A MOLECULAR STUDY OF THE COPPER RESISTANT GENES IN THE MICROBIAL POPULATION OF INDUSTRIAL BIOREACTORS

BY

ABIDEMI OLURANTI OJO

Submitted in fulfillment of the requirements for the degree

MAGISTER SCIENTIAE

In the Faculty of Natural and Agricultural Sciences,
Department of Microbial, Biochemical and Food Biotechnology
University of the Free State,
Bloemfontein

May 2009

Supervisors: Dr. L.A Piater
Prof. E. van Heerden

ACKNOWLEDGEMENTS

Table of contents

iii

v

List of figures

vii

List of tables

Abbreviations

A

A	Absorbance
AIX	Ampicillin/IPTG/X-Gal
ADP	Adenosine diphosphate
ATPase	Adenosine triphosphatase
ATP	Adenosine triphosphate
At. Caldus	*Acidithiobacillus caldus*

B

bp	basepair
BSA	Bovine serum albumin
BLAST	Basic Local Alignment Search Tool

C

copA	Copper translocating P-type ATPase
Cu^{1+}	Copper(I)
Cu^{2+}	Copper(II)
$CuSO_4$	Copper sulphate
cus	<u>Cu-s</u>ensing
cue	<u>Cu-e</u>fflux

D

DAPI	4 '- 6-diamidino-2-phenylindole
DGGE	Denaturing gradient gel electophoresis

| DNA | Deoxyribonucleic acid |

<div align="center">**E**</div>

| EDTA | Ethylene diaminetetraacetic acid |
| EtBr | Ethidium bromide |

<div align="center">**F**</div>

| FP | Forward primer |

<div align="center">**G**</div>

g	Acceleration due to gravity
g	Gram
gDNA	Genomic DNA
Glc	Glucose
GSH	Glutathione
GSSG	Glutathione disulfide

<div align="center">**H**</div>

Hepes	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
H_2O_2	Hydrogen peroxide
Hr	Hour

I

IPTG   Isopropyl β-D-1-thiogalactopyranoside

K

kb   kilobasepair

L

LB   Luria-Bertani

Log   Logarithm

M

Mg^{2+} Magnesium ion

M   Molar

mM   Millimolar

MIC   Minimum inhibitory concentration

N

NADH   Reduced nicotinamide adenine dinucleotide

nm   Nanometer

NCBI   National Center for Biotechnology Information

N   Normal

O

OH⁻	Hydroxyl radical
OD	Optical density

P

PCR	Polymerase chain reaction
ppm	Part-per-million
pco	Plasmid-borne copper resistance

R

RFLP	Restriction Fragment Length Polymorphism
RPM	Revolution per minute
rDNA	Ribosomal Deoxyribonucleic acid
RP	Reverse primer

S

sp.	Species

T

TAE	Tris-Acetic acid-EDTA
Temed	N, N, N', N'-tetramethylethylenediamine
Tris	2-Amino-2-(hydroxymethyl)-1,3-propandiol

U

UV   Ultra violet

UF   Urea-formamide

V

v/v   Volume per volume

W

w/v   Weight per volume

X

X-Gal   5-bromo-4-chloro-3-indolyl-beta-D-galactopyranosidehosphate

X g   Times gravity

Abstract

Micro-organisms were enumerated and identified from industrial bioreactors operated at 37°C and 70°C with pH ranging from 1-1.8 respectively. These bioreactors contained charlcopyrite in which micro-organisms were exposed to bioleaching operation. The bioreactors were assessed by characterising the micro-organisms present and the studies showed little diversity. Separation of polymerase chain reaction (PCR)-amplified 16S rDNA gene products using denaturing gradient gel electrophoresis (DGGE) confirmed the microbial community composition of these samples.

The BLAST results obtained from sequencing analysis revealed the presence of three different isolates in each of the bioreactors, namely; *Leptospirillum* sp., *Sulfobacillus* sp. and *Acidithiobacillus* sp. (37°C bioreactor) while 70°C bioreactor contains *Sulfolobus* sp., *Metallosphera* sp, and *Acidianus* sp. Control tests were done to see if archaea was present in this 37°C bioreactor and bacteria was present in a 70°C bioreactor but the results obtained showed that 37°C bioreactor did not contain archaea and the 70°C bioreactor did not contain bacteria.

The 37°C bioreactor sample was used for further investigations and the minimum inhibitory copper concentration of consortium bacteria as well as for the individual isolates of this bioreactor was determined. The result showed a higher minimal inhibitory concentration of copper at 400 mM MIC for the consortium of bacteria while minimal inhibitory concentrations of copper exhibited by *Sulfobacillus* sp. was at 6 mM; *Leptospirillum* sp. was 3mM and *Acidithiobacillus caldus* was 10 mM.

The copper resistance mechanisms of these bacteria were determined and the results obtained from the consortium of the bioreactor bacteria showed an active efflux mechanism(s), while the copper resistance mechanism exhibited by individual isolate was also studied. The results obtained suggested the possibility of *Acidithiobacillus* sp. being responsible for the efflux of copper ion as the profile obtained for *Acidithiobacillus caldus* resembled that of the bioreactor's profile.

Also, PCR amplification of a *copA* (copper-translocating P-type ATPase) gene was performed and the result obtained showed the PCR amplification of a *copA* (copper-translocating P-type ATPase) fragment from *Acidithiobacillus caldus* which confirmed the possible "protective" role this organism plays in the consortium of bacteria present in the 37°C bioreactor. This study has shown that *Acidithiobacillus caldus* possesses a copper-translocating P-type ATPase which was amplified during PCR and can be characterized with an active efflux resistance mechanism which releases excess copper from the cell with the possibility of intracellular reduction of copper(II) to copper(I) by NADH dehydrogenase.

Chapter 1

Literature Review

1.1. General introduction

Copper occurs naturally in the environment and is dispersed throughout as a result of human activities. These activities include industrial and agricultural applications of copper such as the release of copper from the metal mining industries and continuous application of copper-containing compounds (Anderson *et al.,* 1991; Cooksey *et al.,* 1990). Copper occurs in three valence states namely metallic copper(0), copper (II) and copper(I) [cupric and cuprous ions], with the latter copper ion being more toxic and less stable. Copper is a heavy metal found in group 11 and period 4 of the Periodic Table. Copper has an atomic number of 29 and atomic mass of 63.546 g/mol (WebElements). Metallic copper is a very extremely good conductor of heat and electricity and this property makes it widely used in industrial applications (European Copper Institute).

1.1.1. Physicochemical properties of copper

Table 1.1. Periodic Table of copper (WebElements)

Atomic no	29
Atomic mass	63.546 g/mol
Electronegativity (Pauling)	1.9
Density	8.9 g/cm3
Melting point	1083
Boiling point	2595
Vanderwaals radius	0.128 nm
Ionic radius	0.096 nm (+1) or 0.069 nm (+3)
Isotope	6

High copper concentrations in the environment usually lead to contamination which adversely affects all living organisms present in the vicinity. Studies have shown that micro-organisms are the first affected as this influences microbial diversity of a particular environment. Thus, as a result of copper contamination, microbial populations are affected as this induces copper resistance in some micro-organisms (Jain, 1990; Silver, 1996).

While copper ion is an essential trace element, it can be toxic at elevated concentrations in living cells (Nies, 1999). Micro-organisms that are capable of withstanding elevated concentrations of metal ions are referred to as metallophiles (Nies, 1999) and have evolved several mechanisms that protect the cells from intoxication bycopper ions.

These resistance mechanisms include active efflux which involves the export of excess copper ion from the micro-organisms' cytoplasm (Ge and Taylor, 1996; Odermatt *et al.,* 1992; Rouch *et al.,* 1989), cell wall modification and sequestration (Cha and Cooksey, 1991; Gilotra and Srivastava, 1997). Sequestration can be classified into two namely, intracellular and extracellular sequestration. Intracellular sequestration occurs in copper resistant micro-organisms due to the production of copper complexing-ligands or chelating agents, while extracellular sequestration occurs when chelating agents are released by the resistant micro-organisms to the environment which usually results in bioprecipitation (Blindauer *et al.*, 2002; Choudhury and Srivastava, 2001; Lutsenko and Kaplan, 1996;).

Studies have shown that micro-organisms capable of resisting high copper concentrations may possess more than one resistance mechanism (Nies, 1999). The resistance mechanisms of copper in micro-organisms can be identified in various ways and include: (i) isolation of copper resistance genes from the resistant micro-organisms as these resistance genes can be located on the chromosome or can be due to plasmid borne genes (Bröer *et al.,* 1993) and (ii) determination of copper speciation in the environment.

1.1.2. Chemical states of copper

The oxidation states of copper as a cellular component is important since this metal ion is involved in biological redox reactions by cycling between copper(I) and copper(II) hence, gaining and losing electrons. Unlike copper(I), on exposure to the atmosphere, free copper(II) ions in neutral, aqueous solutions are stable. The latter copper ion can be maintained in solutions at acidic pH or in complexed forms. Stable copper(I) complexes can be formed with acetonitrile. However, these two valence states of copper are able to bind to some biological molecules such as thiol (Solioz and Stoyanov, 2003).

1.1.3. Copper in the environment

Copper is usually released in the environment through natural sources and human activities. The latter, which is anthropogenic sources, include the dispersion of copper into air during combustion of fossil fuel and mining activities, and this settles and binds to water sediment or soil particles (Plant Industry Division, Alberta Agriculture, Food and Rural Development; McCall *et al.,* 1995); building and construction materials, domestic products, copper-based fungicides on agricultural crops as well as manure. Weathered rock from which the soil develops, and erosion and run-off of copper containing minerals are examples of natural sources of copper (Plant Industry Division, Alberta Agriculture, Food and Rural Development). Copper cannot be destroyed but tends to accumulate resulting in high levels of copper in the environment, leading to contamination. This adversely affects living organisms present in the immediate vicinity (Ackerman *et al.,* 1999; Grobler, 1999).

1.1.4. Toxicity of copper

Copper, an essential element for living cells exists in the soil and is present in diets (Albarracin *et al.,* 2005). Metal ions like copper play an essential role in many biological systems; it was estimated that over half of all proteins are metalloproteins containing metal ions as structural components or catalytic co-factors (Degtyarenko, 2000). Copper ions while required for normal growth, are also involved in respiration (electron transport) and serves as co-factors for oxygenases and hydroxylases (Garcia-Horsman *et al.,* 1994), but above optimum concentrations it becomes toxic (Munson *et al.,* 2000). The

involvement of copper ions in redox reactions and the ability of copper to generate free hydroxyl radicals as in Fenton-type reaction usually accounts for the toxicity of copper (Solioz and Stoyanov, 2003).

$$Cu^+ + H_2O_2 \leftrightarrow Cu^{2+} + OH^- + OH^{\cdot} \qquad \text{Equation 1}$$
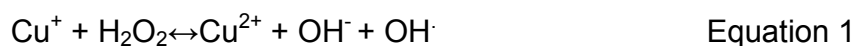
Highly reactive hydroxyl radicals damage DNA by attacking guanine residues and breaking phosphodiester bonds in single-stranded DNA which eventually leads to the modification of the deoxyribose sugars. Also, these reactive hydroxyl radicals can impair lipid membranes and enzymes in living cells (Hoshino *et al.,* 1999). Redox cycling of copper by the reactions shown below is more favourable as these reactions happen at the expense of glutathione (GSH) and oxygen which eliminates the toxicity of copper. During redox cycling of copper, copper(II) in the presence of glutathione (GSH) is reduced to copper(I) which in turn reacts with oxygen to form copper(II).

$$Cu^{2+} + 2GSH \leftrightarrow 2Cu^+ + GSSG + 2H^+ \qquad \text{Equation 2}$$

$$2Cu^+ + 2H^+ + O_2 \leftrightarrow 2Cu^{2+} + H_2O_2 \qquad \text{Equation 3}$$
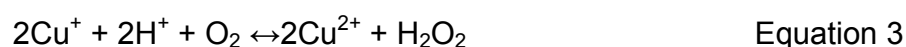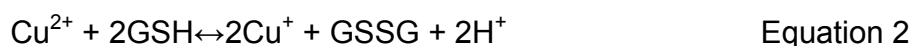
1.1.5. Ion transport

Ion transport can be defined as the movement of ions across energy-transducing cell membranes. The transport can be active or passive. The former is coupled to energy-yielding chemical or photochemical reactions while the latter transport utilizes its energy from the concentration gradient of ions and permits the transport of a solute in one direction. Active transport can be primary or secondary. The primary active transport is referred to as ion pump while secondary active transport uses ion gradient and voltage released by primary transport to drive the co-transport of other ions (Center for Cancer Education, 2007). Figure 1.1 gives an insight into cellular transport activities of charged and uncharged solute molecules in micro-organisms.
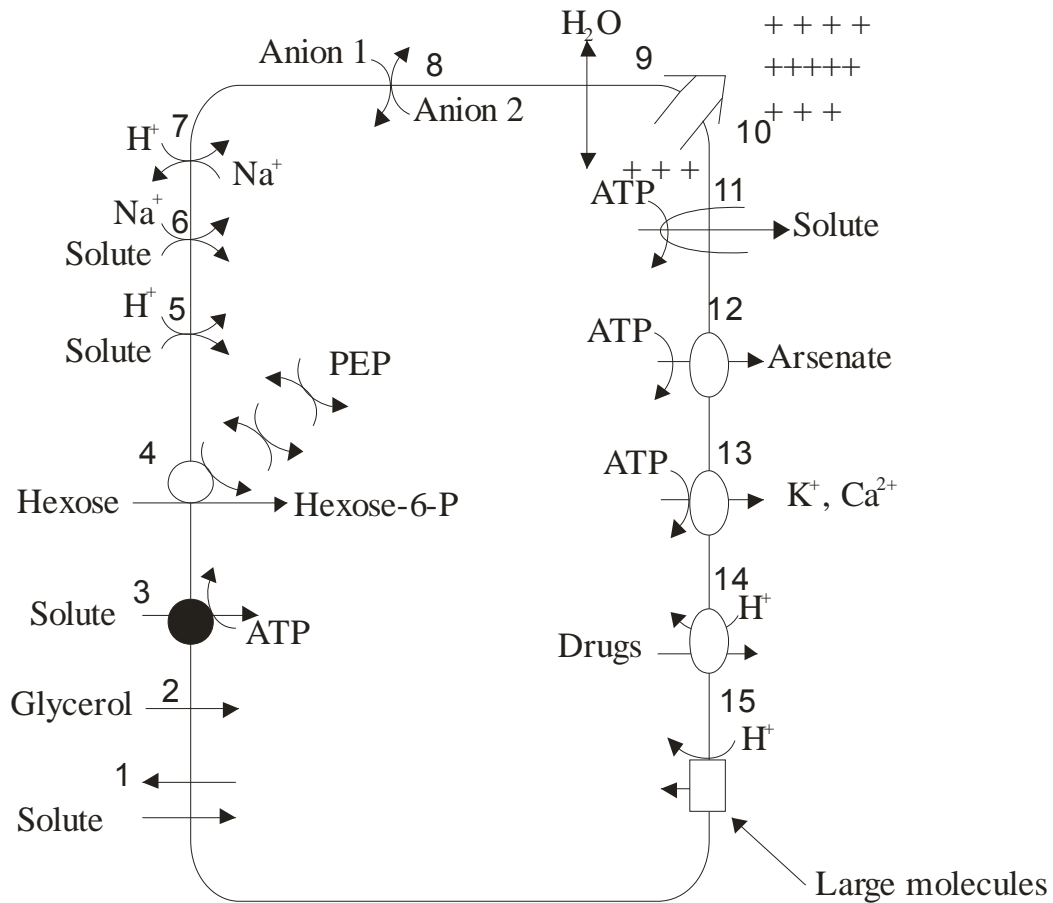
Figure 1.1. Different systems of solute molecule transport in prokaryotes (Taken from Barton, 2005).

Also, ATPases are enzymes that hydrolyse ATP into ADP and phosphate, and are classified into three main categories namely, F-type ATPase, V-type ATPase and P-type ATPase (Center for Cancer Education, 2007). The ATPase is a family of cation transport enzymes that mediate membrane flux of biological cations (Smith *et al.,* 1993). P-type ATPase, is characterized by vanadate sensitivity and a phosphorylated intermediate. There are three main classes of P-type ATPase namely P-1, P-2 and P-3. P-1 transports cadmium ions, copper ions, and zinc ions. Figure 1.2 is the schematic diagram of P-1 (P-type ATPase) (Barton, 2005).
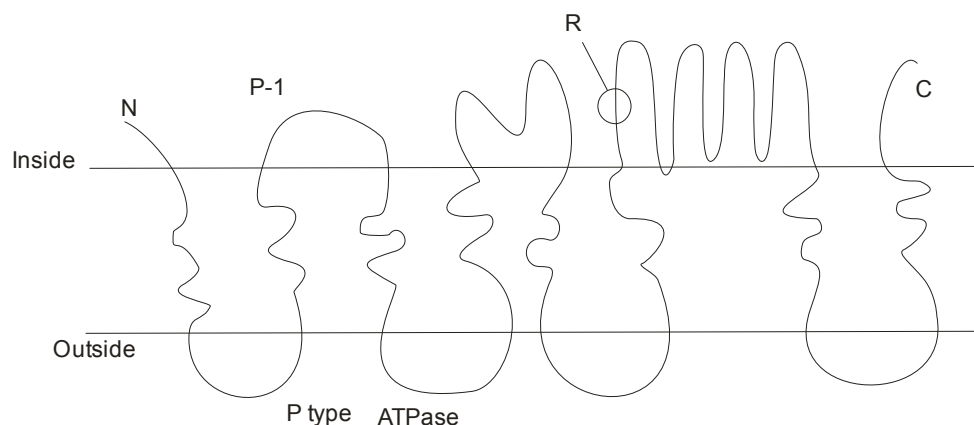
Figure 1.2. R shows the location of an aspartate residue that eventually is phosphorylated by ATP. This is the site for inhibition of vanadate [inside = inner membrane; outside = outer membrane] (Adapted from Barton, 2005).

1.2. Copper resistance mechanisms

As previously mentioned, copper resistant micro-organisms have evolved several mechanisms that protect their cells from deleterious effects of excess copper ions. These resistance mechanisms vary from active efflux (Ge and Taylor, 1996; Odermatt *et al.,* 1992; Rouch *et al.,* 1989), to cell wall modification and sequestration (Cha and Cooksey, 1991; Gilotra and Srivastava, 1997).

1.2.1. Export of excess copper from bacterial cytoplasm

Copper is an essential ion that plays an important role in metabolic processes in some microbial enzymes as it serves as a component (co-factor) of many metalloenzymes. These include cytochrome c oxidase, rusticyanin, nitrite reductase, ammonia monooxygenase, superoxide dismutase and others (Cervantes and Guitierrez-Corona, 1994; Harris, 2000). Copper ion transport into and out of the cell involves P-1 P-type ATPases (Barton, 2005).

The importing P-type ATPase can either import its substrate from the outside or from the periplasm to cytoplasm, while the exporting P-type ATPase exports the ion from the cytoplasm to the outside or periplasm. Regarding homeostasis, P-type ATPases are important because the import systems for macro-elements such as Mg^{2+} may also import heavy metal cations and exporting P-type ATPases may detoxify the toxicity of heavy

6

metal cations by efflux (Snavely *et al.,* 1989). Equation 4 shows catalytic activity of CopA P-type ATPase (UniProtKB/Swiss-Prot).

$$ATP + H_2O + Cu^{1+}[in] = ADP + phosphate + Cu(^{1+})[out]$$

Equation 4
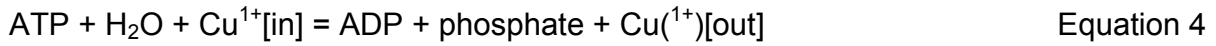
The diagrams (Figure 1.3 A and B) show a model of CopA P-type ATPase and the transport of copper ions in a *E. coli* cell by Copper(I) translocating P-type ATPase (CopA).
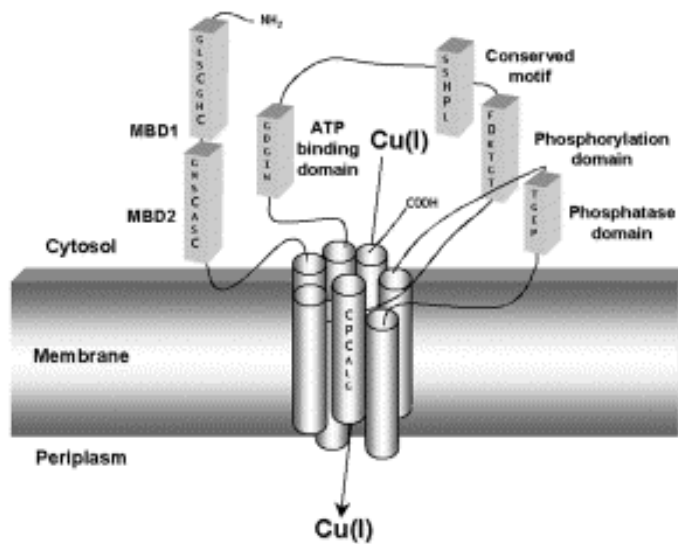
Figure 1.3A. Model of the CopA P-type ATPase. CopA is predicted to have an N-terminal region with two cytosolic CXXC (cysteine or histidine rich metal binding motifs) metal binding domains (MBD1 and MBD2) and eight transmembrane segments (TM). Connecting TM4 and TM5 is the conserved phosphatase domain. TM6 is predicted to be part of the translocation domain and has the consensus CPC (Cys-Pro-Cys or His) sequence. Connecting TM6 and TM7 are the phosphorylation and ATP binding domains and a conserved sequence found only in soft metal P-type ATPases (Rosen, 2002).
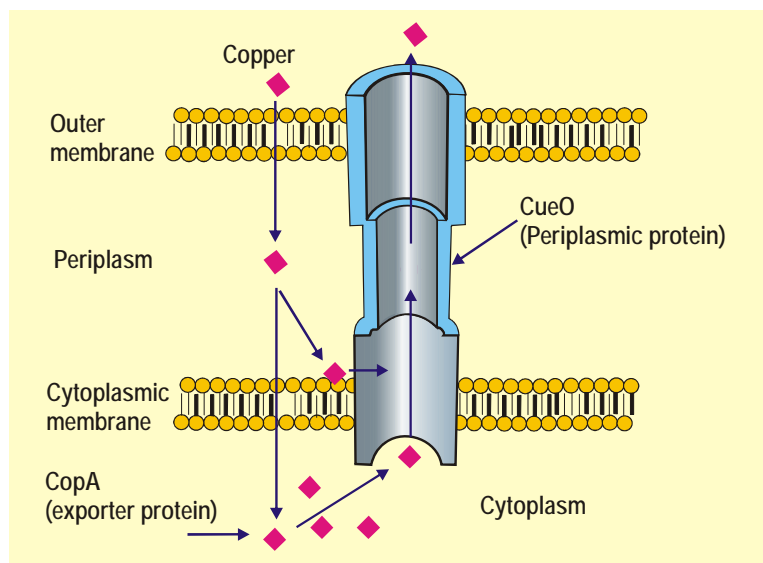
Figure 1.3B. Active efflux mechanism in *E. coli* under aerobic condition (Adapted from Medscape).

1.2.2. Copper ion detoxification by sequestration in bacteria

Sequestration of copper ions can be defined as the seizure of the ion which eventually forms a chelate or other stable complex with the ion so that it is no longer available for any other reaction. Intracellular sequestration of metal ions usually involves chelating agents like proteins or peptides that form a stable complex. These sequestration molecules are cysteine-rich metallothioneins, phytochelatins and sulfide (Dameron and Harrison, 1998).

Metallothioneins are small, 25 to 62 amino acid cysteine-rich proteins where the cysteines are arranged in repetitive Cys-Cys, Cys-Xaa-Cys and Cys-Xaa-Xaa-Cys motifs (Kille *et al.,* 1994). In phytochelatins, the cysteines are arranged in Cys-Xaa-Cys which are the glutamic acid-cysteine polymer, a derivative of glutathione (GSH) (Dameron and Harrison, 1998). Many organisms use sulfide to precipitate excess metals since the cysteinyl sulfurs present in the proteins are ligands for metal ions while addition of sulfide and metal ions to phytochelatin complexes allow the cells to resist metal toxicity (Mutoh and Hayashi, 1988).

Other copper-complexing ligands include amicyanin from *Methylobacterium extorquens* or *Thiobacillus versutus*, rusticyanin from *Thiobacillus ferrooxidans* and a membrane

associated copper binding protein, pseudoazurin, from *Pseudomonas*. Also, proteins containing copper are good electron carriers. Azurin (blue bacterial copper) is an example of a protein that sequesters copper ions. In *Acidithiobacillus ferrooxidans* the blue-copper participates in iron oxidation, while in *Acidithiobacillus versutus* blue-copper is an electron carrier between methylamine dehydrogenase and cytochrome c (Trevors and Cotter, 1989).

1.2.3. Reduced copper import

This resistance mechanism occurs through uptake inhibition or external chelation of copper or reduced permeability to copper owing to the synthesis of new membrane proteins such as *Pseudomonas syringae* Cop proteins and copper-complexing ligands detected in *Vibro* and *Synechococcus* cultures (Cha and Cooksey, 1991; Gordon *et al.,* 2000). Reduction of copper importation to limit a high toxic effect can also occur through copper import machinery (Dameron and Harrison, 1998). The strain of *S. cerevisiae* that can grow at 200 mM exhibits decreased copper uptake (White and Gadd, 1986) while at pH 3-5, the copper uptake in *Penicillium ochro-chloron* is lower than at pH 6 (Gadd and White, 1985).

1.2.4. Bioprecipitation of copper in the environment

Bioprecipitation of copper can be termed as extracellular sequestration. This mechanism uses the ability of bacteria to reduce high concentrations of toxic metal ions by producing volatile compounds such as hydrogen sulfide and metabolic products (Lovely, 2000; Erardi *et al.,* 1987). When the metal ions come into contact with any of these substances there is extracellular chelation of the toxic metal ions as these substances, which serve as metal chelators, prevent the metals from entering the cell thus reducing metal bioavailability and preventing mineral formation (Lovley, 2000).

Erardi and co-workers (1987) demonstrated that a *Mycobacterium scrofulaceum* strain was able to remove copper(II) from the growth medium through the formation of CuS while *Desulfovibrio* sp. protects itself from the toxic effect of copper by producing hydrogen sulfide which subsequently precipitates copper(II) to produce copper sulfide

(Terawaki and Rownd, 1972). Nies (2000) reported that *Ralstonia* sp. CH34, a Gram-negative bacterium with a significant set of resistance determinants, can mediate biochemical reactions that precipitate heavy metals. This occurs when the cell releases a metabolic product such as carbon dioxide during the growth phase. Figure 1.4 is the schematic diagram of a general mechanism of copper ion detoxification.
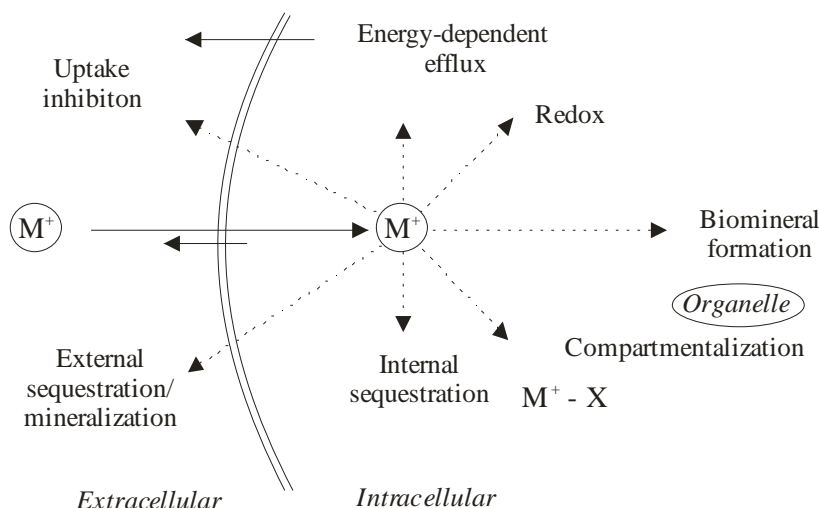
Figure 1.4. General mechanism of copper ion detoxification. Reduced copper import through uptake inhibition and external sequestration usually limits intracellular chelation; sequestration of copper through complexation with proteins such as ligands is also one of the resistance mechanisms used by some micro-organisms and increased copper efflux from the cells by pump which is usually accompanied by redox changes of copper (Dameron and Harrison, 1998).

1.3. Importance of copper resistant micro-organisms

An environment contaminated with heavy metals such as copper can be treated with heavy metal-resistant micro-organisms since some are capable of removing or reducing the availability of copper in the environment. Bioremediation can be described as the use of bioremediators such as plants or micro-organisms to detoxify dangerous chemicals in the environment (Environmental Protection Agency).

Heavy metal-resistant bacteria are of great value in biotechnology as they are desirable from both environmental and economical perspectives. Some of these resistant micro-organisms are used for environmental bioremediation of heavy metals as well as for

10

construction of heavy metal biosensors which are used for detection of the presence of heavy metals (Nies, 1999; Timmis and Pieper, 1999).

Some resistant micro-organisms use sequestration to protect themselves which eventually reduces the bioavailability of copper in the environment. Sequestration of ions can be through bioaccumulation or biosorption. Bioaccumulation of copper involves sequestration of ions by intracellular accumulation while biosorption also involves sequestration of metal ions that depends on the phenomena of adsorption to the cell surface (Qureshi *et al.,* 2001). Some resistant bacteria that sequester heavy metal ions from their surroundings include *Acinetobacter* (Ahmed *et al.,* 1999), *Pseudomonas* sp. (Badar *et al.,* 2001) and *Pseudomonas aeruginosa* (Qureshi *et al.,* 2001) and these are good candidates for bioremediation.

Haung and co-workers (2005) demonstrated that *Enterobacter aerogenes* is a potential organism for remediation as the organism is resistant to both cadmium and copper. The micro-organism promotes adsorption of cadmium and copper thereby reducing the bioavailability of the ions. Miranda and Rojas (2006) have documented that *Vibro* sp. isolated from hatchery-conditioned adult of scallop *Argopecten purpuratus* is capable of accumulating copper within the cell. Figure 1.5 shows metal processing mechanisms in micro-organisms which may affect mobilisation or immobilisation of metal ions.

11
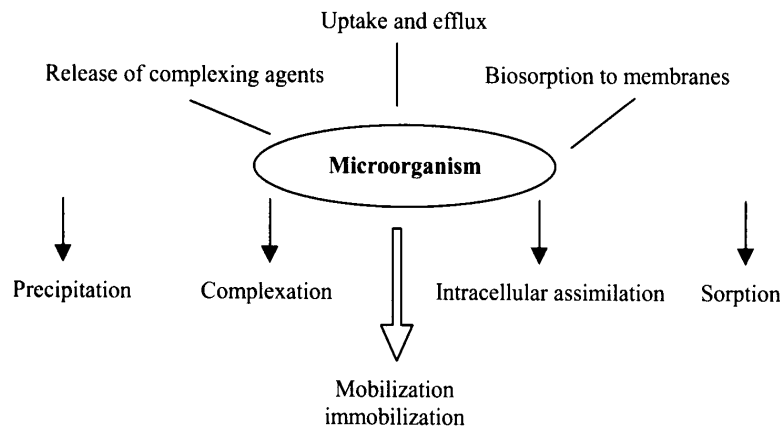
Figure 1.5. Metal-micro-organism interactions (Taken from Gazso, 2001).

For bioremediation to occur there must be metal ion micro-organism interactions. Sequestration, precipitation, or solubilization of the metal such as copper can reduce the availability of the metal ions in the environment. Bioremediation of a copper contaminated environments can be *in situ* or *ex situ*. *In situ* type remediation includes biostimulation and bioaugmentation while *ex situ* involves dig and dump, washing of soil and soil venting (Anh-tu, 2005).

Biostimulation involves the modification of the contaminated environment in order to stimulate the growth of the native microbial population. This method usually presumes that the desired micro-organisms are present (Oppenheimer Biotechnology, 2003). Bioaugmentation can be defined as the addition of pre-grown micro-organisms to contaminated sites to improve the cleaning-up of the contaminant (Innovative Technology Group, 2003). Resistant micro-organisms that are capable of sequestering copper either intracellular or extracellular, are used for this type of bioremediation.

Heavy metal-resistant micro-organisms can also be used as biosensors. A biomarker or gene marker is a DNA sequence that is introduced into an organism to confer a distinct genotype or phenotype that allows environmental monitoring (Jansson and de Bruijn, 1999). Heavy metal-resistant bacteria are useful in the construction of these biosensors or biomarkers irrespective of the resistance mechanisms these micro-organisms may possess. This is due to the fact that all metal determinants in the resistant bacteria are

inducible hence, their regulatory systems are used to construct biosensors that determine the concentration of heavy metal in the environment (Nies, 1999).

Various heavy metal biomarkers have been constructed; Kilinc *et al.* (1990), constructed a biosensor that measures copper(I) and copper(II) speciation. Biomarkers were used to monitor the efficacy of bioremediation (Jansson and de Bruijn, 1999) while Holmes and co-workers (1994) developed biosensors for detecting the bioavailability of mercury and copper in environmental samples.

1.4. Conclusions

Metal ions like copper play an essential role in many biological systems through its involvement in some metabolic processes but above optimum concentrations becomes toxic. Some micro-organisms counter toxicity by resisting the deleterious effect of excess copper. Copper resistance mechanisms possessed by these resistant organisms are dependent on the resistance systems present in the organisms.

Although active exportation of excess copper ion is the most common mechanism that the resistant micro-organisms use, some exhibit other resistance mechanisms which include extracellular sequestration or intercellular sequestration. Also, some micro-organisms use reduced copper import as a means of protecting themselves from high copper concentrations, cell wall modification usually results in reduced copper intake.

These resistant micro-organisms are of great value in biotechnology applications. They can be used in environmental bioremediation of heavy metal contaminated sites and for construction of biomarkers for detection of the metal ions in the desired environment. Some of these heavy metal resistant micro-organisms possess sets of remarkable resistance determinants. For example, *Acidithiobacillus caldus* and *Acidithiobacillus ferrooxidans* are acidophiles and *A. caldus,* a sulphur oxidizer and *A. ferrooxidans,* an iron oxidizer used in bioleaching and are resistant to copper due to resistance genes they possess. The genome of *Acidithiobacillus ferroxidans* was sequenced and this revealed the presence of copper(I) translocating P-type ATPase while a copper resistance gene in *Acidithiobacillus caldus* is yet to be characterized.

13

Since there are increases in environments contaminated with copper as a result of the continuous use of copper-containing pesticides and fertilizers as well as the addition of copper to animal feed, acidophiles such as *Acidithiobacillus* sp., *Sulfobacillus* sp., a sulphur and iron oxidizer and *Leptospirillum* sp., an iron oxidizer used for bio-oxidation of copper sulfide under acidic conditions may be used for removal of excess copper from the environments. Characterizing copper resistance mechanisms of the consortium of these bacteria may reveal the resistance mechanism these bacteria are using to protect themselves from copper toxicity.

1.5. References

Ackerman, D.J., Reineeke, A.J., Els, H.J., Grobler D.G. and Reineeke S.A. (1999). Sperm abnormalities associated with high copper levels in impala (*Aepyceros melampus*) in the Kruger National park, South Africa. *Ecotoxicology*. pp. 261-266.

Ahmed, N., Badar, U., Hassan, M.T. and Raihan, S. (1997). Isolation of local copper tolerant bacteria in Pakistan for biofilm formation on various supports and subsequent biosorption. *Resource and Environmental Biotechnology*. pp. 65-72.

Albarracín,V.H., Amoroso, M.J. and Abate, C.M. (2005). Isolation and characterization of indigenous copper-resistant actinomycete strains. *Geochemistry*. pp 145-156.

Anderson, G.L., Menkissoglou, O. and Lindow, S.E. (1991). Occurrence and properties of copper-tolerant strains of *Pseudomonas syringae* isolated from fruit trees in California. *Phytopathology*. pp. 648-656.

Anh-tu, D.K. (2005). http://courses.washington.edu/rherwig/FISH490-2005/Student%20Presentation/Group2-Metals.ppt

Badar, U., Abbas, R. and Ahmed, N. (2001). Characterization of copper and chromate resistant bacteria isolated from Karachi tanneries effluents. In Ahmed, N., Qureshi, F.M.,

and Khan, O.Y. (eds.). Industrial and Environmental Biotechnology. Horizon Scientific Press, Norfolk. pp. 43-53.

Barton, L. (2005). Structural and functional relationships in prokaryotes. *Science*. pp. 468-489.

Blindauer, C.A., Harrison, M.D., Robinson, A.K., Parkinson, J.A., Bowness, P.W., Sadler, P.W., Sadler, P.J. and Robinson, N.J. (2002). Multiple bacteria encode metallothioneins and SmtA-like zinc fingers. *Molecular Microbiology*. pp. 1421-1432.

Bröer, S. Ji, G. Bröer A. and Silver, S. (1993). Arsenic efflux governed by the arsenic resistance determinant of *Staphylococcus aureus* plasmid PI258. *Journal of Bacteriology.* pp. 3480–3485.

Center for Cancer Education (2007). www.cancerweb.ncl.ac.uk/cgi-bin/omd [Accessed November 12, 2008].

Cervantes, C. and Gutierrez-Corona, F. (1994) Copper resistance mechanisms in bacteria and fungi. *FEMS Microbiol*ogy. pp. 121-37.

Cha, J.S. and Cooksey, D.A. (1991). Copper resistance in *Pseudomonas syringae* mediated by perisplasmic and outer membrane proteins. *National Academy of Sciences, USA*. pp. 8915-8919.

Choudhury, R. and Srivastava, S. (2001). Zinc resistance mechanisms in bacteria: *Current Science*, pp.7.

Cooksey, D.A., Azad, H.R., Cha, J.S. and Lim, C.K. (1990). Copper resistance gene homologs in pathogenic and saprophytic bacterial species from tomato. *Applied and Environmental Microbioliology.* pp. 431-435.

Dameron, C.T. and Harrison, M.D. (1998). Mechanisms for protection against copper toxicity. *Am. Journal of Clininical Nutrition* pp. 1091S-7S.

15

Degtyarenko, K. (2000). Bioinorganic motifs towards functional classification of metalloproteins. *Bioinformatics.* pp. 851-864.

Environmental Protection Agency. (www.epa.gov/superfund/sites) [Accessed on October 14 2008].

Erardi, F.X., Failla, M.L. and Falkinham, J.O. (1987). Plasmid-encoded copper resistance and precipitation by *Mycobacterium scrofulaceum. Applied Environmental Microbiology.* pp. 1951-1954.

European copper institute (www.eurocopper.org/copper) [Accessed August 20, 2008].

Gadd, G.M. and White, C. (1985). Copper uptake by *Penicililium ochro-chloron*: influence of pH on toxicity and demonstration of energy-dependent copper influx using protoplasts. *Journal of General Microbiol*ology. pp. 1875-1879.

Garcia-Horsman, J.A., Barquera, B., Rumbley, R., Ma, J. And Gennis, R.B. (1994). The superfamily of heme-copper respiratory oxidases. *Journal of Bacteriology.* pp. 5587-5600.

Gazso, L.G. (2001). The key microbial processes in the removal of toxic metals and ranuclides from the environment. *Central European Journal of Occupational and Environmental Medicine.* pp. 178-185.

Ge, Z. and Taylor, D.E. (1996). *Helicobacter pylor*i genes hpcopA and hpcopP constitute a cop operon involved in copper export. *FEMS Microbiology. Letter.* pp. 181–188.

Gilotra, U. and Srivastava, S. (1997). Plasmid-encoded sequestration of copper by *Pseudomonas pickettii* strain US321. *Current Microbiology.* pp. 78–381.

Gordon, A.S., Donat, J.R., Kango, R., Dyer, B. and Stuart, L. (2000). Dissolved copper-complexing ligands in cultures of marine bacteria and estuarine water. *Marine Chemistry.* pp. 149-160.

Grobler, D.G. (1999). Copper poisoning in wild ruminants in Kruger National Park: Geobotanical and environmental investigation. *Onderstepoort Journal of Veterinary Research.* pp. 81-93.

Harris, E.D. (2000). Cellular copper transport and metabolism. *Annual Review of Nutrition.* pp. 291-310.

Haung, Q. Chen, W. and Xu, L. (2005). Adsorption of copper and cadmium by Cu and Cd resistant bacteria and their composites with soil colloids and kaolinite. *Journal of Geomicrobiology.* pp. 227-236.

Holmes, D.S., Dubey, S.K. and Gangolli, S. (1994). Development of biosensors for the detection of mercury and copper ions. *Environmental Geochemistry. Health.* pp. 229-233.

Hoshio, N., Kimura, T., Yamaji, A. and Ando, T. (1999). Damage to the cytoplasmic membrane of *Escherichia coli* by catechin-copper complexes. *Free Radical Biology and Medicine.* pp. 1245-1250.

Innovative Technology Group (2003). Bioaugmentation. Conestoga-Rover and Associates. pp.1-2.

Jain, R.K. (1990). Copper resistant micro-organisms and their role in the environment. *World Journal of Microbiology and Biotechnology.* pp. 356-365.

Jansson, J.K. and de Bruijn, F.J. (1999). Biomarker and bioreporters. In:Davies, J. (Ed.). Manual of Industrial Micrbiology and Biotechnology, second Edition. ASM Press, Washington, DC, pp. 651-655.

Kilinc, A.S., Senol, A., Banu, K., Ozge, O. and Baha, B.H. (1990). Development of biosorption-based algal biosensor for Cu(II) using *Tetraselmis chuii. Sensors and actuators, B: Chemical.* pp.273-278.

Kille, P. Hemmings, A. and Lunney, E.A. (1994). Memories of metallothionein. *Biochimical and Biophysica Acta*. pp. 151-212.

Lovley, D.R. (2000). Environmental Microbe-Metal Interactions, ASM Press, Washington, DC. pp. 257-258.

Lutsenko, S. and Kaplan, J.H. (1996). Organization of P-type ATPases: Significance of structural diversity. *Biochemistry.* pp. 607–613.

McCall, J., Gunn, J. and Struik, H., (1995). Photo interpretative study of recovery of damaged lands near the metal smelters of Sudbury, Canada. *Water, Air and Soil Pollution.* pp. 847–852.

Medscape – http://www.medscape.com [Accessed January 30, 2008].

Miranda, C.D. and Rojas, R. (2006). Copper accumulation by bacteria and transfer to scallop larvae. *Marine pollution bulletin.* pp. 293-300.

Munson, G.P., Lam, D.L., Outten, F.W. and O'Halloran, T.V. (2000). Identification of a copper-responsive two-component system on the chromosome of *Escherichia coli* K12. *Journal* of *Bacteriology*. pp. 5864-5871.

Mutoh, N. and Hayashi, Y. (1988). Isolation of mutants of *Schizosaccharomyces pombe* unable to synthesis cadystin, small cadium-binding peptides. Biochemical and *Biophysical Research Communication*. pp. 32-41.

Nies, D.H. (1999). Microbial heavy metal resistance. *Applied Microbiology and Biotechnology.* pp. 730-750.

Nies, D.H. (2000). Heavy metal-resistant bacteria as extremophiles: molecular physiology and biotechnological use of *Ralstonia* sp. CH34. *Extremophiles*. pp. 77-82.

Odermatt, A., Suter, H., Krapf, R. and Solioz, M. (1993). Primary structure of two P-type ATPases involved in copper homeostasis in *Enterococcus hirae. Journal of Biology and Chemistry.* pp. 12775–12779.

Oppenheimer Biotechnology (2003). www.obio.com/bioaugvsbiostim [Accessed December 02, 2008].

Plant Industry Division, <u>Alberta Agriculture, Food and Rural Development</u>. *Elston Solberg, Ieuan Evans, Doug Penny and Denise Maurice.* http://www.saanendoah.com/cudefsoil.html [Accessed October 06, 2008].

Qureshi, F.M., Badar, U. and Ahmed, N. (2001). Biosorption of copper by bacterial biofilm on PVC flexible conduit. *Applied and Environmental Microbiology.* pp.4349-4352.

Rosen, B.P. (2002). Transport and detoxification systems for transition metals, heavy metals and metalloids in eukaryotic and prokaryotic microbes. *FEMS Microbiology Review.* pp. 197-213.

Rouch, D., Camakaris, J. and Lee, B.T.O., in Metal–Ion Homeostasis: Molecular Biology and Chemistry (eds Hamer, D. H. and Winge, D. R.), Alan R. Liss, Inc., New York, 1989, pp. 45–105.

Silver, S. (1996). Bacterial resistance to toxic metal ions - a review. *Gene.* pp. 9-19.

Smith, D.L., Tao, T. and Maguire M.E. (1993). Membrane topology of a P-type ATPase. The MgB magnesium transport protein of *Salmonella typhimurium. Journal of Biology and Chemistry.* pp. 22469-22479.

Snavely, M.D., Florer, J.B., Miller, C.G. and Maguire, M.E. (1989). Magnesium transport in *Salmonella typhimurium*: $^{28}Mg^{2+}$ transport by CorA, MgtA, and MgtB systems. *Journal of Bacteriology.* pp. 4761–4766.

Solioz, M. and Stoyanov, J.V. (2003). Copper homeostasis in *Enterococcus hirae. FEMS Microbiology,* Reviews. pp. 183-195.

Terawaki, Y. and Rownd, R. (1972). Replication of R factor Rts1 in *Proteus mirabilis. Journal of Bacteriology.* pp. 492-498.

Timmis, K.N. and Pieper, D.H. (1999). Bacteria designed for bioremediation. *Division of Microbiology. NRCB.* pp. 200-204.

Trevors, J.T. and Cotter, C.M. (1989). Copper toxicity and uptake in microorganisms. *Journal of Industrial Microbiology.* pp 77-84.

UniProtKB/Swiss-Prot (2008). www.uniprot.org/uniprot/Q59385 [Accessed November 14, 2008].

WebElements www.webelements.com/copper [Accessed October 14, 2008]

White, C. and Gadd, G.M. (1986). Uptake and cellular distribution of copper, cobalt and cadmium in strains of *Saccharomyces cerevisiae* cultured on elevated concentrations of these metals. *FEMS Microbiology and Ecology.* pp. 277-283.

Chapter 2

Microbial diversity studies

2.1. Introduction

Owing to evolutionary distinctiveness of archaea, bacteria and eukarya, all known forms of life can be categorized into three primary domains namely, archaea, bacteria and eukarya (Woese, *et al.,* 1990). Furthermore, the evolutionary relationships of forms of life can be represented as a universal tree as shown in Figure 2.1. Analysis involved in nucleic acid-based information processing yields three domains of life and this three-domain tree has remarkable implications (Delong and Pace, 2001).
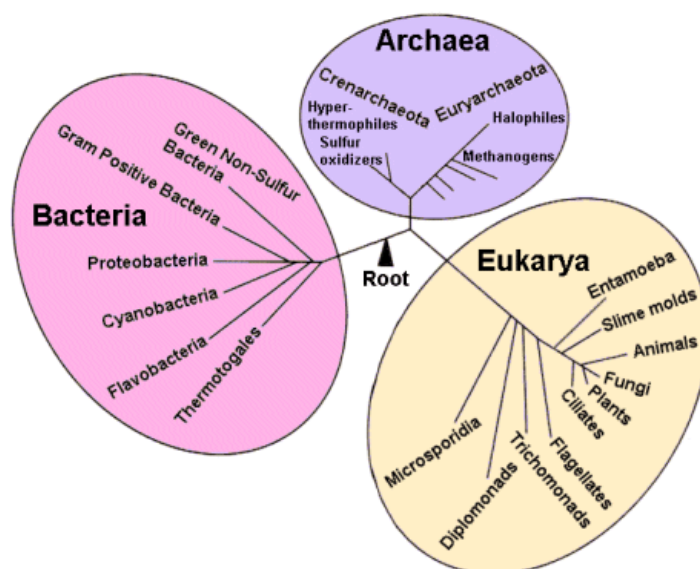
Figure 2.1. Three domains of life (Taken from Scienceblogs.com).

When an environment is contaminated with heavy metals, the function and structure of indigenous microflora automatically changes while the state of the environment depends on the activities of the micro-organisms (Madigan *et al.,* 1996). Geochemical properties of an environment are also very important parameters that should be included for the

characterization of a particular biosphere. These reveal the types and levels of minerals present as they influence microbial morphology and diversity (Madigan *et al.,* 1996).

Microbial species capable of resisting heavy metals such as copper will flourish in the contaminated site while those that cannot withstand the contamination diminish with time (Moon *et al.,* 2006). Copper resistant microbial species are able to colonize copper contaminated environments as metallophytes do (Whiting *et al.,* 2002). However, there is the possibility of abiotic factors besides elevated concentrations of copper that may limit the growth of micro-organisms. Revegetation might change the environment and affect microbial life (De la Iglesia *et al.,* 2006) which might lead to microbial diversification.

2.1.1. Molecular view of microbial diversity

The vast majority of reported studies on copper resistant micro-organisms were based mainly on classical methods such as isolation and cultivation techniques but were also limited by low cultivability of micro-organisms (Amann *et al.,* 1995). The molecular approach in the assessment of natural microbial ecosystems has on the other hand led to the discovery of many evolutionary lineages which are distantly and closely related to known organisms (Pace, 1997). This approach has a great advantage as it involves direct extraction of DNA from any environment which overcomes the limitations that are imposed by conventional cultivation methods (Moon *et al.,* 2006) since there are micro-organisms that are viable but not culturable. Culture-independent approaches have resulted in the effective study of effects of increase in copper concentrations on the whole microbial community (Osborn *et al.,* 2000).

The use of automated sequencing technology has helped in the discovery of new micro-organisms. Molecular sequences through molecular phylogeny are the basic tools that reveal the distribution and roles of organisms in the particular environment (Pace, 1997).

The objectives in this chapter were thus to use molecular techniques in determining the microbial populations of laboratory scale reactor simulating commercial scale processes samples and to identify the micro-organisms present in each reactor.

2.2. Materials and methods

2.2.1. Microbial diversity studies

The microbial populations of the industrial bioreactor samples were assessed and identified using denaturing gradient gel electophoresis (DGGE) and sequence analysis of 16S rDNA fragments amplified by PCR from extracted genomic DNA using universal bacterial and archaeal-specific 16S rDNA primers.

2.2.1.1. Samples

Two samples were received from MINTEK and were used for this study. (i) Sample 1: slurry from a bioreactor operated continuously at 70°C at pH 1-1.2, copper concentration of 16-18 g/l, and iron concentration of 20-25 g/l; and (ii) sample 2: slurry from a semi-batch bioreactor operated at 37°C at pH 1.3-1.8, copper concentration of 18-20 g/l, and iron concentration of 4-5 g/l.

2.2.1.2. 4 ', 6-diamidino-2-phenylindole (DAPI) staining of the samples

Slurry samples were filtered using 0.20 µm filters. Prior to filtering, the samples were mixed with a buffer solution [0.4 g/l $MgSO_4.7H_2O$, 0.2 g/l $(NH_4)_2SO_4$, 0.1 g/l KCl and 0.1 g/l K_2HPO_4] at pH 1.6 and fixed with 4% (final concentration) formaldehyde. These samples were then incubated at 4°C for 2 hrs followed by filtering. DAPI staining was performed by adding 10 µl of DAPI solution (10 µg/ml) onto each filters containing the fixed samples and left in the dark for 2 min at room temperature. The filters were then rinsed with sterile milliQ water and allowed to dry followed by the addition of 10 µl citifluor to reduce bleaching of the samples prior to viewing under an epifluorescence microscope equipped with a filter set 02 operating at wavelength 390 nm or above (Porter and Feig, 1980).

2.2.2. DNA preparations

2.2.2.1. Genomic DNA extraction

Cells from the bioreactor samples were concentrated by centrifugation at 5,000 X g at room temperature for 5 minutes, the supernatant was discarded and the genomic DNA was extracted using the FastDNA soil kit (Promega, Madison, USA) following the manufacturer's instructions as well as a method described by Labuschagne and Albertyn (2007). Loading dye (2 µl) (Fermentas) was added to each 5 µl extracted genomic DNA and the mixture was loaded onto a 0.8% agarose gel. The MassRuler[TM] DNA ladder (Fermentas) was used to visualize the extracted genomic DNA on the gel using the ChemDoc XRS (Biorad Laboratories) gel documentation system.

2.2.2.2. Preparation of agarose gels

Agarose powder was weighed off and 1 X TAE buffer (0.04 M Tris, 0.021 mM glacial acetic acid, 1 mM EDTA, pH 8) added to yield a 0.8% (w/v) agarose gel. This was boiled in a microwave oven until the agarose powder was completely dissolved. The mixture was cooled down to approximately 45°C and 3 µl Ethidium Bromide (EtBr) [10 mg/ml] added for visualization under UV illumination.

2.2.2.3. PCR reactions and conditions

16S rDNA fragments were amplified by PCR from extracted genomic DNA using universal bacterial primers and archaeal-specific primers respectively as set out in Table 2.1.

Table 2.1. Bacterial and archaeal 16S rDNA primers

PRIMERS	SEQUENCE	REFERENCE
Bacterial universal forward primer (27F)	5'-AGA GTT TGA TCM TGG CTC AG-3'	Lane 1991
Bacterial universal reverse primer (1492R)	5'-GGT TAC CTT GTT ACG ACT T-3'	Lane 1991
Archaeal universal forward primer (20bF)	20bF 5'- YTC CSG TTG ATC CYG CSR GA 3'	Rincon *et al.,* 2008
Archaeal universal reverse primer (1090R)	5'-TGG GTC TCG CTC GTT G-3'	Barns *et al.,* 1994

Each of the PCR reaction mixtures contained: 1 µl forward primer (10 µM), 1 µl reverse primer (10 µM), 5 µl 10X buffer, 1 µl template DNA (100 ng/ml), 1 µl dNTPs (10 µM) and the volume was adjusted to a final volume of 50 µl with sterile milliQ water. PCR cycling started with a 5 min hot start step at 95°C of the gDNA-containing master mix. Thereafter, the cycling was stopped and 0.5 µl *Taq* polymerase (5 units/µl) enzyme added and cycling resumed. Amplifications were run for 30 cycles in a thermal cycler pXe 0.2 Thermal Cycler (Thermo electron) after an initial denaturation at 95°C for 5 min. Each cycle was run at 95°C for 30 sec, 51°C for 45 sec and 72°C for 90 sec and final extension, 72°C for 10 min. The same conditions were used for the archaeal amplification except for annealing temperature which was 55°C.

Each PCR product (10 µl) was added to 2 µl loading dye (Fermentas) before loading onto a 1% agarose gel. The MassRuler[TM] DNA ladder (Fermentas) was used to determine the size of bands visualized on the gel using the ChemDoc XRS (Biorad Laboratories) gel documentation system.

The PCR products (20 µl) were again loaded onto 1% agarose gel, the bands corresponding to 1500 bp excised under a low frequency UV-light and purified using GFX^{TM} PCR DNA Purification Kit (Amersham Biosciences, UK).

2.2.2.4. Cloning into pGEM$^®$–T Easy vector and restriction fragment length polymorphism

The 16S PCR fragments were purified and ligated into a pGEM$^®$–T Easy vector according to the manufacturer's instruction (Promega) using T4 DNA ligase from Fermentas Life Sciences (Vilnius, Lithuania). For high efficiency, the ligation reaction was performed overnight at 4°C. The ligation reactions were then transformed into *E. coli* Top10 competent cells, followed by small scale plasmid isolation using the Gene JET ^{TM} plasmid Miniprep Kit according to the manufacturer's instruction (Fermentas). This was followed by a restriction digest on the purified plasmids containing the inserts of interest using *Eco*RI endonuclease as well as a double digest with *Hin*dIII *and Eco*RI endonucleases for assessment of restriction patterns and insert sizes which partially reveal the diversity or microbial population of the samples. The digests were thereafter evaluated.

Table 2.2. Ligation mixture composition for the pGEM®–T Easy vector system

Component	Sample Volume (µl)	Control Volume (µl)
2X Rapid ligation buffer	5	5
pGEM®–T Easy vector (50 ng)	0.8	1
PCR product (75 ng)*	0.8	-
pUC DNA	-	1
T4 DNA ligase (3 Weiss units/µl)	1	1
Sterilised Milli Q H_2O	2.4	1
Total	10	10

* Depending on the fragment length and using the molar ratio 1:3 of insert DNA : vector.

A positive control test was performed to test the efficiency of the *E. coli* Top10 competent cells (host cell) and ligated in the same manner as the PCR products of interest.

2.2.2.4.1. Transformation

E. coli competent cells were prepared using rubidium chloride method (Hanahan, 1983). A single colony was used to inoculate 100 ml Psi media (5 g/l yeast extract, 20 g/l tryptone and 5 g/l magnesium sulfate, pH 7.6) followed by incubation at 37°C until the optical density OD_{550nm} was 0.48. The cells were cooled on ice for 15 min before centrifugation (5000 x *g* for 5 min) and then washed with 40 ml Tfb 1 buffer (potassium acetate 30 mM, rubidium chloride 100 mM, calcium chloride 10 mM, manganese chloride 50 mM and glycerol 15% v/v, pH 5.8). The pelleted cells were resuspended in 4 ml Tfb II buffer (MOPS 10 mM, rubidium chloride 10 mM, calcium chloride 75 mM, manganese chloride 50 mM and glycerol 15% v/v, pH 6.5), and 50 µl aliquots were stored at -80°C. For transformation, *E. coli* competent cells (50 µl) were mixed with 2 µl of the plasmid or ligation mixture and incubated on ice for 30 min. The transformation mixture was then heat-shocked at 42°C for 40 sec, followed by cold shock on ice for 2 min after which 700 µl LB broth containing 50 µl 2 M Magnesium and 100 µl glucose was added and the mixture incubated for 1 hour with shaking at 37°C. The cells (50 µl) were plated onto LB

plates supplemented with ampicilin (60 mg/l); IPTG [isopropylthio-β-D-galactoside] (48 mg/l) and X-gal [5-bromo-4-chloro-3-indolyl-β-D-galactoside] (40 mg/l). Ampicillin was used as the antibiotic of choice for the selection of positively transformed colonies plated out on AIX plates. The plates were incubated at 37°C for 16 h followed by selection of white colonies. The transformants were selected and the representative colonies (10 per plate) inoculated into 5 ml LB medium supplemented with 50 µl of the 10 mg/ml stock ampicillin to provide the adequate antibiotic pressure. These were incubated at 37°C for 16 h while shaking at 175 rpm.

2.2.2.4.2. Plasmid isolation

The plasmids from transformed cells were isolated according to the Gene JET ^{TM} plasmid Miniprep Kit manual (Fermentas) provided prior to restriction fragment length polymorphism analysis.

2.2.2.4.3. Restriction Fragment Length Polymorphism (RLFP)

The restriction digest was performed on the purified plasmids containing the inserts of interest using *Eco*RI endonuclease, as well as the mixture of *Eco*RI and *Hin*dIII endonucleases in order to assess the inserts through the sizes of the bands obtained.

A 1% (w/v) agarose gel was prepared and loading dye (Fermentas) added to 10 µl restriction digest. The MassRuler^{TM} DNA ladder (Fermentas) was used to determine the size of bands visualized on the gel using the ChemDoc XRS (Biorad Laboratories) gel documentation system.

2.2.2.4.4. Sequencing analysis

The selected clones were amplified using the SP6 forward and T7 reverse sequencing primers. The PCR products were then purified using the SigmaSpin™ Post-Reaction Clean-up Column (Sigma) and sequenced with the ABI 377 Genetic Analyser (Applied

Biosystems) at the Department of Microbial, Biochemical and Food Biotechnology, University of the Free State.

2.2.2.5. Microbial population studies using DGGE

Separation of PCR amplified 16S rDNA products using denaturing gradient gel electophoresis (DGGE) was done as a means to study microbial composition of the industrial bioreactor samples as this helps to enumerate the micro-organisms present. The PCR-DGGE steps include: extraction of total community DNA from samples; PCR-controlled amplification using specific synthetic oligonucleotides as set out in Table 2.3 and separation of PCR amplicons using DGGE. The different bands obtained were excised followed by reamplification using a set of bacterial and archaeal primers. These were then sequenced and the identities were revealed using BLAST at NCBI alogarithm.

Table 2.3. Bacterial and archaeal primers for DGGE

PRIMERS	SEQUENCE	REFERENCE
Bacterial forward primer (341F-GC clamped)	5'- CCT ACG GGA GGC AGC AG -3'	Muyzer *et al.*, 1993
Bacterial reverse primer (517R)	5'- ATT ACC GCG GCT GCT GG -3'	Muyzer *et al.,* 1993
Archaeal forward primer (344F)	5'- ACG GGG YGC AGC AGG CGC GA -3'	Muyzer *et al.,* 1993
Archaeal reverse primer (517R)	5'- ATT ACC GCG GCT GCT GG -3'	Muyzer *et al.,* 1993

* Bacterial GC-clamp: 5'- CGC CCG CCG CGC GCG GCG GGC GGG -3'

* Archaeal GC-clamp: 5'-CGCGCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG-3'

2.2.2.5.1. Nested PCR

Amplification of partial 16S rDNA fragments was done using primer sets as shown in Table 2.3 and amplified 16S rDNA for both bacteria and archaea were used as templates. The PCR conditions for the short fragments were: initial denaturation at 95°C for 5 min, followed by 25 cycles of amplification, which consisted of the three steps each

having 95°C denaturation for 45 min, annealing at 55°C for 45 min and extension of primers at 72°C for 1 min. Final extension was done at 72°C for 10 min. The same PCR conditions were used for archaea except for the annealing temperature which was 50°C. The PCR products obtained were first evaluated on a 1% agarose gel, followed by DGGE as described in section 2.2.2.5.2.

2.2.2.5.2. Denaturing gradient gel electophoresis (DGGE)

The principle of denaturing gradient gel electrophoresis is based on separation of the PCR amplicons of equal length in a sequence-specific manner using polyacrylamide gel which contains a denaturing gradient of urea and formamide. DGGE detects melting patterns of small DNA fragments (200-700 bp) that differs by as little as a single base substitution. Denaturing conditions usually allow the fragment to completely dissociate into single strands.

The prepared stock solutions include: 0% urea-formamide [40% acrylamide/bis (10 ml), 50x TAE (1 ml), and sterile milliQ water (39 ml)]; 80% urea-formamide [40% acrylamide/bis (10 ml), 50x TAE (1 ml), formamide (16 ml), urea (16.8 g) and was filled up to 50 ml with sterile milliQ water]. The gradient solutions used for this study were 30% UF [6.25 ml 0% UF stock solution and 3.75 ml 80% UF stock solution] and 70% UF [1.25 ml 0% UF stock solution and 8.75 ml 80% UF stock solution] with a stacking 0% UF. To each gradient solution, APS (63 µl) and TEMED (7 µl) were added. The 8% acrylamide/bis gel was cast, after gel polymerization, the inserted comb removed and the polymerized gel released from the casting stand and placed into the pre-heated buffer [140 ml filtered 50x TAE]. The PCR products were loaded and allowed to run for a minimum of 3 h and followed by gel staining with SYBR Gold solution for 15 min, washed with distilled water subsequently evaluated using ChemDoc XRS (Biorad Laboratories) gel documentation system under a short UV light.

Different bands obtained were excised, autoclaved milliQ water (50 µl) was added to each band in a 1.5 ml tube and incubated overnight at 55°C. To increase the concentrations of the amplicons, reamplification using bacterial and archaeal primers

sets as in Table 2.3 but without the GC-clamp was performed followed by sequencing and identification using BLASTn alogarithm at NCBI.

2.3. Results and discussions

2.3.1. DAPI staining

DAPI staining was completed as described in section 2.2.1.2, and the epifluorescence microscopy revealed the presence of cells in both samples A (70°C bioreactor) and B (37°C bioreactor). No counts were done but the same volume was used for analysis.
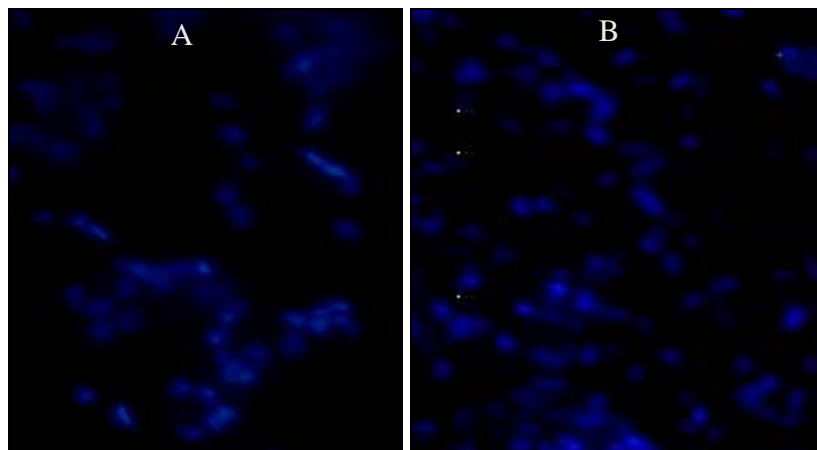
Figure 2.2. DAPI staining showing the cells obtained from the (A) 70°C and (B) 37°C industrial bioreactors (Magnification X100).

2.3.2. Genomic DNA extraction

The FastDNA Spin soil kit as described in section 2.2.2.1 was used for the extraction of genomic DNA from these samples. The extracted genomic DNA was loaded onto a gel and visualized under low UV light as shown in Figure 2.3.
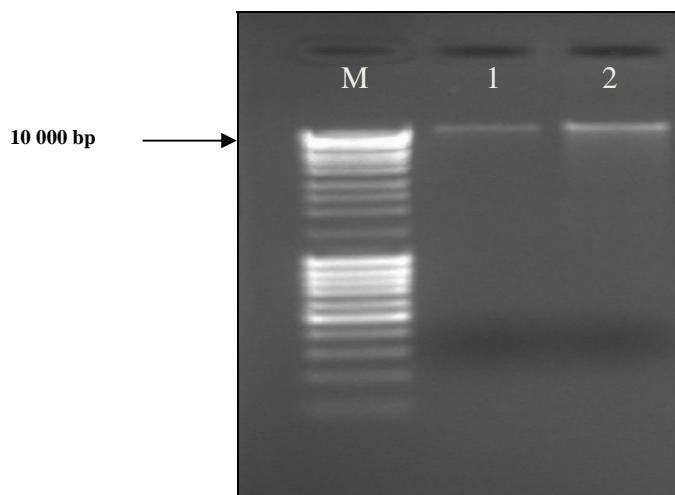
Figure 2.3. Genomic DNA extracted from bioreactors samples. Lane M, MassRulerTM DNA ladder (Fermentas); Lanes 1 and 2, genomic DNA from the 37°C and 70°C bioreactors.

The concentrations of the extracted genomic DNA were measured using a NanoDrop Spectrophotometer (ND-1000). The concentrations obtained from the genomic DNA extracted from 37°C and 70°C bioreactor samples were 109 ng/ml and 111 ng/ml. After separation of the DNA on a gel, the genomic DNA was successfully extracted at sufficient concentrations and these were used for further studies.

2.3.3. 16S rDNA characterisation

The 16S rDNA fragments were amplified as described in section 2.2.2.3 using universal bacterial primers as well as universal archaeal primers and the corresponding bands were obtained. The size for the bacterial amplicon was 1,500 base pairs and 1,030 base pairs of 16S rDNA archaeal amplicon with the universal primers (Figure 2.4A and 2.4B).
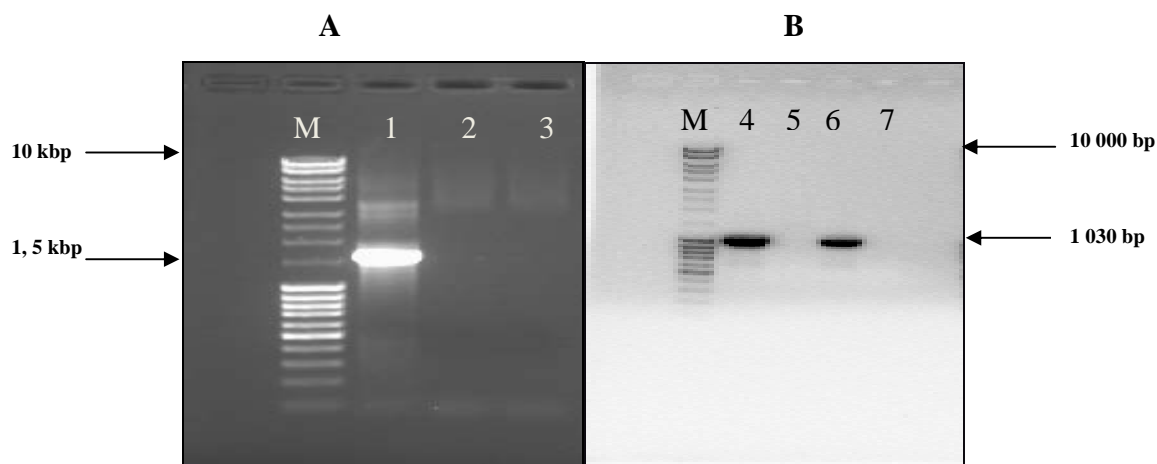
Figure 2.4A. PCR amplification of the bacterial 16S rDNA fragments using total genomic DNA isolated from industrial bioreactor. Lane M, MassRulerTM DNA ladder (Fermentas); Lane 1, sample from 37°C; lane 2, sample obtained from 70°C bioreactors and lane 3, negative control (sterile milliQ water). Figure 2.5B. PCR amplification of archaeal 16S rDNA using total genomic DNA isolated from industrial bioreactor. Lane M, MassRulerTM DNA ladder (Fermentas); lane 4, genomic DNA sample containing archaeal DNA (positive control) lane 5, genomic DNA isolated from 37°C bioreactor; lane 6, archaeal 16S rDNA amplicon obtained from genomic DNA isolated from 70°C bioreactor and lane 7, non-template control (negative control).

As mentioned previously, 16S rDNA fragments of the bacteria and archaea were amplified, there was no amplification of 16S rDNA fragment in the second lane as no archaea should be present in the 37°C bioreactor and fifth lane where no bacteria is expected in the 70°C bioreactor.

2.3.4. Restriction Fragment Length Polymorphism (RFLP)

RFLP was performed on selected clones containing the bacterial and archaeal 16S PCR products as described in section 2.2.2.4.3. Bacterial clones digested with *Eco*RI gave two RFLP patterns while double digesting using both *Eco*RI and *Hin*dIII endonucleases yielded three bands. Also, the results obtained from the digest of archaeal clones with *Eco*RI, and the combination of *Eco*RI and *Hin*dIII endonucleases resulted in one band pattern. Figure 2.5A shows the possibility of having at least two species present in the 37°C bioreactor while the results obtained from double digest as depicted in figure 2.5B confirms the presence of three organisms.

33

A
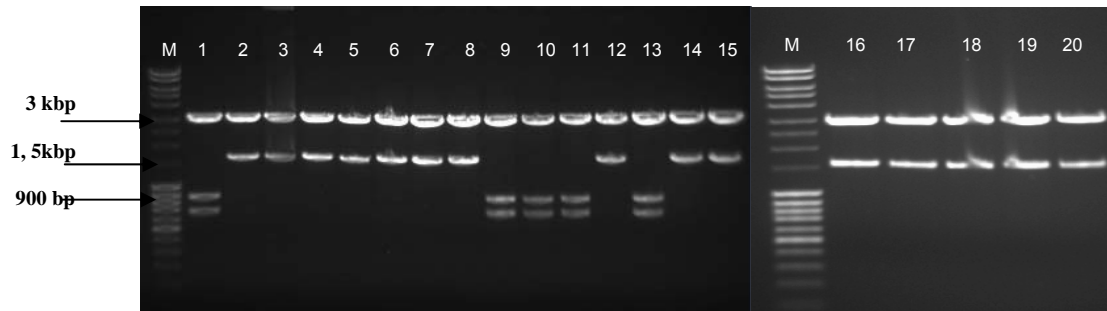
Figure 2.5A. Gel electrophoresis of RFLP patterns obtained from bacterial clones. Gel electrophoresis of RFLP patterns obtained from bacterial clones when digested with *Eco*RI. Lanes M, MassRulerTM DNA ladder (Fermentas) and 1-20, 16S rDNA restriction digest.

B

B

Figure 2.5B. Gel electrophoresis of RFLP patterns obtained from bacterial clones when digested with *Eco*RI and *Hin*dIII. Lane M, MassRulerTM DNA ladder (Fermentas) and lanes 1-20, 16S rDNA restriction digests.

Besides the backbone (pGEM®–T Easy vector) corresponding to 3,000 bp, the first RFLP band corresponds to 850 bp and 650 bp while the second band has one restriction site that corresponds to 1,500 bp and the third banding pattern corresponds to approximately 1,300 bp and 250 bp.

The results obtained from RFLP analysis performed on archaeal 16S clones suggested that there may be one species present in the 70°C bioreactor or one species dominating the bioreactor. As shown in figure 2.6A and 2.6B, one banding pattern was obtained with the single digest as well as double digest.

A

Figure 2.6A. Gel electrophoresis of RFLP patterns obtained from archaeal clones using *Eco*RI as a restriction digest enzyme. Lanes M, MassRulerTM DNA ladder (Fermentas) and lanes 1-20, 16S rDNA restriction digests.

B
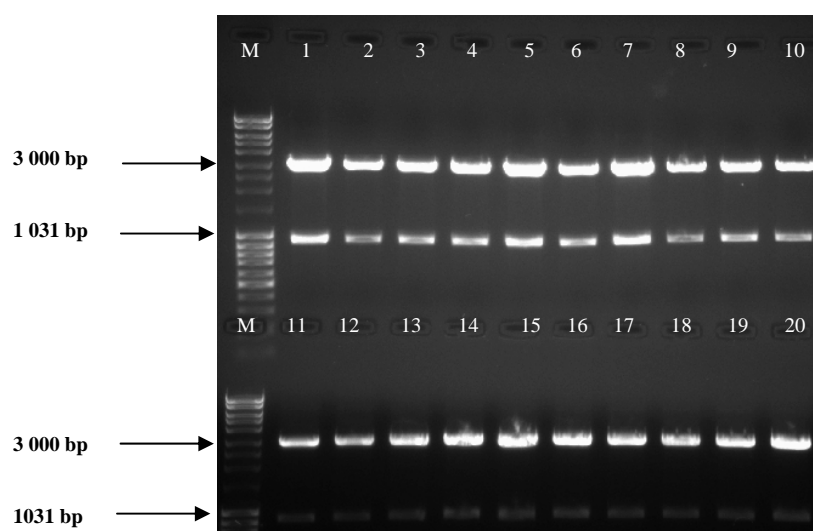
Figure 2.6B. Gel electrophoresis of RFLP patterns obtained from archaeal clones using *Eco*RI and *Hin*dIII as restriction digest enzymes. Lanes M, MassRulerTM DNA ladder (Fermentas) and lanes 1-20, 16S rDNA restriction digests.

The banding pattern obtained corresponds to 1,030 bp while 3,000 bp is pGEM®–T Easy vector usually referred to as the backbone.

2.3.5. Denaturing gradient gel electophoresis (DGGE)

The PCR fragments are allowed to separate on a low to high denaturant gradient acrylamide gel where initially the fragments move according to molecular weight. As

36

these fragments progress into higher denaturing conditions, depending on its sequence composition, each reaches a certain point that the DNA begins to melt. The partial melting retards the progress of the molecule in the gel, and there is a mobility shift which can differ for slightly different sequences.

Nested PCR was performed and 200 base pairs of bacterial and archaeal 16S rDNA fragments amplified with sets of primers specific for bacteria and archaea prior to DGGE (Figure 2.7A and 2.7B).
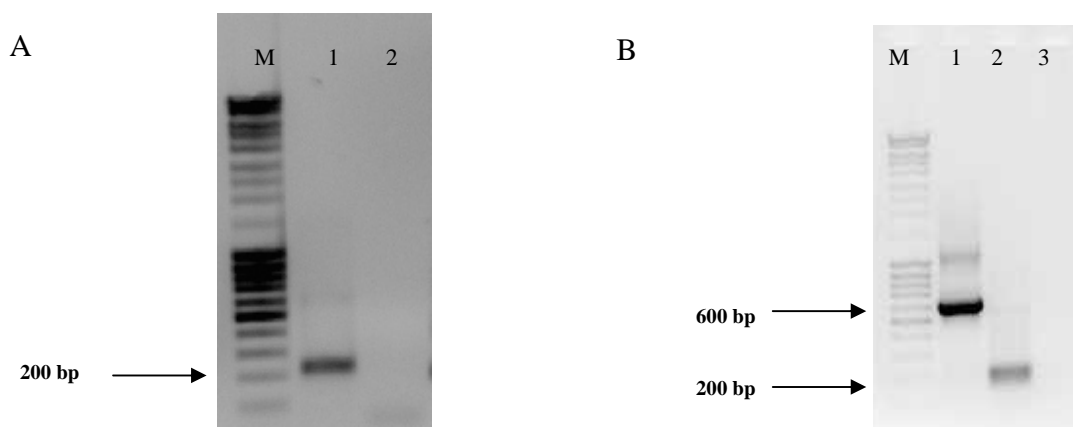
Figure 2.7A. Amplicons obtained with bacterial 341F and 517R GC-clamped primers. Lane M, MassRulerTM DNA ladder (Fermentas); Lane 1 bacterial amplicon and lane 2, non-template control. Figure 2.7B. Amplicons obtained using archaeal 344F, 915R and 517R GC-clamped primers. Lane M, MassRulerTM DNA ladder (Fermentas); Lane 1 archaeal amplicon (344F and 915R) Lane 2 archaeal amplicon (344F and 517R) and Lane 3, non-template control.

The fingerprint obtained from DGGE analysis of the domain bacteria has three prominent bands and two faint bands (Figure 2.8A) hence, the possibility of having at least three organisms present in the 37°C bioreactor while one prominent band and some faint bands (Figure 2.8B) were obtained for archaeal domain, indicating there could be one organism dominating the 70°C bioreactor.
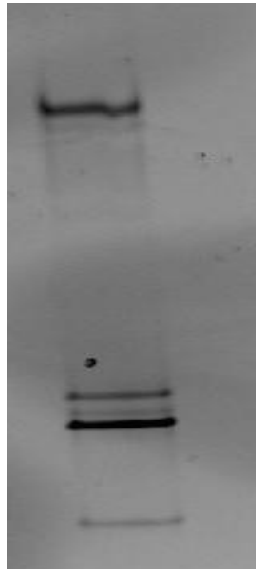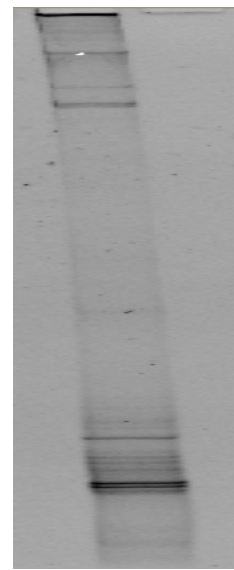
Figure 2.8. Banding patterns from DGGE analysis. A: domain bacteria, and B: domain archaea.

Each band obtained from DGGE was excised from the gel and reamplified to obtain high concentrations of the amplicons with specific primer sets (Figure 2.9A and 2.9B) followed by sequencing analysis.
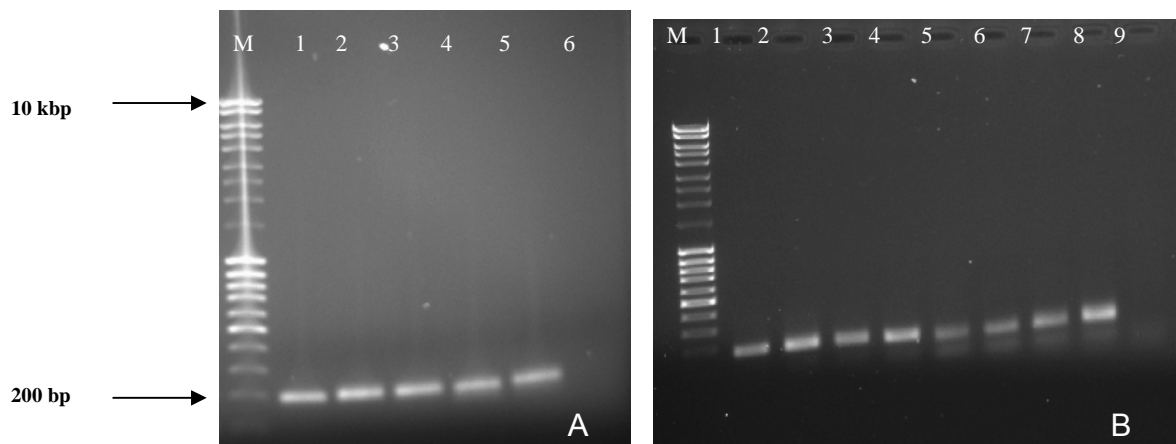
Figure 2.9A. Gel electrophoresis of amplicons obtained with bacterial 341F and 517R primers. Lanes M, MassRulerTM DNA ladder (Fermentas); lane 1-5, bacterial amplicons; lane 6, non-template control and 2.9B. Electrophoresis of amplicons obtained using archaeal 344F and 517R primers. Lane 1- 8, archaeal amplicons; lane 9, negative control (sterile milliQ water).

Expected sizes of bacterial and archaeal amplicons which were 200 base pairs were obtained after reamplification using respective set of primers, while no amplification was obtained for each of the negative control, since sterile milliQ water was used as a template for the negative control. The bacterial and archaeal amplicons were then prepared for sequencing analysis.

2.3.6. Identification of micro-organisms from industrial bioreactors

2.3.6.1. Sequencing analysis

Sequencing PCR was performed on the selected 16S rDNA clones and amplicons obtained from DGGE bands and sequenced with the ABI 377 Genetic Analyser (Applied Biosystems) at the Department of Microbial, Biochemical and Food Biotechnology, University of the Free State. The files were retrieved and compared to sequences deposited in the NCBI (National Center for Biotechnology Information) database. The BLASTn analysis yielded sequences similar to acidophiles as shown in Tables 2.4 and 2.5. Also, the same results were obtained for 16S rDNA clones and DGGE amplicons.

Table 2.4. Sequences producing significant alignments for DGGE analysis and 16S rDNA clones of the 37°C bioreactor sample

Band	Accession	Description	Max score	Total score	Query coverage	E value	Max ident
1	AF137503.2	*Sulfobacillus thermosulfidooxidans* subsp. Asporogenes 16S ribosomal RNA gene, partial sequence	252	252	95%	3e-64	96%
2	AF137503.2	*Sulfobacillus thermosulfidooxidans* subsp. Asporogenes 16S ribosomal RNA gene, partial sequence	250	250	91%	1e-63	97%
3	EF025342.1	*Leptospirillum ferriphilum* strain FTH 16S ribosomal	187	187	93%	1e-44	88%
4	AY427958.1	*Acidithiobacillus caldus* strain MTH-04 16S ribosomal RNA gene, partial sequence	896	896	96%	0	100%

The result obtained showed the presence of at least three bacterial species in the 37°C bioreactor sample which corresponds to the suggested inoculum MINTEK uses.

Table 2.5. BLASTn alogarithm results for DGGE and 16S rDNA clones sequencing analysis of the 70°C bioreactor sample

Band	Accession	Description	Max score	Total score	Query coverage	E value	Max ident
1	D26489.1	*Acidianus brierleyi* strain 16S rRNA gene	101	101	98%	6e-19	96%
2	EF522786	*Metallosphaera sedula* 16S rRNA	121	121	97%	7e-25	97%
3	DQ350777.1	*Sulfobales Archaeon* KOS01 16S rRNA gene partial sequence	127	127	96	2e-22	96

The BLASTn analysis also showed that at least three archaeal species were present in the 70°C bioreactor sample which correlated with the suggested organisms by MINTEK.

2.4. Conclusions

Molecular approaches were used to characterize micoflora present in the industrial bioreactors that are used for bioleaching of metals such as copper. Samples from industrial bioreactors operated at 37°C and 70°C contained acidophiles which suggests that the reactors are operated at acidic pH. After 16S rDNA amplification both reactor communities were defined. The BLASTn results obtained from the selected clones of the 37°C bioreactor revealed the presence of *Leptospirillum* sp. an iron-oxidising micro-organism in 37°C bioreactor, together with a sulphur-oxidising bacterium namely, *Acidithiobacillus caldus* and *Sulfobacillus* sp., an iron and sulphur oxidising micro-organism. This was confirmed by RFLP analysis which showed different digesting patterns. The 70°C bioreactor sample contained S*ulfolobus* sp., *Acidianus* sp. and *Metallosphaera* sp. as confirmed by the BLASTn analysis results. The DGGE analysis, however, indicated that a wider population may be present in these two biorectors.

Also, the results obtained from 16S rDNA amplification showed that the bioreactor operated at 37°C does not contain archaea while no bacteria were present in the

70°C bioreactor. The presence of these organisms in a consortium usually increases the efficiency of bioleaching (Zeng *et al.,* 2008).

2.5. References

Amann, R.I., Ludwig, W.and Schleifer, K.H. (1995). Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiology Review.* pp. 143-169.

Barns, S.M., Fundyga, R.E., Jeffries, M.W. and N.R. Pace. (1994). Remarkable archaeal diversity detected in Yellowstone National Park hot spring environment. *Proceedings of the National Academy of Sciences of the United States of America.* pp. 1609–1613.

De la Iglesia, R., Castro,D., Ginocchio, R., van der Lelie, D. and Gonza´ lez, B. (2006). Factors influencing the composition of bacterial communities found at abandoned copper-tailings dumps. *Journal of Applied Microbiology.* pp. 537-544.

DeLong, E.F. and Pace N.R. (2001). Environmental diversity of bacteria and archaea. *Systematic Biology.* pp. 470-478.

Hanahan, D. (1983). Studies on transformation of *E. coli* with plasmid. *Journal of Molecular Biology.* pp. 557-580.

Labuschagne, M. and Albertyn, J. (2007). Cloning of an epoxide hydrolase-encoding gene from *Rhodotorula mucilaginosa* and functional expression in *Yarrowia lipolytica. Yeast.* pp. 69-78.

Lane, D.J. (1991). 16S/23S rRNA sequencing. In nucleic acid techniques in bacterial systematics, Ed. Stackenbrandt, E. and Goodfellow, M. New York, NY: John Wiley & Sons. pp. 115–175.

Madigan, M.T., Martinko, J.M. and Parker, J. (1996). Brock Biology of micro-organisms. Prentice-Hall, Upper Saddle River, NJ, Ed.8. pp. 983-985.

Moon, H.S., Kahng, H.Y., Kim, J.Y., Kukor, J.J. and Nam, K. (2006). Determination of biodegradation potential by two culture-independent methods in PAH-contaminated soils. *Environmental Pollution.* pp. 536-545.

Muyzer, G., De Waal, E.C., and Uitterlinden, A.G. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied Environmental Microbiology.* pp. 695-700.

Osborn, A.M., Moore, E.R. and Timmis K.N. (2000). An evaluation of terminal-restriction fragment length polymorphism (T-RFLP) analysis for study of microbial community structure and dynamics. *Environmental Microbiology.* pp39-50.

Pace, M.R. (1997). A molecular view of microbial diversity and the biosphere. *Science.* pp. 734-740.

Porter, K.S. and Feig, Y.S. (1980). The use of DAPI for identifying and counting aquatic microflora. *Society of Limnology and Oceanography.* pp. 943-948.

Rincón, B., Borja, R., González, J.M., Portillo, M.C. and Sáiz-Jiménez, C. (2008). Influence of organic loading rate and hydraulic retention time on the performance, stability and microbial communities of one-stage anaerobic digestion of two-phase olive mill solid residue. *Biochemical Engineering.* pp. 253-261.

Scienceblogs.com [Accessed 19 February, 2009].

Whiting, S.N., Reeves, R.D. and Baker, A.J.M. (2002). Mining, metallophyes and land reclamation. *Mining Environmental. Management.* pp. 11-16.

Woese, C.R., Kandler, O. and Wheelis, M.L. (1990). Towards a natural system of organisms: Proposal for the domains archaea, bacteria and eucarya. *Proceedings of the National Academy of Sciences* of the United States of America. pp. 4576-4579.

Zeng, W.M., Zhou, H.B., Wan, M.X. Chao, W.L., Xu, A.L., Liu, X.D. and Qiu, G.Z. (2008). Preservation of *Acidithiobacillus caldus*: A moderately thermophilic bacterium and effect on susbsequent bioleaching of chalcopyrite. *Hydrometallurgy*. pp. 333-336.

Chapter 3

Copper resistant micro-organisms

3.1. Introduction

Although the mode of toxicity of copper is not fully understood, it was noted that toxicity of copper ions in living organisms such as micro-organisms varies with the environment as well as the organisms. For example, the South African water quality standard prescribes the concentration of copper safe for human consumption to be <30 ppm (0.472 mM) Sheldon and Menzies (2004) reported that the minimum range of copper concentrations in soil found to cause the death or prevention of plant growth is <60 - 125 ppm (0.944 - 1.96 mM), while 62 ppm (0.976 M) of copper ions present in water is safe for micro-organisms to dwell (Domestic water use, 1996).

Micro-organisms capable of resisting high copper concentrations have varying capacities. This can be evaluated by obtaining the minimum inhibitory concentrations (MICs) of copper exhibited by the micro-organisms. MIC is defined as the minimum concentration that inhibits growth of micro-organisms (Miranda and Rojas, 2006). Nies (1999) demonstrated that copper resistant *Escherichia coli* exhibited a MIC at 1.0 mM while Bender and Cooksey (1986) showed that from 1.6 to 2.0 mM there was growth inhibition of *Pseudomonas syringae* pv. tomato strain. There are some factors that may influence the toxicity of copper and these include culture media amongst others. Ramamoorthy and Kushner (1975) showed that copper ions present in the rich media are complexed by some media components thereby reducing the availability of copper ions in the medium. Hence, increasing the value obtained for minimum inhibitory copper

concentrations exhibited by the resistant micro-organisms. Trevors and Cotter (1989) stated with reference to Heukekle and Gellman (1955) findings that another factor that may affect availability of copper is pH. Adjustment of pH from pH 4 to pH 6 results in the detection of only 43% of initial copper(II).

The objectives of this chapter were therefore to determine the minimum inhibitory copper concentration exhibited by the consortia of micro-organisms present in the industrial bioreactor samples as well as the MIC of copper exhibited by individual isolates.

3.2. Materials and methods

3.2.1. Media design

The samples were filtered using 0.20 µm filters to concentrate the cells and inoculated into the media described in Table 3.1. All of the prepared media were autoclaved before use.

Table 3.1. Media composition used for inoculation of the 37ºC and 70ºC bioreactor samples

37ºC microbial selective media			
(A) *Sulfobacillus* DMSZ, medium 812.	Components	g	Suitable medium for *Sulfobacillus* sp., and *Acidithiobacillus* sp.
	$(NH_4)_2SO_4$	3.0	
	KCl	0.1	
	KH_2PO_4	0.5	
	$MgSO_4.7H_2O$	0.5	
	$Ca(NO_3)_2.4H_2O$	0.1	
	yeast extract	0.1	
	All components are dissolved in distilled water and made up to 1000 ml at pH 2		
(B) *Leptospirillum* DMSZ, medium 882.	Solution A: Components	g	Suitable medium for *Leptospirillum*
	$(NH_4)_2SO_4$	0.153	
	$MgCl_2.6H_2O$	0.053	
	KH_2PO_4	0.027	

	CaCl$_2$·2H$_2$O	0.147	
	Components dissolved in distilled water, made up to 950 ml and adjusted to pH 1.8 with 10N H$_2$SO$_4$		
	Solution B: Components	g	
	FeSO$_4$·7H$_2$O	20.0	
	Dissolved 50 ml H$_2$SO$_4$, 0.25N adjusted to pH 1.2		
	Trace element solution: Components	g	
	MnCl$_2$· 2H$_2$O	0.062	
	ZnCl$_2$	0.068	
	CoCl$_2$·6H$_2$O	0.064	
	Na$_2$MoO$_4$	0.010	
	H$_3$BO$_3$	0.031	
	CuCl$_2$·2 H$_2$O	0.067	
	Components dissolved in distilled water, made up to 1000 ml and the pH adjusted to 1.8 with 10N H$_2$SO$_4$		

70ºC microbial selective media			
(C) *Sulfolobus* DMSZ, medium 88.	Components	g	
	(NH$_4$)$_2$SO$_4$	1.3	
	KH$_2$PO$_4$	0.28	
	MgSO$_4$·7H$_2$O	0.25	
	CaCl$_2$·2H$_2$O	0.07	
	FeCl$_3$·6H$_2$O	0.02	Suitable medium for *Sulfolobus* sp. and *Metallosphaera* sp.
	MnCl$_2$·4H$_2$O	1.8	
	Na$_2$B$_4$O$_7$·10H$_2$O	4.5	
	VoSO$_4$·2H$_2$O	0.03	
	ZnSO$_4$·7H$_2$O	0.22	
	CuCl$_2$·2H$_2$O	0.05	
	Na$_2$MoO$_4$·2H$_2$O	0.03	
	CoSO$_4$	0.01	

		Yeast extract	1.0	
		pH 2.0		
(D) *Acidianus* DMSZ, medium 150		Components	g	Suitable medium for *Acidianus* sp.
		$(NH_4)_2SO_4$	3.0	
		$K_2HPO_4.3H_2,O$	5.0	
		$MgSO_4.7H_2O$	5.0	
		KCl	1.0	
		$Ca(NO_3)_2$	0.01	
		Sulfur flowers	10.0	
	pH adjusted to 1.5 - 2.5 with 6N H_2SO_4			
	Yeast extract (10% w/v in distilled water) was autoclaved separately			

3.2.2. Molecular approach for selection of common cultivation media

3.2.2.1. DAPI staining

DAPI staining (4´-6-diamido-2-phenylindole) is a nonspecific fluorescent dye used to stain micro-organisms in a natural sample. Cells stained with DAPI usually fluorescence bright blue when bound to DNA at wavelength 390 nm or above.

DAPI staining was performed as in section 2.2.1.2 to check for the presence of cells in these cultures harvested on the ninth day. This was followed by extraction of genomic DNA and PCR amplification of 16S rDNA fragments.

3.2.2.2. Genomic DNA extraction and amplification of 16S rDNA

The bead beating method was used as described by Labuschagne and Albertyn (2007) to extract genomic DNA from the cultures from 37ºC and 70ºC incubators, followed by gel visualization as explained in section 2.2.2.1. Only bacterial 16S rDNA fragments were amplified by PCR using universal bacterial primers as listed in Table 2.1. (Lane *et al.,* 1991). The PCR reaction mixtures contained the following: 1 µl forward primer (10 µM), 1 µl reverse primer (10 µM), 5 µl 10X buffer, 1 µl template DNA, 1 µl dNTPs (10 µM) and the volume was adjusted to 50 µl with sterile milliQ water. PCR cycling started

with a 5 min hot start step at 95ºC of the gDNA-containing master mix. The cycling was paused; 0.5 µl *Taq* polymerase (5 units/µl) enzyme added and cycling resumed. Amplifications were run for 30 cycles in a thermal cycler pXe 0.2 Thermal Cycler (Thermo electron) after an initial denaturation at 95ºC for 5 min. Each cycle was run at 95ºC for 30 sec, 51ºC for 45 sec and 72ºC for 90 sec, and the final extension, 72ºC for 10 min. This was followed by gel visualization as described in 2.2.2.3.

3.2.2.3. Nested PCR and denaturing gradient gel electrophoresis

Amplification of partial 16S rDNA fragments was done using bacterial primer sets as mentioned in Table 2.1 and the PCR conditions for the short bacterial fragment as mentioned in section 2.2.2.5.1 was observed. The PCR product obtained was evaluated on a 1% agarose gel, followed by DGGE. The protocol for DGGE was followed as described in section 2.2.2.5.2, and the gel stained with SYBR Gold solution for 15 min, washed with distilled water followed by evaluation using ChemDoc XRS (Biorad Laboratories) gel documentation system under UV light.

Different bands obtained from the DGGE analysis were excised; the DGGE products present in the excised bands were eluted by the addition of autoclaved milliQ water (50 µl) and overnight incubation at 55ºC. To increase the concentrations of the amplicons, reamplification using bacterial primer sets 341F and 517R as described in Table 2.4 was done following the same PCR conditions as mentioned in section 2.2.2.5.1.

3.2.3. Determination of copper MIC of the micro-organisms

3.2.3.1. Inoculation into copper containing media

Copper sulphate solution was prepared and filter sterilized using 0.20 µm filter prior to the addition at different concentrations into the selected medium. Each pre-inoculum was inoculated into copper-containing medium with different concentrations varying from 20 mM – 300 mM and 400 mM – 1 M copper sulphate; a control solution (no copper sulphate) was also included. These were incubated at 37ºC for 7 days in a shaker rotating at 175 rpm.

3.2.3.2. Evaluation of micro-organisms present in the copper medium

After 9 days, genomic DNA was extracted as described by Labuschagne and Albertyn (2007). This was followed by PCR amplification of the 16S rDNA fragment and gel evaluation using ChemDoc XRS (Biorad Laboratories) gel documentation system. Nested PCR was also performed using bacterial primer sets as given in Table 2.3. The same PCR protocol as described in section 2.2.2.5.1 was followed and the PCR products obtained were evaluated on a 1% agarose gel, followed by DGGE. The protocol for DGGE was followed as discussed in section 2.2.2.5.2 and the gel was evaluated using ChemDoc XRS (Biorad Laboratories) gel documentation system under UV light.

3.3. Results and discussions

3.3.1. Media and growth

Optical density was not a viable option for monitoring the growth due to components that were not completely dissolved in media used. As a result, DAPI staining was used to confirm the presence of cells in each media used.

3.3.2. Molecular approach for media selection

3.3.2.1. DAPI staining

The 37ºC inoculums showed possible growth after 9 days in *Sulfobacillus* DMSZ medium 812 (medium A) and *Leptospirillum* DMSZ medium 882 (medium B) (Figure 3.1) and all attempts to grow communities at 70ºC were unsuccessful and this was confirmed with DAPI staining.

Genomic DNA was extracted followed by PCR amplification of 16S rDNA fragments.
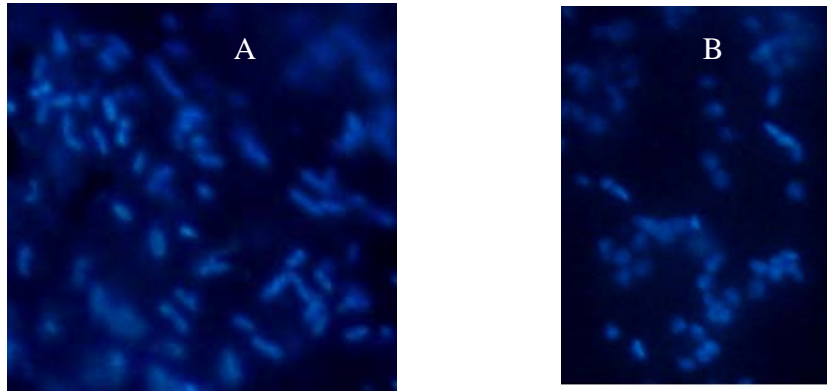
Figure 3.1. DAPI staining of cells obtained from growth in (A) *Sulfobacillus* DMSZ medium 812 and (B) *Leptospirillum* DMSZ medium 882.

3.3.2.2. Genomic DNA extraction

Genomic DNA extraction was performed directly on the 37ºC bioreactor sample, while an appropriate amount (10 ml) of this sample was inoculated into (90 ml) *Sulfobacillus* DMSZ medium 812, *Leptospirillum* DMSZ medium 882, and 70ºC bioreactor sample (10 ml) inoculated into (90 ml) *Sulfolobus* DMSZ medium 88 as well as *Acidianus* DMSZ medium 150. Using a NanoDrop Spectrophotometer (ND-1000), the concentrations of the extracted genomic DNA were measured and the results obtained showed that inoculums of *Sulfobacillus* DMSZ medium 812 had 115 ng/ml genomic DNA, 6 ng/ml from the *Leptospirillum* DMSZ medium 882, 0.1 ng/ml from the *Acidianus* DMSZ medium 150 and there was no DNA for the *Sulfolobus* DMSZ medium 88 (Figure 3.2).
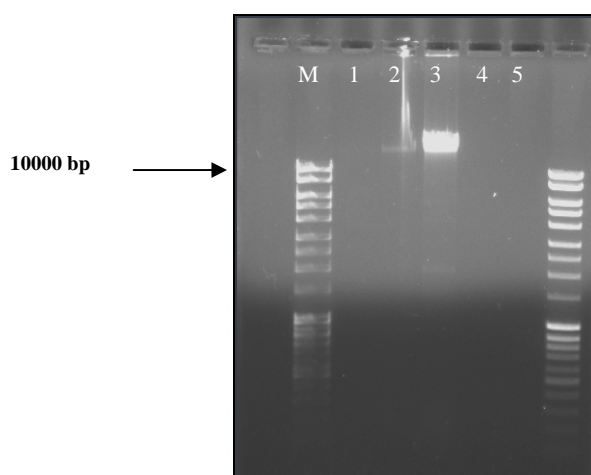
Figure 3.2. Genomic DNA extracted from 37ºC bioreactor and various culture media. Lane M, MassRulerTM DNA ladder (Fermentas); Lane 1, sample from 37ºC bioreactor; lane 2, *Leptospirillum* DMSZ medium 882; lane 3, *Sulfobacillus* DMSZ medium 812, lane 4, *Acidianus* DMSZ medium 150 and lane 5, *Sulfolobus* DMSZ medium 88.

Sulfobacillus DMSZ medium 812 presented at least all three dominant bacterial species and thus was selected for further growth experiments. The concentration of genomic DNA obtained from *Leptospirillum* DMSZ medium 882 containing bacterial culture was low and no cells or growth was obtained from the archaeal cultures despite attempts using different inoculums. Thus no further characterization was done.

3.3.2.3. PCR amplification of 16S rDNA fragments

The expected size (approximately 1,500 bp) of a 16S rDNA fragment was obtained when using genomic DNA extracted from *Sulfobacillus* DMSZ medium 812 containing bacterial cells as a template for the PCR. There was unfortunately no amplification of 16S rDNA fragment when using this genomic DNA of *Leptospirillum* DMSZ medium 882 containing bacterial cells as a PCR template. This may be due to the low concentration of genomic DNA that was obtained from *Leptospirillum* DMSZ medium 882 containing bacterial cells (Figure 3.3).
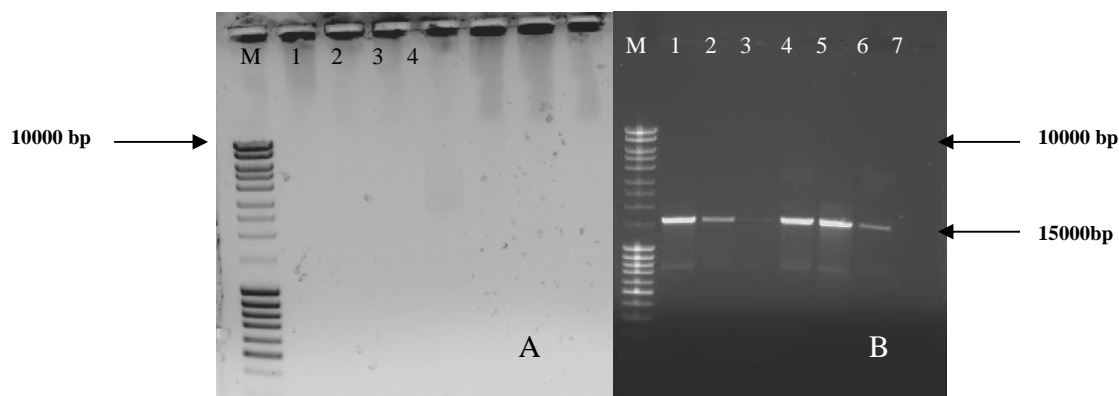
Figure 3.3A. Amplification of bacterial 16S rDNA fragments using genomic DNA from *Leptospirillium* medium. Lane M, MassRulerTM DNA ladder (Fermentas); Lanes 1, *Sulfobacillus* DMSZ medium 812; 2, *Leptospirillium* DMSZ medium 882, 3, non-template control; and 4, non-template control with BSA. Figure 3.3B. Amplification of bacterial 16S rDNA fragments using diluted genomic DNA from *Sulfobacillus* medium. Lane M, MassRulerTM DNA ladder (Fermentas); Lanes 1, 1:10; 2, 1:100; 3, 1:1000; 4, 1:10 + BSA dilutions; 5, undiluted template; 6, 1:100 dilution + BSA and 7, non-template control (negative control).

3.3.2.4. Nested PCR and denaturing gradient gel electrophoresis

The microbial population was accessed, and the sequencing results showed that all the three dominating organisms present in the 37°C bioreactor are all present in this culture medium hence *Sulfobacillus* DMSZ medium 812 was selected for further studies. The results showed that there was amplification and the expected size corresponding to 200 bp was obtained for subsequent DGGE (Figure 3.4 and Figure 3.5).
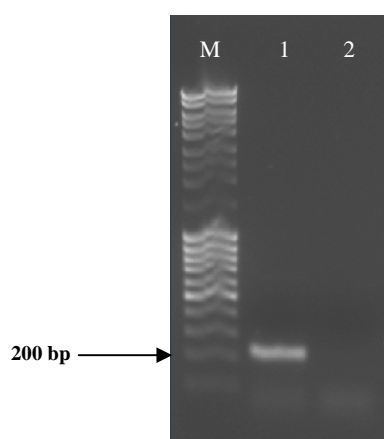
Figure 3.4. Nested-PCR using 341F-GC clamped and 571R primers. Lane M, MassRulerTM DNA ladder (Fermentas); Lane 1, amplified fragment using 16S rDNA template; and lane 2, non template control.
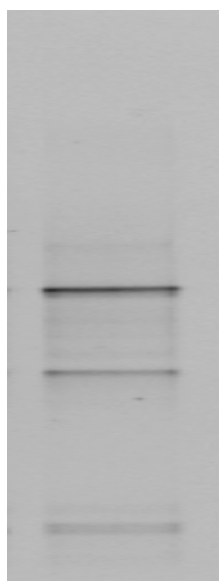
Figure 3.5. Fingerprint obtained from DGGE analysis using medium A.

The DGGE fingerprints showed three distinctive bands that were excised, gel eluted, reamplified with a bacterial set of primers and prepared for sequencing. The results obtained showed that the three organisms present in the $37^{o}C$ are in the selected culture using BLASTn alogarithm at NCBI (Table 3.2).

Table 3.2. Sequences producing significant alignments using the BLASTn alogarithm at NCBI result

Band	Accession	Description	Max score	Total score	Query coverage	E value	Max ident
1	AF137503.2	*Sulfobacillus thermosulfidooxidans* subsp. Asporogenes 16S ribosomal RNA gene, partial sequence	250	250	91%	1e-63	97%
2	EU371536.1	Uncultured *Leptospirillum* sp. clone B31 16S ribosomal RNA gene, partial sequence	187	187	93%	1e-44	88%
3	EU368981.1	Uncultured *Acidithiobacillus* sp. clone spx-B9 16S ribosomal RNA gene, partial sequence	198	198	76%	5e-48	93%

Even though short fragments were sequenced (DGGE analysis), similarity searches showed that *Sulfobacillus* sp. *Leptospirillum* sp. and *Acidithiobacillus* sp. were present.

3.3.3. Determination of copper tolerance of the micro-organisms

3.3.3.1. Determination of minimum inhibitory concentrations (MIC) of copper for the 37ºC bioreactor consortium

The same volume of a homogenous sample was used for comparison. Genomic DNA was extracted from culture (medium A) containing varying copper concentrations ranging from 20 mM – 300 mM (Figure 3.6A) and 400 mM – 1 mM (Figure 3.6B) copper sulphate a after 7 days incubation period. Genomic DNA extraction from culture containing 400 mM – 1 mM copper was unsuccessful. Hence, the results obtained showed a growth inhibition at 400 mM – 1 mM copper while DAPI staining was also performed to ascertain if there were cells present (data not shown) and the stain confirmed that no cells were present in the culture containing 400 mM – 1 mM copper. The MIC of copper for the consortium by definition is therefore at 400 mM.

Figure 3.6A. Genomic DNA extracted from culture containing copper using *Sulfobacillus* medium. Lane M, MassRulerTM DNA ladder (Fermentas); Lane 1, 20 mM; lane 2, 100 mM; lane 3, 150 mM; lane 4, 300 mM CuSO$_4$ and lane 5, control (no copper added). Figure 3.6B. Lane M, MassRulerTM DNA ladder (Fermentas); lane 1, control (no copper added); lane 2, 400 mM; lane 3, 800 mM and lane 4, 1 M CuSO$_4$.

3.3.3.2. Evaluation of micro-organisms present in the copper medium

Since a consortium of bacteria was used, denaturing gradient gel electrophoresis analysis was performed to evaluate the organisms present in the copper medium and it was found that there was no shift in microbial population due to selective pressure from higher copper concentrations. The same banding patterns were observed for cultures containing 20 mM – 300 mM copper and the control which had no copper (Figure 3.7A

and Figure 3.7B). Thus, copper was not selective to any of the three dominating species in the 37ºC bioreactor.
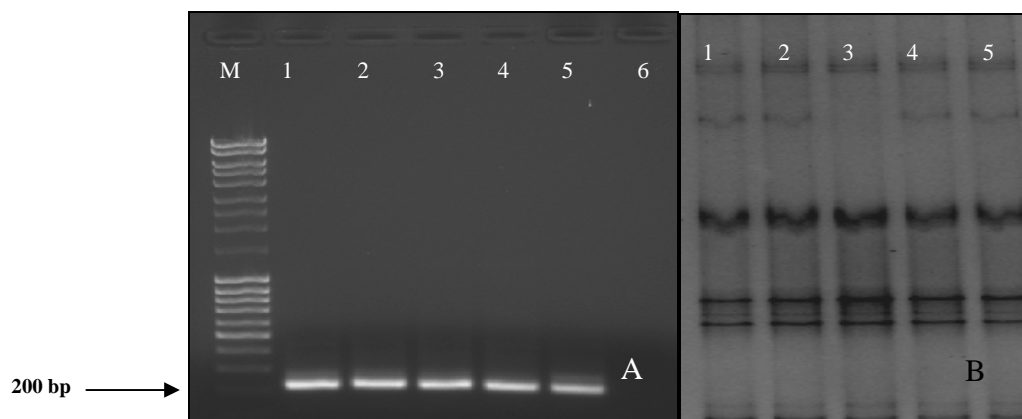
Figure 3.7A. Nested PCR using bacterial 341F-GC clamped & 517R primers. Lane M, MassRulerTM DNA ladder (Fermentas); lane 1, control; lane 2, 20 mM; lane 3, 100 mM; lane 4, 150 mM, lane 5, 300 mM CuSO$_4$ and lane 6, NTC. Figure 3.7B. Banding pattern obtained from denaturing gradient gel electrophoresis analysis. Lane 1, control; lane 2, 20 mM; lane 3, 100 mM; lane 4, 150 mM and lane 5, 300 mM CuSO$_4$.

3.3.3.3. Determination of the minimum inhibitory copper concentrations of individual isolates:

Isolation of each pure culture (*Sulfobacillus* sp., *Leptospirillum* sp. and *Acidithiobacillus* sp.) was achieved through passage of the consortium of bacteria into respective media as in DMSZ medium 812 and DMSZ medium 882 (MINTEK), this was confirmed by the restriction fragment length polymorphism analysis done on each 16S rDNA clones. The identity of each of the isolates was obtained from sequencing analysis using BLAST alogarithm at NCBI and the growth studies were done on the single species.

3.3.3.3.1. Sulfobacillus *sp.*

The same procedures as with the consortium were followed for the determination of copper MIC. Genomic DNA was extracted as described by Labuschagne and Albertyn (2007) after a 7 day incubation period; the pure isolate of *Sulfobacillus* sp. grew at 3 mM copper sulphate and no growth was observed from 6 mM copper concentration and DAPI staining performed also confirmed that there were no cells present in the culture containing 6 mM – 20 mM copper. Hence, *Sulfobacillus* sp. exhibited copper MIC at 6

mM while the minimum inhibitory copper concentration of the consortium of these bacteria was 400 mM (Figure 3.8).
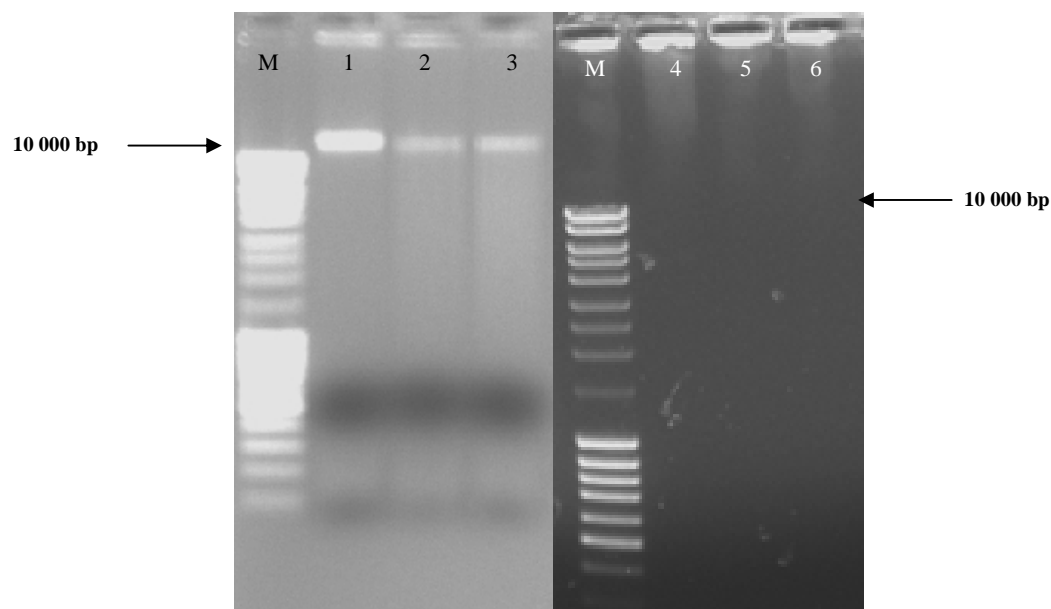
Figure 3.8. Genomic DNA extracted from culture containing copper using *Sulfobacillus* DMSZ medium 812. Lane M, MassRulerTM DNA ladder (Fermentas); Lane 1, 0.00 mM; lane 2, 1 mM; lane 3, 3 mM; lane 4, 6 mM $CuSO_4$ and lane 5, 10 mM and lane 6, 20 mM $CuSO_4$.

3.3.3.3.2. Leptospirillum *sp.*

The same procedures were used to access the MIC of copper of a pure *Leptospirillum* sp. that was isolated through inoculation into *Leptospirillum* sp. DMSZ medium 882 and successive passing to obtain a pure *Leptospirillum* sp.. After 7 days of incubation into a copper medium with different concentrations of copper, genomic DNA was extracted according to the method by Labuschagne and Albertyn (2007). At 3 mM copper, there were no sufficient cells for genomic DNA extraction probably as a result of growth inhibition (Figure 3.9). It could therefore be deduced that the minimum inhibitory copper concentration for this organism was 3 mM and again, the results obtained from DAPI staining confirmed this observation (data not shown).
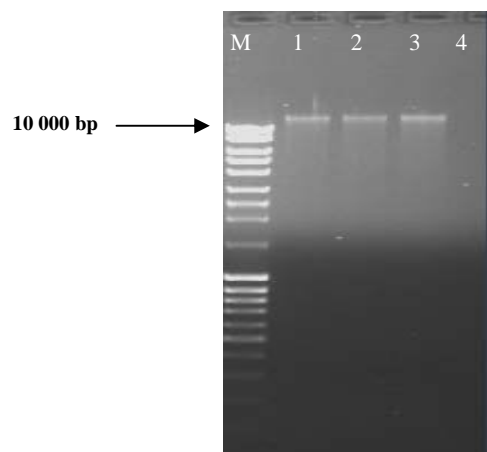
Figure 3.9. Genomic DNA extracted from culture containing copper using *Sulfobacillus* DMSZ medium 812.Lane M, MassRulerTM DNA ladder (Fermentas); Lane 1, 0.00 mM; lane 2, 1 mM; lane 3, 1.5 mM and lane 4, 3 mM CuSO$_4$.

3.3.3.3.3. Acidithiobacillus sp.

Isolation through inoculation into medium A was done and a pure *Acidithiobacillus* sp. was obtained. The same procedures were used to determine the MIC of copper of this pure *Acidithiobacillus* sp. Varying concentrations of copper were added to each pre-inoculum, incubated at an appropriate temperature in a shaker and after 7 days, genomic DNA was extracted as described by Labuschagne and Albertyn (2007) and there was growth inhibition at 10 mM (Figure 3.10). There was no fluorescence observed under an epifluorescence microscope equipped with a filter set 02 operating at wavelength 390 nm when the culture was stained with DAPI solution. This was used to confirm that there was no live cells present (data not shown).
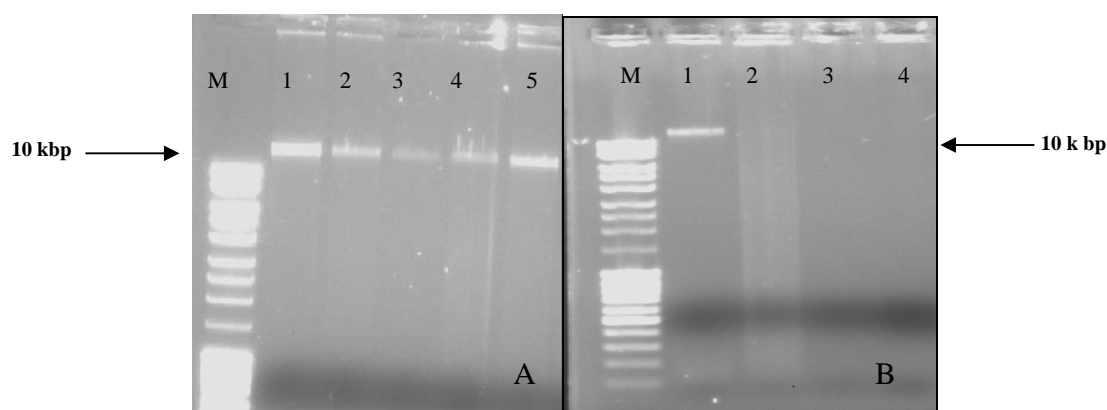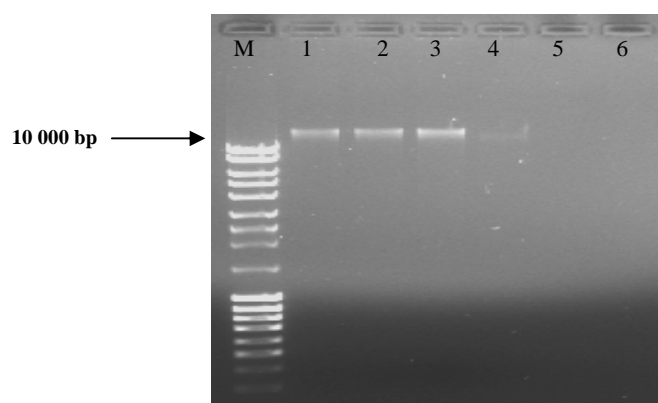
Figure 3.10. Genomic DNA extracted from culture containing copper using *Sulfobacillus* DMSZ medium 812. Lane M, MassRulerTM DNA ladder (Fermentas); Lane 1, 0.00 mM; lane 2, 3 mM; lane 3, 6 mM; lane 4, 10 mM, lane 5, 20 mM and lane 6, 30 mM CuSO$_4$.

The single species was not able to tolerate a high copper concentration such as 300 mM copper as the consortium was able to tolerate.

3.4. Conclusions

Suitable media were selected for the organisms present in the 70ºC and 37ºC bioreactor samples. Biomass was obtained when *Sulfobacillus* DMSZ medium 812 was used as a cultivation medium for the 37ºC bioreactor sample while there were no sufficient cells obtained with *Leptospirillum* DMSZ medium 882. *Acidianus* DMSZ medium 150 and *Sofolobus* DMSZ medium 88 were used as the cultivation media for the 70ºC bioreactor sample. *Sulfobacillus* DMSZ medium 812 yielded an active growing consortium of the three dominating species in the 37ºC bioreactor. Each species was isolated and the MIC of copper determined.

The minimum inhibitory copper concentration of the consortium of bacteria (organisms from 37ºC bioreactor sample) was determined to be 400 mM, while after isolation of each of the organisms present in the 37ºC bioreactor sample led to a drastic drop in copper MIC; *Sulfobacillus* isolate exhibited MIC of copper at 6 mM, *Leptospirillum* isolate a minimum inhibitory copper concentration at 3 mM and *Acidithiobacillus* sp. showed a minimum inhibitory copper concentration at 10 mM. Literature suggests these organisms in a consortium enhance one another because of the presence of sulphur oxidizing organisms and iron oxidizers as the mixed culture increases the dissolution of sulfide

mineral and the recovery of copper from chalcopyrite (Norris, 1990; Yan-sheng, *et al.,* 2008). Here, it is believed that having consortium of bacteria may enhance resistance to toxicity of copper at high concentrations. It has been shown that the presence of *Acidithiobacillus caldus* strain in a co-culture with *Leptospirillum ferrooxidans*-like strain increases bioleaching rate compared with only *L. ferrooxidans* (Norris, 1990). Dopson and Lindström (1999), have also shown that *Acidithiobacillus caldus* enhanced oxidation of arsenopyrite by the moderately thermophilic *Sulfobacillus thermosulfidooxidans.*

3.5. References

Bender, C.L. and Cooksey, D.A. (1986). Indigenous plasmids in *Pseudomonas syringae* pv. tomato and conjugative transfer and role in copper resistance. *Journal of Bacteriology.* pp. 534-541.

Department of Water Affairs and Forestry, (1996). South African Water Quality Guidelines (Second Edition). Domestic water use. pp. 58.

Dopson, M. and Lindström, E.B. (1999). Potential role of *Thiobacillus caldus* in arsenopyrite bioleaching. *Applied Environmental Microbiology.* pp.36–40.

Heukeklekian, H. and Gellman, I. (1955). Studies of biochemical oxidation by direct methods (IV). Effect of toxic metal ions on oxidation. *Sewage Industrial Wastes.* pp. 70-84.

Labuschagne, M. and Albertyn, J. (2007). Cloning of an epoxide hydrolase-encoding gene from *Rhodotorula mucilaginosa* and functional expression in *Yarrowia lipolytica. Yeast.* pp. 69-78.

Lane, D.J., Pelletier, D.A., Barns, S.M. and Weisburg, W.G. (1991).16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology.* pp. 697-703.

Miranda, C.D. and Rojas, R. (2006). Copper accumulation by bacteria and transfer to scallop larvae. *Marine pollution bulletin*, pp. 293-300.

Nies, D.H. (1999). Microbial heavy metal resistance. *Applied Microbiology and Biotechnology.* pp. 730-750.

Norris, P.R. (1990). Acidophilic bacteria and their activity in mineral sulfide oxidation. In: Ehrlich HL, Brierley CL (Eds). Microbial mineral recovery. McGraw Hill, New York, pp. 3–27.

Ramamoorthy, S. and Kushner, D.J. (1975). Binding of mercuric and other heavy metal ions by microbial growth media. *Microbiology Ecology* pp. 162-176.

Sheldon, A. and Menzies, N.W. (2004). Effect of copper toxicity on growth and morphorlogy of Rhodes grass (*Chloris gayana*) in solution culture. School of Land and Food Sciences, University of Queensland, St Lucia, Qld, 4072, Australia.

Trevors, J.T. and Cotter, C.M. (1989). Copper toxicity and uptake in micro-organisms. *Journal of Industrial Microbiology*. pp. 77-84.

Yan-sheng, Z., Wen-qing, Q., Jun, W., Shi-jie, Z., Cong-ren, Y., Jian-wen, Z., Shao-shi, N. and Guan-zhou, Q. (2008). Bioleaching of chalcopyrite by pure and mixed culture. *Transcactions of Nonferrous Metals Society of China.* pp. 1491-1496.

Chapter 4

Characterization of copper resistance mechanisms in bacteria

4.1. Introduction

Metal ions such as copper have a dual role in living organisms while required at low concentrations, copper becomes toxic to cells above optimum concentrations (Resing *et al.,* 2000). Hence, copper presents the cell with a dual challenge (Gordon *et al.,* 1994). As such, some micro-organisms have developed detoxification systems to protect themselves from the toxic effects of excess copper while ensuring that this ion meets their nutritional requirements (Voloudakis, *et al.,* 2005). These detoxification systems can be plasmid borne or chromosomal borne (Rosen, 2002).

The resistance systems in copper resistant micro-organisms may differ functionally (Cooksey, 1994). There are general mechanisms that are proposed for heavy metal resistance in micro-organisms. These include (i) exclusion by active export of the metal from the cell (Nies, 1992; Choudhury and Srivastava, 2001), (ii) exclusion of the heavy metal by a permeability barrier (Gowri and Srivastava, 1996), (iii) intracellular physical sequestration of metal by binding proteins which prevent it from damaging metal-sensitive cellular targets (Gilotra and Srivastava, 1997), (iv) extracellular sequestration (Nies, 2000), and (v) transformation and detoxification (Srivastava *et al.,* 1999). Also, micro-organisms such as fungi also have diverse mechanisms which involve trapping of the metal by cell-wall components, altered uptake of copper, extracellular chelation or precipitation by secreted metabolites and intracellular complexing by metallothioneins and phytochelatins (Cervantes and Gutierrez, 1994).

Although copper resistant mechanisms in bacteria include active efflux, intracellular or extracellular sequestration, and cell wall modification (Choudhury and Srivastava, 2001), efflux systems are a common resistance mechanism (Teitzel and Parsek, 2003). Some micro-organisms respond to elevated copper concentrations by producing copper-

complexing ligands such as amicyanin, a membrane-associated copper binding protein pseudoazurin and rusticyanin which have copper binding strength (Gordon, *et al.,* 2000; Schreiber, *et al.,* 1990). Cha and Cooksey (1991) demonstrated that *Pseudomonas syringae* accumulated copper in the periplasm and outer membrane and this sequesters part of the available copper.

Also, Nies (1999) has shown that *Ralstonia* sp. CH34 was able to live in extreme environments contaminated with heavy metal ions as a result of resistance mechanisms this organism possesses. These mechanisms include active efflux which detoxifies the cytoplasm of the cell and precipitation of heavy metals with the carbon(IV) oxide produced during growth.

4.1.1. Genes involved in copper resistance

It has been reported that some micro-organisms are able to resist the deleterious effects of copper ions due to metal resistance determinants found on their chromosomes as well as within plasmids-borne genes (Bröer *et al.,* 1993; Silver and Phung, 1996). Studies on copper resistance and the genes involved have been done on many organisms. However, since *Escherichia coli* serves as a model bacterium for some purposes (Nies, 2000), studies on copper resistance of some micro-organisms will be done in comparison to copper resistance of *E. coli.*

Copper resistance determinant *copA* found in bacterial chromosomes as in *E. coli* (Silver and Phung, 1996) encodes CopA, a copper-translocating P-type ATPase, (Resing *et al.,* 2000; Solioz and Vulpe, 1996) which transports excess copper or other heavy metal cations from the cytoplasm of a cell to the periplasm. In addition, *E. coli* possesses a plasmid-borne copper resistance determinant *pco* (plasmid-borne copper resistance). The copper resistance specified by this plasmid contains seven genes, *pco ABCDRSE* which encodes Pco proteins, including the regulators encoded by *pcoR* and *pcoS* (Brown and Rouch, 1995). A multi-copper oxidase CueO, chromosomally encoded by *cue* (Cu-efflux), is also a periplasmic protein that protects periplasmic components from copper-mediated toxicity in *E. coli* (Roberts *et al.,* 2002; Grass and Rensing, 2001). Lee *et al.* (2002) showed that plasmid encoded PcoA, a putative multi-copper oxidase can

functionally substitute CueO. Furthermore, *E. coli* is equipped with an additional system namely, *cus* (Cu-sensing) which allows the cell to resist toxic effects of copper under anaerobic conditions. This system consists of the *cusCFBA* operon, whose gene products work on copper efflux (Resing and Grass, 2003). The *cus* determinant does not transport cytoplasmic Cu(I) (Grass and Rensing 2001).

Studies have also shown that *Enterococcus hirae* has copper resistance genes. This copper system contains four genes arranged in a *cop* operon namely, *cop*A, *cop*B, *cop*Y and *cop*Z. The encoded products copA and copB are responsible for the transport of copper and are called copper transporting ATPases. *cop*A functions by encoding CopA which serves in copper uptake when is limiting while the encoded product (CopB) of *cop*B functions as a copper or silver ion-ATPase for the export of copper or silver ions (Solioz and Stoyanov, 2003). This encoded product CopB extrudes copper from the cell when the copper concentration is above optimum concentration. *cop*Y encodes a copper-responsive repressor and *cop*Z encodes a chaperone that catalyzes intracellular copper activity.

Cooksey (1987) characterized a copper resistance mechanism of *Pseudomonas syringae*, a phytopathogen, at a molecular level. This characterization revealed that *Pseudomonas syringae* possesses a single operon, *copABCDRS,* which encodes its proteins that function in increased uptake and sequestration of excess copper. CopA, one of the encoded proteins, belong to the multicopper oxidase protein family which is similar to PcoA in *E. coli*, also a multicopper oxidase. CopA and CopC are outer membrane proteins that bind copper in the periplasm while CopB and CopD transport copper across the membrane (Silver, 1996).

Furthermore, Teixeira and co-workers (2007) demonstrated that *Xanthomonas axonopodis* pathovar citri (Xac) which causes citrus canker is equipped with *copAB* that is involved in the mechanism of Xac copper resistance. These genes are arranged in an operon in which transcription is induced by copper and is specific for this ion. The gene products namely CopA (a cytosolic protein) and CopB were also found in the cytoplasmic

membrane. These proteins allow growth at 1 mM copper sulfate, although the copper resistance mechanism of this organism is yet to be determined.

Copper resistance determinants in some of these resistant bacteria may appear to be related to each other, organized into a regulatory network but senses and responds to copper concentrations in different ways (Teixeira *et al.,* 2007).

4.1.2. Regulation of copper resistance genes in bacteria

The plasmid-borne resistance gene *pco* that confers copper resistance in *E. coli* is regulated by two copper-inducible promoters. One of these two promoters namely, P_{pcoA} is responsible for the expression of *pcoABCDRS* and the other P_{pcoE} for expression of only *pcoE*. A constitutive low-level promoter also expresses the *pcoR* and *pcoS* which are the regulatory genes borne on the *pco* determinant (Brown and Rouch, 1995). The products of these latter two genes show close homology to the family of two-component sensor and responder phosphokinase regulatory system (Brown *et al.,* 1995).

Stoyanov *et al.* (2001) demonstrated that <u>Cu</u> <u>e</u>xport <u>R</u>egulator, CueR (YbbI), a copper-responsive protein of *Escherichia coli,* is a MerR family regulator that controls expression of two genes namely, copper translocating P-type ATPase, CopA and multi-copper oxidase, CueO (Franke, *et al.,* 2001). Rensing and coworkers (2000) have shown that CopA is a copper transporter and Solioz and Vulpe (1996) stated that this transporter is a P-type ATPase containing a CPx motif. This has eight transmembrane regions encoded by an open reading frame (ORF) at position 510603-508099 in the *E. coli* genome sequence (Blattner *et al.,* 1997). Since *E. coli* is equipped by multiple systems under aerobic conditions, CueR is essential for copper tolerance and *cusRS* system is required for copper tolerance under anaerobic condition (Outten *et al.,* 2001).

The regulation of resistance to copper in *Xanthomonas axonopodis* pathovar *vesicatoria* (*Xav*) was described by Voloudakis and coworkers (2005). Here, a novel open reading frame, *copL*, was found to be required for copper-inducible expression of the downstream multicopper oxidase copA in *Xanthomonas axonopodis. copL* is a protein product of 122 amino acids which is rich in histidine and cysteine residues. On the other

hand, the *cop* operon of *Enterococcus hirae* is regulated by CopY, the copper-responsive repressor which is a protein with 145 amino acids. CopY repressor is coded by its gene *cop*Y. *Enterococcus hirae* also possesses chaperone CopZ, a protective protein coded by *copZ* (Solioz and Stoyanov, 2003).

4.1.3. Cupric-reductase activity in micro-organisms

The type II NADH dehydrogenase (NDH-2), being a member of the pyridine nucleotide disulfide reductases family (PNDR), is involved in reductase activities. PNDR is a large and heterogeneous protein family characterized with a disulfide redox-active site including NAD(P)H and FAD binding sites (Argyrou and Blanchard, 2004). Generally, type II NADH dehydrogenase oxidizes NADH without generating a proton-motive force (Melo *et al.,* 2004). For example, it catalyzes electron transfer from NAD(P)H to quinones without energy-transduction. It was noted that type II NADH, quinone oxidoreductases, also referred to as rotenone-in-sensitive NADH dehydrgenases (NDH-2) are always present in bacteria electron transfer chains, and are the energetic factories of respiratory organisms (Melo *et al.,* 2004). NDH-2 plays additional important roles in some micro-organisms under certain conditions. Such roles include cupric reductase activity (Rapisarda *et al.,* 1999), mediation of the electron transfer to membrane-bound methane monooxygenase in *Methlococcus capsulatus* (Cook and Shiemke, 2002) and protection of nitrogenase complex against oxygen inhibition in *Azotobacter vinelandii* (Bertsova *et al.,* 2001).

Rapisarda *et al.* (1999) demonstrated that in *E. coli*, NDH-2 has Cu(II)-reductase activity, which makes the cell more stable to high or low copper concentrations in culture media. NADH dehydrogenase reduces intracellular Cu(II) to Cu(I) *in vivo* in *E. coli* (Rodriguez, *et al.,* 2006; Rapisarda *et al.,* 1999). Although the monovalent state of copper is more toxic and less stable (WebElements), when this ion enters the periplasm, in the absence of multicopper oxidase, the monovalent copper ion oxidizes thereby forming a stable copper(II). As a result, the periplasmic components are impaired. Multicopper oxidase, CueO, usually prevents this damage by transferring its molecular oxygen to copper(I) which eventually becomes stable copper(II). This periplasmic protein can also repair the

damaged periplasmic components if impairment occurs (Grass and Rensing, 2001). With more evidence, Rensing and co-workers (2000) have shown that the monovalent state of copper is transported out of the cell since this is the substrate for the pump. At physiological pH and in standard culture media, copper(I) ions are insoluble in a free form. It was suggested that copper ligands and copper reductases are involved in the copper homeostatic process in some micro-organisms (Solioz and Stoyanov, 2003).

Although literatures describe mostly non-acidophilic microbial copper resistance mechanisms this chapter deals with organisms from acidophilic environments. Very little is described in literature about their copper resistance mechanisms.

The aims of this chapter were thus to determine and characterize the copper resistance gene(s) present in the resistant bacteria and to determine the activity of copper during the transport in the cells.

4.2. Materials and methods

4.2.1. Copper assay

Copper ions having two valence states as mentioned previously can be determined using various methods which include a spectrophotometric method. The method utilizes the reaction of non-absorbent 2,5-Dimercapto-1,3,4-thiadiazole (DMTD) in an acidic medium with copper to give a highly absorbent greenish-yellow chelate product which has an optimum absorbance at 390 nm (Ahmed *et al.,* 2002).

Prior to the assay, stock solutions of 1 M Cu(II) [392.9 mg $CuSO_4.5H_2O$ dissolved in 90 ml of autoclaved deionised water and made up to 100 ml], 4.42 mM DMTD solution [0.067g was dissolved in 97 ml of autoclaved deionised water, the pH adjusted with KOH until the solution is clear (usually pH 8.5), and filled to 100 ml], 2 - 14 mM diluted sulfuric acid [to 199.890 ml autoclaved deionised water, 110 µl H_2SO_4 was added], 1 M copper(I) standard solution [225.83 mg Cu(I)Br in acetonitrile was dissolved in autoclaved deionised water and made up to 100 ml] and 0.01% tartrate [19 mg $KNaC_4H_4O_6.4H_2O$ was dissolved in autoclaved deionised water, filled to 100 ml]. All the stock solutions were filter-sterilized using 0.2µm filters.

Graded concentrations of CuSO₄ ranging from 0.197 mM – 1.5 mM were prepared and the assay was started by transferring of 12.5 µl of 1 M copper(II) to a test tube, 1 ml of a 2 mM diluted sulfuric acid was added, followed by the addition of 4.42 mM DMTD stock solution and made up to 10 ml with autoclaved deionised water and a small amount of perchloric acid was added when measuring copper(II). The same steps were followed when transferring 0.39 mM, 0.79 mM and 1.57 mM copper(II) to a test tube. Readings were taken after 1 min and measured against a reagent blank. Wavelength scan ranging from absorbance 360 nm to 390 nm was done using a spectrophotometer. Also, for construction of a standard curve for copper(I), the same procedure was followed.

This was followed by determining Cu(II) and Cu(I) speciation in the mixture of copper ions. Tartrate (0.01%) was used as the masking agent for Cu(I); total copper (CuT) absorbance readings at 390 nm was determined before the addition of a masking agent and Cu(II) was determined after addition of 0.01% tartrate. Cu(I) absorbance readings were obtained by subtracting Cu(II) absorbance readings from Cu(T) absorbance readings.

Concentrations of copper(I) and copper(II) ranged from 0.2 mM to 12.8 mM; wavelengths ranging from 350 nm to 390 nm were used and the wavelength from 378 nm to 390 nm indicated R^2 of 0.999 for copper(I) and the R^2 of 1 was obtained for copper(II). Hence, wavelength 390 nm was used.
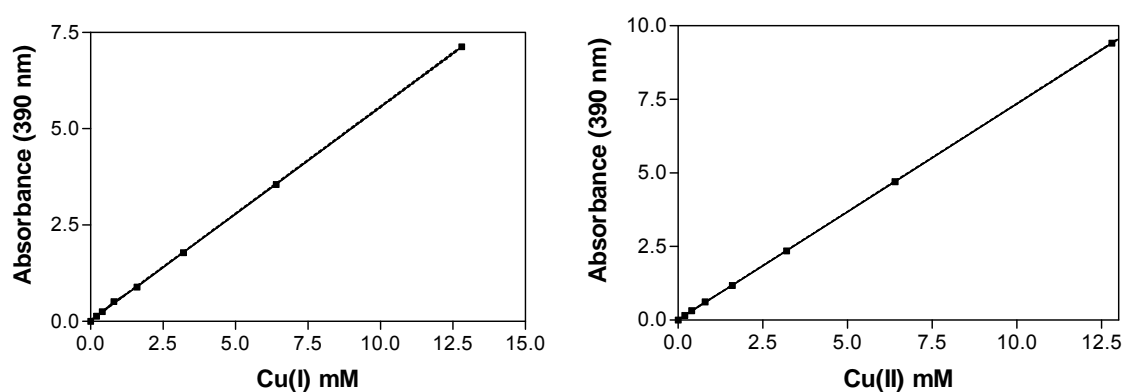
Figure 4.1. Standard curves for copper(I) and copper(II)

4.2.2. Growth study for control organism and individual isolates

4.2.2.1. Proteus mirabilis

A growth study of *Proteus mirabilis* (the bench-marker for the study) was done by inoculating 1 ml of pre-inoculum into 49 ml of LB broth (tryptone 10 g/l; yeast extract 5 g/l; and sodium chloride 10 g/l, pH 7) in a 500 ml shaking flask and incubating at 37°C in a rotating shaker at 175 rpm. This was done in duplicate and the optical density monitored at 600 nm until the stationary phase was reached.

4.2.2.2. Sulfobacillus *sp.*

To 40 ml of the selected medium (medium A as in section 3.2.1) 10 ml pre-inoculum was added and the optical density at 600 nm was taken at time intervals starting from zero while the culture incubated at 37°C in a rotating shaker at 175 rpm. Readings were taken in duplicate until the stationary phase was reached.

4.2.2.3. Leptospirillum *sp.*

A growth study was done by inoculating 10 ml of pre-inoculum into 40 ml medium B (components as in section 3.2.1) and growth was followed by measuring the appearance of iron oxidation over time at 405 nm (Romero *et al.,* 2003). The culture was incubated at 37°C in a rotating shaker at the same speed at 175 rpm. At time intervals (2 days), readings were taken until the stationary phase was reached.

4.2.2.4. Acidithiobacillus *sp.*

To 40 ml of medium A with the same components in section 3.2.1, 10 ml pre-inoculum was added and the growth study was done as in section 4.2.2.3 but the OD readings were taken at 600 nm.

4.2.2.5. Growth study for consortium of bacteria (37°C bioreactor sample)

The 37°C bioreactor sample was filtered using 0.20 µm filter and 5 g of the filtered slurry was inoculated into 95 ml of medium A, 10 ml of the inoculum at time zero was withdrawn into a tube and the remaining culture incubated at 37°C for 15 days. At 3

day intervals, the pH value of the culture medium was monitored for measurement of active growth since optical density could not be used due to slurry interference. Hence, DAPI staining as described in section 2.2.1.2 was employed.

4.2.3. Whole cell interaction with copper

4.2.3.1. Determination of residual copper in the P. mirabilis culture

To confirm the resistance mechanism that *P. mirabilis* possesses, a copper environment was designed using a less complex medium, Vätäänen-Nine-Salts-Solution (VNSS) medium [peptone 1.0 g/l; yeast extract 0.5 g/l; glucose 0.5 g/l; $FeSO_4.7H_2O$ 0.01 g/l and $NaHPO_4$ 0.01 g/l dissolved in nine-salts-solution (NaCl 17.6 g/l; Na_2SO_4 1.47 g/l; $NaHCO_3$ 0.08 g/l; KCl 0.25 g/l; KBr 0.04 g/l; $MgCl_2.6H_2O$ 1.87 g/l; $CaCl_2.2H_2O$ 0.41 g/l; $SrCl_2.6H_2O$ 0.01 g/l; H_3BO_3 0.01 g/l, pH 7,] (Hermansson *et al.,* 1987). A copper concentration of 0.08 mM was added to the VNSS medium containing overnight grown *P. mirabilis*. At a certain period of time, starting from time zero, 1.5 ml culture containing copper was collected in a tube and centrifuged at 10, 000 *X g* for 15 min at 4°C to collect the cells. 1 ml supernatant was collected in triplicate depending on the concentration of the residual copper since the assay is very sensitive to small amounts of copper. The composition was made up and the readings were taken as described in section 4.2.1 at certain time intervals starting from time zero, against a reagent blank. Negative control test (no copper) was also done along with this in order to monitor accurately *P. mirabilis* copper activity.

4.2.3.2. Determination of copper speciation in a 37ºC bioreactor sample

The 37ºC bioreactor sample was cultured in the selected common medium (medium A) for 15 days at 37ºC, 10 ml of the pre-inoculum was added to medium A containing 1.26 mM copper and the same procedure as in 4.2.1 was followed; positive and negative control tests were included in this assay since copper was already present in the bioreactor sample.

4.2.3.3. Determination of residual copper in individual isolates culture media

In order to have an idea of how individual isolates namely, *Sulfobacillus* sp., *Leptospirillum* sp. and *Acidithiobacillus* sp. respond to elevated copper concentrations (0.2 mM – 0.5 mM), determination of copper speciation in a designed copper environment was done following the procedure in 4.2.1.

4.2.3.4. Copper reduction ability using bacterial resting cells

Growth studies were done for these pure isolates in order to determine if individual isolates can reduce the availability of copper under resting conditions. *P. mirabilis* was used as the bench-marker.

Bacterial cells were grown in different media namely, medium A and medium B until stationary phase was reached and cells harvested at an OD reading 0.8, washed three times with 20 mM K_2HPO_4 (pH was adjusted using phosphoric acid to pH 1.8), and the cells suspended in 1 ml of 20 mM K_2HPO_4. The amount of 2.56 ml 20 mM K_2HPO_4 was added again followed by the addition of 400 µl of a 10 mM glucose solution (electron donor), this was left for 5 min at room temperature. Concentrations of copper for this assay were selected according to the minimum inhibitory copper concentrations exhibited by these individual isolates (the selected copper concentrations were few folds lower than the obtained MICs of copper). Thus 0.2 mM final concentration of copper sulphate for *Sulfobacillus* sp. and *Leptospirillum* sp. and 0.5 mM for *Acidithiobacillus* sp. was added making a total volume of 4 ml in a crimp-sealed Balch tube which had a headspace filled with air. A control test was set up except that the cells were not added. At time zero, 120 µl sample was collected for the reaction and the control respectively, centrifuged at 7 000 *X g* for 3 min and the supernatant analyzed for residual copper as described in section 4.2.1. The same procedure was followed for *P. mirabilis* but 20 mM HEPES pH 7 was used as a buffer since *P. mirabilis* has an optimal pH of 7.

4.2.4. Characterization of copper resistance genes

Sequences of copper resistance genes available from the NCBI database were used for primer design. Sets of primers were designed for both Gram-positive and Gram-negative bacteria, while *P. mirabilis* was used as the control for this study since one of the copper

```
E_coli                 CACCTTTAAGGTTAAGCAGAACGGGACTTACTGGTACCACAGCCATTCCG 442
Pseudomonas_syringae   TAAAATCCACGTCAAGCAGAATGGCACTTACTGGTACCACAGCCATTCCG 436
Pseudomonas_fluorescens CCAGTTCAAGGTTCGCCAGCACGGCACCTACTGGTATCACAGCCATTCCG 436
Xanthomonas_campestris TCGCTTCACTGTCCAGCAGGGTGGCACCTACTGGTATCACAGTCATTCGG 487
Sulfitobacter          TCGCTTCCCGATCCAGCAGGCCGGGACCTATTGGTTCCACAGCCATTCAG 472
                            *        *    ***     ** ** ** ****   ***** ***** *


E_coli                 ATGATGACTCACCCCATTCACCTGCATGGTATGTGGAGCGATCTGGAAGA 1658
Pseudomonas_syringae   ATGATGACCCACCCCATTCACCTGCACGGGATGTGGAGCGACCTCGAGGA 1712
Pseudomonas_fluorescens ATGATGACTCACCCCATCCACCTGCACGGCATGTGGAGCGATCTGGAAGA 1553
Xanthomonas_campestris ATGATGTCGCACCCCATCCATCTGCATGGCATGTGGAGCGACCTGGAAGA 1742
Sulfitobacter          ATGATGTCGCATCCGATGCACCTGCACGGGATGTGGTCGATCATCGATGC 1811
                       ****** * ** ** ** ** ***** ** ******         * ** *
```

Figure 4.2A. The alignment results following data mining of the copper resistance gene sequences for Gram-negative bacteria.

```
Staph_CopA    GACATTACAGGTATGACTTGTGCTGCATGCTCAAGCCGTATTGAAAAAGTGTTAAATAAA 288
Bacillus_CopA GCGGTTTCCGGCATGACGTGTGCGGCTTGCGCCAACCGGGTTGAAAAGCGCCTCAACAAG 300
Liseria_CopA  ---GTTTTTGGGATGACCTGTGCGGCGTGTTCCACGAGAATTGAAAAATCATTAAATAAA 90
                   **    ** ***** ***** ** **   * *    *  *******    * ** **


Staph_CopA    AAATTGATACCATCGTTTTAGATAAGACGGGTACCATTACAAATGGTCGTCCAGTCGTGA 1522
Bacillus_CopA GCTTGGAGACGATCGTGCTTGACAAGACGGGGACGGTCACAAATGGGAAGCCTCAGCTGA 1549
Liseria_CopA  AAGTTGATACAATTGTTTTTGATAAAACTGGGACTTTGACAGAAGGTAAATTAGAAGTTA 1336
                 * ** ** ** **   * ** ** ** ** ** **   * *** * **          * *
```

Figure 4.2B. The alignment results following data mining of the copper resistance gene sequences for Gram-positive bacteria.

```
copA_Staphy      AAGCCTATGCAGCTATAGGGATACCGGCAATATTATAGCCGAATGCCCAAAATAGATTTT 171
copA_pyogenes    AAGCCCATG--GCCACTGGAATCATGG-GACATTGTATATGAATGCCCAAAATAAATTTT 171
copA_ferrox      CATCCGGCA--GCAAGGGGGATCGCCACGATGTTGTAGCCGGCGCCCCACCACAGGTTCT 174
                   * **      ** *  ** **      *  ** **  *  ****  *  * ** *

copA_Staphy      GAC-GAAATTACGAATGGTTGCTTTACTTGCATAAATGGCTTTAGGAATAAGCATCAAGT 230
copA_pyogenes    CCTTGACAATCCTCATAGTCGCTCGACTCA-ATGACAGGCTTTCACCAAATCCAGCATAT 230
copA_ferrox      GGA-CATCTTGCGATAGGTGGCGCGGGAAAGTTCCAGGATGGCCACCACGTCCCGGGGAT 233
                   *   * *    ** **        *       *       *   *       *

copA_Staphy      AGTACCGTTTTTGTTCATCGTGAACCAATTACAGTATCATCAACATTCTTCTCAACAGGG 1338
copA_pyogenes    GGCGCCTTTCCCATTCAAGCTGCCGCAAAGACATTATCTTTTTCTCCCTTTTCAACAGGA 1347
copA_ferrox      ACTTGACTCCCCGTTGACGAACCACCGATAACTTCGTCACCGCTGCCCTTCTCTACGGGC 1341
                       *     ** *      * *  **     **      *** ** ** **

copA_Staphy      ATAGATTCACCTGTTAACAAG-ATTCGTCGATGGCAGTCATACCTTTAATAATTTTGCCA 1397
copA_pyogenes    AAACTTTCCCCTGTTAAAAAGGATTCATCCA-AATGCTATGTCCTTCCACGATAATACCG 1406
copA_ferrox      CGACTTTCGCCAGTGACATAG-CCTGGTTGACGGTGGTTCGCCCATCGACGATCAGACCA 1400
                   *  *** ** ** *    **    *  *  *      ** *  *  **      **
```

Figure 4.2C. The alignment results following data mining of the *copA* gene sequences for both Gram-negative and Gram-positive bacteria.

resistance genes in *P. mirabilis* has been previously characterized (Ojo *et al.,* 2008). Genomic DNA was extracted from the 37ºC bioreactor sample as well as individual isolates according to Labuschagne and Albertyn (2007) and were used for the amplification.

4.2.4.1. Primers designed for copper resistance gene(s)

Sequences of copper resistance genes available from the NCBI database for Gram-negative bacteria (*Escherichia coli, pcoA; copA from Pseudomonas syringae, Pseudomonas fluorescens, Xanthomonas campestris,* and *Sulfitobacter* sp. NAS-14.1); and for Gram-positive bacteria (*Staphylococcus* sp., *Listeria* sp. and *Baccillus* sp.) as well as *copA* genes from the mixture of both Gram-negative and Gram-positive bacteria (*Staphylococcus* aureus, *Streptococcus pyogenes* and *Acidithiobacillus ferroxidans*) were obtained from GenBank and their amino acid sequences used to identify conserved regions. Based on these, the corresponding nucleotide sequences were aligned and primers designed for amplification of a similar copper resistance gene present in the 37ºC bioreactor sample as well as individual isolates.

Table 4.1. Copper resistance gene primers designed for Gram-negative and Gram-positive bacteria

PRIMERS	SEQUENCE
Gram-negative bacteria copper resistance genes forward primer	5'-ACY TAC TGG TAY CAC AGC CAT TC-3'
Gram-negative bacteria copper resistance genes reverse primer	5'-CCA CAT VCC RTG CAG GTG-3'
Gram-positive bacteria copper resistance genes forward primer	5'-GGB ATG ACB TGT GCG GCD TG-3'
Gram-positive bacteria copper resistance genes reverse primer	5'-CCA TTT GTV AHV GTC CCC GTC TTA TC-3'
Gram-negative and Gram-positive bacteria *copA* genes forward primer	5'-CCA MMA YAG TTY TGV HAS MT-3'
Gram-negative and Gram-positive bacteria *copA* genes reverse primer	5'-ATT GWC CVC TTW CAD AVG GAC-3'
Gene specific *copA* forward primers	5'-ATG GAC CAT TCG GGA CAT CAG AAA-3'
Gene specific *copA* reverse primers	5'-GGC GGT GAG CCG AAC CCT C-3'

4.2.4.2. PCR amplification of copper resistance gene(s)

PCR amplification of copper resistance gene(s) using the extracted genomic DNA with all the designed gene-specific primers was done. The PCR conditions included initial denaturation at 94ºC for 2 min, amplifications were run for 35 cycles in a thermal cycler pXe 0.2 (Thermo electron corporation). Each cycle was run at 94ºC for 30 sec, annealing temperature ranging from 50ºC - 58ºC for 1 min and 72 ºC for 90 sec and final extension, 72 ºC for 7 min.

5 µl of each PCR product was added to 2 µl loading dye (Fermentas) before loading onto a 1% agarose gel. The MassRuler[TM] DNA ladder (Fermentas) was used to determine the size of bands visualized on the gel using the Chem doc XRS gel documentation system.

4.2.4.3. Purification of PCR products

Purification of the PCR products was done where 20 µl PCR products were loaded onto a 1% agarose gel, the bands corresponding to the expected size excised under a low frequency UV-light and purified using GFX^{TM} PCR DNA Purification Kit (Amersham Biosciences, UK).

4.2.4.4. Identification of copper resistance genes

The purified fragments were ligated with a pGEM$^®$–T Easy vector according to the manufacturer's instructions as described in 2.2.2.4, transformed as described in 2.2.2.4.1 and small scale plasmid isolation (as described in section 2.2.2.4.2) performed followed by restriction fragment length polymorphism (RLFP) as previously described. The selected clones were sequenced as described in section 2.2.2.4.4 and the files retrieved and compared to sequences deposited in the NCBI (National Center for Biotechnology Information) database.

4.3. Results and discussions

4.3.1. Calibration of a standard curve for the copper assay

Maximum absorbance was obtained at the wavelength ranging from 378 nm to 390 nm which could be used for copper(I) or copper(II) assay.

4.3.2. Growth study for control organism and isolates

4.3.2.1. Proteus mirabilis

From the growth study, *P. mirabilis* has an average μ_{max} of 1.92 hr^{-1} with a doubling time (T_d) of 0.33 hr and short lag phase. Figure 4.3 shows the growth pattern achieved for *P. mirabilis* in a VNSS medium after 6.5 hours.
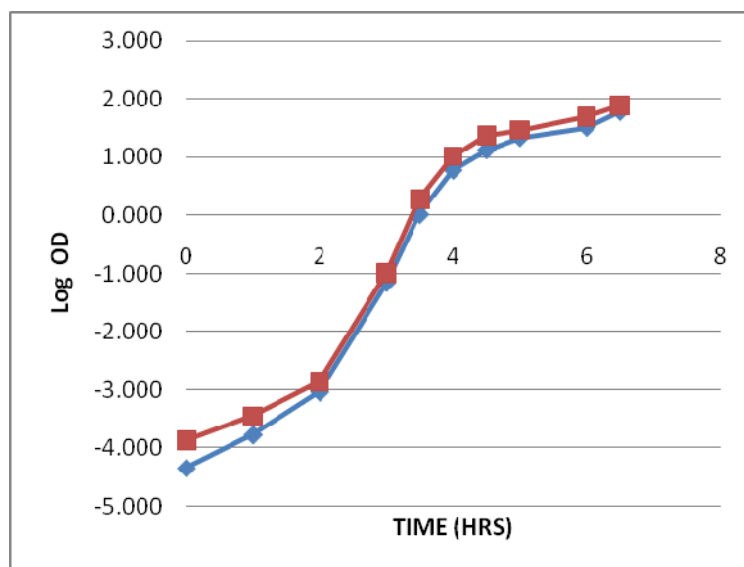
Figure 4.3. Growth curve for *P. mirabilis*

4.3.2.2. Sulfobacillus *sp.*

From the growth study, it can be deduced that *Sulfobacillus* sp. is not a fast growing organism. Linear growth was obtained during seven days and stationary phase started thereafter (Figure 4.4). *Sulfobacillus* sp. has an average μ_{max} of 0.011 hr^{-1} with a doubling time (T_d) of 63 hr.
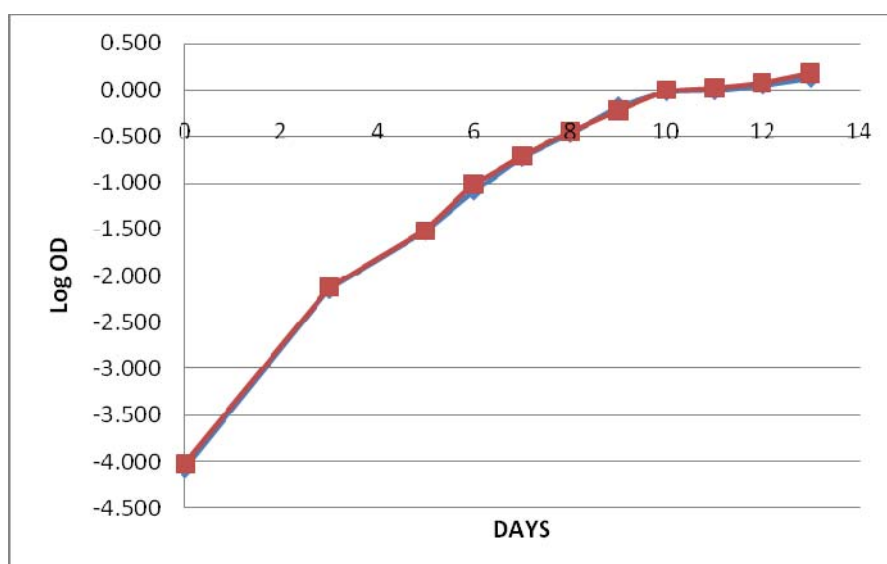
Figure 4.4. Growth curve for *Sulfobacillus* sp.

4.3.2.3. Leptospirillum *sp.*

Leptospirillum exhibited an average μ_{max} of 0.031 hr^{-1} with a doubling time (T_d) of 22.35 hr. Initially a lag phase was exhibited during growth, followed by exponential phase and stationary phase was reached after 17 days.
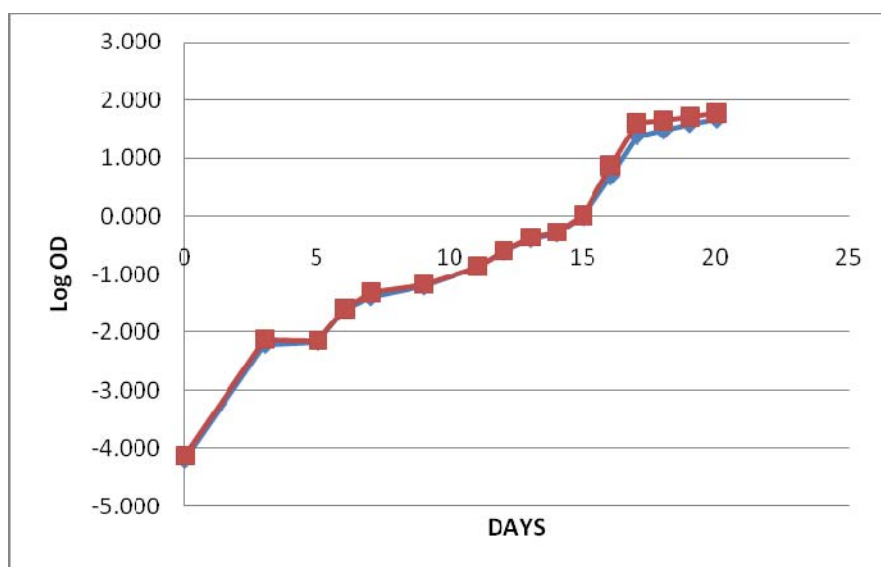
Figure 4.5. Growth curve for *Leptospirillum* sp.

4.3.2.4. Acidithiobacillus *sp.*

It can be deduced that *Acidithiobacillus caldus* has an average μ_{max} of 0.0269 hr^{-1} with a doubling time (T_d) of 25.77 hr. Lag phase was exhibited and exponential phase, and stationary phase was reached after 13 days.
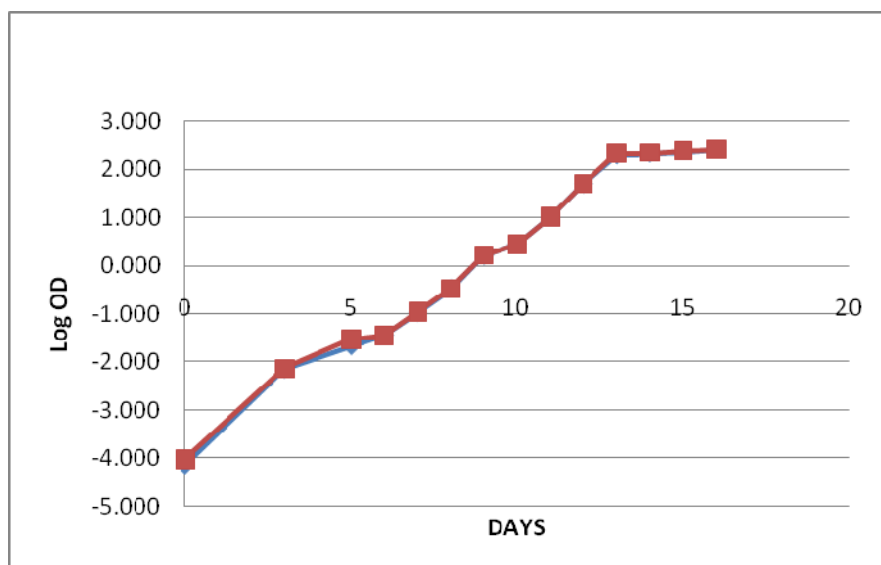
Figure 4.6. Growth curve for *Acidithiobacillus* sp.

When comparing the growth of the three dominant species from the 37°C bioreactor, *Leptospirillum* sp. and *Acidithiobacillus* sp. grow faster than *Sulfobacillus* sp.

4.3.2.5. The 37°C bioreactor sample

The growth for the 37°C bioreactor sample was monitored by checking the pH values over a period of time since the OD cannot be measured as the culture contains some components such as chalcopyrite which contains copper, iron and sulphide. Reduction in pH values reflects the oxidation of iron and sulphide that can be used to show active growth (Trummer *et al.,* 2006).

Table 4.2. Growth monitoring using pH values

DAY	pH
0	2.01
3	1.95
6	1.90
9	1.88
12	1.80
15	1.80

4.3.3. Whole cell interaction with copper

4.3.3.1. P. mirabilis

Initially, there was a considerable influx of copper where during the first 15 min most of the copper(II) was reduced to copper(I) during this period and thereafter the rate of copper(II) uptake was slower and reduction decreased. This may be due to (i) the response of copper translocating P-Type ATPase (CopA) to a high copper concentration and that there was an active efflux of copper, or (ii) saturation of the pump hence less copper(II) uptake and reduction decreased. DAPI staining was used to confirm the growth (data not shown).
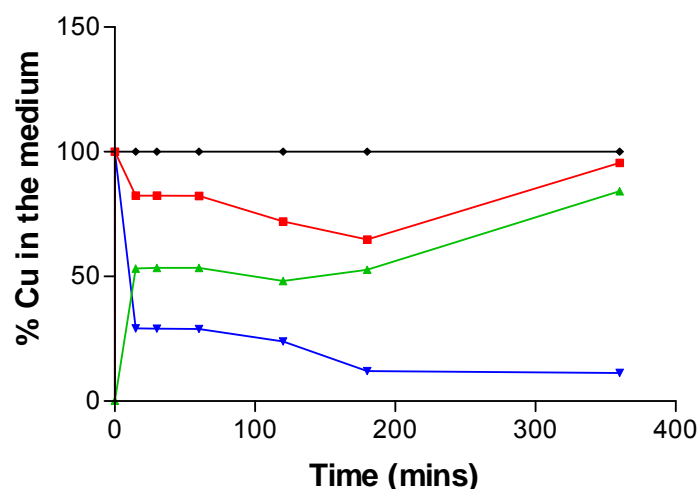
Figure 4.7. Percentage residual copper following growth of *P. mirabilis.* Control (♦; Total copper (Cu[T]) (■); copper(I) (Cu[1]) (▲) ; copper(II) (Cu[II]) (▼).

Also, it has been reported that NADH dehydrogenase reduces intracellular Cu(II) to Cu(I) *in vivo* in *E. coli* (Rodriguez, *et al.,* 2006; Rapisarda *et al.*, 1999). The figure could indicate NADH dehydrogenase reductase activity, since copper(II) was present in the medium but copper(I) was released from the cell. Rensing and co-workers (2000) have also shown that the monovalent state of copper is transported out of the cell since this is the substrate for the CopA.

4.3.3.2. The 37ºC consortium

The 37ºC bioreactor sample contains a consortium of bacteria namely, *Sulfobacillus* sp., *Leptospirillum* sp. and *Acidithiobacillus* sp.*,* and the assay done showed the appearance of copper(I) and disappearance of copper(II) in the medium. After 3 hr of inoculation into copper medium, there was a decrease in copper(II) concentration in the medium and after a further 2 hr the copper concentration decreased more while there was an appearance and increase in copper(I) in the medium. This showed slower copper(II) uptake in comparison to *P. mirabilis* but better continuous reduction.
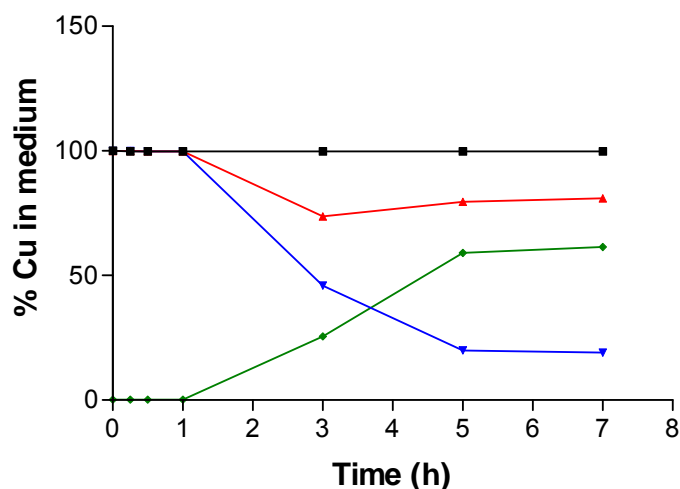
Figure 4.8. Percentage residual copper following growth of the 37ºC bioreactor sample. Control (■); Total copper [Cu(T)] (▲); copper(II) [CuII)] (▼) ; copper(I) [Cu(I)] (◆).

For the first 1 hr, there was no uptake of copper but thereafter, copper(II) was taken up by the cell. As copper(II) disappeared in the medium, there was an appearance of copper(I) in the medium which could be due to a reductase activity of NADH dehydrogenase. Also, the transport of copper(I) to the outside (medium) may be as a result of the presence of CopA pump as it has been shown that copper(I) is a substrate for CopA pump (Rensing *et al.,* 2002). These two reduction experiments were done during active growth.

4.3.4. Copper assay for resting cells of individual isolates:

4.3.4.1. Sulfobacillus *sp.*

There was a slight reduction (1.7 percent) of copper availability in the buffer after 0.5 h of inoculation (data not shown) after which the available copper total in the buffer remained constant. Copper(I) availability test was further done and the result obtained showed no presence of copper(I) in the buffer. Thus, there was no copper reduction activity observed possibly due to low NADH dehydrogenase activity. Individual isolates were subjected to whole cell reduction in the absence of media to assess the ability to interact with copper.
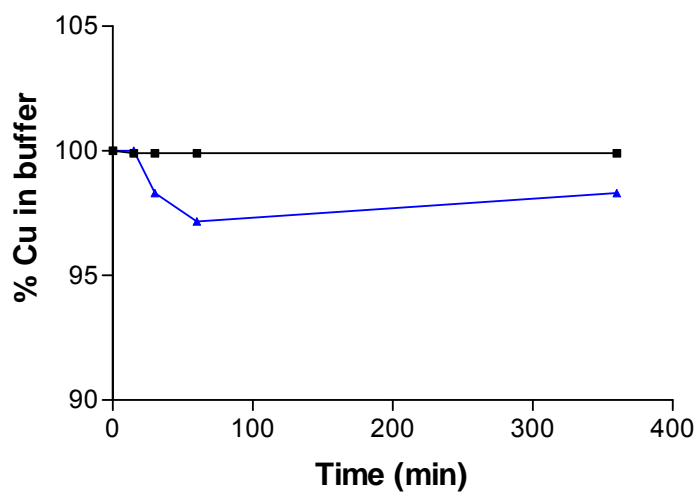
Figure 4.9. Copper assay for *Sulfobacillus* sp. resting cells. Control (■) and Total copper [Cu(T)] (▲).

4.3.4.2. Leptospirillum *sp.*

During this assay using *Leptospirillum* sp. resting cells, it was observed that there was a low intake of copper(II) from 0 h to 0.25 h and at 0.50 h, only 2.3% of copper(II) was taken up by the cell , there was no release of copper(I) and the result obtained from further tests showed that there was no Cu(I) present.
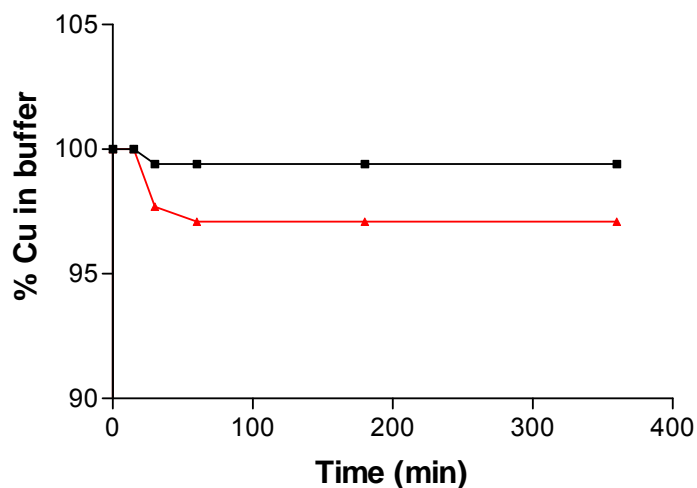
Figure 4.10. Copper assay for *Leptospirillum* sp. resting cells. Control (■) and Total copper [Cu(T)] (▲).

4.3.4.3. Acidithiobacillus sp.

In contrast, *Acidithiobacillus* sp. was able to take up copper(II) and actively reduce it to copper(I) as these cells were able to process the glucose to form NADH and reduction proceeded. *Acidithiobacillus* sp. may be dominant in the ability to reduce copper(II) in the consortium.

Figure 4.11 shows the reduction of total copper by 25 percent; the percentage copper(II) concentration in the buffer was 49 and copper(I) 25 percent at 3 h and thereafter, the total copper concentration in the buffer increased (approximately 80 percent); 20 percent for copper(II) and 60 percent for copper(I).
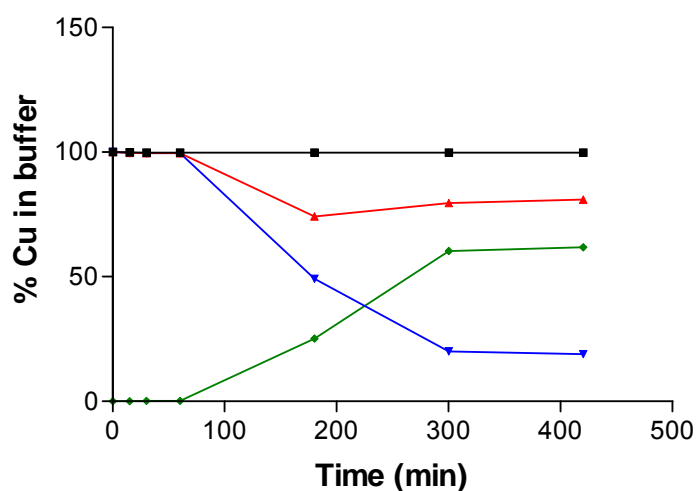
Figure 4.11. Copper assay for *Acidithiobacillus caldus* resting cells. Control (■); Total copper [Cu(T)] (▲); copper(II) [CuII)] (▼) ; copper(I) [Cu(I)] (◆).

4.3.5. Primers designed for copper resistance gene(s) and amplification of copper resistance fragment(s)

Sets of gene-specific primers for both Gram-positive and Gram-negative bacteria were designed and used for the amplification of *copA* fragments using extracted genomic DNA from all samples. There was amplification of a *copA* fragment using the bioreactor genomic DNA. The amplification and sequencing of these product results searches showed similarity to *Acidithiobacillus ferrooxidans* copper-translocating P-type ATPase.

Using individual isolates' gDNA as template, there was no amplification of *copA* fragment for *Sulfobacillus* sp. and *Leptospirillum* sp. but there was *copA* fragment amplification using *Acidithiobacillus* sp. which confirms that this organism in the consortium of bacteria is actively the one reducing copper availaility.

Since the BLASTn result showed similarity to *A. ferrooxidans copA* fragment, a set of primers were designed from *A. ferrooxidans copA* gene but the entire attempt to amplify the full length gene proved abortive. This may be as a result of high GC-content of the designed primers. As a result, the previous set of primers used to amplify *Acidithiobacillus* sp. *copA* fragment was used to check if there could be amplification of a *copA* fragment in *A. ferrooxidans*. This served as a quality control check since the results obtained from BLAST search showed similarity to *A. ferrooxidans copA* gene and there was amplification. The annealing temperature was determined to be 55ºC.
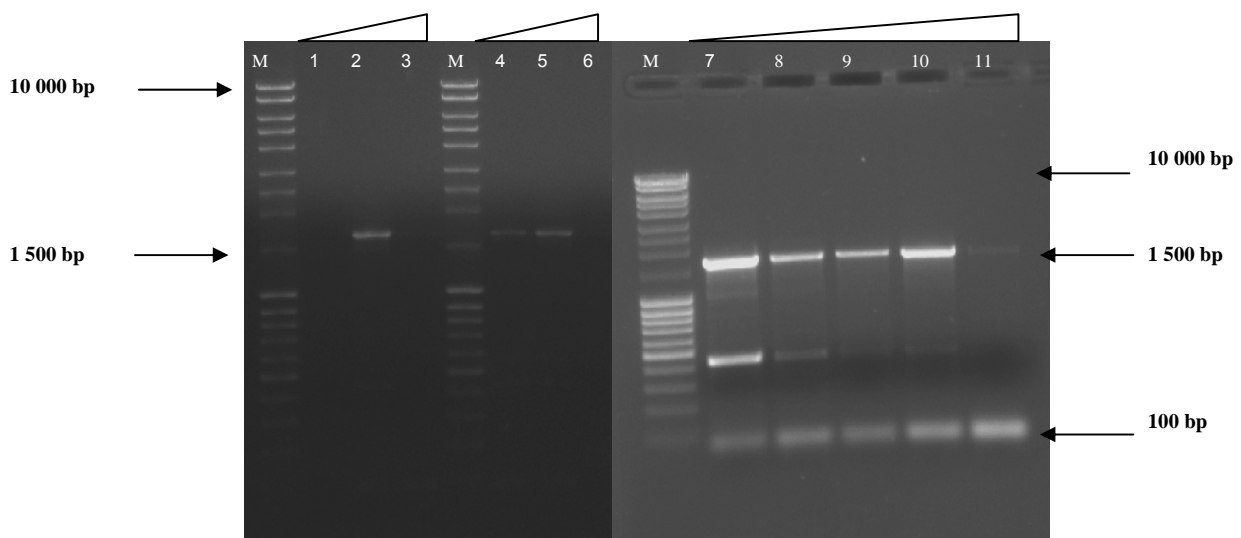
Figure 4.12. Amplification of *copA* gene using the primer sets C. Lane 1-3 *A. ferroxidans*; lane 4-6, *Acidithiobacillus* sp. and lane 7-11, 37ºC bioreactor sample. Gradient temperature: 50ºC, 55ºC and 58ºC for lanes 1-6 and 51ºC, 52ºC 54ºC, 55ºC and 58ºC.

Figure 4.13 shows the alignment of the amplified *copA* fragments of the consortium of bacteria, *Acidithiobacillus* sp. and *Acidithiobacillus ferrooxidans.* This again confirms the *Acidithiobacillus* sp.'s ability to interact with copper.

```
A_caldus      -----------------------------------------CCACAGGTTCTGGACCA 17
CONSORTIUM    -----------------------------------------CCACAGGTTCTGGACCA 17
copA_ferrox   CGGCAGCAAGGGGGATCGCCACGATGTTGTAGCCGGCGCCCCACCACAGGTTCTGGAC-A 179
                                                       ***************

A_caldus      TCTTGCGATAGGTCGCCCGGGAAAGTTCCAGGATGACCGCCACGTCTCGGGGATCGGAGC 77
CONSORTIUM    TCTTGCGATAGGTCGCCCGGGAAAGTTCCAGGATGACCGCCACGTCTCGGGGATCGGAGC 77
copA_ferrox   TCTTGCGATAGGTGGCGCGGGAAAGTTCCAGGATGGCCACCACGTCCCGGGGATCGG-GC 238
              ************* ** ***************** ** ******* ********* **

A_caldus      GAACCAGCACGATGTCCGCCGACTCGATGGCGACGTCCGTGCCCGCCCCGATGGCGATCC 137
CONSORTIUM    GAACCAGCACGATGTCCGCCGACTCGATGGCGACGTCCGTGCCCGCCCCGATGGCGATCC 137
copA_ferrox   GGACCAGCACGATGTCCGCCGACTCGATGGCGACGTCCGTGCCTGCCCCGATGGCGA-CC 297
              * ********************************************* ************* **

A_caldus      CGACATCCGCCTCCACCAGGGCAGGCGCATCGTTGACGCCGTCGCCGACCATGGCCACGC 197
CONSORTIUM    CGACATCCGCCTCCACCAGGGCAGGCGCATCGTTGACGCCGTCGCCGACCATGGCCACGC 197
copA_ferrox   CGACGTCCGCCTCCACTAGGGCGGGCGCATCGTTGACACCGTCGCCAACCATGGCCA-GC 356
              **** ********** ***** ************** ******* ********** **

A_caldus      GCAATCCCCGCGCCTTTACCTCCTTGATCTTCTCCGCCTTTTGTCCAGGGAGCACTTCGG 257
CONSORTIUM    GCAATCCCCGCGCCTTTACCTCCTTGATCTTCTCCGCCTTTTGTCCAGGGAGCACTTCGG 257
copA_ferrox   GCAACCCCCGCGCCTGCACTGCCTTGATCTTCTCCGCCTTTTGATCCGGCAGCACTT-GG 415
              **** ********** ** ********************* * ********* **

A_caldus      CGAAATAGTCGTCCAGCCCCATTTCCTGGGCCACCCAACGGGCCACCGCTGCGGAGTCGC 317
CONSORTIUM    CGAAATAGTCGTCCAGCCCCATTTCCTGGGCCACCCAACGGGCCACCGCTGCGGAGTCGC 317
copA_ferrox   CGAAATAATCGTCAAGCCCCATTTCCTGGGCCACCCAGCGGGCCACCGCTTCCGAGTC-C 474
              ******* ***** ******************** ************ ** ***** *

A_caldus      CGGTCAACATCATCACCTGTACGCCCATCCCCTTCAGCCGCGCGAGGGCTTCCCGCGACT 377
CONSORTIUM    CGGTCAACATCATCACCTGTACGCCCATCCCCTTCAGCCGCGCGAGGGCTTCCCGCGACT 377
copA_ferrox   CGGTCAGTATCATCACCTGTACGCCCATCCCCTTCAGCCGTGCGAGGGCTTCCCGCG-CT 533
              ****** ******************************** ***************** **

A_caldus      CCGCTCGAATGATGTCGGCCAGCGCGACAGCCCCCGCCGGTTTCTCGTCAACGAGCAGGT 437
CONSORTIUM    CCGCTCGAATGATGTCGGCCAGCGCGACAGCCCCCGCCGGTTTCTCGTCAACGAGCAGGT 437
copA_ferrox   CTGCTCGAATGATATCGGCCAGCGCGACCGCCCCTGCCGCTTTCTCGTCGACGAGCAG-T 592
              * *********** ************** ***** **** ********* ******** *

A_caldus      ATACCACCGTTTTGCCCTGGGATGCCAAGGCCGCGACCCGCTCATCGCTGACATCAAGGC 497
CONSORTIUM    ATACCACCGTTTTGCCCTGGGATGCCAAGGCCGCGACCCGCTCATCGCTGACATCAAGGC 497
copA_ferrox   ATACCACCGTTTTGCCCTGGGATGCCAAGGCTCCGATCCGCTCATCGCTGGCATCGAG-C 651
              *****************************   *** ************* **** ** *

A_caldus      CATGCTCCTTGAGATAGCCGGGGCTAACGACCTTCACATTGCGCCCATTTACAACGGCTT 557
CONSORTIUM    CATGCTCCTTGAGATAGCCGGGGCTAACGACCTTCACATTGCGCCCATTTACAACGGCTT 557
copA_ferrox   CATACTCCTTGAGATAGCCGGGGCTCACGACCTTCACGTTGCGTCCTTCCACGATGG-TT 710
              *** ********************* ********** ***** ** * ** * ** **

A_caldus      GCGCACCCTTGCCGGGGATGGCCTTGAATTCCTTGGGAGGCTCGAAGCCGATGCCCTTCG 617
CONSORTIUM    GCGCACCCTTGCCGGGGATGGCCTTGAATTCCTTGGGAGGCTCGAAGCCGATGCCCTTCG 617
copA_ferrox   GTGCGCCCTTGCCGGGGATGGCCTTGAATTCCTTGGGAGGCTCGAAGCTTATGCCCC-CT 769
              * ** ****************************************** ****** *

A_caldus      CTTTGGCATATGCCACGATGCCGGCGGCGATGGGGTGCTCCGACCGACTTTCGAGGGCCG 677
CONSORTIUM    CTTTGGCATATGCCACGATGCCGGCGGCGATGGGGTGCTCCGACCGACTTTCGAGGGCCG 677
copA_ferrox   CTTTGGCATCTGCCACGATGCCGGCGGCGATGGGGTGCTCCGACTGGCTTTCGAGGGT-G 828
              ********* ************************************ * ********* *

A_caldus      CGGCGAGCCGTACGACCTCATCTTCGGCGTATCCGCCGAGCACGACCACATCCGTA--TC 735
CONSORTIUM    CGGCGAGCCGTACGACCTCATCTTCGGCGTATCCGCCGAGCACGACCACATCCGTA--TC 735
copA_ferrox   CGGCGAACCGCACGACCTCATCTTCGGCATATCCGCCCAGCACGACCACATCCGTGAGCC 888
              ****** *** ***************** ******** ******************  *

A_caldus      GAGCGGGGTTTATTTCTACGGCGGTTGGCCCTTCCTGAAGGGGCTGTTTGCCG--AGCTC 793
CONSORTIUM    GAGCGGGGTTTATTTCTACGGCGGTTGGCCCTTCCTGAAGGGGCTGTTTGCCG--AGCTC 793
copA_ferrox   GAACCGCCCCTCCGTCAAGGTC--CCGGTCTTGTCGAACACCACCGCCTGCAGTTGGCCG 946
              ** * *     *    ** * * *     ** * * *     * * *** *    **
```

```
A_caldus      GAAACCC----GCAAGCCAGCCATGATGACGCTGATCGCCCTG-GCCATCAGCGTGGC-- 846
CONSORTIUM    GAAACCC----GCAAGCCAGCCATGATGACGCTGATCGCCCTG-GCCATCAGCGTGGC-- 846
copA_ferrox   CCCGCTCAAAAGCAGACCGGTCCCGGATAAGCAGCCCATGCTGCGCCGCCATGGCGGGGA 1006
                 * *    *** ** * * *  * ** *  *** *** *** ** * **

A_caldus      --CTATTTTTACTCG-AGTGCCGTCGTGTTCGGGGTTCAGGGTGAAGTGTTCTTCTGGGA 903
CONSORTIUM    --CTATTTTTACTCG-AGTGCCGTCGTGTTCGGGGTTCAGGGTGAAGTGTTCTTCTGGGA 903
copA_ferrox   CACGGCGACGACCAGCGGAATCGCTAGGCCCAGAGCATGGGGACAGGCAATCA-CCAAAC 1065
                *         ** *  *    **    * * * *    *** * *   ** *

A_caldus      ACTCGCCACGCTGATCGATGTGATGCTGCTCGGCCACTGGATCGAGATGAAATCGGTCCT 963
CONSORTIUM    ACTCGCCACGCTGATCGATGTGATGCTGCTCGGCCACTGGATCGAGATGAAATCGGTCCT 963
copA_ferrox   TGTGACCATGCGGGCCAGGGCAAACTCGAAGTGCTGGCCAATGACCAGCCAAGTGGAAGG 1125
                *  *** ** *  *   *  *   *    **    **    *   ** **

A_caldus      GGGGGCTTCCGGCGCACTGGAAAAACTGGTGCAAATGATGCCATCCGAGGCCCAT--CTC 1021
CONSORTIUM    GGGGGCTTCCGGCGCACTGGAAAAACTGGTGCAAATGATGCCATCCGAGGCCCAT--CTC 1021
copA_ferrox   GTAAGCCCCCCG-ACACTCAA-----CGCGGTAAGGGTCAGCATCAGGGCCGCACGGTCG 1179
                *   **  ** * ****  *     *  * ** *  **** * * **

A_caldus      GCAACCCCGGAGGGCATGAAAGACGTGCCGGTCGCCAATCTGC-AGCGCGGTGACC---G 1077
CONSORTIUM    GCAACCCCGGAGGGCATGAAAGACGTGCCGGTCGCCAATCTGC-AGCGCGGTGACC---G 1077
copA_ferrox   GCAAGTCCTGGGTCCGGGAGCGGGACTCCTGTGCCCGGCGCACCATCTCGATGACCTGTG 1239
              ****  ** * *  * ** *   ** ** **     * * * ** *****   *

A_caldus      GGTGTTGGT---GAAGCCCGGCGAAAAAATCCCTACGGACGGACGTGTCATCGAGG-GAC 1133
CONSORTIUM    GGTGTTGGT---GAAGCCCGGCGAAAAAATCCCTACGGACGGACGTGTCATCGAGG-GAC 1133
copA_ferrox   AAGATAGGTTTCACTGCCGGTCTTGTGGACCTTGACGGTGATACTTGACTCCCCGTTGAC 1299
                * ***    *** * *     * *  ****   ** ** *  *   *   ***

A_caldus      GGACCACCATCAACCAGGCCA----TGCTC------ACCGGCGAAAGCCAGCCCGTGGAG 1183
CONSORTIUM    GGACCACCATCAACCAGGCCA----TGCTC------ACCGGCGAAAGCCAGCCCGTGGAG 1183
copA_ferrox   GAACCACCGATAACTTCGTCACCGCTGCCCTTCTCTACGGGCCGACTTTCGCCAGTGACA 1359
              * ******   ***    * **    *** *     ** ***  *     *** ***

A_caldus      AAG----GGGGGCGGCGACGAAGTTATCGGCGGTTCGGTTAAC---GGGGA------GTC 1230
CONSORTIUM    AAG----GGGGGCGGCGACGAAGTTATCGGCGGTTCGGTTAAC---GGGGA------GTC 1230
copA_ferrox   TAGCCTGGTTGACGGTGGTTCGCCCATCGACGATCAGACCATCTGTAGGGATTTTTTGCC 1419
                **    *  * *** *      **** ** *  *  * *    ****      * *

A_caldus      GAGTATCACC--------GTCGAGGTCCACAAGACCGGCAGTGAAACCTATCTTGCACAG 1282
CONSORTIUM    GAGTATCACC--------GTCGAGGTCCACAAGACCGGCAGTGAAACCTATCTTGCACAG 1282
copA_ferrox   GGGCTTCACCAGCACCCGGTCACCGCGCTGCAGATTGGCGACCGG-CACGTCTTTCAACC 1478
              * *  *****        ***   *  *   *** ***        *  **** **

A_caldus      GTCATCGAGATGGTGCGCCGGGCGCAGGAGTCCCGCTCCCGGACCCAGGACTTGGCCGAC 1342
CONSORTIUM    GTCATCGAGATGGTGCGCCGGGCGCAGGAGTCCCGCTCCCGGACCCAGGACTTGGCCGAC 1342
copA_ferrox   GTC--CGGGGCCACGAGATGGGCCTCGGAAGGCATCATTTGCACCAGTTTTTTCCAGTGGC 1536
              ***   ** *     * * ****   ***   * *   * ***     *    * *

A_caldus      CGCGCGGCCCTGGTGCTGACCCTTACCGCGTTGAGCG-TCGGGGGGCTTACCCTTGCCAT 1401
CONSORTIUM    CGCGCGGCCCTGGTGCTGACCCTTACCGCGTTGAGCG-TCGGGGGGCTTACCCTTGCCAT 1401
copA_ferrox   CGGAAGCCCCCATGACCGATTTCATCTCGATCCAGTGGCCGAGCAACATCACATCGACAG 1596
              **    * ***     * **     *   * ** *  ** **    * * * * * **

A_caldus      T-TGGCTGGTCATTGGCC-AGCACTTCGAGTTCG-CCCTGCCCGCATGGTCACCGTTATG 1458
CONSORTIUM    T-TGGCTGGTCATTGGCC-AGCACTTCGAGTTCG-CCCTGCCCGCATGGTCACCGTTATG 1458
copA_ferrox   CGTAGCGAGTTCCCAGAAGAACACTTCGCCTTGAACCCCGAACACGACGGCACT-CGGTA 1655
                * **  **      *  * ******* **  *** *  *  * ***    *

A_caldus      GTGATTGCCTGTCCCCATGCTCTGGGCC------------------------------- 1486
CONSORTIUM    GTGATTGCCTGTCCCCATGCTCTGGGCCCG----------------------------- 1488
copA_ferrox   AAAATAGGCCACGCTGATGGCCAGGGCGATCAGCGTCATCATGGCTGGCTTGCGGGTTTG 1715
              ** * *     *   *** * **** * ****
```

Figure 4.13. Alignment of amplified copA fragments of the consortium of bacteria from 37ºC bioreactor, *Acidithiobacillus* sp. and *Acidithiobacillus ferrooxidans.*

84

4.4. Conclusions

Standard curves for copper(I) and copper(II) were set up at 390 nm to distinguish between the two valence states.

Prior to the copper assay, growth studies for *P. mirabilis* (bench-marker) and individual isolates were done. The results obtained conclusively showed that *P. mirabilis* is a fast growing organism with the doubling time of 0.33 hr while the remaining isolates might not be fast growing organisms since each of these took 15 to 20 days to grow. *Sulfobacillus* sp. has an average μ_{max} of 0.011 hr^{-1} with a doubling time (T_d) of 63 hr while *Leptospirillum* sp. exhibited an average μ_{max} of 0.031 hr^{-1} with a doubling time (T_d) of 22.35 hr and *Acidithiobacillus* sp. average μ_{max} was 0.0269 hr^{-1} with a doubling time (T_d) of 25.77 hr. Colour change was observed for these pure isolates starting from the exponential phase for example, for *Sulfobacillus and Leptospirillum* cultures, the colour changed from light green to brown. Also, according to Romero and co-workers (2003) *Leptospirillum* growth is usually monitored as a function of the production of ferric iron, measured by spectrophotometric absorption at 405 nm.

The spectrophotometric method used for determination of residual copper in the *P. mirabilis* culture, the 37ºC bioreactor sample and individual isolates gives an idea of how the organisms behave in copper environments. The complete genome sequence of *P. mirabilis* was done and it was revealed that this organism possesses a copper translocating P-type ATPase (Pearson *et al*., 2008), and amplification of a *pcoA* gene was achieved with the bench-marker organism (Ojo *et al*., 2008) which it uses to protect itself from the deleterious effect of high copper concentrations. The results obtained from the copper assay for *P. mirabilis* confirmed the presence of a copper translocating P-type ATPase in the organism as there was an uptake of copper at a certain period of time and, thereafter, an efflux of excess copper from the cell to the environment. Also, there is the possibility of NADH dehydrogenase reductase activity of copper in *P. mirabilis* since only copper(II), a stable ion, was initially present in the medium but after the efflux process, the assay showed the presence copper(I) in the medium.

Furthermore, when the bioreactor sample was used for the assay, there was a disappearance of copper after 3 hours when grown into copper medium; there was a decrease in copper(II) concentration in the medium and there was appearance of copper(I). After a further 2 hours the total copper concentration increased to 19.9% copper(II) and 59% copper(I) in the medium. Also, there is the possibility of a NADH dehydrogenase reductase activity in the consortium of bacteria. At this stage it was not possible to identify which of the organism(s) out of the consortium was responsible for this efflux. The consortium of these bacteria had a much better ability to interact with copper (MIC of copper was exhibited at 400 mM) in comparison to isolates *Sulfobacillus* sp. (MIC of copper: 6 mM), *Leptospirillum* sp. (MIC of copper: 3 mM) and *Acidithiobacillus* sp. (MIC of copper: 10 mM). The *copA* gene is just a start to elucidate why interaction is more effective when organisms are grown together, this needs more investigation.

The whole cell reduction showed that *Acidithiobacillus* sp. identified as *Acidithiobacillus caldus* was the organism responsible for active efflux of copper and was confirmed by amplification and sequencing of *copA* fragment present in *Acidithiobacillus caldus*.

In support of the above hypothesis, this study could not elucidate or confirm the resistance mechanism(s) of *Sulfobacillus* sp. and *Leptospirillum* sp.. No drastic reduction in the availability of copper in the buffer was seen and there was no amplification of a *pcoA* or *copA* fragment during PCR. With further investigations, the copper resistance mechanism(s) being utilized by each of these isolates can be elucidated.

4.5. References

Ahmed, M., Jahan, I. and Banoo, S. (2002). A simple spectrophotometric method for determination of copper in industrial, environmental, biological and soil samples using 2,5-Dimercapto-1,3,4-thiadiazole. *Analytical Sciences*. pp.805-810.

Argyrou, A. and Blanchard, J.S. (2004). Flavoprotein disulfide reductases; advances in chemistry function. *Progress in Nucleic Acid Research and Molecular Biology*. pp. 89-142.

Bertsova, Y.V., Bogachev, A.V. and Skulachev V.P. (2001). Ubiquinone oxidoreductase of *Azotobacter vinelandii* is required for diazotrophic growth at high oxygen concentrations. *Journal of Bacteriology*. pp. 6869-6874.

Blattner, F.R., Plunkett III, G. Craig, A. B., Perna, N.T., Burland, V., Riley, M., Collado-Vides, J., Glasner, J.D., Rode, C.K., Mayhew, G.F., Gregor, J., Davis, N.W., Kirkpatrick, H.A., Goeden, M.A. Rose, D.J., Mau, B and Ying, S. (1997). The complete genome sequence of *Escherichia coli* K-12. *Science*. pp. 1453-1462.

Bröer, S. Ji, G. Bröer A. and Silver, S. (1993). Arsenic efflux governed by the arsenic resistance determinant of *Staphylococcus aureus* plasmid PI258. *Journal of Bacteriology.* pp. 3480-3485.

Brown, N.L. and Rouch, D.A. (1995). Regulation of copper resistance genes in *Escherichia coli*. *Journal of Inorganic Biochemistry*. pp. 244-244.

Brown, N.L., Barrett, S.R., Camakaris, J., Lee, B.T., and Rouch, D.A. (1995). Molecular genetics and transport analysis of the copper-resistance determinant (*pco*) from *Escherichia coli* plasmid pRJ1004. *Molecular Microbiology*. pp. 1153-1166.

Cervantes C. and Gutierrez-Corona F. (1994). Copper resistance mechanisms in bacteria and fungi. *FEMS Microbiology*. pp. 121-37.

Cha, J.S. and Cooksey, D.A. (1991). Copper resistance in *Pseudomonas syringae* mediated by periplasmic and outer membrane proteins. *National Academy of Sciences, USA.* pp. 8915-8919.

Choudhury, R. and Srivastava, S. (2001). Zinc resistance mechanisms in bacteria: *Current Science*, pp.7.

Cook, S.A. and Shiemke, A.K. (2002). Evidence that a type-2 NADH: quinone oxidoreductase mediates electron transfer to particulate methane monoxygenase in *Methylcoccus capsulatus. Archives of Biochemistry and Biophysics.* pp. 32-40.

Cooksey, D.A. (1987). Characterization of a copper resistance plasmid conserved in copper-resistant strains of *Pseudomonas syringae* pv. tomato. *Applied Environmental Microbiology.* pp. 454–456.

Cooksey, D.A. (1994). Molecular mechanisms of copper resistane and accumulation in bacteria. *FEMS Microbiology. Review.* pp. 381-386.

Franke, S., Grass, G. and Nies D.H. (2001). The product of the *ybdE* gene of *Escherichia coli* chromosome is involved in detoxification of silver ions. *Microbiology.* pp. 965-972.

Gilotra, U. and Srivastava, S. (1997). Plasmid-encoded sequestration of copper by *Pseudomonas pickettii* strain US321. *Current Microbiology.* pp. 78–381.

Gordon, A.S., Donat, J.R., Kango, R., Dyer, B. and Stuart, L. (2000). Dissolved copper-complexing ligands in cultures of marine bacteria and estuarine water. *Marine Chemistry.* pp. 149-160.

Gordon, A.S., Howell, L.D. and Harwood, V. (1994). Responses of diverse heterotrophic bacteria to elevated copper concentrations. *Canadian Journal of Microbiology.* pp 408-411.

Gowri, P.M. and Srivastava, S. (1996). Reduced uptake based zinc resistance in *Azospirillum brasilense* sp7. *Current Science.* pp. 139–142.

Grass, G. and Resing, C. (2001). Genes involved in copper homeostasis in *Escherichia coli. Journal of Bacteriology.* pp. 2145-2147.

Hermansson, M., Jones, G.W. and Kjelleberg, S. (1987). Frequency of antibiotic and heavy metal resistance, pigmentation and plasmids in bacteria of the marine air-water interface. *Applied and Environmental Microbiology.* pp. 2338-2342.

Labuschagne, M. and Albertyn, J. (2007). Cloning of an epoxide hydrolase-encoding gene from *Rhodotorula mucilaginosa* and functional expression in *Yarrowia lipolytica*. *Yeast.* pp. 69-78.

Lee, S.M., Grass, G., Resing, C., Barrett, S.R., Yates, C.J.D., Stoyanov, J.V. and Brown, N.L. (2002). The Pco proteins are involved in periplasmic copper handling in *Escherichia coli. Biochemical and Biophysical Research Communications*. pp. 616-620.

Melo, A.M., Bandeiras, T.M. and Teixeira, M. (2004). New insights into Type II NAD(P)H: Quinone oxidoreductases. *Microbiology and Molecular Biology Reviews*. pp. 603-616.

Nies, D. H. (1992). CzcR and CzcD, gene products affecting regulation of resistance to cobalt, zinc and cadmium (*czc* system) in *Alcaligenes eutrophus. Journal of Bacteriology.* pp. 8102–8110.

Nies, D.H. (1999). Microbial heavy metal resistance. *Applied Microbiology and Biotechnology.* pp. 730-750.

Nies, D.H. (2000). Heavy metal-resistant bacteria as extremophiles: molecular physiology and biotechnological use of *Ralstonia* sp. CH34. *Extremophiles*. pp. 77-82.

Ojo, A.O., van Heerden, E. and Piater, L.A. (2008). Initial characterization of a copper resistant South African mine isolate. *African Journal of Microbiology Research*. pp. 281-287.

Outten, F.W., Huffman, D.L., Hale, J.A. and O'Halloran, T.V. (2001). The independent *cue* and *cus* systems confer copper tolerance during aerobic and anaerobic growth in *Escherichia coli. Journal of Biology and Chemistry.* pp. 30670–30677.

Pearson, M.M., Sebaihia, M., Churcher, C., Quail, M.A., Seshasayee, A.S. Luscombe, N.M., Abdellah, Z., Arrosmith, C., Atkin, B., Chillingworth, T., Hauser, H., Jagels, K., Moule, S., Mungall, K., Norbertczak, H., Rabbinowitsch, E., Walker, D., Whithead, S., Thomson, N.R., Rather, P.N., Parkhill, J. and Mobley, H.L. (2008). Complete genome sequence of uropathogenic *Proteus mirabilis*, a master of both adherence and motility. *Journal of Bacteriology.* pp. 4027-4037.

Ramamoorthy, S. and Kushner, D.J. (1975). Binding of mercuric and other heavy metal ions by microbial growth media. *Microbiology Ecology* pp. 162-176.

Rapisarda, V.A., Rodriguez M.L., Farias, R.N. and Massa, E.M. (1999). Characterization of an NADH-linked cupric reductase activity from *Escherichia coli* respiratory chain. *Archives Biochemistry and Biophysics.* pp. 143-150.

Rensing, C. and Grass, G. (2003). *Escherichia coli* mechanisms of copper homeostatis in a changing environment. *FEMS Microbiology Review.* pp. 197-213.

Rensing, C., Fan, B., Sharma, R., Mitra, B. and Rosen, B.P. (2000). CopA: an *Escherichia coli* Cu(I)-translocating P-type ATPase. *Proceedings of the National Academy of Sciences of the United States of America.* pp. 652-656.

Roberts, S.A., Weichsel, A., Grass, G., Thakali, K., Hazzard, J.T., Tollin, G., Rensing, C. and Montfort, W.R. (2002). Crystal structure and electron transfer kinetics of CueO, a multicopper oxidase required for copper homeostasis in *Escherichia coli. Proceedings of the National Academy of Sciences of the United States of America.* pp. 2766–2771.

Rodriguez, M.L., Volentini, S.I., Farias, R.N., Massa, E.M. and Rapisarda, V.A. (2006). The Cu(II) reductase NADH dehydrogenase-2 of *Escherichia coli* improves the bacterial

growth in extreme copper concentrations and increases the resistance to the damage caused by copper and hydroperoxide. *Archives Biochemistry and Biophysics*. pp. 1-7.

Romero, J. Yañez, C., Vásquez, M., Moore, E.R.B. and Espejo, R.T. (2003). Characterization and identification of an iron-oxidising *Leptospirillum* bacterium present in the high sulfate leaching solution of a commercial bioleaching plant. *Research in Microbiology*. pp. 353-359.

Rosen, B.P. (2002). Transport and detoxification systems for transition metals, heavy metals and metalloids in eukaryotic and prokaryotic microbes. *FEMS Microbiology Review*. pp. 197-213.

Schreiber D.R., Millero, F.J. and Gordon, A.S. (1990). Production of an extracellular copper-binding compound by the heterotrophic marine bacterium *Vibrio alginolyticus. Marine Chemistry.* pp. 275–284.

Silver, S. (1996). Bacterial resistance to toxic metal ions - a review. *Gene.* pp. 9-19.

Silver, S. and Phung, L.T. (1996). Bacterial heavy metal resistance: New surprises. *Annual Review Microbiology.* pp. 753-789.

Solioz, M. and Stoyanov, J.V. (2003). Copper homeostasis in *Enterococcus hirae. FEMS Microbiology. Review*. pp.183-195.

Solioz, M. and Vulpe, C. (1996). CPX-type ATPases: a class of P-type ATPases that pump heavy metals. *Trends in Biochemical Science*. pp. 237-241.

Srivastava, S., Choudhury, R. and Joshi, N. (1999). Mechanisms of zinc resistance in *Pseudomonas putida* strain S4. *Advances in Microbial Biotechnology* (eds Tewari, J.P., *et al.*), APH Publishing corporation, New Delhi.

Stoyanov, J.V., Hobman, J.L. and Brown, N.L. (2001). CueR (Ybb1) of *Escherichia coli* is a MerR family regulator controlling expression of copper exporter CopA. *Molecular Microbiology* pp 502-511.

Teitzel, G.M. and Parsek, M.R. (2003). Heavy metal resistance of biofilm and planktonic *Pseudomonas aeruginosa. Applied and Environmental Microbiology.* pp 2313-2320.

Teixeira, E.C., Franco de Oliveira, J.C., Novo, M.T.M. and Bertolini, M.C. (2007). The copper resistance operon *copAB* from *Xanthomonas axonopodis* pathovar citri: gene inactivation results in copper sensitivity. *Microbiology.* pp. 402-412.

Trummer, E., Fauland, K, Seidinger, S., Schriebl, K., Lattenmayer, C., Kunert, R., Vorauer-Uhl, K., Weik, R., Borth, N., Katinger, H., and Müller, D. (2006). Process parameter shifting: Part I. Effect of DOT, pH, and temperature on performance of Epo-Fc expressing CHO cells cultivated in controlled batch bioreactors. *Biotechnology and Bioengineering.* pp. 1033-1044.

Voloudakis, A.E., Reignier, T.M. and Cooksey, D.A. (2005). A copper resistance genetic trait carried by *Xanthomonas axonopodis* pv*. vesicatoria* plasmids. *Applied Environmental Microbiology.* pp. 782-789.

WebElements www.webelements.com/copper [Accessed October 14, 2008].

Chapter 5

Summary

Copper ions play an essential role in many biological systems but above optimum concentrations it exerts toxic effects. Some micro-organisms mediate the toxic effects of copper through resistance mechanisms the organisms possess. These vary with the resistance systems.

Microbial diversity studies for 37°C and 70°C bioreactors were done to characterize the organisms present in the industrial copper bioreactors used for bioleaching of copper from its ore. After 16S rDNA amplification both reactors communities were defined. The BLASTn results obtained from the selected clones of the 37°C bioreactor revealed the presence of *Sulfobacillus thermosulfidooxidans, Leptospirillum ferriphilum* (these were associated) and *Acidithiobacillus caldus* (which was 100% similar), and *Acidianus* sp., *Solfobales* archaeon and *Metallospaera* sp. were present in the 70°C bioreactor. This conforms to the suggested organisms present in the bioreactor by MINTEK.

Prior to determination of MIC of copper exhibited by the organisms, suitable medium was selected for the 37°C bioreactor organisms and the organisms present in this selected medium was assessed through amplification and sequencing analysis of the DGGE products. The result obtained showed the presence of the three organisms present in the 37°C reactor. Each species was isolated through selective media and the minimum inhibitory copper concentrations were determined thereafter.

The single species were not able to tolerate the high copper concentrations the consortium was able to withstand. The MIC of copper of the consortium of bacteria (organisms from 37ºC bioreactor sample) were determined to be 400 mM, while isolation of each of the organisms present in the 37ºC bioreactor sample led to a drastic drop in copper MIC; *Sulfobacillus* isolate exhibited MIC of copper at 6 mM, *Leptospirillum* isolate

a minimum inhibitory copper concentration at 3 mM and *Acidithiobacillus* sp. showed a minimum inhibitory copper concentration at 10 mM.

Standard curves for copper(I) and copper(II) were set up at 390 nm to distinguish between the two valence states. Whole cell interaction with copper showed the ability of *Proteus mirabilis* and the consortium of bacteria to take up copper(II) and release copper copper(I) at a certain period of time. The individual isolates were subjected to copper(II) environment to assess the ability to interact with copper. The results obtained showed less uptake of the divalent state of copper in *Sulfobacillus* sp. and *Leptospirillum* sp. and no copper(I) was detected. In contrast, *Acidithiobacillus* sp. was able to take up copper(II) and actively reduce it to copper(I). *Acidithiobacillus* sp. may be dominant in the ability to reduce copper(II) and in the consortium. Efflux of copper(I) has been demonstrated by Rensing and co-workers (2000).

The copper resistance mechanism of these organisms was further characterized and the whole cell reduction showed that *Acidithiobacillus* sp. identified as *Acidithiobacillus caldus* was the organism responsible for active efflux of copper(I) and was confirmed by amplification and sequencing analysis of the *copA* fragment.

There was no PCR amplification of a copper resistance fragment in *Sulfobacillus* sp. and *Leptospirillum* sp. but amplification thereof in *Acidithiobacillus* sp. was obtained, while there was also amplification of the fragment in the consortium of bacteria. The BLASTn results obtained after sequencing analysis showed similarity to *Acidithiobacillus ferrooxidans copA* gene. As a result, full length gene specific primers were designed using the *Acidithiobacillus ferrooxidans copA* sequence. The efforts to amplify a full length copA fragment from *Acidithiobacillus caldus* were fruitless. As a result, for quality control, the previous set of designed primers was used for amplification of a *copA* in *Acidithiobacillus ferrooxidans* and there was amplification. The results confirm that *Acidithiobacillus caldus* possesses a copper translocating P-type ATPase which it uses to protect itself from copper toxicity and the remaining organisms in the bioreactor.