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).

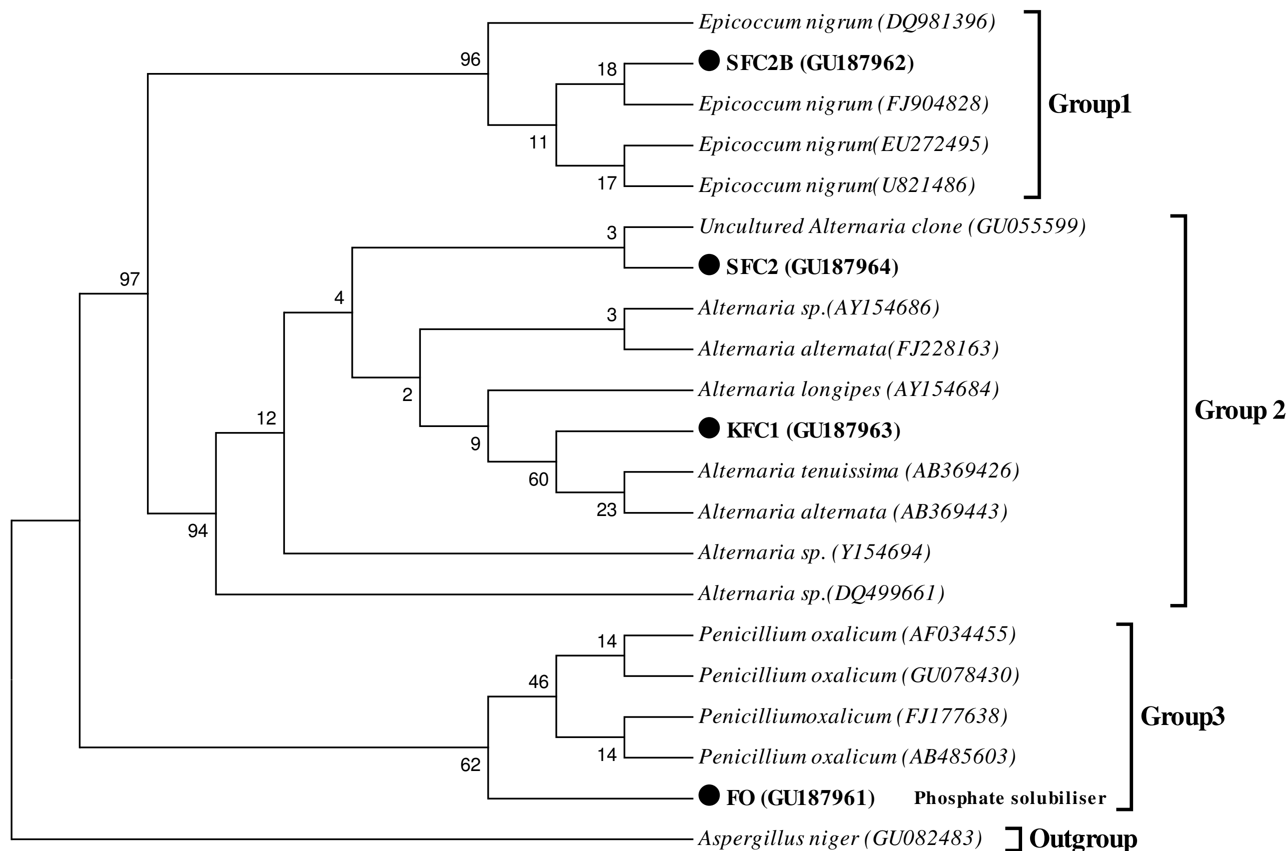

Figure 4.1: Neighbour joining tree constructed from ITS sequences of isolates obtained from iron ore minerals (bold letters) and other sequences obtained from GenBank. The NJ tree was rooted with *Aspergillus niger* as outgroup.

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).

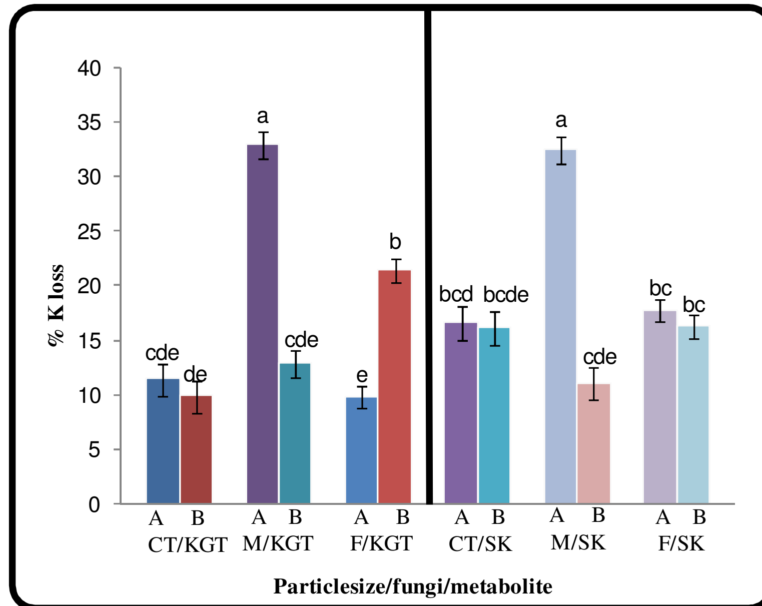

Figure 4.2: Percentage of K reduction from mineral type KGT and mineral type SK in response to fungal (F), metabolite (M) and water – control (CT) treatments two different particle sizes (A and B) of the iron ore materials. Bars with the same letter are not significantly different (P<0.0001).

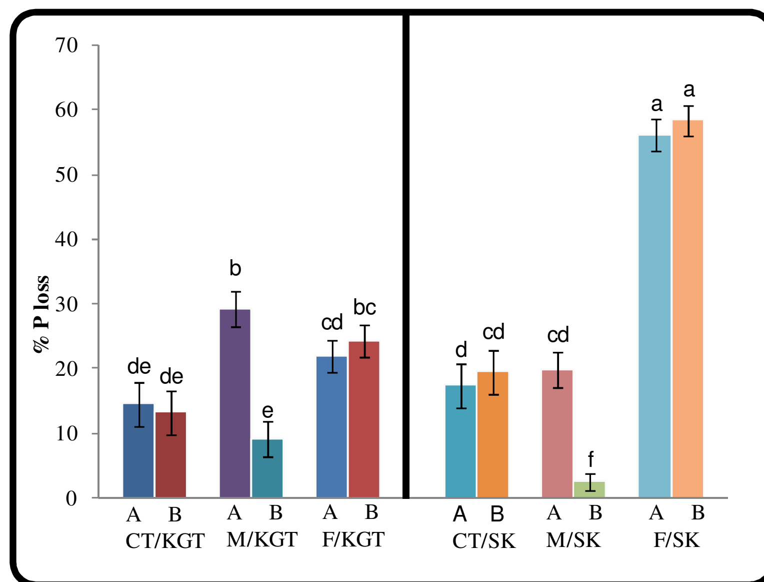

Figure 4.3: Percentage of P reduction from mineral type KGT and mineral type SK in response to fungal (F), metabolite (M) and water – control (CT) treatments two different particle sizes (A and B) of the iron ore materials. Bars with the same letter are not significantly different. (*P*<0.0001)

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.

4.5 References

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