ISOLATION AND CHARACTERIZATION OF BIOOXIDIZING BACTERIA FROM THE OBUASI GOLD MINING SITE

BY

RICHARD HARRY ASMAH

ISOLATION AND CHARACTERIZATION OF BIOOXIDIZING BACTERIA FROM THE OBUASI GOLD MINING SITE

A Thesis Presented to the

The board of Graduate studies University of Ghana, Legon.

Ghana.

In Partial Fulfillment of the Requirement for the Degree

of Master of Philosopy (M.Phil.) in Biochemistry.

By

RICHARD HARRY ASMAH BSc. (Hons.)

Department of Biochemistry,

Faculty of Science,

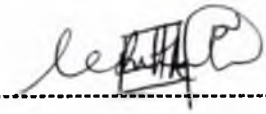
University of Ghana,

Legon, Accra, Ghana.

September, 1998

DECLARATION

I do hereby declare that except for references to other people's work which I have duly acknowledged, this exercise is a result of my own research, and this thesis, either in whole, or in part has not been presented for another degree elsewhere.

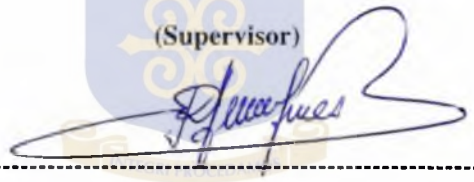


RICHARD HARRY ASMAH

(Student)

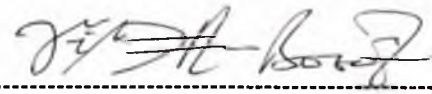
Dr. Y. D. OSEI

(Supervisor)



Dr. F. K. RODRIGUES

(Supervisor)



Dr. K. M. BOSOMPEM

(Supervisor)

DEDICATION

To my mother Beatrice Ellis and in memory of my late father, Harry Benjamin Asmah and late aunties Victoria, Sarah and Christina Ellis. And to my brothers, sisters, cousins and entire family. Thank you for all your love and care.

ACKNOWLEDGEMENTS

I am glad to have this opportunity to express my deepest gratitude to my supervisors, Drs. Y. D. Osei and F. K. Rodrigues of the Department of Biochemistry and Dr. K. M. Bosompem of the Noguchi Memorial Institute For Medical Research (NMIMR). This work was completed through their expert guidance, unfailing courtesy, help and co-operation. Their invaluable comments and critical evaluation enabled me to greatly improve the quality of this work.

I also wish to express my sincere appreciation to Prof. M. E. Addy, the former head of Department of Biochemistry, University of Ghana, for suggesting to me the project area as well as helping me to obtain the samples used in the work. I am also appreciative of her invaluable comments and help in shaping the work. My deepest gratitude goes to Mr. C. Clement for the invaluable assistance in initiation of the project as well as for his continued interest and encouragement. I also express my appreciation to Dr. J. P. Adjimani and all the lecturers of the Department of Biochemistry for their help in difficult times. I wish to acknowledge my indebtedness to all individuals and institutions who in various ways, contributed to the formulation, execution and submission of the work described in this thesis. My sincerest thanks go particularly to the Ashanti Goldfields Company Limited for permitting me to collect the samples used in project.

I really appreciated the help and co-operation of the technical staff, Mr. F. O. Bosompem, the chief technician, Mr. S. A. Y. Feyi, Mrs. Doris Amanor and Mrs. R. Nyarko, the administrative staff, Mr. S. Dramanu, Mrs. C. Nettey, and Mrs. H. Antwi-Boasiakoh, thank you all. I also appreciate the companionship and fruitful discussions with my mates Messrs J. Asiedu-Larbi, H. Asare-Anane, R. M. Yawson, D. Akakpo. To my roommate Mr. Kennedy Acquaah I say thank you for your support through tough times. I wish also to express my sincere thanks to Mr. Appawu, Dr. Armah, Dr. Wilson and Dr. Akanmori of the NMIMR for their help and encouragement, and for allowing me to

use their laboratory facilities. I am extremely grateful to Mrs. Irene Ayi, Mr. William Anyan, Mrs. Bridgette Ogoe, Miss Anita Ofosu-Okyere, Mr. Mike Osei-Atweneboana of the Parasitology Unit, Mr. Mike Ofori of the Immunology Unit of the NMIMR and Mr. Ismaela Abubakar of the computer room for their helpful advice, encouragement and assistance during the work described in this thesis.

To my friends at Campus Christian Family, Miss Juliana Dei-Frempong, Mr. Parker Yarney and Mr. Charles Brown I wish to express my appreciation for their encouragement and prayers. I finally acknowledge the help of all those whose names I might have not mentioned but contributed in making this project a success. May God bountifully bless you.

TABLE OF CONTENTS

LIST OF FIGURES

LISTS OF TABLES

ABBREVIATIONS

APS	-Ammonium per sulphate
2-ME	-2-mercaptoethanol
NMIMR	-Noguchi Memorial Institute for Medical Research
SDS-PAGE	-Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
TEMED	-N, N, N'- N-tetramethylethylenediamine
RFLP	-Restriction Fragment Length Polymorphism
PMSF	-Phenyl methyl sulfonyl fluoride
TPCK	-Tosyl phenyl alanine chloromethyl ketone
TLCK	-Tosyl lysine alanine chloromethyl ketone

ABSTRACT

Processing of gold arsenopyrite and sulphide ores is currently done with biooxidizing bacteria. This procedure is preferred because of its environmental friendliness and efficiency of gold recovery from ores, compared to the conventional methods. The efficiency of biomining is, however, largely influenced by the origin of biooxidizing bacteria used and local organisms have been found to be better adapted to extracting gold from the ore from which they were isolated.

The work reported in this thesis was conducted with the objective of isolating and characterizing local acidophilic bioleaching bacteria from surface arsenopyrite and underground sulphide gold ores and underground mine water at the Ashanti Goldfields Company (AGC) in Ghana, one of the world's richest gold mines. Biooxidizing bacteria were also isolated from slurry from the commercial bioleaching tank at AGC and characterized for comparative purposes.

Local biooxidizing bacteria were isolated from surface arsenopyrite and underground sulphide gold ores and underground mine water and identified using cultural, physiological, morphological and biochemical criteria. In all, eleven bacterial isolates were obtained from the samples collected. Their cell morphology showed that seven isolates were straight rods while the others were curved rods. All the bacterial isolates were physiologically either ferrous iron (Fe^{2+}) oxidizers or sulpho (S^{o})-oxidizers, Gram negative, mesophiles as well as aerobes.

Three representative pure biooxidizing bacteria isolates were obtained from the surface ore (SO). These were, SO1-Fe^{2+} and SO2-Fe^{2+} both of which were ferrous iron oxidizers and SO3-S^{o} which was a sulpho oxidizer. Similarly, three pure isolates were obtained from the underground ore (UO1-Fe^{2+}, UO2-Fe^{2+} and UO3-S^{o}). However, only two representative ferrous iron oxidizers were purified from underground mine water (UW1-Fe^{2+} and UW2-Fe^{2+}). The ferrous iron bacterial isolate 2 (SO2-

Fe^{2+}, UO2-Fe^{2+} and UW2-Fe^{2+}) from each of the samples failed to grow on solid media whilst, the ferrous isolate 1(SO1-Fe^{2+}, UO1-Fe^{2+}, UW1-Fe^{2+}) as well as the sulpho oxidizers (SO3-S° and UO3-S°) grew on solid media. Biooxidizing bacterial isolates (BT1-Fe^{2+}, BT2-$Fe^{2+}$and BT3-S°) obtained from the commercial bioleaching tanks exhibited similar characteristics. Characterization of the purified bacterial isolates using cultural, physiological, morphological and biochemical criteria revealed that the ferrous iron oxidizers, isolate 1 (BT1-Fe^{2+}, SO1-Fe^{2+}, UO1-Fe^{2+} and UW1-Fe^{2+}) were *T. ferrooxidans* and the second isolates (BT2-Fe^{2+}, SO2-Fe^{2+}, UO2-Fe^{2+} and UW2-Fe^{2+}) were *L. ferrooxidans*. The sulpho oxidizers (BT3-S°, SO3-S° and UO3-S°) were identified as *T. thiooxidans*.

In addition to the absence of sulpho-oxidizing bacterium (*T. thiooxidans*) in the underground mine water, the iron oxidation rates of *T. ferrooxidans* and *L. ferrooxidans* isolates obtained from the sample were slower compared to isolates of the same bacteria from the other samples. Furthermore, *T. ferrooxidans* isolated from underground mine water did not exhibit pleomorphism and had a unique colony size (2-5mm) as compared to the other isolates (0.5-5mm) thus suggesting that it could be of a different strain. Attempts to characterize the bacterial isolates and detect strain differences by restriction fragment length polymorphism of their DNA, however, proved difficult because of smearing of bands.

Soluble proteins from crude bacterial cell lysates of all the purified isolates (BT1-Fe^{2+}, SO1-Fe^{2+}, UO1-Fe^{2+}, UW1-Fe^{2+}, BT3-S°, SO3-S° UO3-S° BT2-Fe^{2+}, SO2-Fe^{2+}, UO2-Fe^{2+} UW2-Fe^{2+}) were electrophoresed on 15-20 % acrylamide gradient gels, using the SDS-tris-glycine discontinuous buffer system, and the protein profiles analyzed. This electrophoretic analysis showed that the *T. ferrooxidans* isolates (BT1-Fe^{2+}, SO1-Fe^{2+}, UO1-Fe^{2+} and UW1-Fe^{2+}) had similar protein profiles. Similarly, *T. thiooxidans* isolates (BT3-S°, SO3-S° and UO3-S°) and *L. ferrooxidans* isolates (BT2-Fe^{2+}, SO2-Fe^{2+} and UO2-Fe^{2+}) had unique protein profiles. The protein profiles clearly differentiated

the bacterial isolates at the species level. This study has therefore demostrated the presence of local isolates of the 3 important biooxidizing bacteria utilized in biomining and appears to suggest that some of the local isolates are different strains that may be used for more effficent gold extraction at the AGC.

CHAPTER 1

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Trading in precious metals especially gold has for centuries been an important economic activity in Ghana. Today, mining, processing and export of mineral ores including gold, bauxite, manganese and diamond constitute a sizeable percentage of the total export earnings of Ghana. Presently, gold, the largest income generating export commodity in Ghana accounts for over 50% of the national foreign exchange earnings (AGC Annual report, 1997). This contributes to the financing of health, education and infrastructural development amongst other developmental priorities of the nation.

With civilization and technological advances, the consumption of diverse metal products has increased immensely. Unfortunately, just like in other parts of the world, there is an apparent exhaustion of the supply of high grade mineral ore in Ghana due to the successive mining of such mineral deposits (Rawlings and Woods, 1995). There are, however abundant quantities of low-grade ore but this is difficult to process using conventional technology or traditional methods. The conventional technology used in processing this type of ore (roasting at high temperatures and pressure oxidation) cannot efficiently handle extraction of the metal. These methods are known to be inefficient, expensive and environmentally degrading (Ehrlich and Holmes, 1985; Barrett *et al.*, 1993). It is therefore, necessary to shift to the use of more efficient extraction methods such as biotechnological applications, if the industry is to survive into the next century (Barrett *et al.*, 1993; Morin, 1995; Pizzaro *et al.*, 1996). The applicability of biotechnological methods such as processing mineral ore with microbes (biomining) in some of the major gold mining industries of the world was communicated by Rawlings and Silver (1995). Microbial mining (biohydrometallurgy) began in 1963 when results of laboratory studies confirmed the involvement of bacteria in the solubilization of copper from sulphidic ore (Beck, 1967). Subsequently, various sulpho-oxidizing and ferrous

oxidizing bacteria were reported to enhance the extraction of gold, uranium and copper from their respective low grade ores (Rawlings and Silver, 1995). Today, biomining is known to be easy to implement, cost effective and has minimal effects on the environment (Barrett *et al.*, 1993).

Chemolithotrophic bacteria of the genera *Thiobacilli* and *Leptospirilli* are used in the extraction of gold from refractory ore. In refractory ore, gold is trapped in a compound matrix with sulphur, ferrous iron and arsenic (Barrett *et al.*, 1993). This process which is known as bioleaching, gives 95 to 99% efficiency in the recovery of gold as compared to conventional methods of roasting and cyanide treatment which give 30 to 50% recovery (Norman and Snyman, 1988; Morin, 1995). A synergistic mixture of *Leptospirillium ferrooxidans, Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans* are extensively used in the gold mining industries of the world (Morin, 1995; Jerez *et al.*, 1995). These bacteria obtain energy by oxidizing sulphide and ferrous iron components of the ore thereby releasing chemically trapped gold particles. Other products of these reactions are sulphuric acid and ferric compounds.

The importance of biomining at one of the richest gold mines in the world, the Ashanti Goldfields Company Limited (AGC) in Ghana cannot be over-emphasized. AGC has proven reserves of about 21 million ounces of gold and it currently operates the world's largest bioleaching plant for refractory gold ore (Osae *et al.,* 1995; AGC Annual Report, 1996). The plant processes about 800 tons of ore per day and provides about 30 to 40% of the total gold ore processed by the company (AGC Annual Report, 1996). Microbial mining activity at AGC mining site is likely to increase in the future because of the progressive depletion of high grade oxide ore in the presence of large quantities of low grade arsenopyrite and sulphide ores (AGC Annual Report, 1994). It is, therefore important to ensure that the efficiency of the bioleaching plant is maintained at a high level. One way to ensure such efficiency is to use local strains of bioleaching organisms (Rawlings and Woods, 1995). This is

because efficiency in biooxidization depends on the bacterial strains found in the particular environment. It has also been observed that every ore deposit is unique with respect to its mineralogy and chemical composition (Rawlings and Woods, 1995) thus bacterial populations that rapidly oxidize one ore deposit may not be very active on another deposit which has higher concentrations of heavy metals such as arsenic and silver which may be toxic to the bacteria (Pakniker and Agate, 1987). Interestingly, bacterial strains used by the AGC in the sulphide treatment plant (STP) were obtained from Gencor Company in South Africa and they are being used to process surface arsenopyrite and underground sulphide ores that have different characteristics.

These biooxidizing bacteria are known to exist in soils containing sulphur compounds (Waksman and Joffe, 1922), refractory gold ore (Livesey-Goldblatt *et al.*, 1983; Livesey-Goldblatt, 1986), acid mine drainage and underground mine water (Colmer *et al.,* 1950; Beck, 1960; Rawlings and Woods, 1995). It is therefore possible to obtain local isolates of biooxidizing bacteria from the AGC (Obuasi) mining site, which may be more efficient than the strains currently in use at the sulphide treatment plant. It is equally important to characterize any local isolates that might be found since efficiency in biooxidation depends on the bacterial strain found in a particular environment (Rawlings and Woods, 1995).

It was based on these observations that the work described in this thesis was conducted to isolate and characterize biooxidizing bacteria from different sources at the Obuasi mining site.

JUSTIFICATION FOR THE WORK:

The isolation and characterization of local biooxidizing bacteria from the Obuasi mining site (Ghana) would provide valuable information on local biooxidizing organisms from a part of the world that had previously not been explored. Furthermore, the characterization of local biooxidizing bacteria may lead to the identification of bacterial strains which are better suited for more efficient extraction of gold from surface arsenopyrite and underground sulphide ores of different characteristics.

1.2 Objectives of Study

(a) To isolate and identify the iron and sulpho-oxidizing bacteria (*T. ferrooxidans, T. thiooxidans* and *L. ferrooxidans*) from the Sulphide Treatment Plant (STP) reactor tanks at the Obuasi mining site.

(b) To isolate and identify local strains of these organisms from the surface arsenopyrite and underground sulphide ores, and from underground mine water at Obuasi.

(c) To establish pH, temperature and substrate concentration profiles for optimum growth of the local biooxidizing bacterial isolates.

(d) To determine possible similarities and differences in protein profiles between local biooxidizing bacterial isolates and those present at the STP using Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE).

(e) To determine possible similarities and differences in DNA fingerprints between local biooxidizing bacterial isolates and those present at the STP using Restriction Fragment Length Polymorphism (RFLP) of genomic DNA.

1.3 Literature Review

1.3.1 Biomining

Micro-organisms have been used as biotechnological tools for many purposes. Biomining is the process of metal extraction which uses micro-organisms such as bacteria in commercial mining operations (reviewed by Brierley, 1978; Lundgren and Silver, 1980). The bacteria attack and help dissolve mineral ore, by converting insoluble metal deposits such as metal sulphides or oxides of gold, copper and uranium through metabolic processes to soluble metal sulphates. According to Rawlings and Silver (1995) and Barrett *et al.* (1993) this exploitation of micro-organisms simplifies metal extraction from low grade mineral ore. The involvement of bacteria in metal leaching is reported to be physiological and is as a consequence of the bacteria's mode of metabolism which results in their growth and survival in the mining environment (Lundgren and Silver, 1980).

1.3.2 Biohydrometallurgy and Conventional Methods of Low-Grade Ore Processing

Hydrometallurgy is the process of dissolving metals from mineral ore and the recovery of the desired metals. The involvement of bacteria in this process is called biohydrometallurgy. Two main conventional methods were developed for commercial extraction of gold from low grade ore. These were (1) pressure oxidation, involving digestion of the ore with acid in a pressure cooker in an oxygen enriched atmosphere and (2) roasting the ore in a furnace at 700°C in the presence of oxygen (Barrett *et al.*, 1993; Morin, 1995). The application of these mining methods were found necessary because of several reasons. For example, the depletion of high grade ore deposits forced mining companies to work low grade ores and also to develop more efficient methods of recovering small quantities of metals left after physical processing of richer ore materials (Rawlings and Woods, 1995).

Nevertheless, the use of these conventional mining methods led to other related problems such as high cost of metal extraction compared to value of metal recovered. Also there was the need for highly skilled operators to run processing plants (Morin, 1995) and environmental pollutants were

created (Rawlings and Silver, 1995). Mining companies therefore had to look for alternative methods that were preferably more cost effective and environmentally friendly for recovering valuable metals from low grade ores (Livesey-Goldblatt *et al.*, 1983; Ehrlich and Holmes). The most suitable alternative to conventional mining techniques so far introduced is bioleaching (Morin, 1995).

Bioleaching is a hydrometallurgical process in which mineral ore is differentially or collectively solubilized (Barrett *et al.*, 1993). By this process insoluble metal sulphides and oxides are made soluble (leached from ores) through the natural action of autotrophic bacteria under suitable growth conditions. These bacteria called biooxidizers, carry out oxidation of the mineral ores as a means of generating energy for cell growth, cell division and other cellular metabolic processes. Most commercial bioleaching is however, carried out with a consortium of chemolithotrophic bacteria which efficiently oxidize ferrous compounds and reduced sulphur compounds present in mineral ores. However, heterotrophic bacteria and other micro-organisms such as yeast, fungi, algae and mould are believed to play significant but undetermined roles which enhance the biooxidation of mineral ore. According to Barrett *et al.* (1993) the overall significance of the heterotrophic species in metal leaching systems is not yet clear because of lack of suitable data.

1.3.3 Bacterial Leaching of Mineral Ore

Some inorganic ion oxidizing chemolithotrophic bacteria have the ability to oxidize insoluble metal sulphides and solubilize a wide range of metals including copper, uranium and more recently gold bearing arsenopyrite ores in great tonnages. Other ore types processed using bacterial leaching include sulphides of zinc, lead, cobalt, nickel, bismuth, molybdenum and manganese (Brierley, 1978; Brierley, 1982; Barrett *et al.*, 1993; Rawlings and Silver, 1995). It is reported that bacterial leaching activity can achieve 100% recovery of metal from ores containing as low as 0.03% to 0.3% metal (Rawlings and Silver, 1995). Most strains of bacteria being used in bioleaching operations have been

isolated from sites where natural leaching takes place (Lundgren and Silver, 1980; Harrison, 1984) and the bacteria are therefore classified on the basis of the ore they oxidize. Commercial bioleaching processes include dump, *in situ*, heap, vat, and agitated tank leaching methods (Barrett *et al.,* 1993). In bioleaching of refractory gold ore, the agitated or stirred reactor method is mostly used (Brierley, 1978; Barrett *et al.,* 1993; Rawlings and Silver, 1995).

1.3.4 Classification of Biooxidizing Bacteria used in Mining Operations

(i) *Ferrous Iron and Sulpho-oxidizing Bacteria*

Ferrous iron bacteria oxidize iron in the +2 oxidation state (Fe^{2+}) to its +3 state (Fe^{3+}). Those used in mining operations belong to the genera *Thiobacillus* and *Leptospirillum*. The ore types oxidized by these bacteria include arsenopyrite (FeAsS), pyrite (FeS_2), mascasite (Fe_2S), pyrrhotite (FeS) and chalcopyrite ($FeCuS_2$) (Lundgren and Silver, 1980; Barrett *et al.,* 1993; Rawlings and Silver, 1995).

Unlike ferrous iron oxidizers, sulpho-oxidizing bacteria oxidize elemental sulphur as well as sulphur in other oxidation states (reduced sulphur and partially reduced sulphur as found in sulphidic ore) to +4 oxidation state to form sulphuric acid. Sulpho-oxidizers used in the mining industry belong mainly to the genus *Thiobacillus* (Rawlings and Silver, 1995).

(ii) *Bacterial Growth Temperatures*

The optimum growth temperature of biooxidizing bacteria is one of the criteria used in the classification of the microbes utilized in biohydrometallurgical processes. The important groups based on temperature are: (a) mesophiles of the genera *Thiobacillus* and *Leptospirillum* which grow at temperatures from 4 to 40 °C, (b) moderate thermophiles of the genus *Sulfobacillus* together with a number of unidentified strains, which grow at temperatures from 40 to 55°C, (c) extreme thermophiles of the genera *Sulfolobus, Acidanus, Metallosphaera* and *Sulfurococcus* which grow at

temperatures greater than 55°C and (d) heterotrophic micro-organisms of the genera *Acidophilum* and *Acetobacter* among others with growth temperatures of 59°C and 5 to 42 °C, respectively. Some of the heterotrophic bacteria have commensal relationship with the mesophilic biooxidizing bacteria (Brierley, 1978; Lundgren and Silver, 1980; Harrison, 1984).

However, most bacteria presently used in commercial mining are mesophiles of the genera *Thiobacillus* and *Leptospirillum,* (Morin, 1995) even though, the other types of micro-organisms mentioned above are also believed to play important roles in the biooxidation of mineral ore (Brierley, 1978; Rawlings and Silver, 1995).

1.3.5 Chemolithotrophic Bacteria as Catalytic Agents in Mining

Chemolithotrophic bacteria make use of inorganic salts in deriving energy for their life processes. They thus act as biological catalysts in mineral ore processing by helping in the oxidation of the ore leading to the release of metals. Most bacteria used in catalytic oxidation of mineral ore are mixed cultures which share synergistic benefits, making the oxidation of low grade ore more efficient than the conventional methods used (Barrett *et al.,* 1993; Rawlings and Silver, 1995). Presently, the most extensively (commercially) exploited bacterial species which efficiently and rapidly attack mineral ore are *T. thiooxidans* and *T. ferrooxidans* from the genus *Thiobacillus* and *L. ferrooxidans* from the genus *Leptospirillum* (Rawlings and Woods, 1995).

(i) *Genus Thiobacillus*

Sulphur oxidizing bacteria were discovered in 1902 by Beijerinck (cited by Vishnac, 1975). Since then at least 14 species have been reported. These microbes utilize elemental sulphur, sulphur compounds and sulphuric ore as substrates for energy metabolism (Vishnac, 1975; Barrett *et al.,* 1993). The most studied of the *Thiobacillus* associated with metal extraction are *T. thiooxidans* and

T. ferrooxidans. Although there is evidence that most of the remaining 12 species may participate in biooxidation of mineral ore, only the two species mentioned are important in biomining (Rawlings and Silver, 1995). An important characteristic of the two species is that they can better tolerate ions of heavy metals like copper, nickel, zinc, uranium and arsenic. The high resistance of these bacterial strains to metal ions cannot be explained by only physiological and genetic properties of the organisms (Barrett *et al.,* 1993). It is reported that this property may just be a pseudo-resistance which depends on the state of the metallic ions in the different environments such as: (1) complexing of the ions with dead bacterial cells or organic metabolites, (2) precipitation of the metal ions, (3) low pH of the media which makes the bacteria surface binding sites less available for metals and (4) presence of other substances that compete with the toxic or metalloid species (Barrett *et al.,* 1993; Rawlings and Kusano 1994). Nevertheless, it is known that other heavy metals such as silver, mercury, antimony, cadmium, chromium and molybdenum may inhibit *T. thiooxidans* and *T. ferrooxidans* (Brierley, 1978; Barrett *et al.,* 1993; Rawlings and Silver, 1995).

(a) *Thiobacillus thiooxidans*

Thiobacillus thiooxidans was first isolated in 1922 (Waksman and Joffe, 1922) from compost soil. Three species types (1, II and III) are classified under genus *Thiobacilli* based on their cellular lipid composition (Vishnac, 1975). The *T. thiooxidans* utilized in biooxidation is placed under the type III species. Their growth temperature ranges from 2 to 40 °C, with the optimum between 25 to 35 °C (Barrett *et al.,* 1993). The organisms are reported to grow at pH ranging from pH 0.5 to 6 (Vishnac, 1975; Konishi *et al.,* 1995) with an optimum from pH 1.5 to 2.0 (Rawlings and Silver, 1995). Thus, *T. thiooxidans* is able to grow under very high acidic conditions (pH $\leq$1) mostly because of its ability to rapidly oxidize elemental sulphur or partially reduced sulphur compounds, such as sodium thiosulphate ($Na_2S_2O_3$) to sulphuric acid (Vishnac, 1975).

T. thiooxidans organisms are strict autotrophs and aerobes. Strains of the bacteria have been isolated from acid mine waters, corroding steel and concrete, as well as from sulphidic ore (Vishnac, 1975; Lane *et al.,* 1992; Padival *et al.,* 1995). The organisms are not able to withstand environmental temperatures above 45°C due to their low resistance to desiccation (Starkey, 1925; Harrison, 1982; Barrett *et al.,* 1993). In the laboratory, ammonium sulphate serves as their nitrogen source during cultivation, though urea and nitrates can also be used (Newburgh, 1954; Brierley and Brierley, 1968; Vishnac, 1975; Brierley, 1978; Harrison, 1984). The identification of *T. thiooxidans* is based partly on the mole % of guanine and cytosine (G+C) content of their DNA which is reported to range from 50 to 65 % depending on the type of isolate (Harrison, 1984; Lane *et al.,* 1992). *T. thiooxidans* strains have been found to exhibit various morphological forms and behaviour in liquid medium supplemented with sulphur.

(b) *Thiobacillus ferrooxidans*

T. ferrooxidans was first isolated in 1949 (Colmer *et al.,* 1950) and characterized in 1951 (Temple and Colmer, 1951). The bacterium has the ability to oxidize ferrous iron, elemental sulphur, as well as partially reduced sulphur compounds to derive energy for metabolism (Silverman and Lundgren, 1959; Unz and Lundgren, 1961; Harrison, 1984; Rawlings and Silver, 1995). Leathen *et al.* (1956) isolated an iron oxidizing bacterium which they named *Ferrobacillus ferrooxidans* based on its inability to utilize sulphur as energy substrate. Kinsel (1960) also isolated a biooxidizing bacterium which he named *Ferrobacillus sulfooxidans* based on its ability to utilize ferrous iron and sulphur but not sodium thiosulphate. However, careful work by Kelly and Tuovinen (1972) showed that both *Ferrobacillus ferrooxidans* (Leathen *et al.,* 1956) and *Ferrobacillus sulfooxidans* (Kinsel, 1960) could indeed utilize ferrous iron, as well as sulphur and sodium thiosulphate, thus invalidating the different names given and confirming the organisms to be *T. ferrooxidans*. Interestingly, many

strains of *T. ferrooxidans* have also been found to fix nitrogen (Temple and Colmer, 1951; Mackintosh, 1978; Harrison, 1984; Rawlings and Kusano, 1994).

Many strains of *T. ferrooxidans* have been isolated from different geographical areas (Lane *et al.*, 1992). The bacteria are mainly autotrophs that preferentially utilize ferrous iron in the presence of both ferrous iron and sulphur compounds. Nevertheless, in the presence of large amounts of sulphide ore it oxidizes sulphur rather than ferrous iron (Margalith *et al.*, 1966; Harrison, 1984; Shrihari *et al.*, 1991). For *T. ferrooxidans*, the amount of dissolved oxygen available for growth plays a critical factor in its oxidation of mineral ore (Lui *et al.*, 1988; Barrett *et al.,* 1993), thus most strains of *T. ferrooxidans* isolated are aerobes but some are anaerobes (Rawlings and Kusano, 1994; Das and Mishra, 1996).

T. ferrooxidans has been shown to be involved in the oxidation of pyrite, marcasite and coal seams to ferric sulphate and sulphuric acid (Colmer and Hinkle, 1947; Das and Mishra, 1996; Nyavor *et al.,* 1996). The bacterium is considered to play a principal role in bioleaching operations because it oxidizes both ferrous and sulphuric ore, and is found in most mining sites where a form of leaching occurs. *T. ferrooxidans* is also useful in desulphurization of coal (Ehrlich and Holmes, 1985; Harrison, 1986; Rawlings and Silver, 1995; Gaylarde and Videla, 1995; Hallberg *et al.,* 1996). Its growth temperature ranges from 2 to 40 °C (Barrett *et al.*, 1993) with an optimum from 25 to 35 °C (Ferroni *et al.*, 1986; Ahonen and Tuovinen, 1991). Its range of pH adaptability is similar to that of *T. thiooxidans* (pH 0.5 to 6) with an optimum from pH 1.5 to 2.5 (Harrison, 1984; Barrett *et al.*, 1993; Rawlings and Silver, 1995).

(ii) *Genus Leptospirillum*

The genus *Leptospirillum* is classified under the family *Spirillaceae*. The genus includes mesophilic species and moderate thermophiles of which *L. ferrooxidans* and *L. thermoferroxidans* are, respectively the most important in biooxidation. Members of the genus *Leptospirillum* have been isolated from mining environment (Harrison, 1986). *L. ferrooxidans* are acidophiles which utilize ferrous iron, pyrite and some types of mineral ore as energy substrate (Harrison, 1984; Harrison and Norris, 1985; Sand *et al.*, 1992).

(a) *Leptospirillum ferrooxidans*

L. ferrooxidans was first isolated by Markosyan in an Armenian copper deposit (Markosyan, 1972) and characterized in 1974 (Balashova *et al.,* 1974). The bacterium oxidizes ferrous iron slowly, however, in the presence of cysteine in its growth medium the bacterium rapidly oxidize Fe^{2+} to Fe^{3+} (Harrison, 1984). It grows between a temperature range of 4 to 45 °C with an optimum from 25 to 35 °C. It also grows at a pH range of pH 1 to 4 with an optimum pH between pH 1.5 to 2.0 (Barrett *et al.*, 1993; Rawlings and Silver, 1995). Most strains of *L. ferrooxidans* are reported to have a mole % (G+C) above 50% (Lane *et al.,* 1992).

(b) *Leptospirillum thermoferrooxidans*

L. thermoferrooxidans is an obligate autotroph. It is a moderate thermophile (Barrett *et al.,* 1993) with optimum growth temperature ranging from 45 to 50 °C and optimum pH from pH 1.65 to 1.9. Even though this bacterium plays an important role in commercial bioleaching of gold ore it is not used extensively because of its thermophilic properties (Barrett *et al.*, 1993; Rawlings and Silver, 1995). *L. thermoferrooxidans* is a strict aerobe that obtains energy solely by oxidizing Fe^{2+} in aqueous solution (Lane *et al.,* 1992; Sand *et al.,* 1992). Its mole % (G+C) is 65%.

1.3.6 Mechanisms of Biooxidation

Two main mechanisms of oxidation of iron and sulphur by biooxidizing bacteria have been reported (Silverman, 1967; Brierley, 1978; Lundgren and Silver, 1980; Barrett *et al.,* 1993). These are: (1) the direct contact mechanism and (2) the indirect contact mechanism. Both processes of oxidation have oxygen as the terminal electron acceptor that is reduced to water.

$$O_2 + 4H^+ + 4e^- \rightarrow 2H_2O$$

(i) *Direct Contact Mechanism of Biooxidation*

In the direct contact mechanism of biooxidation there is an intimate physical contact between the bacteria and the sulphidic mineral ore. Under aerobic conditions bacterial leaching occurs through the action of bacterial enzymes on the components of the mineral ore that are susceptible to oxidation. This process of solubilization of the metal sulphate had been confirmed by observing the rate of bacteria acceleration of the oxidation of pyrite (FeS_2), chalcocite (CuS), covelite (CuS) as well as other types of mineral ore (Razzell and Trussell, 1963; Silverman, 1967; Mustin *et al.,* 1992; Sand *et al.*, 1992). Scanning electron micrographs have also revealed that numerous bacteria attach themselves to the surface of sulphide minerals in solutions supplemented with nutrients (Bennett and Tributsch, 1978; Brierley, 1982). The direct contact mechanism is, however, difficult to demonstrate using iron containing minerals such as arsenopyrite (2FeAsS), chalcopyrite ($CuFeS_2$) and bornite (Cu_3FeS_4) owing to the release of soluble iron during oxidation and probable occurrence of the indirect contact mechanism at the same time (Silverman, 1967). Metals in these ores are thus released through oxidative metabolism of micro-organisms or solubilized indirectly by chemical oxidant produced as metabolic products of the micro-organisms (Lundgren *et al.*, 1985).

T. ferrooxidans and *L. ferrooxidans* are reported to utilize the direct contact mechanism of biooxidation during leaching of mineral ore (Beck and Brown, 1968; Pinches, 1975; Brierley, 1978; Sand *et al.*, 1992; Rawlings and Silver, 1995). However, an investigation by Silverman (1967) revealed that oxidation of pyrite by *T. ferrooxidans* involves not only the direct attack of the ore by the bacteria but also chemical oxidation of the ore by ferric iron produced by the bacteria and released in the leacheate. Similarly, *T. ferrooxidans* selectively attacks sulphide containing minerals and converts the insoluble metal compounds of copper, lead, zinc, nickel, cadmium and cobalt to their soluble metal sulphates (Brierley, 1978; Rawlings and Kusano, 1994). In this type of reaction ferric iron present in the ore acts as a primary oxidant in the oxidation of elemental sulphur by *T. ferrooxidans.* It has also been reported that *T. ferrooxidans* is selective in choosing sites in mineral ores that are favourable for energy extraction depending upon availability of substrate (Bennett and Tributsch, 1978; Lundgren and Silver, 1980; Rodriguez-Leiva and Tributsch, 1988).

T. thiooxidans also utilizes the direct contact mechanism in its oxidation of the sulphur component of sulphide ore (Konishi *et al.*, 1995). The bacteria play an indispensable role in oxidizing sulphur to sulphuric acid thus exposing the constituent metals for further leaching (Brierley, 1982; Mustin *et al.*, 1992; Curutchet *et al.*, 1995). It does this by attaching itself to the sulphur particle before oxidizing it (Starkey, 1937; Harrison, 1984; Rawlings and Silver, 1995). It was reported that most of the oxidation processes involving sulphide minerals occur through the direct contact mechanism as they involve attachment of the various kinds of bacteria to the mineral lattice and subsequent oxidation of the iron and the sulphur components (Sakaguchi *et al.,* 1976; Barrett *et al.,* 1993). The involvement of the bacterial pili in their attachment to the sulphide mineral surface has been demonstrated by electron microscopy (Lizama and Suzuki, 1988). Generally, however, all the processes involved in the direct contact mechanism are not fully understood (Bennett and Tributsch, 1978; Mustin *et al.,* 1992).

(ii) *Indirect Contact Mechanism of Biooxidation*

In the indirect contact mechanism of biooxidation, the bacteria generate ferric iron (a powerful oxidizing agent) by oxidizing soluble ferrous iron. The ferric iron in turn reacts with other metals in the sulphidic mineral ore transforming them into soluble oxidized forms in an acidic (sulphuric acid) solution, and is itself reduced to ferrous iron. The biooxidizing bacteria then oxidize the ferrous iron to the ferric state thereby regenerating the primary oxidant. This mechanism is also referred to as bacterial assisted leaching (Brierley, 1982). It is, however, difficult to determine the roles of the direct and indirect leaching mechanisms quantitatively because most mineral ores contain some iron that could initiate indirect leaching alongside the direct process (Brierley, 1982). Hence, in practice, leaching of metals by bacteria is far more complex than the theory may suggest. This is especially so as there are many other minor processes in addition to the direct and indirect contact mechanisms described above (Barrett *et al.,* 1993). Some of the minor processes lead to the formation of secondary compounds and elemental sulphur from sulphide ore which can inactivate the reactive surfaces of the ore where bacterial leaching is taking place, thereby inhibiting the process. Nevertheless, as reported by Lundgren *et al.* (1985) and Rawlings and Silver (1995), the oxidation of pyrite ($4FeS_2$) and arsenopyrite (4FeAsS) could be summarized in the following reactions:

(a) $$4FeS_2 + 15O_2 + 2H_2O \xrightarrow{\text{(bacteria)}} 2Fe_2(SO_4)_3 + 2H_2SO_4$$

(b) $$4FeAsS + 14O_2 + 4H_2O \xrightarrow{\text{(bacteria)}} 4FeAsO_4 + 4H_2SO_4$$

1.3.7 Isolation of Biooxidizing Bacteria from Natural Sources

Micro-organisms can be selectively obtained from natural habitats such as soil or water either by direct or by enrichment isolation. Direct isolation involves using a selective medium solidified with

a gelling agent and allowing colonies to develop. On the other hand enrichment isolation involves the use of an inoculum in a liquid selective medium which selects for the micro-organism of interest (Stainer *et al.,* 1992). Isolation of bacterial cultures for use in biomining is carried out by inoculating appropriate selective nutrient media with bacteria containing samples such as mineral ore, acid mine water and underground mine water. The medium consists essentially of nutrients that support growth of the organisms that are expected in the samples. Inhibitory media could also be used to help select for the organisms of interest (Cowan, 1985). The need for isolation of a single or mixed culture determines the choice of medium. However, for the purpose of identification and characterization, it is essential that pure isolates be obtained. Several workers have communicated specific procedures for isolation of specified types of organisms (Salle, 1967; Pelczar and Chan, 1977; Joklik *et al.,* 1980; Cowan, 1985; Collin and Lyne, 1989). The most important factors to consider in an isolation protocol include source of sample, energy source, optimum temperature, pH and the suitability of liquid or solid media.

(i) *Important Factors Considered in Isolating Biooxidizing Bacteria*

(a) *Sample source*

The initial process of isolation of a microorganism, must take into consideration the possible habitats of the organisms of interest. For biooxidizing bacteria the environment of interest is normally a leaching environment, a habitat in which oxidizable forms of metals and sulphur occur. For example, *Thiobacilli* are widespread in ore deposits, sulphur springs and compost soils (Harrison, 1986; Said and Johnson, 1988; Barrett *et al.,* 1993), while *T. ferrooxidans* and other acidophiles produce large quantities of sulphuric acid in leaching environments which makes their ecological niche inhospitable to most organisms. The local temperature and pH of the micro-environment also influence the character as well as composition of bacterial species in any habitat.

Most strains of *T. ferrooxidans* have been isolated from acid mine drainage, coal spoils, copper deposits, mine effluents, uranium mines and gold mining sites (Livesey-Goldblatt *et al.*, 1983; Murayama *et al.*, 1985; Harrison, 1986; Lane *et al.,* 1992; Rawlings and Woods, 1995). *T. thiooxidans* strains have been isolated from compost of soil (Waksman and Joffe, 1922), acidic sulphate soil (Lane *et al.*, 1992), sulphur springs, acid mine waters, corroding steel or concrete and most uranium, copper and gold mining sites (Vishnac, 1975; Harrison 1982; Harrison, 1986; Goebel and Stackebrandt, 1994). Similarly, *L. ferrooxidans* have been isolated in copper deposits, coal dumps, uranium mines and generally mine ore samples but rarely mine waters (Sand *et al.*, 1992; Barrett *et al.*, 1993; Goebel and Stackebrandt, 1994).

(b) *Energy substrate, pH and temperature*

The type of energy substrate serves as one of the most effective selective factors in isolating biooxidizing bacteria. Biooxidizing bacteria have a wide range of specific energy requirements for growth and cellular metabolic processes. For example, the acidophiles oxidize inorganic materials to obtain energy, while the autotrophic bacteria use the energy generated out of the oxidation process to fix carbon from atmospheric CO_2 in their cellular matter. At the species level important biooxidizing bacterium like *T. thiooxidans* oxidizes elemental sulphur and reduced sulphur compounds such as sodium thiosulphate to produce sulphuric acid. Other nutrients needed for growth are obtained from inorganic salts (Unz and Lundgren, 1961; Otero *et al.*, 1995). On the other hand, *T. ferrooxidans* uses ferrous iron compounds either in aqueous form or the iron (II) content of pyrite ore as well as the oxidation of sulphur even though ferrous iron utilisation is reported to be preferred to sulphur when both substrates are present (Beck, 1960; Harrison, 1984). All strains of the other ferrous iron oxidizer, *L ferrooxidans,* described up to date use only iron (II) in aqueous solution or the ferrous iron content of mineral ore as their energy substrate (Sand *et al.,* 1992; Barrett *et al.*, 1993; Rawlings

and Woods, 1995). Since the energy sources used by these microbes are vital to their survival, their inclusion in liquid or solid media enable the cultivation and isolation of biooxidizing bacteria.

Biooxidizing bacteria are known to grow within defined temperature ranges with an optimum between 25 to 35 °C (Vishnac, 1975: Barrett *et al.,* 1993). It is therefore convenient to isolate these bacteria at ambient temperatures (Ahonen and Tuovinen, 1991; Rawlings and Silver, 1995). Most biooxidizing organisms are extreme acidophiles growing best between pH 1 to 3.5 (Vishnac, 1975; Barrett *et al.,* 1993; Battaglia *et al.*, 1994; Rawlings and Silver, 1995). This pH range is highly selective because most micro-organisms are known to grow around pH 7 (Vishnac, 1975; Joklik *et al.*, 1980). The required acidic condition therefore greatly reduces or in some cases eliminates the need for special sterilization methods, thus making biomining economical.

(c) *Liquid medium*

Whilst all biooxidizing bacteria grow in liquid media some are unable to grow on solid media (Harrison, 1984). Most isolates of *T. thiooxidans* have been obtained in liquid medium with elemental sulphur or sodium thiosulphate as the energy source. However, high concentrations of sodium thiosulphate is reported to inhibit the growth of the organism in liquid medium (Starkey, 1925; Unz and Lundgren, 1961), however, while some strains of *T. thiooxidans* do not utilize sodium thiosulphate at all (Waksman and Joffe, 1922; Adair, 1966; Harrison, 1982; Harrison, 1984). The pH of the medium is initially lowered (pH <1) to eliminate contaminating micro-organisms (Harrison, 1982; Harrison, 1984; Sand *et al.*, 1992) even though it may result in a decrease in the rate of oxidation (Starkey, 1925). The culture medium used in growing *T. thiooxidans* is reported to develop a characteristic greyish colour which intensifies as growth proceeds (Starkey, 1925; Konisihi *et al.*, 1995).

Similar to *T. thiooxidans,* most isolates of *T. ferrooxidans* and *L. ferrooxidans* have been obtained using liquid medium but with ferrous iron as the energy source although elemental sulphur and sodium thiosulphate have also been used for *T. ferrooxidans* (Colmer *et al.*, 1950; Temple and Colmer, 1951; Kinsel, 1960; Colmer, 1962; Harrison, 1984; Schrader and Holmes, 1988; Sand *et al.,* 1992; Barrett *et al.*, 1993). However, the propagation of *L. ferrooxidans* in liquid medium requires the addition of cysteine to enhance their growth (Harrison, 1984). In growing *T. ferrooxidans* the conversion of Fe^{2+} to Fe^{3+} changes the initial colour of the medium from colourless or pale blue, depending on pH, through amber to reddish brown. This colour change and the depletion of iron (II) may be used as growth indicators. Other methods used include: (1) polarographic assay, (2) chemostat reactor measurements, (3) determination of bacterial nitrogen, (4) rate of carbon dioxide fixation, (5) manometric measurements involving the measurement of small amounts of oxygen uptake using a respirometer or an oxygen electrode and (6) most probable number (MPN) of bacterial cells. The oxygen uptake has been reported to correlate directly with the oxidation of Fe^{2+} to Fe^{3+} and is generally applicable, but the MPN is not suitable in situations where the bacteria are attached to solid substrates or entrapped in ferric iron precipitates (Silverman and Lundgren, 1959; Beck, 1960; Dugan and Lundgren, 1965; Smith *et al.,* 1972; Brierley, 1978; Harrison, 1982; Kulpa *et al*, 1985; Barron and Leuking, 1990; Skoog *et al*, 1992; Mustin *et al.*, 1992; Barrett *et al*, 1993; Hallberg *et al.*, 1996). General microbiological methods such as the determination of the turbidity of cultures and direct counting of the bacteria are not used for determining the growth of biooxidizing bacteria because their energy substrates are inorganic salts that result in the formation of inorganic precipitates (Brierley, 1978).

(d) Solid medium

The common method used for obtaining a pure isolate of an organism from culture is to get it to grow as a colony in or on solid medium (Veldkamp, 1970). Solid media prepared using the same

constituents as for liquid media and solidified with gelling agents such as purified agar, silica, silicic acid, agarose or polyacrylamide (Harrison, 1984; Barrett *et al.*, 1993) have been used to isolate biooxidizing bacteria (Harrison, 1982; Barrett *et al.*, 1993; Peng *et al.*, 1994). Some types of conventional agar are however, known to be inhibitory to some strains of *T. thiooxidans* and *T. ferrooxidans* (Harrison, 1984). Nevertheless, Vishnac (1975), Harrison (1982, 1986) reported the isolation of *T. thiooxidans* on sodium thiosulphate agar. Agarose has been reported to be a good gelling agent for isolating single colonies of *T. ferrooxidans* (Vishnac, 1975; Harrison, 1984; Barron and Leuking, 1990). Yeast extract and tryptone soya are added to solid media where the aim is to isolate heterotrophic organisms (Harrison, 1984). *L. ferrooxidans* has also been isolated using this particular type of medium (Johnson, 1995).

(ii) *Purification of Isolates*

Pure cultures are needed for identification and characterization of bacterial isolates. Methods used to obtain pure cultures include plating, selection by acid and use of statistical dilution, single cell isolation using a micro-manipulator and selection with heavy metals. Plating is normally used for strains that produce colonies in or on solid medium. A single colony is usually selected and cultivated in liquid medium. The plating procedure is then repeated and a single colony is eventually selected for cultivation as a pure culture. This procedure is, however, not applicable to some strains of biooxidizing bacteria that do not form well defined colonies (Harrison, 1984). Such bacteria may be purified by the acid selection method. This technique is based on the observation that biooxidizing bacteria (autotrophs) of interest have the ability to grow at pH below 2 whilst many heterotrophs which are likely contaminants in the isolation procedure are unlikely to grow at such low pH. Hence rapid serial cultivation of the bacteria in 9K liquid medium (pH below 2) enriches the isolation of iron and sulpho-oxidizers of interest (Silverman and Lundgren, 1959; Harrison, 1986; Sand *et al.*, 1992).

In the statistical dilution method the cell density of a culture is determined using microscopic assay and serial dilutions made using sterile 9K liquid medium to obtain ≤ 1 cell per specified volume. After a period of growth the culture is assayed as before using microscopy and the procedure repeated several times. This method makes it possible to even separate two strains of the same species (Harrison, 1984). Single cell isolation has also been achieved using a micro-manipulator, even though the method is subject to some form of contamination (Unz and Lundgren, 1961; Harrison, 1984). The method of selection with heavy metals is designed to exploit the ability of autotrophic biooxidizers to readily adapt to high concentrations of heavy metals like copper, arsenic and uranium (Brierley, 1978; Harrison, 1984; Barrett *et al.*, 1993). The presence of such heavy metals therefore eliminates a broad range of contaminating organisms. A combination of the methods described above are however normally used in the purification of biooxidizing bacteria.

(iii) *Maintenance and Preservation of Bacterial Cultures*

Biooxidizing bacterial isolates have been maintained in the laboratory using the procedure of subculturing in liquid medium (Harrison, 1984). A working stock of these organisms may also be maintained at 4°C on agarose slant under mineral oil (Barron and Leuking, 1990; Vandepitte *et al.*, 1991). Long term storage methods employed include mixing the organisms with sterile ore (Larpage *et al.,* 1970; Gupta and Agate, 1986) and freezing aliquots of the bacteria at 4°C, -20°C or -70°C (Norris and Ribbons, 1970; Barron and Leuking, 1990), or freeze-drying the organisms (Norris and Ribbons, 1970; Murakami *et al.,* 1986; Wakao *et al.,* 1990). Freeze-drying have been a preferred method for long term storage of biooxidizing bacteria (Wakao *et al*, 1990). The method maintains large numbers of viable organisms over long periods of time and allows for easy recovery. For instance, *T. ferrooxidans* has been stored in the freeze-dried state for periods up to 2 years and *T. thiooxidans* up to 6 years (Norris and Ribbon, 1970; Wakao *et al*, 1990). The method of choice for

long term preservation, however, depends on the nature of the organism being preserved and the type of storage medium used.

1.3.8 Identification of Biooxidizing Bacteria

To identify a micro-organism, it is important to determine which characteristics have the greatest differentiating capacity. Morphological, cultural, physiological, ecological features and biochemical utilization of organic or inorganic substances have all been used in the identification of biooxidizing bacteria (Goodfellow and Board, 1980 ; Stainer *et al.*, 1992). Tang *et al.* (1997) in a review described such biological characteristics or profiles as biograms, and the process of determination of the relatedness of different organisms on the basis of their biological profiles as biotyping.

(i) *Morphological Characteristics*

Chemolithotrophic and acidophilic bacteria of the genus *Thiobacillus* are all straight rods or ovoid in shape (Harrison, 1982; Harrison, 1984). Rod cells of *T. ferrooxidans* have rounded ends and measure 0.3 to 0.5 μm X 1 to 1.7 μm in size (Barrett *et al.*, 1993). They are non-sporing Gram negative organisms and their cell sizes depend on the type of liquid medium in which they are cultured. Colmer *et al.* (1950) reported that in liquid medium where sodium thiosulphate was the energy source, *T. ferrooxidans* cells were larger compared to when ferrous iron sulphate was used. Some strains of *T. ferrooxidans* move by means of flagella (Barrett *et al.*, 1993) and the cells occur mostly in singles and pairs, but rarely in short chains (Beck, 1960; Vishnac ,1975; Harrison, 1982; Barrett *et al.*, 1993). On the other hand, *T. thiooxidans* cells are short rods measuring 0.5μm X 1 to 2μm in size and they occur in singles, pairs or short chains. In young cultures some strains of *T. thiooxidans* are motile whilst others are not (Waksman and Joffe, 1922; Unz and Lundgren, 1961; Harrison, 1984).

L. ferrooxidans cells are vibrio or comma shaped when young but as they grow older they join together becoming spiral like in appearance (Harrison, 1986; Sand *et al.,* 1992) and by accumulating iron precipitates, the cells may assume coccoid-like appearance (Harrison, 1982; Harrison, 1986; Sand *et al.,* 1992; Barrett *et al.*, 1993). They form aggregates or clusters in medium made slimy by capsular excretes from their outer cell envelopes (Harrison and Norris, 1985; Harrison, 1986; Barrett *et al.*, 1993) and they are highly motile organisms. *L. ferrooxidans* cells measure 0.3 to 0.6 μm in diameter and 1 to 3.5μm in length. Generally, each bacterium usually exhibits a characteristic morphology in young cultures and in media where conditions are favourable for growth (Salle, 1967). Consequently, variation of their cell size and overall morphological characteristics depend to a large extent on parameters such as, temperature of incubation, age of culture, concentration of substrate, composition of medium and the presence of waste products. According to Salle (1967), changes in these parameters lead to corresponding decrease in bacterial cell size.

(ii) *Cultural Characteristics*

The distinctive characteristics of biooxidizing bacteria on or in solid medium depend on constituents such as the type of gelling agent used in solid medium (Beck, 1960; Harrison, 1984). The important characteristics on solid media are the form, color, margin, surface texture and elevation of colonies. Various colony forms of biooxidizing bacteria including punctiform, circular, filamentous, irregular, rhizoid or spindle-like shapes have been observed on solid media prepared with different gelling agents and energy substrates (Harrison, 1984). On thiosulphate agar *T. thiooxidans* colonies are punctiform whilst *T. ferrooxidans* colonies appear as punctiform or filamentous (frosty) (Colmer *et al.,* 1950; Colmer, 1962; Vishnac, 1975). However, on ferrous agarose, *T. ferrooxidans* colonies are irregular in form but filamentous on ferrous agar. The colony colour intensity has also been reported to vary with age and is influenced by the type and concentration of energy substrate in solid media (Kinsel, 1960; Vishnac, 1975). For example, young colonies of *T. ferrooxidans* on ferrous agar are

creamish or whitish with a brown pigment in the center, but become reddish brown with age. However, on ferrous agarose *T. ferrooxidans* colonies are orange or yellow, while on ferrous silica the young colonies are brown and age to become reddish (Vishnac, 1975; Harrison, 1984; Kawarazaki *et al.,* 1986). On sodium thiosulphate agar *T. thiooxidans* colonies are initially pale yellow but become transparent as the colonies age. Colony elevation of bacteria may be flat, raised, convex, pulvinate or umbonate. Most colonies of *T. ferrooxidans* are raised whilst those of *T. thiooxidans* are flat. Margins of bacteria colonies can be entire, undulate, lobate, erose, filamentous or curled. *T. thiooxidans* colonies have entire margins whilst, *T. ferrooxidans* colonies are either filamentous, entire or undulate (Vishnac, 1975; Harrison, 1984). In liquid media, biooxidizing organisms form pellicles, flocculent or flaky sediments with their substrates and waste products (Vishnac, 1975). *T. ferrooxidans* and *L. ferrooxidans* cells form pellicles with ferric compounds such as ferric hydroxysulphate when the pH of the culture medium is ≥ 1.9 (Harrison, 1984).

(iii) *Physiological Characteristics*

Bacterial physiology essentially constitutes bacterial cell functions that enable the interrelationship between the organisms and the environment through utilization of various substances, such as, carbon, nitrogen, oxygen and energy used for metabolic processes. Other important bacterial physiological properties are determined by the influence of different organic compounds and stimulants on bacterial growth, the ability to grow at certain temperatures and production of certain metabolites (Waksman and Joffe, 1922; Harrison, 1984; Harrison and Norris, 1985; Barrett *et al.,* 1993; Goebel and Stackebrandt, 1994; Rawlings and Kusano, 1994). In general, differences in bacterial physiological properties provide a basis for characterization, differentiation and identification of bacterial genera and to some extent their species.

It is the unique physiological characteristics of biooxidizing bacteria that enable them to grow in mining environments. Pronk *et al.* (1991b), reported that, even though most strains of *T. ferrooxidans* use atmospheric CO_2 as their carbon source, some strains can utilize formic acid. *T. ferrooxidans* is also reported to diazotropic; possessing genes for nitrogen fixation (Pretorius *et al.,* 1986) thus, it can fix atmospheric nitrogen (Mackintosh, 1978; Pretorius *et al.,* 1986). The organism is also capable of utilizing nitrates as a source of nitrogen (Vishnac, 1975). *T. ferrooxidans* normally grows best in an aerobic environment where it uses oxygen as a terminal acceptor in metabolic oxidation. However, if oxygen is lacking, reduced sulphur compounds or formate act as the electron donor and ferric iron becomes the terminal electron acceptor. It is this property that enables some strains of *T. ferrooxidans* to live under anaerobic conditions (Pronk *et al.*, 1991a; Pronk *et al.,* 1992; Sugio *et al.*, 1992; Das and Mishra , 1996). Drobner *et al.* (1990) and Fischer *et al.* (1996) have also reported that other substrates such as hydrogen can be used as energy source by some strains of *T. ferrooxidans* although, their ability to do this is limited by the presence of ferrous iron or sulphur under aerobic conditions. Despite the wide range of energy sources, most strains of *T. ferrooxidans* cannot utilize organic compounds for energy (Harrison, 1984; Rawlings and Kusano, 1994). Trace elements used by biooxidizing bacteria for metabolic purposes are usually present as impurities in water or in the ore. Biooxidizing bacteria, therefore, have modest nutritional requirements that can be supplied by water, air and oxidizable iron or sulphur. For this reason, the organisms can adapt to severe adverse conditions in their environment (Rawlings and Kusano, 1994; Morin, 1995).

(iv) *Biochemical Characteristics*

Several biochemical characteristics of biooxidizing bacteria have been used in their identification. These distinguishing characteristics include: formation of metabolites, DNA base composition differences, gene sequencing, ribosomal RNA (rRNA) sequencing, susceptibility to antibiotics

(chemotaxonomy) and presence of certain types of plasmids (Harrison, 1982; Harrison, 1986; Visca *et al.,* 1988; Lane *et al.,* 1992; Stoner *et al.,* 1996, Pizarro *et al.,* 1996).

(a) *Formation of Metabolites*

During the oxidation of sulphur and metal sulphides biooxidizing bacteria release organic substances into their culture medium. These are exometabolites and are high molecular mass substances such as lipids and phospholipids and low molecular mass substances (LMM) like acids of the tricarboxylic acid cycle, amino acids and ethanolamine. Their secretion is reported to correlate with bacterial growth and reaches a maximum in the exponential phase of their growth. The function of these exometabolites have not yet been extensively studied (Adair, 1966; Harrison, 1984; Barrett *et al.,* 1993). However, Schaeffer *et al.* (1963) and Harrison (1984) suggested that the lipids and phospholipids act as moisturizing agents and contribute to the oxidation of sulphur by converting it to colloidal states that can easily be metabolized by the bacteria. LMM substances also participate as complexing agents for ionic species such as aqueous iron (III) and arsenic (III) (Harrison, 1984). The exometabolites contribute significantly to the adhesion of the bacteria to the surface of mineral ore (Barrett *et al.,* 1993). Also, exometabolites are beneficial to the organisms as they form complexes with toxic metal ions, thereby reducing the bioavailability of these metal ions (Barrett *et al.,* 1993). Nevertheless, a build-up of the metabolites is toxic and affects the growth of the bacteria.

(b) *Genetic Relatedness of Biooxidizing Bacteria*

The overall DNA base composition of an organism has been used as a valuable preliminary tool for assessing the relatedness of one strain of a microbe to another. It is also used in assigning newly isolated species to their specific genera (Lane *et al.,* 1992). Organisms that are closely related at the species level are reported to have similar or nearly similar DNA base composition (Harrison, 1982;

Harrison, 1986). Harrison (1986) and Lane *et al.* (1992) demonstrated genomic diversity amongst micro-organisms using their DNA base composition. Other methods that have been used to study genetic relatedness of biooxidizing bacteria include (1) DNA hybridization analysis (homology), (2) 5S rRNA assay and (3) determination of mole % of guanine and cytosine (G+C) (Marmur *et al.*, 1963; Harrison, 1982; Harrison, 1986; Lane *et al.*, 1992; Rawlings and Kusano, 1994). On the basis of inter-strain DNA hybridization analysis *T. ferrooxidans* strains were divided into seven homologous groups (Harrison, 1982), and Stoner *et al.* (1996) used 5S rRNA assay to differentiate between strains of *T. ferrooxidans, T. thiooxidans, L. ferrooxidans* and other acidophiles.

Determination of (G+C) in mole % has been used in the characterization of different strains of biooxidizing bacteria (Vishnac, 1975). Barrett *et al.* (1993) reported that the (G+C) in mole % varied depending on the bacterial strain. For example, the mole % (G+C) for *T. ferrooxidans* strains ranged from 55 to 65 %, whilst that for *T. thiooxidans* strains was 52 to 65 %, and 50 to 56 % for *L. ferrooxidans*. However, Brierley (1978) and Rawlings *et al.* (1991) reported that variations existed in DNA base composition of *T. ferrooxidans* strains and other acidophiles grown on different energy substrates. Nevertheless, Lane *et al.* (1992) showed that there is no quantitative relationship between mole % (G+C) and genotype of organisms.

(c) *Chemotaxonomy*

The differentiation of bacterial strains using their susceptibility to various types of antibiotics is termed chemotaxonomy. The identification profiles obtained by the method are called antibiograms (Tang *et al.*, 1997). Interestingly, all mesophilic isolates of biooxidizing bacteria described up to date areGram negatives and sensitive to antibiotics such as ampicillin, vancomycin and kanamycin (Huber *et al.*, 1985; Barrett *et al.*, 1993).

1.3.9 Comparative Characterization of Biooxidizing Bacteria

Morphological, cultural, physiological and biochemical characteristics (biograms) of micro-organisms are used normally in their identification. However, Tang *et al.* (1997) in a review reported that in some cases these characteristics are unstable and therefore not reliable for identification of bacterial strains. This is mainly because the characteristics may be influenced by genetic regulation, technical manipulation and gain or loss of plasmids by the microbes. Other properties are therefore usually used in conjunction with these biograms to accurately identify biooxidizing bacteria strains. These include their antigenic properties, mineral ore oxidation rates, the optimum pH, temperature and substrate concentration, and protein/ DNA profiles.

(i) *Use of Antigenic Properties*

In vitro reactions between antigens and their homologous antibodies have been widely used in the identification and characterization of micro-organisms (Harlow and Lane, 1988; Tang *et al.*, 1997). Serological reactions are reported to reveal marked differences among bacterial cultures that appear to be similar on the basis of morphology and physiology (Weir, 1986). This type of analysis distinguishes bacterial species on the basis of their antigenicity. For example, Apel *et al.* (1976) adapted the fluorescent antibody staining technique to identify *T. ferrooxidans* isolates using rabbit anti-*T. ferrooxidans* immunoglobulin G (IgG) against 23 bacterial isolates. They observed fluorescence with *T. ferrooxidans* isolates grown with both iron and sulphur as energy substrates but noted cross-reactivity for two other unrelated bacterial species. Their antibodies, however, failed to react with known *T. thiooxidans* isolates. Nevertheless, Brierley (1978) reported in a review that the application of the fluorescent antibody staining technique in the field using mineral ore samples was not very successful because of low populations of biooxidizing bacteria in the ore. Muyer *et al.* (1987) also demonstrated that antiserum raised against whole cells of *T. ferrooxidans* reacted with a variety of acidophiles and non-acidophiles in an enzyme-linked immunoabsorbent assay. They also

observed reactivity of their antiserum with biooxidizing bacteria in another experiment where an indirect immunofluorescence assay was combined with a DNA-fluorescence staining technique (Muyer *et al.*, 1987). The above experiments thus demonstrated that polyclonal antisera raised against biooxidizing bacteria were not specific at the species level (Muyzer *et al.*, 1987; Arrendondo and Jerez, 1989). The success so far achieved with polyclonal antibodies in identification of some biooxidizing bacteria may however suggest that the use of specific monoclonal antibodies would lead to more accurate characterization.

(ii) *Use of Mineral Ore Oxidation Rate, pH, Temperature and Substrate Concentration Profiles*

The rate of mineral ore solubilization by biooxidizing bacteria had been used in their characterization because of its economic importance in commercial mining. The ability of biooxidizing bacteria to leach different types of mineral ores efficiently varies because of environmental conditions such as pH, temperature and the concentrations of various substrates (Ahonen and Tuovinen, 1991; Barrett *et al.,* 1993). Hence, a mixed culture of *T. ferrooxidans, T. thiooxidans* and *L. ferrooxidans* is normally used in bioleaching of mineral ore since these bacteria have a sort of synergistic relationship which enhances the biooxidation process rather than pure individual cultures of the organisms (Barrett *et al.*, 1993; Rawlings and Silver, 1995; Morin, 1995). Even with the same species of biooxidizing bacteria it has been observed that organisms isolated from a particular mineral ore oxidize that ore better than isolates from a different ore (Rawlings and Woods, 1995). According to Lundgren and Silver (1980), bioleaching of mineral ore is influenced by the chemical nature of both the aqueous and solid crystal phases of the ore. Morin (1995) explained that this was so, since mineral ore deposits from different geographical sources were unique with respect to their mineralogy and chemical composition which in turn affects the morphology and surface features of the ore (Agate and Khinvasara, 1985; Baldi *et al.,* 1992; Barrett *et al.,* 1993; Rawlings and Woods, 1995; Morin, 1995). In general, however, the characterization of biooxidizing bacteria using the mineral ore

oxidation rates is enhanced by comparative measurement of pH, temperature and substrate concentration profiles.

(iii) *Use of Protein Profiles in Characterization*

Shapes and functions of cells are determined by their proteins (Wilson and Walker, 1995). Some of the protein molecules (enzymes) catalyze reactions which synthesize cell membranes, pigments and drive the mechanisms of energy production from substrates. Indeed, it is the differences in the structure and composition of these proteins that provide the basis of species and strain designation of microorganisms. For this reason, several electrophoretic methods are available for analysis of proteins made by micro-organisms. In this respect, zone electrophoresis of a mixture of bacterial cell proteins in well defined standardized conditions produce protein profiles which are considered as fingerprints of the different strains under investigation (Kersters and De Ley, 1980; Copeland, 1994). On the other hand, similar strains of bacteria grown under standardized culture conditions have the same set of proteins (Kersters and De Ley, 1980). Electrophoresis of total soluble or outer membrane proteins of bacterial strains therefore yield complex polypeptide protein bands that are similar for the same species or strains of organisms. The protein bands usually consist of a number of structurally different molecules with identical electrophoretic mobilities (Copeland, 1994; Wilson and Walker, 1995; Tang *et al.,* 1997). Several workers have as a result used protein band analysis by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) to identify and differentiate between bacterial isolates (Swings *et al.*, 1976; Huber *et al.,* 1985; Chamorro *et al.,* 1988; Arrendondo and Jerez, 1989; Deutscher, 1990; Hames and Rickwood, 1994; Teixaeira *et al.,* 1995). The accuracy of the method was shown by Rawlings *et al.* (1991) who differentiated between *T. ferrooxidans* grown with ferrous iron as energy substrate and the same organism grown with elemental sulphur.

(iv) *Use of DNA Profiles in Characterization*

Rapid progress in the molecular genetics of procaryotes has made it possible to differentiate between bacteria up to the strain level using polymorphism in their nucleic acids. The genome of most Gram negative bacteria consists of a single circular molecule of DNA with size ranging from 6.0 X 10^5 to 7.8 X10^7 base-pairs (Cavalier-Smith, 1985; Li and Graur, 1991). Some bacteria also contain additional small circular DNA molecules called plasmids that encode for a variety of cellular functions (Hames and Higgins, 1991; Watson *et al.,* 1992). Differences in DNA of organisms may however arise from point mutations (insertions and deletions) which alter the sequence of nucleotides or genes comprising genomic DNA. These mutations produce variations in the number of tandemly repeated DNA sequences and alters the length of DNA fragment between two recognition sites for restriction endonucleases (Watson *et al.*, 1992; Pingoud *et al.,* 1993), thereby providing the basis for differentiation between strains of bacteria by analysis of the electrophoretic mobility of nucleic acids (Patterson and Hyypia, 1985). Variations detected this way serve as extremely valuable genetic markers (Rothwell, 1988) and is termed Restriction Fragment Length Polymorphism (RFLP). The digestion of genomic DNA with type II restriction enzymes have been used in the characterization of biooxidizing bacteria (Southern, 1979; Yates *et al.,* 1986; Tang *et al.*, 1997). According to Tang *et al.* (1997) the advantage of restriction endonuclease analysis is that it is highly reproducible and very accurate in determining the relatedness of microbial strains.

CHAPTER 2

2.0 MATERIALS AND METHODS

2.1 Samples

The samples used in this study were collected from the mining site of the Ashanti Goldfields Company Limited, Obuasi. These were;

(1) slurry from the biooxidation reactor tanks (BT) (see Figure 1A, 1B),

(2) gold arsenopyrite ore (S2) from a surface mining site (see Figure 2),

(3) gold sulphide ore (S3) from an underground mining site (see Figure 3) and

(4) underground mine water (UW).

Gold ore samples (mainly crushed ore) and 50ml of underground mine water or slurry were collected into polythene bags or sterile 50ml centrifuge tubes, respectively and transported to the laboratory within 24 hours. The samples were subsequently stored at 4°C until use.

2.2 Isolation, Purification and Maintenance of Ferrous Iron Oxidizing Bacteria

(i) *Isolation*

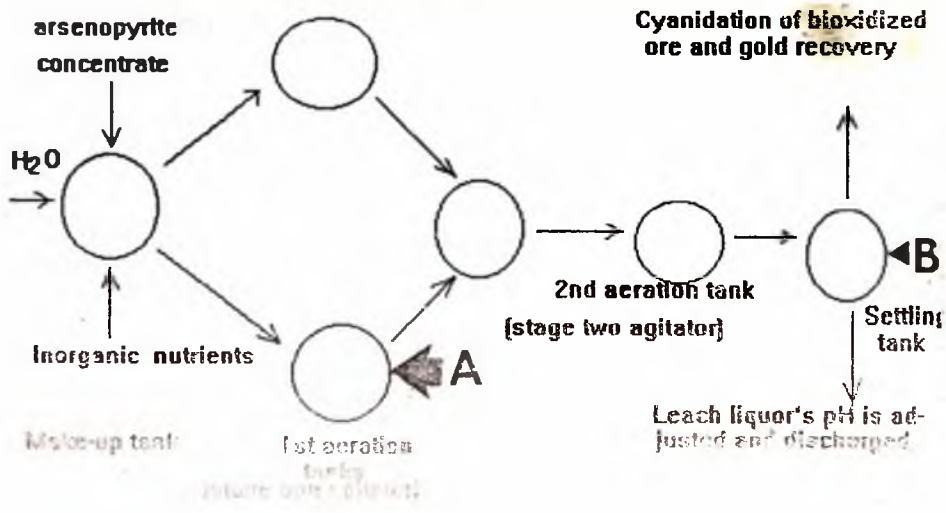
Five millilitres of slurry from the biooxidation tank were pipetted into 100ml of 9K enrichment medium (Appendix A) supplemented with ferrous iron sulphate (9K-Fe^{2+}), pH ≤1.8 in a 500ml Erlenmeyer flask. The culture was then incubated at room temperature (25 to 30 °C) for 8 to 10 days with continuous agitation at a speed of 100 rpm on a rotary shaker (Vishnac, 1975; Harrison, 1984; Goebel and Stackebrant, 1994).

A

Figure 1 Photograph showing the sulphide treatment plant (STP) at Obuasi. A= reactor tank, B= settling tank.

Flow diagram showing system of operation of STP at Obuasi.

B



Schematic diagram of the STP at Obuasi. Sample BT was collected from reactor tank 'A'.

Figure 2 Photograph showing surface mining site at Obuasi where the surface gold ore sample (SO) was collected.

Figure 3 Photographs showing underground mining sites where the underground gold ore sample (UO) was collected.

Similarly, 10ml of underground mine water were added to 30ml of enrichment medium in a 250ml conical flask or 3 grams of surface and underground gold ore were added separately to 100ml of the medium in a 500ml Erlenmeyer flask and cultured as described above (Barrett *et al.,* 1993). The underground mine water was cultured for 2 to 3 weeks instead of 10 days.

(ii) *Purification*

Purification of ferrous iron oxidizing bacterial isolates were done using the methods of enrichment dilution and colony isolation (Unz and Lundgren, 1961; Beck, 1960; Barron and Leuking, 1990) in either solid or liquid media.

(a) *T. ferrooxidans-like Bacteria*

Primary isolation of *T. ferrooxidans*-like bacteria was done by spreading 100µl of bacteria suspension at the logarithmic phase of growth as indicated by medium colour (deep amber) and pH of the medium (pH ≥1.8) on ferrous agar (FA) or on ferrous agarose (FAR) in petri plates (Appendix B). The plates were incubated at 37°C for 4 to 6 days during which bacterial cell colonies appeared on the solid medium. Single colonies were then transferred with a sterile Pasteur pipette back into 2ml of 9k-Fe^{2+} liquid medium in a 24 well tissue culture plate (Becton Dickinson and Company, USA). The culture plate was incubated at room temperature (25 to 30 °C) with continuous agitation on a rotary shaker for 1 to 3 weeks during which growth of bacteria was observed. The above procedure was repeated 3 times to ensure the purity of the isolates. Light microscopy was used (mag. X 1000, objective X100) to ascertain purity of the isolates by their morphological characteristics (Sand *et al.,* 1992; Goebel and Stackebrant, 1994).

(b) *L. ferrooxidans-like Bacteria*

Ten millilitres of inoculum from the stationary phase of enrichment cultures prepared with samples (Section 2.2.1) were pipetted into 100ml of 9k-Fe^{2+} liquid medium added with L-cysteine (0.01%) which selects for *L. ferrooxidans* in 500ml Erlenmeyer flasks separately (Harrison, 1984). The individual cultures were incubated at room temperature (25 to 30 °C) with continuous agitation on a rotary shaker at a speed of 100 rpm. An aliquot (10ml) of each culture was then pipetted into fresh liquid medium in a 500ml Erlenmeyer flask under aseptic conditions in a laminar flow cabinet at 2 weeks intervals. Sub-culturing was repeated at least 10 times. Light microscopy was used to ascertain purity of the isolates by their morphological characteristics (Sand *et al.,* 1992; Goebel and Stackebrandt, 1994).

Further purification was achieved using the method of statistical dilution (Harrison, 1984). Briefly, a suspension of *L. ferrooxidans* in culture at the logarithmic phase of growth was titrated using 9K-Fe^{2+} medium with L-cysteine from 2 X 10^{-2} to 2 X 10^{-20} at 50 fold dilution. One hundred microlitres volumes of each dilution was then pipetted into the wells of a sterile 96 well tissue culture plate. The plates were then incubated at room temperature (25 to 30 °C) under continous agitation at a speed of 30 rpm on a rotary shaker (Thomas Kagaku, Co. Ltd, Japan). The micro-plates were inspected at weekly intervals for signs of growth as indicated by medium colour change. After every 30 days 100µl of fresh sterile 9k-Fe^{2+}/L-cysteine medium were added under aseptic conditions to make up for evaporative losses. Light microscopy was used to ascertain purity of the isolates by their morphological characteristics (Sand *et al.*, 1992; Goebel and Stackebrandt, 1994).

(iii) *Maintenance*

T. ferrooxidans-like bacterial isolates were maintained using 9k-Fe^{2+} liquid medium. Fresh cultures of the bacterial isolates were prepared every two weeks by sub-culturing 10ml of an on going culture into 100ml of 9k liquid medium in a 500ml Erlenmeyer flask. However, sub-culturing of purified bacterial isolates from underground mine water were done monthly. The cultures were continuously agitated on a rotary shaker at a speed of 100 rpm at room temperature (25 to 30 °C) and opened every 5 days to allow fresh supply of air into the flasks. On the other hand, purified *L. ferrooxidans*-like bacteria were maintained in 9k liquid medium with 0.01% L-cysteine, by sub-culturing every 2 weeks.

2.3 Isolation, Purification and Maintenance of Sulpho-Oxidizing Bacteria

(i) *Isolation*

One hundred millilitres of 9K medium (pH ≤ 1) supplemented with elemental sulphur (9k-S°) in a 500ml Erlenmeyer flask was inoculated with 5ml of sample BT or 3 grams of surface and underground gold ores (Barrett *et al.*, 1993). The resulting enrichment medium was then continuously agitated at 100 rpm on a rotary shaker (Eyela Tokyo Rikikai Co., Ltd., Tokyo, Japan) for 30 days at room temperature (25 to 30 °C). Growth of the bacteria was monitored by medium colour change which developed from colourless to intense grey (Sand *et al.*, 1992; Goebel and Stackebrandt, 1994).

(ii) *Purification of T. thiooxidans-like Bacteria*

T. thiooxidans-like bacteria were purified by the method of selection by acid described by Harrison (1984) and by colony isolation. Briefly, the acid selection method utilizes a low pH medium which enabled only *T. thiooxidans*-like bacteria to grow (Torma, 1985). An aliquot (100µl) of the resulting

culture was spread on sodium thiosulphate agar (Appendix B) and incubated for 8 to 10 days at 37°C. During this period bacteria cell colonies appeared on the plates. Single colonies were picked with the tip of a sterile needle because of the minute sizes of the colonies that developed and put back in 9k-S° medium. The transfer of single bacterial colonies into liquid phase was repeated 2 to 3 times. Light microscopy was used to ascertain purity of the isolates by their morphological characteristics (Sand *et al.*, 1992; Goebel and Stackebrandt, 1994).

(iii) *Maintenance*

Sub-culturing of sulpho-oxidizing bacterial isolates was done every 30 days by pipetting 10ml of the on going culture into 100ml of fresh 9k-S° liquid medium in a 500ml Erlenmeyer flask. The culture flask was opened every 5 days to allow a fresh supply of air in a sterilized laminar flow cabinet.

2.4 Preservation of Ferrous Iron and Sulpho-oxidizing Bacterial Isolates

Ferrous iron or sulpho-oxidizing bacteria in 100ml cultures at the stationary phase of growth were harvested by centrifugation at 10,000*g* for 1 hour. The pellet obtained was re-suspended in 2ml of 9k liquid medium pH 6.5 (Appendix A). One hundred microlitres (100µl) of re-suspended bacterial cells were then pipetted into 5ml glass vials (Nichiden-Rika-garasu Co. Ltd, Japan) and frozen at -20°C before freeze-drying using a Yamato freeze drying machine (Yamato Co. Ltd, Japan). Freeze-drying was done at -105°C for one hour. The freeze-dried samples were stored at 4°C until use.

The viability of freeze-dried bacterial cells was tested as follows: immediately after freeze-drying some of the samples were re-suspended in 100µl of 9k liquid medium supplemented with ferrous iron or elemental sulphur as energy substrate for ferrous and sulpho-oxidizing bacteria respectively. The entire 100µl bacterial suspensions were then pipetted separately into 2ml of the respective media and agitated continuously on a rotary shaker at a speed of 100 rpm in a 24 well tissue culture plate

(Becton Dickinson and Company, USA). One hundred microlitres of bacterial cell suspensions stored at 4°C and -20°C transferred into culture plates similarly were used as positive controls whilst 100µl of 9k liquid medium without any bacteria cells served as negative control. Growth of the prepared cultures were monitored by medium colour change over a period of 3 months.

2.5 Characterization of Biooxidizing Bacteria

2.5.1 Staining of Bacterial Isolates

(i) *Basic Dye and Gram's Staining*

Basic dye stains were used in staining biooxidizing bacteria (Salle, 1967; Cowan, 1985; Lillie, 1990). Ten microlitres (10µl) of bacterial culture were pipetted and smeared evenly on a cleaned (grease free) labelled slide. The smear was air dried and then heat fixed by passing the slide through a bunsen flame 5 to 6 times. Prepared slides were flooded separately with any of the basic dye solutions for 1 to 3 minutes (Appendix C). The slides were then washed with distilled water to remove all excess stain and air dried at room temperature. The stained cells were observed under oil immersion using a light microscope at a magnification of X 1000 (objective X100, ocular X10). Photomicrographs of stained bacterial cells were taken using an Olympus model phase-contrast light microscope (Olympus Optical Co. Ltd., Tokyo, Japan) with a camera attached.

Gram's staining was performed as described by Collins and Lyne (1989) with modification. Briefly, a suspension of bacteria (approximately 1.5ml) at logarithmic phase of growth was pelleted by centrifuging in a microfuge for 1 hour. The supernatant was decanted and the pellet re-suspended in 100µl of 9k liquid medium pH 6.5 (Appendix A). The bacterial cells were then fixed onto microscope slides as described above before flooding with crystal violet solution. The slides were washed with excess distilled water and Logol's iodine applied by dropwise addition for 1 minute (Appendix C). This was followed by washing with acetone until colour ceased to come out of the

preparation. The slides were again washed with distilled water and the smear counter-stained with carbol fuschin for 3min. The excess stain was then washed with distilled and the slide air dried. Microscopic examination was performed as described earlier.

2.5.2 SDS-PAGE Analysis of Bacterial Cell Lysates

Electrophoresis of bacterial cell lysates were performed with an ATTO CORPORATION slab gel apparatus (Bunkyo-Ku, Tokyo, Japan) using the SDS-tris-glycine discontinuous buffer system (Laemmli, 1970). All bacterial samples were electrophoresed on 15 – 20% resolution acrylamide gradient gels with a 3% stacking gel.

(i) *Preparation of Crude Bacterial Cell Lysates for SDS-PAGE and Electrophoretic run*

Crude bacterial cell lysates were prepared from the isolates obtained from different samples. Bacterial cells were harvested from a 100ml culture at stationary phase of growth by centrifugation at 2900 *Xg* for 60 mins at 4°C using a Hitachi CF7D2 Centrifuge (Hitachi Co., Tokyo, Japan). The cell pellet was re-suspended in 100µl of sample buffer (62.5mM, Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% 2-Mercaptoethanol) in a 1.5ml Eppendorf tube. Protease inhibitors [2.5µl Leupeptin (10mg/ml), 2.5µl E64 (10mg/ml), 5ul mixture containing 100mM PMSF, 20mM TPCK and 5mM TLCK] were then added. The tubes were rapidly frozen in liquid nitrogen and transferred immediately to a water-bath at 37°C to thaw before vortexing briefly for 2min to break up the cells. The freeze-thaw process was repeated 20 times to enhance disruption of the cells. The prepared crude bacterial cell lysates were boiled for 15min after the addition of loading buffer (Huber *et al.*, 1985) at 100°C in a water-bath before loading onto acrylamide gels for electrophoresis.

Crude bacterial cell lysates were loaded into the wells of the polymerized stacking gel alongside standard low molecular weight markers (Sigma Co, Ltd, St Louis, MO, USA) prepared as described

by the manufacturer. A constant current of 20mA was supplied by an electrophoresis power supply pack ATTO AE-3121 (ATTO Corporation, Japan) until the tracking dye (bromophenol blue) had travelled to the interface between the stacking and resolution gels. The current was then increased to 30mA to improve the sharpness of resolution of the peptide bands in the separating gel.

A vertical strip of the gel containing the standard molecular weight markers and resolved sample proteins was cut using a surgical blade and transferred into a plastic tray containing staining solution [0.5% (w/v) Coomassie blue, 10% (v/v) acetic acid, 50% (v/v) methanol] for 15min with slow shaking on a rotary shaker. The staining solution was discarded and the gel rinsed with first destaining solution [50% (v/v) methanol, 10% (v/v) acetic acid] for 1min. Destaining was continued using a second solution [10% (w/v) methanol, 7% (v/v) acetic acid] until the stained protein bands were visible in the gel. The destained gel was then visualized under an ordinary light illuminator and stored in distilled water to prevent it from drying up.

The destained gels were soaked in 25% glycerol for 2-5 min and transferred onto chromatographic filter papers 3mm thick (Toyo Roshi Kaishi, Ltd, Tokyo, Japan) and overlaid with a cellophane membrane previously softened by immersion in distilled water. The gels were dried in an ATTO gel drying machine (ATTO Corporation, Tokyo, Japan) at 60°C for 14 hours. The molecular weights of prominent protein bands observed in the resolved bacterial proteins were determined using a relative mobilty curve (Appendix H).

2.5.3 Preparation and Restriction Endonuclease Cleavage of Biooxidizing Bacterial Genomic DNA

(i) *Preparation of Genomic DNA*

e protocol described by Sambrook *et al.* (1989) and Flook *et al.* (1992) for extraction of genomic DNA, was adapted with slight modifications. Bacterial cells (100ml cultures) were harvested at the

stationary phase of growth by centrifuging at 2900*g* for 1 hour in a centrifuge at 4°C. After centrifugation the cell pellet was rinsed 3 times with 1M Tris-HCl pH 7.6 and re-suspended in 200µl of Blender buffer (0.1M NaCl, 0.2M sucrose, 0.1M Tris-HCl pH 7.5, 0.05M EDTA pH 9.1, O.5% SDS stored at 4°C) in an Eppendorf tube. The homogenate was incubated at 65°C for 1 hour and spun in a microfuge for 10mins (Kubota 1120 Kubota Corporation, Bunkyo-Ku Tokyo, Japan). Thirty microlitres (30µl) of pre-chilled 8M potassium acetate were added, mixed well by tapping and left to stand on ice for 45min before centrifuging at 12,000g for 10min. The supernatant (230µl) was transferred into a fresh Eppendorf tube and 2X volume cold absolute ethanol added. The sample was then kept at -50°C for 30min to precipitate DNA. The DNA was recovered in a pellet following centrifugation at 12,000g for 10min and air dried.

The DNA pellet was then dissolved in 50µl of Tris-EDTA (TE) buffer pH 7.6 with 10mg/ml, RNAse solution (Appendix E) and further purified by phenol chloroform extraction. To the sample was added an equal volume of phenol-chloroform mixture (1:1 v/v) and the contents mixed till an emulsion formed. The mixture was centrifuged for 5mins in a microfuge to separate the organic and aqueous phases, and the aqueous phase containing the DNA transferred into a fresh tube. This phenol chloroform extraction procedure was repeated two more times. The procedure was again repeated using only chloroform. Purified genomic DNA was then precipitated by the addition of 1/10 volume of sodium acetate pH 5.2 and 2 X volume cold absolute ethanol to the aqueous phase and keeping the mixture at -20°C overnight. The DNA was recovered by centrifugation at 12,000 *X*g for 10min. The DNA pellet obtained was washed with 70% ethanol, air dried at room temperature then dissolved in 30µl TE buffer (1mM EDTA, 10 mM Tris-HCl pH, 7.6, defined in appendix E) and stored at -20°C.

The purity and yield of extracted DNA was determined as described by Rodriguez and Tait (1983). DNA samples (10µl each) were diluted with 1.9ml of distilled water and the absorbance at 260, 280

and 300 nm read using silica cuvettes with distilled water as blank in a double-beam spectrophotometer (Shimadzu UV 190 Double beam, Japan). From the absorbance readings obtained, the quantity and purity of DNA samples were calculated. The absorbance at 260nm was used to calculate the concentration of DNA (OD 0.2 = 10µg DNA/ml) and the absorbance ratio A_{260}/A_{280} provided an estimate of the purity of DNA (Sambrook *et al.*, 1989).

(ii) *Cleavage of Genomic DNA with Restriction Endonucleases*

Cleavage of genomic DNA with restriction endonucleases was done using the method of Sambrook *et al.* (1989) and modified following the manufacturer's recommendations. DNA solutions (10µl each) were placed in sterile tubes and mixed with 8µl of sterile distilled water to give 18µl DNA solution with concentration ranging from 5 to 10 µg. To these tubes were added 2µl of 10 X restriction digestion buffer (Appendix F) and the contents mixed by tapping gently. The appropriate enzyme (1µl) (Appendix F) was added directly into the reaction tubes and incubated at 37°C overnight. The tubes were heated at 70°C to stop the enzyme reaction and 4µl of 6X gel loading buffer added to each reaction mixture. Digested DNA samples were stored at -20°C until use.

Electrophoresis of digested and undigested DNA was performed as described by Sambrook *et al.* (1989). Undigested and digested DNA samples with restriction endonuclease mixed with loading buffer (Appendix F) were carefully dispensed using a micropipette into the slots in a 0.8% agarose gel submerged in electrophoresis buffer [1X Tris acetate EDTA (TAE) buffer] (Appendix G). Electrophoresis was performed using a mini-gel system (Biorad Model 200/2.0). The gels which contained 0.5µg/ml ethidium bromide were run for about 1 hour at 80V and photographed using a UV trans-illuminator and a polariod camera fitted with an orange filter of wavelength 302nm. The size of genomic DNA and digested DNA fragments were determined using a standard calibration curve (Appendix H).

CHAPTER 3

3.0 RESULTS

3.1 Bacterial Isolates obtained from the Different Samples

3.1.1 Ferrous Iron Oxidizing Bacterial Isolates

Enrichment cultures containing ferrous iron (Fe^{2+}) as energy substrate were used to isolate ferrous iron oxidizing bacteria. All four sample types (slurry from biooxidation tank, surface arsenopyrite ore, underground gold sulphide ore and underground mine water) were found to contain ferrous iron oxidizing bacteria. The growth of these organisms was monitored by the characteristic change of medium colour from pale green to deep amber or reddish brown within 4 to 10 days of incubation (Figure 4). Ferrous iron oxidizing bacterial isolates in the slurry, surface arsenopyrite and underground sulphide ores produced more intense medium colour change. Phase-contrast microscopical examination of the culture revealed a diverse population of straight rods and curved or spiral shaped cells.

3.1.2 Sulpho-oxidizing Bacterial Isolates

Enrichment cultures containing elemental sulphur as energy substrate were used to isolate sulpho-oxidizing bacterial isolates. Sulpho-oxidizing bacterial isolates were obtained from only 3 sample types namely, slurry from biooxidation tank, surface arsenopyrite ore and underground gold sulphide ore. The growth of the sulpho-oxidizing organisms was indicated by the change of medium colour from colourless to intense grey after 30 days incubation. Phase-contrast microscopy revealed a population of straight rods cell.

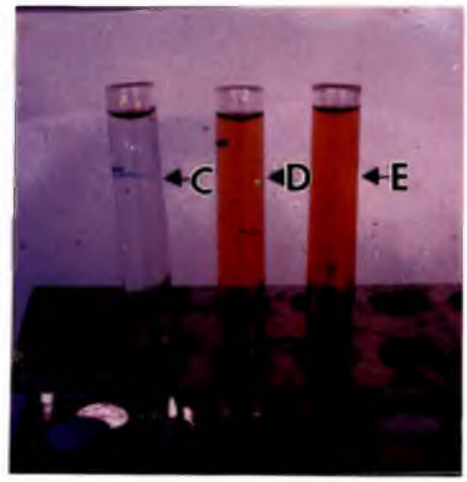


Figure 4 Photograph showing stages of growth of the ferrous iron oxidizing bacteria.

Colouration of the medium indicated the growth phase of the organisms.

C: lag phase (plain in colour).

D: logarithm phase (amber in colour).

E: stationary phase (reddish brown or deep amber in colour).

3.2 Designations of Bacterial Isolates

Table 1 contains a list of selected representative bacterial isolates, their sources and designated abbreviated names.

3.3 Identification of Purified Bacterial Isolates

3.3.1 Ferrous Iron Oxidizing Bacteria

Purified isolates of two main ferrous iron oxidizing bacteria were obtained from all the samples analyzed. One of the ferrous iron oxidizing bacteria from each of the sample sources could grow on ferrous agar or agarose whilst the other grew only in liquid medium (Table 2 and Figures 5-10). The bacteria that grew on solid media showed as pinpoint iron encrusted colonies (Figures 5 and 6) or larger spreading colonies with opaque iron encrusted centres and transparent edges (Figures 7 and 9). These bacteria also grew on sodium thiosulphate agar (Figure 8) or ferrous/sodium thiosulphate agar (Figures 9 and 10). Transfer of single colonies of the organisms back into fresh liquid medium resulted in the oxidation of ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}). These isolates were also able to grow in 9K medium supplemented with elemental sulphur or sodium thiosulphate (Table 2). Microscopical examination of the cells obtained from single colonies revealed a homogenous culture of straight rods that appeared in singles, pairs and in clusters (Figures 11, 12 and 13). The isolates were Gram negative. Representative isolates selected from the various sample sources for further characterization were BT1-Fe^{2+}, SO1-Fe^{2+}, UO1-Fe^{2+}, and UW1-Fe^{2+}.

Serial cultivation of crude ferrous iron oxidizing bacteria in 9K-Fe^{2+} liquid medium with L-cysteine produced bacteria that did not grow on solid media (Table 2) but oxidized Fe^{2+} to Fe^{3+} as indicated by change of medium colour. Microscopical examination of cultures revealed a homogenous sample of curved or comma shaped rod cells and occasional spiral shaped cells (Figure 14). As shown the cells appeared in singles, pairs and in clusters. These cells were also Gram negative (Table 4).

Representative isolates selected from the various sample sources for further characterization were BT2-Fe^{2+}, SO2-Fe^{2+}, UO2-Fe^{2+} and UW2-Fe^{2+}.

3.3.2 Sulpho-oxidizing Bacteria

Purified isolates of two different sulpho-oxidizing bacteria were obtained from three of the sample sources namely, slurry, surface arsenopyrite and underground sulphide ore. After growth, it was confirmed that one group of bacteria were identical to ferrous iron oxidizers (section 3.3.1) so were designated BT1-Fe^{2+}, SO1-Fe^{2+}, UO1-Fe^{2+}. These isolates grew on sodium thiosulphate agar, as well as ferrous agar or agarose and ferrous/sodium thiosulphate agar (Figures 5, 6, 8 and 9). The other isolates grew only on sodium thiosulphate agar forming minute pale yellow colonies (Figure 15). Single colonies transferred back into fresh 9K-S° liquid medium grew elemental sulphur as energy substrate. Microscopic examination of the sulpho-oxidizing bacterial culture revealed a homogenous sample of straight rods that appeared in singles, pairs and in chains (Figure 16). The organisms were Gram negative (Table 4).

Table 1 Selected biooxidizing bacterial isolates and their designated codes

Source of sample	Designated sample code	Selected biooxidizing bacterial isolates		
		1[a]	2[b]	3[c]
Biooxidation reactor tank	BT	BT1-Fe^{2+}	BT2-Fe^{2+}	BT3-S^0
Surface arsenopyrite ore	SO	SO1-Fe^{2+}	SO2-Fe^{2+}	SO3-S^0
Underground gold sulphide ore	UO	UO1-Fe^{2+}	UO2-Fe^{2+}	UO3-S^0
Underground mine water	UW	UW1-Fe^{2+}	UW2-Fe^{2+}	*

[a] Rod shaped ferrous iron oxidizers.

[b] Curved shaped ferrous iron oxidizers.

[c] Rod shaped sulpho-oxidizing bacteria.

* There was no sulpho-oxidizing bacteria in the sample.

Table 2 Physiological characteristics of biooxidizing bacterial isolates

Bacterial Isolate	Use of Fe^{2+}	Use of sulphur	Use of CO_2	Use of $(NH_4)_2SO_4$	Growth at RT[a] (25 to 30 °C)	Growth on FA[b]	Growth on FAR[c]	Growth on STA[d]	Growth on FSTA[e]
BT1-Fe^{2+}	+	+	+	+	+	+	+	+	+
BT2-Fe^{2+}	+	-	+	+	+	-	-	-	-
BT3-S°	-	+	+	+	+	-	-	+	+
SO1-Fe^{2+}	+	+	+	+	+	+	+	+	+
SO2-Fe^{2+}	+	-	+	+	+	-	-	-	-
SO3-S°	-	+	+	+	+	-	-	+	+
UO1-Fe^{2+}	+	+	+	+	+	+	+	+	+
UO2-Fe^{2+}	+	-	+	+	+	-	-	-	-
UO3-S°	-	+	+	+	+	-	-	+	+
UW1-Fe^{2+}	+	+	+	+	+	+	+	+	+
UW2-Fe^{2+}	+	-	+	+	+	-	-	-	-

[a] RT = room temperature.
[b] FA = ferrous agar.
[c] FAR = ferrous agarose.
[d] STA = sodium thiosulphate agar.
[e] FSTA = ferrous /sodium thiosulphate agar.
+ = bacterium utilizes it or grows on solid medium.
- = bacterium does not utilize it or does not grow on solid medium.

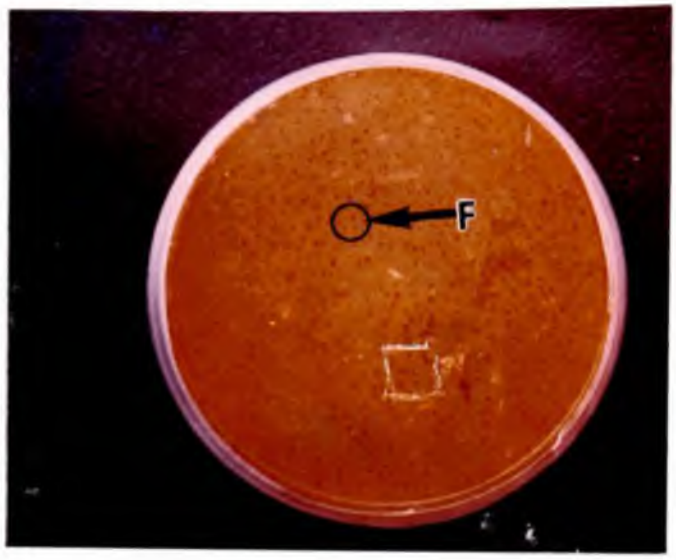


Figure 5 Isolate BT1-Fe^{2+} grown on ferrous agarose showing orange, small pinpoint colonies 'F'.

Figure 6 Small, pinpoint, reddish brown (iron encrusted) colonies 'G' of isolate BT1-Fe^{2+} grown ferrous agar.

Figure 7 Large and circular colony 'A' of isolate BT1-Fe^{2+} grown on ferrous agar.

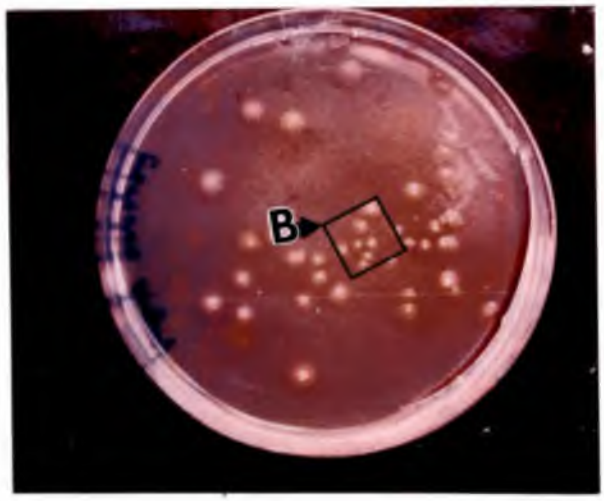


Figure 8 Characteristic "frosty" colonies 'B' of isolate BT1-Fe^{2+} grown on sodium thiosulphate agar.

Figure 9 Large, spreading colonies 'E' of isolate BT1-Fe^{2+} grown on ferrous/sodium thiosulphate agar. 'D' shows iron encrusted center and 'G' small, pinpoint colonies.

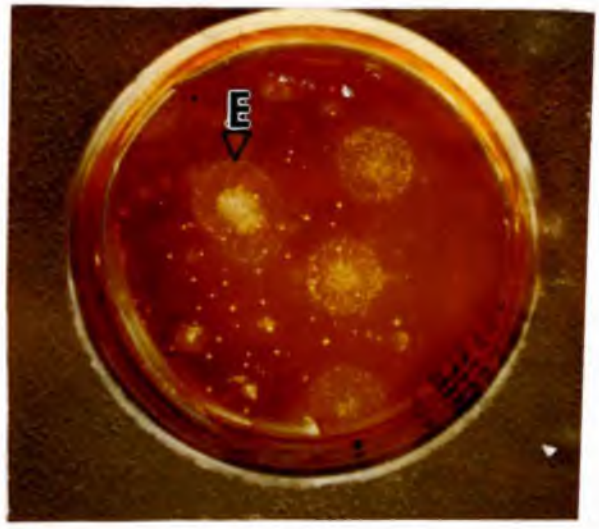


Figure 10 Large, spreading colony 'E' of isolate UW1-Fe^{2+} grown on ferrous/sodium thiosulphate agar.

Figure 11 Phase-contrast photomicrograph (mag. X 1000) showing straight rod cells 'N' of isolate BT1-Fe^{2+}.

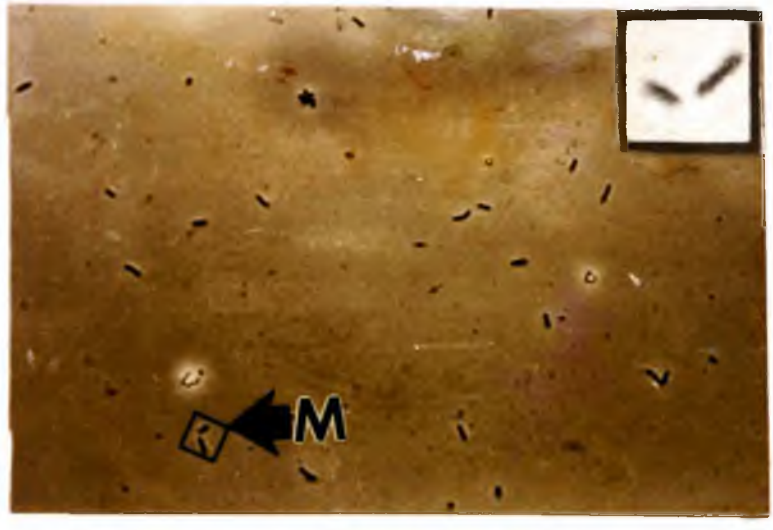


Figure 12 Phase-contrast photomicrograph (mag. X 1000) showing straight rod cells 'M' of isolate UW1-Fe^{2+}.

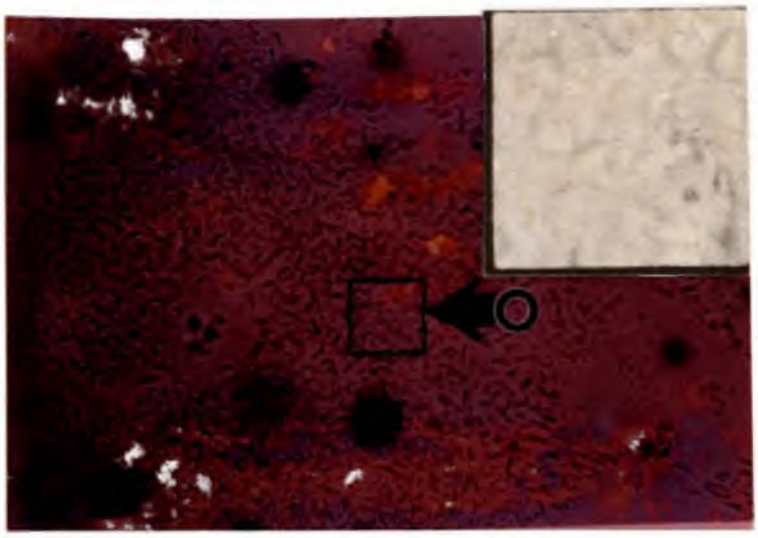


Figure 13 Phase-contrast photomicrograph (mag. X 1000) showing clustered straight rod cells 'O' isolate SO1-Fe^{2+}.

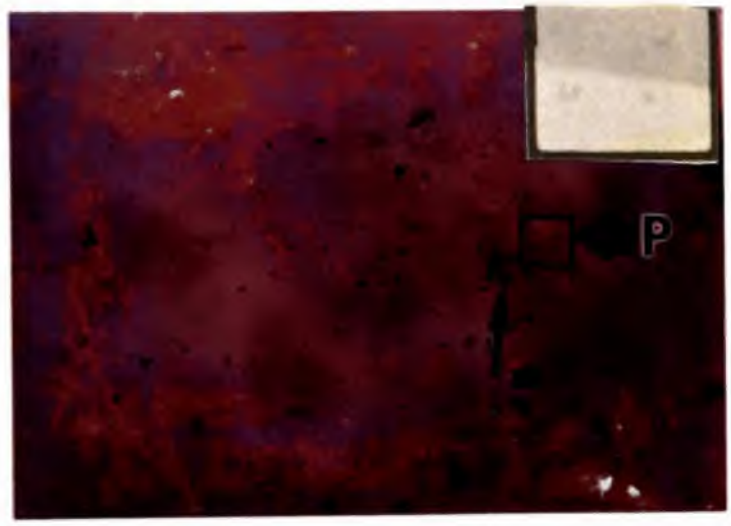


Figure 14 Phase-contrast photomicrograph (mag. X 1000) showing curved or comma rod cells 'P' of isolate BT1-Fe^{2+}. Slender arrow shows spiral shaped cells.

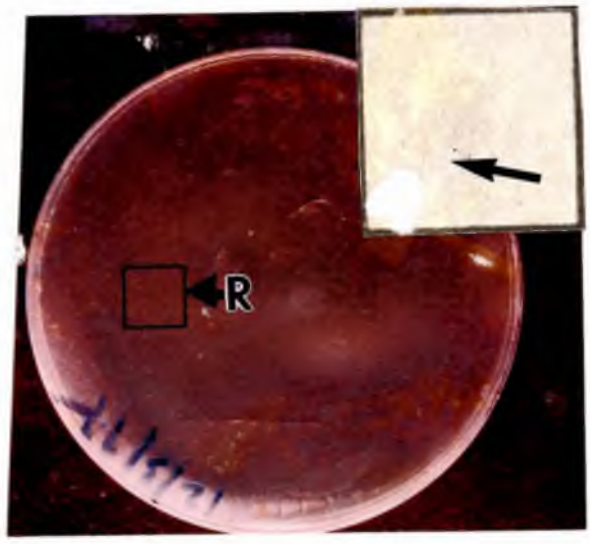


Figure 15 Punctiform or minute colonies 'R' of isolate BT3-S° grown on sodium thiosulphate agar. Insert shows enlarged colonies in box with the arrow pointing to a single colony that appears as a white spot.

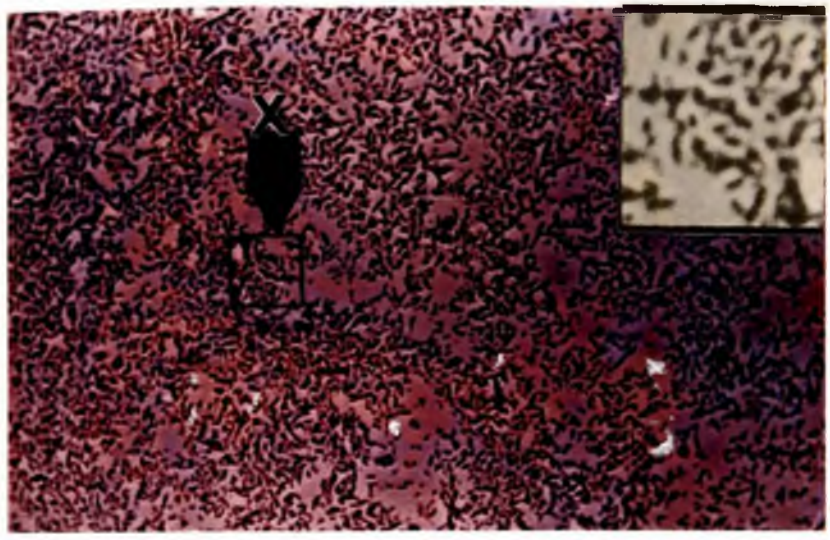


Figure 16 Phase-contrast photomicrograph (mag. X 1000) showing straight rod cells 'X' of isolate BT3-S°.

Representative isolates selected from the various sample sources for further characterization were BT3-S°, SO3-S° and UO3-S°

3.4 Physiological, Cultural, Morphological and Biochemical Characteristics of Selected Bacterial Isolates

Tables 2, 3 and 4 summarizes the physiological, cultural, morphological and biochemical characteristics of selected bacterial isolates. The ferrous iron and sulpho-oxidizing bacterial isolates utilized atmospheric CO_2 as their carbon source and $(NH_4)_2SO_4$ as their nitrogen source (Table 2). The organisms were Gram negative (Table 4). Ferrous iron oxidizers oxidized ferrous iron to ferric iron, however isolates BT1-Fe^{2+}, SO1-Fe^{2+} and UO1-Fe^{2+} also oxidized sulphur to sulphuric acid (Table 4). Sulpho-oxidizing bacteria oxidized sulphur to sulphuric acid (Table 4) and on solid media colonies of isolates BT3-S°, SO3-S° and UO3-S° had forms that were punctiform and pale yellow in colour. Their colony surfaces were smooth, colony elevation flat, margin entire and colony diameter 0.5mm (Table 3).

Isolates BT1-Fe^{2+}, SO1-Fe^{2+}, UO1-Fe^{2+}, and UW1-Fe^{2+} appeared were circular (Figure 7), punctiform (Figures 5 and 6), filamentous (Figures 8, 9 and 10) and irregular (Figure 5). Their colony elevations were either raised or smooth, colony margin filamentous, undulate and entire (Table 3). Colonies of BT1-Fe^{2+}, SO1-Fe^{2+} and UO1-Fe^{2+}, were 0.5-5mm in diameter whilst UW1-Fe^{2+} had colony diameter of 2-5mm (Table 3). Isolates BT2-Fe^{2+}, SO2-Fe^{2+}, UO2-Fe^{2+} and UW2-Fe^{2+} did not grow on solid media. Based on these characteristics BT1-Fe^{2+}, SO1-Fe^{2+}, UO1-Fe^{2+} and UW1-Fe^{2} were identified as *T. ferrooxidans,* BT2-Fe^{2+}, SO2-Fe^{2+}, UO2-Fe^{2+} and UW2-Fe^{2+} *L. ferrooxidans,* BT3-S°, SO3-S° and UO3-S° *T. thiooxidans*.(Harrison, 1984; Goebel and Stackebrandt, 1994)

Table 3 **Cultural and morphological characteristics of bacterial isolates on agar and agarose media**

Bacteria Isolate designation	Colony Form [a]	Colony Color [b]	Colony Surface [c]	Colony elevation [d]	Colony margin [e]	Colony diameter [f]
BT1-Fe^{2+}	C, P, F, IR	WC,R,O, Y,BA	r or s	raised	F, U and E	0.5 - 5mm
BT3-S^{0}	P	PY	s	flat	E	0.5mm
SO1-Fe^{2+}	C, P, F, IR	R,O,Y,BA	r or s	raised	F, U and E	0.5 - 5mm
SO3-S^{0}	P	PY	s	flat	E	0.5mm
UO1-Fe^{2+}	C, P, F, IR	R,O,Y,BA	r or s	raised	E, U and E	0.5 - 5mm
UO3-S^{0}	P	PY	s	flat	E	0.5mm
UW1-Fe^{2+}	F, IR	WC	r	raised	F	2 - 5mm

[a] C= circular, P= punctiform, F= filamentous, IR= irregular.
[b] WC= whitish cream(frosty), R= reddish brown, O= orange, Y= yellow, BA= black with amber surrounding, PY= pale yellow.
[c] r = rough, s = smooth.
[e] F= filamentous, U= undulate, E= entire.

Table 4 **Cultural, morphological and biochemical characteristics of biooxidizing bacterial isolates in 9k liquid media**

Bacteria Isolate designation	Energy source	Metabolite produced	Initial pH of medium	Final pH medium	Cell shape[a]	Reaction to Gram's stain	Initial colour of medium[b]	Final colour of medium[c]	Reaction to basic stains[d]
BT1-Fe^{2+}	Fe^{2+}	*Fe^{3+}	≤1.8	≥2	SR	-	PG	DA, RB	+
BT2-Fe^{2+}	Fe^{2+}	Fe^{3+}	≤1.8	≥2	CR	-	PG	DA, RB	+
BT3-S^o	S^o	Sulphuric acid	≤1	≥1	SR	-	C	IG	+
SO1-Fe^{2+}	Fe^{2+}	*Fe^{3+}	≤1.8	≥2	SR	-	PG	DA, RB	+
SO2-Fe^{2+}	Fe^{2+}	Fe^{3+}	≤1.8	≥2	CR	-	PG	DA, RB	+
SO3-S^o	S^o	Sulphuric acid	≤1	≥1	SR	-	C	IG	+
UO1-Fe^{2+}	Fe^{2+}	*Fe^{3+}	≤1.8	≥2	SR	-	PG	DA, RB	+
UO2-Fe^{2+}	Fe^{2+}	Fe^{3+}	≤1.8	≥2	CR	-	PG	DA, RB	+
UO3-S^o	S^o	Sulphuric acid	≤1	≥1	SR	-	C	IG	+
UW1-Fe^{2+}	Fe^{2+}	Fe^{3+}	≤1.8	≥1.9	SR	-	PG	DA	+
UW2-Fe^{2+}	Fe^{2+}	Fe^{3+}	≤1.8	≥1.9	CR	-	PG	DA	+

[a] SR= straight rods, CR= curved rods. [b] PG= pale green ,C= colorless.
[c] DA= deep amber, RB= reddish brown, IG= intense grey. [d] = basic stains used were crystal violet, neutral red, safranin and carbol fuschin.
S^o = elemental sulphur, Fe^{2+}= ferrous iron, Fe^{3+} = ferric iron. * Isolate also oxidized elemental sulphur

3.5 Viability of Stored Bacterial Isolates

Viability of stored bacteria was assessed using aliquots of representative bacterial isolates in suspension kept at 4°C and -20°C, as well as freeze-dried samples stored at 4°C (Table 5). The results indicated that the organisms retained viability under the various storage conditions.

3.6 Comparison of Bacterial Isolates using SDS-PAGE

Bacterial cells lysates of the isolates were analyzed by SDS-PAGE to determine their protein patterns. As shown in Figure 17, the organisms had different polypeptide band profiles. Whereas the *T. ferrooxidans* sample showed prominent bands ranging from 28 to 68 kDa, the *T. thiooxidans* sample had prominent bands ranging from 34 to 66 kDa with a large polypeptide band at 36 kDa. *L. ferrooxidans* on the other hand had 3 major clusters of polypeptide bands ranging from 10 to 24, 24 to 29 and 29 to 66 kDa (Figure 17).

The protein profile in lane 2 (Figure 18) illustrates a typical pattern seen for all isolates of *T. ferrooxidans* from the different samples. Figures 19 and 20 show typical polypeptide band patterns for the different isolates of *L. ferrooxidans* and *T. thiooxidans* from the samples analyzed.

Table 5 **Viability of stored bacterial isolates**

Selected bacterial isolate	Growth of bacterial preparations stored at different temperatures and periods				
	Bacteria in suspension		Freeze dried specimen stored at 4°C		
	3 mons (4°C)	3 mons (-20°C)	1 mon	2 mons	3 mons
BT1-Fe^{2+}	+	+	+	+	+
BT2-Fe^{2+}	+	+	+	+	+
BT3-S^{o}	+	+	+	+	+
SO1-Fe^{2+}	+	+	+	+	+
SO2-Fe^{2+}	+	+	+	+	+
SO3-S^{o}	+	+	+	+	+
UO1-Fe^{2+}	+	+	+	+	+
UO2-Fe^{2+}	+	+	+	+	+
UO3-S^{o}	+	+	+	+	+
UW1-Fe^{2+}	+	+	+	+	+
UW2-Fe^{2+}	+	+	+	+	+

mons = period of storage of samples in months.

+ = Biooxidizing bacterial cells were able to grow when put back in 9k liquid media.

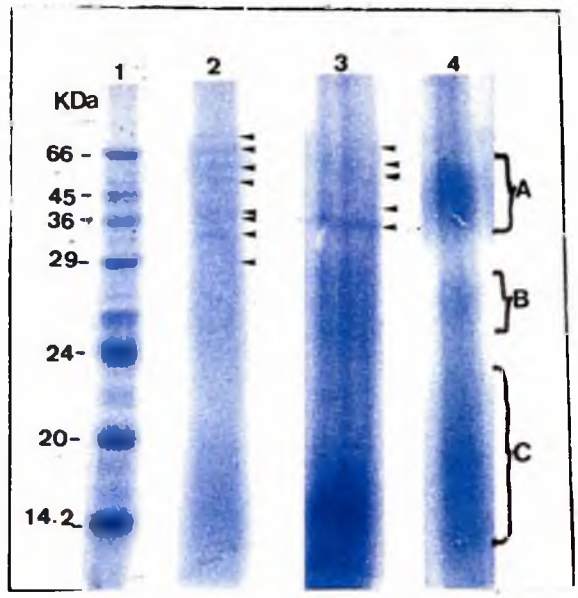


Figure 17 Comparison of bacterial cell lysates of isolates by SDS-PAGE.

Lane 1 shows the standard molecular weight marker, lanes 2, 3 and 4 show protein band profiles of *T. ferrooxidans, T. thiooxidans and L. ferrooxidans* respectively, from the biooxidation tank. Arrowheads and brackets indicate prominent bands.

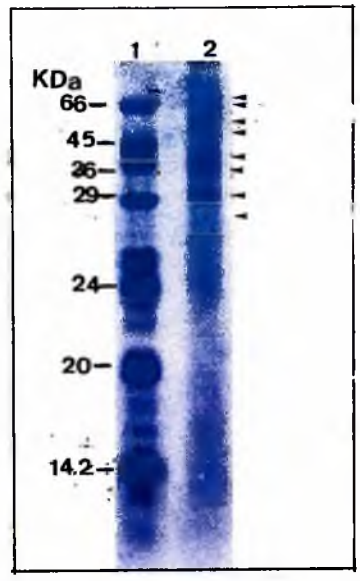


Figure 18 SDS-PAGE of bacterial cell lysates of *T. ferrooxidans* from biooxidation tank (lane 2). Lane 1 shows standard molecular weight marker and arrowheads indicate prominent polypeptide bands.

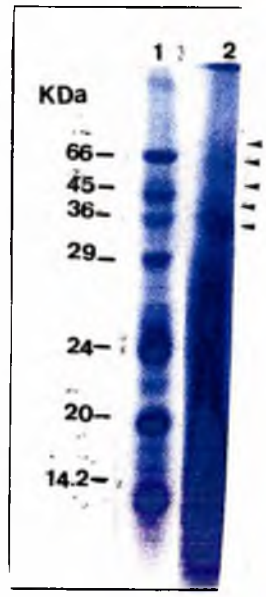


Figure 20 SDS-PAGE of bacterial cell lysates of *T. thiooxidans* isolates from the biooxidation tank.

Lane 1 shows standard molecular weight marker and arrowheads indicate prominent polypeptide bands. Lane 2, ***T. thiooxidans*** **isolate.**

3.7 Comparison of Bacterial Isolates by Genomic DNA Analysis using RFLP

DNA obtained from the bacterial isolates were analyzed using Restriction Fragment Length Polymorphism (RFLP). The yield and purity of DNA for a 100ml culture (*T. ferrooxidans, T. thiooxidans* and *L ferrooxidans* isolates) were similar (5-10 μg and purity approximately 1.8). As shown in Figure 21, the different bacterial isolates had similar DNA size (approximately 23 kb). Restriction endonuclease digestion of the DNA gave mostly smears (Figure 22).

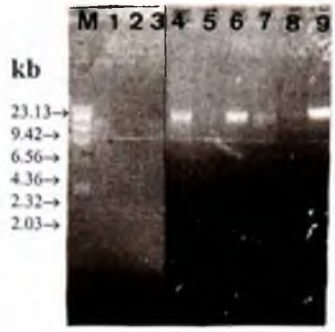


Figure 21 Agarose gel electrophoresis of undigested biooxidizing bacteria DNA.

Lane M shows a Lambda Hind III digested DNA marker. Lanes 1-3 undigested DNA of *T. thiooxidans* isolates lanes 4-6 undigested DNA of *T. ferrooxidans* isolates and Lanes 7-9 undigested DNA of *L. ferrooxidans* isolates from biooxidation tank, underground gold sulphide and surface arsenopyrite ores respectively.

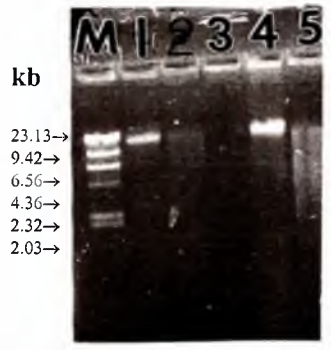


Figure 22 Restriction endonuclease (Hind III) digested DNA of biooxidizing bacterial isolates from biooxidation tank.

(M) Lambda Hind III digested DNA marker. (1) Undigested DNA of *L. ferrooxidans*. (2) Digested DNA of *L. ferrooxidans*. (4) Undigested DNA of *T. ferrooxidans*. (5) Digested DNA of *T. ferrooxidans*.

CHAPTER 4

4.0 DISCUSSION AND CONCLUSIONS

The main purpose of the study described in this thesis was to isolate and characterize biooxidizing bacteria from different sites at the Obuasi gold mining concession using microbiological methods, SDS-PAGE analysis of bacterial cell lysates and RFLP of their genomic DNA using restriction endonuclease digestion.

The biooxidizing bacterial isolates obtained in this study could be placed in three main groups based on morphological, biochemical, and physiological characteristics. The first group consisting of isolates, BT1-Fe^{2+}, SO1-Fe^{2+}, UO1-Fe^{2+} and UW1-Fe^{2+} were initially identified as *T. ferrooxidans*-like organisms due to reported characteristics such as being straight rods, Gram negative, ability to grow on solid media and oxidize iron II to iron III (Vishnac, 1975; Harrison, 1984; Torma, 1985; Harrison, 1986; Goebel and Stackebrandt, 1994). The ability of these *T. ferrooxidans*-like bacteria to utilize both iron II and elemental sulphur as energy substrates and their pleomorphic behaviour on solid media indicated that they were indeed *T. ferrooxidans* (Vishnac, 1975; Colmer *et al.*, 1950, Leathen *et al.,* 1956; Kinsel, 1960; Colmer, 1962; Harrison, 1984; Wakao *et al.,* 1991; Junior, 1991; Goebel and Stackebrandt, 1994). According to Schrader and Holmes (1988) the pleomorphic property of *T. ferrooxidans* is due to a phenotypic switching mechanism which enables the organism to survive adverse environmental conditions. It is indeed this property that enables *T. ferrooxidans* to grow on solid medium and utilize both ferrous iron or sodium thiosulphate as energy substrate (Rawlings *et al.,* 1991). The second group of isolates (BT2-Fe^{2+}, SO2-Fe^{2+}, UO2-Fe^{2+} and UW2-Fe^{2+}) which among other characteristics were, curved rods, Gram negative and oxidized iron II to iron III were primarily considered as *L. ferrooxidans* based on earlier observations (Harrison and Norris, 1985; Harrison, 1986; Sand *et al.,* 1992).

Their inability to grow on solid media confirmed the identification (Harrison 1984; Goebel and Stackebrandt, 1994). Goebel and Stackebrandt (1994) isolated Gram negative, straight rod bacterial cells that grew at pH < 1 and utilized elemental sulphur as energy substrate, and identified them as *T. thiooxidans*. The third group of isolates (BT3-S°, SO3-S° and UO3-S°) identified in this study exhibited similar characteristics as *T. thiooxidans*. The present isolates also shared other *T. thiooxidans* characteristics such as the formation of pale yellow minute colonies on sodium thiosulphate agar reported by earlier workers (Waksman and Joffe, 1922; Vishnac, 1975; Harrison , 1986; Konishi *et al.,* 1995).

The absence of sulpho-oxidizing bacteria in underground mine water was not surprising since most isolates, especially *T. thiooxidans* had been obtained from soil samples (Waksman and Joffe, 1922; Vishnac, 1975; Harrison, 1986; Konishi *et al.,* 1995). Lundgren and Silver (1980) reported that biooxidizing bacteria were likely to be found mostly in samples where their energy substrate is readily available. The absence of *T. thiooxidans* in underground mine water is therefore attributable to a deficiency of the energy substrate (elemental sulphur and reduced sulphur compounds). Rawlings and Kusano (1994) explained that low concentrations of iron in the sample may result in small populations of ferrous iron oxidizing bacteria with slow iron oxidation rate. This may also explain the slow ferrous iron oxidation rates of *T. ferrooxidans* and *L. ferrooxidans* isolates obtained from underground mine water as compared to isolates of the same bacteria from the other samples. However, the difficulty of the *T. ferrooxidans* isolate from underground mine water to undergo pleomorphism and its unique colony size (2-5mm) as compared to the other isolates (0.5-5mm) may suggest that the isolate was of a different strain.

The mineralogy and chemical composition of ore has been reported to determine the population of biooxidizing bacteria present and their efficiency of mineral ore oxidation (Murayama *et al.,*

1987; Suzuki *et al.*, 1990; Baldi *et al.*, 1992; Morin, 1995; Rawlings and Silver, 1995). This may suggest that different strains of biooxidizing bacteria could be isolated from the surface arsenopyrite and underground sulphide ores at Obuasi. Isolates of biooxidizing bacteria obtained in this study were therefore characterized using SDS-PAGE analysis of bacterial cell lysates and RFLP of their genomic DNA with the objective of revealing possible strain similarities and differences within the same species of organism isolated from the different ecological niches. Rawlings *et al.* (1991) used the SDS-PAGE and noted its high degree of resolution of bacterial cell proteins. Also, Kersters and De Ley (1980) reported that this method was particularly suited for comparing cell envelopes and ribosomal proteins.

In this work it was possible to differentiate between *T. ferrooxidans, T. thiooxidans* and *L. ferrooxidans* using their protein profiles. Similar studies by Huber *et al.* (1985), Harrison and Norris (1985) and Chamorro *et al.* (1987) revealed differences in the protein bands of the three organisms. The protein profiles obtained showed prominent bands that differentiated one bacterium from the other. The bands observed were comparable to those obtained by Huber *et al.* (1985). Harrison and Norris (1985) also identified individual strains of biooxidizing bacteria using the SDS-PAGE. However, no differences in protein profiles were observed between the various isolates of *T. ferrooxidans, T. thiooxidans* and *L. ferrooxidans* analyzed in this study. Even though the surface and underground ores used in this work had different mineralogy and chemical composition, the lack of detectable strain differences by SDS-PAGE was not surprising since different biooxidizing bacterial strains identified earlier were obtained from completely different metal ores namely, copper and uranium ores(Huber *et al.*, 1985; Harrison and Norris, 1985; Chamorro *et al.*, 1987).

Previous workers have characterized biooxidizing bacteria by RFLP (Shiratori *et al.*, 1989; Rawlings, 1995). Tang *et al.* (1997) pointed out that a large number of fragments obtained in the DNA digests of bacteria had small size differences that resulted in overlapping separation normally observed as a smear. This may explain the inability to differentiate between the bacterial isolates in this study because of smearing of digested DNA fragments. Rawlings (1995) overcame this problem by amplifying specific DNA sequences before analysis by RFLP. Earlier on, Shiratori *et al.* (1989) employed Southern hybridization of genomic DNA to identify strains of biooxidizing bacteria. It was however not possible to use any of these improved methods in this study due to resource constraints. Nevertheless, the similarities between *T. ferrooxidans*, *T. thiooxidans* and *L. ferrooxidans* isolates from the biooxidation tank and the local ores based on cultural, physiological and morphological characteristics as well as protein profiles may suggest that the organisms from the biooxidation tanks are not different from the local isolates. This is an interesting observation since there is always the possibility of local biooxidizing bacterial strains overgrowing and replacing seeded organisms in long term culture systems. Such a situation may lead to inefficient gold recovery since not all the local biooxidizing bacterial strains are efficient oxidizers of gold ore. If this observation were true, then there is an urgent need to characterize and select efficient local strains for the biooxidation process at the sulphide treatment plant at Obuasi.

In conclusion, this study confirms the presence of biooxidizing bacteria currently exploited in biomining namely, *T. ferroxidans* *T. thiooxidans* and *L. ferrooxidan* in the biooxidation tank at AGC, Obuasi. Furthermore, local isolates of the three organisms with potential use for more efficient gold extraction from local ores were obtained from surface and underground gold ores as well as underground mine water.

RECOMMENDATIONS

1) There is the need to conduct further studies to determine the gold recovery efficiency of the isolated local biooxidizing bacteria.

2) More work is needed to determine the taxonomic significance of bacterial isolates from the underground mine water which had some unique morphological and physiological properties.

3) Further characterization of the local biooxidizing bacterial isolates should emphasize DNA analytical methods, such as; nucleic acid hybridization, ribotyping (probing restriction patterns with labelled bacterial ribosomal operons that code for 16s or 23s rRNA) and amplification of known segments of genomic DNA.

REFERENCES

Adair, F. W. (1966). Membrane sulfur oxidation by the autotrophic *Thiobacillus thiooxidans*. *J. Bacteriol.*, 92, 899-905.

Agate, A. D. & Khinvasara N. J. (1985). Bioleaching of copper ores and the concentrate of Malanjkhand area, India. In: Ehrlich, H. L. & Holmes, D. S. (eds) Biotechnology for the Mining, Metal-refining and Fossil Fuel Processing Industries. John Wiley & Sons Inc., New York. pp. 83-90.

Ahonen, L. & Tuovinen, O. H. (1991). Temperature effects on bacterial leaching of sulphide minerals in shake flask experiments. *Appl. Environ. Microbiol.*, **57,** 138-145.

Apel, W. A., Dugan, P. R., Flippi, J. A. & Rheins, M. S. (1976). Detection of *Thiobacillus ferrooxidans* in an acid mine environment by indirect flourescent antibody staining. *Appl. Environ. Microbiol.*, 32, 159.

Arrendondo, R. & Jerez, C. A. (1989). Specific dot-immunobinding assay for the detection and enumeration of *Thiobacillus ferrooxidans*. *Appl. Environ. Microbiol.*, **55**, 2025-2029.

Ashanti Goldfields Company Limited (1994). Annual report.Royle Printing press, England. pp1-33

Ashanti Goldfields Company Limited (1996). Annual report.). Annual report.Royle Printing press, England, pp1-33

Ashanti Goldfields Company Limited (1997). Annual report.). Annual report.Royle Printing press, England, pp1-33

Balashova, V. V., Vederina, I. Y., Markosyan, G. E. & Zarvarzin, G. A. (1974). The auxotrophic growth of *Leptospirillum ferrooxidans*. *Microbiol.*, **43**, 581-585.

Baldi, F., Clack, T., Pollack, S. S. & Olson, G. J. (1992). Leaching of pyrite of various reactivities by *Thiobacillus ferrooxidans*. *Appl. Environ. Microbiol.*, **58,** 1853-1856.

Barrett, J., Hughes, M. N., Karavaiko, G. I. & Spencer, P. A. (1993). Metal extraction: Bacterial oxidation of minerals Ellis Harwoods Limited, England. pp. 1-189.

Barron, J. L. & Leuking, D. R. (1990). Growth and maintenance of *Thiobacillus ferrooxidans* cells. *Appl. Environ. Microbiol.*, **56,** 2801-2806.

Battaglia, F., Morin, D., Garcia, J. L & Ollivier, P. (1994). Isolation and study of two strains of Leptospirillum-like bacteria from a natural mixed population on a cobaltiferous pyrite substrate. *Antonie-Van-Leeuwenhoek*, **66,** 295-302.

Beck, J. V. (1960). A ferrous iron oxidizing bacterium: Isolation and some general physiological characteristics. *J. Bacteriol.*, 79, 503-509.

Beck, J. V. (1967). The role of bacteria in copper mining operations. *Biotechnol. Bioeng.*, **9,** 487-497.

Beck, J. V. & Brown, D. G. (1968). Direct sulfide oxidation in the solubilization of sulfide ores by *Thiobacillus ferrooxidans*. *J. Bacteriol.*, **96,**1433-1434.

Bennett, J. C. & Tributsch, H. (1978). Bacterial leaching patterns on pyrite crystal surfaces. *J. Bacteriol.*, 134, 310-317.

Brierley, J. A. & Brierley, C. L. (1968). Urea as a nitrogen source for *Thiobacilli*. *J. Bacteriol.*, **96,** 573.

Brierley, C. L. (1978). Bacterial leaching. *Crit. Rev. Microbiol.*, **6,** 207-263.

Brierley, C. L. (1982). Microbiological Mining. *Sci. Am.*, **247,** 42-51.

Cavalier-Smith, T. (1985). The Evolution of Genome Size (eds) John Wiley Company New York.

Chamorro, D., Arrendondo, R., Peirano, I. & Jerez, C. A. (1987). The programme of proteins synthesized by *Thiobacillus ferrooxidans* under different environmental conditions;

Analysis by two dimension gels: In Norris, P. R. & Kelly, D. P. (eds). Biohydrometallurgy; Proceedings of the International Symposium, Warwick, Conventry, UK. pp. 558-560.

Collins, C. H. & Lyne, P. M. (1989). Microbiological Methods, 5th. ed. Butterworth and Co. (publishers) Ltd, UK.

Colmer, A. R. & Hinkle, M. E. (1947). The role of microorganisms in acid mine drainage: a preliminary report. *Science*, 106, 253.

Colmer, A. R., Temple, K. L. & Hinkle, M. E. (1950). An iron-oxidizing bacterium from the acid drainage of some bituminous coal mine. *J. Bacteriol.*, **59**, 317-328.

Colmer, A. R. (1962). Relation of the iron oxidizer. *Thiobacillus ferrooxidans* to thiosulphate. *J. Bacteriol.*, 83, 761-765.

Copeland, R. A. (1994). Methods for Protein Analysis. 1st ed. Chapman and Hall Ltd, New York. Pp59-99.

Cowan, S. T. (1985). Identification of Medical Bacteria. 2nd ed. Cambridge University Press, London, UK.

Curutchet, G., Pogliani, C. & Donati, E. (1995). Indirect bioleaching of covellite by *Thiobacillus thiooxidans* with an oxidizing agent. *Biotechnol. Lett.*, **17**, 1251-1256.

Das, A. & Mishra, A. K. (1996). Role of *Thiobacillus ferrooxidans* and sulphur (sulphide)-dependent ferric-iron reducing activity in the oxidation of sulphide minerals. *Appl. Microbiol. Biotechnol.*, 45, 377-382.

Deutscher, M. P. (1990). Guide to Protein Purification. In: *Methods in Enzymology* **182** Academic Press, Inc., UK. pp. 425-488.

Drobner, E. Huber, H. & Stetter, K. O. (1990). *Thiobacillus ferrooxidans,* a new facultative hydrogen oxidizer. *Appl. Environ. Microbiol.*, **56**, 2922-2923.

Dugan, P. R. & Lundgren, D. G. (1965). Energy supply for the Chemoautotroph *Ferrobacillus ferrooxidans*. *J. Bacteriol.*, 89, 825-834.

Ehrlich, H. L. & Holmes, D. S. (1985). Biotechnology for the mining, metal-refining and fossil fuel processing industries. (ed.) John Wiley & Sons Inc., New York.

Ferroni, G. D., Leduc, L. G. & Todd, M. (1986). Isolation and temperature characterization of psychotropic strains of *Thiobacillus ferrooxidans* from the environment of a uranium mine. *J. Gen. Appl. Microbiol.*, **32,** 169-175.

Fischer, J., Quentmeier, A., Kostka, S., Kraft, R. & Friedrich, C. G. (1996). Purification and characterization of the hydrogenase from *Thiobacillus ferrooxidans*. *Arch. Microbiol.*, 165, 289-296.

Flook, P. K., Wilson, M. D. & Post, R. J. (1992). The use of repetitive DNA probes in the analysis of natural populations of insects and parasites.. In: Berry, R. J., Crawford, T. J. & Hewitt, G. M. (eds) Genes in ecology British Ecological Society/Blackwell scientific Publication, Oxford. pp. 484-486

Gaylarde, C. C. & Videla, H. A. (1995). Bioextraction and Biodeterioration of metals. (eds.) Cambridge University Press. Oxford.

Goebel, B. M. & Stackebrandt, E. (1994). Cultural and phylogenetic analysis of mixed microbial populations found in natural and commercial bioleaching environments. *Appl. Environ. Microbiol.*, 60, 1614-1621.

Goodfellow, M. & Board, R. G. (1980). Microbiological classification and identification. (eds.) Academic Press Inc., New York.

Gupta, S. G. & Agate, A. D. (1986). Preservation of *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans* with activity checks. *Antonie-Van-Leeuwenhoek* **52,** 121-128.

Hallberg, K. B., Dopson, M. & Lindstrom, E. B. (1996). Reduced sulfur compound oxidation by *Thiobacillus caldus*. *J. Bacteriol.*, **178,** 6-11.

Hames, B. D. & Higgins, S. J. (1991). Nucleic Acid Hybridization, a Practical Approach. (eds.) IRL Press Inc., Oxford, UK. pp. 21.

Hames, B. D. & Rickwood, D. (1994). Gel Electrophoresis of Proteins. 2nd. ed. IRL Press Inc., Oxford, UK.

Harlow, E. & Lane, D. (1988). Antibodies, a laboratory manual. (eds) Cold Springs Harbour Laboratory .New York, USA.

Harrison, A. P. (1982). Genomic and physiological diversity amongst strains of *Thiobacillus ferrooxidans* and genomic comparison with *Thiobacillus thiooxidans*. *Arch. Microbiol.*, **131**, 68-76.

Harrison, A. P (1984). The acidophilic *Thiobacilli* and other acidophilic bacteria that share their environment. *Ann. Rev. Microbiol.*, **38**, 265-292.

Harrison, A. P. & Norris, P. P. (1985). *Leptospirillum ferrooxidans* and similar bacteria: Some characteristics and genomic diversity. *FEMS. Microbiol. Lett.*, **30**, 99-102.

Harrison, A. P. (1986). Characteristics of *Thiobacillus ferrooxidans* and some other iron-oxidizing bacteria, with emphasis on nucleic acid analyses. *Biotechnol. Appl. Biochem.*, **8**, 249-257.

Huber, G., Huber, H. & Stetter, K. O. (1985). Isolation and characterization of metal-mobilizing bacteria. In: Ehrlich, H. L. & Holmes, D. S. (eds.) Biotechnology for the Mining, Metal-refining and Fossil Fuel processing Industries. John Wiley & Sons Inc., New York pp. 239-252.

Jerez, C. A., Vargas, T. Toledo, H. & Wiertz, J. V. (1995). Biohydrometallurgical Processing. Proceedings of the International Biohydrometallurgy Symposium IBS-95 Vina del Mar, Chile.

Johnson, D. B. (1995). Selective media for isolating and enumerating acidophilic bacteria. *J. Microbiol. Methods*. **23**, 205-218.

Joklik, W. K., Willet, H. P. & Amos, D. B. (1980). Microbiology, 17th ed. Appleton-Century-Croft, New York.

Junior, O. G. (1991). Isolation and Purification of *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans* from some coal and uranium mines of Brazil. *Rev. Microbiol.*, **22**, 1-6.

Kelly, D. P. & Tuovinen, O. H. (1972). Recommendation that *Ferrobacillus ferrooxidans* Leathen and Braley and *Ferrobacillus sulfooxidans* Kinsel be regarded as synonyms of *Thiobacillus ferrooxidans* Temple and Colmer. *Int. J. Syst. Bacteriol.*, **22**, 170-172.

Kersters, K. & De Ley J. (1980). Identification and classification of bacteria by electrophoresis of their proteins. In: Goodfellow, M. & Board, R. G. (eds.) Microbiological Classification and Identification. Academic Press Inc., New York. pp. 273-294.

Konishi, Y., Asai, S. & Yoshida, N. (1995). Growth kinetics of *Thiobacillus thiooxidans* on the surface of elemental sulfur. *Appl. Environ. Microbiol.*, **61**, 3717-3622.

Kinsel, N. A. (1960). New sulfur oxidizing bacterium: *Ferroobacillus sulfooxidans* sp. n. *J. Bacteriol.*, 80, 628-632.

Kulpa, C. F., Mjoli, N. & Roskey, M. T. (1985). Comparison of Iron and Sulphur oxidation in *Thiobacillus ferrooxidans*: Inhibition of iron oxidation by growth on sulfur. In: Ehrlich, H. L. & Holmes, D. S. (eds.) Biotechnology for the Mining, Metal-refining and Fossil Fuel Processing Industries. John Wiley & Sons Inc., New York. pp. 289-298.

Kawarazaki, M., Yagisawa, M. & Yamaguchi, M. (1986). The viable count of iron- oxidizing bacteria, *Thiobacillus ferrooxidans,* with new silica gel plate method. *Rep. Fermen. Res. Inst.*, 65, 45-49.

Laemmli, U. K. (1970). Cleavage of structural proteins during assembly of the head of bacteriaphage T4. *Nature*, 227, 680-685.

Lane, D. J., Harrison, A. P., Stahl, D., Pace, B., Giovannoni, S. J., Olsen, G. J. & Pace, N. R. (1992). Evolutionary relationship among sulfur and iron-oxidizing eubacteria *J. Bacteriol.*, 174, 269-278.

Larparge, S. P., Shelton, J. E., Mitchell, T. G. & Markenzie, A. P. (1970). Culture collection and the preservation of bacteria. In: Norris, J. R. & Ribbon, D. W. (eds.) Methods in Microbiology, Vol. 3A, Academic Press Inc., London. pp. 135-228.

Leathen, W. W., Kinsel, N. A & Braley Sr, S. A. (1956). *Ferrobacillus ferrooxidans*: a new chemosynthetic autotrophic bacterium. *J. Bacteriol.*, **72**, 700-704.

Li, W.-H and Graur, D. (1991). Fundamentals of molecular evolution (eds) Sinauer associates Inc, Massachusett.

Lillie, R. D. (1990). H. J. Conn's Biological stains. 9th ed. Sigma Chemical Company, USA.

Lui, M. S., Branion, R. M. R. & Duncan, D. W. (1988). Oxygen transfer to Thiobacillus cultures. In: Norris, P. R. & Kelly, D. P. (eds). Biohydrometallurgy; proceedings of the International symposium, Warwick, Conventry, UK. pp. 375-384.

Livesey-Goldblatt, E., Norman, P. & Livesey- Goldblatt, D. R. (1983). Gold recovery from arsenopyrite/pyrite ore by bacterial leaching and cyanidation. In: Rossi, G. & Torma, A. E. (eds.) Recent Progress in Biohydrometallurgy, Azzociazione Moneria Sardia, Iglesia, Italy.

Livesey-Goldblatt, E (1986). Bacterial leaching of gold, uranium pyrite bearing compacted mine tailing slimes. In: Lawrence, R. W., Branion, R. M. R. & Ebner, H. G. (eds.) Fundamental and Applied Hydrometallurgy. Elsevier, Amsterdam. pp. 89-96.

Lizama, H. M. & Suzuki, (1988). Bacterial leaching of a sulfide ore by *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans*. I Shake flask studies. *Biotechnol. Bioeng.*, **32,** 110-116.

Lundgren, D. G. & Silver, M. (1980). Ore leaching by bacteria. *Ann. Rev. Microbiol.*, **34**, 263-283.

Lundgren, D. G., Volkova-Valchanova, M. & Reed R. (1985). Chemical reactions important in bioleaching and bioaccumulation. In: Ehrlich, H. L. & Holmes, D. S. (eds.) Biotechnology for the Mining, Metal-refining and Fossil Fuel Processing Industries.), John Wiley & Sons Inc., New York. pp. 7-22.

Mackintosh, M. E. (1978). Nitrogen fixation by *Thiobacillus ferrooxidans*. *J. Gen. Microbiol.*, **105**, 215-218.

Margalith, P., Silver, M. & Lundgren, D. G. (1966). Sulfur oxidation by the iron bacterium *Ferrobacillus ferrooxidans*. *J. Bacteriol.*, **92**, 1706-1709.

Markosyan, G. E. (1972). A new oxidizing bacterium *Leptospirillum ferrooxidans* gen. et. nov. *Biol. Zh. Arm.* 25, 26. (In English).

Marmur, J., Falkow, S. & Mandel, M. (1963). New approaches to bacterial taxonomy. *Ann. Rev. Microbiol.*, 17, 329-372.

Morin, D. (1995). Bacterial leaching of gold sulfide ore pp 25-62. In: Gaylarde, C. C. & Videla, H. A. (eds.) Biocxtraction and biodeterioration of metals. Cambridge University Press. Oxford.

Murakami, Y., Aikawa, T., Kimura, K., Ito, J. & Nakmura, Y. (1986). Preservation of *Thiobacillus ferrooxidans* by freeze drying. *Rep. Ferment. Res. Inst.*, **66**, 9-12.

Maruyama, T., Konno, Y., Sakata, T. & Imaizumi, T. (1987). Application of immobilized *Thiobacillus ferrooxidans* for large scale treatment of acid mine drainage. *Methods in Enzymology*, 136, 531-541.

Mustin, C., Berthelin, J., Marion, P., & De Donato, P. (1992). Corrosion and Electrochemical oxidation of a pyrite by *Thiobacillus ferrooxidans*. *Appl. Environ. Microbiol.*, **58**, 1175-1182.

Muyzer, G., De-Bruyn, A. C., Schmedding, D. J. M., Bos, P., Westbroek, P. (1987). A combined Immunofluorescence- DNA-fluoresence staining technique for the enumeration of *Thiobacillus ferrooxidans*. Appl. Environ. Microbiol., **53,** 660-664.

Newburgh, R. W. (1954). Phosphorylation and chemosynthesis by *Thiobacillus thiooxidans. J. Bacteriol.*, 68, 93-97.

Norman, P. F. & Snyman, C. P. (1988). The biological and chemical leaching of an auriferous pyrite/arsenopyrite floatation concentrate: A Microscopic examination. *Geomicrobiol. J.*, 6, 1-10

Norris, J. R. & Ribbons, D. W. (1970). Methods in microbiology. Vol. 3A, Academic Press Inc., London. pp. 135-228.

Norris, J. R. (1980). Introduction. In: Goodfellow, M. & Board, R. G. (eds.) Microbiological Classification and Identification. Academic Press Inc., New York. pp. 1-5.

Nyavor, K., Egiebor, N. O. & Fedorak, P. M. (1996). Suppression of microbial pyrite oxidation by fatty acid treatment. *Sci. Total-Environ.,* **182,** 75-83.

Osae, S., Kase, K. & Yamamoto, M. (1995). A geochemical study of the Ashanti gold deposits at Obuasi, Ghana. (Okayama University) *Earth Science Reports*, **2,** 81-90.

Otero, A. P., Curutchet, G., Donati, E. & Tedesco, P. (1995). Action of *Thiobacillus thiooxidans* on sulfur in the presence of a surfactant agent and its application in the indirect dissolution of phosphorus. *Process. Biochem.*, **30,** 747-750.

Padival, N. A., Weiss, J. S. & Arnold, R. G. (1995). Control of *Thiobacillus* by means of microbial competition: Implication for corrosion of concrete sewer. *Water-Eniviron. Res.* 67, 201-205.

Paknikar, K. M. & Agate, A. D. (1987). Occurrence of a *Thiobacillus ferrooxidans* strain tolerating an unusual high concentration of metal and an associated metal-tolerant acidophilic heterotrophic medium. In: Norris, P. R. & Kelly, D. P. (eds).

Biohydrometallurgy; proceedings of the international symposium, Warwick, Conventry, UK. pp. 558-560.

Patterson, U.& Hyypia, T. (1985). Nucleic acid hybridization, an alternative tool in diagnostic microbiology. *Immuno. Today.* **6**, 268-272.

Pelczar, M. J. & Chan, E. C. S. (1977). Laboratory Exercises in Microbiology. 4th ed. McGraw-Hill Inc.

Peng, J., Yan, W. & Bao, X. (1994). Expression of heterogenous arsenic resistance genes in the obligate autotrophic biomining bacterium *Thiobacillus ferrooxidans*. *Appl. Environ. Microbiol.*, **60**, 2653-2656.

Pinches, A. (1975). Bacterial leaching of an arsenic bearing sulphide concentrate. In: Burkin, A. R. (eds.) Leaching and reduction in Hydrometallurgy. Institution of Mining and Metallurgy. London. pp. 28.

Pingoud, A., Alves, J., Geiger, R. (1993). Restriction enzymes. In: Burell, M. M. (eds.) Enzymes of Molecular Biology. Humana Press Inc., Tatowa, New Jersey. pp. 107.

Pizarro, J. Jedlick, E., Orellana, O. Remero, J. & Espejo, R. T. (1996). Bacterial populations in samples of bioleached copper ore as revealed by DNA obtained before and after cultivation. *Appl. Environ. Microbiol.*, **62**, 1323-1328.

Pretorious, L. M., Rawlings, D. E. & Woods, D. R. (1986). Identification and cloning of *Thiobacillus ferrooxidans* structural nif genes in *Escherichia coli*. *Gene.* **45**, 59-65.

Pronk, J. T., De Bruyn, J. G., Bos, P. & Kuenen, J. G. (1992). Anaerobic growth of *Thiobacillus ferrooxidans*. *Appl. Environ. Microbiol.*, **58**, 2227-2230.

Pronk, J. T., Liem, K. Bos, P. & Kuenen, J. G. (1991a). Energy transduction by anaerobic ferric iron respiration in *Thiobacillus ferrooxidans*. *Appl. Environ. Microbiol.*, **57**, 2057-2062.

Pronk, J. T., Meijer, W. M., Hazeu, W., Van-Dijken, J. P., Bos, P. & Kuenen, J. G. (1991b). Growth of *Thiobacillus ferrooxidans* on formic acid. *Appl. Environ. Microbiol.*, **58**, 2057-2062.

Rawlings, D. E., Woods, D. R. & Mjoli, N. P. (1991). The cloning and structure of genes from the autotrophic biomining bacterium *Thiobacillus ferrooxidans*. *Advances in Gene Technology*. **2**, 215-237.

Rawlings, D. E. & Kusano, T. (1994). The molecular genetics of *Thiobacillus ferrooxidans*. *Microbiol. Rev.*, 58, 39-55.

Rawlings, D. E. (1995). Restriction enzyme analysis of 16SrRNA Genes for the rapid identification of *Thiobacillus ferrooxidans, Thiobacillus thiooxidans* and *Leptospirillum ferrooxidans* strains in leaching environments. In: Jerez, C. A., Vargas, T. Toledo, H. & Wiertz, J. V. (eds.) Biohydrometallurgical Processing. Proceedings of the International Biohydrometallurgy Symposium IBS-95 Vina del Mar, Chile. pp. 9-17.

Rawlings, D. E. & Silver, M. (1995). Mining with microbes. *Biotechnol.*, **3**, 773-778.

Rawlings, D. E. & Woods, D. R. (1995). Development of improved biomining bacteria. In: Gaylarde, C. C. & Videla, H. A. (eds.) Bioextraction and Biodeterioration of metals. (eds.) Cambridge University Press. Oxford. UK. pp. 63-84.

Razell, W. E. & Trussel, P. C. (1963). Isolation and properties of an iron oxidizing *Thiobacillus*. *J. Bacteriol.*, 85, 595-603.

Rodriguez, R. L. & Tait, R. C. (1983). Recombinant DNA Techniques An Introduction. The Benjamin/Cumming Publishing Company Inc., California, USA.

Rodriguez-Leiva, M. & Tributsch, H. (1988). Morphology of the bacterial leaching patterns by *Thiobacillus ferrooxidans* on synthetic pyrite. *Arch. Microbiol.*, **49**, 401-405.

Rothwell, N. V. (1988). Understanding Genetics. 4th. ed. Oxford University Press, New York. pp. 557-579.

Said, M. F. & Johnson, D. B. (1988). Copper tolerance in acidophilic microbial populations. In Norris, P. R. & Kelly, D. P. (eds). Biohydrometallurgy; Proceedings of the International Symposium. Warwick. Conventry, UK. pp. 561-562.

Sakaguchi, H., Torma, A. E. & Silver, M. (1976). Microbiological oxidation of synthetic chalcopyrite and covellite by *Thiobacillus ferrooxidans*. *Appl. Environ. Microbiol.*, **31**, 7-10.

Salle, A. J. (1967). Fundamental principles of bacteriology. 6th. ed. McGraw-Hill Inc., New York.

Sambrook, J., Fritsch, E. F. & Maniatis, T (1989). Molecular Cloning: a Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbour, New York.

Sand, W., Rohde, K., Sobotke, B. & Zenneck, C. (1992). Evolution of *Leptospirillum ferrooxidans* for leaching. *Appl. Environ. Microbiol.*, **58**, 85-92.

Schaeffer, W. I., Holert, P. E. & Umbreit, W. W. (1963). Attachment of *Thiobacillus thiooxidans* to sulphur crystals. *J. Bacteriol.*, **85**, 492-493.

Schrader, J. & Holmes, D. S. (1988). Phenotypic switching of *Thiobacillus ferrooxidans*. *J. Bacteriol.*, 170, 3915-3923.

Shiratori, T., Inoue, C., Sugawara, K., Kusano, T. & Kitagawa, Y. (1989). Cloning and expression of *Thiobacillus ferrooxidans* mercury resistance genes in Escherichia coli. *J. Bacteriol.*, 171, 3458-3464.

Shrihari, T., Kumar, R., Gandhi, K. S. & Natarajan, K. A. (1991). Role of cell attachment in leaching of chalcopyrite mineral by *Thiobacillus ferrooxidans*. *Appl. Microbiol. Biotechnol.*, 36, 278-282.

Silverman, M. P. (1967). Mechanism of bacterial pyrite oxidation. *J. Bacteriol.*, **94**, 1046-1051.

Silverman, M. P. & Lundgren, D. G. (1959). Studies on the chemoautotrophic iron bacterium *ferrobacillus ferrooxidans*: I, An improved medium and a harvesting procedure for securing high cell yields. *J. Bacteriol.*, **77,** 642-647.

Skoog, D. A., West, D. M. & Holler, F. J. (1992). Fundamentals of analytical chemistry 6th ed. Saunders Publishers, Inc., New York.

Smith, D. W., Flierman, C. B. & Brock, T. D. (1972). Technique for measuring $^{14}CO_2$ uptake by soil microorganism *in situ*. *Appl. Microbiol.*, **23,** 595.

Southern, E. (1979). Gel electrophoresis of restriction fragments. In Wu, R. ed. *Methods in enzymology* 68, Academic Press Inc., pp. 152-176.

Stainer, R. Y., Ingraham, J. L., Wheelis, M. L. & Painter, P. R. (1992). General microbiology, 5th ed. Macmillan Education Ltd. London.

Starkey, R. L. (1925). Concerning the physiology of *Thiobacillus thiooxidans* an autotrophic bacterium oxidizing sulfur under acidic conditions. *J. Bacteriol.*, **10,** 135-163.

Starkey, R. L. (1937). Formation of sulfide by some sulfur bacteria. *J. Bacteriol.*, **33,** 545-571.

Stoner, D. L., Browning, C. K., Bulmer, D. K., Ward, T. E., & MacDonell, M. T. (1996). Direct 5S rRNA assay for monitoring mixed-culture bioprocesses. *Appl. Environ. Microbiol.*, **62,** 1969-1976.

Sugio, T., White, K. J. Shute, E., Choate, D. & Blake. R. C. (1992). Existence of a hydrogen sulfide : ferric iron oxidoreductase in iron-oxidizing bacteria. *Appl. Environ. Microbiol.*, 58, 431-433.

Suzuki, I., Takeuchi, T. L., Yuthasastrakosol, T. D. & Oh, J. E. (1990). Ferrous iron and sulphur iron oxidation and ferric iron reduction activities of *Thiobacillus ferrooxidans* are affected by growth on ferrous iron, sulfur or a sulfide ore. *Appl. Environ. Microbiol.*, **56,** 1620-1626.

Swings, J., Kersters, K. & De Ley, J. (1976). Numerical analysis of electrophoretic patterns of Zymomonas strains. *J. Gen. Microbiol.*, **93,** 266-271.

Tang, Y., Procop, G. W. & Persing, D. H. (1997). Molecular diagnosis of infectious diseases. *Clin. Chem.* **43**, 2021-2038.

Teixaeira, M. C., Brandao, R. L., Barbi, S. N. & Nicoli, J. R. (1995)., Evidence for two arsenate resistance mechanisms in *Thiobacillus ferrooxidans. Rev. Microbiol.*, **26,** 16-21.

Temple, K. L. & Colmer, A. R. (1951). The autotrophic oxidation of iron by a new bacterium *Thiobacillus ferrooxidans. J. Bacteriol.*, **62,** 605-611.

Torma, A. E. (1985). Biohydrometallurgy as a emerging Technology. In: Ehrlich, H. L. & Holmes, D. S. (eds.) Biotechnology for the Mining, Metal-refining and Fossil Fuel Processing Industries. John Wiley & Sons Inc., New York. pp. 49-64.

Unz, F. & Lundgren, D. G. (1961). A comparative nutritional study of 3 chemoautotrophic bacteria: *Ferrobacillus ferrooxidans, Thiobacillus ferrooxidans. and Thiobacillus thiooxidans. Soil Sci.* **92**, 302-313.

Vandepitte, J., Engback, K., Piot, P. & Henk, C. C. (1991). Basic laboratory procedures in clinical bacteriology, W.H.O. Geneva, pp. 14-15.

Veldkamp, H. (1970). Enrichment cultures of prokaryotic organisms. In: Norris, J. R. & Ribbon, D. W. (eds.). Methods in Microbiology. Vol. 3A, Academic Press Inc., London. pp. 305-312.

Visca, P., Valetti, P., Bianchi, E., Cosentino, S. & Orsi, N. (1988). Comparative plasmid analysis in *Thiobacillus ferrooxidans* strains. In: Norris, P. R. & Kelly, D. P. (eds). Biohydrometallurgy; Proceedings of the International Symposium, Warwick, Conventry, UK. pp. 565-568.

Vishnac, W. V. (1975). Genus *Thiobacillus*. In: Buchanan, R. E. & Gibbions, N. E. (eds.) Bergey's manual of Determinative Bacteriology. 8th ed. Williams and Wilkins Company, Baltimore, USA. pp. 456-462.

Wakao, N., Konno, J., Sakurai, Y. & Shiota, H. (1990). Survival of freeze-dried *Thiobacillus ferrooxidans* NCIB 8455 after long-term storage *J. Gen.. Appl. Microbiol.*, **36**, 283-286.

Wakao, N., Panada, K., Takahashi, A., Sakurai, Y. & Shiota, H. (1991). Morphological, physiological and chemotaxonomical characteristics of iron and sulfur oxidizing bacteria isolated from acid mine drainage waters. *J. Gen. Appl. Microbiol.*, **37**, 35-48.

Waksman, S. A. & Joffe, J. S. (1922). Microorganisms concerned in the oxidation of sulfur in the soil. *J. Bacteriol.*, 7, 239-255.

Watson, J. D., Gilman, M., Witkowski, J. & Zoller, M. (1992). Recombinant DNA. 2nd ed. Scientific America Books Ltd. New York.

Weir, D. M. (1986). Application of Immunological Methods in Biomedical sciences. 4th ed. Blackwell Scientific Publications Oxford, UK.

Wilson, K. & Walker, J. M. (1995). Practical Biochemistry: Principles and techniques. 4th ed. Cambridge University Press, Cambridge, UK.

Yates, J. R., Lobos, J. H & Holmes, D S. (1986). The use of genetic probes to detect microorganisms in bioleaching operations. *J. Ind. Microbiol.*, **1**, 129-135.

APPENDICES

Appendix A

Composition and preparation of 9k liquid medium (after Silverman and Lundgren, 1959)

The composition and preparation of 9k liquid medium used in isolation and cultivation of biooxidizing bacteria is presented below.

Solution A

Ammonium sulphate	3.0g
Potassium chloride	0.1g
Dipotassium hydrogen phosphate	0.5g
Magnesium sulphate heptahydrate	0.5g
Calcium nitrate	0.01g
Distilled water	700ml

Solution B1 (energy substrate for ferrous iron oxidizers)

Iron (II) sulphate heptahydrate	14.74g
Sulphuric acid (5M)	400µl
Distilled water	100ml

Solution B2 (energy substrate for sulphur oxidizing bacteria; Unz and Lundgren, 1961)

Elemental sulphur	1.0g
Sulphuric acid (5M)	1.0ml
Distilled water	20ml

Solution A was sterilized by autoclaving for 15min at a temperature of 121°C and a pressure of 1.5 atmospheres. It was then filtered through a Whatman number 1 filter paper into a sterilized 1litre volumetric flask, cooled to room temperature and stored at 4°C until required.

Solution B1 was prepared by filter-sterilizing the dissolved constituents through a 0.45µm millipore filter (Millipore Co., Ltd, Bedford, Ireland) into a sterilized 100ml volumetric flask and stored at 4 C. To prepare 9k medium supplemented with ferrous iron (Fe^{2+}), 4 parts of solution A was added to 1 part solution B1 in a 500ml Erlenmeyer flask rinsed with 5M H_2SO_4. On the otherhand 9k medium supplemented with elemental sulphur (S^0) was prepared by adding 4 parts of solution A to 1 part solution B2 in an acid washed 500ml Erlenmeyer flask and sterilized by heating to 105 C in a water bath for 45min on two successive days (Harrison, 1984).

Appendix B

Composition and preparation of solid media

To prepare solid media, the following solutions were used:

Solution 1:

Sodium thiosulphate	0.5g
Distilled water	2.5ml

Solution 2:

Iron(II) sulphate heptahydrate ($FeSO_4 \cdot 7H_2O$)	0.5g
Distilled water	2.5ml
5M H_2SO_4	10µl

Solution 3:

Ammonium sulphate	1.125g
Potassium chloride	0.04g
Magnesium sulphate heptahydrate ($MgSO_4 \cdot 7H_2O$)	0.1875g
Distilled water	125ml

pH of solution was adjusted to 3.0 at room temperature (25-30°C) using dilute H_2SO_4.

Solution 4 (autoclaved):

Agar	2.5g
Distilled water	120ml

(a) Ferrous iron sodium thiosulphate agar (FSTA) (After. Peng *et al.*, 1994)

To prepare FSTA, solutions 1 and 2 were sterilized by filtration, separately, through 0.45µm millipore filters. Solutions 3 and 4 were each autoclaved for 30min at 121°C and the four solutions, at the volumes specified above, mixed together. 30ml of the resulting solution (final pH 3) were poured into 9cm diameter sterile petri dishes to set at room temperature and stored at 4°C until required.

(b) Preparation of ferrous agar (FA) plates

The amount of Iron (II) sulphate heptahydrate and sulphuric acid in solution 2 above was doubled and solution 1 eliminated. The procedure for preparation was the same as described in (a).

(c) Preparation of sodium thiosulphate agar (STA)

The amount of sodium thiosulphate and distilled water in solution 1 above was doubled and solution 2 eliminated. The procedure for preparation was the same as described (a).

(d) Preparation of ferrous agarose (FAR): Manning's Modified ISP 9k solid medium (Manning, 1975; Manning, 1982)

Solution 1

Ammonium sulphate	0.5g
Potassium chloride	0.02g
Magnesium sulphate heptahydrate	0.02g
Calcium nitrate	0.001g
Iron(II) sulphate heptahydrate ($FeSO_4.7H_2O$)	3.5g
Distilled water	50ml

Solution 1 (pH 2.5) was mixed and filter-sterilized through a 0.45µm millipore filter and then warmed to 45°C in a waterbath.

Solution 2 (autoclaved)

Agarose	0.5g
Distilled water	50ml

Solution 2 was autoclaved for 15min at 121°C and cooled to 45°C and solution 1 (at the same temperature) added. The resulting mixture was aseptically poured into 9cm diameter sterile petri dishes and left to set in a laminar flow cabinet and stored at 4°C until required.

Appendix C

Preparation of reagents for staining bacterial isolates; (after Salle, 1967 and Collin and Lyne, 1989)

(1) Crystal violet solution

Two grammes of crystal violet and 0.8g of ammonium oxalate were dissolved in 20ml of 95% ethanol and 80ml of distilled water respectively. The two solutions were mixed and left to stand for 24 hours before filtering into a dark bottle using Whatman number 1 filter paper and stored at room temperature.

(2) Iodine solution

Two grammes of potassium iodide and 1.0g of iodine were grounded together in a motar and dissolved in 20ml of distilled water. The solution was made up to 100ml with distilled water when the solutes have completely dissolved.

(3) Carbol fuschin (Cowan, 1985):

Solution A

Basic Fuschin	10g
Ethanol (95%)	100ml

This solution (A) was kept at 37°C overnight.

Solution B

Phenol	5g
Distilled water	100ml

To prepare stock Carbol fuschin stain, 10ml of solution A was added to 100ml of solution B. Carbol fuschin used in staining was prepared by diluting one volume of stock solution with two parts distilled water.

(4) Neutral red solution (Collin and Lyne, 1989):

Neutral red (0.1g) was dissolved in 100ml of distilled water and 0.2ml of 1%(v/v) acetic acid added. The prepared stain was kept at room temperature until use.

(5) Safranin stock solution

Safranin (2.5g) was dissolved in 95% ethanol to make a stock solution which was stored at room temperature. Dilute safranin solution used in staining was prepared by making 1 in 10 dilution of the stock.

Appendix D

Preparation of reagents for Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE);

Solution 1 (Monomer Solution)

Acrylamide	30.0g
N,N--methylene Bis-acrylamide (Bis-acrylamide)	0.8g
Distilled water	100ml.

The solution was filtered through a 0.22µm millipore filter membrane into a dark bottle.

Solution 2 (1.5M Trisaminomethane, pH 8.8)

Tris-aminomethane (Tris)	18.16g
Distilled water	100ml

The pH of solution 2 which was used in preparing the resolution gel was adjusted to pH 8.8 with dilute HCl.

Solution 3 (0.5M Tris HCl, pH 6.8)

Tris-aminomethane (Tris)	4.56g
Distilled water	100ml

The pH of solution 2 which was used in preparing the stacking gel buffer was adjusted to pH 6.8 with dilute HCl.

Solution 4 (acrylamide gel polymerization initiator)

Ammonium persulphate (APS) stock solution (10%)was always prepared fresh before use.

Solution 5 (catalyst)

N, N ,N',-N'-tetramethylethylenediamine (TEMED).

Solution 6 (running buffer) [X10 stock pH 8.3]

Tris-aminomethane (Tris)	3.03g
Glycine	14.4g
SDS	1g [0.1%(w/v)]
Distilled water	1000ml

The pH of Solution 6 was adjusted to pH 8.3 with dilute HCl and a working stock prepared by diluting the X10 stock to obtain a X1 solution.

Solution 7 [20% stock Sodium Dodecyl Sulphate (SDS)]

SDS (20g) was dissolved in 100ml distilled water in a 100ml bottle and stored at room temperature.

Constituents of resolution and stacking gels

Solution A: (15% resolution gel):

30%(w/v) acrylamide	
0.8%(w/v) bis-acrylamide	4.25ml
1.5M Tris-HCl pH 8.8	2.125ml
20%(w/v) SDS	42.5µl
10%(w/v) APS	33.5µl
TEMED	11.3µl
Distilled water	2.055ml

Total volume	8.5ml

Solution B (20% resolution gel)

30%(w/v) acrylamide	
0.8%(w/v) bis-acrylamide	5.667ml
1.5M Tris-HCl pH 8.8	2.125ml
20%(w/v) SDS	42.5µl
10%(w/v) APS	33.5µl
TEMED	11.3µl
Distilled water	663.7µl
Total volume	8.5ml

Solution C (3% Stacking gel)

30%(w/v) acrylamide	
0.8%(w/v) bis-acrylamide	0.4ml
0.5M Tris-HCl pH 6.8	1ml
20%(w/v) SDS	20.0µl
10%(w/v) APS	32.8µl
TEMED	6.6µl
Distilled water	2.560ml
Total volume	**4.0ml**

Standard molecular weight markers

Low molecular weight standard markers (Sigma Chemical Company, USA.) used were; Albumin from bovine (66 kDa), Albumin from egg (45 kDa), Glyceraldehyde-3-phosphate Dehydrogenase from rabbit muscle (36 kDa), Carbonic anhydrase from bovine erythrocytes (29 kDa), Trypsinogen from bovine pancreas (24 kDa), Trypsin inhibitor from soybean (20 kDa) and -Lactalbumin from bovine milk (14.2 kDa).

Appendix E

Preparation of reagents for isolation and purification of DNA

(1) 0.5M EDTA stock solution pH 8.0

Disodium ethylene diamine tetra-acetate (9.306g) was added to 40ml of distilled water and vigorously stirred whilst the pH was adjusted to 8.0 by the addition of NaOH crystals. The volume was then made up to 50ml with distilled water and the solution aliquoted and sterilized by autoclaving at 121°C for 30minutes.

(2) 10% (w/v) sodium dodecyl sulphate (SDS)

SDS (10g) was rapidly dissolved in 90ml of distilled water by heating to 68°C. The pH was adjusted to 7.2 by the addition of a few drops of concentrated HCl and the volume made up to 100ml with distilled water.

(3) 5M Potassium acetate

Potassium acetate (24.53g) was dissolved in 50ml of distilled water and the solution kept at -20°C.

(4) Potassium acetate (5M)

5M potassium acetate solution (10ml) was mixed with 11.5ml glacial acetic acid and made up to 100ml with distilled water. The solution was stored at 4°C.

(5) 1M Tris-HCl stock solution pH 7.6

Tris base (12.11g) was dissolved in 70ml of distilled water. The pH was adjusted to 7.6 by the addition of concentrated HCl. The solution was allowed to cool to room temperature before final

adjustment to the pH was made. The volume was then made to 100ml with distilled water. The solution was dispensed into aliquots and sterilized by autoclaving.

(6) TE buffer (pH 7.6) (10mM Tris-Cl, pH 7.6: 1mM EDTA, pH 8.0)

This buffer was prepared by diluting 10ml of the EDTA stock solution and 10ml of 1M Tris-Cl pH 7.6 and making up the volume to 500ml with distilled water. The solution was dispensed into aliquots, autoclaved (121°C for 15minutes) and kept at room temperature.

(7) 3M sodium acetate (pH 5.2)

Sodium acetate.3H$_2$O (40.81g) was dissolved in 80ml of water and pH of the solution adjusted to 5.2 with glacial acetic acid. The volume of the solution is then made up with distilled water, dispensed into aliquots and sterilized by autoclaving.

(8) Phenol chloroform

An equal amount of phenol and chloroform were mixed and equilibrated by extracting the mixture several times with 0.1M Tris-Cl (pH 7.6). This solution was under an equal volume of 0.01M Tris-Cl (pH 7.6) and stored in a dark bottle at 4°C.

(9) 10% NaOH

NaOH (4g) was dissolved in 100ml distilled water, sterilzed by autoclaving then stored at room temperature.

Appendix F

Composition and preparation of reagents for restriction endonuclease digestion;

(1) Restriction enzyme stock solutions

Restriction Endonuclease	Prokaryotic source	**Recognition sequence**	**Activity (units/µl)**
Hind III	*Haemophilus influenzae Rd*	5'-A/AGCTT-3'	10
Bam HI	*Bacillus amyloliquefaciens*	5'-G/GATCC-3'	10
Pst I	*Providencia stuartii*	5'-CTGCA G-3'	12
Eco RI	*Escherichia coli*	5'-G/AATTC-3'	10
Sal I	*Streptomyces albus G*	5'-GTCGA/C-3'	10

The enzymes were stored at -20 C. The enzymes were obtained from Gibco BRL.

(2) Restriction Enzymes Digestion Buffers

The buffers were supplied by the manufacturers, each enzyme has its specific buffer and at concentrations that were 10% the final concentration used. The buffers were stored at -20°C.

(3) Gel - loading buffer (5 X Orange G)

20% (w/v) Ficoll, 25mM EDTA, 2.5% (w/v) orange G, stored at room temperature. Final concentration used in DNA samples is 1X.

Appendix G

***Reagents** for agarose gel electrophoresis*

(1) Electrophoresis buffer, stock solution; 50X Tris acetate (TAE)

This solution consists of 242g Tris base, 57.1ml glacial acetic acid, 100ml 0.5ml EDTA, with pH adjusted to 7.7 (with glacial acetic acid) in 1 litre water. Working solution was 1X TAE.

(2) Ethidium bromide (10mg/ml)

Ethidium bromide powder (0.1g) was added to 10ml of distilled water. The solution was then stored in a container wrapped in aluminium foil and stored at room temperature.

(3) Nucleic acid markers [Lambda DNA/Hind III Digest (Gibco BRL)]

The sizes (bp) of the fragments are as follows; 23,130, 9,416, 6,557, 4,361, 2,322, 2,027, 564, and 125.

Appendix H