

CHAPTER 3

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

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

3.1 Abstract

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

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

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3.2 Introduction

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

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

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

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3.3 Materials and Methods

3.3.1 Sample Selection and Processing

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

3.3.2 Chemical Analysis of the Process- and Groundwater

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

3.3.3 Total Plate Counts of the Process- and Groundwater

Heterotrophic plate counts of the process- and groundwater of the mine were conducted using the pour plate method (Health Protection Agency, 2004a). A dilution series for each water sample was prepared in sterile test tubes using distilled water (dH₂O) (Health Protection Agency, 2004b). One millilitre of each dilution was pipetted into a 90 mm Petri dish (Concorde Plastics, Johannesburg, South Africa), followed by the addition of 20 ml of liquid (50°C) Standard Nutrient Agar (Appendix A) to each Petri dish. Once the agar had solidified, the agar plates were incubated for 48 h at 28°C. Each process- and groundwater sample was analysed in triplicate. Following the incubation period, the bacterial colonies were enumerated and the Simpson's index of diversity ($1-D = 1 - \sum pi^2$), as well as the Equitability index ($E_D = D/D_{max}$) calculated (D : Simpson's diversity index; pi : proportion of species made up of the i th species; D_{max} : the maximum value D could assume if individuals in the community were completely evenly distributed).

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3.3.4 Preparation of Pure Cultures

Pure cultures of each morphological distinct bacterial colony, which was isolated on the standard plate count agar plates, were prepared. Each colony was inoculated separately onto agar plates containing solidified Standard Nutrient Agar (Appendix A). The agar plates were incubated for 48 h at 28°C in order to obtain single bacterial colonies. The procedure was repeated, followed by the bacterial identification. Suspensions from the pure cultures isolated from the groundwater sample were prepared, using sterile dH₂O, for molecular analysis.

3.3.5 Bacterial Identification of the Bacteria Isolated from the Process Water

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

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

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

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

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

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All PCR reagents were manufactured by Bio-Rad Laboratories (Hercules, CA, USA), unless otherwise stated. The PCR reaction was performed in a reaction volume of 25 $\mu\ell$ containing, 150 mM KCl, 30 mM Tris-HCl (pH 9.0), 0.3% Triton X-100, 50 mM MgCl₂, 10 μM PCR nucleotide mix, 5 pmol primer PRUN518r (Whitehead Scientific, Cape Town, South Africa), 5 pmol primer PA8f-GC (Whitehead Scientific), 1.5 units of Taq polymerase and 0.5 $\mu\ell$ bacterial suspension. Denaturation of extracted DNA at 95°C for 10 min was followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 51°C for 30 s, and extension at 72°C for 1 min (Bio-Rad Thermal Cycler, Bio-Rad Laboratories). A final extension at 72°C for 10 min concluded the PCR amplification of the DNA. A reaction containing no DNA template was included as a negative control. The amplified PCR products were separated using 1% agarose gel electrophoresis in Tris-Acetate-EDTA (TAE) buffer.

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

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

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

3.4 Results and Discussion

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

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

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TABLE 3.1

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

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

*ND – Not Detected

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

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TABLE 3.2

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

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

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

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

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TABLE 3.3	
Bacteria isolated and identified from the different process water samples collected at the Sishen Iron-Ore Mine	
Identification of Isolated Bacteria	Average Bacterial Count (cfu.mℓ⁻¹)
Water from the Process Dam	
<i>Aeromonas hydrophila</i>	4.67 × 10 ³
<i>Alcaligenes faecalis</i>	3.21 × 10 ²
<i>Brevundimonas vesicularis</i>	1.39 × 10 ²
<i>Acinetobacter junii</i>	0.33 × 10 ¹
Water flowing from the Slimes Dam	
<i>Aeromonas hydrophila</i>	7.99 × 10 ²
<i>Pantoea spp.</i>	7.01 × 10 ¹
<i>Flavobacterium meningosepticum</i>	5.35 × 10 ¹
Water flowing to the Slimes Dam	
<i>Aeromonas hydrophila</i>	1.08 × 10 ³
<i>Chryseomonas luteola</i>	1.72 × 10 ²
<i>Enterobacter sakazakii</i>	8.60 × 10 ¹

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

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

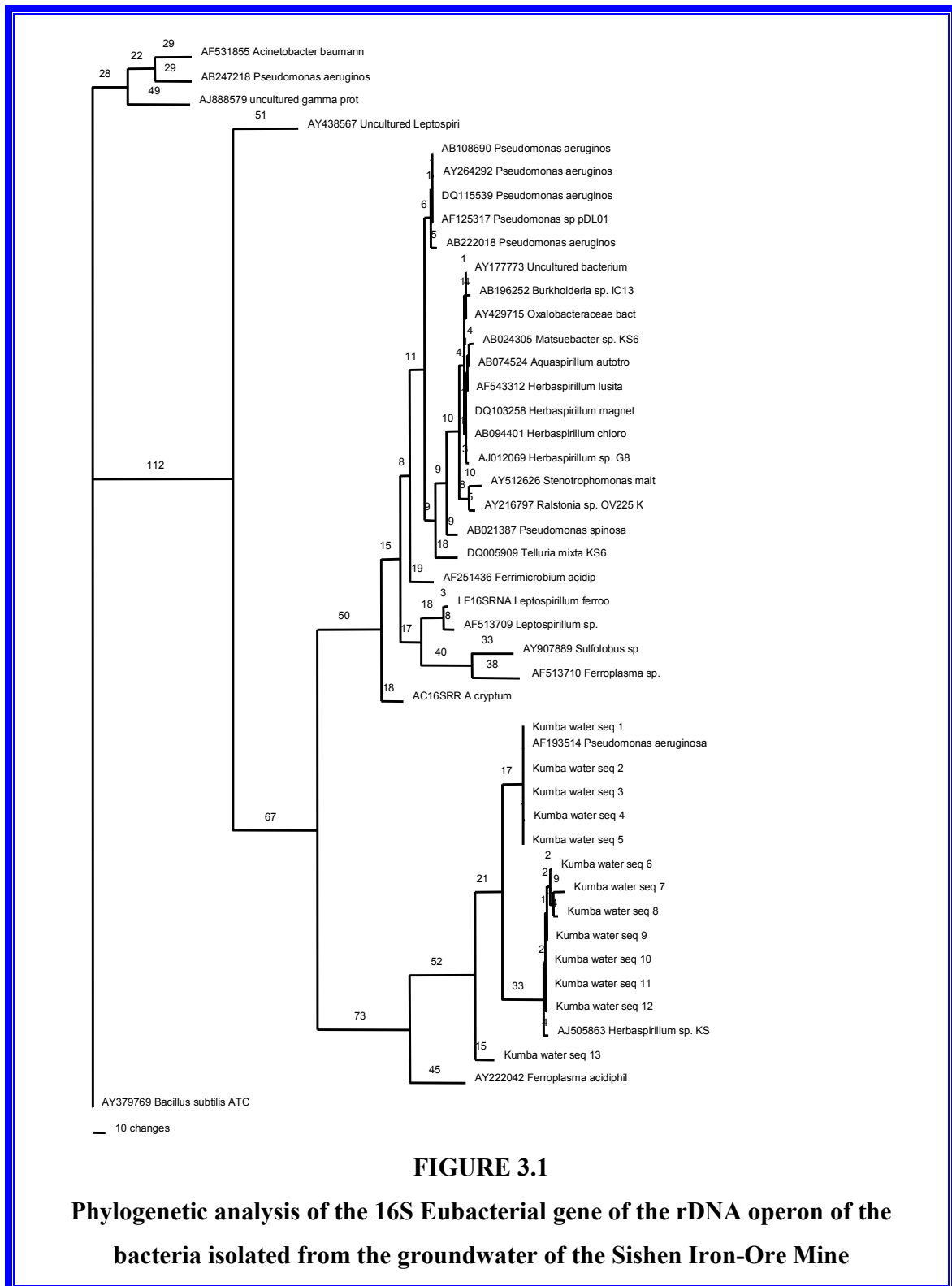
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infections associated with water systems in hospitals (Squier *et al.*, 2000). *Chryseobacterium meningosepticum*, which is typically found in water and soil, generally has low pathogenicity, but may be of clinical importance in immunocompromised patients,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

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

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

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

3.5 Conclusions

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

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CHAPTER 4

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

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

4.1 Abstract

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

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



4.2 Introduction

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

Microorganisms are capable of converting nutrients in their surrounding environment to biochemical compounds, such as organic acids, required by their metabolism (Gupta and Sharma, 2002; Lesniak *et al.*, 2002). The processes by which this happens can be invaluable when applied in industrial practice. For example, *Acidithiobacillus ferrooxidans* and *At. thiooxidans* are capable of converting ferrous sulphate (FeSO_4) to ferric sulphate [$\text{Fe}_2(\text{SO}_4)_3$], while producing sulphuric acid (H_2SO_4) (Glazer and Nikaido, 1995; Rawlings, 2002). Ferric sulphate and H_2SO_4 both play an important role during the overall leaching of certain minerals from ore bodies (Glazer and Nikaido, 1995). Ferric sulphate is a strong oxidising agent, which is reduced to ferrous sulphate (FeSO_4), leading to the oxidation of certain insoluble metal sulphides to the corresponding soluble metal sulphate (Glazer and Nikaido, 1995). Sulphuric acid is responsible for maintaining the low pH in the environment in order to maintain the optimal metabolic activity of the bacteria in the system (Glazer and Nikaido, 1995).



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

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

4.3 Materials and Methods

4.3.1 Sample Selection

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



4.3.2 *Bacterial Isolation*

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

4.3.3 *Fungal Isolation*

Fungi were isolated from the soil/iron ore by adding 100 g of each sample to 100 ml of sterile distilled water (dH₂O) in a different 500 ml Erlenmeyer flask. The flasks and their contents were incubated at 25°C for 48 h. One millilitre of the suspension was plated onto 20 g.l⁻¹ water agar (Merck, Darmstadt, Germany) in 60 mm Petri-dishes (Concorde Plastics, Johannesburg, South Africa). The Petri-dishes were incubated at 25°C and monitored at 24 h intervals for fungal spore formation. Single fungal spores were then selected and transferred onto half-strength Potato Dextrose Agar (PDA) (Appendix A) (Merck) in 60 mm Petri-dishes (Concorde Plastics). The Petri-dishes were incubated at 25°C for approximately 2 weeks.

4.3.4 *Bacterial DNA Extraction*

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



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

4.3.5 *Fungal DNA Extraction*

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

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

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



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

All PCR reagents were manufactured by Bio-Rad Laboratories (Hercules, CA, USA), unless otherwise stated. The PCR reaction was performed in a reaction volume of 25 μ l containing, 150 mM KCl, 30 mM Tris-HCl (pH 9.0), 0.3% Triton X-100, 50 mM MgCl₂, 10 μ M PCR nucleotide mix, 5 pmol primer PRUN518r (Whitehead Scientific, Cape Town, South Africa), 5 pmol primer PA8f-GC (Whitehead Scientific), 1.5 units of Taq polymerase and 0.5 μ l bacterial suspension. Denaturation of extracted DNA at 95°C for 10 min was followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 51°C for 30 sec, and extension at 72°C for 1 min (Bio-Rad Thermal Cycler, Bio-Rad Laboratories). A final extension at 72°C for 10 min concluded the PCR amplification of the DNA. A reaction containing no DNA template was included as a negative control. The amplified PCR products were separated using 1% agarose gel electrophoresis in Tris-Acetate-EDTA (TAE) buffer.

4.3.7 Denaturing Gradient Gel Electrophoresis (DGGE)

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

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

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



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

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

All PCR reagents were manufactured by Bio-Rad Laboratories (Hercules, CA, USA), unless otherwise stated. The PCR reaction was performed in a reaction volume of 25 μ l containing, 150 mM KCl, 30 mM Tris-HCl (pH 9.0), 0.3% Triton X-100, 50 mM MgCl₂, 10 μ M PCR nucleotide mix, 5 pmol primer ITS1 (Whitehead Scientific, Cape Town, South Africa), 5 pmol primer ITS4 (Whitehead Scientific), 1.5 units of Taq polymerase and 0.5 μ l bacterial suspension. Denaturation of extracted DNA at 92°C for 10 min was followed by 30 cycles of denaturation at 92°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min (Bio-Rad Thermal Cycler, Bio-Rad Laboratories). A final extension at 72°C for 10 min concluded the PCR amplification of the DNA. A reaction containing no DNA template was included as a negative control. The amplified PCR products were separated using 1% agarose gel electrophoresis in Tris-Acetate-EDTA (TAE) buffer.

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

Sequencing the PCR products from the 16S PCR and ITS PCR using the K and ITS1 primers above, respectively, provided tentative species identification. Each isolate was sequenced in an Eppendorf tube containing 1 μ l of clean PCR product, 2 μ l "Big Dye" (Roche) sequence mix, 0.32 μ l primer and 1.68 μ l filter-sterilised dH₂O. The sequence PCR products were cleaned by the addition of 15 μ l of sterile dH₂O, followed by the transfer of the entire volume to a 0.5 μ l Eppendorf sequencing tube. Two microlitres of 3 M sodium acetate and 50 μ l of 95% ethanol were added to each sequencing tube and allowed to stand on ice for 10 min, followed by centrifugation at 10 000 rpm for 30 min. The supernatant was removed, followed by washing of the DNA with 150 μ l of 70% ethanol. After centrifugation at 10 000 rpm for 5 min, the supernatant was aspirated and the DNA pellet vacuum dried for 10 min. DNA sequences were determined using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit using AmpliTaq® DNA Polymerase (Applied Biosystems, UK). Partial sequences of the 16S eubacterial gene, as well as of the ITS region of the rDNA of the bacteria and fungi, respectively, were obtained using the K and ITS1 primers, respectively. Nucleotide sequence order was confirmed by



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

4.4 Results and Discussion

An attempt was made to isolate bacterial DNA directly from the iron ore concentrate by adding iron ore to sterilised dH₂O, followed by vigorous shaking using a vortex. This proved, however, to be ineffective as no bacterial identification was possible due to a very low DNA quantity in the sample. Therefore, it was decided to use enrichment techniques in attempt to obtain efficient DNA quantities for molecular identification of the bacterial species present in the iron ore concentrate samples. The shortcoming of this method is that certain heterotrophic bacteria may proliferate to such an extent that possible autotrophic bacteria may be suppressed, with the consequence that they may not be detected. The DNA extracted from the iron ore enrichment cultures using the CTAB method proved to be of a high quality, displaying no protein and no RNA contamination (Figure 4.1). The 16S PCR of the DNA extracted from the iron ore enrichment cultures yielded a *ca.* 500bp band on a 1.5% TAE agarose gel (Figure 4.2).

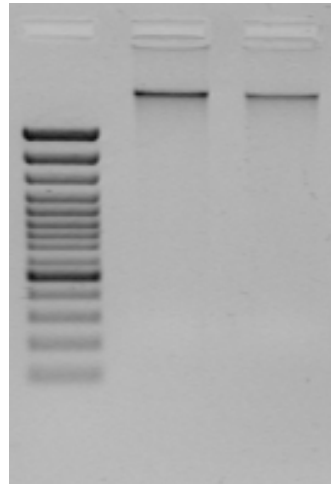


FIGURE 4.1

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

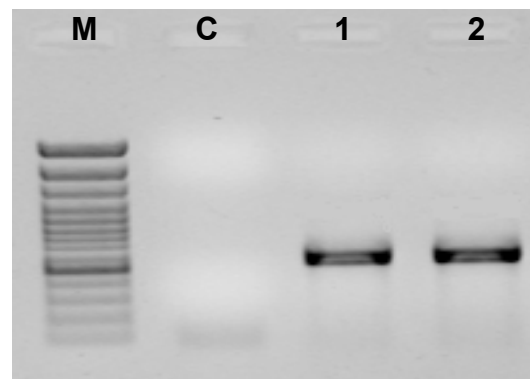


FIGURE 4.2

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

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

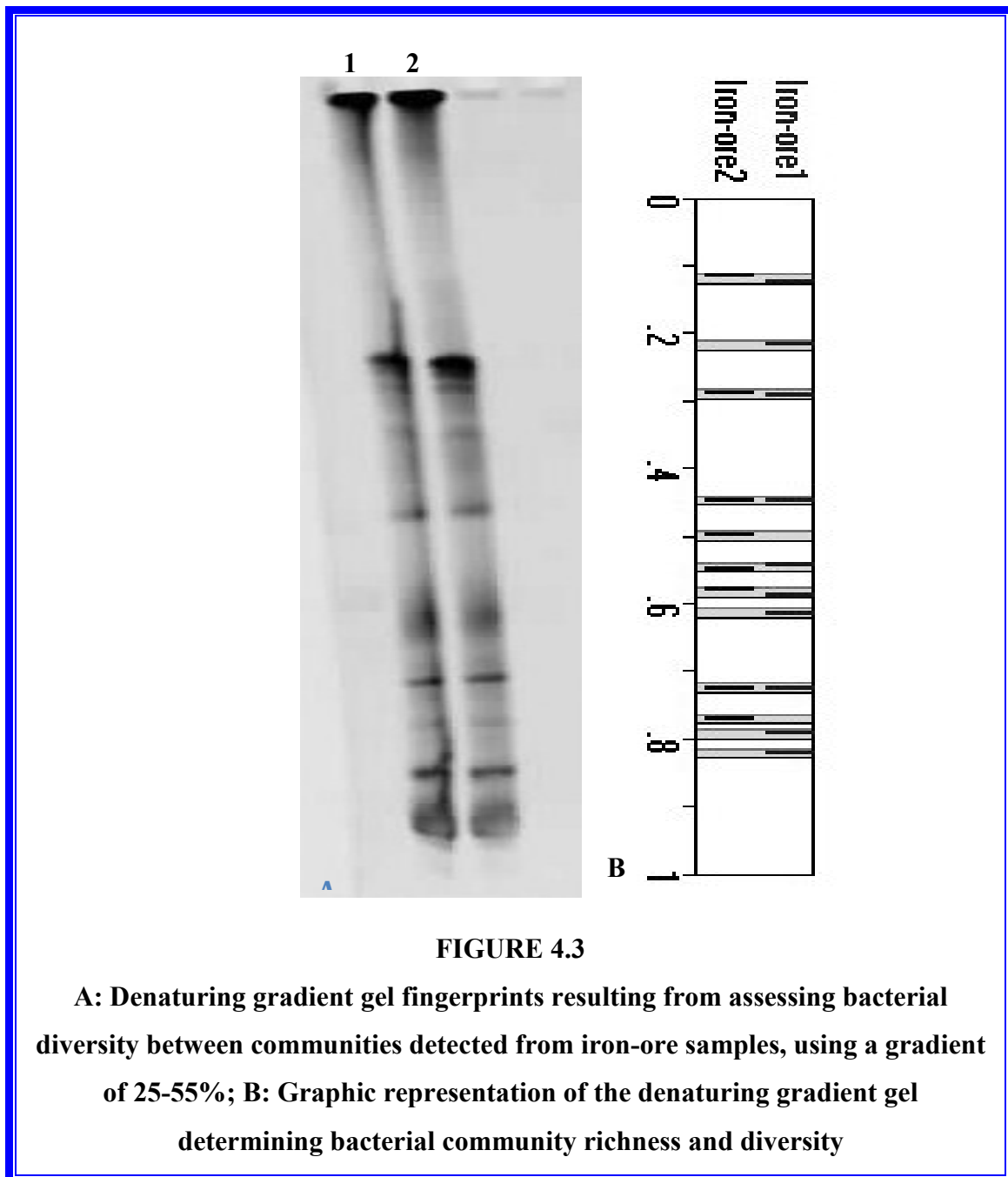


FIGURE 4.3

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

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



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

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

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

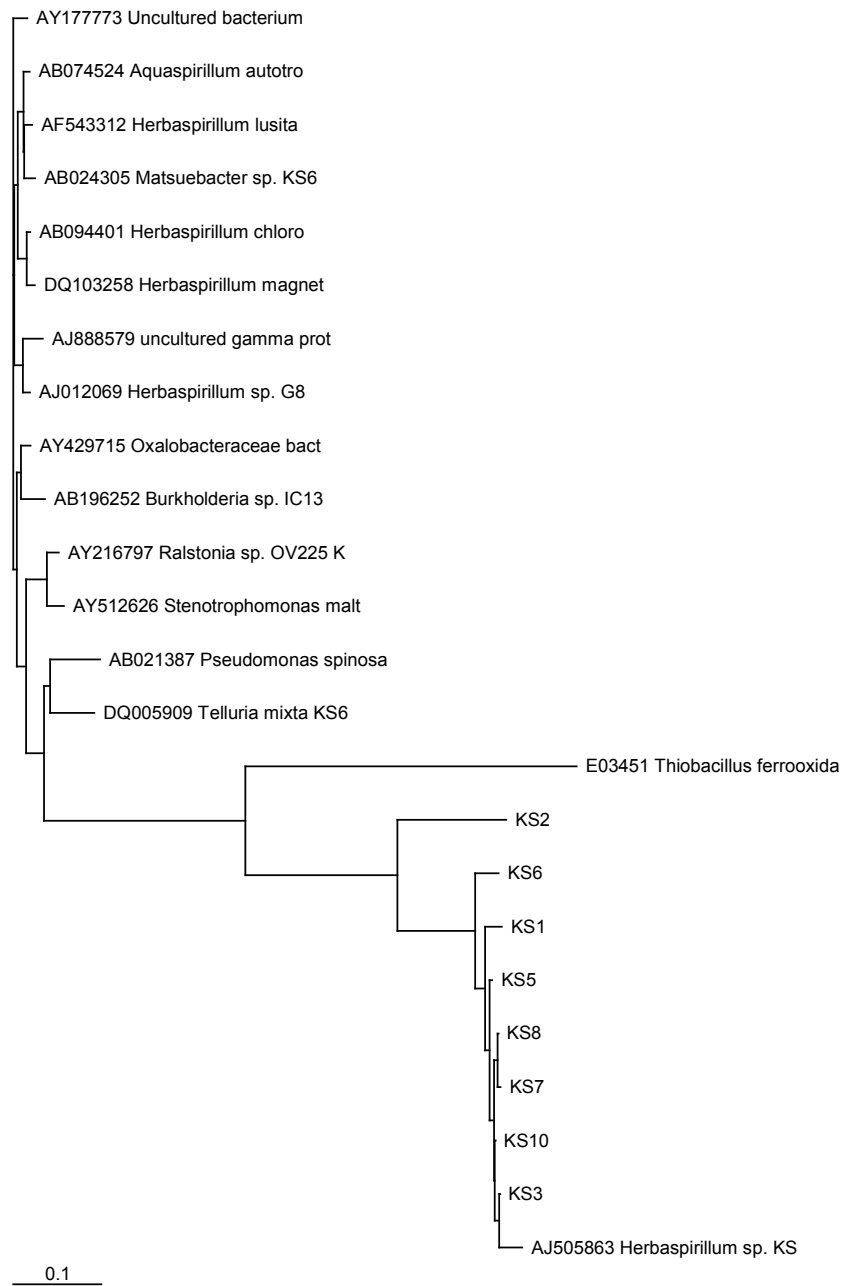


FIGURE 4.4

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

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

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

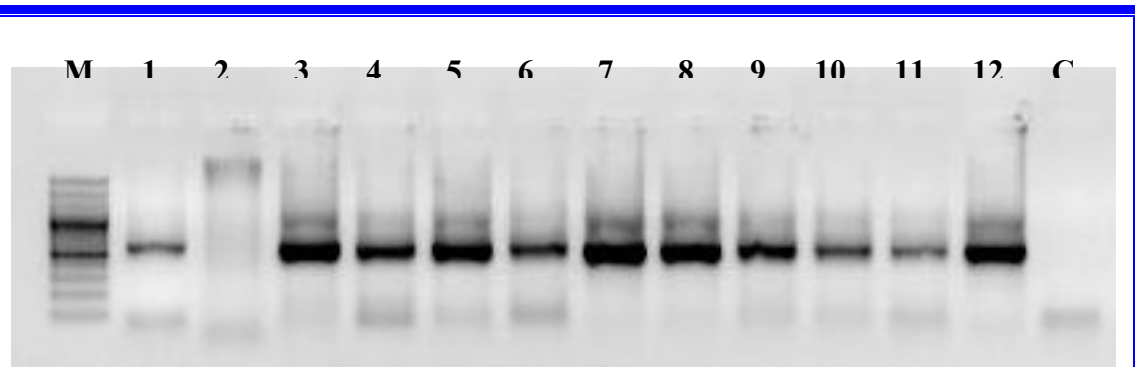


FIGURE 4.5

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

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

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

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

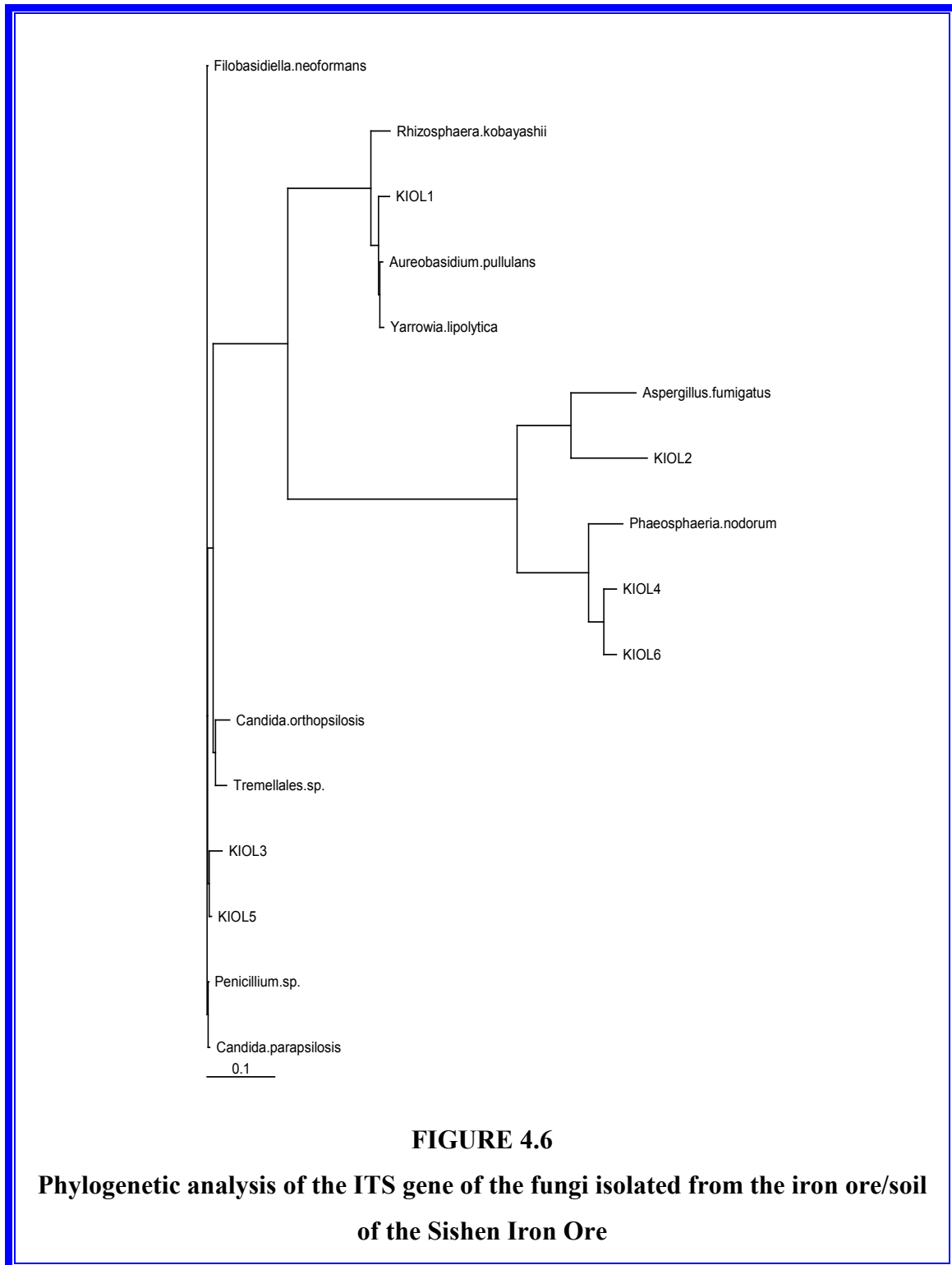


FIGURE 4.6

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



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

4.5 Conclusions

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



4.6 References

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