

Isolation and Molecular Characterization of Sulphur- and Iron-oxidizing bacteria from Kalahari Goldridge Mine, in the North West Province, South Africa

By

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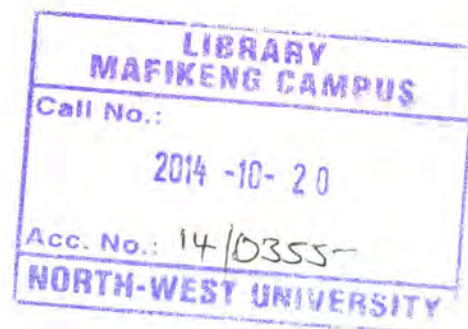


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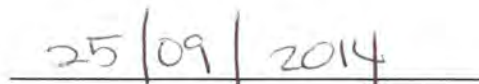
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Declaration

I declare that, the dissertation for the degree of Master of Science in Biology (Microbial Biotechnology) at the North-West University, Mafikeng Campus hereby submitted, has not been submitted by me for a degree at this or any other university. This is my own work in design and execution and that all material contained herein has been duly acknowledged.



Keletso Magdeline Sebogodi



Date

Dedication

This work is dedicated to my husband Sello Mashile, my baby girl Nthabiseng Owethu Moratiwa, my parents Mrs. Dorothy Sebogodi and Mr. Morris Sebogodi, my brother Mr. Thebe Sebogodi, my sister Mrs. Dineo Mogodiri and her husband Mr. Sello Mogodiri.

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Abstract

The mining industry is faced with the challenge of high grade ores being scarce or diminishing at an alarming rate, thus leaving behind low grade ores. In this situation, traditional methods such as roasting and smelting, which involve the use of high temperatures, cannot be applied. Application of heat to these ores leads to an increased production of sulphur dioxide which harms the environment thus contributing to climate change. The use of sulphur- and iron-oxidizing bacteria has been previously identified as an environmentally friendly method of extracting metals from ores. The major contribution of sulfur and iron oxidizing bacteria is the ability to effectively solubilize the sulfidic material contained in ores and to mobilize the insoluble metal sulphides and convert them to soluble metal sulfates.

Two water samples (S7 & S8) and six soil samples (S1, S2, S3, S4, S5 and S6) were collected randomly around the Kalahari Goldridge mine. It was observed that the water samples had high iron content that was indicated by an orange coloured deposit at the bottom of the dam where the water samples were collected. *Thiobacillus* agar was the chosen medium used to isolate and determine growth patterns of isolates of this study. The isolates were designated as Kal1, Kal2, Kal3, Kal4, Kal5, Kal6, Kal7 and Kal8. Microscopic investigations revealed colonies of different morphologies and the majority of them were identified as *Pseudomonas* spp. of different strains which grew at an optimum pH of 7.61 and an optimum temperature of 30°C. The isolates that were identified were *Pseudomonas aeruginosa* MP14, *Endophytic bacterium*, *Enterobacter hormaechei*, *Pseudomonas* sp. *P. aeruginosa* strain B1, *P. aeruginosa* strain 21R, *Enterobacter* sp. and *Pseudomonas* strain HY13 KR. Biochemical characteristics of the bacteria were determined using API 20NE and they revealed that *Pseudomonas* spp. were positive for the nitrate/nitrite reduction test. All the isolates also gave positive results for the biochemical tests: tyryptophan, arginine, esculin, gelatin, capric acid and malic acid tests. They gave a negative result for the tests: urea and phenylacetic acid. Phenotypic characteristics were determined by growing the isolates in the presence of inorganic and organic compounds; *Thiobacillus* broth 0.02% yeast extract and glucose, respectively, to determine the growth pattern of each isolate in each compound. The results showed that a significant effect on cell growth of all the isolates occurred when a carbon source; yeast extract was added to the *Thiobacillus* medium, thus the isolates exhibited chemoautotrophic and chemoheterotrophic characteristics. Detection of *soxA*,

rusA, *thio* and *iro* genes was done by PCR amplification. It was observed that all 8 isolates produced no amplification using the *rusA* primers but showed amplification for primers *thio*, *sox* and *iro* when using plasmid DNA. Universal primers; 27F and 1492R were used to amplify the 16S rRNA gene and the 16S rRNA phylogeny tree show that the isolates were highly related to each other but were less related to the species of *Thiobacillus ferrooxidans*.

List of abbreviations

AMD:	Acid Mine Drainage
API:	Analytical Profile Index
BIOX:	Biooxidation
HiPIP:	High-potential iron-sulphur protein
PCR:	Polymerase Chain Reaction
RISC:	Reduced Inorganic Sulphur Compound
SOB:	Sulphur Oxidizing Bacteria
BLAST:	Basic Local Alignment Search Tool

CHAPTER 1

1. Introduction

Mining is a key activity that contributes to South Africa's economic development and the minerals are considered to be natural assets (Davis & Tilton, 2005). South Africa produces manganese, chrome, vanadium and it is the world's fourth largest producer of diamonds. Due to the high demand for metals, reserves of high grade ores are diminishing at a fast rate. However, there are large stockpiles of low and lean grade ores containing metal sulphides which may yet to be mined (Devasia & Natarajan, 2004). The use of sulphur- and iron-oxidizing bacteria to recover metals from the ores is now an established method that has proven to have its advantages over the traditional physicochemical methods (Rawlings, 2002).

Sulphur- and iron-oxidizing bacteria have been found to be helpful in enhancing the leaching of metals from insoluble metal sulphides. These bacteria have been isolated from different areas of industrial operations, such as the acid mine drainage (AMD) of metal and coal mines, sulphide mineral leach dumps and heaps as well as from sites of natural leaching (Johnson and Hallberg, 2003). These areas are characterized by low pH conditions which are ideal for acidophiles which comprise a wide diversity of autotrophic and heterotrophic prokaryotes with a limited range of eukaryotes (Hallberg & Johnson, 2001).

The role of sulphur- and iron-oxidizing bacteria is to oxidize sulfidic minerals to free metals and this process is known as biomining or bioleaching. It is defined as a process whereby bacteria are used to extract metals from their ores. It may also be defined as the solubilization of metals by applying bacteria on mineral ores to recover metals from solution (Rohwerder & Sand, 2003). In these processes, the bacteria catalyze reactions which would otherwise take very long periods (Norris, 1990). This is an economical method for recovering metals from mineral ores, especially low grade ores that are considered as 'waste' from the mining operations (Watling, 2006).

The bacteria involved in this process (biomining), share many physiological features being; Gram-negative and Gram-positive chemolithotrophs that fix carbon dioxide from the atmosphere and obtain energy by using either ferrous iron or reduced inorganic sulphur compounds as an electron donor and oxygen as the electron acceptor. They can grow in low pH environments and

are tolerant to a wide range of metal ions (Zeng *et al.*, 2009; Foucher *et al.*, 2003; Dopson *et al.*, 2003). Rittenberg (1969) described their nutritional requirement as being obligate chemolithotrophic heterotrophs that acquire their energy from an inorganic compound but require an organic substrate to fulfill their carbon requirement. Shi *et al.*, (2011) described these bacteria as aerobic, mesophilic, non-sporulating, Gram-negative, rod-shaped bacteria which utilize elemental sulphur or compounds containing oxidizable sulphur as sources of energy. They are not harmful to humans, animals or plant life. They thrive under the harsh conditions presented by bioleaching and can do in six days what would normally take 20 years to achieve. These bacteria are important in metal recovery from sulphide ores, particularly in large scale heaps or bioreactors (Fu *et al.*, 2008).

Bioleaching is a method that can be used to recover metals from low grade ores, mine tailings and heap leaching (Watling, 2006) in which conventional methods of extracting metals such as smelting generate a lot of SO₂ in the environment (Stott *et al.*, 2000). Bioleaching therefore is considered as an environmentally friendly method as compared to traditional extraction methods (Fu *et al.*, 2008). The method does not need any heat and it is used on the type of material that had been considered waste or too expensive to mine by using traditional methods; these are ores that require mining companies to mine large quantities of it, to extract small amounts of metal value (Fraser *et al.*, 1991).

For a mineral ore to be biologically leached, it should contain iron or a reduced form of sulphur. If it lacks these compounds, then the mineral may be leached if it occurs together with another mineral that contains iron and reduced sulphur, provided that the mineral is subject to attack by ferric iron and/or sulphuric acid (Rawlings, 2005). Metals in certain non-sulphide minerals may be solubilized by a process of complexation with oxalic, citric or other organic acids which are typically produced by certain types of fungi (Bosecker, 2006). Sulphur- and iron-oxidizing bacteria can either be used to convert insoluble metal sulphides to water soluble metal sulfates (bioleaching) i.e. the conversion of covellite (CuS) to a soluble copper sulfate (CuSO₄), or can be used for pre-treatment of opening up the structure of a mineral ore (biooxidation), thereby making the metal available for further treatment (Rawlings, 2005).

Biooxidation is a term referring to the recovery of metal, where only the metal is recovered and not solubilized, and is mainly applied in gold extraction. Thus, bioleaching is an inappropriate term to use when referring to gold recovery (Rawlings, 2002). Gold may be recovered from gold-bearing iron (arsenopyrite), where sulfidic material is removed by bacteria to expose the gold and further treated with cyanide so that it can be easily extracted (Rawlings, 2005). Nevertheless, the term biomining or bioleaching is frequently used for both processes (bioleaching and biooxidation). The mining industry is now exploiting microbial activity, for instance the BacTech Mining Corporation uses bacteria to extract gold from their ores. It owns commercially-proven bioleaching technology that liberates metals from rock (Clark *et al.*, 2006).

Bioleaching can also be used as a bioremediation tool; as treatment for contaminated sewage sludge, for solid waste and any other industrial waste that may contain metals (Krebs *et al.*, 2006). It has been reported as an efficient and economical method for removal of metals from sewage sludge, because chemical methods require large amounts of chemicals, high operating costs and have secondary pollution problems associated with them (Blais *et al.*, 2005). *Acidithiobacillus thiooxidans* has been found to be the key bacterium in bioleaching of sewage sludge at pH less than 2 (Bouchez *et al.*, 2006). The sulphur- and iron-oxidizing bacteria are not only useful in the mining and waste industry but are also useful in various industries where discarding of metal-containing waste is a challenge such as; petroleum catalysts, electronic scraps, lithium battery wastes, sewage sludge and nuclear waste, to mention a few, are some of the major industrially-produced wastes which may contain Ni, V, Mo, Co, Cu, Pb, Zn and some bacteria are employed to leach the metals from solid to aqueous state (Mishra & Rhee, 2010).

Though extensive work has been done on acidophiles that are capable of oxidizing iron, little is known about neutrophilic sulphur- and iron-oxidizers (Auernik *et al.*, 2008), due to the difficulties associated with growing, obtaining and maintaining them in pure culture (Edwards *et al.*, 2003). An example of a neutrophile is *Thiobacillus intermedius*, which grows best at temperatures of 25-37°C and pH of up to 7. It gets energy from the oxidation of elemental sulphur and sulphur-containing compounds. A few of the members of *Thiobacilli* can assimilate organic matter but only to a limited extent, and *Thiobacillus intermedius* is one such organism. Other examples of neutrophilic, obligate chemolithotrophs in the genus *Thiobacillus* include *Thiobacillus thioparus* and *Thiobacillus neapolitanus*. They grow best at neutral pH and may

grow at pH values above 9 and require inorganic sulphur as a source of energy (Vidyalakshmi *et al.*, 2009). The neutrophilic mixotrophs include *Thiobacillus novellus*, a soil organism named and originally described by Starkey (1934 & 1935).

Molecular phylogenetics aims to know the relationship among these bacteria by isolating and determining their DNA, RNA or protein sequences, and by forming a phylogenetic tree that will depict their relationship to one another. There are various methods of phylogenetic analysis and there is no one right method for determining the relationship of their genes or species and all depends on the nature of the study and the data that is used (Hall & Barlow, 2006). The interest in the sulphur- and iron-oxidizing bacteria's evolution stems from the realization of how their chemolithotrophic metabolism is primitive and how it may have shaped our planet Earth (Peck, 1968; Van Niel, 1946). According to Lane *et al.*, (1985), the phylogenetic diversity of sulphur- and iron-oxidizing phenotypes suggests a closer look at the genes that encode the enzymes of the pathways which provides important information on the evolution of bacteria carrying these genes.

This work describes the isolation of sulphur- and iron-oxidizing bacteria and identifying genes that are responsible for oxidation of iron and sulphur, from samples obtained from the Kalahari Goldridge mine in the North West Province of South Africa.

1.1 Significance of the research

Metals may be recovered from their respective ores through pyro- and hydro-metallurgical routes or a combination of both. As high grade metal ores (usually sulphides) are now getting depleted and stricter environmental rules are being implemented, there exist large stockpiles of low grade ores that cannot be treated effectively using traditional methods (Mishra & Rhee, 2010). Cheap and efficient methods have to be employed to extract the little that is left in these low grade ores (Zeng *et al.*, 2009) and this includes the use of bacteria. The study was designed to identify bacteria from the Kalahari Goldridge mine for the possibility of utilizing them in the extraction of metals by oxidizing sulphur and iron.

1.2 Objectives

- ✓ To isolate and identify bacteria responsible for oxidation of sulphur and iron.
- ✓ To detect and amplify genes responsible for the oxidation of sulphur and iron from the isolates.

1.3 Aims of the study

The aim of the study was to determine which bacteria were present in the Kalahari Goldridge' open cast mine, as well as to determine which ones carry the sulphur- and iron-oxidizing genes and to determine the possible relationship between using a phylogenetic tree. To date, no information regarding the microbial community of the Kalahari Goldridge mine exists.

CHAPTER 2

2. Literature review

Some moderately and extremely thermophilic organisms are being considered for bioleaching as a means to improve mineral sulphide oxidation rates (Rodriguez *et al.*, 2003). Therefore, biomining or bioleaching is focusing on the use of microorganisms suitable for acidic and high temperature environments to improve bioleaching processes (Rawlings, 2002).

It has been established that microorganisms capable of being used for biomining must have the qualities of being able to oxidize sulphur and iron, they must be autotrophic and they must be able to fix carbon dioxide from the atmosphere. In fact, it has been shown that some microorganisms grow better when provided with air that has been enriched with 0.5–5.0% (v/v) carbon dioxide (Rawlings *et al.*, 2003). In addition to fixing carbon dioxide, they should be able to obtain their energy from reduced inorganic sulphur compounds or ferrous compounds or both, as an electron donor with oxygen as the electron acceptor. In the process, sulphuric acid and ferric ions are formed, leading to sulphuric acid creating a low pH environment and ferric ions being used by sulphur oxidizing bacteria in place of oxygen as electron acceptor (Clark & Norris, 1996).

The leaching of copper from its ores (bioleaching) and the precipitation of copper from solution (bioaccumulation) is an ancient technology which the Chinese practiced as far back as 100–200BC and possibly even earlier. However, metal solubilization using specific bacteria was not practiced until the 1940's. Since then, however research has helped to clarify the mechanism behind the process. The Rio Tinto mine cannot be excluded in the bioleaching discussions because it is considered as the cradle of biohydrometallurgy (Mishra *et al.*, 2005). The biooxidation pre-treatment plant with the longest history is commissioned at Goldfields' BIOX process at the Fairview Mine in South Africa. This plant has been operating since 1986 and it treats refractory arsenopyrites, pyrite and pyrrhotite gold bearing ores, in large, stirred-tank, and aerated continuous reactors (Clark *et al.*, 2006).

The advantages of the BIOX process are; improved rates of gold recovery, lower capital costs, and low running technology suited to remote areas, low skills required for operation and reduced environmental impact. The process uses a combination of three bacteria that occur naturally, *Acidithiobacillus (Thiobacillus) ferrooxidans*, *A. thiooxidans* and *Leptospirillum ferrooxidans* (helical curved rods), to break down these sulfidic materials. The bacteria attach themselves to the metal sulphide surfaces in the ore resulting in the accelerated oxidation of the sulphides (Clark *et al.*, 2006).

The potential benefits of bioleaching are that metals can be recovered from ores that may be considered as 'waste' which are uneconomical to smelting. There are no noxious gases that are released. It requires simple technology in terms of equipment and conditions of operation at ambient pressure and temperature, though the latter mainly applies in heap and dump leaching, thus the use of bacteria is more environmentally friendly and there is less consumption of energy (Raheb *et al.*, 2009). Biological (environmentally friendly) techniques for the treatment of heavy metals present in waste are more suitable than compared to the use of traditional technologies. It is important to note that bacteria do not destroy metals but can influence metal mobility in the environment by modifying their chemical or physical characteristics (Mishra & Rhee, 2010). Conventional processes can be considered to treat the waste materials for recycling purposes. However, the overall process costs, both operational and financially is high. The general cost of an operation depends on; the concentration of the metal in solution, operational mode of the equipment, the need for secondary treatment and disposal of secondary waste such as sludge (Eccles, 1995).

Industries need to invest huge sums of money in these areas and therefore any new form of technology can significantly reduce such challenges in both economic and ecofriendly ways.

Bioleaching can also be used or applied in industries that have metal-filled waste such as; fly ash treatment, electronic scraps or waste, spent batteries, spent petroleum catalysts etc., where sulphur- and iron-oxidizing bacteria (*A. thiooxidans*, *A. ferrooxidans*) may be used to leach out the metals present in the waste. Mesophilic and moderately thermophilic acidophiles have been considered for leaching of metals from E-waste and such bacteria have been named 'computer munching' microbes (Ilyas *et al.*, 2010).

In a study conducted by Mishra *et al.*, (2008), pure cultures of *A. ferrooxidans* were used to leach out cobalt and lithium from lithium-ion batteries and other major solid waste coming from batteries containing lithium, nickel and cadmium, especially those used in digital cameras, cellular phones and laptops. A similar study was conducted by Xin *et al.*, (2009) but with a mixed culture of acidophilic sulphur- and iron-oxidizers. Even though the spent batteries have neither iron nor sulphur content within them, elemental sulphur may be added as an energy source, as sulphuric acid is produced, allowing metal dissolution to occur (Zhao *et al.*, 2008).

Bioleaching has been developed and demonstrated to be a cost effective and efficient way of removing heavy metals from sewage sludge without seriously affecting the soil conditions and its fertilizing conditions (Xiang *et al.*, 2000). The processes of iron-oxidizing bacteria are quite efficient in removing metals from sewage sludge, which requires the addition of industrial grade iron sulfate as an energy source (Tyagi *et al.*, 1996). This process requires acidification of the sludge to pH 4.0, so as to create an acidic environment. However, in application of sulphur-oxidizing bacteria, acidification of the sludge is not required because the sludge pH decreases, thus creating an environment where metals can be effectively removed (Chan *et al.*, 2003).

A comparison was made between the efficiency of sulphur- and iron-oxidizing bacteria and, according to the statistical comparisons of the metal yield, it was revealed that microbial leaching with iron-oxidizing bacteria was less efficient than sulphur-oxidizing bacteria for aluminum, chromium, nickel and lead, because of its lower acidification capacity. However, Xiang *et al.*, (2000) showed that the indigenous iron-oxidizing bacteria isolated from an anaerobically digested sludge was effective in removing zinc, copper, nickel and chromium from sludge of pH 5 with the addition of Fe^{2+} as the energy source. In addition, iron-based bioleaching is considered superior to sulphur-based bioleaching because there is no residual sulphur formed in the decontaminated sludge. This eliminates the risk of secondary pollution caused by acidification of soil due to sulphur oxidation (Wong *et al.*, 2002).

Sewage sludge contains a large amount of organic compounds which may be inhibitory to the growth of bioleaching bacteria (Cho *et al.*, 2002). However, the presence of other heterotrophic microorganisms can enhance the activity of bioleaching, as these microorganisms break down the organic materials. For example, heterotrophic fungi identified as *Cladosporium* was found in

sewage sludge and was found to break down organic matter from the sludge, thus increasing the rate of bioleaching (Picher *et al.*, 2002).

2.1 Types of bacteria in bioleaching

The types of bacteria involved in the process of bioleaching, include a variety of autotrophic, heterotrophic, mesophilic, aerobic sulphur- and iron-oxidizing, thermophilic sulphur- and iron-oxidizing and anaerobic sulfate-reducing bacteria. Some are heterotrophic, which indirectly affect solubilization of metal sulphides by affecting the growth and activity of metal-solubilizing bacteria (Devasia & Natarajan, 2004).

Heterotrophic bacteria that live off waste products that are produced by the autotrophic bacteria contribute tremendously to bioleaching by removing toxic organic compounds that may inhibit autotrophic activity. In fact, the growth of primary acidophilic bacteria (i.e. *Acidithiobacillus ferrooxidans*, *Acidithiobacillus thiooxidans*) has been measured during bacterial leaching of metals from municipal sewage sludge (Gamache *et al.*, 2001) and said to be enhanced by the presence of heterotrophic bacteria (Pronk & Johnson, 1992). These heterotrophs may include species from the genera *Paracoccus*, *Xanthobacter*, *Alcaligenes* and *Pseudomonas* and they may also show chemolithotrophic growth on inorganic sulphur (Kuenen *et al.*, 1982). The leaching mechanisms of these heterotrophs involve an indirect process in which their metabolic products dissolve metals from the mineral ores by formation of soluble metal complexes and chelates (Rezza *et al.*, 2001). Lingling *et al.*, (2012) isolated a microorganism from the acidic mine tailing and identified the isolate as *Pseudomonas aeruginosa* and performed bioleaching experiments using the bacterium on low grade collophanite ore. They observed that the insoluble phosphorus within the collophanite was dissolved to soluble phosphorus and the bioleaching mechanism was mainly indirect by releasing metabolites (organic acids). Heterotrophs that have been found in the solubilization of gold ores include; *Serratia marcescens*, *Bacillus subtilis*, *Pseudomonas fluorescens*, *Bacillus alvei*, *Bacillus megaterium*, *Bacillus mesentericus*, *Pseudomonas liquefaciens* and *Bacterium nitrificans* (Korobushkina *et al.*, 1974; Boyle & Boyle, 1979).

Bacteria that may be prevalent in bioleaching processes operating at temperatures of 35-40°C are mostly considered to be Gram-negative and these include; *A. thiooxidans*, *A. ferrooxidans*, *A.*

zaldus, *Leptospirillum ferriphilum* (Coram & Rawlings, 2002). For many years, the majority of obligately chemolithotrophic acidophilic bacteria that can oxidize ferrous iron and reduced inorganic sulphur compounds (RISCs) have been considered to be strains of the well documented *A. ferrooxidans* (Amouric *et al.*, 2011).

Thermophiles that have also been reported in bioleaching environments include *Sulfobacillus thermosulfidoxidans* and other extreme thermophiles which use higher temperature of about 70°C for faster bioleaching rate. These include *Sulfobacillus ambivalens*, *Sulfobacillus brierleyi* and *Thiobacter subterraneus* (Kletzin, 2007; Hirayama *et al.*, 2005; Konishi *et al.*, 1998). Organisms that grow at temperatures higher than 65°C are placed in the Archaea domain. Gram-positive acidophilic sulphur- and iron-oxidizing isolates designated as TC-34 and TC-71 in Jiang *et al.*, (2009), were isolated from a solfataric region in China. The isolates (TC-34 & TC-71) were aerobic, spore-forming coccoid, but rods and flagella were not observed.

Traditionally, detection of these bacteria has been possible by using culture dependent techniques, using liquid or solid media (Johnson, 1995). However, this methodology is slow and can take several weeks (Escobar *et al.*, 2008). In the last few years, molecular techniques have been developed using Polymerase Chain Reaction (PCR), for the identification of bacteria, where 16S rDNA sequences are amplified from isolated genomic DNA (Jensen *et al.*, 1993). Molecular techniques that have been used in identification of microbial communities include; 16S rDNA analysis by restriction enzymes (Rawlings, 1995), analysis of spacing regions from ribosomal operons (Pizarro *et al.*, 1996), analysis of phylogenetic groups (Wulf *et al.*, 1997) and Denaturing Gradient Gel Electrophoresis (DGGE) (Demergasso *et al.*, 2005). DGGE and terminal Restriction Fragment Length Polymorphism (t-RFLP) offer a rapid, culture-independent way for detecting and identifying predominant PCR-targeted populations (Bryan *et al.*, 2005; Casamayor *et al.*, 2000). These techniques have their limitations; they tend to be slow and in many cases resulting to irrelevant bacteria being detected in the samples (Escobar *et al.*, 2008). Table 2.1 taken from Robertson & Kuenen (2006) indicates some of the isolated species that have emerged since the genus *Thiobacillus* was first described:

Table 2.1 *Thiobacillus* species that emerged since the genus was first described by Robertson & Kuenen 2006

Sub-division	Name of species	Synonym
α	<i>Acidiphilium acidiphilium</i>	<i>Thiobacillus acidiphilium</i> / <i>organoparus</i>
α	<i>Paracoccus pantotrophus</i>	<i>Thiosphaera pantotrophus</i>
α	<i>Paracoccus versutus</i>	<i>Thiobacillus versutus</i> / <i>rapidicrescens</i>
α	<i>Starkeya novella</i>	<i>Thiobacillus novellus</i>
β	<i>Thiobacillus aquaesulis</i>	
β	<i>Thiobacillus denitrificans</i>	
β	<i>Thiobacillus thioparus</i>	<i>Thiobacillus thiocyanoxidans</i> / <i>Bacterium thioparum</i>
β	<i>Thiomonas cuprina</i>	<i>Thiobacillus cuprinus</i>
β	<i>Thiomonas intermedia</i>	<i>Thiobacillus intermedius</i>
β	<i>Thiomonas perometabolis</i>	<i>Thiobacillus perometabolis</i> / <i>rubellus</i>
β	<i>Thiomonas thermosulfata</i>	
β	<i>Thiobacillus plubophilis</i>	
γ	<i>Acidithiobacillus albertensis</i>	<i>Thiobacillus albertensis</i>
γ	<i>Acidithiobacillus caldus</i>	<i>Thiobacillus caldus</i>
γ	<i>Acidithiobacillus ferrooxidans</i>	<i>Thiobacillus ferrooxidans</i> / <i>Ferrobacillus ferrooxidans</i>
γ	<i>Acidithiobacillus thiooxidans</i>	<i>Thiobacillus thiooxidans</i> / <i>concretivorans</i> / <i>kabobis</i> / <i>thermitanus</i> / <i>lobatus</i> / <i>cretanus</i> / <i>umbonatus</i>
γ	<i>Halothiobacillus halophilus</i>	<i>Thiobacillus halophilus</i>
γ	<i>Halothiobacillus hydrothermalis</i>	
γ	<i>Halothiobacillus kellyi</i>	

α ; alphaProteobacteria, β ; betaProteobacteria, γ ; GammaProteobacteria

Table 2.1 continued: *Thiobacillus* species that emerged since the genus was first described by Robertson & Kuenen 2006

Sub-division	Name of species	Synonym
γ	<i>Halothiobacillus neapolitanus</i>	<i>Thiobacillus neapolitanus</i>
γ	<i>Thermithiobacillus tepidarius</i>	<i>Thiobacillus tepidarius</i>
γ	<i>Thioalcalivibrio denitrificans</i>	
γ	<i>Thioalcalivibrio denitrificans</i>	
γ	<i>Thioalcalivibrio nitratus</i>	
γ	<i>Thioalcalivibrio versutus</i>	
γ	<i>Thioalcalimicrobium aerophilum</i>	
γ	<i>Thioalcalimicrobium sibericum</i>	
γ	<i>Thiomicrospira chilensis</i>	
γ	<i>Thiomicrospira crunogena</i>	
γ	<i>Thiomicrospira frisia</i>	
γ	<i>Thiomicrospira kuenenii</i>	
γ	<i>Thiomicrospira pelophila</i>	
γ	<i>Thiomicrospira thyasirae</i>	<i>Thiobacillus tyasiris</i>
γ	<i>Thiobacillus prosperus</i>	
γ	<i>Thiomicrospira denitrificans</i>	
γ	<i>Thiobacillus delicatus</i>	
γ	<i>Thiobacillus capsulatus</i>	

α; alphaProteobacteria, β; betaProteobacteria, γ; GammaProteobacteria

2.2 Feeding/ nutrition of sulphur- and iron oxidizing bacteria

Bacteria such as *Acidithiobacilli* (*Thiobacilli*) and *Leptospirilli* fix carbon dioxide in the air with the help of the enzyme ribulose 1,5-bisphosphate carboxylase, for their cell mass synthesis (Tyson *et al.*, 2004). *A. ferrooxidans* is considered to be an obligate autotroph and uses low concentrations of formic acid as a carbon source provided that it is grown in continuous culture and kept at low concentrations (Pronk *et al.*, 1991). When formic acid is lysed, formate is assimilated by the Calvin Cycle in much the same way as carbon dioxide. *Leptospirillum* spp. have been reported to possess the genes for the formate hydrogenlyse complex, therefore, they are capable of using formate as a source for growth (Tyson *et al.*, 2004). Nitrogen is the next most important element after carbon for the synthesis of new cell mass. Ammonium levels of 0.2 mM have been reported to provide sufficient nitrogen requirement of *A. ferrooxidans* (Tuovinen *et al.*, 1971). In commercial operations, fertilizer grade ammonium sulfate is typically added to biooxidation tanks or bioleaching heaps to ensure nitrogen availability (Dew *et al.*, 1997). The ability of bacteria to fix nitrogen is due to nitrogenase enzyme and this enzyme is inhibited by oxygen. The nitrogen fixing operon of *L. ferrooxidans* from the Tinto river has been isolated and sequenced (Parro & Moreno-Paz, 2004). With regard to organic compounds on bacterial growth, the acidophile strain ZW-1 was inhibited with the addition of glucose, as cell density decreased, there was no further growth. However, with the addition of yeast extract or peptone, growth was favored. There was an increase in cell density, therefore adding some nitrogenous compounds such as those found in yeast and peptone, promoted the growth of the strain (Zeng *et al.*, 2009).

2.3 Media for isolating and enumerating acidophilic bacteria

Cultivating acidophiles in appropriate liquid medium is not usually problematic although difficulties are experienced using solid media. This can be because of two reasons; firstly strain variation, some strains of acidophiles such as *A. ferrooxidans*, may grow more readily on solid media than other. The other reason is that solid media is more successful when plating mixed population of autotrophs and heterotrophs than pure cultures (Johnson, 1995).

Although culture-based techniques are useful in revealing physiological characteristics of the bacteria, there is a need to validate certain findings using culture independent molecular techniques (Tan *et al.*, 2008). In the last few years, culture dependent and culture independent techniques (Cai *et al.*, 2006) were used to study microbial communities of acidic environments.

Some of these techniques were used in the Tengchong solfataric region, China (Jiang *et al.*, 2009) where some novel species such as *Acidianus tengchongensis*, *Sulfolobus tengchongensis*, *Thermoanaerobacter tengcongensis*, *Meiothermus rosaceus*, *Thermus rehai* (He *et al.*, 2004; Xiang *et al.*, 2003; Lin *et al.*, 2002; Chen *et al.*, 2002; Xue *et al.*, 2001) were cultivated and characterized.

The enrichment medium that was used by Jiang *et al.*, (2009) in isolating sulphur-oxidizing bacteria from Tengchong solfataric region, China, was the Norris broth (Norris *et al.*, 1996) but it was modified by supplementing yeast extract and using gelrite gellum gum to solidify it. The Norris broth contained the basal salts/inorganic: $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, NH_4SO_4 , K_2HPO_4 , $\text{K}_2\text{S}_4\text{O}_6$, KCl and yeast extracts and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ as the energy source for iron-oxidizing bacteria, sulphur was also added for sulphur-oxidizing bacteria. The enrichment and cultivation medium was kept at acidic conditions by adding sulphuric acid and was conducted at 30-37°C under rotary conditions at 200 rpm for the purpose of obtaining acidophilic mesophilic microorganisms.

Filter-sterilized 9K iron medium (Silverman & Lundgren, 1959) of pH 2 containing inorganic salts [$(\text{NH}_4)_2\text{SO}_4$, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, K_2HPO_4 , FeSO_4] and ferrous iron were incorporated for enrichment and maintenance of bacteria. The bacterium was also grown in iron-free 9K iron medium of pH 3 where sulphur was added as the energy source for sulphur-oxidizing properties of the bacterium. The liquid medium was incubated under rotary conditions of about 170 rpm and for isolation of the isolate; the medium was solidified with ferrous-agarose. Overlay media iron, (Fe_o) iron/tetrathionate, (FeS_o) and yeast extracts, (YE_o) (Hallberg & Johnson, 2003), were used in enumerating and isolating acidophilic sulphur- and iron-oxidizing bacteria by Tan *et al.*, (2008) in isolating and determining phylogenetic diversity of microorganisms found in open-dumped, extremely acidic Pb/Zn mine tailings. Agar powder can be used to solidify the enrichment broth instead of ferrous agarose, this technique was used by Chen *et al.*, (2009). Overlay media have been shown to be efficient and selective for cultivating both autotrophic and heterotrophic acidophiles (Tan *et al.*, 2008). Before applying bacteria on overlay media, the samples were first suspended in basal salts at acidic conditions and placed in a rotary incubator at 180 rpm. Serial dilutions were then done using the solution of basal salts, then plated on the solid media and incubated for 10-20 days. Results obtained by Tan *et al.*, (2008) showed growth of iron- and sulphur- oxidizing on Fe_o and FeS_o and the colony count was about $2.2 \pm 0.3 \times 10^3$ and

$2.2 \pm 0.2 \times 10^3$ CFU g⁻¹ and acidophilic heterotrophs grew on YE₀ at pH of 3.0 reaching $1.6 \pm 0.1 \times 10^4$ CFU g⁻¹ higher than autotrophs.

Lavalle *et al.*, (2005) used 9K iron medium solidified with agarose and supplemented with surface-active agent, Tween 20 to improve the colony size on solid medium. Incubation was also done on a rotary shaker at 200 rpm. Vdyalakshmi & Sridar (2007) used the Starkey medium to isolate sulphur-oxidizing around locations such as; rhizosphere, pulse rhizosphere, sewage, biogas slurry, tannery effluent and mine soils. The Starkey broth was composed of KH₂PO₄, MgSO₄·7H₂O, CaCl₂·H₂O, (NH₄)₂SO₄ and traces of FeSO₄. From this, it can be observed that media are mainly composed of basal inorganic salts, and the medium is suspended in acidic conditions under rotary conditions until growth is evident.

2.4 Phylogeny of the chemolithotrophic bacteria

The environment that the sulphur- and iron-oxidizers (low levels of nutrients)/inorganic or lithotrophs strive in, probably caused chemolithotrophic bacteria to evolve in isolation from the more commonly studied bacteria, *Thiobacillus ferrooxidans* (Rawlings, 2001). Since *Thiobacillus* genus was first described, researchers realized that there was a need to regroup and re-organize it because the only criterion for grouping the species belonging to this genus was based on the fact that they were rod-shaped and obtained energy for autotrophic growth from oxidizing inorganic sulphur-containing substances (Kelly & Harrison, 1984). However, the genus includes species that grow at different pH, temperature and have different nutrient requirements from acidophiles, neutrophiles, thermophiles, denitrifiers, facultative heterotrophs (Kuenen *et al.*, 1992).

16S rRNA sequence data has been an important tool in determining how these chemolithotrophic bacteria are related to one another. The 16S rRNA gene analysis emerged as a more accurate, objective and reliable method for identifying bacteria and an added capability of defining taxonomical relationships among them (Clarridge, 2004). These gene sequences, 16S rRNA, are by far the most common housekeeping genetic marker mainly because they are present in almost all bacteria that exist as operons and their function has not changed overtime which makes it possible to measure evolution over time and the size of the gene is large enough for bioinformatics studies (Patel, 2001). In the phylogenetic tree extracted from Williams *et al*

(2010), it was found that *A. ferrooxidans*, *A. thiooxidans* and *A. caldus* (which is considered to be moderately thermophilic with an optimum temperature of 45-48°C), are closely related bacteria and fall within the division Proteobacteria, very close to the α (alpha) and β (beta) subdivisions. There is an indication that the genus *Acidithiobacillus* has generally been considered as member of the γ (Gamma) -Proteobacteria (Williams *et al.*, 2010), even though a recent study suggests that it arose after divergence from the α -Proteobacteria but before the β -Proteobacteria/ γ -Proteobacteria (Rawlings, 2001).

Escherichia coli (*E. coli*) which is a member of the γ -Proteobacteria, was also found to be closely related to obligately chemolithotrophic *Acidithiobacilli* (Williams *et al.*, 2010). This is why *A. ferrooxidans*' genes can be expressed in *E. coli* but in contrast the *Leptospirilli* are placed in the *Nitrospira* division (Hugenholtz *et al.*, 1998). *Leptospirilli* can have more than one species as it may have been observed using the 16S rRNA sequence tool, but only using rRNA sequence data for classification can make the validation of the classification system to be questionable. There have also been numerous reports suggesting that Fe^{2+} oxidizing *Acidithiobacilli* are a heterogeneous group of bacteria that has sufficient genetic variability giving rise to more than one species (Ni *et al.*, 2007). In addition to genetic variability, several phenotypic differences have been observed among the Fe^{2+} oxidizing *Acidithiobacillus*, such as their motility and presence or absence of flagella, as well as optimal pH and temperature for growth (Hallberg *et al.*, 2010; Kupka *et al.*, 2007). In a recent study by Luo *et al.*, (2009), 12 strains isolated from various mining areas in China were identified as *A. ferrooxidans* but were diverse at the genomic level that was due to the geographic distribution.

The α -Proteobacteria include most of the oligotrophic Proteobacteria. This class of the Proteobacteria has seven orders and twenty families; this includes the family of purple non-sulphur bacteria. They may be spirals, rods, cocci and they may form prosthecae and buds, that use anoxygenic photosynthesis and possesses bacteriochlorophylls *a* or *b*. Most are motile by polar flagella. They normally grow anaerobically as photoorganoheterotrophs by trapping light and using organic molecules as both electron and carbon sources. In the absence of light, they can grow aerobically as chemoorganoheterotrophs but others can carry out fermentations

anaerobically. Despite their name, purple non-sulphur bacteria can oxidize sulphide to sulfate but they do not oxidize sulphur to sulfate (Willey *et al.*, 2008).

Some examples of the purple non-sulphur bacteria include those from the family *Rhodospirillaceae* that are capable of forming cysts in response to nutrient limitation. Cysts are similar to endospores in that, they are able to survive under harsh conditions (Willey *et al.*, 2008). γ -Proteobacteria are defined as chemoorganotrophic and facultatively anaerobic and also include aerobic chemoorganotrophs, photolithotrophs, chemolithotrophs or methylotrophs (Willey *et al.*, 2008). Examples include, purple sulphur bacteria (*Thiospirillum*, *Thiocapsa*, *Chloromatum*) that are strict anaerobes and usually photolithoautotrophs which oxidize hydrogen sulphide to sulphur and deposit it externally as sulphur granules (usually within invaginated pockets of the plasma membrane) and eventually oxidize sulphur to sulfate. The nutritionally diverse subgroup of the β -Proteobacteria includes *Nitrosomonas*, a genus of soil bacteria that play an important role in the nitrogen cycle, recycling ammonium (NH_4^+) and producing nitrite as a waste product. Normally, they employ carbon dioxide as their carbon source and thus are chemolithoautotrophs but can also function as chemolithoheterotrophs and use reduced organic carbon sources. β -Proteobacteria also includes the order Hydrogenophiales which contains *Thiobacillus*- one of the best studied chemolithotrophs and most prominent of the colourless sulphur bacteria. Many are unicellular rod-shaped or spiral sulphur-oxidizing bacteria that are non-motile or motile by flagella. *Thiobacillus* grows aerobically by oxidizing a variety of inorganic sulphur compounds (elemental sulphur, hydrogen sulphide and thiosulfate) to sulfate. Although they normally use carbon dioxide as the major source of carbon, a few strains can grow heterotrophically. Even though some are called sulphur-oxidizing bacteria, they do not derive energy from sulphur oxidation for example; *Thiobacterium* and *Macromonas*. They may increase soil fertility when they release elemental sulphur by oxidizing it to sulfate. As previously stated that 5S phylogenetic analyses and 16S rRNA phylogenetic analysis (Goebel & Stackebrandt, 1994; Lane *et al.*, 1985) revealed that the *Acidithiobacillus* species are distributed in 3 subclasses and the results of these analyses may have proven the ability to oxidize sulphur found in many groups of Gram-negative bacteria. Therefore, this ability together with rod-shaped morphology should not be considered a useful taxonomic criterion of new species in the genus *Thiobacillus* (*Acidithiobacillus*) (Moreira & Amils, 1997).

2.5 Genes responsible for sulphur- and iron oxidation

Some of the genes that have been identified responsible for oxidation of sulphur and iron are the *sox*, *rus*, and the *iro*. Previous literature has established some of these genes to be present in some of the sulphur- and iron-oxidizing bacteria. The genes encoding sulphur-oxidizing ability were first described from the alphaproteobacterium *Paracoccus pantotrophus* a close relative of *Paracoccus versutus* formerly known as *Thiobacillus versutus* (Von Heijne, 1985), which is a facultative chemolithotroph and grows with thiosulfate as an energy source (Friedrich *et al.*, 2005). The ability of oxidation of sulphur and reduced inorganic sulfidic compounds via the sulphur oxidation enzyme system *sox* is found in both aerobic chemotrophic bacteria and anaerobic phototrophic bacteria (Rother *et al.*, 2005). The *sox* pathway is the most widespread and the best studied pathway of sulphur oxidation and is used by various bacterial genera (Friedrich *et al.*, 2001). The sulphur oxidation process was studied in detail in the bacterium *Paracoccus pantotrophus* which possesses a periplasmic enzyme complex known as the *sox* enzyme system that is able to oxidize thiosulfate to sulfate. The bacterium is a Gram-negative, neutrophilic lithotroph that grows in the presence of thiosulfate. Its *sox* enzyme system entails 15 genes of which 7 code for proteins that are important in sulphur oxidation (Friedrich *et al.*, 2000).

The rusticyanin (*rus*), a low molecular weight copper-containing protein which is a part of an operon (*rus*) consisting of aa₃-type cytochrome oxidase, a high molecular weight outer membrane located cytochrome-c, a c₄-type cytochrome and an open reading frame of unknown function, is proposed to encode for the electron transport chain that is used during the oxidation of ferrous iron (Rawlings, 2005). Yarzabal *et al.*, (2004), studied the iron- and sulphur- oxidizing bacteria *Acidithiobacillus ferrooxidans* at the RNA and protein level and confirmed that the *rus* operon was expressed more in ferrous iron grown cells; an indication that *rus*-operon expression is induced by ferrous iron.

The *iro* gene, which encodes for a HiPIP; a high-potential iron-sulphur protein is said to be an iron oxidase (Kusano *et al.*, 1992) and was first purified to homogeneity and characterized by Yamanaka *et al.*, (1991) The high-potential iron-sulphur protein (HiPIP) present in *A. ferrooxidans* has been identified and is said to be involved in sulphur-oxidation respiratory chain in a number of strains (Quatrini *et al.*, 2009) and was described as the first electron acceptor

from Fe^{2+} in some other strains (Cavazza *et al.*, 2006). HiPIP are soluble bacterial ferredoxins which are commonly found in the purple photosynthetic bacteria and contain a single redox-active four iron-four sulphur cluster in a peptide chain of 54-85 amino acid residues (Kusano *et al.*, 1992).

Other strains of *A. ferroxidans* have been reported to have two distinct types of the blue copper protein rusticyanin, the *rusA* and *rusB* and some strains may carry only one type of the rusticyanin gene while others may carry both of them (Sasaki *et al.*, 2003). The *rusA* gene belongs to the *rus* operon which encodes two cytochromes *c* and the aa_3 cytochrome oxidase, all of which play a central role in the Fe^{2+} respiratory chain (Quatrini *et al.*, 2009), while the function of *rusB* has not yet been described.

2.6 Metal sulphides types

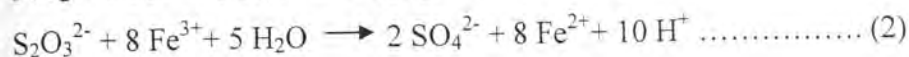
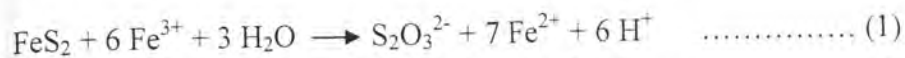
2.6.1 The acid-insoluble metal sulphide

Not all metal sulphides are the same and therefore they do not undergo identical mineral dissolution processes (Schippers & Sand, 1999). There are acid-soluble metal sulphides and there are acid-insoluble metal sulphides. The acid-insoluble metal sulphides use the thiosulfate mechanism where ferric (Fe^{3+}) ions are used to attack these metals to solubilize them, with thiosulfate being the main intermediate and sulfate the main end-product, while the acid-soluble metal sulphides use the polysulfide mechanism, where Fe^{3+} ions and acid attack the sulphide with elemental sulphur being the main intermediate. The sulphur will in turn be oxidized to sulfate by sulphur-oxidizing microbes (Rawlings, 2005). The iron and sulphide are microbially oxidized to produce ferric iron and sulphuric acid, and these chemicals convert the insoluble sulphides of metals such as copper, nickel and zinc to soluble metal sulfates that can be readily recovered from solution. In the case where gold is present in the ore, gold is inert to microbial action so, microbes are used to recover gold by opening the ore structure to allow the cyanide to penetrate the mineral ore (Swamy *et al.*, 2005).

2.6.1 (a) The mechanism of oxidization of acid-insoluble metal sulphide

In the thiosulfate mechanism, solubilization is through ferric ion attack. Ferric sulfate is considered as a strong oxidizing agent and is usually used in bioleaching processes (Devasia &

Natarajan, 2004). It can also be used as an energy source for bacteria in bioleaching processes (Chan *et al.*, 2003). Leaching by ferric sulfate is considered to be an indirect process because it is not solely dependent on the activity of bacteria and it can occur in the absence of oxygen. This is depicted below by the following equation using pyrite as an example, (Rawlings, 2005).

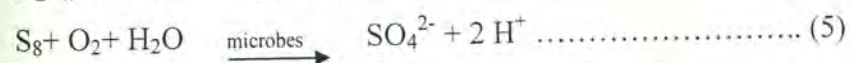
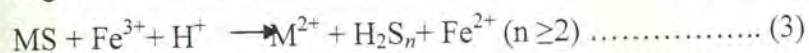


2.6.2 The acid-soluble metal sulphide

In the polysulphide mechanism, the solubilization is of an acid-soluble metal sulphide and the process is through a combination of ferric ion and a proton (acid). The elemental sulphur that is formed as an intermediate is stable and may be oxidized to sulfate by sulphur- oxidizing bacteria such as *A. thiooxidans* or *A. caldus*.

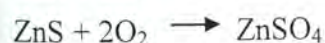
2.6.2 (a) The mechanism of oxidation of acid-soluble metal sulphide

A generalized formula is used to express the following reactions:



The ferrous ion that is produced in all these steps may be re-oxidized to ferric ion by iron-oxidizing bacteria such as *A. ferroxidans*, *Leptospirillum* spp. and *Sulfobacillus* spp. From these reactions, it is evident that sulphur-and iron-oxidizing bacteria provide sulphuric acid to keep the iron in the oxidized ferric state for an attack on the sulphide mineral (Rawlings, 2005).

In the direct mechanism, the bacteria have intimate contact and adhesion to the sulphide mineral ore for the solubilization process to occur whereby the bacteria directly oxidize the metal sulphide to soluble metal sulfate (Chan *et al.*, 2003):



CHAPTER 3

3. Materials and methods

3.1 The study area

This study was carried out at the North-West University, Mafikeng Campus, in the North-West Province. All samples were collected from an open pit gold mine; Kalahari Goldridge, that is owned by Harmony Gold Mining Company Limited. The mine is situated about 60 km south of Mahikeng, 14 km from the Kraaipan village and about 10 km from Mareetsane village. The type of ore in the mine is of quartz within magnetite-chert iron.

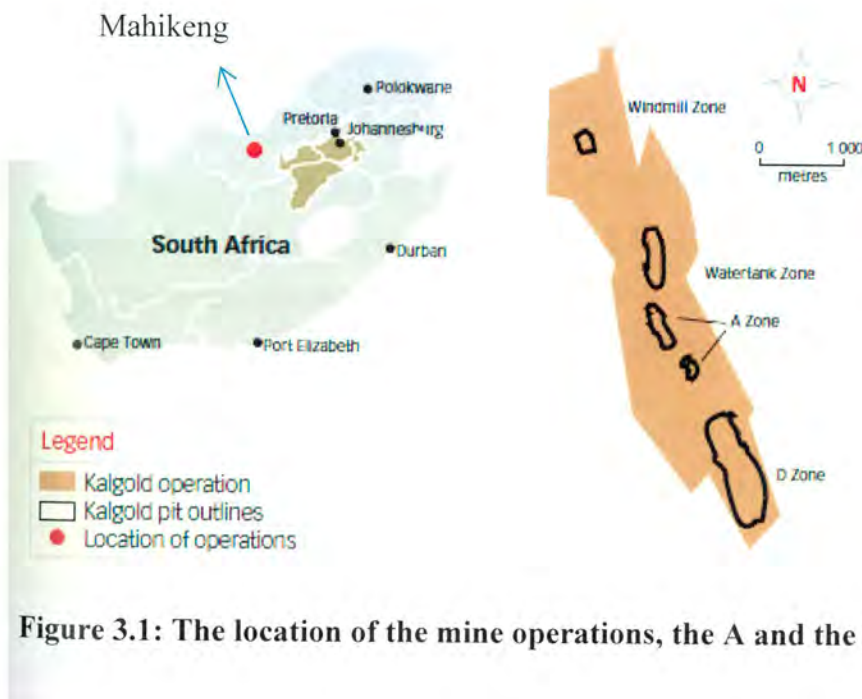


Figure 3.1: The location of the mine operations, the A and the D zone

3.2 Site selection

The criterion used for selecting the sampling sites was an area with high mining activities. This was motivated by the fact that the conditions in this area which include high metal content, optimal pH and temperature favor the growth of sulphur- and iron-oxidizing bacteria.

3.3 Collection of samples

(a) Soil samples

All samples were collected in 31 May 2011. Using aseptic techniques, soil samples were collected from 6 different locations and placed in sterile paper bags. pH of the soil was measured according to Dick *et al.*, (2000)

(b) Water samples

Water samples (Sample 7 and 8) were collected from the water tank zone, from two of the dams located around the A and the D zone. The samples were collected into a 500 ml-capacity low density polyethylene bottles, maintained at 4°C, marked properly and transported to the North-West University Laboratories for further analysis.

In total 8 samples were collected. Table 3.1 shows the depth, hole number and block number where 3 of the soil samples were collected from.

Table 3.1: Depth, hole number and block number of where the samples were collected

Samples	1	2	3
Block Number	418-011	418-011	418-011
Hole Number	342	309	150
Depth (m)	10.9	10.6	11.0

Block number; specific locations in the mine designated as numbers

Sample 4: Soil collected from dyke: sulphidic material contaminated with surface soil (block number 416).

Sample 5: Soil collected from the diabase dyke: sulphidic material not contaminated with surface soil.

Sample 6: Collected from block number 418-011, 100 m from blasting area.

3.4 Analytes (Iron & Sulphur) analyses

Analysis was carried out by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) to measure the concentration of sulphur and iron in the samples obtained.; the procedure was adopted from Sandroni & Smith (2002). Soil samples were placed in fluoroplastic vessels which were mounted in ceramic supporting vessels. The reaction vessels were cleaned before each digestion using 5 ml of HNO_3 , heated for 30 minutes. Three replicate samples were taken from each soil sample. The covers to the reaction vessels were screwed on and hand-tightened, positioned in the rotor and placed in the multiwave and the safety door shut. The acids selected for use in the sample digestion were 3 ml HNO_3 , 9 ml HCl and 1 ml H_2O_2 , using a Microwave reaction system multiwave 300 Perkin-Elmer. When digestion was complete, the samples were transferred to volumetric flasks and filled to the mark. Perkin-Elmer NexION 300 ICP-MS was used for the analysis.

3.5 Isolation of bacterial strains, media composition and culture conditions

About 10.16 g *Thiobacillus* broth reagent was dissolved in 1000 mL distilled water, stirred and autoclaved at 121°C for 15 minutes. One gram of a soil sample was inoculated into a 50 mL Falcon tube with 20 mL of *Thiobacillus* broth made up of 0.40 g $(\text{NH}_4)_2\text{SO}_4$, 4.00 g KH_2PO_4 , 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25 g CaCl_2 , 0.01 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and 5.0 g $\text{Na}_2\text{S}_2\text{O}_3$. The medium with the soil samples was placed in a shaking incubator shaken at 180 rpm at a temperature of 30°C . Isolation of the bacteria was achieved by spreading about 100 μL of the sample from the shaking incubator on the *Thiobacillus* agar which had been solidified using 10 g/L of Molecular Biology Agarose (Certified™ BIO-RAD Laboratories, Inc).

3.6 Biochemical and physiological characteristics

The following experiments were performed in triplicates.

a) Biochemical characteristics of the isolates

The biochemical characteristics were determined by reduction of nitrates, enzyme activities like gelatin hydrolysis, esculin hydrolysis, indole production tests, glucose fermentation, catalase reaction and assimilation tests for L-arabinose, D-mannose, D-mannitol, N-acetyl-glucosamine, D-maltose, potassium gluconate, capric acid, malic acid, trisodium citrate and phenyl acetic acid.

b) Physiological experiments

Effects of organic compound on bacterial growth

The methods were adapted from Zeng *et al.*, (2009). Bacterial growth was monitored in *Thiobacillus* broth which was supplemented with 0.02% yeast extracts and glucose, respectively and *Thiobacillus* broth without any supplementation. The broth cultures were incubated at 27-30°C in rotary shaker and growth recorded every 24 hours for up to 3 days using a spectrophotometer (Thermo Spectronic Heλios □, Merck) at an absorbance of 600 nm. All data presented were mean values of three determinations and statistically compared using LSD (Lowest Significant Difference) at a significance level of $P < 0.05$.

3.7 Oxidation experiments

a) Demonstration of iron oxidation

The method was adopted from Lavalley *et al.*, (2005). *Thiobacillus* broth solidified with agarose supplemented with 10 g of ferrous sulfate was used for this test. The isolated bacteria were plated and incubated for a period of a month in broth supplemented with yeast extract in the one experiment and no supplement in the other.

(b) Demonstration for sulphur (thiosulfate) oxidation

The method was adopted from Vidyalakshmi & Sridar (2007). Sulphur oxidation was determined under chemolithotrophic conditions that is, in the presence of only the basal salts $(\text{NH}_4)_2\text{SO}_4$, KH_2PO_4 , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, CaCl_2 , $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{Na}_2\text{S}_2\text{O}_3$ without addition of any organic compounds. pH was measured using a Crison pH Meter Basic 20⁺, for any formation of sulphuric acid.

3.8 DNA extraction, amplification, sequencing and phylogenetic analysis

To extract DNA from our isolates the Zymo Research (ZR-96) Soil Microbe DNA Kit™ was used. Following the manufacturer's protocol 10-20 mg of the isolated bacterial cells that had been suspended in 50 µL of water was added to the tubes of a ZR bashing bead lysis rack. About 750 µL of lysis solution was added to the bacterial cells. The ZR bashing bead lysis rack together with the lysis solution were centrifuged at $\geq 3,000 \times g$ (5,000 $\times g$ max.) for 5 minutes. About 250 µL of the supernatant was added to a clean collection tube and 750 µL of soil DNA binding

buffer was added to the supernatant in the collection tube from the previous step and vortexed for 2 minutes then centrifuged in the collection tube at $\geq 3,000 \times g$ ($5,000 \times g$ max.) for 5 minutes. About 500 μL of the supernatants obtained from the previous step was transferred to a clean collection tube or column and centrifuged again at $\geq 3,000 \times g$ ($5,000 \times g$ max.) for 5 minutes. The flow-through was discarded from the collection tube and 200 μL of the DNA pre-wash buffer was added to the column and centrifuged at $\geq 3,000 \times g$ for 5 minutes. Then 500 μL of soil DNA wash buffer was added and centrifuged at $\geq 3,000 \times g$ for 5 minutes. The column was transferred into an elution tube and added 100 μL of the DNA elution buffer and centrifuged at $\geq 3,000 \times g$ for 5 minutes. The DNA was now ready for PCR for amplification which was carried out using PCR Master Mix which consisted of 1X PCR buffer with MgCl_2 , 200 μM dNTPs, 0.6 μM , 1.4 U DNA Polymerase and 1.2 μL template DNA. Cycling conditions for each primer are found in tables 4.1 and 4.2

For plasmid extraction, we followed the manufacture's protocol; Zyppy™ Plasmid Miniprep Kit. About 600 μL of our bacterial culture grown in *Thiobacillus* broth (supplemented with yeast extract), was added into 1.5 mL microcentrifuge tube and 100 μL of the 7X blue lysis buffer was added and mixed by inverting the tube about 4-6 times. To neutralise the solution, which was seen by a colour change from blue to yellow, 350 μL of cold neutralization buffer (yellow) was added and mixed thoroughly for 2-3 minutes to ensure complete neutralization. The solution was then centrifuged at 11000-16000 $\times g$ for 2-4 minutes. After centrifugation, 900 μL of the supernatant was transferred into the provided Zymo-Spin™ IIN column, placed the column into a collection tube and centrifuged at 11000-16000 $\times g$ for 15 seconds. The flow-through was discarded and the column was placed into the same collection tube and 200 μL of the endo-wash buffer was added to the column and centrifuged for 30 seconds. 400 μL of the Zyppy™ wash buffer was added to the column and centrifuged for 1 minute. The column was then transferred into a clean 1.5 mL microcentrifuge tube and then 30 μL of Zyppy™ elution buffer was added allowed to stand for 60 seconds at room temperature. The DNA was eluted after centrifugation for 30 seconds and was now ready for PCR.

Successful amplification of the DNA was confirmed by running 10 μL of the PCR reaction and about 5 μL of loading dye on a 1% agarose gel in Tris/acetate buffer and analysed by staining with ethidium bromide under UV light. The bands of the expected size were cut off and purified with a commercial kit and submitted to Inqaba Laboratories, Pretoria, for sequencing and

Table 3.2: PCR conditions for primers selected for study

Primer	Sequence (5' – 3')	References	PCR Conditions	Cycling	Expected size (bp)
27F	AGA GTT TGA TCC TGG CTC AG	Khamis <i>et al.</i> , (2005)	30× ^a	94°C for 30s	1500
1492R	TGA CTG ACT GAG GCT ACC TTG			61°C for 30s	
	TTA CGA			72°C for 5min	
<i>soxA-F</i>	TGG GAG AAG GGC AAG GAG CT	Mukhopadhyaya <i>et al.</i> , (2000)	30× ^a	94°C for 30s	286
<i>soxA-R</i>	GTC ACA TAG ACC TCA AGC GC			55°C for 30s	
				68°C for 180s	
					436
<i>rusA-F</i>	ACT GGT ATG TAA CTG TTG GTG	Amouric <i>et al.</i> , (2011)	30× ^a	94°C for 30s	
	CG			55°C for 30s	
<i>rusA-R</i>	GTG TAT CCG AAC TTG CCA TCT			72°C for 420s	

^a; Initial denaturing steps of (95°C for 5 min)

Table 3.2 continued: PCR conditions for primers selected for study

Primer	Sequence (5' – 3')	References	PCR	Cycling Conditions	Expected size (bp)
F1_Thio	ATG CGT AGG AAT CTG TCT TT	Urbanova <i>et al.</i> , (2011)	40× ^a	95 °C for 10 min	985
R1_Thio	GGA CTT AAC CCA ACA TCT CA			95 °C for 15 s 60 °C for 1 min	
Iro (purA)	CGTACCGAGATCCTCTGA	Bruscella <i>et al.</i> , (2005)	30× ^a	58°C for 30 s	385
Airo3	TGCTGACTTAGGAACAAAGGC			72°C for 30 s 72°C for 2 min and 30 s	

^a; Initial denaturing steps of (95°C for 5 min) ^b; Initial denaturing steps of (56°C for 2 min)

Chapter 4

4. Results

The pH of the soil samples = 7.0

The pH of water samples = 7.0

4.1 Analytes (Iron & Sulphur) analyses

The analytes analyses of samples revealed that sample S1 to S7 which were soil samples were lower in iron concentration compared to water samples S8 and S9 (Figure 4.1). The iron concentration in the water samples was more than five-fold higher than sample 5, 6 and 7 which were collected from dykes and 100 m away from the blasting area, respectively. The concentration of sulphur ranged between 0.03 g/L in S5 to 0.64 g/L in S4 (Figure 4.1). Similar to iron, the concentrations of sulphur collected from dykes and 100 m away from the blasting area were lower compared to the water samples and samples from holes. The sulphur concentration in S4 was more than double the concentration of any other sample.

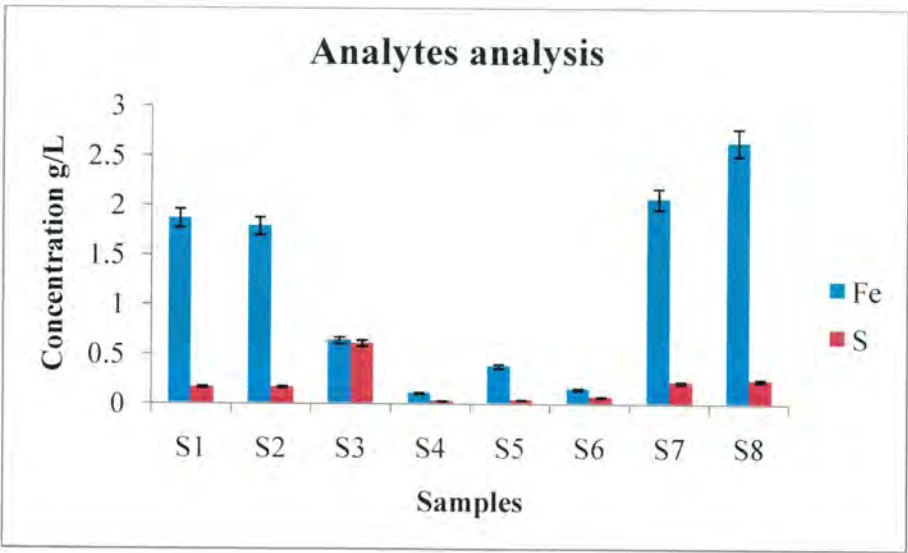


Figure 4.1 Analytes analysis of samples

Fe: Iron; S=sulphur

4.2 Colony morphology of the isolates

The pH of the *Thiobacillus* agar medium used was 4.84. The isolates were able to grow at this pH, though it was slow. The optimal pH of the growth of the isolates was measured around 7, that is, when the medium was supplemented with 0.02% yeast extract powder. Colony morphologies that were prevalent in the collected samples were cream-white, yellow, round, smooth with raised elevation and smooth glistening isolates. In samples 7 and 8, isolates that were predominantly present gave unique morphology from the rest; a transparent colour, irregularly shaped, with a flat elevation and they resembled 'fried-eggs'. It was particularly noticed that when the isolates were grown on yeast extract agar the colour changed to blue green (Figure 4.3). When cultures were viewed under microscope after Gram-staining the cells were short rod-shaped without endospores.

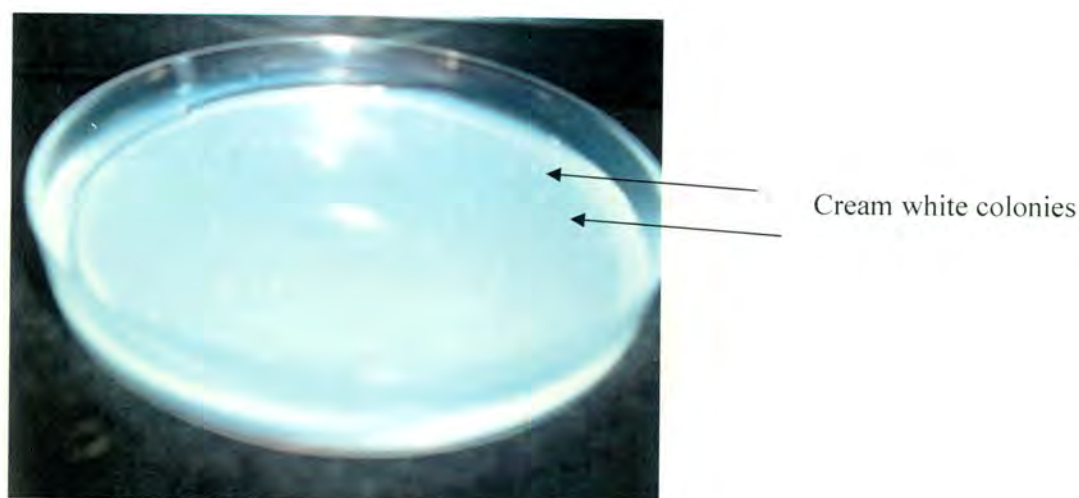


Figure 4.2: Colony morphology of isolates grown on *Thiobacillus* agar

Table 4.1 Colony morphology of the isolates

Isolate	Colour	Shape	Edge	Elevation	Surface
<i>P. aerugi</i> MP14	c/white	round	smooth	raised	S,G
<i>Endophytic bacterium</i>	white	round	smooth	raised	S,G
<i>Enterobacter hormaechei</i>	c/white	round	smooth	raised	S,G
<i>Pseudomonas</i> sp.	c/white	round	smooth	raised	S,G
<i>P. aerugi</i> B1	c/white	round	smooth	raised	S,G
<i>P. aerugi</i> 21R	yellow	round	undulate	raised	S,G
<i>Enterobacter</i> sp.	transparent	irregular	irregular	flat	S,G
<i>Pseudomonas</i> sp.13KR	transparent	irregular	irregular	flat	S,G

P. aerugi; *Pseudomonas aeruginosa*, SG; smooth and glistening, c/white; cream white

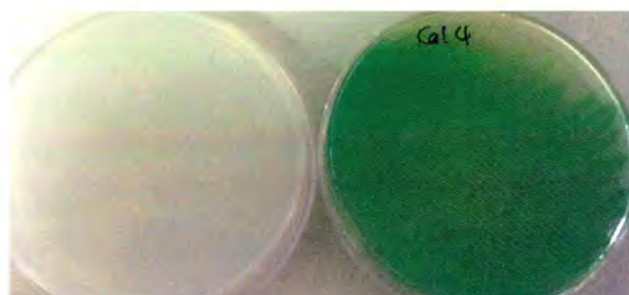


Figure 4.3: Colour change produced by the isolates grown on yeast extract agar

4.3 Biochemical characteristics of the isolates

The biochemical tests that were conducted were used to identify the organisms observed in the *Thiobacillus* broth/agar, (Table 4.2). A total of 8 species were identified. All the isolates were positive for catalase, esculin, gelatin, tryptophan, arginine, capric acid and malic acid and all but one (Kal4) were negative for phenylacetic acid, adipic acid and urea. The isolates that gave a pink-red colour for the nitrogen test were: Kal1, Kal3 Kal4 and Kal6 after. Isolates that gave a negative result were the ones that were identified as: Kal2, Kal5, Kal7 and Kal8. Isolates Kal1 and Kal6 were the only two that were negative for the test for p-nitrophenyl-beta-D-glucopyranoside and for the mannose, adipic acid and maltose test, while Kal4 was the only one that gave positive results.

Table 4.2 Biochemical characterization of the isolates

Test	Kal1	Kal2	Kal3	Kal4	Kal5	Kal6	Kal7	Kal8
Catalase	+	+	+	+	+	+	+	+
NO ₂	+	-	+	+	-	+	-	-
NO ₃ N ₂	-	+	-	-	+	-	+	+
Tryptophan	+	+	+	+	+	+	+	+
Glucose	-	+	+	+	+	-	+	+
Arginine	+	+	+	+	+	+	+	+
Urea	-	-	-	-	-	-	-	-
Esculin	+	+	+	+	+	+	+	+
Gelatin	+	+	+	+	+	+	+	+
PNPG	-	+	+	+	+	-	+	+
Mannose	-	-	-	+	-	-	-	-
Mannitol	+	+	-	+	+	-	-	-
NAG	-	+	-	+	+	-	-	-
Maltose	-	-	-	+	-	-	-	-
GNT	+	+	-	+	+	-	-	+
CAP	+	+	+	+	+	+	+	+
ADI	-	-	-	+	-	-	-	-



Table 4.2 continued: Biochemical tests of the isolates

Test	Kal1	Kal2	Kal3	Kal4	Kal5	Kal6	Kal7	Kal8
MLT	+	+	+	+	+	+	+	+
CIT	+	+	-	+	+	-	-	+
PAC	-	-	-	-	-	-	-	-

PNPG; p-nitrophenyl-beta-D-glucopyranoside, NO₂; reduction of nitrates to nitrites, N₂; reduction of nitrates to nitrogen, NAG; N-acetyl-glucosamine, GNT; potassium gluconate, CAP; capric acid, ADI; adipic acid, MLT; malic acid, CIT; trisodium citrate, PAC; phenylacetic acid

4.4 Physiological characteristics of the isolates

The addition of yeast generally increased the growth of all the organisms compared to the addition of glucose or TB without any organic supplement. This growth pattern was noticed in almost all of the organisms. Growth of Kal1 was significant in the yeast medium at 48 and 72 hours, (Figure 4.4). In Kal2, there was no significance increase in growth when addition of yeast was compared to glucose for the period of 72 hours (Figure 4.3b). However, addition caused a significant increase when compared to TB from 24 hours. At 72 hours, the addition of yeast and glucose caused significant increase in the growth of Kal2. A slight decrease of growth of Kal3 was observed at 48 hours when it was grown in TB medium. This growth pattern was also seen when the isolate was grown in the presence of glucose. For isolate Kal4, there was a significant growth when it was grown in TB at 72 hours and there was an increase in growth for Kal5 when glucose was added. The growth of Kal6, Kal7 and Kal8 started to decrease from 24 hours, when grown in the presence of glucose. It was particularly noticeable that the growth of Kal8 started to pick up after 24 hours in TB medium. The control included all the substrates respectively, and was subjected to the same conditions as the isolates.

From Figure 4.5 to Figure 4.5h, it is clear that in all the isolates, when grown in yeast extract, the pH increased and the highest value was recorded at 7.9 for Kal4 after 72 hours. The lowest pH read in the yeast medium was read at 7.61 after 72 hours for Kal6. The lowest pH recorded was for Kal5 when it was grown in glucose. From the figures it was observed that when all the isolates were grown in glucose and TB and their growth was plotted in a graph, their standard error bars were overlapping indicating that their average points (mean) are

likely to be the same and thus there was no significant difference in the pH. However, for the isolates Kal2 and Kal3, there was a significant difference of pH after 72 hours.

The control for both the absorbance and pH included all the substrates respectively without inoculation and was subjected to the same conditions as with the inoculated medium. There was no significant change for both the absorbance and the pH.

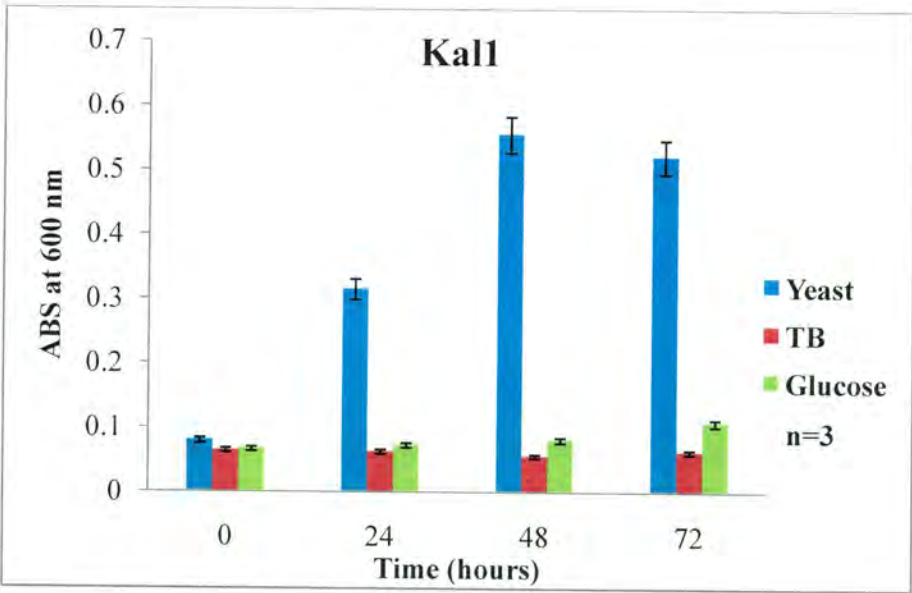


Figure 4.4: The effects of organic/inorganic compounds on the growth of Kal1

TB; *Thiobacillus* broth (without any supplements), ABS; Absorbance; n; independently performed experiments for each compound

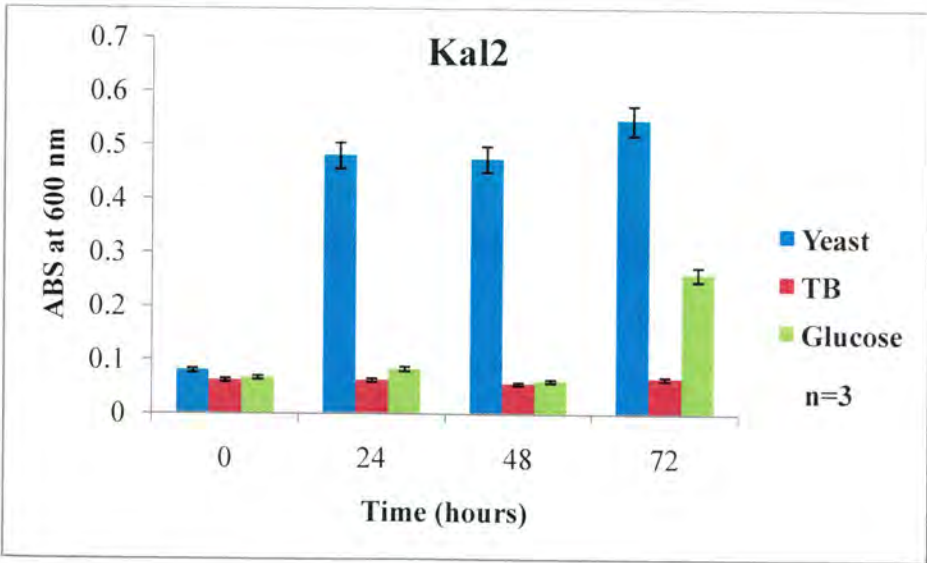


Fig 4.4 a: The effects of organic/inorganic compounds on the growth of Kal2

TB; *Thiobacillus* broth (without any supplements), ABS; Absorbance; n; independently performed experiments for each compound

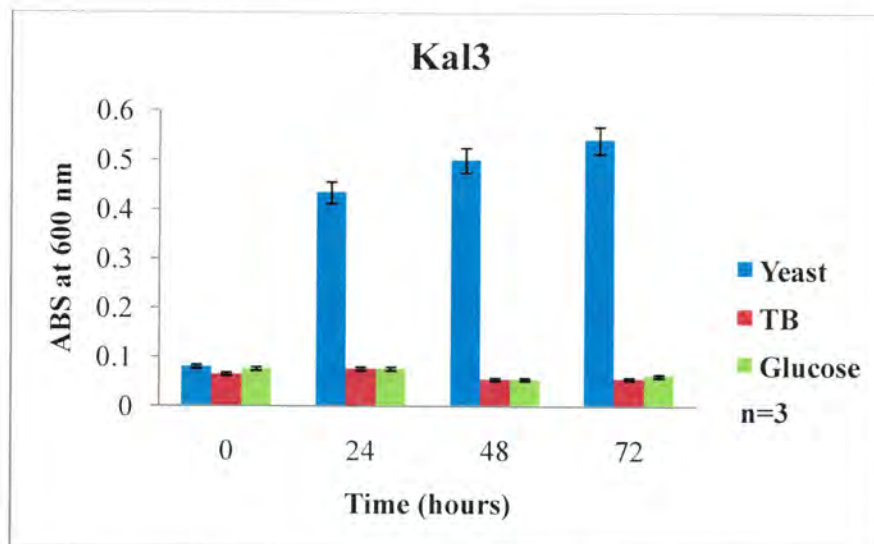


Figure 4.4b: The effects of organic/inorganic compounds on the growth of Kal3

TB; *Thiobacillus* broth (without any supplements), ABS; Absorbance n; independently performed experiments for each compound, Glu; glucose, Yeast; yeast extract powder

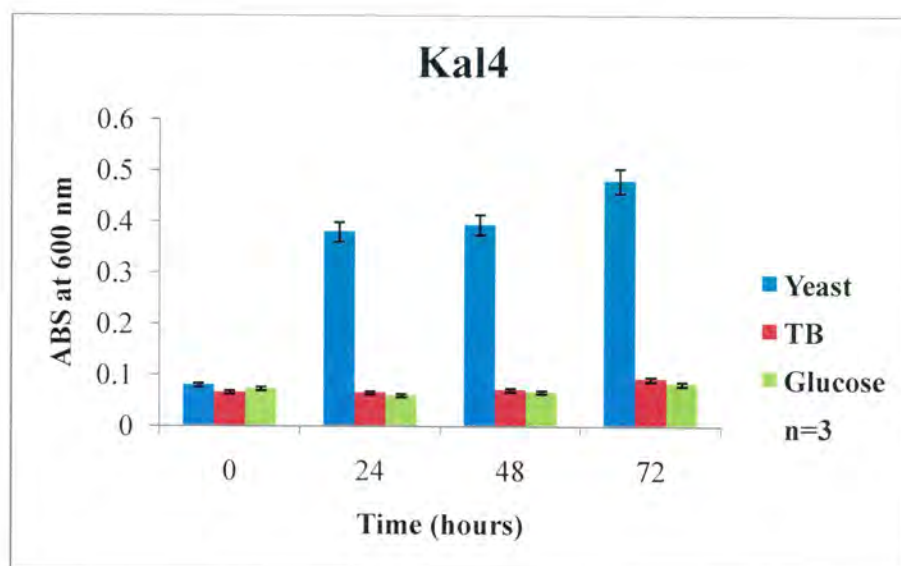


Figure 4.4c: The effects of organic/inorganic compounds on the growth of Kal4

TB; *Thiobacillus* broth (without any supplements), ABS; Absorbance, n; independently performed experiments for each compound, Glu; glucose, Yeast; yeast extract powder

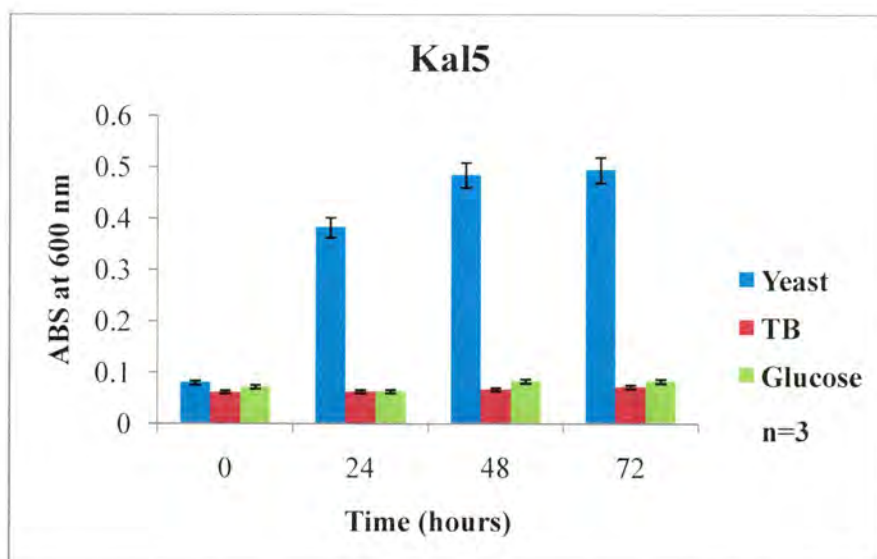


Figure 4.4d: The effects of organic/inorganic compounds on the growth of Kal5

TB; *Thiobacillus* broth (without any supplements), ABS; Absorbance, n; independently performed experiments for each compound, Glu; glucose, Yeast; yeast extract powder

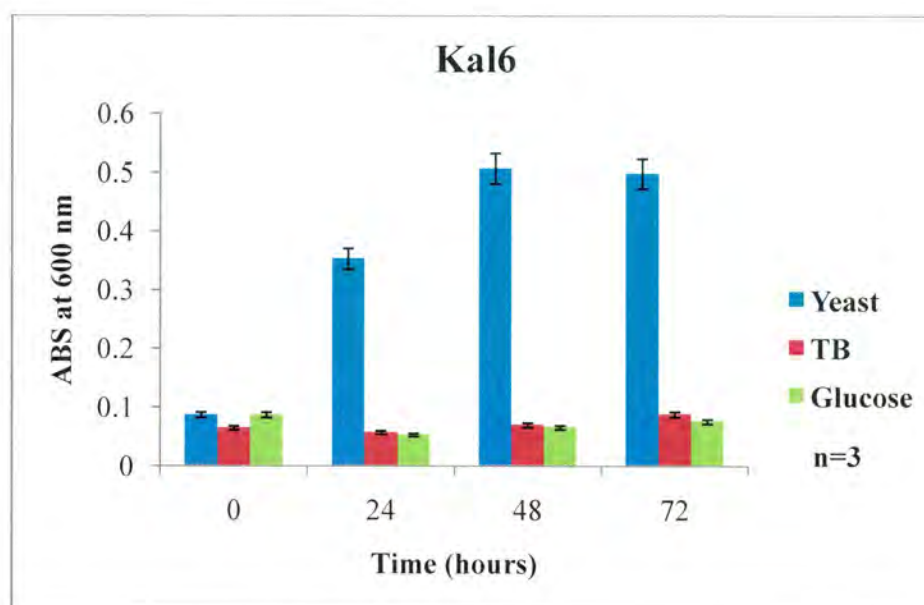


Figure 4.4e: The effects of organic/inorganic compounds on the growth of Kal6

TB; *Thiobacillus* broth (without any supplements), ABS; Absorbance, n; independently performed experiments for each compound, Glu; glucose, Yeast; yeast extract powder

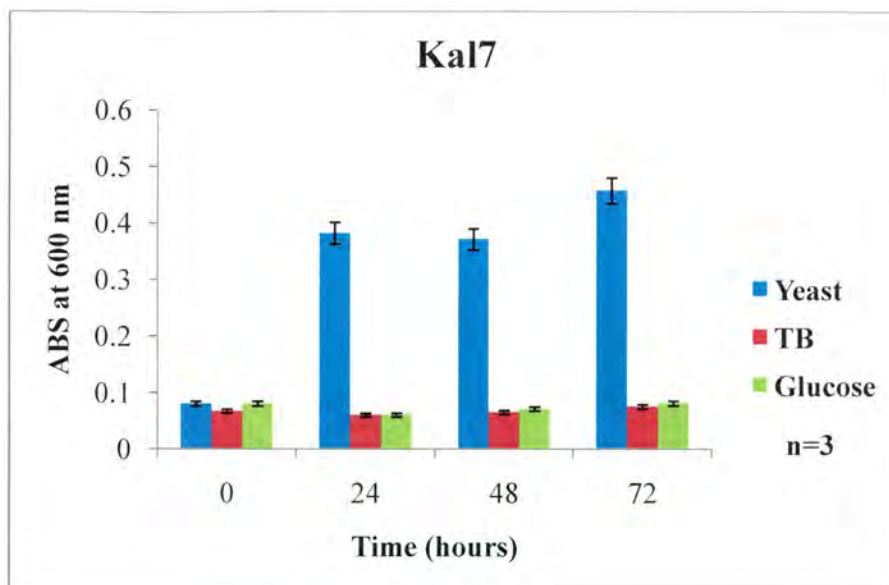


Figure 4.4f: The effects of organic/inorganic compounds on the growth of Kal7

TB; *Thiobacillus* broth (without any supplements), ABS; Absorbance, n= independently performed experiments for each compound, Glu; glucose, Yeast; yeast extract powder

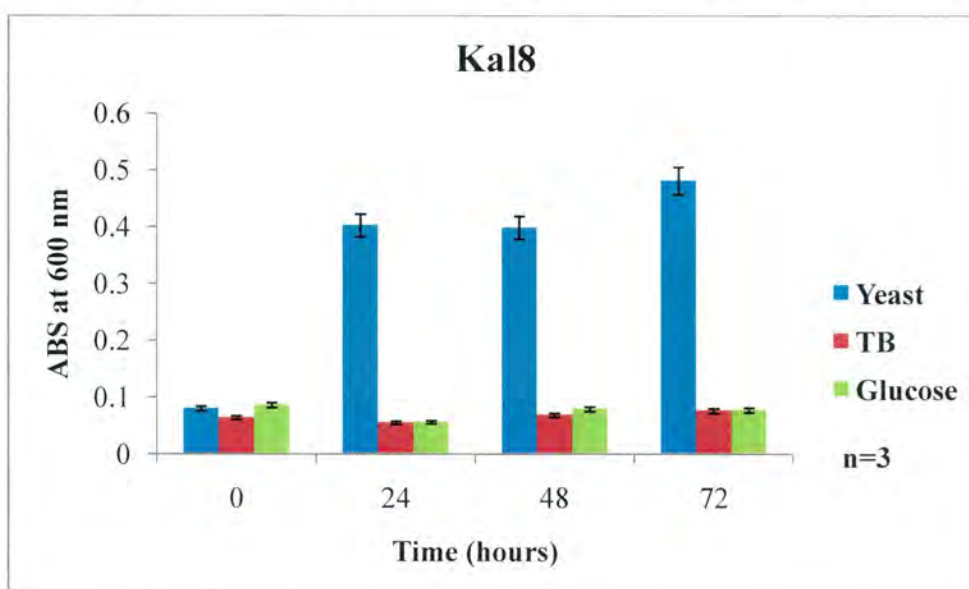


Figure 4.4g: The effects of organic/inorganic compounds on the growth of Kal8

TB; *Thiobacillus* broth (without any supplements), ABS; Absorbance, n= independently performed experiments for each compound, Glu; glucose, Yeast; yeast extract powder

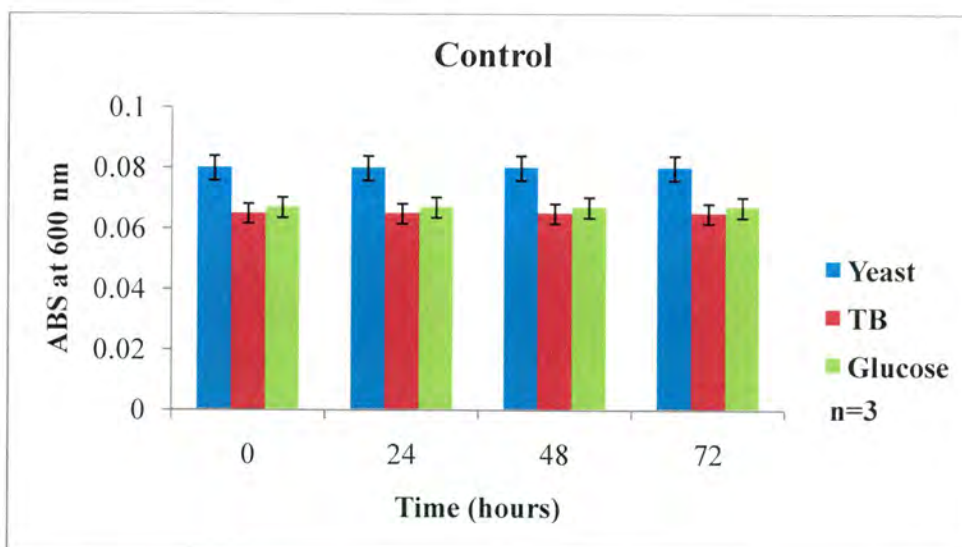


Figure 4.4h: The effects of organic/inorganic compounds without inoculation

TB; *Thiobacillus* broth (without any supplements), ABS; Absorbance, n= independently performed experiments for each compound, Glu; glucose, Yeast; yeast extract powder

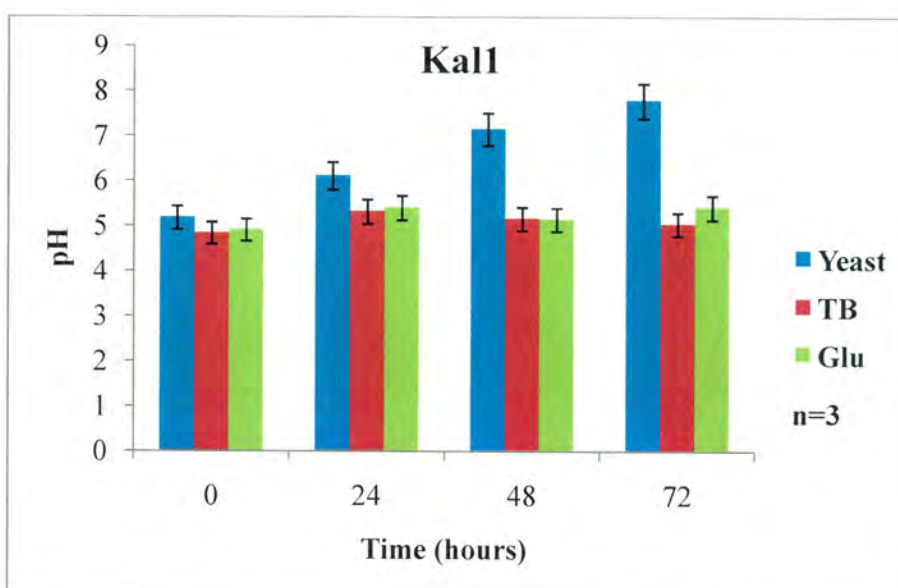


Figure 4.5: pH changes of media in which Kal1 grew in the presence of organic/inorganic compounds

TB; *Thiobacillus* broth (without any supplements), n; independently performed experiments for each compound, Glu; glucose, Yeast; yeast extract powder

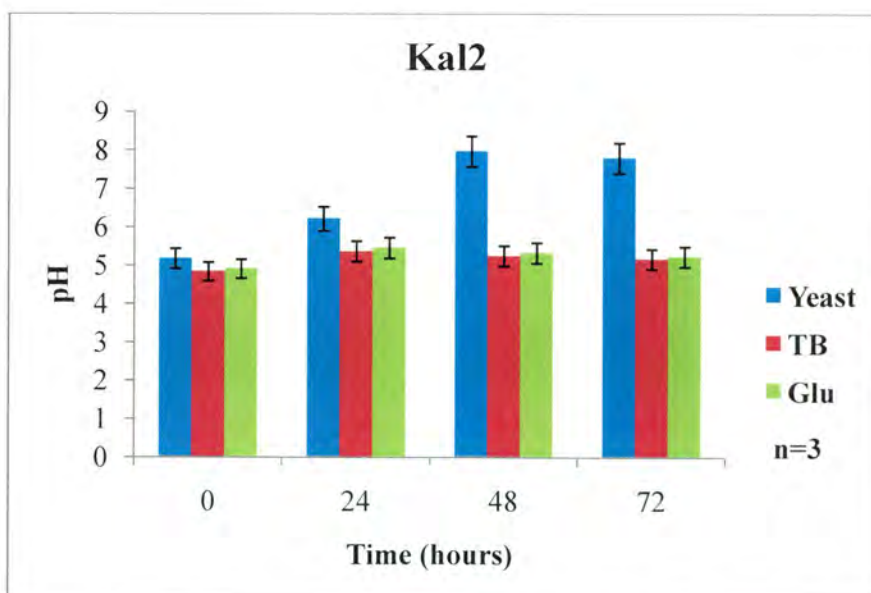


Figure 4.5a: pH changes of media in which Kal2 grew in the presence of organic/inorganic compounds

TB; *Thiobacillus* broth (without any supplements), ABS; Absorbance, n; independently performed experiments for each compound

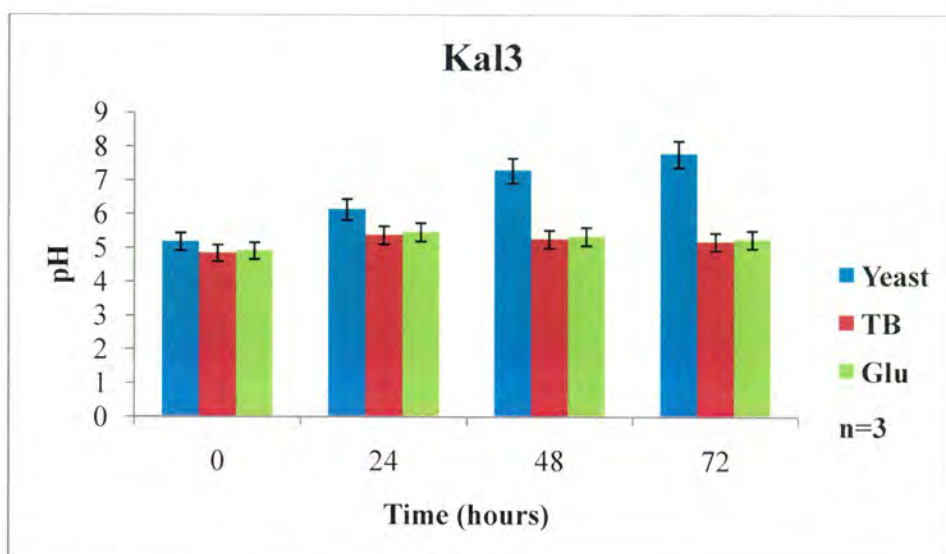


Figure 4.5b: pH changes of media in which Kal3 grew in the presence of organic/inorganic compounds

TB; *Thiobacillus* broth (without any supplements), n; independently performed experiments for each compound, Glu; glucose, Yeast; yeast extract powder

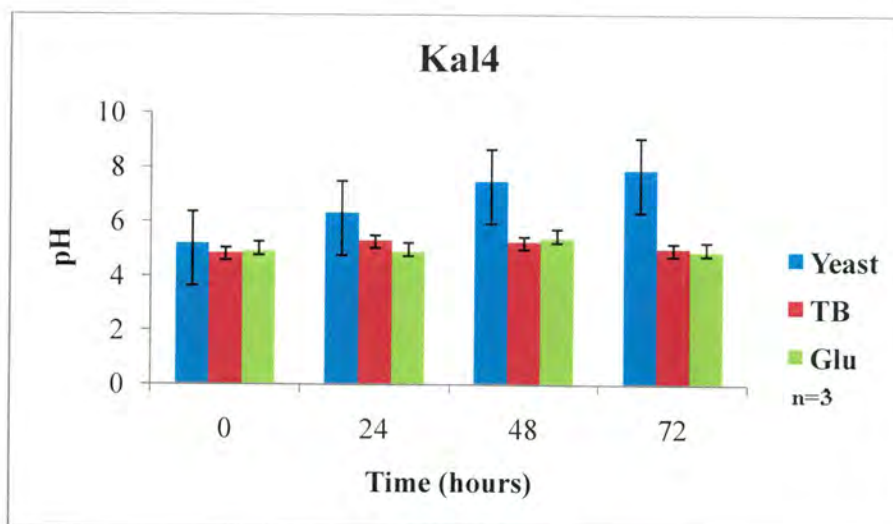


Figure 4.5c: pH changes of media in which Kal4 grew in the presence of organic/inorganic compounds

TB; *Thiobacillus* broth (without any supplements), n; independently performed experiments for each compound, Glu; glucose, Yeast; yeast extract powder

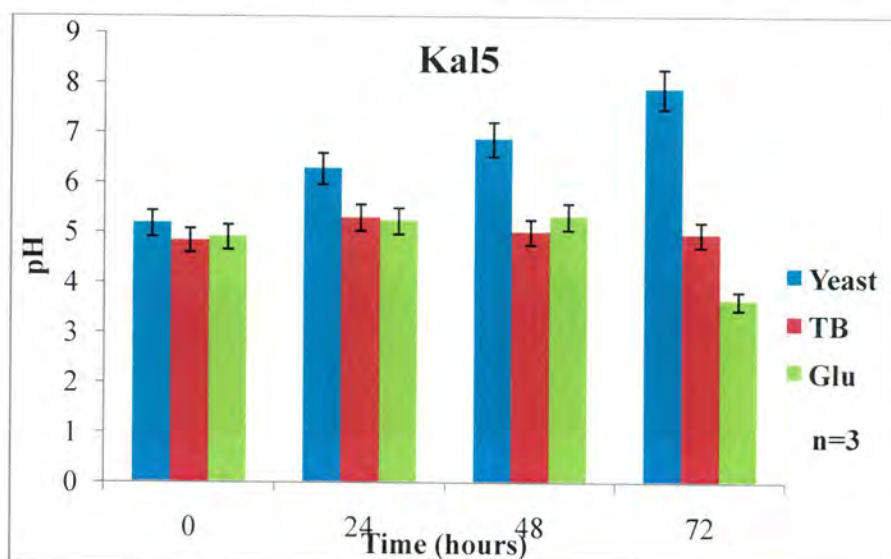


Figure 4.5d: pH changes of media in which Kal5 grew in the presence of organic/inorganic compounds

TB; *Thiobacillus* broth (without any supplements), n; independently performed experiments for each compound, Glu; glucose, Yeast; yeast extract powder

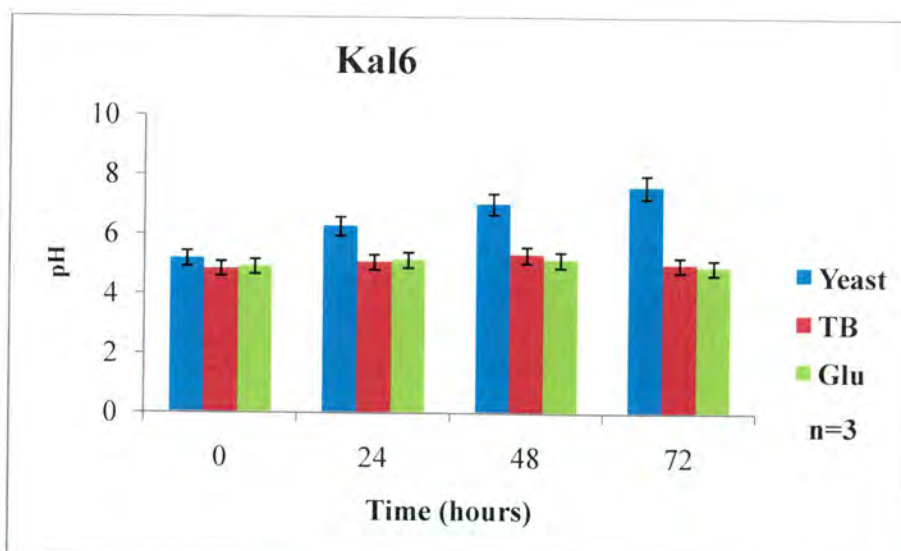


Figure 4.5e: pH changes of media in which Kal6 grew in the presence of organic/inorganic compounds

TB; *Thiobacillus* broth (without any organic supplements), n; independently performed experiments for each compound, Glu; glucose, Yeast; yeast extract powder

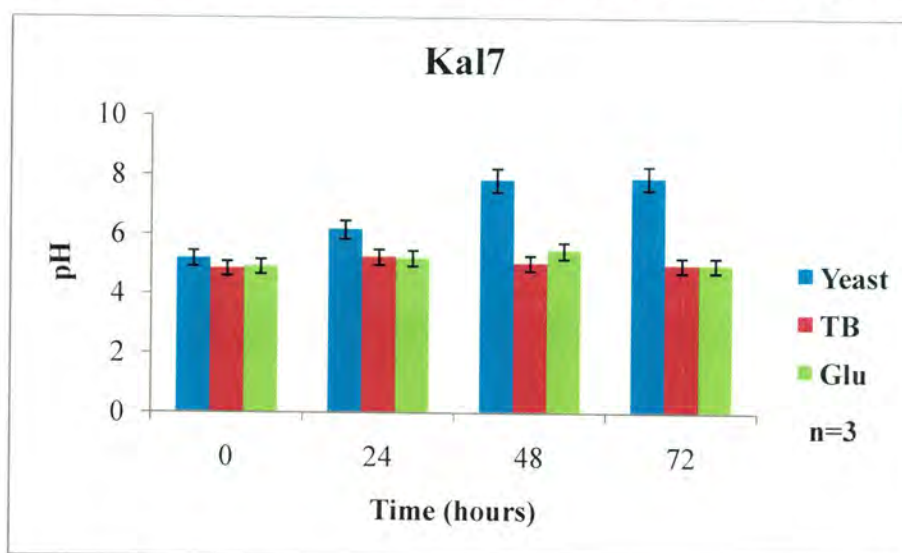


Figure 4.5f: pH changes of media in which Kal7 grew in the presence of organic/inorganic compounds

TB; *Thiobacillus* broth (without any organic supplements), n; independently performed experiments for each compound, Glu; glucose, Yeast; yeast extract powder

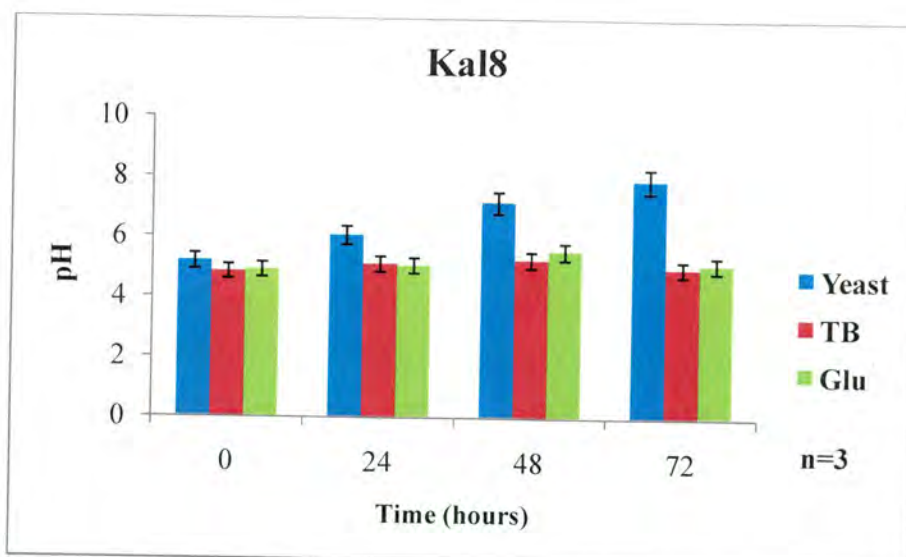


Figure 4.5g: pH changes of media in which Kal8 grew in the presence of organic/inorganic compounds

TB; *Thiobacillus* broth (without any organic supplements), n; independently performed experiments for each compound

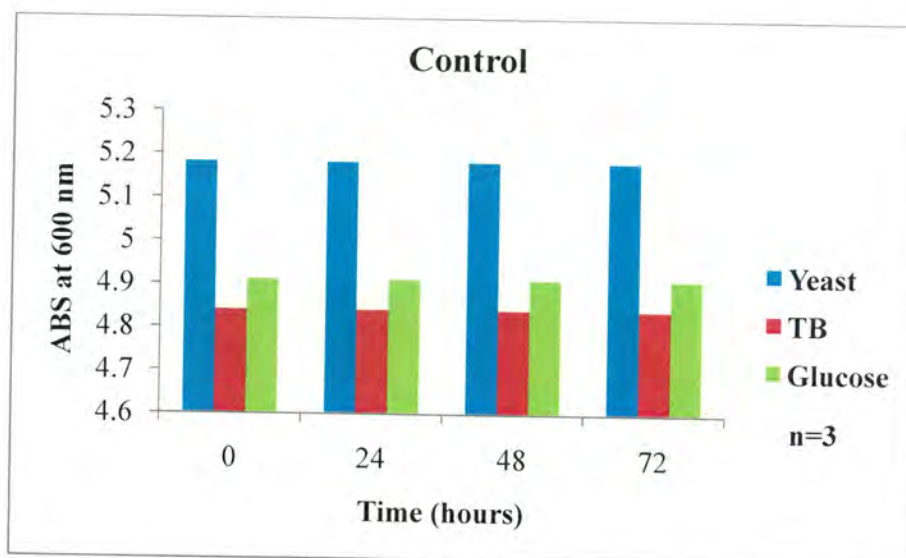


Figure 4.5h: pH of media in which the medium was not inoculated (control)

TB; *Thiobacillus* broth (without any organic supplements), n; independently performed experiments for each compound

4.5 Oxidation experiments

4.5a Demonstration of iron oxidation

We were able to grow our isolates on *Thiobacillus* agar (without any organic supplementation), and the colour of the medium changed from clear to orange and it was observed that in the duration of this experiment, the medium supplemented with 0.02% yeast extract showed improved growth. The growth of the isolates was compared on both the solidified *Thiobacillus* agar and *Thiobacillus* broth supplemented with ferrous sulfate as an iron source and 0.02% yeast extract. Within two weeks both the *Thiobacillus* broth and *Thiobacillus* agar media that were supplemented with 0.02% yeast extract changed colour to orange, as an indication that Fe^{2+} had been oxidized to Fe^{3+} and there was an orange precipitate at the bottom of the tubes with *Thiobacillus* broth supplemented with 0.02% yeast extract. The medium which was not supplemented with yeast extract that consisted of only basal salts ($(\text{NH}_4)_2\text{SO}_4$, KH_2PO_4 , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, CaCl_2 , $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{Na}_2\text{S}_2\text{O}_3$) took more than two weeks to indicate a colour change to a point where the medium dried up. In the course of the oxidation reaction, the control which was just the media without inoculation of cultured isolates took about 4 months to change colour. It was also observed that isolates that were capable of changing the colour of the medium but when they were sub-cultured again on agar that was supplemented with yeast extracts, could not change the colour of the medium again because they had lost the ability to oxidize Fe^{2+} .



Figure 4.6: Demonstration of oxidation of iron under heterotrophic conditions

4.5b Demonstration of sulphur oxidation

In the sulphur oxidation experiments, *Thiobacillus* broth was used (without addition of yeast); we examined thiosulfate oxidation using the isolates with thiosulfate being the sole source of energy and inorganic sulphur source. The ability to oxidize sulphur compounds during chemolithotrophic growth has traditionally been attributed to *Thiobacilli*. Yellow deposits were observed after a month (Figure 4.7). Thiosulfate was oxidized to sulfate which is the major product of inorganic sulphur compounds.

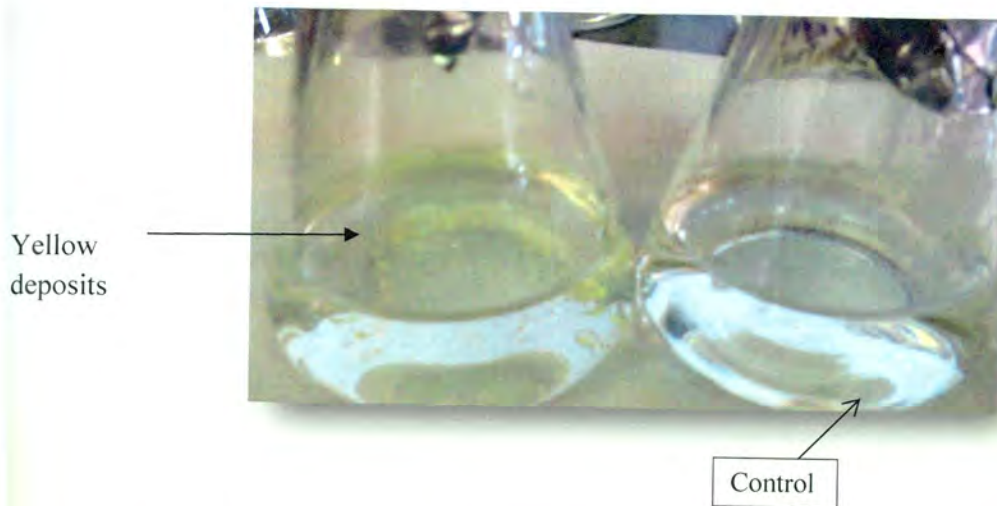


Figure 4.7: Demonstration of sulphur oxidation under chemolithotrophic conditions

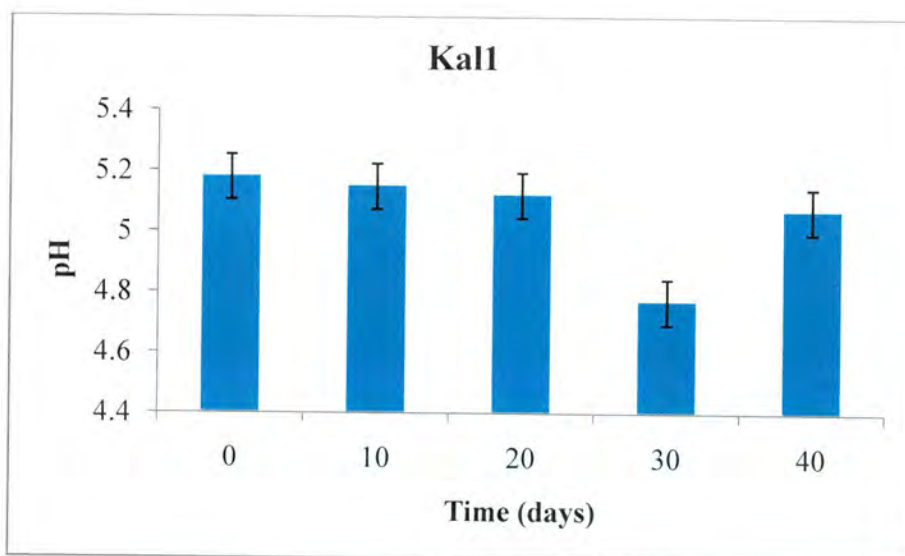


Figure 4.8: pH changes of medium where Kal1 was grown in chemolithotrophic conditions over 40 days

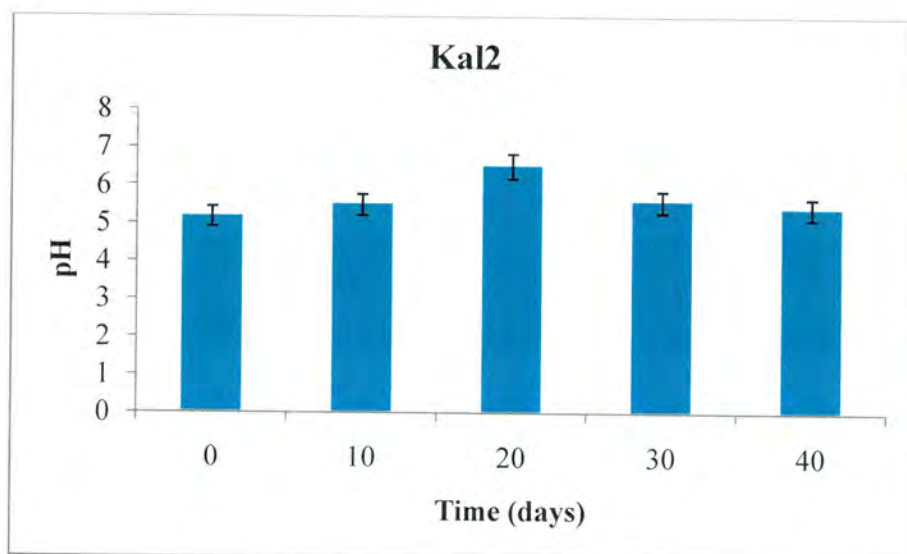


Figure 4.8a: pH changes of medium where Kal2 was grown in chemolithotrophic conditions over 40 days

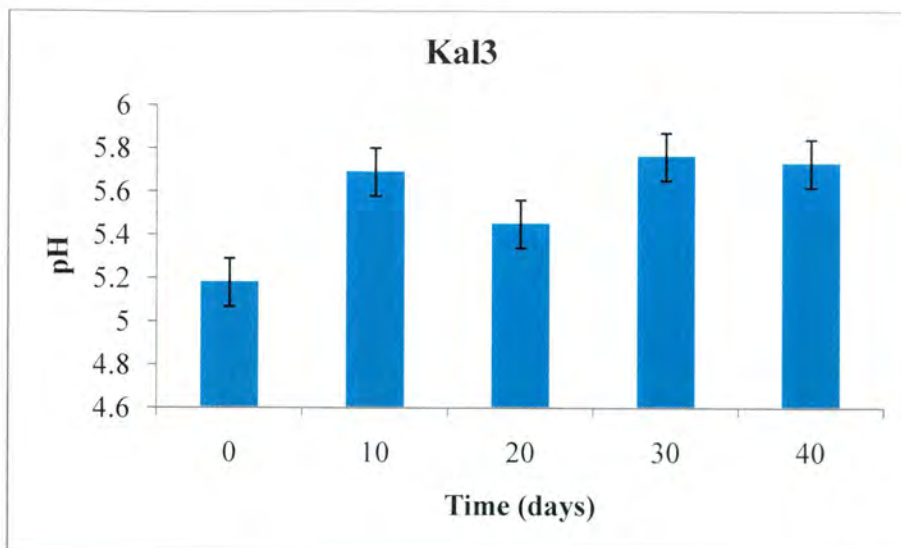


Figure 4.8b: pH changes of medium where Kal3 was grown in chemolithotrophic conditions over 40 days

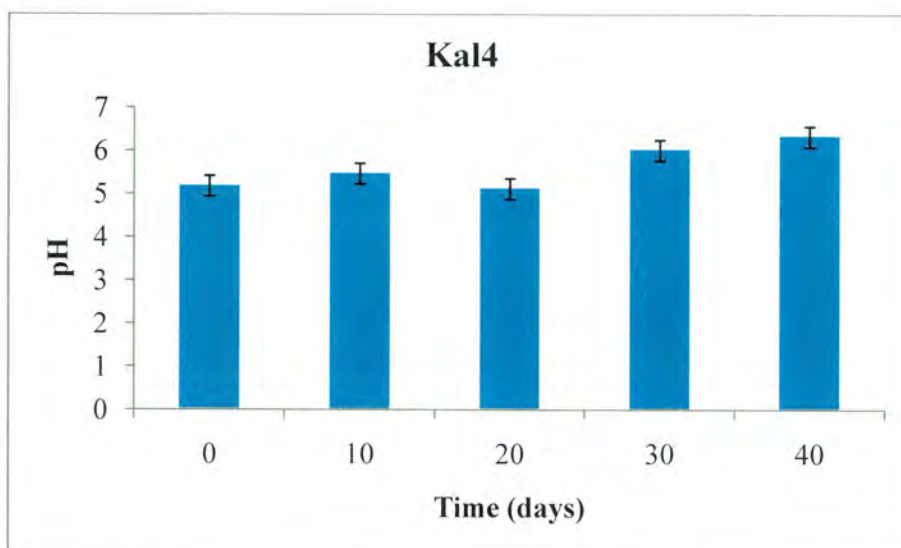


Figure 4.8c: pH changes of medium where Kal4 was grown in chemolithotrophic conditions over 40 days

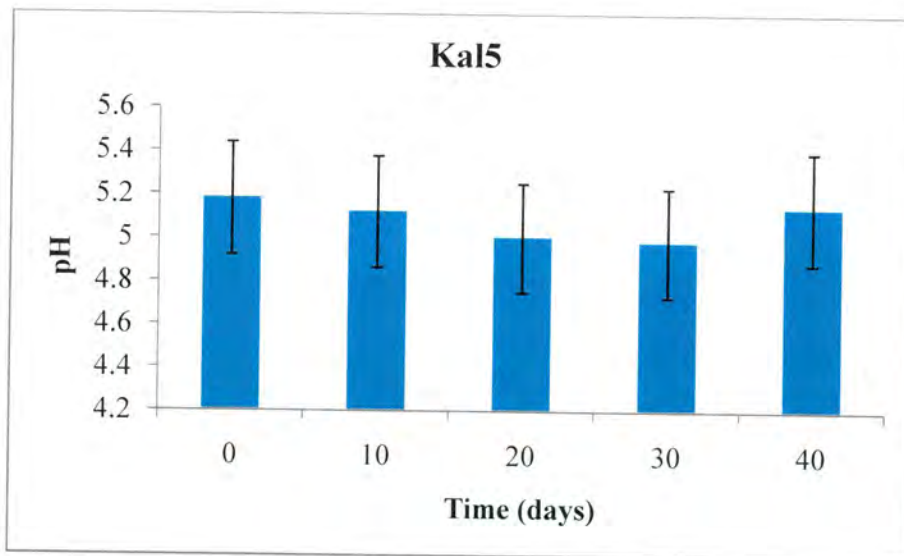


Figure 4.8d: pH changes of medium where Kal5 was grown in chemolithotrophic conditions over 40 days

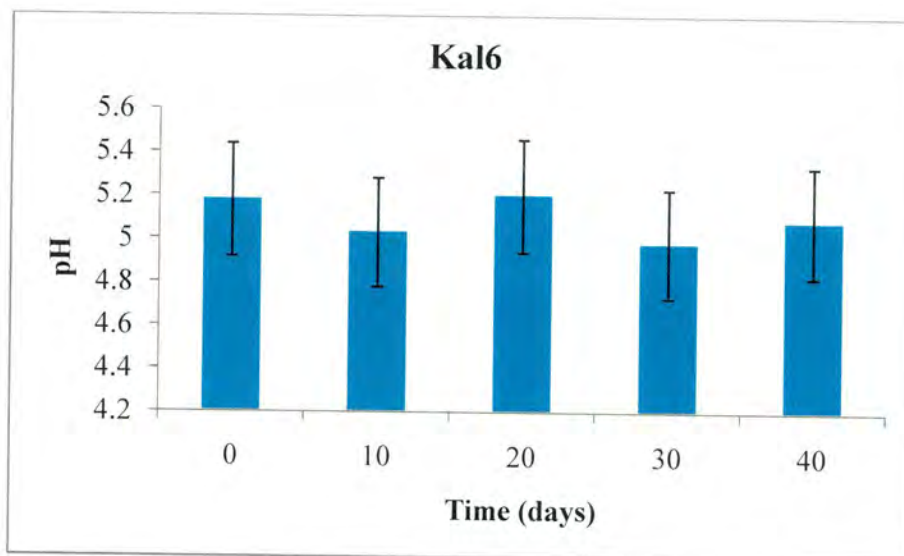


Figure 4.8e: pH changes of medium where Kal6 was grown in chemolithotrophic conditions over 40 days

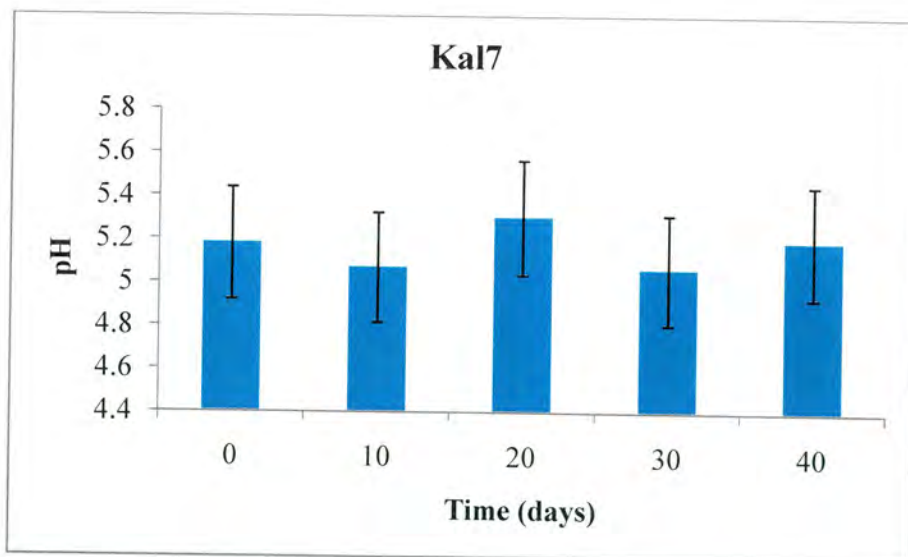


Figure 4.8f: pH changes of medium where Kal7 was grown in chemolithotrophic conditions over 40 days

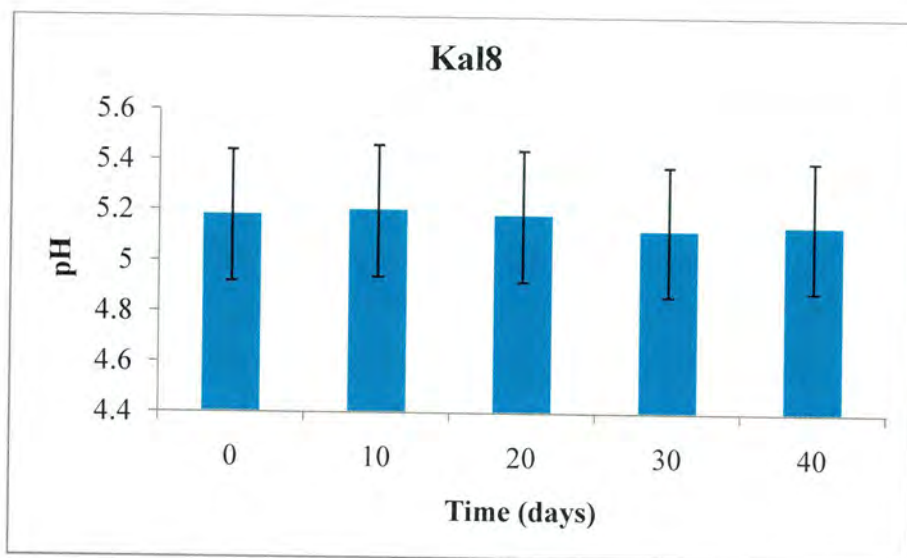


Figure 4.8g: pH changes of medium where Kal8 was grown in chemolithotrophic conditions over 40 days

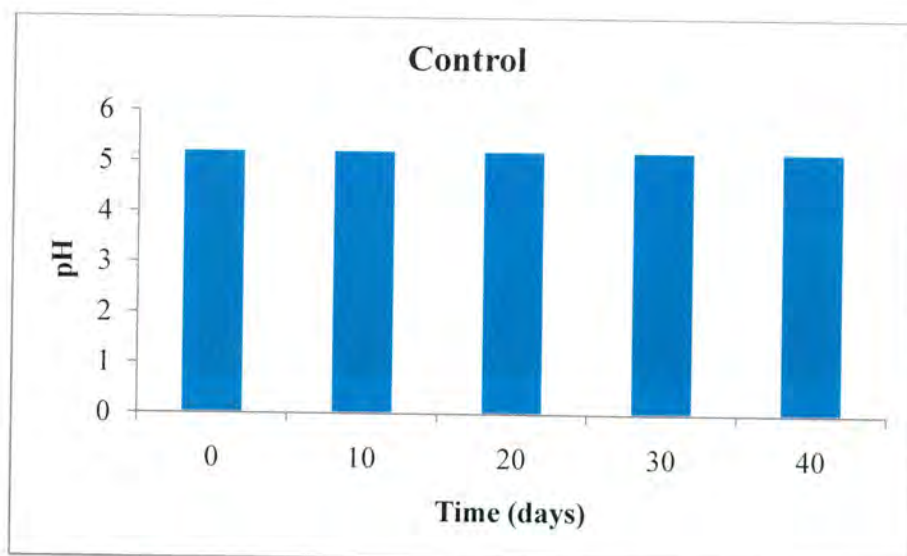


Figure 4.8h: pH changes of control (without inoculation) in chemolithotrophic conditions over 40 days

4.6 DNA extraction, amplification, sequencing and phylogenetic analysis

After DNA extraction, 16S universal primers were used for amplification and Figure 4.9 depicts the results we obtained based on the 16S rRNA gene sequences. The DNA from our isolates amplified at 1500 bp.

Table 3.2 outlines the primer pairs chosen for our experiments to detect genes responsible for sulphur- and iron-oxidation with their sequences and suitable amplification conditions. All 8 isolates produced no amplification products using the *rusA* primers. *rusA* was however not detected in our isolates, though they were capable of growth in Fe(II) as it was seen in the oxidation experiments.

From Figure 4.9, it is evident that the plasmid DNA from our isolates amplified using respective specific primers. Our isolates amplified for *thio*, *sox* and *iro* genes with the use of a negative control.

The isolates were subjected to a BLAST search and phylogenetic analysis indicated how the bacteria were related to one another. GenBank accession numbers were also provided Table 4.3. The 16S rRNA gene sequence analysis demonstrated higher percentages identity of the isolates' sequences with the corresponding bacteria obtained from the NCBI. 16S rRNA phylogeny showed that the isolates were highly related to each other, but were less related to the strain of *Thiobacillus ferrooxidans* (Figure 4.11)



Figure 4.9 Electrophoresis of the PCR products of the 16S rRNA gene sequence amplified from genomic DNA extracted from the isolates

M

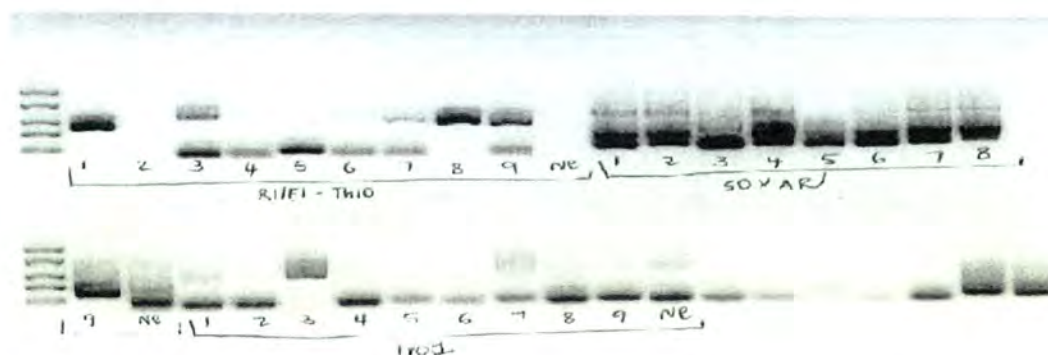


Figure 4.10 Electrophoresis of the PCR products amplified from plasmid DNA extracted from the isolates

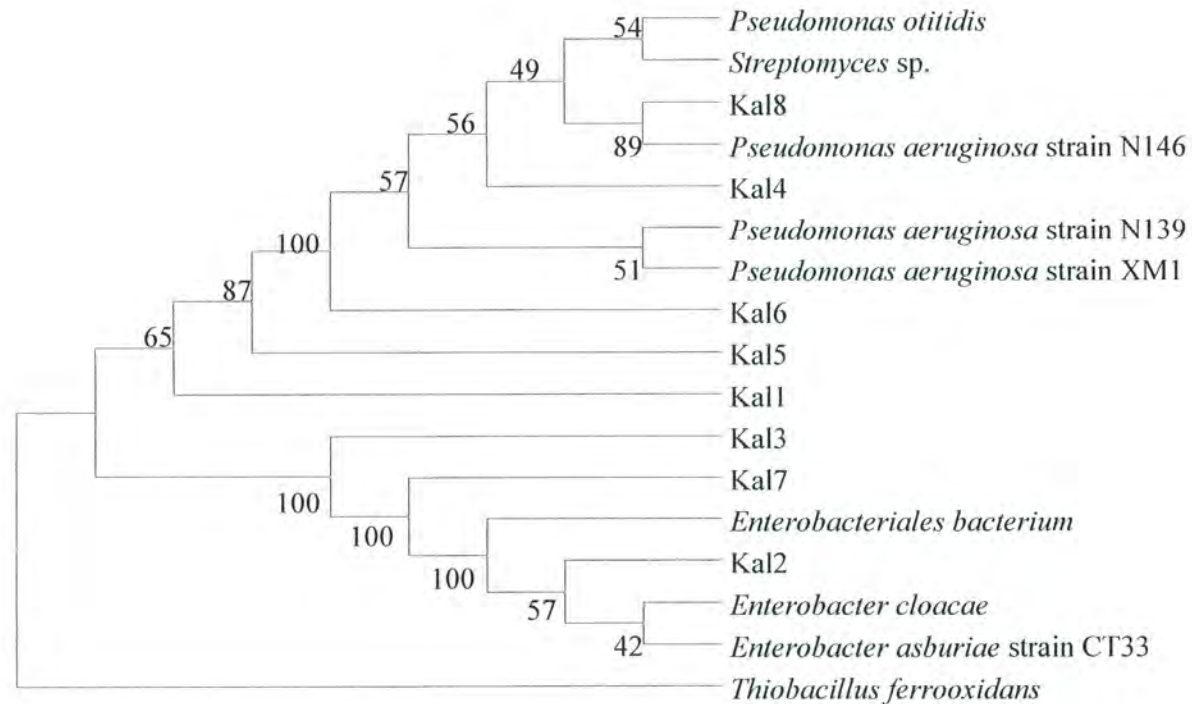
M; Marker, 1; Kal1, 3; Kal2, 4; Kal3, 5; Kal4, 6; Kal5, 7; Kal6, 8; Kal7, 9; Kal8, ne; negative control

The marker that was used for amplification of the genes from the plasmid DNA was the Thermo scientific fast ruler middle range. From Figure 4.10, it was observed that for the detection of the *sox* gene, all the isolates amplified at 100 bp. For the detection of *thio* gene Kal1 and Kal8 amplified at 1150 bp while the rest of the isolates amplified at 100 bp. For the detection of *iro* gene, all the isolates amplified at 100 except for isolates Kal3 which amplified at around 1150 bp.

Table 4.3: Identified isolates according to NCBI database

Isolate name	Genbank accession	Percentage identity	NCBI database species
Kal1	HM597242	82	<i>Pseudomonas aeruginosa</i> strain MP14
Kal2	JF901358	99	<i>Endophytic bacterium</i>
Kal3	KF224910	99	<i>Enterobacter hormaechei</i>
Kal4	KF307201	100	<i>Pseudomonas</i> sp.
Kal5	KC107733	90	<i>Pseudomonas aeruginosa</i> strain B1
Kal6	GU263805	91	<i>Pseudomonas aeruginosa</i> strain 21R
Kal7	GQ284539	97	<i>Enterobacter</i> sp.
Kal8	KF307201	99	<i>Pseudomonas</i> HY 13KR

Figure 4.11: A consensus phylogenetic tree derived from the 16S rRNA gene sequence data showing 8 isolates from this study and their relationship to other species obtained from the Gene bank. Bootstrap values of each major division are given at the respective nodes. The accession numbers for the sequences used to make this tree are given and the tree was rooted with the sequence from the *Thiobacillus ferrooxidans*.



CHAPTER 5

5. Discussion

5.1 Analytes analysis of the samples

Upon collection of the water samples from the mine's shallow dams, orange deposits were observed at the bottom of the dams. Iron hydroxide is what makes the water source to turn orange (Bruns & Sweet, 2004). This might explain why the two water samples had high iron content. The iron hydroxide may be formed from the oxidation of the type of ore (sulphidic) that is mined in the Kalahari Goldridge mine which may have resulted from the exposure to both water and oxygen (Johnson & Hallberg, 2005).

5.2 Colony morphology of the isolates

The isolates Kal1, Kal2, Kal3...to Kal8, were identified as *Pseudomonas aeruginosa* MP14, *Endophytic bacterium*, *Enterobacter hormaechei*, *Pseudomonas* sp. *Pseudomonas aeruginosa* strain B1, *Pseudomonas aeruginosa* strain 21R, *Enterobacter* sp. and *Pseudomonas* strain HY13 KR. Their optimum pH seemed to be around 6-7 when the medium was supplemented with 0.02% yeast extract powder. Though there was growth it was poor when they were subjected to obligate acidophilic chemolithotrophic conditions, that is, when grown in *Thiobacillus* broth without organic compounds in which the pH was recorded at 4.84. Therefore it cannot be concluded that the isolates of this study are obligate chemolithotrophic acidophiles.

Although the aim of the study was to isolate *Thiobacillus* species as we had used a selective growth medium it was found that the isolates that were predominantly present in the ore and water samples were identified as *Pseudomonas* sp. which agreed with the results of previous researchers on the subject matter. Mackintosh, (1978) reported on the 'impurity' of *Thiobacillus ferrooxidans* and that whether *Thiobacillus ferrooxidans* is isolated in a liquid culture or plated out, there will be at least two types of colonies seen. Amouric *et al.*, (2011) also observed this when they re-streaked the same colony on two different media (the ferrous sulfate and the yeast extract) colonies of differing morphologies were obtained. Johnson & Kelso (1983) reported that *Thiobacillus* spp. are almost always detected with heterotrophic species. Harrison *et al.*, (1980) also reported that microscopic examination of some allegedly pure *Thiobacillus ferrooxidans*

cultures revealed more than one morphological type. Two biotypes were seen; small motile rods and larger nonmotile rods, one an obligate heterotroph incapable of utilizing either ferrous iron or elemental sulfur and the other as non-motile strict autotroph.

Assuming that *Thiobacillus* sp. might have been present at initial stages of isolation in the study using the *Thiobacillus* broth (without any organic supplementation), when the same isolates were re-streaked on *Thiobacillus* agar (supplemented with yeast extract) the conditions might have favored the growth of the heterotroph. This can be seen as an advantage in bioleaching process as most commercial bioleaching operations are carried out with a consortium of highly acidophilic chemolithotrophic bacteria as well as heterotrophic bacteria. Mixed cultures of bacteria are often more efficient at ore decomposition than pure cultures of a single bacterium (Rawlings & Kusano, 1994).

Belly & Brock (1974), isolated a yellow-pigmented, non-sporeforming heterotroph growing at a pH of 2, while Harrison *et al.*, (1980) also isolated a yellow-pigmented heterotroph from coal spoils as a heterotrophic 'contaminant' of *Thiobacillus ferrooxidans* which grew at pH 6.7. The yellow-pigmented heterotroph that was isolated from our work, which was identified as *Pseudomonas aeruginosa* strain 21R, grew at an initial pH of 5.18 at day 0 and the highest growth was recorded at a pH of 7.61 and an absorbance of 0.498 at 600 nm at day 3. The sticky texture of the yellow-pigment of *P. aeruginosa* is owed to biofilm formation of *P. aeruginosa* and *Enterobacter* sp. which is useful in bioleaching as it mediates the attachment of the bacteria and for the dissolution of the metal sulphides (Rohwerder *et al.*, 2003; Vu *et al.*, 2009). Biofilms may be defined as an aggregation of bacteria encased in a matrix which consist of polymeric compounds, polysaccharides referred to as extracellular polymeric substances-EPS (Vu *et al.*, 2009). *Enterobacter* and *Pseudomonas* species have been applied in the biosorption of toxic heavy metals in bioremediation processes because of their ability to form biofilms. EPS has also been detected in *Thiobacillus ferrooxidans* and is useful particularly because of the insolubility of metal sulphides, thus direct bacterial attack is initiated by cells to the surface of the mineral ore (Yu *et al.*, 2011).

It was particularly noticed that when isolates were grown on yeast extract agar, all the species designated as *Pseudomonads*, *Enterobacter* and the *Endophytic* produced a blue-green colour in the medium. This colour is due to a combination of two metabolites of *P. aeruginosa*, pyocyanin and pyoverdine. Pyocyanin is a blue pigment belonging to the group of phenazine compounds which are low-molecular weight, heterocyclic compounds with redox-activity produced by *Pseudomonas* species and some other *Pseudomonads* (Parsons *et al.*, 2007). The presence of pyocyanin turns stationary growth phase cultures of *P. aeruginosa* green and its most important function is to assist iron uptake from the environment (Cox, 1986). The combination of the two pigments imparts the blue-green characteristic colour of cultures and when cells were viewed under microscope, they showed short rod-shaped without endospores inside the cell and they stained Gram-negative. This is characteristic of *Pseudomonas* sp. and the results obtained were similar to that of Lingling *et al.*, (2012).

5.3 Biochemical tests of the isolates

All the isolates were screened for nitrate and nitrite reduction by growing them in tubes of potassium nitrate found in API 20NE, under aerobic conditions and it was observed that most of the *Pseudomonas* isolated from this work, gave positive results. The ability to break down nitrates is very important in bioleaching processes because nitrates and nitrites are eutrophic which means that they promote production of organic nutrients and thus inhibit the growth of bioleaching chemoautotrophs and ultimately metal solubilization efficiencies (Xiang *et al.*, 2003). The presence of these heterotrophic bacteria assists chemoautotrophic bacteria such as *Thiobacillus ferrooxidans* to effectively mobilize metals from their respective ores (Xiang & Wong, 2000). Thus heterotrophic bacteria involved in denitrification are often implicated in enhanced metal acquisition (Kuba *et al.*, 1997). The *Pseudomonas* are considered as incomplete denitrifiers as they were only capable of reducing nitrates to nitrites with no further reduction of the nitrites as they lack the key nitrite reductase enzymes which enable complete denitrification to reduce nitrites (Cao *et al.*, 2013). A significant amount of incomplete denitrifiers are mostly *Pseudomonas* and are very important and more predominant in aerobic regions of the medium and did not grow very well in oxygen limited regions of the medium (Drysedale *et al.*, 1999), which confirmed with the results obtained in this study.

Pseudomonads have been known for their ability to breakdown arginine, by the enzyme arginine deaminase (hydrolase) (Hashim *et al.*, 2004). The purpose of the test was to determine if the bacteria can use the amino acid as a sole carbon source for energy and growth. All the results for L-arginine tests were positive which makes these isolates to be good candidates for bioleaching process. This type of nutritional category was earlier described by Akashi & Gojobori (2002), Johnson *et al.*, (2006). Various scientists (Uğur *et al.*, 2012) observed that *Pseudomonas* spp. do not degrade tryptophan to produce indole, which is in contrast with the findings of this study.

Heterotrophic isolates obtained from this study degraded the organic acids; capric and adipic acid provided by the API 20 NE strips. This is important in bioleaching because heterotrophic bacteria are believed to assist the growth of *Thiobacillus ferrooxidans*. They assist them through their ability to remove organic acids which tend to be toxic and inhibit the growth of the primary iron- and sulphur-oxidizers (*Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans*), thus increasing the mineral oxidation rate (Rawlings, 2005).

5.4 Effects of organic compounds on bacterial growth

A significant effect on cell growth was indicated when a carbon source, yeast extract was added as seen from the results. Köhler *et al.*, (2000) revealed that the presence of an organic source promoted optimal cell propagation and swarming motility of *Pseudomonads* and this motion is dependent on carbon sources. Isolates obtained from this study may be characterized as heterotrophs but it was observed that they exhibited both chemoautotrophic and chemoheterotrophic characteristics or they can be described as mixotrophs, which is the ability of the organisms to utilize both inorganic and organic compounds (Eiler, 2006). The latter characteristic was observed when yeast extracts powder was added to the *Thiobacillus* broth. Adding glucose as a carbon source did not yield the same result as the yeast extract powder. This type of nutritional category is unlike *Thiobacillus ferrooxidans*, which is an obligate autotrophic chemolithotroph obtaining its carbon from CO₂ or low amounts of formic acid can be used as an organic compound. Other than that, reports on the ability of *Thiobacillus ferrooxidans* to use organic compounds are considered to be erroneous, this mistake has been made numerously for the reason that *Thiobacillus ferrooxidans* cultures are notoriously difficult to free from heterotrophic bacteria and it is the growth of these types of bacteria that have been mistaken for growth of *Thiobacillus ferrooxidans* (Harrison, 1984). Generally, organic compounds inhibit the

growth of *Thiobacillus ferrooxidans* (Fang & Zhou, 2006). Colmer and co-workers considered this bacterium as a strict autotroph but the first report that this species is capable of heterotrophic growth came from Lundgren's laboratory (Harison *et al.*, 1980).

5.5 Oxidation experiments of the isolates

The first prokaryotes among many, to be observed and recorded by pioneer microbiologists in the 19th century were the iron bacteria. The phrase 'Iron bacteria' has been used to accommodate bacteria that not only can catalyze the oxidation of Fe^{2+} to Fe^{3+} ions but also those that can catalyze the dissimilatory reduction of Fe^{3+} to Fe^{2+} ions such as *Geobacter* spp. Iron bacteria have continued to be the focus of a large amount of research due to their importance in biomining/bioleaching of metal sulfide ores (Hedrich *et al.*, 2011). Most microbial bioleaching processes involve the oxidation of both iron and sulfur (Suzuki, 2001). Johnson & Hallberg, (2005), stated that many metals mainly occur as sulphide ores and they tend to associate with pyrite which is the most abundant metal sulfide ore on the planet. The process of oxidation of metal sulfide involves two steps; the oxygen-independent reaction where Fe^{3+} is the oxidant that attacks the metal sulfide. The other step is the oxygen-dependent which is the re-oxidation of Fe^{2+} to Fe^{3+} , which also involves the oxidation of inorganic sulphur compounds which are converted to sulfate eventually (Johnson & Hallberg, 2005). The orange colour that was seen in the oxidation experiments for iron was due to the ferric ion that was present. The Rio Tinto (Red River) is a typical example of ferric ion accumulation which obtained its name from the deep orange colour imparted to the water by the high concentration of ferric iron. This dissolved ferric iron was due to natural microbial activity. From earliest records, the Rio Tinto has been known as a river devoid of fish and with water that is undrinkable (Hosseiniabadi, 2009). The ability to oxidize sulphur compounds during chemolithotrophic growth has traditionally been attributed to *Thiobacilli* (Kelly *et al.*, 1997). Yellow deposits were observed after a month, which indicated that thiosulfate was oxidized to sulfate which is the major product of inorganic sulphur compounds (Friedrich, 2001; Johnson & Hallberg, 2005). The ability to oxidize various inorganic sulfur compounds is of fundamental interest because they are of economic importance such as in oxidation of metal sulphide (Johnson *et al.*, 2006). According to Beller *et al.*, (2006), *Pseudomonas* sp. particularly *Pseudomonas aeruginosa* possess an extensive biochemical system similar to that of the *Thiobacilli* for utilization of inorganic sulphur sources such as thiosulfate

($\text{Na}_2\text{S}_2\text{O}_3$). This could probably explain why *Pseudomonas* sp. were able to grow on *Thiobacillus* medium where thiosulfate was the main source for sulphur. Biological oxidation of thiosulfate to sulfate is exclusively oxidized by prokaryotes in the domain Bacteria and includes genera such as *Bacillus* (Aragno, 1992), *Pseudomonas* and *Paracoccus* which are mainly mesophilic (Friedrich & Mitrenga, 1981). Two major pathways have been proposed for these sulfur-oxidizing bacteria, the first one being the sulfur oxidation pathway which involves a *sox* gene cluster found in the *Paracoccus*. It includes seven genes *soxXYZABCD* which code for proteins essential for sulphur oxidation in vitro and are induced by thiosulfate (Chandra & Friedrich, 1986).

5.6 DNA extraction, sequencing and amplification

RusA is considered to play a central role in the oxidation of Fe(II) (Ida *et al.*, 2003) and belongs to the *rus* operon depicted in Figure 5.1 which encodes two cytochromes *c* and *aa₃* which have been shown to be involved in Fe(II) oxidation (Quatrini *et al.*, 2009).

Iron acquisition in *Pseudomonas* is also achieved by siderophores as it was established by Meyer *et al.*, (1996), though detection of siderophores was not carried out in this study. They not only assist in acquiring iron but they assist in the reduction of ferric to ferrous ions, and production of iron-chelating compounds (Llamas *et al.*, 2006).

Isolates obtained from this study did not amplify at their expected sizes. This could have been due to various parameters. These may include soil physics (physicochemical characteristics), chemical properties and the environment (Dommergues & Mangenot, 1970). Soil is a very complex system with different physicochemical gradients and varying environmental conditions in which the bacteria adapt to these conditions interacting with one another (Torsvik & Øvreås, 2002). The wide range of substrates available, the physical chemical gradients, as well as the interactions between different bacterial populations may have been responsible for the different band size obtained from this study. Another crucial factor is that the organisms isolated from our study are mostly *Pseudomonas* spp., and not obligate chemolithotrophic acidophilic bacteria such as *Thiobacillus* which come from an environment which could be predicted to be genetically more isolated (Butcher *et al.*, 2000). Another factor might be that the isolates may have been the heterotrophic 'contaminant' bacteria that were previously described by Johnson & Kelso (1983), Harrison *et al.*, (1980). This was evident when the isolates were sent for

sox and *thio*) to isolates of this study might have assisted them to be able to oxidize both sulphur and iron and to survive under metal-rich environment such as the mine where the samples were collected. Soil is considered one of the bacterial habitats where bacteria could potentially transfer genes by one or several transfer mechanisms, which is a part of the normal mechanisms of prokaryotes with evolutionary potential (Arber, 2000; de Vries & Wackernagel, 2005)

CHAPTER 6

6. Conclusion and Recommendations

In this work, *Pseudomonas* spp. together with *Enterobacter* and *Endophytic* spp. contain genes that render them useful in bioleaching and bioremediation industries. It will be a worthy venture to come up with an improved broth medium specific to only *Thiobacillus* spp. and thus *Pseudomonas* spp. can be used together with *Thiobacillus* spp. to improve bioleaching rates.

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APPENDIX 1

Media

API AUX Medium

Composition

Agar	1.5 g
Ammonium sulfate	2 g
Vitamin solution	10.5 mL
Trace elements	10 mL
Monosodium phosphate	6.24 g
Potassium chloride	1.5 g
Demineralized water to make 1000 ml	
Final pH	7.0±0.2

APPENDIX 2

Basal salts of media

2.1 Magnesium sulfate (Unilab, Saarchem)

Assay	99%
Chloride	0.01%
Heavy metals (Pb)	0.001%
Arsenic (As)	0.0005%
Iron (Fe)	0.001%
Calcium	0.02%

2.2 Ferrous sulfate (Unilab, Saarchem)

Assay	99.5%
Chloride (Cl)	0.03%
Heavy metals (Pb)	0.005%
Manganese (Mn)	0.1%
Zinc (Zn)	0.005%

2.3 Ammonium sulfate (Univar Saarchem)

Assay	min	99%
Loss on drying		0.4%
Residue on ignition		0.005%
Water-insoluble matter		0.002%
Chloride (Cl)		0.0005%
Nitrate (NO ₃)		0.001%
Heavy metals (Pb)		0.0005%
Iron (Fe)		0.0005%

2.4 Sodium sulfate anhydrous (UniverSaarchem)

Assay	min	99.0%
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Maximum limits of impurities

Chloride (Cl)	0.02%
Loss on ignition	0.5%
Insoluble matter	0.1%
Arsenic (As)	0.001%
Calcium (Ca)	0.01%
Iron (Fe)	0.001%
Lead (Pb)	0.001%

2.5 Potassium chloride (SMM chemicals)

Assay (after ignition) not less than 99.8%

Maximum limits of impurities

Insoluble matter	0.003%
Free acid (HCl)	0.0018%
Free alkali	0.05 mL
Bromide and Iodide (Br)	0.005%
Nitrate (NO ₃)	0.001%
Phosphate (PO ₄)	0.001%
Sulfate (SO ₄)	0.001%
Ammonium (NH ₄)	0.001%
Arsenic (As)	0.00004%
Barium (Ba)	0.001%
Calcium (Ca)	0.001%
Copper (Cu)	0.0002%
Iron (Fe)	0.0002%
Lead (Pb)	0.001%
Magnesium (Mg)	0.001%
Sodium (Na)	0.05%

Loss at 150	0.1%
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2.6 Sodium chloride (UnilabSaarchem)

Assay	99.5%
Sulfate (SO ₄)	0.01%
Phosphate (PO ₄)	0.001%
Total Nitrogen (N)	0.0005%
Heavy metals (Pb)	0.0005%
Iron (Fe)	

2.7 Potassium Dihydrogen Orthophosphate (Univar, Merck)

Assay (calculated on dried substance)	min	99%
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Maximum limits of impurities

Chloride (Cl)	0.002%
Sulfate (SO ₄)	0.02%
Heavy metals (as Pb)	0.001%
Iron (Fe)	0.002%
Sodium (Na)	0.1%

APPENDIX 3

Molecular reagents

PCR Master Mix

Composition

<i>Taq</i> DNA polymerase	0.05 u/ μ l
Reaction buffer	
MgCl ₂	4 mM
dNTP (dATP, dCTP, dGTP and dTTP)	0.4 mM

Primers

1. 27F

T_m(50mM NaCl): 54.3°C

GC Content: 50%

Molecular Weight: 6148.0

Nmoles/OD260: 5.2

ug/OD260: 32.0

Ext. Coefficient: 192100 L/(mole·cm)

2. 1492R

T_m(50mM NaCl): 60.6°C

GC Content: 48.1%

Molecular Weight: 8920.4

nmoles/OD260: 3.9

ug/OD260: 32.3

Ext. Coefficient: 256800 L/(mole·cm)

3. SoxA-F

T_m (50mM NaCl): 61.0°C
GC Content: 60%
Molecular Weight: 6296.1
nmoles/OD260: 4.8
ug/OD260: 30.4
Ext. Coefficient: 206800 L/(mole·cm)

4. *SoxA*-R

T_m (50mM NaCl): 55.7°C
GC Content: 55%
Molecular Weight: 6071.0
nmoles/OD260: 5.2
ug/OD260: 31.5
Ext. Coefficient: 192800L/(mole·cm)

5. *RusA*-F

T_m (50mM NaCl): 57.3°C
GC Content: 47.8%
Molecular Weight: 7125.7
nmoles/OD260: 4.5
ug/OD260: 32.1
Ext. Coefficient: 221900 L/(mole·cm)

6. *RusA*-R

T_m (50mM NaCl): 54.8°C
GC Content: 47.6%
Molecular Weight: 6372.2
nmoles/OD260: 5.2
ug/OD260: 32.9
Ext. Coefficient: 193700 L/(mole·cm)

7. *F1_Thio* (Sense)

T_m (50mM NaCl): 51.4°C

GC Content: 40%

Molecular Weight: 6138.0

nmol/OD260: 5.2

μg/OD260: 32.1

Ext. Coefficient: 191400 L/(mol·cm)

6. *R1_Thio*(Antisense)

T_m (50mM NaCl): 51.9°C

GC Content: 45%

Molecular Weight: 6030.0

nmol/OD260: 5.2

μg/OD260: 31.2

Ext. Coefficient: 193300 L/(mol·cm)

Induction Certificate, Permission Letter and Visitors Induction Assessment form

Induction certificate

	KALGOLD OPERATIONS
KALGOLD INDUCTION CERTIFICATE	
This is to certify that <u>KELETSO SEBOLLE</u> ID No: <u>850923 0205 0815</u>	
Coy No: _____ has attended the Generic Induction Programme	
Issue date: <u>31.05.2011</u> (Valid for one year) Expiry Date: <u>31.05.2012</u>	
Holder's Signature: <u>[Signature]</u>	
Assessor/ Trainer Name: <u>VEP ONIKA</u>	Assessor/ Trainer Signature: <u>[Signature]</u>
Reference: G KALGOLD IND	Cert. No: _____

Permission letter

To Whom it May Concern

Please allow Dr. Babalola Olubukola O and her student Keletso to take away ore and waste samples for scientific research purposes at North – West University.

Thanks



Edgar Chandomba

Visitors induction assessment form

	VISITORS INDUCTION ASSESSMENT	REVIEW NUMBER 1.0	REVIEW IMPLEMENTATION DATE 16 August 2010
		DOCUMENT TYPE	ASSESSMENT
		REFERENCE NUMBER	KAL 8

NAME & SURNAME	:	
SIGNATURE	:	
IDENTITY NUMBER	:	
COMPANY NAME	:	
ASSESSOR/ TRAINER NAME	:	
ASSESSOR/ TRAINER SIGNATURE	:	
MODERATOR NAME	:	
MODERATOR SIGNATURE	:	

DATE : SITE/
AREA-
VISITED :

COMPETENT	NOT YET COMPETENT	Yes	No
Please answer the following questions with a x:-			
1	At Kalgold, we have a Zero tolerance attitude regarding the not wearing of PPE		
2	Are you allowed to drink water at any place at Kalgold?		
3	Should you take any valuable possessions on your visit?		
4	A visitor may leave the working place if there is a threat to his/ her health and safety		
5	A risk to health and safety must be reported immediately to the supervisor.		
6	You are responsible for your own safety at the work place.		
7	When abandoned explosives have been identified- should you report it?		
8	Do you have to wear a full body harness when working at heights?		
9	Should you adhere to all the symbolic signs at Kalgold?		
10	Are you allowed to take weapons/ cameras or cell phones into the different work areas?		
11	Should hazardous ground conditions be reported?		
12	Does it matter if the weather worsens whilst on your visit?		
13	Are you allowed to operate any equipment or machinery without permission?		
14	Should you adhere to the traveling speeds at Kalgold?		
15	Is uncontrolled/ unprotected electricity dangerous?		
16	Do you have to wear your seat belt whilst travelling in the vehicles at Kalgold?		
17	Can you handle hazardous chemicals/ materials if you have not being trained?		
18	Are you allowed to be in possession of any mine property whilst leaving the property?		
19	May a security official search you?		
20	Is it important to know where the assembly point is at the site visited?		

Reference No: KAL 8

Page 1 of 3

Version 1.0

Name 4 cause of ground, air and water pollution:-

1	Smoke		
2	Gas / Fumes		
3	Dust		
4	Slime Dumps		
5	Rain water		
6	Process sewerage		
7	Clouds		

State whether the following answers are true or false:

		True / False	
1	The natural environment consists of buildings, cars and trees	T	F
2	We pollute the air through dust from our slimes dam	T	F
3	We need soil to throw our waste in	T	F
4	We can reduce water pollution by not drinking any water	T	F
5	Metals, tyres and electricity are not natural resources	T	F
6	Dirty water, chemicals and oils makes the water toxic for fish and cattle	T	F
7	Plants, animals and humans are the three living things on earth	T	F
8	One of the things we can do to reduce harm to humans are to be nice to my neighbour	T	F
9	We can reduce our impact on plants and animals by eating them	T	F
10	We pollute soil by spilling oil, grease and diesel on it.	T	F
11	Air pollution can cause lung diseases and acid rain	T	F
12	We need water to drink	T	F
13	ISO 14001 is a system that has a set of rules	T	F
14	We need ISO 14001 because it sounds cool	T	F
15	ISO 14001 says we must identify the things which can harm the environment	T	F
16	If you see something that harms the environment, you must scream and run away	T	F
17	We need metals to build structures and provide an income	T	F
18	An impact is a deep hole in the ground	T	F
19	We do not only have to look after the people working for Harmony, we must also look after the people living around our sites	T	F
20	Vehicles and blasting can cause noise that can hurt people's ears	T	F