Isolation and Characterization of Acidophilic sulphur and iron oxidizing *Acidithiobacillus thiooxidans* and *Acidithiobacillus ferrooxidans* from black shale

By

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Isolation and Characterization of Acidophilic sulphur and iron oxidizing *Acidithiobacillus thiooxidans* and *Acidithiobacillus ferrooxidans* from black shale

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Microbiology

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DECLARATION

The material contained in this thesis is my original work and I have not presented any part of this work elsewhere for any other degree.

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CERTIFICATE

This thesis, submitted by **Shahroz khan** is accepted in its present form by the Department of Microbiology, Faculty of Biological Sciences, Quaid-i-Azam University Islamabad, as fulfilling the thesis requirement for the degree of **Master of Philosophy** in Microbiology.

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.....To My Parents.

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LIST OF ABBREVIATONS

At. ferrooxidans	*Acidithiobacillus ferrooxidans*
$(NH_4)_2SO_4$	Ammonium sulfate
µL	Micro litre
µm	Micrometre
At. thiooxidans	*Acidithiobacillus thiooxidans*
ATCC	American type culture colonies
Ca $(NO_3)_2$	Calcium Nitrate
$CaCO_3$	Calcium carbonate
CO_2	Carbon dioxide
Cu	Copper
$CuFeS_2$	Copper iron sulfate
CuS	Copper sulfate
Cyt	Cytochrome
EPS	Exopolysaccharide
Fe^{2+}	Ferrous
Fe^{3+}	Ferric
FeS_2	Ferous sulfate
$FeSO_4.7H_2O$	Iron sulfate heptahydrated
g/l	Gram per Litre
Gel$^+$	Gelrite growing strain
Glu$^+$	Glucose growing strain
H	Hydrogen
H_2O	Dihydrogen oxide
H_2O_2	Hydrogen peroxide
H_2S	Hydrogen sulfide
H_2SO_4	Sulfuric acid
K_2HPO_4	Dipotassium hydrogen phosphate
KCl	Potassium chloride
L	Litre

MeS_2	**Metal sulfide**
Mg	**Magnesium**
$MgSO_4.7H_2O$	**Magnesium sulfate heptahydrogen**
mL	**Millilitre**
mm	**Millimetre**
$Na_2S_2O_3.H_2O$	**Sodium thiosulfate**
NADH	**Nicotinamide dihydrogen**
Ni	**Nickl**
O_2	**Molecular oxygen**
PbS	**Lead sulfide**
rpm	**Rotation per minute**
S	**Sulphur**
S°	**Elemental sulphur**
SDO	**sulfur dioxygenase**
SO_4^{2-}	**Sulfate**
SOR	**sulfite acceptor oxidoreductase**
Thio$^+$	**Thiosulfate growing strain**
U	**Uranium**
V	**Vanadium**
w/v	**Weight per volume**
ZnS	**Zinc sulfate**

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Shahroz Khan Yousafzai

Abstract

Although much of the research work has been done on *Acidithiobacillus thiooxidans* and *At. ferrooxidans* in the rest of the world, unfortunately significant study has not been reported in Pakistan. The principal objective of the present investigations was to isolate and characterize the acidiphilic sulphur and iron oxidizing *Acidithiobacillus thiooxidans* and *Acidithiobacillus ferrooxidans* bacteria from black shale and to grow the isolates on solid and liquid medium. The indigenous strains of *At. thiooxidans* and *At. ferrooxidans* were isolated from the black shale, Tarbela (Ghazi) and water samples from Chamyari. These strains were found Gram-negative, motile, and rod-shaped bacteria. *At. ferrooxidans* oxidized iron and reduced sulfur compounds like thiosulfate and tetrathionate, otherwise *At. thiooxidans* were also grown on glucose medium. The bacterial oxidation of pyrite and reduced sulfur compounds produced sulfuric acid in shake flasks bioleaching which followed a drop in initial pH value of the medium. *At. thiooxidans* oxidized only sulfur and reduced sulfur compounds to sulfuric acid through its metabolic activity. Different biochemical activities like starch hydolysis, gelatin hydrolysis, hydrogen sulfide production, catalase reaction, urease test, indole production, methyl red test, voges proskauer, citrate utilization and triple sugar iron tests of the isolates were performed and picturized. Effects of carbon sources like glucose, sucrose, fructose, raffinose, d-sorbitol, galactose, lactose, maltose, rahammanose and mannose, were studied to check the growth of isolates. Glucose and fructose were found to be the best carbon sources for the growth of isolates.

INTRODUCTION

Introduction

The ability of *Acidithiobacillus ferrooxidans* to oxidize iron (II) plays an important role in the recovery of metals from low-grade sulphide ores. In addition to iron oxidation, this autotrophic bacterium catalyses the oxidation of sulphur compounds to sulphuric acid (Tuovinen *et al.*, 1996). Several studies have been carried out regarding *At. ferrooxidans* cell growth using inocula taken from media containing the same or another energy source. Different studies on the rate of iron (II) oxidation by sulphur grown cells have reported contradictory results. (Sugio *et al.*, 1996). The acidiphilic microorganisms that take part in dissolution of metals from the sulfide ores are autotrophic in nature. They can grow in inorganic medium having low pH values. They can tolerate high metal ion concentrations. The two main functions of this type of bacteria are oxidation of Fe^{2+} to Fe^{3+} and S to H_2SO_4. The acidiphilic microorganisms that actively take part in oxidizing Fe (II) to Fe (III) and S to H_2SO_4 are *Acidithiobacillus. Acidithiobacillus* species are rod-shaped, gram-negative, non-spore forming and mesophilic except the thermophilic *Acidithiobacilli*, which can grow at a higher temperature (Buchanan *et al.*, 1974).

Characteristics of mineral degrading bacteria

The most important microbes involved in the biooxidation of minerals are those that are responsible for producing the ferric iron and sulfuric acid required for the bioleaching reactions. These are the iron and sulfur oxidizing chemolithrophic bacteria and archaea (Rawling *et al.*, 2000). Irrespective of the type of process or temperature at which they are employed, these microbes have a number of features in common that make them especially suitable for their role in mineral solubilization. Four of the most important characteristics are;

a) They grow autotrophically by fixing CO_2 from the atmosphere.

b) They obtain their energy by using either ferrous iron or reduced inorganic sulfur compounds (some use both) as an electron donor, and generally use oxygen as the electron acceptor.

c) They are acidophiles and grow in low pH environments (pH.1.4 to 1.6 is typical).

d) They are remarkably tolerant to a wide range of metal ions (Dopson *et al.*, 2003).

The modest nutritional requirements of these organisms are provided by the aeration of an iron- and/or sulfur-containing mineral suspension in water or the irrigation of a heap. Small quantities of inorganic fertilizer can be added to ensure that nitrogen, phosphate, potassium and trace element limitation does not occur. A further advantageous characteristic of mineral biooxidation operations is that they are usually not subject to contamination by unwanted microorganisms. In the case of continuous-flow tank leaching processes, the continual wash-out of mineral together with their attached microbes as well as the organisms in suspension provides strong selection for improved microorganisms (Rawlings *et al.*, 2005).

Mesophiles are those microorganisms which grow at a prevailing room temperature, i.e. 28–37^{0}C. Among the mesophiles, the most popular and widely used strain is *Acidithiobacillus ferrooxidans* (Bhattacharya *et al.*, 1990). Although many strains of *Acidithiobacillus ferrooxidans* have been isolated from different sources, most of the strains showed the following optimum growth conditions, i.e. pH 1.5–2.5 and a temperature range of 28–37^{0}C (Ahonen *et al.*, 1989). *At. ferrooxidans* being a lithotroph derives energy for its growth by oxidizing Fe^{2+} to Fe^{3+} and sulfur, sulfide and different oxyanion of sulfur to sulfate. Bioleaching, a general term refers to the conversion of an insoluble metal *(e.g.* CuS) into a soluble form (usually SO_4^{2-}) by biological oxidation and by applying microbes (Rawlings *et al.*, 2002). Metals for which this technique is mainly employed for recovery includes, copper, cobalt, nickel, iron, sulphur, zinc and uranium. For recovery of gold and silver the activity of leaching bacteria is applied only to remove interfering metal sulfides from ores bearing the precious metals prior to cyanidation treatment (Rohwerder *et al.*, 2003).

The application of bacterial leaching to metal recovery from mineral ores has progressed steadily in the last 20 years (Olson *et al.*, 2003).

Bioleaching refers to the mobilization of metal ions from insoluble ores by biological oxidation and complexation (Rohwerder *et al.*, 2003). The application of bacterial leaching to metal recovery from mineral ores has progressed steadily in the last 20 years (Olson *et al.*, 2003).

It is assumed that heterotrophic leaching should provides better extraction of metals ions from organometallic compounds in shale ores. Bioleaching is carried out by

astonishing diverse groups of bacteria. At least 11 putative prokaryote divisions can be related to this phenomenon (Rohwerder *et al.*, 2003). The most common microorganisms belong to the genera *Acidithiobacillus* and *Leptosprillium* which are mesophile, acidiphilic and chemolithoautotrophe. They obtain energy from oxidation of either ferrous ion to ferric or reduction of sulfur compounds to sulfuric acid (Rawlings *et al.*, 2003).

Acidithiobacillus

Scientific classification

Kingdom:	Eubacteria
Phylum:	Proteobacteria
Class:	Gamma Proteobacteria
Order:	*Acidithiobacillales*
Family:	*Acidithiobacillaceae*
Genus:	*Acidithiobacillus*

Species

Acidithiobacillus ferrooxidans
Acidithiobacillus thiooxidans
Acidithiobacillus albertensis
Acidithiobacillus caldus
Acidithiobacillus cuprithermicus

Acidithiobacillus is a genus of proteobacteria. The members of this genus used to belong to *Thiobacillus*, before they were reclassified in the year 2000.

- *Acidithiobacillus ferrooxidans* (syn. *Thiobacillus ferrooxidans*) lives in pyrite deposits, metabolizing iron and sulfur and producing sulfuric acid.
- *Acidithiobacillus thiooxidans* (syn. *Thiobacillus thiooxidans*) consumes sulfur and produces sulfuric acid. Though first isolated from the soil. It has also been observed corroding concrete sewer pipes, altering hydrogen sulfide sewage gas into sulfuric acid.

Both of these bacteria are used in a mining technique called bioleaching whereby metals are extracted from their ores through oxidation. The bacteria are used as catalysts (Kelly *et al.*, 2000).

Thinly bedded shale that is rich in carbon, sulfide, and organic material formed by anaerobic decay of organic matter. A dark, thinly laminated carbonaceous shale,

exceptionally rich in organic matter (5% or more carbon content) and sulfide (esp. iron sulfide, usually pyrite), and more commonly containing unusual concentrations of certain trace elements (U, V, Cu, Ni). Fossil organisms (principally planktonic and nektonic forms) are commonly preserved as a graphitic or carbonaceous film or as pyrite replacements. Shale (also called mudstone) is a fine grained sedimentary rock whose original constituents were clay minerals or muds. It is characterized by thin laminae breaking with an irregular curving fracture, often splintery and usually parallel to the often indistinguishable bedding plane. This property is called fissility. Non-fissile rocks of similar composition but made of particles smaller than 1/16 mm are described as mudstones. Rocks with similar particle sizes but with less clay and therefore grittier are siltstones. Shale is the most common sedimentary rock. The process in the rock cycle which forms shale is compaction. The fine particles that compose shale can remain in water long after the larger and denser particles of sand have deposited. Shales are typically deposited in very slow moving water and are often found in lake and lagoonal deposits, in river deltas, on floodplains and offshore of beach sands. They can also be deposited on the continental shelf, in relatively deep, quiet water. Black shales are dark, as a result of being especially rich in unoxidized carbon. Common in some Paleozoic and Mesozoic strata, black shales were deposited in anoxic, reducing environments, such as in stagnant water columns. Fossils, animal tracks/burrows and even raindrop impact craters are sometimes preserved on shale bedding surfaces. Shales may also contain concretions (Blatt *et al.*, 1996).

Methods

Heap leaching is the most common method for bioleaching and is mainly used for secondary copper ores. Stirred tank leaching is used for refractory gold concentrates where gold is locked into the pyrite/arsenopyrite matrix. As the microbes do not necessarily need to contact the valuable metal bearing material that is bioleached, they can be physically separated from it (Dixon *et al.*, 2003).

Direct bioleaching

The microbes are kept together with the valuable metal-bearing material.

$$MeS_2 + H_2O + 3.5O_2 \xrightarrow{\text{Bacteria}} Me^{2+} + 2SO_4^{2-} + 2H^+ \qquad (1)$$

During direct bioleaching, bacteria directly interact with minerals and enhance the rate of mineral dissolution above the rate of chemical leaching driven by Fe^{3+} (e.g. equation (1).

Indirect bioleaching

The microbes are kept in a pond external to the valuable metal-bearing material and provide the leaching chemicals at a distance.

$$14Fe^{2+} + 3.5O_2 + 14H^+ \xrightarrow{\text{Bacteria}} 14Fe^{3+} + 7H_2O \quad (2)$$

$$MeS_2 + 8H_2O + 14Fe^{3+} \rightarrow Me2^+ + 14Fe^{2+} + 2SO_4^{2-} + 16H^+ \quad (3)$$

The reaction represented by equation (2) requires the participation of bacteria, where the equation (3) is a purely chemical process. Since Fe^{2+} produced in the reaction of (3) is recycled to equation (2), highly amount of heavy metals can be continuously from solid substrate. Which mechanism is dominant between the two remains controversial, but a recent experiment showed that the indirect mechanism might play a critical role in the microbial leaching process (Kim *et al.*, 2005).

Bioleaching involves abiotic and biotic reactions, often with different physicochemical requirements. Indirect bioleaching is a way of satisfying the requirements independently by separating the biotic and abiotic reactions. In direct bioleaching the challenge is to select microbes whose living conditions are as closabiotic leaching reactions as possible.

Leaching reaction means leaching mechanism which is done by these Microorganisms.

Mechanism of Bacterial Bioleaching

Metal leaching is now recognized as being mainly a chemical process in which ferric iron and protons are responsible for carrying out the leaching reactions. The role of the microorganisms is to generate the leaching chemicals and to create the space in which the leaching reactions take place. Microorganisms typically form an exopolysaccharide (EPS) layer when they adhere to the surface of a mineral (Sand *et al.*, 1995).

But not when growing as planktonic cells. It is within this EPS layer rather than in the bulk solution that the biooxidation reactions take place most rapidly and efficiently and therefore the EPS serves as the reaction space (Rohwerder *et al.*, 2003).

The mineral dissolution reaction is not identical for all metal sulfides and the oxidation of different metal sulfides proceeds via different intermediates (Rawlings *et al.*, 2003). Briefly, a thiosulfate mechanism has been proposed for the oxidation of acid insoluble metal sulfides such as pyrite (FeS_2) and molybdenite (MoS_2), and a polysulfide mechanism for acid soluble metal sulfides such as sphalerite (ZnS), chalcopyrite ($CuFeS_2$) or galena (PbS). In the thiosulfate mechanism, solubilization is through ferric iron attack on the acid-insoluble metal sulfides with thiosufate being the main intermediate and sulfate the main end-product. Using pyrite as an example of a mineral, the reactions may be represented as:

$$FeS_2 + 6\ Fe^{3+} + 3\ H_2O \rightarrow S_2O_3^{2-} + 7\ Fe^{2+} + 6\ H^+ \quad (1)$$

$$S_2O_3^{2-} + 8\ Fe^{3+} + 5\ H_2O \rightarrow 2\ SO_4^{2-} + 8\ Fe^{2+} + 10\ H^+ \quad (2)$$

In the case of the polysulfide mechanism, solubilization of the acid-soluble metal sulfide is through a combined attack by ferric iron and protons, with elemental sulfur as the main intermediate. This elemental sulfur is relatively stable but may be oxidized to sulfate by sulfur oxidizing microbes such as *Acidithiobacillus thiooxidans* or *Acidithiobacillus caldus* (reaction 5 below).

$$MS + Fe^{3+} + H^+ \rightarrow M^{2+} + 0.5\ H_2S_n + Fe^{2+}\ (n \geq 2) \quad (3)$$

$$0.5\ H_2S_n + Fe^{3+} \rightarrow 0.125\ S_8 + Fe^{2+} + H^+ \quad (4)$$

$$0.125S_8 + 1.5O_2 + H_2O \rightarrow SO_4^{2-} + 2H^+ \quad (5)$$

The ferrous iron produced in reactions (1) to (4) may be reoxidized to ferric iron by iron oxidizing microorganisms such as *Acidithiobacillus ferrooxidans* or bacteria of the genera *Leptospirillum* or *Sulfobacillus*.

$$2Fe^{2+} + 0.5O_2 + 2H^+ \rightarrow 2Fe^{3+} + H_2O \quad (6)$$

The role of the microorganisms in the solubilization of metal sulfides is, therefore, to provide sulfuric acid (reaction 5) for a proton attack and to keep the iron in the

oxidized ferric state (reaction 6) for an oxidative attack on the mineral (Rawlings *et al.*, 2003).

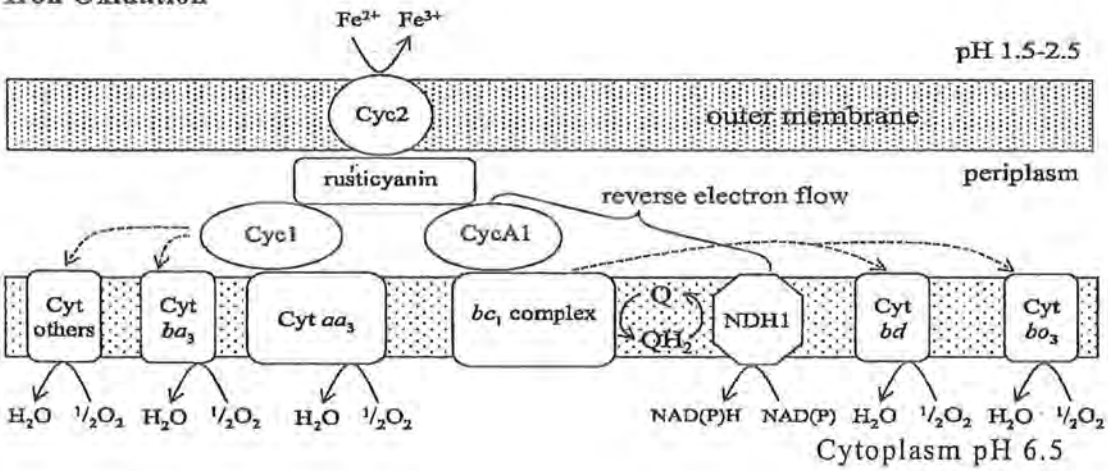
Mineral sulfide oxidation pathways

Acid soluble sulfides and acid stable sulfides are highly reduced compounds. The type of sulfide mineral will affect by which mechanism the oxidation will proceed.

- Acid-soluble metal sulfides are leached by both of the leaching chemicals Fe^{3+} and H^+ via the polysulfide pathway.
- Acid-nonsoluble metal sulfides are leached by the leaching chemical Fe^{3+} alone via the thiosulfate pathway.

This difference in mechanisms explains why sulfur oxidizers are able to leach some minerals but not others (Brassuer *et al.*, 2004).

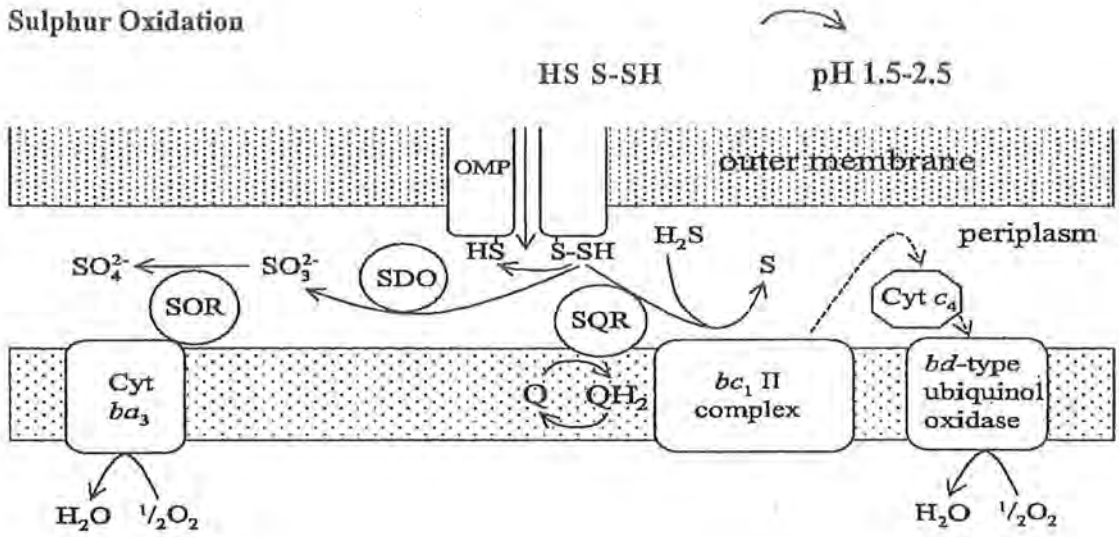
Iron Oxidation



Model Figure. 1.

Model of the iron oxidation electron transport pathway of *At. ferrooxidans* based partly on references below. Electrons are transferred from the membrane located cytochrome *c* 2, rusticyanin and then along one of two paths. The downhill path is via cytochrome *c*4 (Cyt1) to cytochrome *aa*3 or the uphill, reverse electron transport path via cytochrome *c*4 (CytA1) to a *bc*1 I complex and a NADH-Q oxidoreductase. *At. ferrooxidans* has up to twelve cytochromes *c* and a variety of cytochrome oxidizes some of which appear to play different roles depending on whether iron or sulfur is being oxidized. The NADH is responsible for mercury reduction using a MerA mercuric reductase and the cytochrome *aa*3 is required to reduce mercury via the

unique iron dependent mechanism discovered in *At. Ferrooxidans* (Brassuer *et al.*, 2004).



A Figure 2

A composite model of sulfur oxidation electron transport pathway of *At. ferrooxidans* based on references below. Thiol groups of outer membrane proteins are believed to transport the sulfur to the periplasm where it is oxidized by a periplasmic sulfur dioxygenase (SDO) to sulfite and a sulfite acceptor oxidoreductase (SOR) to sulfate. Although other cytochrome oxidases are present, a *ba*3 cytochrome oxidase and a *bc*1 II complex together with a *bd*-type ubiquinol oxidase are believed to play the major roles during sulfur oxidation. Rusticyanin and an iron oxidizing protein (not shown) might also be involved during sulfur oxidation but their exact role is still to be determined (Brassuer *et al.*, 2004).

Contact, non-contact and cooperative leaching

Bioleaching microbes interact with the metal-containing material by direct contact or by affecting the water-solution holding the metal-containing material.

- Non-contact leaching: Free microbes produce the leaching chemicals Fe^{3+} and H^+.
- Contact leaching: Attached microbes produce the leaching chemicals Fe^{3+} and H^+.

Acid-insoluble sulfides are attacked by ferric iron produced by iron oxidizing bacteria and archaea whether they are attached to the mineral surface or in the liquid phase, where ferric iron oxidises the mineral following transport by diffusion of mass action.

Bioleaching compared with other extraction techniques

Traditional extractions involve many expensive steps such as roasting and smelting, which require sufficient concentrations of elements in ores and are environmentally unfriendly. Low concentrations are not a problem for bacteria because they simply ignore the waste which surrounds the metals, attaining extraction yields of over 90% in some cases. These microorganisms actually gain energy by breaking down minerals into their constituent elements. The company simply collects the ions out of the solution after the bacteria have finished (Brandl *et al.*, 2001).

Some advantages associated with bioleaching are

- **Economical**

Bioleaching is generally simpler and therefore cheaper to operate and maintain than traditional processes, since fewer specialists are needed to operate complex chemical plants.

- **Environmental**

The process is more environmentally friendly than traditional extraction methods. For the company this can translate into profit, since the necessary limiting of sulfur dioxide emissions during smelting is expensive. Less landscape damage occurs, since the bacteria involved grow naturally, and the mine and surrounding area can be left relatively untouched. As the bacteria breed in the conditions of the mine, they are easily cultivated and recycled.

- *Ores* and concentrates of lower metal concentration can be treated economically. Therefore the concentrating process can stop earlier, before the concentrate is sent for leaching. This means that loss of metal value during concentration is avoided.
- *"Difficult"*- refractory concentrates can be processed.
- Concentrates with containments like arsenic, bismuth and magnesia are often expensive to treat in conventional metal-production. Mining companies often

have to pay penalties for these difficult-to-treat contaminants when they sell concentrate to a smelter.

- *The arsenic* in the concentrates can be removed in an environmentally stable form.
- *Possible* to make use of existing plant capacities once the oxidic ore cap has been mined out.
- *Economic exploitation* of smaller deposits, in remote locations, becomes viable because of reduced infrastructural costs.
- *Rapid start-up*. Easy and reliable process when it comes to maintenance.
- *The process* takes place at atmospheric pressure and low temperatures.
- *Water-based* process means less dust.
- *No emissions* of sulfur dioxide. Therefore, purification of smoke gas and sulfuric acid plants are superfluous.

Despite the advantages with bioleaching it is not always easy to choose among the different methods of metal extraction in order to explore a potential mine. The Techno-Economic factors of a resource need to be evaluated from case to case (Rawlings *et al.*, 2002).

This procedure was introduced in studies on leaching of polymetallic shale ore, called Polkowice black shale. Tube bioreactors designed by our research team were used in these bioleaching experiments (Farbiszewska *et al.*, 2006).

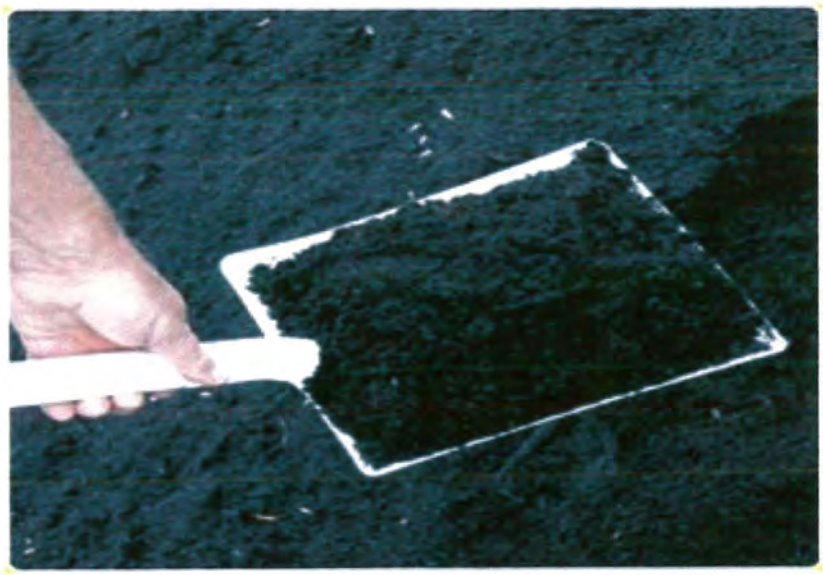


Fig. 3. Black shale in soil form

Fig. 4. Electron microscopic Picture of iron oxidizing *Acidithiobacillus ferrooxidans*

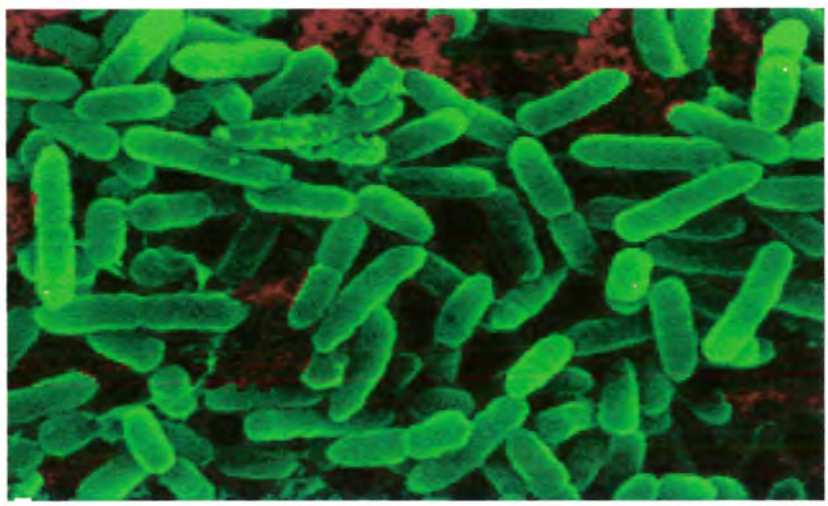


Fig. 5.Electon microscope picture of sulfur oxiziding *Acidithiobacillus thiooxidans*

Aims and Objectives

The aims and objectives of these researches were

- Isolation of *At. ferroxidans* and *At. thioxidans* from black shale.
- Evaluate bioleachability of Polkowice black shale ore.
- Establish reliable lab scale pilot operations of process.
- Optimize configuration and settings.

LITERATURE REVIEW

Review of Literature

Black shales are dark, as a result of being especially rich in unoxidized carbon. Common in some Paleozoic and Mesozoic strata, black shales were deposited in anoxic, reducing environments, such as in stagnant water columns. Fossils, animal tracks/burrows and even raindrop impact craters are sometimes preserved on shale bedding surfaces. Shales may also contain concretions (Blatt *et al.*, 1996). The black shale present in Polish ores (Lubin region) differs from others in mineralogical and chemical properties and in susceptibility to enrichment. The ores are characterized by high content of copper and others metals like silver. Some metals in the shale ore are present as bituminous organo-metallic compounds such as porphyrinus. The presence of these compounds reduces metals recovery by classical acid bioleaching methods. The bioleaching process was carried out in neutral medium using *Bacillus cereus* and *Bacillus amyloliquefaciens* strains. The progress of process was followed by analysis of copper, zinc and nickel bioleaching kinetics. The bioleaching experiments with copper suggest different organic forms of this metal in the shale ore. Some of them are highly susceptible to bioleaching by heterotrophic bacteria. Organic compounds of the nickel appeared to be more resistant to bioleaching than copper compounds. Bioleaching of zinc was negligible. Also, the bioleaching process was carried out on a batch scale using "Biomel" reactor (kiczma *et al.*, 2004).

Acidophilic properties

From an industrial perspective it is essential that biomining microorganisms are able to grow at low pH and tolerate high concentrations of acid. Two important reasons for this are to enable iron cycling and to permit reverse electron transport to take place. A low pH is required for the iron cycle whereby ferrous iron serves as an electron donor under aerobic conditions and ferric iron as an energetically favourable alternate electron acceptor if the concentration of oxygen falls. This has been described above. Ferric iron is almost insoluble at a neutral pH, whereas in acid solutions its solubility is increased. The possibility of using ferric iron as an alternate electron acceptor is therefore readily available to acidophiles but less available to aerobic neutrophiles or moderate acidophiles

because ferric iron is almost totally insoluble in neutral, aerobic environments. The external pH of the environment in which extreme acidophiles such as biomining microbes grow is low (e.g. pH 1.0–2.0), whereas the internal cellular pH remains close to neutral (Cox *et al.*, 1979). This difference results in a steep pH gradient across the cell membrane. This pH gradient is important for nutritional purposes, especially when using a weak reductant such as ferrous iron as an electron donor. Autotrophic organisms have a high requirement for compounds such as NAD(P)H to reduce their carbon source (CO_2) to produce the sugars, nucleotides, amino acids and other molecules from which new cell mass is synthesized. Heterotrophic bacteria do not have as high a demand for NAD(P)H as their carbon source is more reduced than CO_2 and hydrogen atoms removed from their source of nutrition may be used to satisfy their lower NAD(P)H requirement. Chemolithotrophic autotrophs require a large transmembrane proton gradient to generate the required proton motive force to energise the synthesis of NAD (P) H. This process is known as reverse electron transport or the 'uphill' electron transfer pathway (Brassuer *et al.*, 2002). Although this phenomenon has not been studied in many iron or sulfuroxidizing chemolithotrophs, strong evidence has been presented that when grown on iron, *At. ferrooxdians* contains a unique cytochrome bc1 complex that functions differently from the bc1 complex used during the oxidation of sulfur and is specifically involved in the 'uphill' pathway (Elbehti *et al.*, 2000). One way of viewing this is that growth in acid solutions is a nutritional necessity as a large transmembrane pH gradient is required to produce the hydrogen atoms needed to reduce CO_2 to cell mass (Doglous *et al.*, 2005).

Microorganisms are used in large scale heap or tank aeration processes for the commercial extraction of a variety of metals from their ores or concentrates. These include copper, cobalt, sulphur, iron, gold and, in the past, uranium. The metal solubilization processes are considered to be largely chemical with the microorganisms providing the chemicals and the space (exopolysaccharide layer) where the mineral dissolution reactions occur. Temperatures at which these processes are carried out can vary from ambient to 80°C and the types of organisms present depends to a large extent on the process temperature used. Irrespective of the operation temperature, biomining

microbes have several characteristics in common. One shared characteristic is their ability to produce the ferric iron and sulfuric acid required to degrade the mineral and facilitate metal recovery. Other characteristics are their ability to grow autotrophically, their acid-tolerance and their inherent metal resistance or ability to acquire metal resistance.

Although the microorganisms that drive the process have the above properties in common, biomining microbes usually occur, in consortia in which cross feeding may occur such that a combination of microbes, including some with heterotrophic tendencies may contribute to the efficiency of the process. The remarkable adaptability of these organisms is assisted by several of the processes being continuous flow systems that enable the continual selection of microorganisms that are more efficient at mineral degradation. Adaptability is also assisted by the processes being open and non sterile thereby permitting new organisms to enter. This openness allows for the possibility of new genes that improve cell fitness to be selected from the horizontal gene pool. Characteristics that biomining microorganisms have in common and examples of their remarkable adaptability are described (Rawlings *et al.*, 2005).

Solubilization of metals due to the action of microbes and the subsequent recovery of the metals from solution has deep historical roots that have been extensively reviewed (Olson *et al.*, 2003). Similarly, an indication of the number and sizes of the operations that employ microbes for the recovery of mainly copper, gold, cobalt, iron, sulphur and uranium has also been reviewed. These processes use the action of microbes for one of two purposes. Either to convert insoluble metal sulfides (or oxides) to water soluble metal sulfates or as a pretreatment process to open up the structure of the mineral thereby permitting other chemicals to better penetrate the mineral and solubilize the desired metal. An example of the first type of process is the conversion of insoluble copper present in minerals such as covellite (CuS) or chalcocite (Cu_2S) to soluble copper sulfate. An example of the second is the removal of iron, arsenic and sulfur from gold bearing arsenopyrite so that the gold that remains in the mineral is more easily extracted by subsequent treatment with cyanide. Both are oxidation processes, but where the metal to be recovered is extracted into solution the process is known as bioleaching, whereas

when the metal remains in the mineral, bioleaching is an inappropriate term and the process should strictly be referred to as biooxidation. Nevertheless, the term bioleaching is frequently used for both. Not all types of mineral are amenable to biologically-assisted leaching. In general, the mineral should contain iron or a reduced form of sulfur. Alternately, a mineral lacking in these compounds may be leached if it occurs together with another mineral that contains iron and reduced sulfur, provided that the mineral is subject to attack by ferric iron and/or sulfuric acid. Metals in certain non-sulfide minerals may be solubilized by a process of complexation with oxalic, citric or other organic acids (Bosecker *et al.*, 1997).

Mechanisms of bioleaching

Metal leaching is now recognized as being mainly a chemical process in which ferric iron and protons are responsible for carrying out the leaching reactions. The role of the microorganisms is to generate the leaching chemicals and to create the space in which the leaching reactions take place. Microorganisms typically form an exopolysaccharide (EPS) layer when they adhere to the surface of a mineral but not when growing as planktonic cells. It is within this EPS layer rather than in the bulk solution that the biooxidation reactions take place most rapidly and efficiently and therefore the EPS serves as the reaction space (Rohwerder *et al.*, 2003).

The mineral dissolution reaction is not identical for all metal sulfides and the oxidation of different metal sulfides proceeds via different intermediates. Briefly, Thiosulfate mechanism has been proposed for the oxidation of acid insoluble metal sulfides such as pyrite (FeS_2) and molybdenite (MoS_2), and a Polysulfide mechanism for acid soluble metal sulfides such as sphalerite (ZnS), chalcopyrite ($CuFeS_2$) or galena (PbS). In the thiosulfate mechanism, solubilization is through ferric iron attack on the acid-insoluble metal sulfides with thiosulfate being the main intermediate and sulfate the main end product. Using pyrite as an example of a mineral, the reactions may be represented as:

$$FeS_2 + 6\,Fe^{3+} + 3\,H_2O \rightarrow S_2O_3^{2-} + 7\,Fe^{2+} + 6\,H^+ \quad (1)$$

$$S_2O_3^{2-} + 8\,Fe^{3+} + 5\,H_2O \rightarrow 2\,SO_4^{2-} + 8\,Fe^{2+} + 10\,H^+ \quad (2)$$

In the case of the polysulfide mechanism, solubilization of the acid soluble metal sulfide is through a combined attack by ferric iron and protons, with elemental sulfur as the main intermediate. This elemental sulfur is relatively stable but may be oxidized to sulfate by sulfur-oxidizing microbes such as *Acidithiobacillus thiooxidans* or *Acidithiobacillus caldus* (reaction 5 below).

$$MS + Fe^{3+} + H^{+} \rightarrow M^{2+} + 0.5\ H_2Sn + Fe^{2+}\ (n > 2) \quad (3)$$

$$0.5\ H_2Sn + Fe^{3+} \rightarrow 0.125\ S_8 + Fe^{2+} + H^{+} \quad (4)$$

Microbes

$$0.125S_8 + 1.5O_2 + H_2O \rightarrow SO_4^{2-} + 2H^{+} \quad (5)$$

The ferrous iron produced in reactions (1) to (4) may be reoxidized to ferric iron by iron oxidizing microorganisms such as *Acidithiobacillus ferrooxidans* or bacteria of the genera *Leptospirillum* or *Sulfobacillus*.

Microbes

$$2Fe^{2+} + 0.5O_2 + 2H^{+} \rightarrow 2Fe^{3+} + H_2O \quad (6)$$

The role of the microorganisms in the solubilization of metal sulfides is, therefore, to provide sulfuric acid (reaction 5) for a proton attack and to keep the iron in the oxidized ferric state (reaction 6) for an oxidative attack on the mineral (Rawlings *et al.*, 2003).

Effect of temperature

Bioleaching processes are carried out at a range of temperatures from ambient to a demonstration plant that has been operated at 80°C (Rawlings *et al.*, 2003).

As would be expected, the types of iron and sulfur-oxidizing microbes were different depending on the temperature range. The types of microbes found in processes that operate from ambient to 40°C tend to be similar irrespective of the mineral, as are those within the temperature ranges 45–55°C and 75– 80°C. As described below, there are two broad categories of biologically-assisted mineral degrading processes. An ore or concentrate is either placed in a heap or dump where it is irrigated or a finely milled

mineral suspension is placed in a stirred tank where it is vigorously aerated. In general, mineral solubilization processes are exothermic and when tanks are used, cooling is required to keep the processes that function at 40°C at their optimum temperature. At higher temperatures the chemistry of mineral solubilization is much faster and in the case of minerals such as chalcopyrite, temperatures of 75–80°C are required for copper extraction to take place at an economically viable rate. (Doglous *et al.*, 2003).

Commercial metal extraction operations

Heap leaching processes commercial bioleaching can take place using what may be considered to be a low technology process, the irrigation of waste ore dumps (Brierley *et al.*, 1982). The metal recovery process may be made more efficient by the construction and irrigation of especially-designed heaps rather than by the irrigation of an existing dump that has not been designed to optimize the leaching process (Schippers *et al.*, 1999). When building a heap, agglomerated ore is piled onto an impermeable base and supplied with an efficient leach liquor distribution and collection system. Acidic leaching solution is percolated through the crushed ore and microbes growing on the surface of the mineral in the heap produce the ferric iron and acid that result in mineral dissolution and metal solubilization. Aeration in such processes can be passive, with air being drawn into the reactor as a result of the flow of liquid or active with air blown into the heap through piping installed near the bottom. Metal containing leach solutions that drain from the heap are collected and sent for metal recovery (Schnell *et al.*, 1997). Heap reactors are cheaper to construct and operate, and are therefore, more suited to the treatment of lower grade ores. However, compared with tank reactors, heap reactors are more difficult to aerate efficiently and the undesirable formation of gradients of pH and nutrient levels as well as liquor channeling are difficult to manage. Furthermore, although one can rely on the natural movement of microbes to eventually inoculate the heap, initial rates of bioleaching can be improved by effective heap inoculation, but this is difficult to achieve. Copper is the metal recovered in the largest quantity by means of heap reactors (Olson *et al.*, 2003).

Although comparisons are difficult as data are presented in different ways, examples of large copper leaching operations are those by Sociedad Contractual Minera El Abra and the Codelco Division Radimiro Tomic both in Chile and producing 225 000 and 180 000 tonnes Cu per annum respectively. Gold ore is also pretreated by bioleaching in heaps by New Mont Mining, in the Carlin Trend region, Nevada, USA (Olson *et al.*, 2003).

Tank leaching processes

In stirred tank processes highly aerated, continuous-flow reactors placed in series are used to treat the mineral. Finely milled mineral concentrate or ore is added to the first tank together with inorganic nutrients in the form of ammonia- and phosphate containing fertilizers. The mineral suspension flows through series of highly-aerated tanks that are pH and temperature-controlled (Dew *et al.*, 1997). Mineral solubilization takes place in days in stirred-tank reactors compared with weeks or months in heap reactors. Stirred tank reactors that operate at 40° C and 50°C have proven to be highly robust and very little process adaptation is required for the treatment of different mineral types (Rawlings *et al.*, 1997). A major constraint on the operation of stirred tank reactors is the quantity of solids (pulp density) that can be maintained in suspension. This is limited to about 20%, as at pulp densities >20%, physical mixing and microbial problems occur. The liquid becomes too thick for efficient gas transfer and the shear force induced by the impellers causes' physical damage to the microbial cells. This limitation in solids concentration plus considerably higher capital and running costs in tank compared with heap reactors has meant that the use of stirred reactors has been restricted to high value minerals or mineral concentrates (Doglous *et al.*, 2003).

Stirred tanks are used as a pretreatment press for gold-containing arsenopyrite concentrates with the first of these having been built at the Fairview mine, Barberton, South Africa in 1986 (Rawlings *et al.*, 1997).

The largest is at Sansu in the Ashanti goldfields of Ghana, West Africa. These two operations currently treat 55 and 960 tonnes of gold concentrate per day respectively. Another example is the use of stirred tanks to treat 240 tonnes of cobalt-containing pyrite in 1300 m3 tanks at Kasese, Uganda (Briggs *et al.*, 1997).

Types of Microorganisms

In general, the types of microorganisms found in heap-leaching processes are similar to those found in stirred tank processes, however, the proportions of the microbes may vary depending on the mineral and the conditions under which the heaps or tanks are operated. In processes that operate from ambient temperatures to about 40 ^{0}C, the most important microorganisms are considered to be a consortium of Gram-negative bacteria. These are the iron- and sulfur oxidizing *Acidithiobacillus ferrooxidans* (previously *Thiobacillus ferrooxidans*), the sulfur-oxidizing *Acidithiobacillus thiooxidans* (previously *Thiobacillus thiooxidans*) and *Acidithiobacillus caldus* (previously *Thiobacillus caldus*), and the iron-oxidizing *leptospirilli*, *Leptospirillum ferrooxidans* and *Leptospirillum ferriphilum* (Foucher *et al.*, 2003). If ferrous iron is added to the leaching solutions (lixiviants) that are circulated through a heap or dump, then *At. ferrooxidans* may dominate the iron oxidizers. In continuous flow, stirred tank processes, the steady state ferric iron concentration is usually high and under such conditions *At. ferrooxidans* is less important than a combination of Leptospirillum and *At. thiooxidans* or *At. caldus* (Rawlings *et al.*, 1999). Gram-positive iron and sulfur-oxidizing bacteria related to *Sulfobacillus thermosulfidooxidans* have also been identified (Foucher *et al.*, 2003).

The consortium of bioleaching microbes frequently includes acidophilic heterotrophic organisms such as bacteria belonging to the genus *Acidiphilium* (Harrison *et al.*, 1981). A fluidized-bed reactor operating at 37°C and pH 1.4 was dominated by *L. ferriphilum* with a small proportion of Ferro plasma-like archaea (Kunnunen *et al.*, 2004). Heterotrophically inclined' microbes are believed to assist the growth of iron oxidizing bacteria like *At. ferrooxidans* and the *leptospirilli* (Johnson *et al.*, 1998).

This is thought to be due to their ability to provide essential nutrients or to remove toxic organic compounds or other inhibitory substances. How much this ability contributes to the overall mineral biooxidation efficiency of a microbial consortium in practice is still unclear (Johnson *et al.*, 1997). There are fewer commercial processes that operate in the 45–50 ^{0}C range and therefore, studies on microorganisms that dominate these bioleaching consortia have been less well reported (Rawlings *et al.*, 1999). Identified *At. caldus* and a species of *Leptospirillum* as being the dominant microbes in a continuous-flow

biooxidation tanks processing several mineral ores operating in this temperature range. *At. caldus*, *Sulfobacillus thermosulfidooxidans* and bacteria of the informally recognized species *'Sulfobacillus montserratensis'* together with an uncultured thermal soil bacterium were found to dominate the consortium of organisms oxidizing chalcopyrite concentrate at 45°C. The same bacteria dominated the culture irrespective of whether chalcopyrite, pyrite or an arsenic pyrite concentrate was being oxidized (Dopson *et al.*, 2004). In a pilot scale, stirred tank operation in which three tanks in series were used to treat a polymetallic sulfide ore at 45°C, *At. caldus* like, *L. ferriphilum* like and *Sulfobacillus* like bacteria were found to dominate the first tank (Okibe *et al.*, 2003). The proportions of these bacteria decreased in the second tank with the numbers of *At. Caldus* and Ferro plasma-like archaea being equally dominant. The Ferro plasma-like archaea completely dominated the third tank with the number of leptospirilli being reduced to undetectable levels. When combinations of pure cultures were tested, a mixed culture containing both autotrophic (*Leptospirillum* MT6 and *At. caldus*) and heterotrophic moderate thermophiles (Ferro plasma MT1 7) was the most efficient (Okibe *et al.*, 2004).

The presence of Ferro plasma-like organisms is being increasing recognized in bioleaching processes that operate at very low pH (1.4 or less). These archaea appear to be able to oxidize minerals like pyrite in pure culture although not without a small quantity of yeast extract. Species of the gram-positive genus, *Acidimicrobium* (Clark *et al.*, 1996). There are even fewer reports on types of microbes that occur in mineral treatment processes that operate at temperatures >70° C than at lower temperatures. However, it is clear that these biomining consortia are dominated by archaea rather than bacteria, with species of *Sulfolobus* and *Metallosphaera* being most prominent (Norris *et al.*, 2000). *Sulfolobus metalicus* has been found to dominate at 70°C but this archeaon is probably excluded at higher temperatures with other *Metalosphaera*-like and *Sulfolobus*-like archaea dominating at 80°C. Archaea belong to the genus *Acidianus* such as *Ad. ambivalensi* or *Ad. infernus* are also capable of growing at high temperature (90°C for *Ad. infernus*) on reduced sulfur and at low pH. However, the contribution of these organisms to industrial bioleaching is not well-established (Norris *et al.*, 1997).

Nutrition

1) Autotrophy

Microorganisms that drive the mineral degradation processes are autotrophic and obtain their carbon for cell mass synthesis from the carbon dioxide in the air used to aerate the process. Heterotrophic microorganisms that live off waste products produced by the autotrophs are usually also present and there is some evidence that these heterotrophs might assist the process (Johnson *et al.*, 1997). Mineral degradation processes differ from the vast majority of other commercial processes that employ microorganisms where an organic substrate is necessary to provide the carbon source and energy required for microbial growth. If it were necessary to feed the microorganisms required for mineral degradation with a carbon source (e.g. molasses). Commercial mineral biooxidation processes would be unlikely to be viable. Bacteria such as the *acidothiobacilli* and *leptospirilli*, fix CO_2 by the Calvin reductive pentose phosphate cycle, using the enzyme ribulose 1, 5-biphosphate carboxylase (RuBPCase or Rubisco) (Tyson *et al.*, 2004).

The CO_2 concentration present in air is generally sufficient to avoid carbon limitation when bacteria such as *Acidithiobacillus ferrooxidans* are growing on ferrous iron. This bacterium probably responds to CO_2 limitation by increasing the cellular concentration of RuBPCase (Codd *et al.*, 1987). *At. ferrooxidans* strain Fe1 has been reported to have two identical copies of the structural genes for RuBPCase (although the flanking regions are different, (Kusano T *et al.*, 1991).

Which are separated by more than 5 kb (Kusano *et al.*,1993). The reason for this duplication has not been tested. *At. ferrooxidans* is considered to be an obligate autotroph but has been shown to use formic acid as a carbon source provided that it was grown in continuous culture and the formic acid was fed in sufficiently slowly for the concentration to remain low (Pronk *et al.*, 1991). Similarly, genes for a formate hydrogenlyase complex have been located on the genome of *Leptospirillum* type II and it is therefore likely to also grow on formate (Tyson *et al.*, 2004). However, like CO_2, formic acid has a single carbon atom and when lysed by the cell formate may be assimilated by the Calvin cycle in much the same way as CO_2. Whether the ability to use formate is of value in commercial processes is not clear. In the case of several of the

other bacteria, such as the moderately thermophilic *Sulfobacillus thermosulfidooxidans*, 1% v/v CO_2 enriched air is required for rapid autotrophic growth in pure culture. This may be partly because the solubility of CO_2 is reduced at 50°C and partly because these bacteria are known to be inefficient at CO_2 uptake. *Sulfobacillus* species are nutritionally versatile and also capable of heterotrophic growth (Clark *et al.*, 1996).

2) Nitrogen, phosphate and trace elements

Based on dry weight, nitrogen is the next most important element after carbon for the synthesis of new cell mass. Ammonium levels of 0.2 mM have been reported to be sufficient to satisfy the nitrogen requirement of *At. ferrooxidans* (Tuovinen *et al.*, 1971). High concentrations of inorganic or organic nitrogen are inhibitory to iron oxidation. Exactly how much nitrogen needs to be present in a growth medium will be dependent on the quantity of cell growth to be supported. Ammonia is highly soluble in acid solutions and it has been found that traces of ammonia present in the air can be readily absorbed into growth media. Therefore, determination of the exact nitrogen requirements is difficult to estimate. In commercial operations, inexpensive fertilizer grade ammonium sulfate is typically added to biooxidation tanks or bioleaching heaps to ensure that sufficient nitrogen is available (Dew *et al.*, 1997).The ability of *At. ferrooxidans* to reduce atmospheric dinitrogen to ammonia was reported and the genes for the enzyme nitrogenase (nifHDK) were cloned several years ago (Pretorius *et al.*, 1987). The ability to fix nitrogen is probably a general property of *At. ferrooxidans* as at least fifteen strains of *At. ferrooxidans* have been shown to contain the nitrogenase genes (Rawlings *et al.*, 2006). *L. ferrooxidans* was also shown to contain nifHDK genes, to reduce acetylene to ethylene (a common test for nitrogenase activity) and at the same time to oxidize ferrous to ferric iron at low oxygen concentrations (Norris *et al.*, 1995). This activity was repressed by ammonia, a strong indication of the nitrogen fixing activity. The nitrogen fixing (nif) operon and many of the nif regulatory elements of a *L. ferrooxidans* from the Tinto river have been isolated and sequenced (Parro V *et al.*, 2004). Interestingly analysis of the genome of *Leptospirillum* type II (*L. ferriphilum*) indicated the absence of genes for nitrogen fixation in this species (Tyson *et al.*, 2004). Nitrogenase enzyme activity is inhibited by oxygen. It was found that *At. ferrooxidans* growing on iron did not fix

nitrogen when aerated, but began to fix nitrogen once the oxygen concentration had fallan (Mackintosh *et al.*, 1978).

Therefore how much nitrogen fixation takes place in highly aerated biooxidation tanks or heaps is uncertain. However, the aeration of heaps is not homogenous and nitrogen fixation could take place in parts of a heap where the oxygen is absent or its concentration is sufficiently low. The sensitivity of nitrogenase to oxygen poses a special problem for leptospirilli because, as far as is known, it uses only iron as its electron donor and is probably obligately aerobic. One mechanism by which nitrogenase can be protected against oxygen is respiratory protection, whereby rapid consumption of oxygen by a cytochrome oxidase is maintains a low oxygen concentration compatible with nitrogen fixation. It has been suggested that cytochrome bd is responsible for respiratory protection in *At. ferrooxidans* (Brassuer *et al.*, 2004). It has been found that *Leptospirillum* type II also has genes encoding both ccb3 and bd terminal oxidases even though it has no nitrogenase (Tyson *et al.*, 2004). One can speculate that if cytochrome bd is also present in *L. ferrooxdans*, this cytochrome could be responsible for respiratory protection of its nitrogenase.

Sources of energy

Soluble metal ions are frequently present fairly high concentrations in highly acidic environments. Metal ions which exist in more than one oxidation state and which have redox potentials that are more negative than the O_2/ H_2O redox couple, have the potential to serve as electron donors for acidophilic bacteria. An *At. ferrooxidans* like bacterium was reported to directly oxidize Cu^{+} to Cu^{2+} (Nielsen *et al.*, 1978). And U_{4+} to U_{6+} under aerobic conditions and that these oxidation reactions were coupled to CO_2 fixation (Dispirito *et al.*, 1982). However, whenever ferric iron is present, it is difficult to unequivocally demonstrate the biological oxidation of the metal as opposed to chemical oxidation of the metal by ferric iron. Similarly it has been reported that Mo^{5+} can be oxidized to Mo^{6+} and a molybdenum oxidase has been isolated from cell extracts of *At. ferrooxidans* (Sugio *et al.*, 1992). The potential also exists that the oxidation of oxyanions such as As^{3+} (AsO_3^{2-}) to As^{5+} (AsO_4^{3-}) can serve as an alternate electron donor for acidophilic organisms (Silver *et al.*, 2005). An analysis of the *At. ferrooxidans*

ATCC23270 genome revealed that as many as eleven cytochromes c were present (Yarzabal *et al.*, 2002). One cytochrome c was specific for growth on sulfur, three were specific for growth on iron and several were produced on both substrates. The large number of cytochrome c molecules might also be a reflection of the versatility of electron donors (and electron acceptors) that the bacterium is capable of using.The type strain of *At. ferrooxidans* ATCC23270 as well as the two other *At. ferrroxidans* strains tested were found to grow by hydrogen oxidation but not *At. thiooxidans* or *L. ferrooxidans* (Drobner *et al.*, 1999). When growing on hydrogen they had a broad pH optimum of pH 3.0 to 5.8 with no growth occurring at pH<2.2 or pH>6.5. Hydrogen oxidation appeared to be repressed by the presence of S0, Fe^{2+} and sulfidic ore. In a later study, only one of six *At. ferrooxidans* strains tested could use hydrogen as an electron donor to support CO_2 fixation and cell growth with oxygen as electron acceptor (Ohmura *et al.*, 2002). There is a possibility that some isolates of the genes L*eptospirillum* might be able to use hydrogen as an electron donor although this has not yet been demonstrated.

Bioleaching of heavy metals from contaminated soil was carried out using indigenous sulfur oxidizing bacterium *Acidithiobacillus thiooxidans*. Experiments were carried out by varying sulfur/soil ratio from 0.03 to 0.33 to evaluate the optimum ratio for efficient bioleaching of heavy metals from soil. The influence of sulfur/soil ratio on the bioleaching efficiency was assessed based on decrease in pH, increase in oxidation–reduction potential, sulfate production and solubilization of heavy metals from the soil. Decrease in pH, increase in oxidation–reduction potential and sulfate production was found to be better with the increase in sulfur/soil ratio. While the final pH of the system with different sulfur/soil ratio was in the range of 4.1–0.7, oxidation reduction potential varied from 230 to 629 mV; sulfate production was in the range of 2,786– 8,872 mg/l. Solubilization of chromium, zinc, copper, lead and cadmium from the contaminated soil was in the range of 11–99% (Nareshkumar *et al.*, 2007).

Heterotrophic pretreatment combined with autotrophic bioleaching of Polkowice black shale were studied. Combination of these two processes was introduced as two-stage process which turned out to be more efficient in the terms of metals extraction. The sequence of preliminary heterotrophic and further autotrophic bioleaching was found as most reliable processing manner, since heterotrophic process provides the material's surface area expansion, and therefore strong influences rate and efficiency of autotrophic leaching. Besides, it was found that tube bioreactors designed specially for hereby research ensure better processing condition than Erlenmeyer flasks in the terms of extraction speed and efficiency (Grobelski *et al.*, 2007).

The rate of iron (II) oxidation by sulphur grown *Acidithiobacillus ferrooxidans* cells decreased when the pH of the original growth medium was lowered. This behaviour was observed even after shifting from the original growth pH to a higher pH. After being suspended in medium at a pH higher than the growth pH, sulphur-grown cells could leach covellite at a similar initial rate to iron-grown cells. Sulphur-grown cells exhibited a long lag phase when the original growth pH was low. These results were correlated with the number of protons associated with the cell surface, rather than with cell hydrophobicity or cell capacity to attach to solid particles. Sulphur-grown cells grown in very acidic media (without pH control) were not able to oxidize iron (II) or leach covellite even after shifting to a high pH (Curutchet *et al.*, 2000). The iron-oxidizing bacterium, *Acidithiobacillus ferrooxidans*. was cultivated on a medium without ferrous iron. Molecular hydrogen and air were supplied to the medium. It was found that *A. ferrooxidans* could grow with hydrogen in the pH range between 2.0 and 3.5. A trickle-bed contactor was used to increase the dissolution rate of hydrogen. The doubling time was increased and the cell concentration reached 4.0×10^9 cells ml-1 after 6 days. When the cells taken from the hydrogen medium were transferred back into the medium containing ferrous iron, the growth rate and the iron-oxidizing ability were the same as the predictions assuming that the microorganism grown with hydrogen was *A. ferrooxidans* (Kai *et al.*, 2007).

Although heap leaching has become established as the technology for treatment of some copper, gold, and zinc minerals, as well as its extension to the treatment of other types of minerals such as saltpeter and mine tailings, little study has been made on the optimization of this technology. Usually the operation of heap leaching is carried out until the maximum recovery has been obtained, or until observing that the concentration in the output solution of the heap exhibits no further extraction. The present study makes an analysis to determine if these criteria present the best conditions from the economic standpoint. Two variables are examined including, 1) Leaching time, 2) Height of the heap, at a copper mineral treatment plant. The results of the study showed that the design (height of the heap), and planning of the operation (operational time) were interactive factors, and that maximum recovery was not necessarily the best measure of operational efficiency based on economic considerations (Gonzalo *et al.*, 2008).

In pure culture form, *Acidithiobacillus ferrooxidans* was found to have a higher bioleaching capacity than *Leptospirillum ferrooxidans* and *Acidithiobacillus thiooxidans* with the capability of the latter to bioleach copper being very limited. Mixed cultures, MixA (*At. ferrooxidans*, *L. ferrooxidans* and *At. thiooxidans*) and MixB (*L. ferrooxidans* and *At. thiooxidans*) were shown to perform better than the pure cultures with the highest extraction of copper (62.1% Cu) being achieved by MixA. Copper bioleaching performances of the cultures were observed to agree with their respective growth pattern. The results also indicated that the increase in the pulp density (1–5% wt/vol) adversely affected bioleaching process regardless of the pure and mixed cultures used having led to the decrease in the extent of final copper extraction i.e. 50.3% Cu recovery at 1% wt/vol for *At. ferrooxidans* compared with 38.6% Cu at 5% wt/vol. This study underlines the importance of mixed cultures and, iron and sulphur-oxidising activity of a bacterial culture to efficiently oxidise chalcopyrite (Akcil *et al.*, 2006).

The microbiological leaching of Fe, Al, Zn, S, Cu, Ni and Co from sulfide ore material was evaluated with four percolation regimes involving trickle and flood leaching. Continuous circulation of the leach solution associated with flood leaching resulted in the highest rates of leaching of Ni (44% recovery), Zn (25%), Co (18%), and Cu (8%) over a period of about half a year. Iron and aluminum recoveries remained low because of their precipitation. Bacterial counts increased from 3.2×10^6 to 4.8×10^7 iron-oxidizers and from 6.6×10^6 to 1.8×10^7 glucose-oxidizers per ml leach solution.

Microscopic counts reached a maximum of 4.9×10^8 cells per ml. Neither microscopic nor viable counts reflected the time course and the progress of the leaching. However, both the microscopic and viable counts were highest with the continuous flooding technique which also yielded fastest of metal solubalization (Puhakka *et al.*, 1986).

Obligate autotrophic and acidophilic characteristics of iron oxidizing bacteria were exploited in order to prevent or attenuate the generation of acid mine drainage. Inhibition of biomass growth was performed by variation of 9K medium by increasing ferrous iron concentration (substrate inhibition), by addition of limestone (inhibition by pH increase) and olive pomace (inhibition by organic compounds). Inhibition tests of batch growth were performed according to a full factorial design with three factors (ferrous iron, limestone and olive pomace) taken at two levels. All tests were monitored by measuring cell and ferrous iron concentrations, pH and redox potential. Biomass characterization was performed by determining the sequence of 16S ribosomal RNA gene that denoted a 100% homology with the sequence of *Acidithiobacillus ferrooxidans*, one of the main bacteria responsible for acid mine drainage. Experimental data of cell growth denoted a strong inhibition in all the operating conditions, except the case when larger ferrous iron concentrations were used. Blank tests allowed isolation of the effect of chemical speciation from the effect due to metabolic activities. In particular, inhibition by limestone seemed to be due not only to pH increase, but also to a reduced availability of

ferrous iron substrate, while inhibition by pomace may be related to the antioxidant properties of polyphenols. Interactions among operating conditions, biomass growth and chemical parameters were specifically assessed by analysis of variance. Estimates of significant effects (95%) for cell concentration denoted that only main effects related to limestone and pomace addition determined a significant diminution of cell concentration with a dynamic increasing trend (Pagnanelli *et al.*, 2007).

The microorganisms cultured by pyrrhotite were a mixture of *Acidithiobacillus ferrooxidans* and *Acidithiobacillus thiooxidans*, of which the capability to oxidize ferrous to ferric irons is enhanced by the high mass ratio of Fe to S in pyrrhotite. Three pyrrhotite samples were separated into various parts with corresponding S/Fe ratios by magnetic separation and were used to culture the elective bacteria as the substrate. The association of the cultures could provide a more rapid and complete oxidation of sphalerite than that of bacteria cultivated by conventional methods (Song *et al.*, 2008). Biomining, the use of micro-organisms to recover precious and base metals from mineral ores and concentrates, has developed into a successful and expanding area of biotechnology. While careful considerations are made in the design and engineering of biomining operations, microbiological aspects have been subjected to far less scrutiny and control. Biomining processes employ microbial consortia that are dominated by acidophilic, autotrophic iron- or sulfur-oxidizing prokaryotes. Mineral biooxidation takes place in highly aerated, continuous-flow, stirred-tank reactors or in irrigated dump or heap reactors, both of which provide an open, non-sterile environment. Continuous-flow, stirred tanks are characterized by homogeneous and constant growth conditions where the selection is for rapid growth, and consequently tank consortia tend to be dominated by two or three species of micro-organisms. In contrast, heap reactors provide highly heterogeneous growth environments that change with the age of the heap, and these tend to be colonized by a much greater variety of microorganisms. Heap micro-organisms grow as biofilms that are not subject to washout and the major challenge is to provide sufficient biodiversity for optimum performance throughout the life of a heap (Rawlings *et al.*, 2007).

Economic development causes a huge increase of demand of metals, whose natural supplies quickly diminish. In addition, exploitation and enrichment of poorer and poorer layers of ores leave behind vast quantity of mining wastes. Traditional methods of ore enrichment including hydrometallurgical ones are approaching economical limits. However, the considerable quantities of old wastes are frequently richer in the demanded metals than many natural ore beds. Therefore, the hydrometallurgical methods are presently often supplemented by bio-hydrometallurgical methods, which in the recent years became very popular, especially in mining of gold (Karas *et al.*, 2002). The presently used bio-hydrometallurgical methods are mostly base on bioleaching of sulfometallic ores in acid solutions. Metals in some ores, however, are present also in organometallic chelate compounds, some of them containing cyclic hydrocarbons. The content of the cyclic hydrocarbon chelates is rather small but due to the high demand for metals the exploitation of such ores (using the bioleaching methods) is worth considering. The bioleaching of organometallic ores has to be done with heterotrophic bacteria, which can grow on organic material and degrade compounds such as proteins, fats and carbohydrates. Studies showed that some heterotrophic bacteria in the absence of complex organic compounds can utilize even simple hydrocarbons. Therefore it can be suggested that these bacteria may also efficiently degrade other difficult to exploit organometallic ores (Farbiszewska *et al.*; 1995; Sudoł *et al.*, 1997).

MATERIALS AND METHODS

Materials and Methods

The present research work was conducted at Microbiology Research Laboratory, Department of Microbiology, Quaid-i-Azam University Islamabad. The present aim of the study was to isolate and characterized the acidiphilic sulphur and iron oxidizing bacteria from black shale.

Source of ore and culture

For this purpose soil samples of black shale were collected from Tarbela, (Ghazi) and water samples from Chamyari, N.W.F.P (Pakistan).

Isolation of microorganisms from soil and water samples

Serial dilution of the samples were done, 10 screw capped tubes were used for serial dilution. About 9 ml of sterilized saline was taken in each tube. One gram of soil (black shale) was added to sterilized saline. After shaking it well, 1 ml of the suspension was transferred to tube 1 asceptically. The tube was shaken and from this tube, 1 ml of the dilution was then transferred to tube 2. Similarly, the 10^{th} dilution was prepared by transferring 1 ml to the next tube, under aseptic conditions. Same process was repeated for all other samples. After serial dilutions 0.1 ml was taken from tube 5 of water sample and 0.1 ml from soil samples and spread on the growth medium plates. The plates were incubated on 30^0C for 24 hours.

Microbiological growth media

Liquid Media

Iron medium ($9kFe^{2+}$)

For the growth of bacterial strains, iron Liquid medium ($9kFe^{2+}$) was used (Silverman and Lundgren 1959).

Composition of the (9kFe^{2+}) medium

Content	amount (g/l)
$(NH_4)_2SO_4$	3.00
$MgSO_4.7H_2O$	0.50
K_2HPO_4	0.50
KCl	0.10
Ca $(NO_3)_2$	0.01
pH	1.5
Dist.water	800 ml

These salts were dissolved in 800 ml of distilled water and the pH was adjusted to1.5 by adding 5M H_2SO_4. Aliquots of basal salts solution were sterilized by autoclaving at 121°C and 15 lbs for 20 minutes. Ferrous sulfate ($FeSO_4.7H_2O$) solution was prepared by dissolving 44 g in 200 ml distilled water and pH was adjusted to 1.5 with 5M H_2SO_4. The medium was sterilized by passing through membrane filters (0.22 µm, Millipore GVWP filters). The sterilized ferrous sulfate solution was added aseptically to the cooled basal salts medium to give final ferrous iron concentration of 160 mM.

Sulfur medium (9KS°)

Sulfur medium (9KS°) used for isolation and growth of *At. thiooxidans* was 9K mineral salts medium in which ferrous sulfate was replaced by elemental sulfur. The medium was prepared as described earlier (Silverman and Lundgren 1959). The pH of medium was adjusted to 1.5 with 5M H_2SO_4 and 100 ml of aliquots were taken into 250 ml Erlenmeyer flask. Elemental sulfur was added to each flask. Medium sterilization was done by tyndallization, by heating the flasks at 121°C for 30 minutes for three successive days. This medium was used for growth of *At. ferrooxidans.*

Glucose medium

Glucose medium was prepared as described by Guay and Silver (1975). This glucose medium consists of (9kFe^{2+}) having the same composition as described before, and Fe $SO_4.7H_2O$, 0.01 gm. These salts were dissolved 900 ml of distilled water and pH of

the medium was adjusted to 3.5 with dilute H_2SO_4. Aliquots of basal salt solution in Erlenmeyer flasks (1L) were sterilized by autoclaving at 121 °C for 20 minutes. Glucose solution was sterilized by filtration (0.22 µm Millipore GVWP filters), and added aseptically into basal salt solution as prepared before.

Solid Media

Gelrite-$FeSO_4$ medium

A new efficient (Gelrite-$FeSO_4$) solid medium was developed and successfully employed for isolation and enumeration of *At. ferrooxidans* in mine water, microbial leached solutions and solid samples. Gelrite-$FeSO_4$ solid medium was routinely used in the present studies. Three separate solutions were prepared and mixed after autoclaving.

1) Solution A

(Ferrous sulfate solution), was prepared by dissolving 50.0g $FeSO_4.7H_2O$ in 200 ml distilled water and the pH was adjusted to 2.2 with 5M H_2SO_4. It was sterilized by filtration (0.22 µm Millipore GVWP filters) and collected in a pre-sterilized bottle.

2) Solution B

(Mineral salts medium), which was a modified form of 9K medium having the same amount of contents, (Silverman and Lundgren 1959). All ingredients were dissolved in 500 ml distilled water and the pH was adjusted to 2.3 with 5N H_2SO_4. This solution was sterilized by autoclaving for 20 minutes at 15 lbs and 121 ^{0}C.

3) Solution C (Gelling solution)

About 4.0g of Gelrite was soaked in 300 ml distilled water for 20 minutes and then autoclaved for 20 minutes at 15 lbs and 121°C.When all these solutions had cooled down to 70-75°C, solution B was mixed with solution C aseptically, and then solution A was added to get final concentration of Gelrite medium. These solutions were stirred

constantly to mix at same temperature. The final pH of this medium should be 2.3. Approximately 20 ml of this mixture was poured into pre-sterilized Petri dishes.

Thiosulfate medium

Thiosulfate solid medium was routinely used for isolation and enumeration of *At. thiooxidans* during present study. Three separate solutions were prepared separately, and mixed after sterilization.

1) Solution A

(Sodium thiosulfate solution), was prepared by dissolving 10.0 g ($Na_2S_2O_3.H_2O$) in 100 ml distilled water and it was separately sterilized by filtration (0.22 µm Millipore GVWP filters) and collected in a pre-sterilized bottle.

2) Solution B

Mineral salts solution), which was prepared in a modified form according to 9K medium (Silverman and Lundgren 1959). All ingredients were dissolved in 600 mL distilled water and the final pH was adjusted to 4.0 with dilute H_2SO_4. This solution was sterilized by autoclaving for 20 minutes at 15 lbs and 121°C.

3) Solution C (Gelling solution)

About 4.0 g of purified agarose was soaked in 300 ml distilled water for 20 minutes and then autoclaved for 20 minutes at 15 lbs and 121°C. When all these solutions cooled down to 45-50°C, solution B (Mineral salt solution) was mixed to solution C (Gelling solution) aseptically, and then solution A (Sodium thiosulfate solution) was added to a final concentration of 0.4% agarose (w/v) and $Na_2S_2O_3.5H_2O$ of 1% (w/v). These solutions were stirred constantly to mix and the final pH of the medium was (4.0) at same temperature. Approximately 20 ml of this mixture was poured into pre-sterilized Petri dishes.

Glucose medium (Agarose-Glucose medium)

Glucose solid medium was prepared as described earlier for the preparation of thiosulfate solid medium, except that thiosulfate solution was replaced by glucose solution (10% w/v).

Isolation and enumeration of *Acidithiobacillus ferrooxidans*

After sampling, 1.0 ml aliquot of each liquid sample was inoculated separately into liquid iron (9kFe^{2+}) medium (Silvrman and Lundgren 1959). These flasks were incubated at 30°C at 150 rpm. The presence of iron-oxidizing bacteria in liquid iron medium was indicated by the formation of ferric iron and the medium becoming brick red in color. A serial dilution of each culture was performed using sterile saline. About 0.1 ml amounts from each dilution was spread on solid Gelrite-$FeSO_4$, plates. The inoculated plates were incubated at 30°C in a sealed polyvinyl-bag to block moisture evaporation. Plates were examined with the naked eye to observe the colony size, shape, color, and other morphological features. Of the bacterial growth single colony of iron-oxidizing bacteria (*At. ferrooxidans*), were picked from the plates by using a sterile inoculating loop and inoculated into 25 ml sterilized vials containing 10 ml liquid iron medium, pH 2.0 and was vortexed to spread the colony. All the cultures were incubated at 30°C until the color of the medium changed to brick red indicating ferrous iron (Fe^{2+}) oxidation by iron-oxidizing bacteria. To check the purity of isolated cultures, cells growing in liquid iron medium were spread on solid Gelrite-FeSO4 plates. After 5-7 days, the plates were examined for the required colonies and were sub cultured on fresh liquid iron (9kFe^{2+}) and sulfur medium as described earlier by (Silverman and Lundgren 1959).

Gram's staining

1) Cleaned four glass slides.

2) Using sterile technique, prepared a smear of these culture, by placing a drop of water on the slide and transferring each organism separately to the slide with sterile, cooled needle.

3) Allowed smears to air dry, then heat fixed in the usual manner.

4) Flooded the smears with crystal violet and let stand for one minute.

5) Washed with tap water.

6) Flooded smear with the Gram's iodine mordant and let stand for one minute.

7) Washed with tap water.

8) Decolorized with 95% ethyl alcohol.

9) Washed with tap water.

10) Counterstained with safranin for 45 seconds.

11) Washed with tap water.

12) Blotted dry with bibulous paper and examined microscopically. The cells of iron-oxidizing bacterium (*At. ferrooxidans*) were viewed under compound microscope to examine cell size, shape, motility and the tendency to form filaments. Photograph of the slide were taken through microscopic camera after staining. Other liquid samples were employed directly on Gelrite-$FeSO_4$ plates, which were incubated at 30°C. For residue sample, 1.0 g of each sample was added into a 250 ml Erlenmeyer flask containing 100 ml sterile 9K mineral salts medium (pH 4.0) and vortexed. About 0.1 ml of this solution was spread on solid Gelrite-$FeSO_4$ plates and incubated at 30 ^{0}C.

Isolation and enumeration of *Acidithiobacillus thiooxidans*

One ml aliquot of each liquid sample was inoculated into liquid sulfur medium (9KS°) of pH 2.5 and incubated at 30°C and 100 rpm. The presence of sulfur-oxidizing bacteria (*At. thiooxidans and At. ferrooxidans*) in liquid sulfur media was indicated by a drop in pH of the medium due to the production of sulfuric acid. When pH of the medium dropped to less than 1.0, an aliquot of 1.0 g was taken and subcultured into fresh sulfur liquid medium. Finally, after 5-6 subculturing for 5-6, it was centrifuged at 10,000 rpm in a 40^{0}C for 10 minutes. The pellet was re-suspended in 20 ml of sterilized distilled water, pH 4 adjusted with 1M H_2SO_4. Tenfold serial dilution of isolated culture was prepared using sterile saline water as diluent. About 0.1ml amounts of each dilution was spread on solid tetrathionate plates and incubated at 30 ^{0}C. To avoid drying-up the plates were kept in a sealed polyvinyl bag. Gram's staining was performed as described before.

Photographs of the slides were taken and also plates were examined with naked eye to record morphological features of colonies, such as size, shape, and color. Single colonies were picked from the plates by using a steriled loop and inoculated separately into 25 ml vials containing 10 ml tetrathionate liquid media of pH 4.0. All cultures were incubated at 30°C until the medium became milky and pH dropped to less than 1.0 due to oxidation of tetrathionate by sulfur-oxidizing bacteria. To check the purity of isolated strains, cells growing in liquid tetrathionate medium were spread on solid Gelrite-$FeSO_4$ and glucose plates and observed after 5-7 days of incubation, for the presence of any iron-oxidizing (*At. ferooxidans*) or glucose-oxidizing (*At. acidophilus*) bacteria, respectively. They were picked and streaked onto solid tetrathionate, glucose and Gelrite-$FeSO_4$, media to check its growth on glucose and ferrous iron. Single colonies were also subcultured into ferrous iron, tetrathionate, and glucose liquid media. The slides were prepared by Gram's staining and observed under microscope.

Biochemical characteristics of the microorganisms

Extra cellular Enzymes activities

1) Starch hydrolysis

Starch agar was used to demonstrate the hydrolytic activities of these exoenzymes. Starch in the presence of iodine imparts a blue-black color to the medium, indicating the presence of starch-splitting enzymes and representing a negative result. If the starch has been hydrolyzed, a clear zone of hydrolysis surrounds the growth of the organism. This was a positive result.

2) Gelatin hydrolysis

Nutrient gelatin deep tubes were used to demonstrate the hydrolytic activity of gelatinase. The medium consists of nutrient broth supplemented with 12% gelatin. The high concentration results in a stiff medium and also serves as the substrate for the activity of gelatinase. Following inoculation and incubation for

48 hours, the cultures are placed in refrigerator at 4 ^{0}C for 30 minutes. Cultures that remain liquefied are indicative of slow gelatin hydrolysis.

Intra cellular Enzymes activities

1) Hydrogen sulfide production.
2) Catalase reaction.
3) Urease test
4) Indole production test
5) Methyl red test
6) Voges Proskauer test
7) Citrate utilization
8) Triple sugar- iron test.

All these activities were performed to check the bio chemical activities of *At. thiooxidans* and *At. ferroxidans.*

1) Hydrogen Sulfide Test

This test was performed to determine the ability of *At. thiooxidans* and *At. ferrooxidans* to produce H_2S from inorganic sulfur and sulfur containing amino acids. Isolates were incubated for 72 hours at 30 ^{0}C in SIM agar tubes. Results were determined by observing the color change around the growth area.

2) Catalase Test

This test was used to determine the ability of *At. thiooxidans* and *At. ferrooxidans* to degrade H_2O_2 by means of catalase enzyme. Results were obtained by observing color change after adding few drops of 3% H_2O_2 over 72 hours culture incubated at 30°C in tripticase soya agar slants.

3) Urease Test

This test was performed to check the ability of *At. thiooxidans* and *At. ferrooxidans* to degrade urea by means of urease enzyme. This culture was grown for 72 hours in glass

tubes containing urea broth at 30 ^{0}C. Change in the color of sample with its control.

4) Indole production test

In this experiment, SIM agar which contains the substrate tryptophan was used. The presence of indole was detectable by adding kovac, s reagent, which produces a cherry red reagent color. The absence of red coloration demonstrates that the substrate tryptophan was not hydrolyzed and indicated a negative indole reaction. Using a sterile technique, inoculated each experimental organism into its appropriately labeled deep tubes by means of a stab inoculation. The last tube served as a control, incubated the tubes for 24 to 72 hours at 30 ^{0}C.

5) Methyl red test

The methyl red indicator in the pH range of 4 turned red the tube medium. This was indicative of a positive test. When tube medium was not red, it was a negative result. Incubated all the cultures for 24 to 72 hours at 30 ^{0}C.

6) Voges-Proskaur test

In this test development of a deep rose color in the culture 15 minutes following the addition of barrit, s reagent was the indicative of the presence of acetyl-methylcarbinol and represented a positive result. While the absence of rose coloration was a negative result, incubated the culture for 24 to 72 hours at 30 ^{0}C.

7) Citrate utilization test

Following incubation for 72 hours at 30 ^{0}C, citrate-positive cultures were identified by the presence of growth on the surface of slant, which is accompanied by blue coloration. Citrate negative cultures will show no growth, and the citrate medium will remain green.

8) Triple iron sugar

The TSI agar medium contains sodium thiosulfate, a substrate for hydrogen sulfide

production and ferrous sulfate for detection of this colorless end product. Following incubation for 24 to 72 hours at 30 ^{0}C, only cultures of organism's capable producing hydrogen sulfide, showed an extensive blackening in the butt because of the precipitation of the insoluble ferrous sulfide.

Effect of carbon sources on growth of bacteria

Different carbon sources were used for the growth of *Acidithiobacillus thiooxidans* and *At. ferrooxidans*. The different carbon compounds (1%) were as follows.

1) Glucose
2) Sucrose
3) Fructose
4) Raffinose
5) D-sorbitol
6) Galactose
7) Lactose
8) Maltose
9) Rhammanose
10) Mannose

The O.D of the above carbon sources was taken at 440 nm, after 24, 48, 72, and 96,120,144 and up to 216 hours, to check the growth of isolates and their results were shown in graphs.

RESULTS

Results

Isolation and Characterization of Acidiphilic Iron and Sulfur oxidizing *Acidithiobacillus bacteria*

Isolation of Iron- and Sulfur-Oxidizer (*Acidihiobacillus ferrooxidans*)

For isolation of acidophilic iron and sulfur-oxidizing (*At. ferrooxidans*) bacteria from soil and water samples from Tarbela and Chamyari, an appropriate amount (100 µL) of liquid sample was inoculated onto solid Gelrite-$FeSO_4$ medium (Khalid *et al.* 1993). After 5-7 days of incubation at 30°C, reddish-brown colonies of iron-oxidizing bacteria were developed on the plates. These reddish-brown colonies growing on Gelrite-$FeSO_4$ plates were picked and cultivated separately into liquid iron medium (9KFe^{2+}) as described earlier. After 3-5 days of incubation at 30°C and 150 rpm under shaking condition, the medium became reddish-brown due to bacterial oxidation of Fe^{2+} to Fe^{3+}. The harvested cells of iron-oxidizing bacteria were inoculated onto solid Gelrite-$FeSO_4$ plates. Such ordinary purification procedures were repeated several times, finally pure cultures were obtained (Fig. 11).

Isolation of Sulfur-Oxidizer (*Acidithiobacillus thiooxidans*)

For the isolation of acidophilic sulfur-oxidizing bacteria (*At. thiooxidans*) from soil and water samples. An appropriate amount (100 µL) of liquid sample was streaked onto solid Agarose-S_2O_3 medium. After 12-15 days of incubation at 30 ^{0}C. Some colonies were off-white, circular in shape and relatively medium in size. This became pale-yellow in color after 3-4 weeks of incubation. A characteristics smell of elemental sulfur (S^o) was observed from these colonies. While some were milky white, circular shape and relatively small in size. Each type of single colony was picked and inoculated separately into liquid sulfur (9KS^o) and glucose media for further screening. The flasks were incubated on a shaking incubator at 30 ^{0}C. After 5-7 days of incubation, the sulfur medium became turbid and milky white exhibiting the growth of microbial populations (Fig. 12).

Cell Morphology and Characterization of Isolated Strains

The compound microscopic observations of isolated strains of *At. ferrooxidans* and *At. thiooxidans* revealed that these strains were Gram-negative, motile, and single rod-shaped bacteria, *At. ferrooxidans* oxidized Fe^{2+} to Fe^{3+}. Pyrite, sulfur and reduced sulfur compounds like thiosulfate and tetrathionate. The bacterial oxidation of pyrite, sulfur and reduced sulfur compounds produced sulfuric acid which followed a drop in initial pH-value of the medium. *At. thiooxidans* oxidized only sulfur and reduced sulfur compounds to sulfuric acid through its metabolic activity.

Growth Studies of *Acidithiobacillus* bacteria

Oxidation of Ferrous Iron (Fe^{2+}) by *At. ferrooxidans*

The ferrous iron oxidation by isolated strain of *At. ferrooxidans* was conducted in shake flasks containing iron liquid medium ($9KFe^{2+}$) containing 160 Mm $FeSO_4$ of pH-value of 1.5. It was observed that ferrous iron (Fe^{2+}) was completely oxidized to ferric iron (Fe^{3+}) by the isolated strain of *At. ferrooxidans* during 3-5 days of incubation time at 30°C and 150 rpm (Fig. 1, 2). In chemical control flasks, only a negligibe amount of ferrous iron was oxidized due to air-oxidation under the same experimental condition (Fig. 13, 14). After the gram's staining different biochemical activities were analysed for the both Glu^+ and $Thio^+$ (*Acidithiobacillus thiooxidans*).

These activities were

(1) Starch hydrolysis

In case of $Thio^+$ the medium color was blue-black, indicated the absence of starch-splitting enzymes and represented a negative result while the in case of Glu^+ the starch was hydrolyzed, and a clear zone of hydrolysis arround the growth of organism, showed a positive result (Fig. 8, 9).

(2) Gelatin hydrolysis

In cases of *Thio*$^+$ the gelatin medium remained solid on refrigeration at 4 ^{0}C indicating the lack of gelatinase while in case of *Glu*$^+$ vice versa (Fig. 16, 22).

(3) Hydrogen sulfide

The hydrogen sulfide gas is colorless and was not visible. In case of *Glu*$^+$ ferrous ammonium sulfate in the medium served as an indicater by combining with gas, forming an insoluble black ferrous sulfide precipitate which showed the production of H_2S. While in case of *Thio*$^+$ the absence of precipitate was the sign of a negative result (Fig. 17, 25).

(4) Catalase test

For both *Thio*$^+$ and *Glu,*$^+$ the catalase production was determined when the 3% H_2O_2 was added as a substrate to an appropriately incubated tripticase soya agar slant culture. Catalase was present and the chemical reaction mentioned was indicated by bubbles of free oxygen gas ($O_2\uparrow$). This was positive catalase test while the absence of bubble formation was a negative catalase test (Fig. 24).

(5) Urease test

When the organisms *Thio*$^+$ and *Glu*$^+$ were grown in the urea broth medium containing the pH indicator phenol red, the substrate urea was not split into its products, the presence of ammonia did not create an alkaline environment the phenol red did not turn to a deep pink. This was a negative reaction for the presence of urea (Fig. 18, 23).

(6) Indole production (A)

The absence of red coloration demonstrated that the substrate tryptophan was not hydrolysed by *Thio*$^+$ and *Glu*$^+$ indicated an indole negative reaction (Fig. 17, 19).

(7) Methyl red test (B)

In case of *Glu*$^+$ the Methyl red indicater in the pH range of 4 turned red and showed the presence of acid, which indicate of a positive result. While in case of *Thio*$^+$ with lower

Hydrogen ion concentration the indicator turned yellow and was a negative result (Fig. 19, 23).

(8) Voges proskauer (C)

In both the cases of *Thio*$^+$ and *Glu*$^+$ a deep rose color was not developed, 15 minutes after the addition of Barritt's reagent which did not indicated the presence of acetyl-methylcarbinol and represented a negative result (Fig. 18).

(9) Citrate utilization (D)

After incubation, citrate-positive *Glu*$^+$ was indicated by the presence of growth on the surface of the slant, accompanied by blue coloration. It was shown that *Glu*$^+$ has used citrate as a carbon source. While the *Thio*$^+$ slant color was not changed which indicated that the citrate was not used as a carbon source by *Thio*$^+$ and the result was negative (Fig. 20, 26).

(10) Triple sugar iron

In case of *Thio*$^+$ the color change showed the carbohydrate fermentation activity and the result was positive while in case of *Glu*$^+$ the color was not changed showing that the carbohydrate fermentation has not taken place and the result was negative (Fig. 15, 21).

TEST PERFORMED	*Glu*$^+$	*Thio*$^+$
Starch hydrolysis	+	-
Gelatin hydrolysis	-	+
Hydrogen sulfide test	+	-
Catalase test	+	+
Urease test	-	-
Indole production (A)	-	-
Methyl red test (B)	+	-
Voges proskauer test (C)	-	-
Citrate utililzation test	+	-
Triple sugar iron	-	+

Bergey's Manual of Determinative Bacteriology

Effect of Carbon sources on growth of bacteria

(1) Glucose

The growth pattern of *Glu*$^+$ at 1% glucose concentration in 9k medium indicated that glucose was utilized as carbon source and growth of the isolate was maximum after 48 hours of incubation, stationary phase (Fig. 27). By further incubation the growth decreased after 120 hours (Decline phase). While the *Thio*$^+$ on 1% glucose (9k medium) showed maximum growth after 96 hours of incubation (Fig. 28).

(2) Sucrose

The result indicated that the *Glu*$^+$ has started growth in 1% sucrose (9k medium) after 24 hours of incubation (lag phase). By further incubation at 24 hours intervals up to 120 the growth was increased during log and stationary phases (Fig. 29). The growth of *Thio*$^+$ on the above mentioned medium, after incubation for 24 hours the growth started (lag phase) first in lag phase up to 48 hours. After 96 hours of incubation it was stationary phase growth (Fig. 30).

(3) Fructose

On 1% fructose concentration (9k medium) the growth of *Glu*$^+$ reached stationary phase after 72 hours of incubation. Further incubation up to 120 hours showed the (decline phase) (Fig. 31). While the *Thio*$^+$ on 1% fructose (9k medium) after incubation of 72 hours has optimum growth and after the 96 hours the growth was at peak (Fig. 32). In both cases the phase was stationary as mentioned before.

(4) Raffinose

The growth of *Glu*$^+$ on 1% raffinose (9k medium) after 48 hours of incubation, showed lag phase up to 72 hours. By further incubation up to 96 hours the log phase started where the growth reached to maximum (Fig. 33). In the case of *Thio*$^+$ started the growth after incubation of 120 hours on same medium (stationary phase), (Fig. 34).

(5) D-sorbitol

The growth of *Glu*$^+$ on 1% D-sorbitol (9k medium) started after 24 hours of incubation. The incubation was continued up to 144 hours showing (log, stationary and decline) phases (Fig. 35). In case of *Thio*$^+$ the growth on the same medium was started at 24 hours and peaked after 72, 96 hours (log and stationary) phases (Fig. 36).

(6) Galactose

The results showed that the growth on (mineral salt medium) containing Galactose 1% by the *Glu*$^+$ started after incubation of 24 to 216 hours passing through all lag, log, stationary and decline phases (Fig. 37). While in case of *Thio*$^+$ the growth on same medium was maximum after 96 hours incubation and immediately dropped (Fig. 38).

(7) Lactose

On lactose 1% mineral salt or 9k medium the *Glu*$^+$ started growth after the incubation of 120 hours (stationary) phase, the growth was maintained up to 192 hours. By further incubation the (death phase) started (Fig. 39). While on the same medium *Thio*$^+$ has started growth after 24 hours incubation passing through (lag, log, stationary and decline) phases (Fig. 40).

(8) Maltose

The growth pattern of *Glu*$^+$ at 1% maltose concentration in 9k medium showed clear results, indicated that maltose was utilized as carbon source and growth of the isolate was maximum at 144 hours of incubation. The growth shows both (log and stationary) phases. More incubation was showed the more growth and there was no death phase (Fig. 41). While the *Thio*$^+$ on the above mentioned medium shows all four Phases (Fig. 42).

(9) Rahammanose

The result showed that the *Glu*$^+$ has grown on 1% rahammanose (9k medium) after the incubation of 48 hours. The graphic result passes all the stages of growth (Fig. 43). While in case of *Thio*+ the result is different in which only stationary and death phases after the incubation in same medium (Fig. 44).

(10) Mannose

The growth pattern of *Glu*$^{+}$ on 1% mannose (mineral salt medium) indicated that the growth has been started after the incubation of 48 hours. On the incubation up to 216 hours. Three growth phases have been showed. In stationary phase the growth was on peak. There was no death phase (Fig. 45). Otherwise the *Thio*$^{+}$ on the same medium showed all growth phases (Fig. 46).

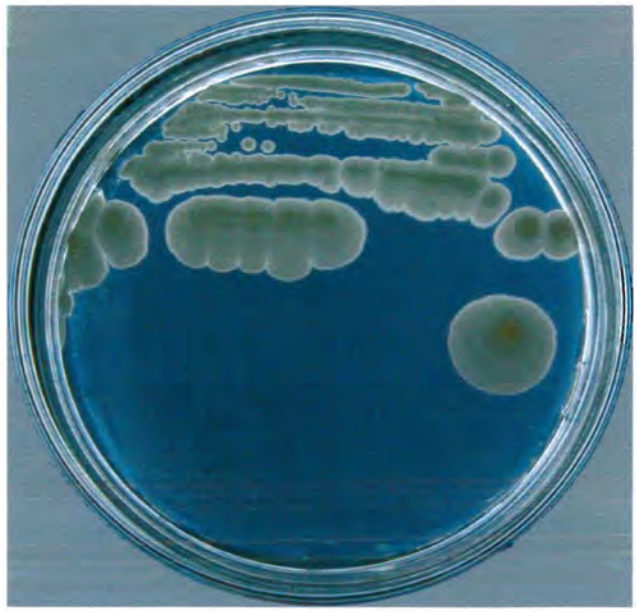


Fig. 6. Colonies of glucose oxidizing bacteria

Fig. 7. Colonies of thiosulfate oxidizing bacteria

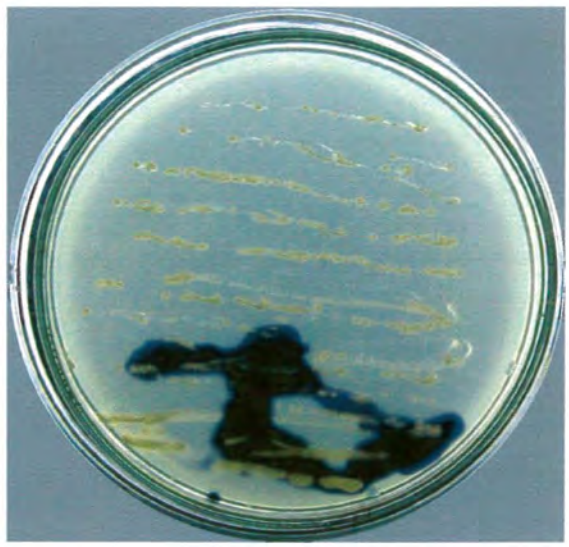


Fig. 8. Starch hydrolysis by *Glu*$^{+}$

Fig. 9. Stach hydrolysis by *Thio*$^{+}$

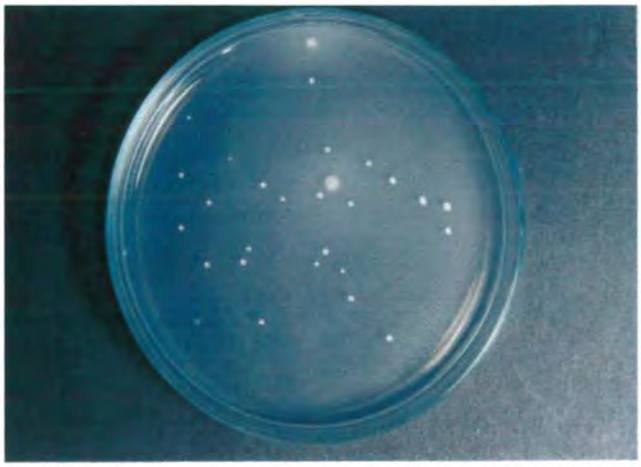


Fig. 10. Oxidation of elemental sulphur by *Acidithiobacillus thiooxidans.*

Fig. 11. Coversion of Fe^{2+} into Fe^{3+}
by *Acidithiobacillus ferrooxidans* at 30^0C, 150 rpm.

Fig. 12. Lowering of pH activity of *Acidithiobacillus thiooxidans* at 30^0C, 150 rpm.

Fig. 13. *Glu*$^+$ bacteria after gram's staining.

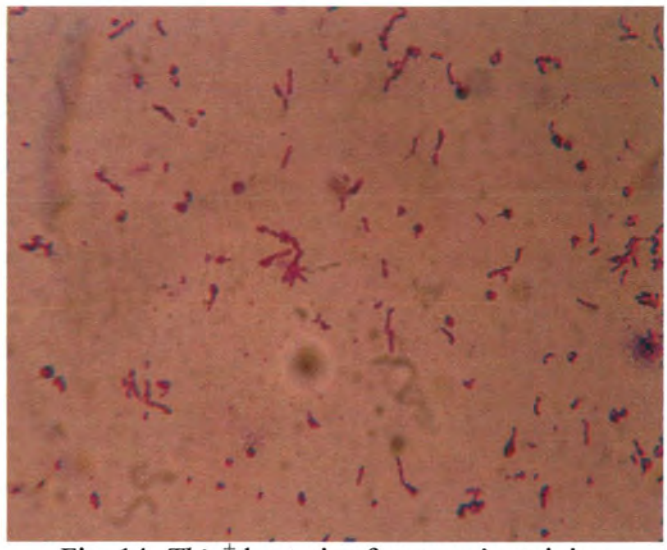


Fig. 14. *Thio*$^{+}$ bacteria after gram's staining

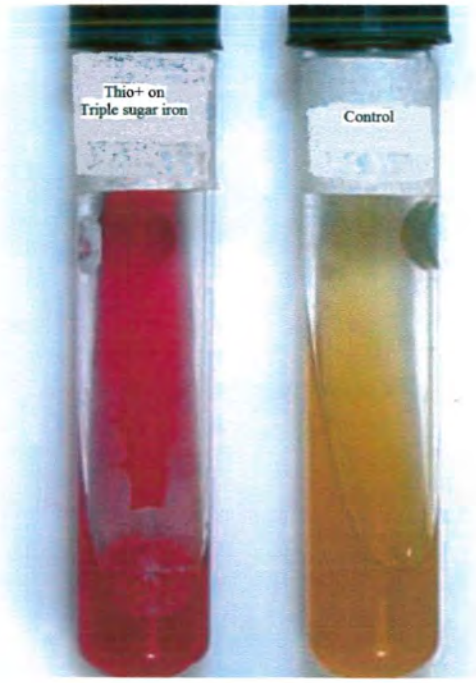


Fig. 15. Triple sugar iron test for *Thio*$^{+}$ bacteria.

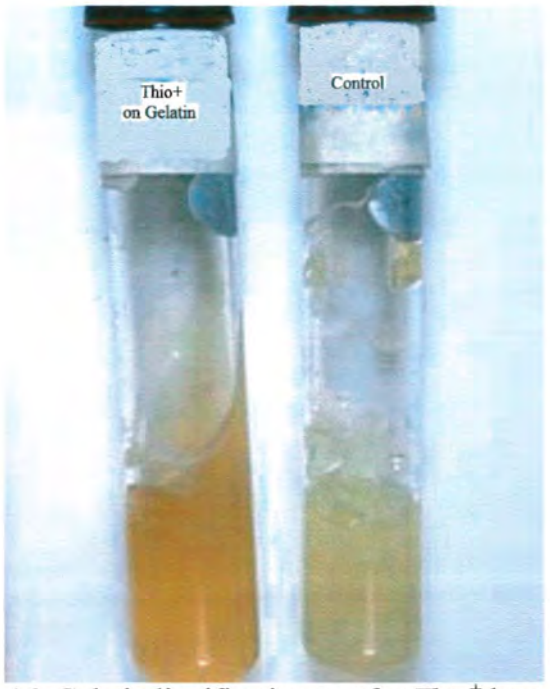


Fig. 16. Gelatin liquification test for *Thio*$^{+}$ bacteria.

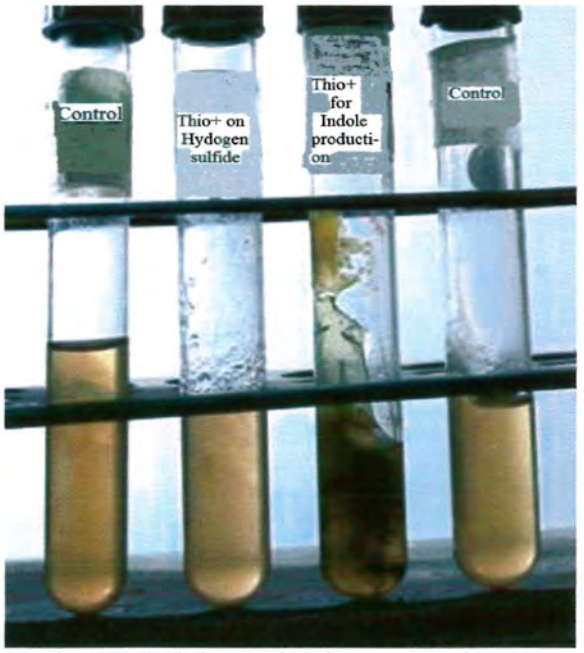


Fig. 17. Indole production and hydrogen sulfide tests for *Thio*$^{+}$ bacteria.

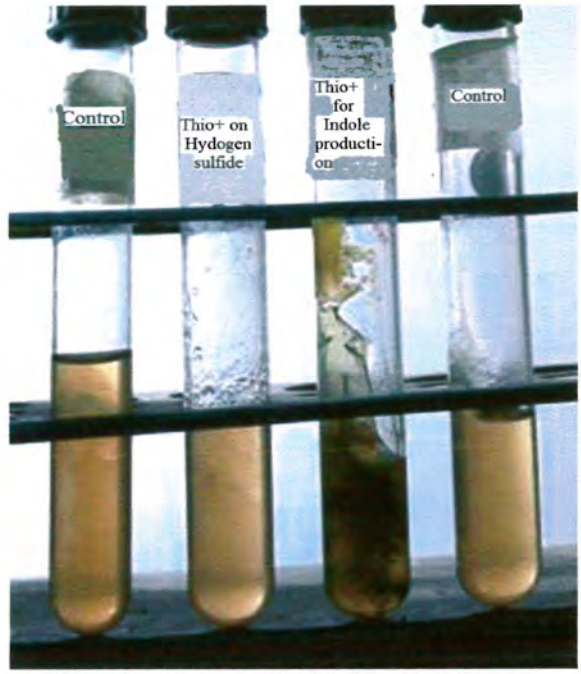


Fig. 18. Urease and Voges-Proskauer tests for *Thio*$^{+}$ bacteria.

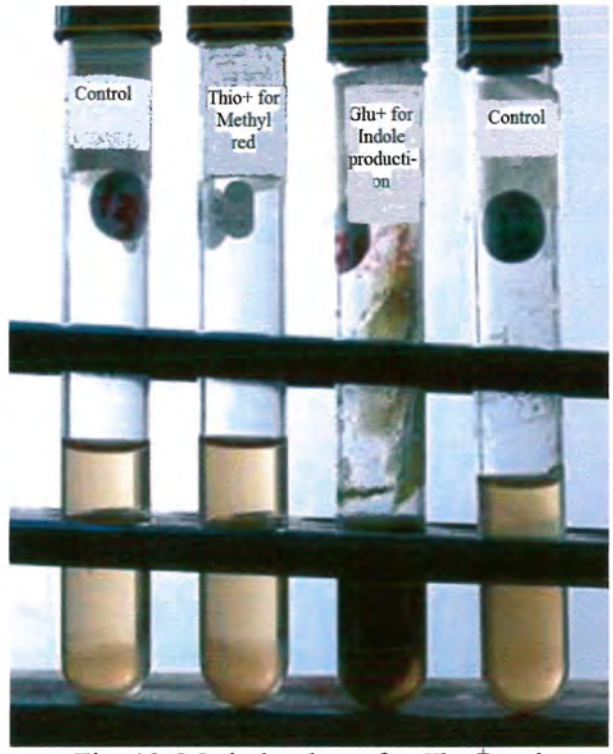


Fig. 19. Methyl red test for *Thio*$^{+}$ and indole production for *Glu*$^{+}$ bacteria.

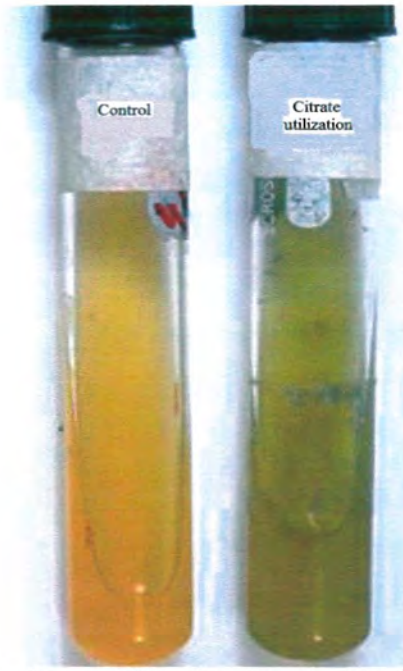


Fig. 20. Citrate utilization test for *Thio*$^{+}$ bacteria.

Fig. 21. Triple sugar iron test for *Glu*$^{+}$ bacteria.

Fig. 22. Gelatin liquification test for *Glu*$^{+}$ bacteria.

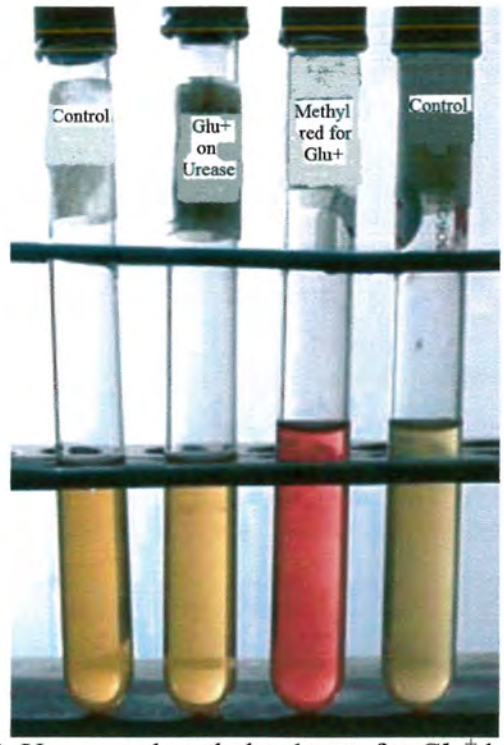


Fig. 23. Urease and methyl red tests for *Glu*$^{+}$ bacteria.

Fig. 24. Catalase test for *Glu*$^{+}$ bacteria.

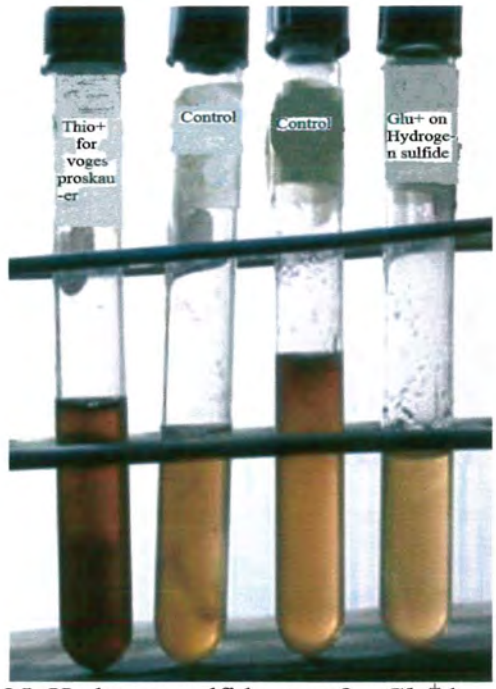


Fig. 25. Hydrogen sulfides test for *Glu*$^{+}$ bacteria.

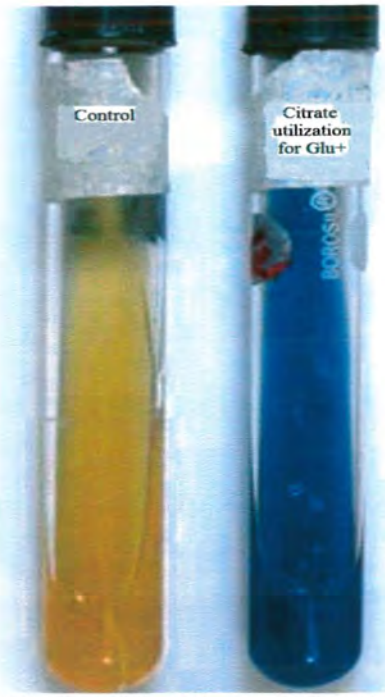


Fig. 26. Citrate utililazation test for *Glu*$^{+}$ bacteria.

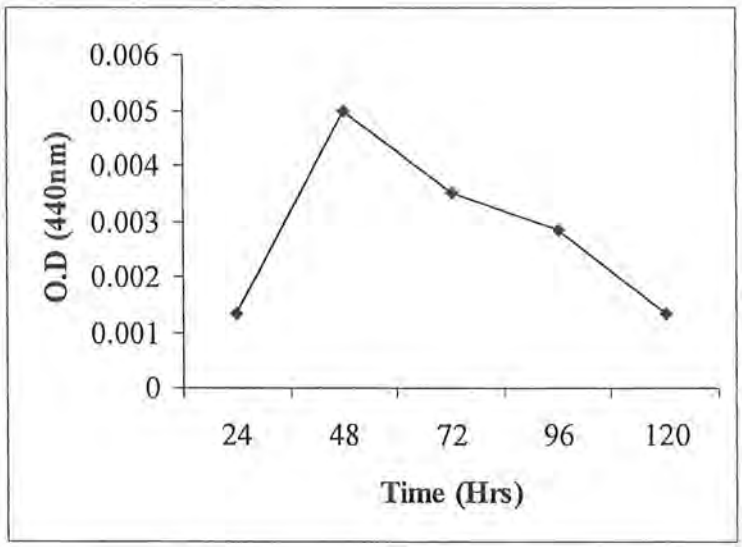


Fig. 27. Growth of *Acidithiobacillus thiooxidans Glu*$^{+}$ on glucose.

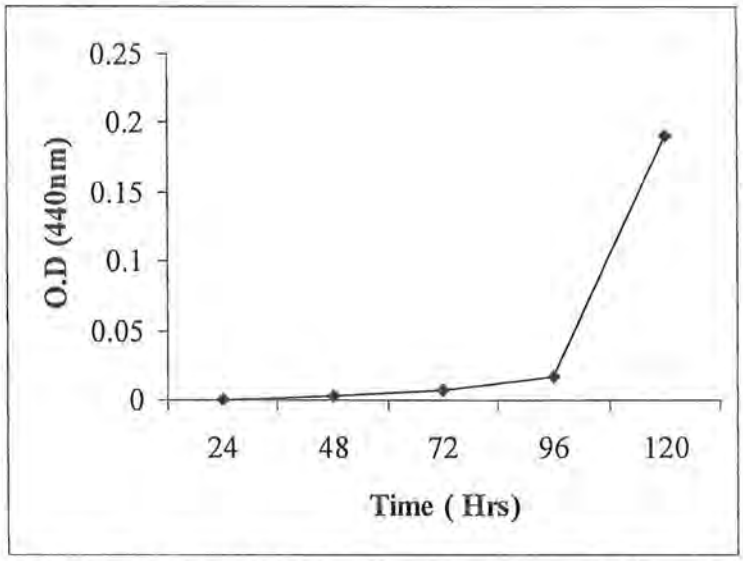


Fig. 28. Growth of *Acidithiobacillus thiooxidans Thio*$^{+}$ on glucose.

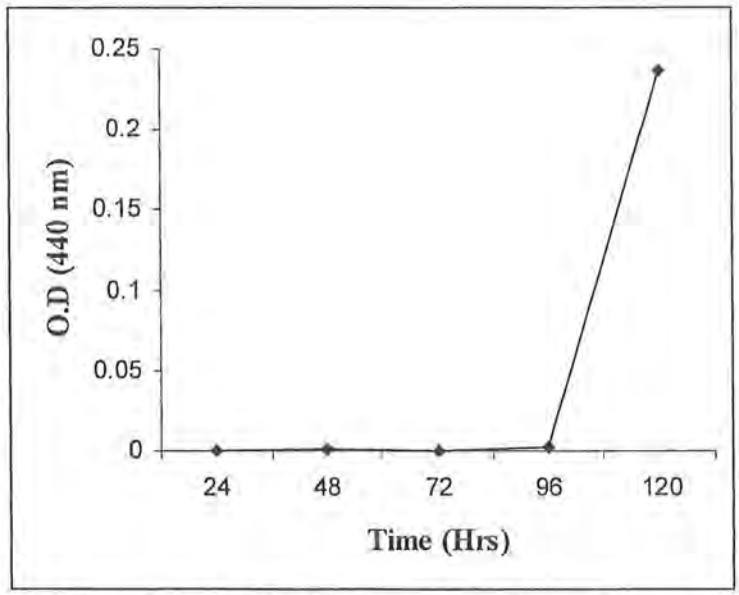


Fig. 29. Growth of *Acidithiobacillus thiooxidans Glu*$^{+}$ on sucrose.

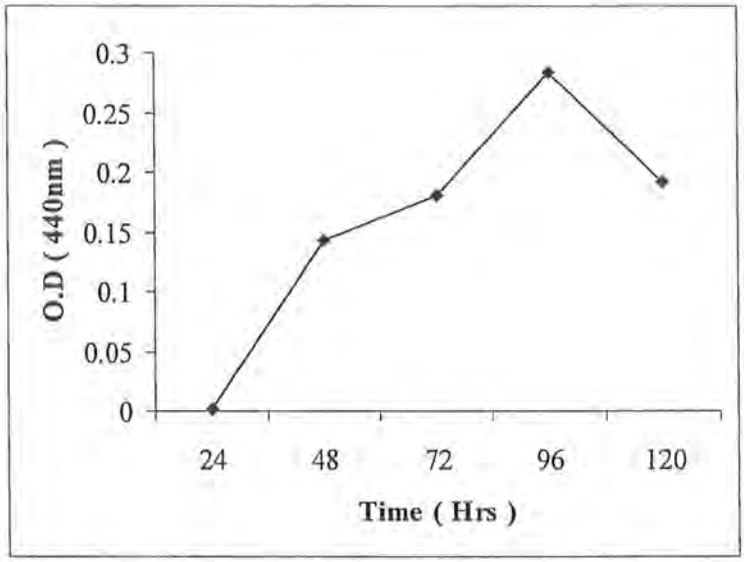


Fig. 30. Growth of *Acidithiobacillus thiooxidans Thio*$^{+}$ on sucrose.

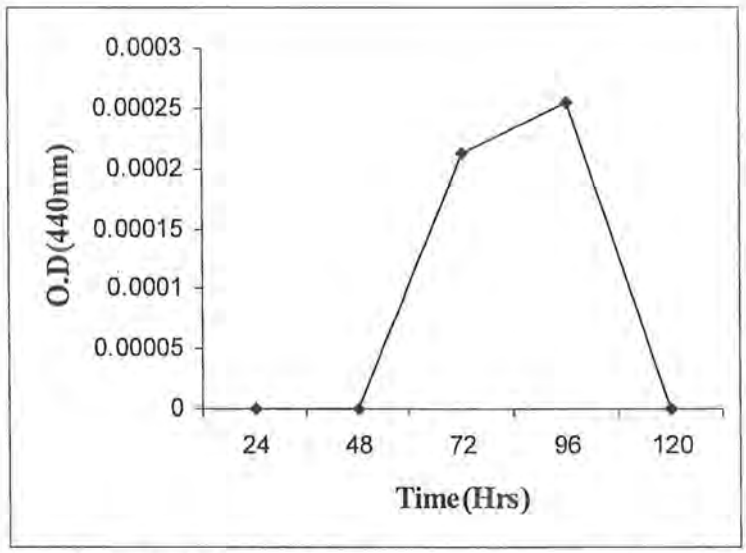


Fig. 31. Growth of *Acidithiobacillus thiooxidans Glu*$^{+}$ on fructose.

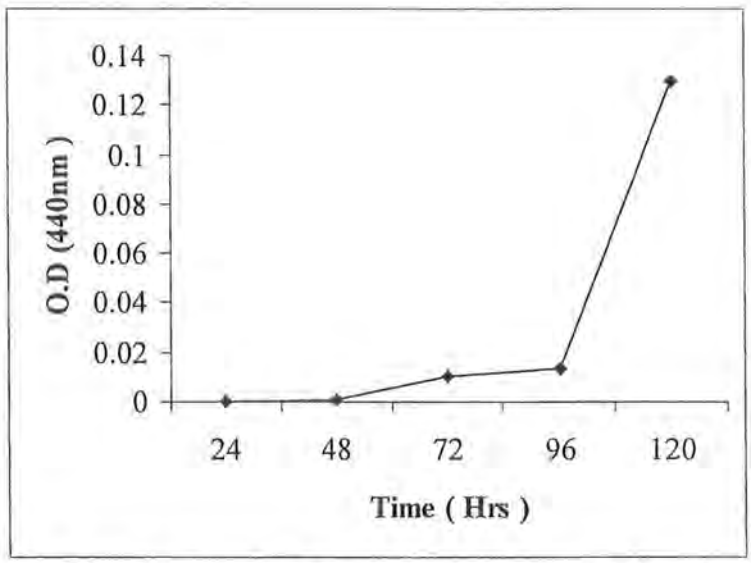


Fig. 32. Growth of *Acidithiobacillus thiooxidans Thio*$^{+}$ on fructose.

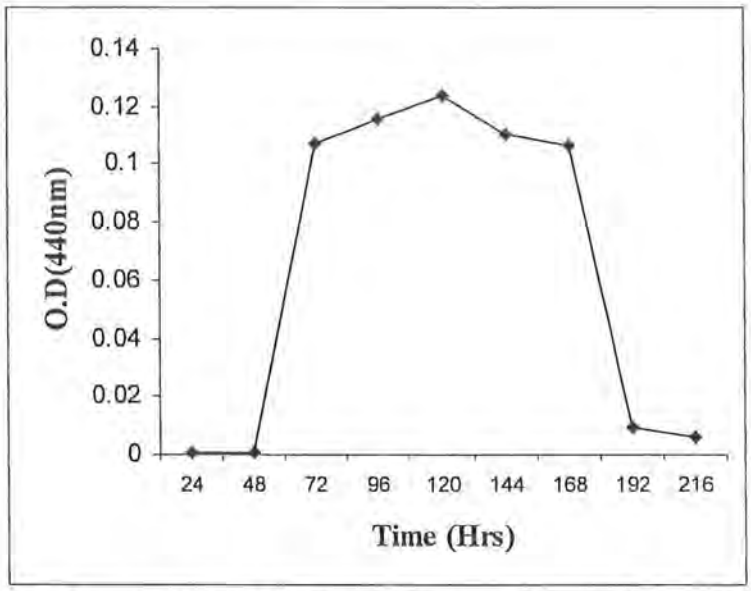


Fig. 33. Growth of *Acidithiobacillus thiooxidans Glu*$^{+}$ on raffinose.

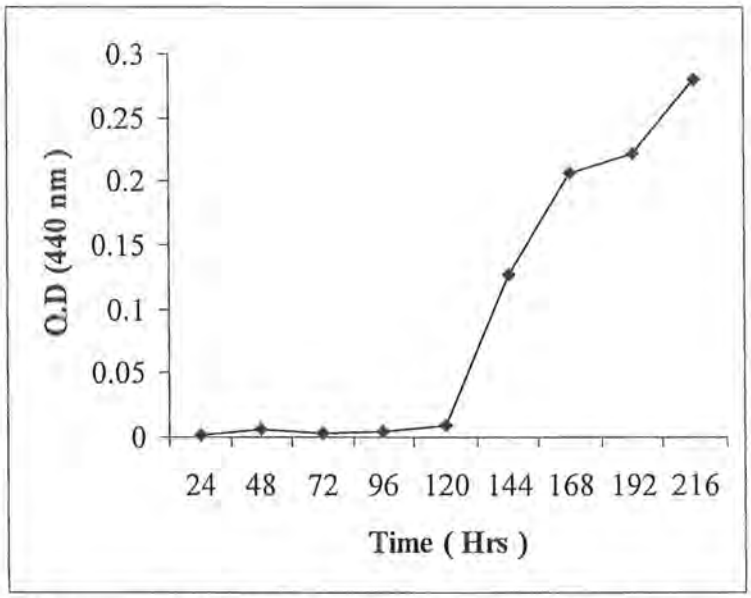


Fig. 34. Growth of *Acidithiobacillus thiooxidans Thio*$^{+}$ on raffinose.

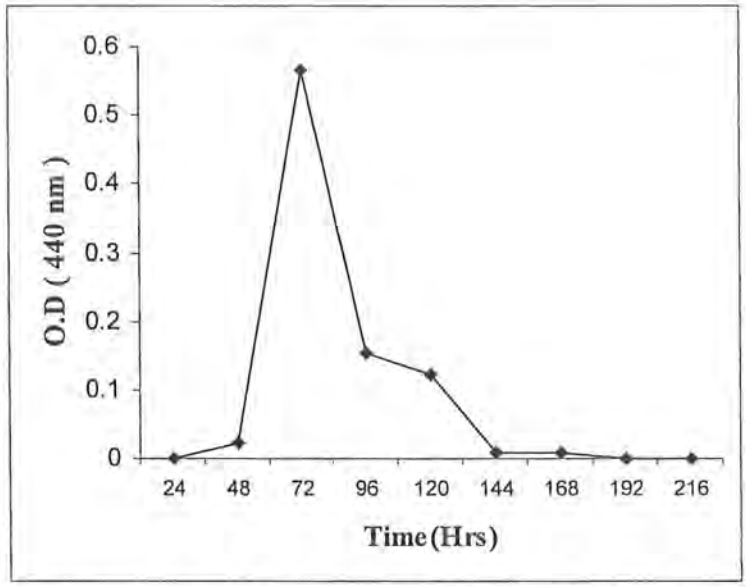


Fig. 35. Growth of *Aidithiobacillus thiooxidans Glu*$^{+}$ on D-sorbitol.

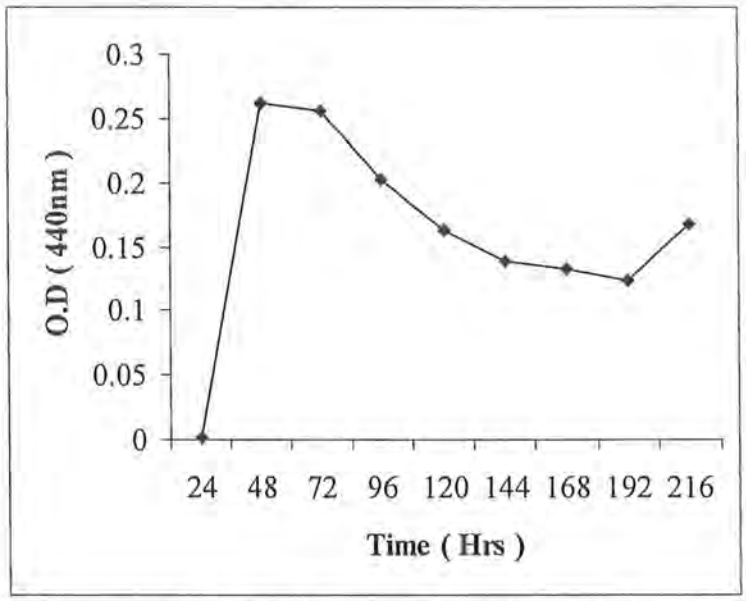


Fig. 36. Growth of *Acidithiobacillus thiooxidans Thio*$^{+}$ on D-sorbitol.

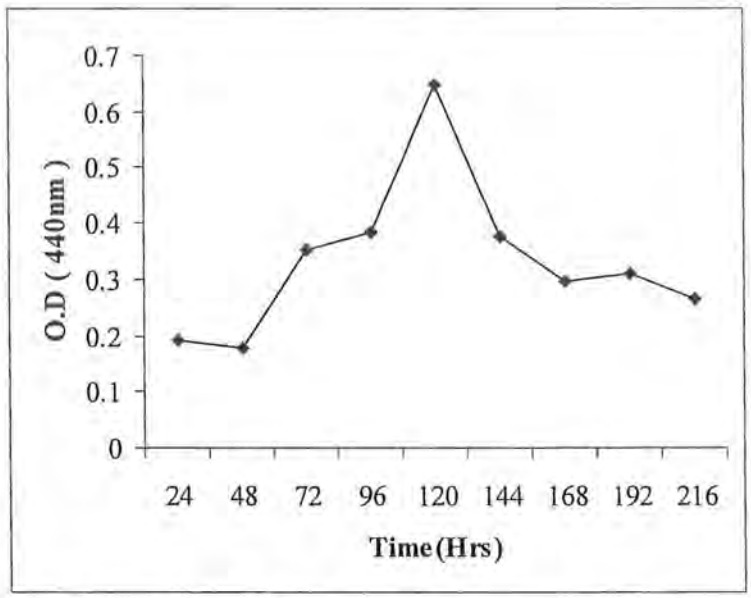


Fig. 37. Growth of *Acidithiobacillus thiooxidans Glu*$^{+}$ on galactose.

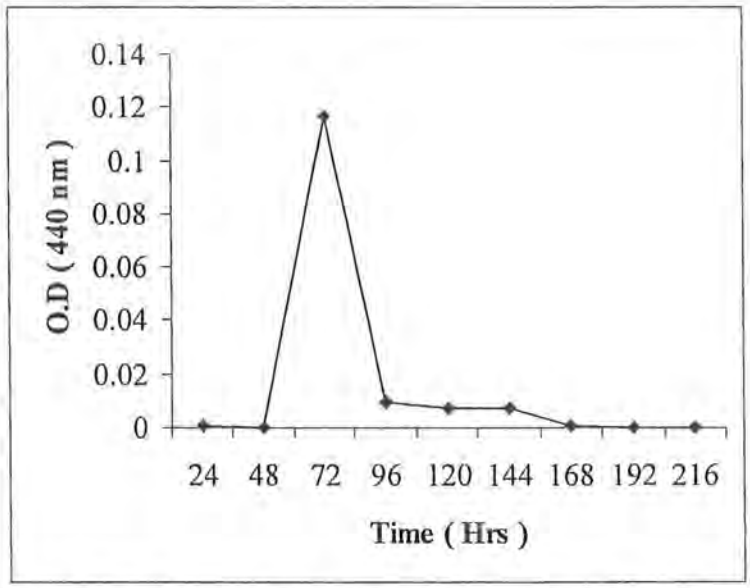


Fig. 38. Growth of *Acidithiobacillus thiooxidans Thio*$^{+}$ on galactose.

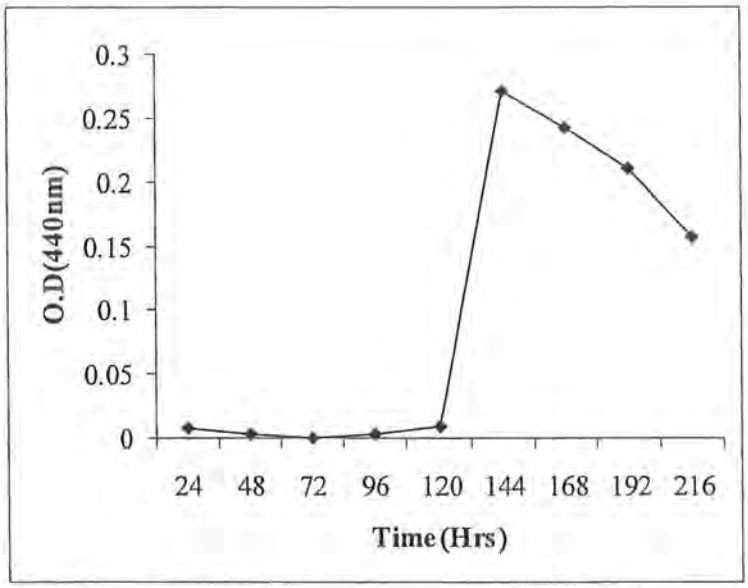


Fig. 39. Growth of *Acidithiobacillus thiooxidans Glu*$^{+}$ on lactose.

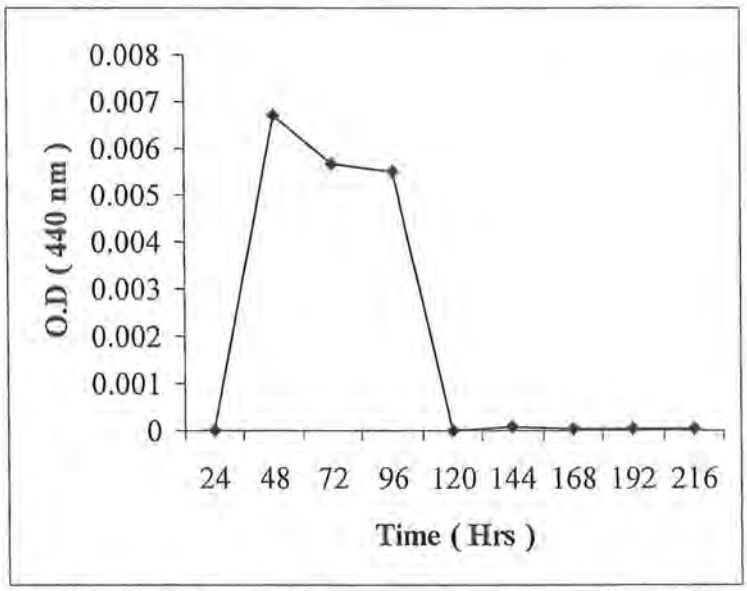


Fig. 40. Growth of *Acidithiobacillus thiooxidans Thio*$^{+}$ on lactose.

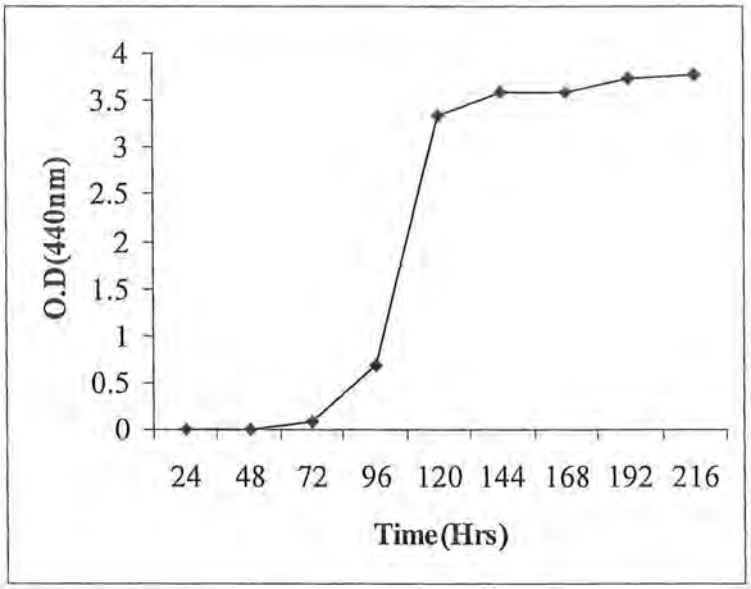


Fig. 41. Growth of *Acidithiobacillus thiooxidans Glu*$^{+}$ on maltose.

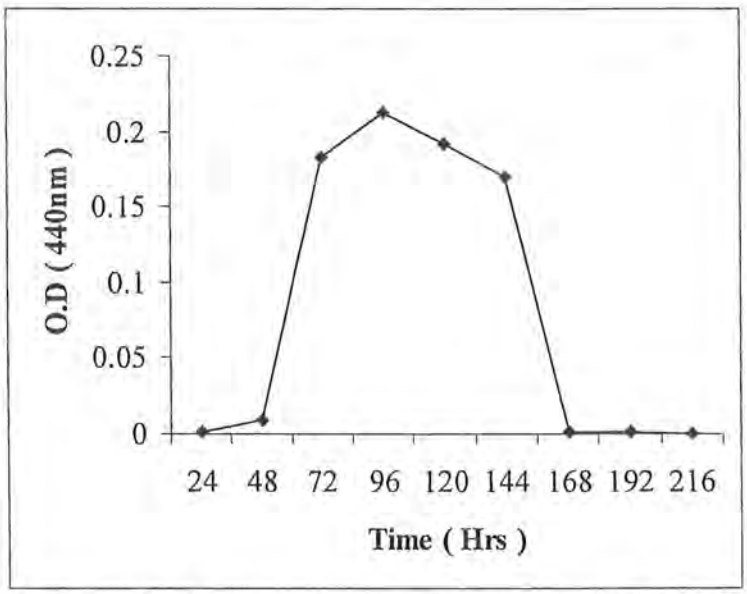


Fig. 42. Growth of *Acidithiobacillus thiooxidans Thio*$^{+}$ on maltose.

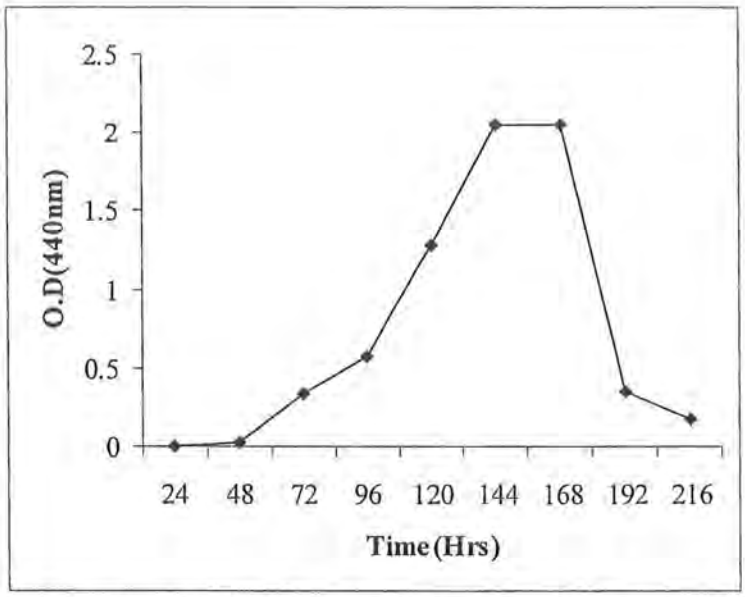


Fig. 43. Growth of *Acidithiobacillus thiooxidans Glu*$^{+}$ on rhammanose.

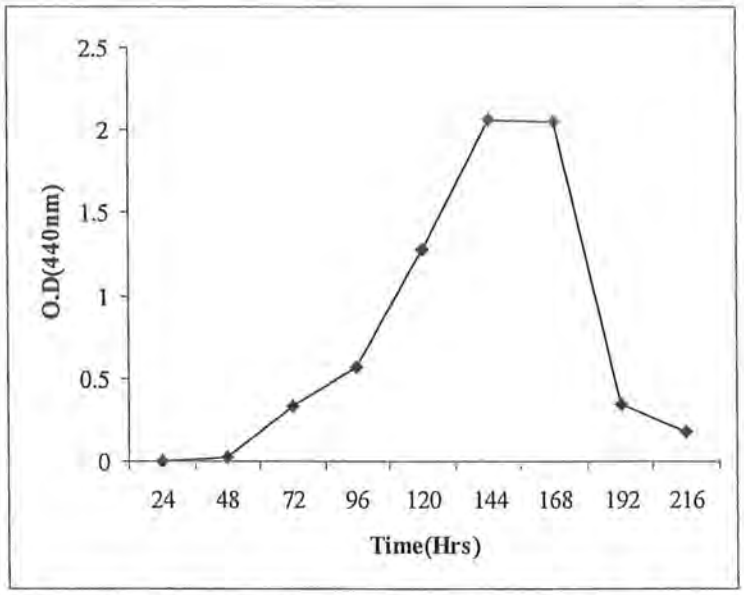


Fig. 44. Growth of *Acidithiobacillus thiooxidans Thio*$^{+}$ on rhammanose.

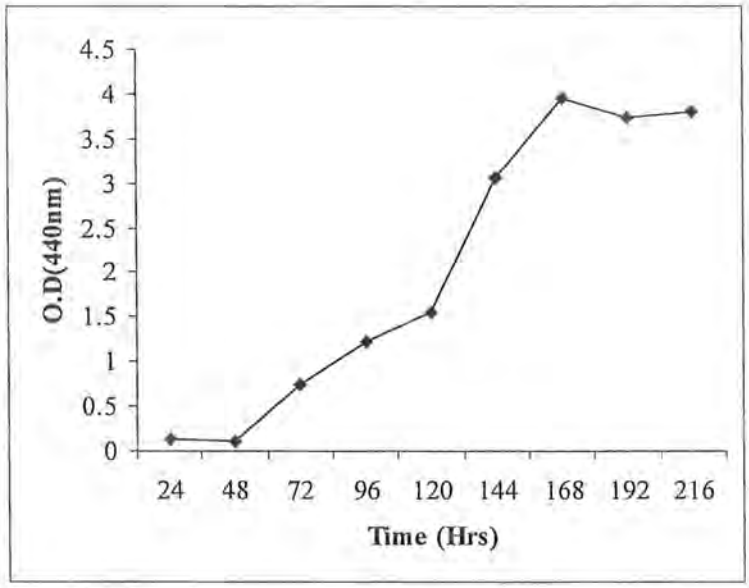


Fig. 45. Growth of *Acidithiobacillus thiooxidans Glu*$^{+}$ on mannose.

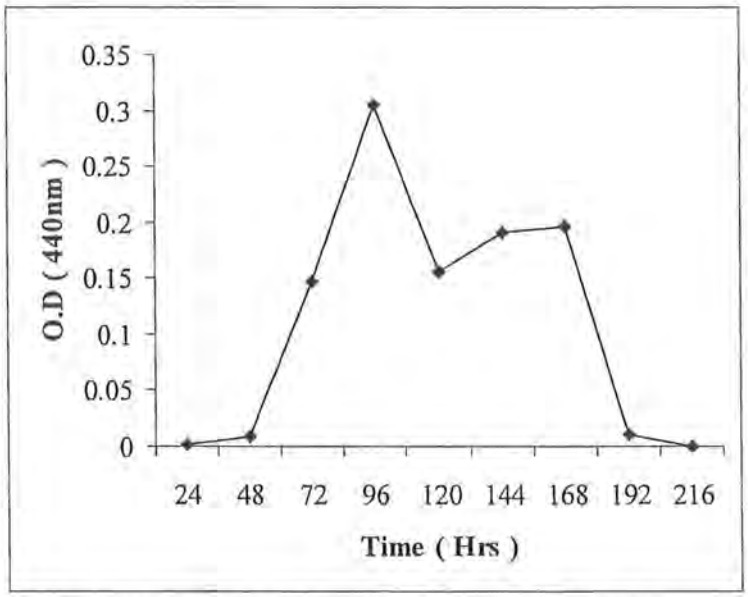


Fig. 46. Growth of *Acidithiobacillus thiooxidans Thio*$^{+}$ on mannose.

DISCUSSION

Discussion

Indigenous *Acidithiobacillus* were isolated from the soil (black shale) and water samples. In the tailing of soil and water samples microbial populations of *At. ferrooxidans*, *At. thiooxidans* and glucose-oxidizing heterotrophs were detected. Similar results have been reported by (Tuovinen *et al.*, 1981).

The isolate oxidized Fe^{2+}, pyrite, sulfur and reduced sulfur compounds to sulfuric acid and acidic ferric/ metal sulfate (Fig. 10 and 11). Different solid media have been used for the isolation and enumeration of *At. ferrooxidans* (Kelly *et al.*, 1972).

Solid medium for simultaneous growth of *At. ferrooxidans* and acidiphilic heterotrophs have been used. A new efficient Gelrite-FeSO4 solid medium was developed and successfully employed for isolation and enumeration of *At. ferrooxidans* in mine water, tailings liquid (Khalid *et al.*, 1993).

Dark reddish-brown and circular colonies which were robust and well differentiated, developed on Gelrite-$FeSO_4$ medium within 72-96 hours. Sulfur-oxidizing strain designated as *At. thiooxidans* was morphologically similar to iron- and sulfur-oxidizing bacterium (*At. ferrooxidans*) except that it did not oxidize Fe^{2+}. The cells of *At. thiooxidans* were motile, rod-shaped and closely resembled to *At. thiooxidans*. Off-white and very tiny colonies developed on thiosulfate medium, with a characteristic smell of elemental sulfur (Fig. 10). The growth of *At. ferrooxidans* was indicated by the drop in initial pH of the liquid media of sulfur, slag and pyrite, and by the acid production and/ or oxidation of ferrous to ferric (Fig. 11 and 12).

Both the strains of *At. ferrooxidans* and *At. thiooxidans* were found in abundance in tailings of water and liquid samples. The microbial population of iron-oxidizing bacteria (*At. ferrooxidans*) was found much higher in the black shale residues which were collected from one and two month old-dried tailings pile. In fact, tailings liquid contained a significant amount of $FeSO_4$ at the time when solid tailings residue was being dumped in the tailings pond of the mill after acid leaching of sandstone ore.

Elemental sulphur can be utilized as an energy source by iron and sulphur oxidizing bacteria to produce H_2SO_4 resulting in a pH drop of the tailings residue. *At. ferrooxidans* can also oxidize elemental sulfur in the presence of $Fe_2 (SO_4)_3$ at low pH (Prank *et al.*, 1992).

Sulfuric acid thus produced can change the physical and chemical characteristics of tailing residues. The presence of glucose-oxidizing heterotrophs was also noted in tailings (black shale) liquid, microbial leach liquors and solid samples obtained from columns and heap. Microbial leaching is a biochemical process involving enzymes as catalyst by which insoluble inorganic substrate is oxidized to a soluble form (Torma *et al.*, 1977).

Metals are released from sulfide minerals directly through oxidative metabolism of microorganisms or solubilized indirectly by chemical oxidants such as ferric sulfate or sulfuric acid produced as metabolic products of microorganisms (Lundgren *et al.*, 1986). In the present studies on bacterial oxidation of ferrous iron, it was observed that ferrous iron was completely oxidized to ferric iron by *At. ferrooxidans* during 72 hrs of incubation (Fig. 11). Bacterial oxidation of pyrite by *At. ferrooridans* resulted in sulfuric acid production followed by a drop in the initial pH of the leaching system (Fig. 12). *At. ferrooxidans* oxidizes ferrous iron at much faster rates as compared to a solely chemicals system. Sulfuric acid thus produced during the bacterial oxidation of sulfides, thereby accelerates the rate of metal solubilization. Sulfuric acid also neutralizes carbonate materials like calcite ($CaCO_3$) and dolomite (Ca, Mg) CO_3 present in the ore matrix (Bhatti *et al.*, 1991a).

Incomplete oxidation of the sulfide entity commonly occurs in the acid leaching process which results in the formation of polythionates and the precipitation of elemental sulfur. The latter effectively coats the metal sulfides and prevents their further oxidation until the sulfur is removed by bacterial oxidation. The microbial degradation of silicate minerals requires the availability of external energy substrates (Ralph *et al.*, 1985).

Different biochemical activities of the isolates were performed. Starch hydrolysis test was performed in the case of *Thio*$^+$ strain, where as *Glu*$^+$ hydrolyzed the starch and indicated a positive result. This showed that *Glu*$^+$ was better hydrolyser of starch than *Thio*$^+$ (Fig. 8 and 9). Both the isolates were not able to hydrolyzed the gelatin (Fig. 16 and 22). *Glu*$^+$ showed the production of hydrogen sulfide, by forming an insoluble black ferrous sulfide while in case of *Thio*$^+$, the absence of precipitate was the sign of negative result (Fig. 17 and 25). Both the isolates were able to hydrolyze catalase as shown by the production of oxygen bubbles (Fig. 24).

Both the isolates were urease negative for production (Fig. 18 and 23). Isolates did not produce a red reagent in both the cases of *Thio*$^+$ and *Glu*$^+$. The absence of red coloration demonstrated that the substrate tryptophan was not hydrolysed and indicated an indole negative reaction (Fig. 17 and 19). In methyl red test, the *Glu*$^+$ strain by the presence of acid indicated positive result. While the *Thio*$^+$ result was negative (Fig. 19 and 25). In both *Thio*$^+$ and *Glu*$^+$ a deep rose color developed which indicated the presence of acetyl-methylcarbinol and represented a positive result for voges proskauer test (Fig. 18). Citrate-positive *Glu*$^+$ was indicated by the presence of growth on the surface of the slant. Which was accompanied by blue coloration and showed that *Glu*$^+$ has used citrate as a carbon source. While in case of *Thio*$^+$ slant color, was not changed, which showed that the result was negative (Fig. 20 and 25). In case of *Thio*$^+$, the color change showed the carbohydrate fermentation activity and the result was positive while in case of *Glu*$^+$ the color was not changed showing that the carbohydrate fermentation has not taken place and the result was negative in triple sugar iron (Fig. 15 and 21). Similar results were reported by (Cappuccino *et al.*, 2005).

The growth pattern of *Glu*$^+$ in the presence of 1% glucose in 9k medium indicated that glucose was utilized as carbon source and growth of the isolate was maximum at 48 hours of incubation. Where as the strain *Thio*$^+$, on 1% glucose (9k medium) started the growth after 72 hours of incubation and after the 96 hours the growth was at peak. For *Thio*$^+$ the glucose was found to be good energy source as compared to that in case of *Glu*$^+$ strain (Fig. 27 and 28).

The result indicated that the *Glu*$^+$ has started growth in the presence of 1% sucrose (9k medium) after 24 hours of incubation. The growth of *Thio*$^+$ on the same medium, after the incubation of 48 hours showed that for both the sucrose was good for growth (Fig. 29 and 30).

In the presence of 1% fructose (9k medium) the *Glu*$^+$ was growed after 72 hours incubation. Where as *Thio*$^+$ strain on 1% fructose (9k medium) after the 96 hours of incubation, the growth was at peak. For *Thio*$^+$ the Fructose was found to be good energy source as compared to that in case of *Glu*$^+$ strain (Fig. 31 and 32).

The strain *Glu*$^+$ growth in the presence of 1% raffinose (mineral salt medium) after 48 hours of incubation was at peak. Where as *Thio*$^+$ has started growth after the incubation of 120 hours on same above medium. On comparison the growth of *Thio*$^+$ was found to be better on raffinose than in case of *Glu*$^+$ strain (Fig. 33 and 34).

The growth of *Glu*$^+$ strain in the presence of 1% D-sorbitol (9k medium) was maximum after 144 hours of incubation. Where as in the case of strain *Thio*$^+$ on the same medium the growth was at peak 96 hours of incubation. It was concluded that *Glu*$^+$ utilized D-sorbitol as a good energy source as compared to strain *Thio*$^+$ (Fig. 35 and 36).

The results indicated that on (mineral salt medium) galactose 1% the growth of strain *Glu*$^+$ after the incubation of 216 hours was maximum. While in case of strain *Thio*$^+$ the growth on same medium shoot up on 96 hours of incubation and immediately dropped. This indicated that the *Glu*$^+$ strain utilized best galactose as a carbon source as compared to that in case of *Thio*$^+$ strain (Fig. 37 and 38).

In the presence of lactose 1% (mineral salt medium) the strain *Glu*$^+$ started growth after the incubation of 120 hours and was maximum after 192 hours. Where as on the same medium strain *Thio*$^+$ growth was maximum after 24 hours incubation. The conclusion was that *Thio*$^+$ strain was found to be better utilizer of Lactose as a carbon source than strain *Glu*$^+$(Fig. 39 and 40).

In the presence of 1% maltose in 9k medium the strain *Glu*$^+$ indicated clear results, that maltose was utilized as carbon source and growth of the isolate was maximum at 144 hours of incubation. Where as the strain *Thio*$^+$ on the same medium indicated maximum growth after 120 hours of incubation. From this it was concluded that *Glu*$^+$ strain was found to be best utilizer of maltose as an energy source as compared to that in case of *Thio*$^+$ strain (Fig. 41 and 42).

Result indicated that the strain *Glu*$^+$ was grown in the presence of 1% rhammanose (9k medium) after the incubation of 48 hours. Where as the result of *Thio*$^+$ was different in which after the incubation of 144 hours, the growth was at peak. This indicated that on rhammanose the *Glu*$^+$ strain growth was found to be better than *Thio*$^+$ strain (Fig. 43 and 44).

The growth pattern of *Glu*$^+$ in the presence of 1% mannose (mineral salt medium) indicated that the growth after the incubation of 216 hours was maximum. Where as the *Thio*$^+$ strain on the same medium showed growth after 96 hours of incubation. The conclusion was that strain *Glu*$^+$ was found to be best utilizer of mannose as compared to that in case of *Thio*$^+$ strain (Fig. 45 and 46). Similar results were reported in (Berge, s manual *et al.*, 2005).

Conclusions

- *Acidithiobacillus thiooxidans* (sulphur oxidizing bacteria) and *Acidithiobacillus ferooxidans* (Iron oxidizing bacteria) were isolated from black shale.

- Both the strains were identified microscopically and biochemically.

- Various carbon sources were checked for growth of bacterial strains.

Future prospects

1. *Acidithiobacillus* other species like *Leptospirillum* can be extracted from black shale.
2. *Acidithiobacillus ferrooxidans* and *At. thiooxidans* can be can be used for the bioleaching of uranium and gold.
3. Molecular identification like 16s RNA can be used for *At. ferroxidans* and *At. thiooxidans.*

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APPENDIX

Tables of *Acidithiobacillus (Glu$^+$)* on different carbon sources

Serial NO	O.D (440 nm)	TIME (Hrs)
1	0.001316	24
2	0.004997	48
3	0.003512	72
4	0.002841	96
5	0.004193	120

Table. 1. Growth of *Acidithiobacillus thiooxidans* (*Glu*$^+$) on 1% glucose as an energy source at 440 nm optical density after 24 hours interval during five consecutive days.

Serial No	O.D (440 nm)	TIME (Hrs)
1	0	24
2	0.0034235	48
3	0.0072867	72
4	0.000037241	96
5	0.19134	120

Table. 2. Growth of *Acidithiobacillus thiooxidans* (*Thio*$^+$) on 1% glucose as an energy source at 440nm optical density after 24 hours interval during five consecutive days.

Serial No	O.D (440 nm)	TIME (Hrs)
1	0	24
2	0.001704	48
3	0	72
4	0.002196	96
5	0.23639	120

Table. 3. Growth of *Acidithiobacillus thiooxidans* (*Glu*$^+$) on 1% sucrose as an energy source at 440 nm optical density after 24 hours interval during five consecutive days.

Serial No	O.D (440 nm)	TIME (Hrs)
1	0.001713	24
2	0.14264	48
3	0.18097	72
4	0.28433	96
5	0.26996	120

Table. 4. Growth of *Acidithiobacillus thiooxidans* (*Thio*$^{+}$) on 1% sucrose as an energy source at 440 nm optical density after 24 hours interval during five consecutive days.

Serial No	O.D (440 nm)	TIME (Hrs)
1	0	24
2	0	48
3	0	72
4	0.000254	96
5	0	120

Table. 5. Growth of *Acidithiobacillus thiooxidans* (*Glu*$^{+}$) on 1% fructose as an energy source at 440 nm optical density after 24 hours interval during five consecutive days.

Serial No	O.D (440 nm)	TIME (Hrs)
1	0.000	24
2	0.0008698	48
3	0.0014500	72
4	0.000316	96
5	0.12966	120

Table. 6. Growth of *Acidithiobacillus thiooxidans* (*Thio*$^{+}$) on 1% fructose as an energy source at 440 nm optical density after 24 hours interval during five consecutive days.

Serial No	O.D (440 nm)	TIME (Hrs)
1	0.000734	24
2	0.000375	48
3	0.10697	72
4	0.11545	96
5	0.12358	120
6	0.1104	144
7	0.10627	168
8	0.009626	192
9	0.005814	216

Table. 7. Growth of *Acidithiobacillus thiooxidans* (*Glu*$^{+}$) on 1% raffinose as an energy source at 440 nm optical density after 24 hours interval during nine consecutive days.

Serial No	O.D (440 nm)	TIME (Hrs)
1	0.14922	24
2	0.00687	48
3	0.002738	72
4	0.004638	96
5	0.008871	120
6	0.12734	144
7	0.20626	168
8	0.22148	192
9	0.27981	216

Table. 8. Growth of *Acidithiobacillus thiooxidans* (*Thio*$^{+}$) on 1% raffinose as an energy source at 440 nm optical density after 24 hours interval during nine consecutive days.

Serial No	O.D (440 nm)	TIME (Hrs)
1	0.21647	24
2	0.021421	48
3	0.5661	72
4	0.15344	96
5	0.12259	120
6	0.00879	144
7	0.009065	168
8	0.1428	192
9	0.004506	216

Table. 9. Growth of *Acidothiobacillus thiooxidans* (*Glu*$^{+}$) on 1% D-sorbitol as an energy source at 440 nm optical density after 24 hours interval during nine consecutive days.

Serial No	O.D (440 nm)	TIME (Hrs)
1	0.23211	24
2	0.26231	48
3	0.2558	72
4	0.20217	96
5	0.1629	120
6	0.13934	144
7	0.1322	168
8	0.124	192
9	0.16744	216

Table. 10. Growth of *Acidithiobacillus thiooxidans* (*Thio*$^{+}$) on 1% D-sorbitol as an energy source at 440 nm optical density after 24 hours interval during nine consecutive days.

Serial No	O.D (440 nm)	TIME (Hrs)
1	0.19135	24
2	0.17611	48
3	0.35102	72
4	0.38479	96
5	0.64757	120
6	0.37633	144
7	0.29467	168
8	0.31000	192
9	0.26362	24

Table. 11. Growth of *Acidithiobacillus thiooxidans* (*Glu*$^{+}$) on 1% galactose as an energy source at 440 nm optical density after 24 hours interval during nine consecutive days.

Serial No	O.D (440 nm)	TIME (Hrs)
1	0.0071904	24
2	0.0018464	48
3	0.11681	72
4	0.0098103	96
5	0.0070980	120
6	0.0072303	144
7	0.10142	168
8	0.008600	192
9	0.13766	216

Table. 12. Growth of *Acidithiobacillus thiooxidans* (*Thio*$^{+}$) on 1% galactose as an energy source at 440 nm optical density after 24 hours interval during nine consecutive days.

Serial No	O.D (440 nm)	TIME (Hrs)
1	0.0070912	24
2	0.0024145	48
3	0.000056977	72
4	0.0031512	96
5	0.0083900	120
6	0.27077	144
7	0.24159	168
8	0.20972	192
9	0.15630	216

Table. 13. Growth of *Acidithiobacillus thiooxidans* (*Glu*$^{+}$) on 1% lactose as an energy source at 440 nm optical density after 24 hours interval during nine consecutive days.

Serial No	O.D (440 nm)	TIME (Hrs)
1	0.18805	24
2	0.0067266	48
3	0.0056634	72
4	0.0054804	96
5	0.000	120
6	0.0042654	144
7	0.0015377	168
8	0.0013507	192
9	0.0036098	216

Table. 14. Growth of *Acidithiobacillus thiooxidans* (*Thio*$^{+}$) on 1% lactose as an energy source at 440 nm optical density after 24 hours interval during nine consecutive days.

Serial No	O.D (440 nm)	TIME (Hrs)
1	0.0074301	24
2	0.0063735	48
3	0.085993	72
4	0.69364	96
5	3.33500	120
6	3.58330	144
7	3.58450	168
8	3.72910	192
9	3.79320	216

Table. I5. Growth of *Acidothiobacillus thiooxidans* (*Glu*$^{+}$) on 1% maltose as an energy source at 440 nm Optical density after 24 hours interval during nine consecutive days.

Serial No	O.D (440 nm)	TIME (Hrs)
1	0.18928	24
2	0.0084773	48
3	0.18231	72
4	0.21245	96
5	0.19117	120
6	0.16934	144
7	0.99686	168
8	0.18030	192
9	0.57805	216

Table. 16. Growth of *Acidithiobacillus thiooxidans (Thio*$^{+}$*)* on 1% maltose as an energy source at 440 nm Optical density after 24 hours interval during nine consecutive days.

Serial No	O.D (440 nm)	TIME (Hrs)
1	0.22787	24
2	0.22433	48
3	0.33156	72
4	0.57322	96
5	1.2788	120
6	2.05730	144
7	2.04680	168
8	0.34267	192
9	0.18024	216

Table. 17. Growth of *Acidithiobacillus thiooxidans* (*Glu*$^{+}$) on 1% rhammanose as an energy source at 440 nm optical density after 24 hours interval during nine consecutive days.

Serial No	O.D (440 nm)	TIME (Hrs)
1	0.11206	24
2	0.0074445	48
3	0.0094829	72
4	0.0095841	96
5	0.18174	120
6	0.05730	144
7	0.1109	168
8	0.11257	192
9	0.0083581	216

Table. 18. Growth of *Acidithiobacillus thiooxidans* (*Thio*$^{+}$) on 1% rhammanose as an energy source at 440 nm optical density after 24 hours interval during nine consecutive days.

Serial No	O.D (440 nm)	TIME (Hrs)
1	0.12652	24
2	0.11039	48
3	0.74809	72
4	0.14488	96
5	1.55176	120
6	3.06570	144
7	3.94850	168
8	3.72660	192
9	3.79350	216

Table. 19. Growth of *Acidithiobacillus thiooxidans* (*Glu*$^{+}$) on 1% glucose as an energy source at 440 nm optical density after 24 hours interval during nine consecutive days.

Serial No	O.D (440 nm)	TIME (Hrs)
1	0.13461	24
2	0.0086341	48
3	0.14731	72
4	0.30430	96
5	0.15634	120
6	0.19069	144
7	0.19569	168
8	0.11032	192
9	0.19134	216

Table. 20. Growth of *Acidithiobacillus thiooxidans* (*Thio*$^{+}$) on 1% mannose as an energy source at 440 nm optical density after 24 hours interval during nine consecutive days.