser". The phosphate solubiliser was identified with molecular method as a *Penicillium* sp. Experiments conducted to compare the direct and metabolite effects of this fungus suggests a good potential in the use of indigenous microbes for the reduction of K and P from iron ore minerals. Mineralogy, particle size and organic acid production all played important roles in the mobilisation of the elements. It is pertinent to mention that the metabolite usage could be more reliable than the direct use of the fungus because of surface attachment that is always experienced with fungi.

7.4 Culturable Microorganisms associated with Sishen iron ore and their potential roles in biobeneficiation

Additonal experiment about the indigenous microbes (bacteria) provided more significant results. There is a high diversity of bacteria inhabiting the surface of the iron ore minerals with a total of 23 different bacteria isolated. Of this group, only eight of them were successfully screened for their bioleaching potentials. One isolate (KU6), identified molecularly as *Acinetobacter calcoaceticus*, was a slime producing bacteria as well as phosphate solubilising bacteria. This isolate was able to remove high amounts of both K and P contents of the iron minerals, which means it is possible to use this type of organism for biobeneficiation of iron ore mineral. The only problem may be about how to use this isolate under non-sterile conditions.

7.5 Fermentation in Biohydrometallurgy, a novel method for K and P removal from iron ore

Biohydrometallurgical methods involving sterile techniques are very expensive and may not be suitable for a cheap mineral like iron ore. To address this problem, this study was rounded up by investigating a cheap method that could be used for the bioleaching of iron ore minerals. This method relied on the ability of bacteria contained in the leaching solution (from spoilt grape fruits) to produce organic acids. The bacteria were later identified as acetic acid bacteria; *Gluconobacter* spp. Due to the high production of organic acid by this group of bacteria, they can afford to grow under very low pH. Fortunately, the acidic environment kept away all other microbes, thereby indirectly keeping the system sterile. The most important advantages of this method seemed to be the effect of such low pH and organic acid production during the leaching process. These two factors were linked to the successful reduction of K and P from the iron ore minerals.

In summary, this study has successfully investigated and highlighted potential roles of different microorganisms from different environments in biobeneficiation of iron ore minerals. The results

indicate that whether plants are colonised by ECM fungi or not, they can participate in deposition and weathering of minerals. With this fact in mind, and the fact that leaching is known to be one of the stages in weathering, ECM fungi can therefore be linked to bioleaching processes. As proposed, in the absence of host plants, ECM fungi were able to mobilise both K and P from the iron ore samples. However, the "bottleneck" of this idea was strong attachment of the fungal hyphae to the surface of the ore. Such attachment could be difficult to remove after bioleaching and may also lead to additional cost. In addition, the slow growth of this group of fungi was also a problem. Meanwhile, a benefit that can be derived from these experiments is the possibility of using these fungi and their host plants in bioremedication of iron ore mines, a situation that can also improve the nutrient cycling conditions of such ecosystems. More studies are therefore encouraged in this direction.

Isolation, identification and the use of indigenous microorganisms for the mobilisation of K and P from iron ore minerals were intended to solve the problems mentioned above. There is definitely a need for fast growing microorganisms that can perform this task without creating another problem. The use of indigenous microorganisms was expected to provide an advantage of easy adaptation to the leaching environment. For the fungi isolated, a *Penicillium* sp. was able to show the attributes of a mineral-solubilsing fungus. After testing this isolate and its metabolite in a shake flask experiment, it was discovered that it has the potential for biobeneficiation of iron ore. The metabolite usage was necessary in order to eliminate the possibility of fungal attachment to the iron ore surface during the leaching process. Similar to the principle behind the fungal experiment, different bacterial isolates obtained from the iron ore surface were successfully investigated. Results indicated potentials of indigenous microorganisms in solubilisation of iron ore especially the *Acinetobacter* isolate that was able to reduce both K and P contents of the iron ore samples at the same time. Critical evaluation of the adopted methods with regards to indigenous microflora can generate two other hypotheses. The first is the underlying reason responsible for the iron ore bioleaching microbes inhabiting the ore surfaces. This is because they are adapted to this environment and it means, there is high possibility for these microbes to utilise the high levels of K and P in the iron ore for their survival. This idea was adopted in this study.

On the other hand, it is also possible that existence of quality iron ore minerals with low levels of K and P is primarily due to the activities of the indigeous microbes through biomineralisation. Under such scenerio, then potential bioleaching microbes will be abundant on the surfaces of quality iron ore minerals where they have probably helped reduced the levels of these elements. As earlier mentioned, the two hypotheses are possible but the former is more feasible. Mineralogy and chemistry of ores depend on so many factors which are mostly geological. However, full understanding of these processes can only be realised if studies could be conducted to compare microbial diversity of quality and low grade ores.

The main problem with the investigation of bioleaching potentials of indigenous microbes is the use of sterile techniques. In the review by Jain and Sharma (2004), it was predicted that future development of a reliable biobeneficiation method will depend on the possibility of using non-sterile techniques and finding a cheap source of carbon. These two points were addressed in the last experiment conducted in this study. Spoilt grape fruits, as source of cheap carbon, which are always available in the wine industries, were fermented and utilised under non-sterile conditions for biobeneficiation of iron ore samples. Finally, this suggests that the secret of developing a dependable biobeneficiation method may be by keeping it “simple and septic”. To ascertain the efficiency of this novel idea, it is however necessary to conduct a field trial with GS.

7.6 References

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APPENDICES

Appendix I (Media and reagents)

Modified Melin Norkrans Medium (MMN) - Marx, 1969

- Malt extract 3g/
- $(NH_4)_2HPO_4$ 0.25g/l
- $MgSO_4.7H_2O$ 0.075g/l
- $CaCl_2.H_2O$ 0.067g/l
- NaCl 0.025 g/l
- $FeCl_3$ 1%
- Thiamine 100µg/l
- Agar 10g

Phosphate solubilising medium - Mehta and Nautiyal, 2001

- $(NH_4)_2SO_4$ 0.10g/L
- $MgSO_4 \cdot 7H_2O$ 0.25 g/L
- $MgCl_2 \cdot 6H_2O$ 5.00 g/L
- KCl 0.20 g/L
- $Ca_3(PO_4)_2$ 2.5 g/L
- Glucose 10 g/L
- Agar 20 g/L

Appendix

TE (Tris/EDTA) buffer pH 8.0

- 100 ml 1 M Tris-HCl pH 7.5
- 20 ml 500 mM EDTA pH 8.0
- 880 ml ultrapure water

Appendix II

Roots clearing and staining protocol/solutions (Smith and Dickson, 1997)

- Root clearing with 5% KOH for 45 mins at 90 °C.
- Rinsing with distilled water
- Root bleaching in alkaline H_2O_2 for 30 mins
- Root acidification with 0.1M HCl solution for 3 hours
- Staining was carried out with 0.5% trypan blue in lactoglycerol at 90 °C for 45 mins
- To destain, roots were covered with lactoglycerol overnight

50% ethanol

- 1000ml ethanol
- 1000 ml distilled water

5% KOH

- 100 g KOH
- 2L distilled water

Alkaline Peroxide H_2O_2

- 3 ml NH_4OH (Ammonia)
- 30 ml 10% H_2O_2
- 567 ml distilled water, prepared only when required.

Appendix

0.1M HCl (32% MW36.46)

- 22.79 ml HCl
- 2L Distilld water

Lactoglycerol trypan blue stain

- Lactic acid: Glycerol: Water (13:12:16)
- 520 ml lactic acid
- 480 ml Glycerol
- 640 ml distilled water
- 082 g Trypan blue

Lactoglycerol Destain

- Lactic acid: Glycerol: Water (13:12:16)
- 520 ml lactic acid
- 480 ml Glycerol
- 640 ml distilled water

Appendix III

1. Aligned (Mafft) sequences of fungal isolates from the iron ore and their related sequences from the GeneBank

Title: mafft aligned.pir

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Appendix

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Appendix

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Appendix

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Appendix

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Appendix

2. Aligned (Mafft) sequences of bacterial isolates from the iron ore and their related sequences from the GeneBank

Title: MAFFT.fas

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Appendix

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Appendix

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Appendix

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Appendix

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Appendix

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Appendix

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Appendix

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Appendix

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Appendix

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Appendix

3. Aligned (Mafft) sequences of bacterial isolates from GS and their related sequences from the GeneBank

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Appendix

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