THE MICROBIOLOGY OF MINING LEACH LIQUOR REGENERATION

BY

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CONTENTS

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ABSTRACT

A method for monitoring the growth of Thiobacillus ferrooxidans independently of iron oxidation was developed. This method involved the analysis of protein using the Coomassie Blue protein-binding assay. It was found to be free from interference by the inorganic ions present in T. ferrooxidans culture medium.

The effect of ferrous iron concentrations and mixtures of ferrous and ferric irons on the rate of iron oxidation by T. ferrooxidans in batch culture was determined. The exponential iron oxidation rate was not affected by the ferric iron concentration *per se* but rather by the ferrous-ferric ratio.

The growth of T. ferrooxidans in the iron medium supplemented with different levels of glucose and yeast extract was investigated. T. ferrooxidans LT2, FD1 and ATCC 19859 strains were able to utilize both organic compounds up to concentrations of 0.1% but 0.5% of either glucose or yeast extract inhibited growth.

Acidophilic heterotrophic bacteria were isolated from LT2 and FD1 cultures of T. ferrooxidans after a stepwise adaptation to grow on glucose. An identical GC content and a high degree of DNA homology was observed between the two heterotrophic bacteria isolated.

The relationship between T. ferrooxidans strains and the heterotrophic bacteria was studied. The heterotrophic bacteria benefitted from T. ferrooxidans by utilizing organic by-products released during the growth of Thiobacillus. These acidophilic heterotrophs did not seem to affect the growth of T. ferrooxidans in the iron media.

Identical DNA base composition and a high degree of DNA homology was obtained for the iron-oxidising Thiobacillus LT2, FD1, ATCC 19859 and TF1-35. A GC content of 60,54 mol% was observed for the FD1 strain grown in the iron and iron-glucose medium (*FD1). A high DNA homology (85%) was obtained between FD1 and *FD1, suggesting that these two bacteria are the same organism. T. ferrooxidans FD1 strain proved to be a facultative autotrophic iron-oxidising bacteria able to grow in iron and to utilize glucose in the presence of iron. Higher bacterial yields were obtained in the iron-glucose medium in contrast to the iron medium. When grown in a mixotrophic medium, the ratio of iron to glucose determined the ability of T. ferrooxidans FD1 strain to use either of the substrates.

The cell densities of different T. ferrooxidans strains were determined by centrifugation in a Percoll gradient. Cells from the facultative autotroph T. ferrooxidans FD1 strain had a much lower density than the cells from the obligate autotrophic strains. This centrifugation method has the potential of separating these two groups of T. ferrooxidans strains.

CHAPTER 1

GENERAL INTRODUCTION

The exhaustion of high grade mineral ores has greatly influenced the development of microbiological leaching as a means of processing lower grade ores. The term 'microbiological leaching' has been defined as a biochemical oxidation process catalysed by living organisms (Torma, 1977). The significance of using bacteria in hydrometallurgical operations is attributed to the higher costs involved in mining lower grade ores by conventional means (Brierley, 1978).

Bacterial oxidation of sulphide minerals is a natural phenomenon that has been known to occur for centuries. It is believed that the Romans were the first to recover copper from sites of natural leaching but, it was only in the 18th century that details of a copper recovery process were documented at Rio Tinto in Spain. During this century large-scale operations have been developed in the United States of America for the recovery of low-grade copper waste material (Kelly, Norris & Brierley, 1979).

Leaching of uranium deposits on a commercial scale has been carried out successfully for a number of years but not to the extent of copper leaching (Brierley, 1978). The first leaching operations of mined-out areas and low-grade heaps at the uranium mines in the Elliot Lake area of Canada began in the early 1960s. The demand for uranium as a nuclear fuel has contributed greatly to the improvement of technology for the recovery of deep and low-grade uranium resources.

The main bacterially assisted leaching methods in which the leaching solution is percolated through heaps of ore include dump, heap and vat leaching. Underground in situ leaching is the only system that does not require the transport of the ore to the surface followed by crushing and grinding. In this method the lixiviant solution is pumped into the ore and allowed to percolate until it reaches the impermeable rock where it collects and is then withdrawn to the surface. When the ore body is not porous it is fractionated by underground explosions so that the leach liquor can penetrate (Brierley, 1978; Kelly, Norris & Brierley, 1979). In situ leaching is applicable to oxide and sulphide minerals of copper and uranium. In the case of copper, leaching occurs by a combination of direct bacterial attack with indirect chemical solubilization. The first method occurs in areas where oxygen is abundant and requires a physical contact between the bacteria and the iron pyrites that exist in association with copper. The second mechanism occurs in anaerobic conditions such as in the centre of a heap of ore, by the chemical action of the highly oxidizing acidic ferric sulphate solution that percolates through the ore. This solution is produced by the bacteria (Manchee, 1979). Leaching of uranium takes place by the indirect method only. Direct attack by the bacteria does not occur with uranium ores because they are often in the form of insoluble oxides (Torma, 1977).

Thiobacillus ferrooxidans is considered to be the most important organism in acid leaching. It is a motile, gram-negative, rod-shaped bacterium. T. ferrooxidans is a strict aerobe growing well at a temperature between 28 and 35°C and it requires an acid environment (pH 1.5 - 3.5). Being an autotrophic organism it uses CO_2 as the sole source of carbon, fixing it via the Calvin-Benson cycle and the carboxylation of phosphoenol pyruvate, simultaneously (Silver, 1978). The energy, in the form of ATP, required for the assimilation of CO_2 and other growth functions is obtained directly from the oxidation of inorganic substances such as sulphur, sulphides and ferrous iron.

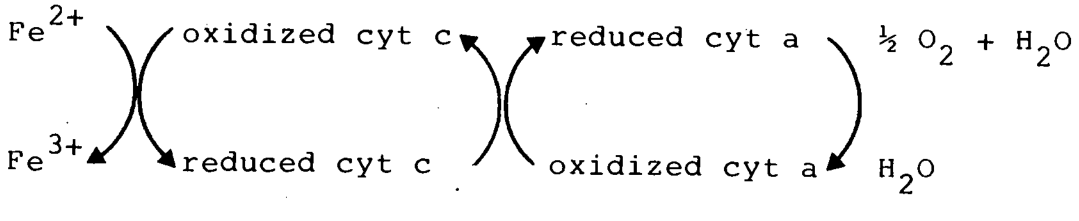
Pyrite, which is found in association with uranium, constitutes one of the most important energy sources (Manchee, 1979). The reactions involved in the oxidation of iron pyrite are shown in the following equations (Manchee, 1979):

$$2\ FeS_2 + 7\ O_2 + 2\ H_2O \xrightarrow{bacteria} 2\ FeSO_4 + 2\ H_2SO_4 \qquad (1)$$

$$4\ FeSO_4 + O_2 + 2\ H_2SO_4 \xrightarrow{bacteria} 2\ Fe_2(SO_4)_3 + 2\ H_2O \qquad (2)$$

Unlike many other chemolithotrophic bacteria, T. ferrooxidans has no protons associated with its energy source. During the oxidation of Fe^{2+} to Fe^{3+} one electron is removed by the cell and, in order to maintain a balance of electrical charge, a proton is obtained from the organism's natural environment. This proton may be ultimately utilized for the reduction of CO_2 and O_2. Apel & Dugan (1978) suggested that the lack of protons associated with the organism's energy source may explain the obligately acidophilic nature of this bacterium. An H^+ gradient exists across *T. ferrooxidans*' cytoplasmic membranes due to the low external pH compared to the relatively high internal pH. It is postulated that this bacterium utilizes this H^+ gradient to generate cellular energy via a chemiosmotic coupling mechanism (Apel, Dugan & Tuttle, 1980). Furthermore, the extent of H^+ uptake is dependent on Fe^{2+} concentration and the initial pH of the suspending medium. This suggested that the primary function of iron oxidation by these cells may be the release of electrons which are utilized for the electrical neutralization of H^+ on the cell interior (Apel, Dugan & Tuttle, 1980).

T. ferrooxidans contains cytochromes as components of electron transport mechanisms for the operation of a complete respiratory chain. The isolation of cytochrome c and cytochrome a from this bacterium suggested that iron oxidation occurs through the cytochrome system with oxygen as the final electron acceptor (Torma, 1977).



T. ferrooxidans obtains its nitrogen from either dissolved ammonia or nitrate. However, if dissolved sources of nitrogen are in short supply, this organism is able to fix atmospheric nitrogen (MacKintosch, 1978).

The main application of bacterially assisted leaching in South Africa is in the recovery of uranium. Being a major supplier of this strategic metal bacterial leaching will enable the recovery, at a much lesser cost, of uranium from high grade ores as well as small amounts of uranium left in the wastes from the processing of richer ores. In addition, pyrite, the major energy source for bacterial growth, is found in South African uranium bearing ores together with other essential factors (Livesley-Goldblatt, 1977).

As mentioned before, <u>T. ferrooxidans</u> plays an indirect role in the uranium leaching process in that it produces suitable chemical conditions for the dissolution of uranium (Tomizuka, & Yagisawa, 1978). This bioleaching process is mediated through diffusion of soluble ferric ions to the uranium particles dispersed in the ore matrix (Guay & Silver, 1980/81). Ferric iron is a powerful chemical solubilizing agent for numerous minerals and its action is enhanced in the presence of sulphuric acid. The ferric iron formed during the oxidation of iron pyrite can produce more acid in a nonbiological reaction:

$$2\ Fe_2\ (SO_4)_3 + 12\ H_2O \longrightarrow 4\ Fe(OH)_3 + 6\ H_2SO_4 \qquad (3)$$

The mixture of ferric sulphate (equation 2) and sulphuric acid (equations 1 and 3) is able to react with the insoluble tetravalent form of uranium present in the ore converting it to the soluble hexavalent uranyl sulphate according to the following equations (Tuovinen & Kelly, 1974; Torma, 1977):

$$UO_2 + Fe_2(SO_4)_3 \longrightarrow UO_2SO_4 + 2\ FeSO_4$$

$$UO_3 + H_2SO_4 \longrightarrow UO_2SO_4 + H_2O$$

The presence of the above iron-oxidizing bacterium ensures that ferric iron is constantly regenerated to maintain a more or less constant supply of chemical oxidants (Guay & Silver, 1977).

The oxidation of ferrous sulphate by T. ferrooxidans in liquid medium is accompanied by the accumulation of insoluble ferric compounds known as jarosite. This jarasite precipitate is composed of ferric hydroxides and basic ferric sulphates. The former is produced according to the following equation (Guay & Silver, 1980/81):

$$Fe_2(SO_4)_3 + 6\ H_2O \longrightarrow 2\ Fe(OH)_3 + 3\ H_2SO_4$$

The basic ferric sulphates are formed by the reaction (Ivarson, 1973):

$$Fe_2(SO_4)_3 + 2\ H_2O \longrightarrow 2\ Fe(OH)SO_4 + H_2SO_4$$

In addition, it has been observed that a considerable proportion of the bacterial population attaches to this deposit of jarosite. The close association of T. ferrooxidans with the inorganic substrates constitute a problem as far as the enumeration of these bacteria is concerned. Often the growth of *T. ferrooxidans* is measured as the oxidation of ferrous iron. However, Kelly & Jones (1978) reported iron oxidation in chemostat and batch cultures of non-growing *T. ferrooxidans* suspensions for certain levels of CO_2 and oxygen supply. It is then necessary to have a means of monitoring the growth of *T. ferrooxidans* that does not depend on the oxidation of iron only because under certain conditions non-growth associated iron oxidation can occur. A detailed account of the problems encountered in the enumeration of *T. ferrooxidans* and the method developed in this study is given in Chapter 2.

As previously discussed, the regeneration of ferric iron liquor plays a central role in the bacterially assisted uranium recovery from ores. Consequently it is important to understand the physiology of *T. ferrooxidans* in relation to ferrous and ferric iron. In Chapter 3 of this manuscript the implications of the relative concentrations of ferrous and ferric irons and the effect of ferric iron accumulation in the growth of this bacterium are discussed. Various types of thermophilic bacteria have been reported to exist in leaching systems (Kelly, Norris & Brierley, 1979; Brierley, 1982). They can derive energy from oxidizing inorganic compounds like ferrous iron, sulphur and soluble or insoluble sulphides. Although generally they can fix carbon dioxide, some are fully autotrophic and others require organic substances for growth. The isolation of the various bacteria present in natural leaching environments is essential in determining the role played by these bacteria in mineral breakdown. A report on the bacteria isolated from mining leach liquors is given in Chapter 4.

CHAPTER 2

DETERMINATION OF T. FERROXIDANS' GROWTH

Summary: The growth of T. ferrooxidans LT2 on different solid media was investigated. No colonies were produced on agar even when membrane filters were used. On agarose media slightly pink colonies were observed after a long period of incubation. These colonies developed over a lawn of Thiobacillus growth.

Indirect estimation of T. ferrooxidans growth in liquid media was determined by various methods of protein analysis. The inorganic ions present in T. ferrooxidans culture media interfered with the Ninhydrin assay and with the Lowry method using Folin-Coicelteu reagent. The Coomassie Blue protein assay was free from interference by the inorganic ions. This assay was found to be a reproducible and sensitive method for monitoring the growth of Thiobacillus cells. The assay correlated well with the direct microscopic bacterial counts and with the amount of bacterial iron oxidation.

2.1 INTRODUCTION

The growth of T. ferrooxidans in ferrous sulphate liquid medium leads to the formation of a yellow precipitate referred to as jarosite. It has been postulated that jarosite is composed of ferric hydroxides and basic ferric sulphates that can serve as substrates to which the bacteria have been found to adhere (Ivarson, 1973). A similar bacterial deposit is formed on ferrous sulphate agar medium. The relationship and significance between the iron-oxidising bacteria and the precipitate formed is not exactly known. The presence of these inorganic substrates constitutes a problem as far as the enumeration of these bacteria is concerned. Techniques that are normally used to measure bacterial growth, namely direct microscopic counting and colorimetric techniques are dependent on bacteria existing as isolated single cell units. It is therefore extremely difficult to apply these methods to T. ferrooxidans. The most probable number (MPN) method requires serial dilutions of liquid medium. Again, a great limitation results from the fact that representative samples cannot be obtained of the mineral particles or the bacteria (Brierley, 1978). The same reasoning applies to the bacterial dry weight method. The culturing of many iron oxidising Thiobacillus strains on solid medium has not been very successful. Agar was found to be toxic and therefore inhibit colony development on ferrous iron medium (Beck, 1960). Tuovinen & Kelly (1973) used membrane filters to grow T. ferrooxidans on solid medium so that the bacteria would be physically separated from the inhibitory materials of the agar. However, some toxicity due to a diffusible soluble agar component was observed. It was suggested that galactose, the main product of the acid hydrolysis of the agar, is the responsible agent. Other solidifying agents have been tried in order to get single-colony growth of T. ferrooxidans. Razzel & Trussell

(1963) managed to isolate this microorganism on silica gel plates and Manning (1975) reported successful growth on agar plates using a low concentration of purified agar. However, only one strain of *T. ferrooxidans* was tested and it is likely that other strains will have slightly different requirements for which optimum conditions have to be established. Although some strains are able to grow as isolated colonies on solid medium there are many strains that do not have this ability. The use of the Coulter Counter for determination of cell number and size of *T. ferrooxidans* strains has also been reported (Brierley, 1978). The problem of the precipitated ferric iron salts was solved by dissolving them with a mixture of NaCl and EDTA. One disadvantage of this method is that cell numbers attached to mineral particles cannot be determined because EDTA does not disintegrate these particles as it does the ferric iron precipitate. The other disadvantage of the Coulter Counter is that it does not differentiate between species such as *Thiobacillus thiooxidans* and *T. ferrooxidans* because their cell sizes are similar (Brierley, 1978). It is crucial to be able to distinguish different species of *Thiobacillus* when one aims either to get a pure culture of *T. ferrooxidans* or to check the purity of a particular culture.

Several indirect techniques have been used for the enumeration of iron-oxidising bacteria. Smith, Fliermans & Brock (1972) used a technique based on $^{14}CO_2$ uptake to study the autotrophic fixation of carbon dioxide by *T. ferrooxidans*. The suitability of this technique is that it allows the determination of bacterial activity associated with solid substrates. However, a limitation to the use of this method is that it does not differentiate between organisms which take up carbon dioxide. Another factor that may contribute to innacurate results is that organic material is utilized by these organisms to a certain extent and it is possible that assimilation of available organic carbon is preferred to carbon dioxide (Brierley, 1978). Often the growth of Thiobacillus strains is measured as the oxidation of ferrous iron. However, a drawback to this method is that some Thiobacillus species which are not able to oxidise ferrous iron have been found in acid mine drainage. In addition, it has been shown that non growth-associated iron oxidation may occur (Kelly & Jones, 1978). It is therefore necessary to obtain a method that does not depend on iron oxidation only so that either of the above parameters can be determined. Other methods used include those where some cell component is measured such as total cellular nitrogen and analysis of protein. Determination of bacterial nitrogen as a means of estimating bacterial cells is of limited value in that it does not distinguish between bacterial nitrogen and nitrogen which precipitates as ammoniojarosite and it will also measure nitrogen containing organic by-products of microbial growth (Brierley, 1978). Estimation of bacterial numbers by protein determination has been used but problems involving the interference from inorganic ions have been reported (Le Roux, North & Wilson, 1973).

This study was undertaken to develop a means of determining bacterial growth of local isolates of T. ferrooxidans in both solid and liquid media. In order to find a suitable plating method, different solidifying agents were tested, namely agarose, methocel, silica gel and different brands of agar. To follow T. ferrooxidans' growth in liquid medium measurements of protein were determined and its values correlated with the readings obtained for the titration of the residual ferrous iron in the medium. If the protein content was to be measured its release from the microorganism had to be ensured. Disintegration of bacterial cells by a physical method (ultrasonic treatment) or their hydrolysis by a chemical mechanism (cold or hot alkali treatment) were tested. Three different protein assays were used, namely the determination of amino-acids with the Ninhydrin reaction after alkaline hydrolysis of the protein, the estimation of bacterial protein with the Folin Coicelteu reagent and the binding of Coomassie Brilliant Blue to protein. To prevent interference by ferric iron in some of the protein assays, two methods of washing the samples prior to protein estimation were followed.

2.2 MATERIALS AND METHODS

All % values are w/v unless otherwise stated. Details of the media are listed in Appendix A. Details of buffers and solutions are listed in Appendix B. Specialised chemicals and their sources are listed in Appendix C.

2.2.1 Bacteria

The T. ferrooxidans strain used was LT2 which was isolated from the BACFOX Unit at the Buffelsfontein Mine, General Mining (Livesley-Goldblatt, 1979).

2.2.2 Growth of T. ferrooxidans on Solid Media

Solid 9K media were prepared according to Silverman & Lundgren (1959) using three different types of agar (Table 2.1). Iron-salts-purified agar medium (ISP) was prepared according to Manning (1975). ISP solid medium was also prepared using agarose, methocel and silica gel instead of agar (Table 2.1). The growth of T. ferrooxidans was also tested on membrane filters placed on 9K and ISP plates solidified with Oxoid Agar 3 and ferrous-iron solid medium (Oxoid Agar 3) prepared according to Tuovinen & Kelly (1973). Oxoid membrane, Millipore AP2504200 and Sartorius SM 11106 filters were used. As the filters might contain inhibitory materials, they were either boiled in sterile distilled water for 10 min and left in water 24h before use or they were soaked for 24h.

T. ferrooxidans strain LT2 was cultured in 9K iron broth medium of Silverman & Lundgren (1959). The culture was incubated at 30°C in a rotary shaker at 150 rev-min^{-1} until the iron in the medium was completely oxidised. The culture (0,1 mℓ) was plated on the solid media and the plates incubated at 30°C for 2 to 4 weeks. The plates were

TABLE 2.1: Concentrations of different solidifying agents used in the preparation of solid ferrous iron media, pH 2.0.

Medium	Type and Concentration of Solidifying Agent
9K	1,6% Oxoid Agar 3
9K	1,6% Difco Bacto Agar
9K	1,6% Difco Noble Agar
ISP	0,7% Oxoid Agar 3
ISP	0,3% Agarose
ISP	0,6% Methocel
ISP	0,2% Silica Gel

incubated in plastic bags to prevent dehydration. A single colony or a loopful of growth was transferred from the plates into flasks containing 9K medium. The flasks were incubated at 30°C in a rotary shaker for a maximum of 6 weeks. A freshly oxidised T. ferrooxidans culture was plated in the membrane filters by filtering through the membranes which were placed on the solid media. The inverted plates containing the membrane filters were incubated at 30°C for 3 weeks. After incubation the membranes were removed and the colonies stained for 15 min by placing them on 5cm Whatman No. 17 absorbant pad soaked with 1% aqueous acid fuchsin. The membrane filters were allowed to dry at room temperature and were examined under a dissecting microscope.

2.2.3 Determination of Ferrous Iron Oxidation in Liquid Media

Ferrous iron was estimated by titrating the residual ferrous iron with standard potassium dichromate solution. Samples (5 mℓ) were pipetted into conical flasks together with 15 mℓ of H_2SO_4-H_3PO_4 water mixture (Appendix B). Sodium diphenylamine sulphonate (6-8 drops) was added as an indicator. These solutions were then titrated with standard potassium dichromate solution to a purple end point.

2.2.4 Direct Counts

Direct microscopic bacterial counts were determined with a haemocytometer.

2.2.5 Protein Determinations

2.2.5.1 Washing of Samples

The methods of Tabita and Lundgren (1971-a) and Berry & Murr (1978) were used to wash *Thiobacillus* cells. In the method of Tabita & Lundgren *T. ferrooxidans* cells were harvested by centrifugation (14 000 rpm, 10 min) from a 40 mℓ oxidised culture. The pellet was resuspended in 6 mℓ of 0.2M Tris-HCl buffer pH 7.9. The mixture was centrifuged at 1 000 rpm for 10 min to remove precipitated ferric iron. The supernatent was decanted and the cells collected by centrifugation at 14 000 rpm. The pellet was resuspended in 6 mℓ of the Tris-buffer and 0,5 mℓ of the resuspended pellet was removed and tested for interference against Ninhydrin and Folin-Coicelteu. The remaining 5,5 ml were centrifuged for 15 min at 14 000 rpm. The pellet was resuspended in 5,5 mℓ of the Tris-buffer, 0,5 mℓ were removed and checked for its effect on Ninhydrin and Folin-Coicelteu while the rest was centrifuged again. The next pellet was resuspended in 5 mℓ Tris-buffer, 0,5 mℓ were removed to be tested and the rest centrifuged at 14 000 rpm for 15 min. The final pellet was resuspended in 4,5 mℓ of the Tris-buffer and 0,5 mℓ were removed and verified for interference against Ninhydrin and Folin Coicelteu.

In the method of Berry & Murr (1978) <u>T. ferrooxidans</u> cells were harvested from an oxidised culture by centrifugation at 15 000 rpm for 10 min. The pellet was resuspended in 6 mℓ cold distilled water acidified to pH 2.5 with H_2SO_4 and allowed to stand for at least 6h at 4°C. The turbid supernatent was carefully decanted from the underlying layer of precipitated iron and allowed to stand overnight at 4°C. Five millilitres of the supernatent containing the cells were decanted and 0,5 mℓ removed and tested for interference against Ninhydrin and Folin-Coicelteu. The remaining 4,5 mℓ was centrifuged at 15 000 rpm and the pellet resuspended in 4,5 mℓ of distilled water pH 2.5. An aliquot (0,5 mℓ) was removed and tested against Ninhydrin and Folin-Coicelteu while the remaining 4 mℓ was centrifuged again and the pellet resuspended in 4 ml of distilled water pH 2.5. An aliquot (0,5 mℓ) was again removed and the rest centrifuged at 15 000 rpm for 10 min. The final pellet was resuspended in 3,5 mℓ of distilled water of which 0,5 mℓ were removed and tested for interference against Ninhydrin and Folin-Coicelteu.

2.2.5.2 Ninhydrin Assay

This indirect method of measuring protein involves the alkaline hydrolysis of the protein with barium hydroxide followed by a Ninhydrin estimation of the amino acids released (McGrath, 1972). The Ninhydrin solution was prepared by dissolving 0,5 g of Ninhydrin in 100 mℓ of ethylene glycol monoethyl ether. Protein solutions (1 mℓ) were pipetted into test tubes, 100 mg of $Ba(OH)_2 8H_2O$ were added and the contents of the tubes well mixed. The mixture was heated at 120°C for 10 min in an autoclave to ensure hydrolysis of the protein and 2 mℓ of sodium acetate buffer was added to dissolve the barium hydroxide completely. The pH was checked and adjusted if necessary to 5.1 ± 0.1 with 2 M HCl. The Ninhydrin solution (3 mℓ) was added and the mixture was heated to 108°C for 5 min in a pressure cooker to ensure complete colour development. The solution was allowed to cool and the blue/purple colour read at 570 nm in a Bauch and Lamb spectrophotometer against a reagent blank. Each sample was assayed in triplicate. The amount of protein in the culture sample was determined from a calibration curve prepared with 10, 20, 50, 75 and 100 µg/mℓ of bovine serum albumin (BSA).

2.2.5.3 Folin-Coicelteu Assay

Reagent A was prepared by dissolving 20 g Na_2CO_3 and 2 g NaK tartrate in 1 000 mℓ of distilled water. Reagent B was made up by adding 50 g $CuSO_4.5H_2O$ to 1 000 mℓ distilled water. Reagent A (50 parts) was mixed with Reagent B (1 part) to make up Reagent C. This was prepared fresh before use. A protein sample (1 mℓ) was pipetted into a test tube. Reagent C (5 mℓ) was added, mixed well and the solution allowed to stand for 10 min at room temperature. Folin-Coicelteu Reagent (0,5 mℓ) was added, mixed well and allowed to stand for 30 min at room temperature for maximum colour development. The optical density was then read at 690 nm. The amount of protein in the sample was determined from a calibration curve prepared with different concentrations of bovine serum albumin ranging from 10 to 100 µg/mℓ.

2.2.5.4 Coomassie Blue Assay

Coomassie Blue reagent was prepared by dissolving 100 mg of Coomassie Brilliant Blue G-250 in 50 mℓ of 90% ethanol. To this solution 100 mℓ of 85% phosphoric acid was added. The resulting solution was diluted to a final volume of 1 ℓ with distilled water. Coomassie Blue reagent (5 mℓ) was added to 1 mℓ of protein sample, the solution was mixed well and allowed to stand for 5 to 30 min before determining the absorbance at 595 nm. The protein in the sample was determined from a calibration curve prepared with different concentrations of BSA ranging from 0,1 to 20 µg/mℓ. Protein assays were carried out in triplicate.

2.2.6 Protein Extraction

2.2.6.1 Alkaline Hydrolysis

Samples of 9K medium (50 mℓ) were centrifuged at 3 000 rpm for 15 min and the pellets were resuspended in 2 mℓ of 1N NaOH. Two millilitres of the mixture were immediately pipetted into test tubes and kept at room temperature for 10, 30, 60 and 120 min or overnight. The samples were then centrifuged at 3 000 rpm for 10 min in order to remove the insoluble cell wall components. The supernatents (1 mℓ) were either assayed for protein or were placed in a boiling water bath for 5, 10, 15 and 20 min respectively. The boiled samples were centrifuged at 3 000 rpm for 10 min before being assayed for protein.

2.2.6.2 Ultrasonic Disintegration

Samples of 9K medium (5 mℓ) were centrifuged (3 000 rpm, 10 min). Half of the pellets were resuspended in 2 mℓ of 1N NaOH and mixed together in a small beaker. The other half was resuspended in distilled water. The two mixtures were then sonicated using a Soniprep 150 Disintegrator at an amplitude of 10 µ for a total of 9 min for periods of 20 sec. Samples were removed from both mixtures after 3, 6 and 9 minutes of sonication, centrifuged and the supernatents assayed for protein.

2.3 RESULTS

2.3.1 Growth of T. ferrooxidans on Solid Media

Attempts to grow single colony cultures of LT2 on solid ferrous sulphate medium were unsuccessful. T. ferrooxidans grew as a yellow lawn in what seemed to be a deposit of oxidised iron surrounding the growth of T. ferrooxidans. No iron oxidation was obtained in 9K liquid medium flasks inoculated with a loopful of this apparent growth. However isolated colonies were recently obtained with another T. ferrooxidans strain (T. ferrooxidans FD1) on 9K medium solidified with Difco noble agar containing 3 g/ℓ of ferrous iron (A. Petersen, personal communication). Single colonies inoculated into 9K liquid medium oxidised the iron.

An unexpected result was the development on Agarose plates of tiny pinkish colonies over the yellow lawn of bacteria and oxidised iron. The colonies were formed some weeks after the plates had been taken out of the incubator and left at room temperature. When these colonies were inoculated into 9K medium no iron oxidation took place. However good growth was obtained when the colonies were inoculated into Tryptone-glucose-yeast extract medium. Growth of T. ferrooxidans LT2 was not observed on membrane filters.

2.3.2 Protein Estimation using Ninhydrin

The inorganic ions present in *T. ferrooxidans* culture medium interfered with the Ninhydrin reaction resulting in the production of additional colour (Table 2.2). The generation of colour by the ions present in the oxidised supernatent fluid seemed to be independent of the concentration of iron since similar interference colour factors were obtained with different samples.

The interference observed on the Ninhydrin reaction by the iron associated with *T. ferrooxidans* cells was eliminated by a series of washings with acidified distilled water according to the method by Berry & Murr (1978). The method suggested by Tabita and Lundgren (1971) did not release all the iron attached to the cells.

2.3.3 Protein Estimation using Folin-Coicelteu

A precipitate was formed after addition of reagent C which became more pronounced upon addition of Folin-Coicelteu reagent. This precipitate strongly interfered with the reading obtained. The interference was eliminated by a series of washings with acidified distilled water according to the method by Berry & Murr (1978). However, this method was not very sensitive in that small amounts of protein (0,5 µg) were not detected.

TABLE 2.2: Effect of inorganic ions present in *T. ferrooxidans* cultures on the Ninhydrin reaction.

Sample	Oxidised supernatant fluid (dilution factor 1) + BSA			Oxidised supernatant fluid (dilution factor 2) + BSA			Oxidised supernatant fluid (dilution factor 4) + BSA		
Expected Concentration of BSA (µg)	Apparent protein concentration	Correction for zero BSA reading	Interference factor	Apparent protein concentration	Correction for zero BSA reading	Interference factor	Apparent protein concentration	Correction for zero BSA reading	Interference factor
0	38,426	0	0	17,76	0	0	4,693	0	0
10	134,693	96,267	9,627	69,493	51,733	5,173	52,56	47,867	4,787
50	159,093	120,667	2,41	124,026	106,266	2,125	122,693	118,0	2,36
100	220,426	182,0	1,82	183,36	165,6	1,656	159,693	154,0	1,54

$$\text{Interference factor} = \frac{\text{Corrected Apparent Protein Concentration}}{\text{Expected Protein Concentration}}$$

2.3.4 Protein Estimation using Coomassie Blue

The Coomassie Blue protein-binding assay was free of interference from the inorganic ions present in *T. ferrooxidans* culture media (Table 2.3). A correlation coefficient of 0,9993 was obtained when the protein concentrations for the two BSA preparations were compared. The effect of the final pH of the sample on the absorbance of Coomassie Blue was determined (Table 2.4). Although there was an increase in absorbance with an increase in pH, this increase was negligible between pH 5 and pH 11. After the extraction of protein the pH of sample should be readjusted to between this range.

TABLE 2.3: Effect of inorganic ions present in *T. ferrooxidans* cultures on the Coomassie Blue protein-binding assay.

Expected Concentration of BSA added (µg)	Apparent Protein Concentration	
	Oxidised Supernatent Fluid + BSA	Water + BSA
10	11,25 (± 0,112)	11,31 (± 0,16)
15	17,03 (± 0,01)	17,03 (± 0,01)
20	20,71 (± 0,18)	21,34 (± 0,56)
25	23,21 (± 0,32)	23,62 (± 0,48)

Standard deviations in brackets.

Different concentrations of Coomassie Blue were tried and 100 mg proved to be the most suitable which is in agreement with Bradford's report (1976). The colour stability of the protein-dye complex was not affected between 5 and 45 min after addition of the protein reagent.

TABLE 2.4: Effect of acid and alkaline conditions on the Coomassie Blue protein-binding assay.

pH	Absorbance 595 mm
1 M HCl	< 0
1	0,172
2	0,260
3	0,269
5	0,277
7	0,273
11	0,276
12	0,282
13	0,545
1 M NaOh	0,77

The optimum time for complete hydrolysis of two identical bacterial samples depended on the temperature of hydrolysis (Table 2.5). Treating the cells for 5 min with boiling NaOH or 60 min at 20°C was sufficient to ensure maximum solubilization of the protein. The decrease in protein absorbance observed when the sample was boiled for longer than

5 min suggested that this treatment was too severe and that hydrolysis for 60 min at room temperature was more suitable.

TABLE 2.5: Effect of temperature and time of hydrolysis of *T. ferrooxidans* cells by 1 N NaOH

Temperature	Time (min)	µg Protein (BSA)
Boiling	0	1,8841
	5	2,1739
	10	1,7713
	15	1,3688
	20	1,1755
Room Temperature (20°C)	10	0,4992
	30	1,4010
	60	2,1578
	120	1,9807
	O/N	0,8374

Sonication of bacterial samples did not prove to be a suitable means of disintegrating *T. ferrooxidans* cells as detectable amounts of protein were not obtained after 6 min of sonication. The growth of iron oxidising *Thiobacillus* measured by the Coomassie Blue protein binding assay, direct bacterial counts and the amount of iron oxidation (Figure 2.1).

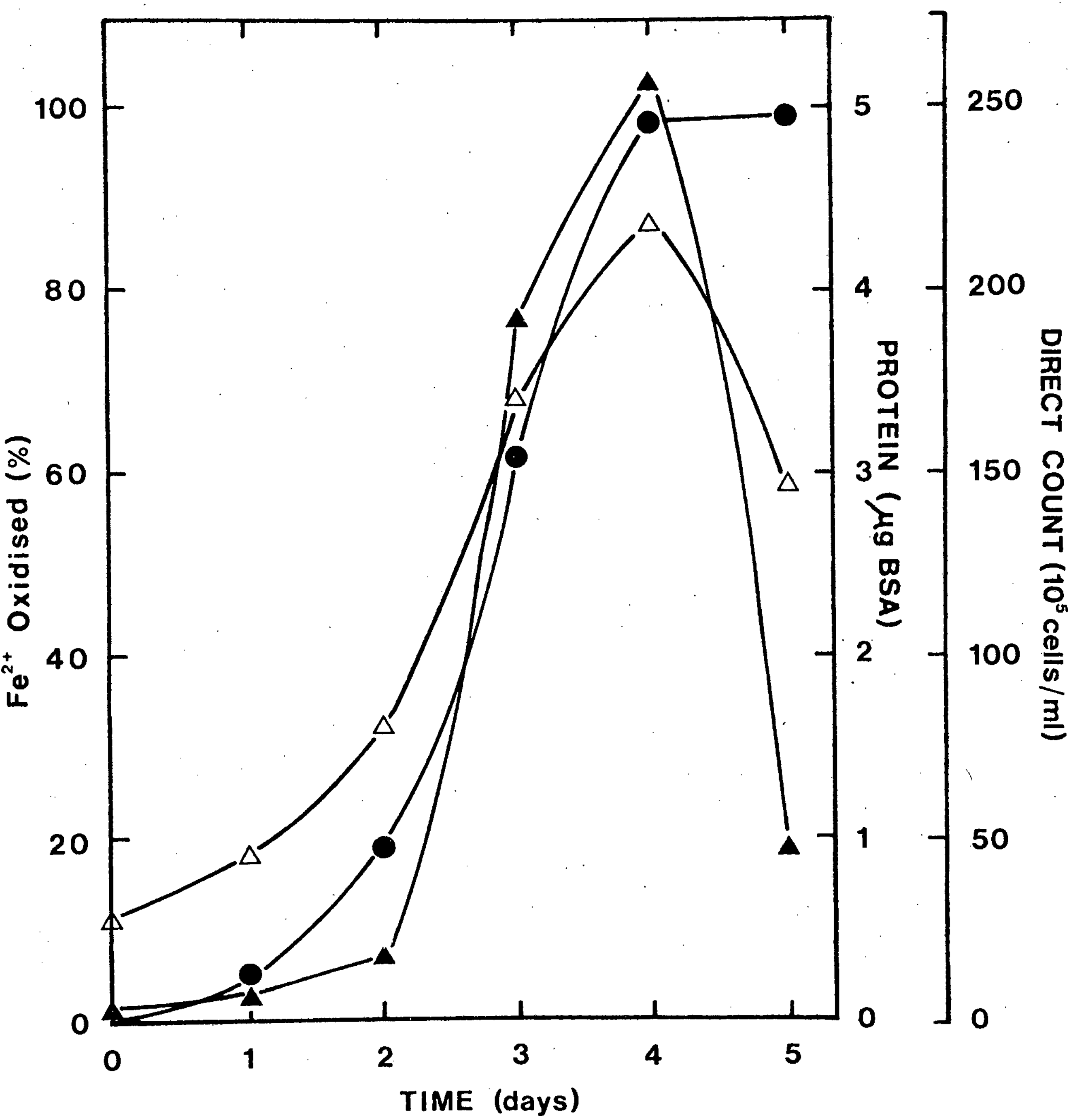


FIGURE 2.1: A comparison of T. ferrooxidans LT2 growth measured by the Coomassie Blue protein-binding assay (△), direct count (▲) and ferrous iron oxidation (●).

After 4 d of aeration, iron oxidation was complete and the number of bacteria as determined by direct count and protein assay had reached a peak. A good correlation between the three methods used to estimate T. ferrooxidans growth was observed at all times. Correlation coefficients for plots of the amount of iron oxidised versus the protein assay, the direct cell count versus the protein assay and the amount of iron oxidised versus the direct count were 0,9924, 0,9823 and 0,9849 respectively. Standard deviations amounted to 2,11% of the protein at the lowest levels of protein determined and 2,22% at peak protein levels. Bacterial cell numbers of $3,6 \times 10^5$ cells/mℓ could be determined with the above reproducibility by means of the protein-binding assay.

2.4 DISCUSSION

The inability to grow T. ferrooxidans strain LT2 on solid medium agrees with previous observations that agar inhibits colony development on ferrous-iron medium (Tuovinen & Kelly, 1973). Manning (1975) reported that potassium phosphate was the component responsible for the inhibition of growth of iron colonies on 9K medium. However, when ISP medium was used no colonies were formed. Toxicity of the agar was also observed when membrane filters were used. This confirmed previous findings that although the bacteria had no physical contact with the agar the inhibition of colony development could have been due to some diffusible toxic soluble component of the agar (Tuovinen & Kelly, 1973).

Free sugars, formed from acid hydrolysis of the agar, were proposed as possible toxic components. An interesting finding was the growth as single colonies of strain FD1 on solid medium and its ability to oxidise iron when transferred into liquid medium. This emphasises the variations among T. ferrooxidans strains and differences in agar tolerance suggested by Mishra & Roy(1979). The growth obtained when T. ferrooxidans FD1 was inoculated onto solid media may indicate that this strain is not as sensitive to agar. The yellow precipitate observed on 9K agar medium is due to the formation of ammoniojarosite instead of potassium or sodium jarosite that is formed in liquid medium (Ivarson, 1973). The preference of ammonium over potassium for the formation of the basic ferric sulphate may result from the conditions of the solid agar medium in a way similar to the observed preferential incorporation of aluminium over iron when acidity is reduced (Ivarson, 1973). The formation of this precipitate which accompanies bacterial oxidation of ferrous iron was retarded by keeping the pH below 1.85. The pink colonies obtained on Agarose plates after some weeks of incubation could either be another Thiobacillus species since it did not oxidise iron when transferred into 9K liquid medium or another organism able to use organic matter released by T. ferrooxidans. The second explanation is more likely as this organism was able to grow rapidly on Tryptone-glucose-yeast extract media in the absence of iron.

An examination of the protein assays used for the indirect estimation of bacterial numbers indicated the sensitivity of colourimetric protein assays to inorganic ions in the T. ferrooxidans culture. Protein estimations by both the Ninhydrin and Folin-Coicelteu assays were very sensitive to interference. Although washing of the samples proved successful in preventing interference, a drawback was the possible loss of bacterial cells which would lead to inaccurate results since small samples are used each time. Washing was also time-consuming. In addition, these two methods were not very sensitive to small amounts of protein. The Coomassie Blue protein-binding assay proved to be completely free of interference from ions and is a convenient and sensitive technique for monitoring the growth of Thiobacillus. A possible drawback to this protein assay method seemed to be the alkaline hydrolysis of T. ferrooxidans cells prior to protein measurement in that according to Bradford (1976), Coomassie Blue was affected by strongly alkaline solutions. However, the addition of acid and buffer to the protein extract was sufficient to maintain the final sample pH within the pH range in which Coomassie Blue was stable. A technique that has also been used successfully to monitor the growth of iron-oxidising Thiobacillus is the incorporation of $^{14}CO_2$ into cellular material (Beck, 1960; Kelly & Jones, 1978). This technique has a drawback in that it requires the use of sealed flasks which interferes with the transfer of gases and hence the partial pressures and supply of O_2 and CO_2 to the bacterial cells. Sealing of containers is not required for the Coomassie Blue protein-binding assay.

CHAPTER 3

PHYSIOLOGY OF IRON-OXIDISING THIOBACILLUS

Summary: The effect of ferrous and ferric iron concentrations on the rate of iron oxidation by T. ferrooxidans was investigated in batch culture using LT2, ATCC 19859 and FW1 strains. The rate of iron oxidation was exponential and maximum rates were obtained at ferrous concentrations of 60-80 mM. Exponential iron oxidation rates were a function of the initial ferrous concentration and were affected by the ferrous-ferric ion ratio. Oxidation rates were almost constant at ferrous-ferric ion ratios greater than 1 and decreased when this ratio was lower than 1.

3.1 INTRODUCTION

The economic potential of T. ferrooxidans in the recovery of metals from their ores and the need to find alternative means for conventional methods of mining and ore processing has greatly contributed to the application of bacterially assisted leaching by the mineral industry. However, it has been reported that the rate of microbiological leaching is slower than the rate obtained with some chemical methods of extraction (Brierley, 1982). Research in this area is mainly concerned with improving the rate of bacterial leaching by studying the factors that influence bacterial growth.

The role of T. ferrooxidans in the extraction of uranium is confined to the generation of ferric sulphate, the oxidising agent, and sulphuric acid, the solvent, according to the equation:

$$2\ FeS_2 + 7\tfrac{1}{2}\ O_2 + H_2O \longrightarrow Fe_2(SO_4)_3 + H_2SO_4$$

The ferric sulphate can react chemically with the insoluble tetravalent form of uranium present in the ore oxidising it to the soluble hexavalent form as follows (Chackrabarty, 1978):

$$UO_2 + Fe_2(SO_4)_3 \longrightarrow UO_2SO_4 + 2\ FeSO_4$$

The ferric iron is constantly regenerated by the micro-organism via the reoxidation of the ferrous iron produced. This ferrous-ferric reoxidation is thought to be one of the rate-limiting steps in the leaching of metals by T. ferrooxidans.

Dugan and Lundgren (1965) proposed a model to explain the mechanism of iron oxidation by T. ferrooxidans. According to their model ferrous iron forms a complex in the growth medium or at the cell surface and it is oxygenated but not oxidised, since no electron transport has taken place. The complex somehow binds to the cell wall or membrane, or to both. At the binding site iron oxidase or oxygenase reacts with this complex oxidising the iron and releasing one electron. After oxidation the ferric ions diffuse away from the cell and react with water or sulphate to form ferric hydroxide or sulphate. The authors suggested that either sulphate or a flavoprotein is involved in the initial electron-transfer link between iron and the cell. A more recent model was proposed by Silver (1978). He suggested that ferrous iron forms a complex with an organic molecule and that coenzyme Q is the intermediate electron carrier between the ferrous iron-sulphate-organic complex associated with the cell envelope. The iron cytochrome c-oxidoreductase responsible for iron oxidation binds sequentially to ferrous and ferric irons by a mechanism elucidated by Din & Suzuki (1967). In this mechanism the enzyme containing one atom of ferric iron binds one atom of ferrous iron resulting in the reduction of the bound iron to ferrous iron and a molecule of ferric iron is released. The oxidised cytochromec then binds to the enzyme and is immediately reduced by its bound ferrous iron. The reduced cytochrome c is then reduced.

Lazaroff (1963) reported the requirement of a relatively high quantity of sulphate ions for growth and iron oxidation. He suggested two possible explanations for this anion requirement, the one being the need for sulphate for the synthesis of a sulphur compound involved in energy transfer from the iron oxidase and the other, a role for sulphate in controlling the entrance of ferrous iron into the cell. The need for sulphate ion in the autotrophic iron oxidation was later confirmed by Lees, Kwok & Suzuki (1969). No iron oxidation took place when $FeSO_4$ was replaced by $FeCl_2$ and the pH was adjusted with HCl instead of H_2SO_4. Similar results were obtained by Beck (1969).

Schnaitman (1969) did some kinetic analyses of ferrous iron oxidation by T. ferrooxidans cells and showed that iron oxidation follows zero order kinetics. An interesting observation was that the major effect of pH on the iron oxidation reaction was on the maximum reaction velocity (V_{max}) and not on the Michaelis-Menten constant (K_m). The author suggested that this pH effect could be due to the loss of iron-binding groups present in the cell envelope or to the ionization of groups around the active sites of the enzymes involved in iron oxidation.

Bodo and Lundgren (1974) reported that the enzymes associated with iron oxidation are located in the cell envelope. They suggested that this enzyme localization would be advantageous to the cell in that any toxic products released during iron oxidation are less liable to enter the cytoplasm. It was proposed by Chackrabarty (1978) that ferric iron represses the rate of iron oxidation by competitively inhibiting the binding of ferrous iron to the enzyme responsible for iron oxidation. Kelly & Jones (1978) investigated this further and reported that the principal factor limiting the growth of T. ferrooxidans in batch culture is the inhibitory effect of ferric iron on the ferrous iron oxidation step. In addition, he regarded both ferrous and ferric irons as potentially toxic metals to T. ferrooxidans. The inhibition by ferric iron on iron oxidation by T. ferrooxidans was also studied by Wong et al. (1973) as quoted by

Brierley, (1978) who proposed a noncompetitive inhibition by ferric iron. The competitive inhibition of ferric iron for the enzyme binding site is easily followed by Silver's model (1978) of iron oxidation.

Brierley (1978) suggested that possible differences in the inhibitory effect of ferric iron on iron oxidation by T. ferrooxidans could result from strain differences.

An important aspect of the economical application of bacterial assisted leaching in the industrial recovery of metals is the microbial recycling of the lixiviant solutions. In the case of uranium leaching, the ferric iron reduced to ferrous is returned to the leach where the ferrous iron is re-oxidised by T. ferrooxidans to ferric iron (Livesley-Goldblatt, 1977). Since the regeneration of leach liquor occurs in the presence of both ferrous and ferric ions it is important to establish the effect of one on the other.

In this study the effect of ferrous iron concentrations and mixtures of ferrous and ferric iron on the rate of iron oxidation by T. ferrooxidans in batch culture was determined. To test the effect of strain diversity in the oxidation of iron, three different T. ferrooxidans strains were used.

3.2 MATERIALS AND METHODS

3.2.1 Bacteria

The T. ferrooxidans strains used are listed in Table 3.1.

TABLE 3.1: Iron oxidising bacteria used in this study.

T. ferrooxidans strain	Source
LT2	BACFOX Unit of the Buffelsfontein Mines General Mining - South Africa
FW1	Fairview Water Leach Residue Ore - General Mining - South Africa
ATCC 19859	American Type Culture Collection.

3.2.2 Effect of Different Ferrous Iron Concentrations on the Rate of Iron Oxidation

Sterile double strength 9K Basal Salts (75 mℓ) were dispensed into 15 sterile 250 mℓ conical flasks. Double strength ferrous sulphate solutions (75 mℓ) containing 18, 70, 139, 268 and 344 mM of ferrous iron were prepared in triplicate. The pH of the iron solutions was adjusted to 1.8 with H_2SO_4 and sterilised through a membrane filter (0,45 µm pore size) and added to the rest of the medium when cool. The final pH of the culture medium was adjusted to 1.7 and kept constant throughout the experiment. The bacterial cultures were inoculated into each set of flasks containing different concentrations of iron. The flasks were incubated at 30°C in a Gallenkamp Orbital Shaker. Samples were withdrawn at regular time intervals and assayed for ferrous iron oxidation by titration with standard potassium dichromate solution.

3.2.3 Effect of Different Ferric Iron Concentrations on the Rate of Iron Oxidation

Sterile double strength 9K Basal Salts (75 mℓ) were dispensed into 12 sterile 250 mℓ conical flasks. Ferrous sulphate solutions (32,5 mℓ) were prepared in triplicate to give final concentrations of 118 mM ferrous iron. The pH of the iron solution was adjusted to 1.8 with H_2SO_4, sterilised through a membrane filter (0,45 µm pore size) and added to the Basal Salts when cool.

Double strength ferric sulphate (32,5 mℓ) containing 36,72 and 140 mM of ferric iron were prepared in triplicate and sterilised by membrane filtration. The ferric iron solution was added to 9 of the 12 flasks. The remaining 3 flasks were used as controls and 32,5 mℓ of sterile distilled water were added to make up the volume to 150 mℓ. Samples were withdrawn at regular time intervals and assayed for ferrous iron oxidation and ferric iron concentration.

3.3 RESULTS

For the three iron oxidising bacterial strains the rate of bacterial growth, determined by iron oxidation at different time intervals, proved to be proportional to the amount of ferrous sulphate present. Figure 3.1 shows the exponential iron oxidation for the *T. ferrooxidans* FW1 strain. In a plot of the log of ferrous iron oxidised versus time (Figure 3.2) it can be seen that the exponential relationship no longer holds when a large proportion of the

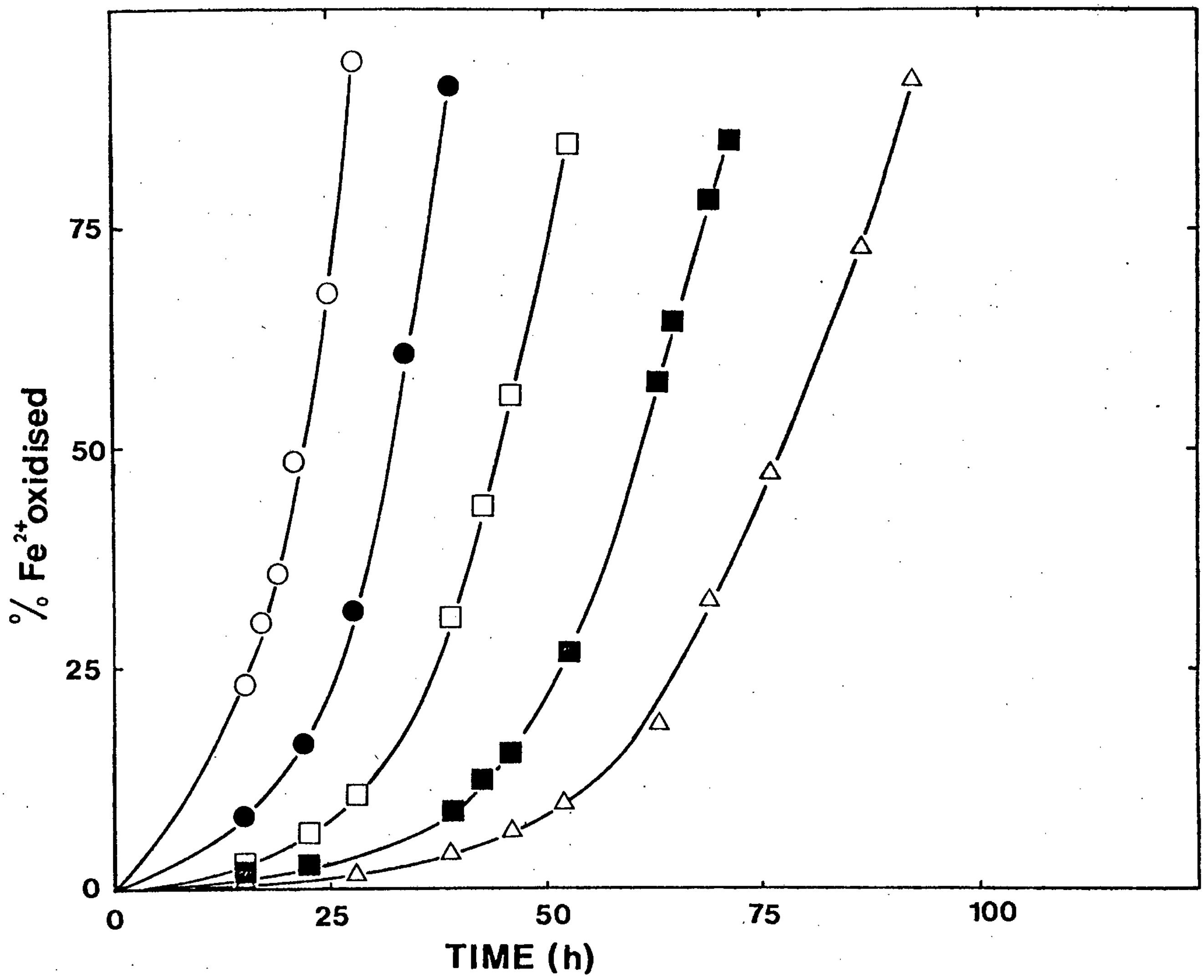


FIGURE 3.1: Exponential rate of iron oxidation by T. ferrooxidans FW1 at different initial ferrous iron concentrations. Concentrations of $FeSO_4$; ○ 18 mM; ● 70 mM; □ 139 mM; ■ 268 mM; △ 344 mM.

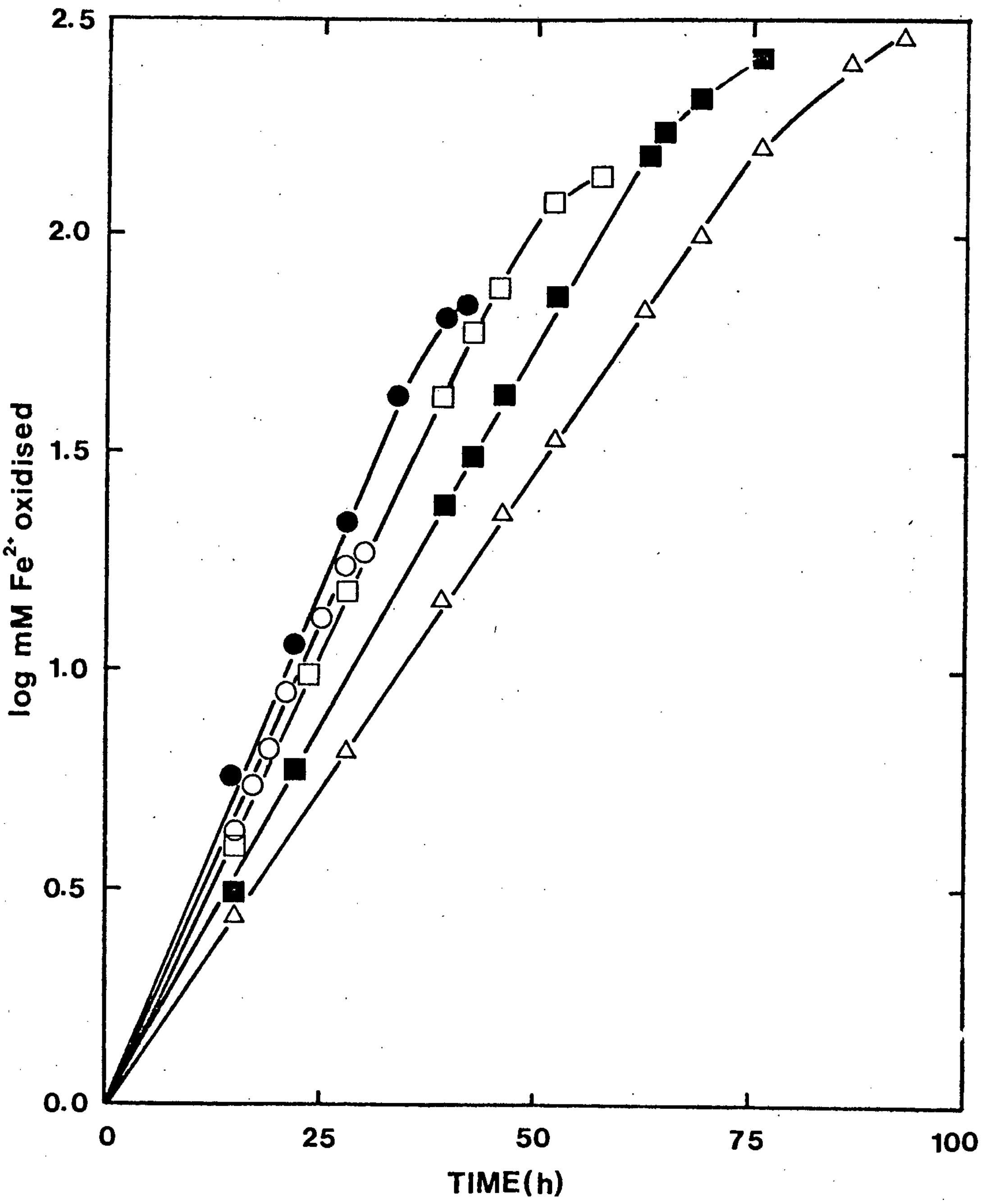


FIGURE 3.2: The rate of iron oxidation by T. ferrooxidans FW1 at different initial ferrous iron concentrations. Concentrations of $FeSO_4$; ○ 18 mM; ● 70 mM; □ 139 mM; ■ 268 mM △ 344 mM.

iron has been oxidised. Figure 3.3 shows the specific growth rate calculated from the linear portion of the exponential rates of ferrous sulphate oxidation, as a function of the initial concentration of ferrous iron. A maximum rate of iron oxidation was obtained at ferrous iron concentrations between 60-80 mM for the three bacterial strains, followed by a decline for ferrous iron values greater than 80 mM. The specific growth rate was calculated for each point of the bent portion of the linear plot of the exponential rate of ferrous sulphate oxidation (Figure 3.2) as a function of the ferrous-ferric ratio (Figure 3.4). Oxidation rates were constant for ferrous-ferric ratios greater than 1. In the presence of additional ferric iron the rate of iron oxidation is still dependent on the ferrous-ferric ratio (Figure 3.5).

3.4 DISCUSSION

Wong et al. (1973), as quoted by Brierley (1978), suggested that ferrous iron oxidation was inhibited by ferric iron at concentrations between 2,58 mM and 15,0 mM. Kelly & Jones (1978) studied the effect of ferric iron on iron oxidation in batch culture and found that ferric iron initially decreased the rate of iron oxidation but appeared stimulatory at higher concentrations of ferrous iron. In this study a similar result was obtained in that the exponential rate of iron oxidation was no longer linear when a certain proportion of oxidised iron was reached.

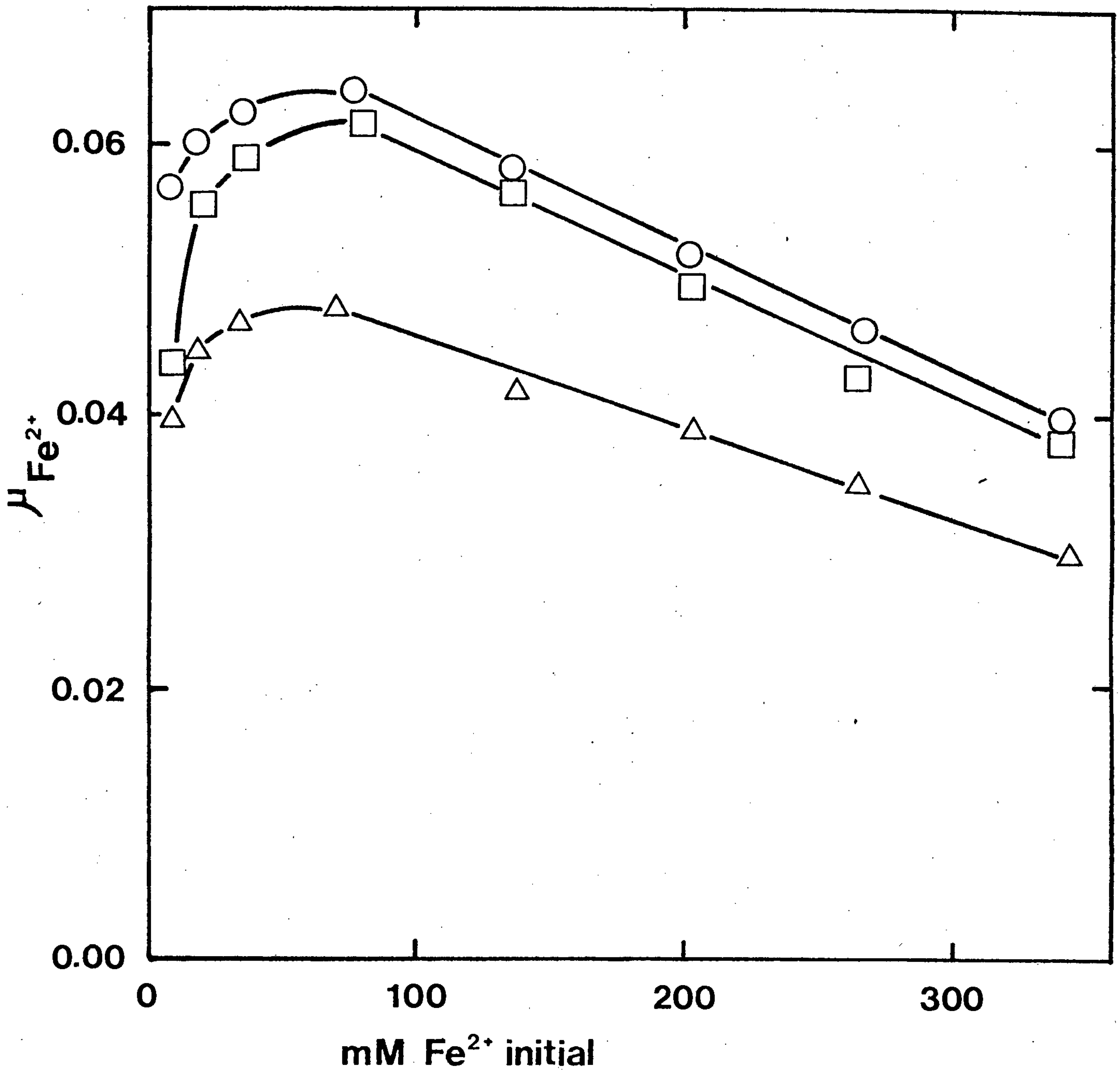


FIGURE 3.3: The effect of initial ferrous iron concentration on the exponential growth rate (μ Fe^{2+}) of three *T. ferrooxidans* strains. △ FW1; □ LT2; ○ ATCC 19859.

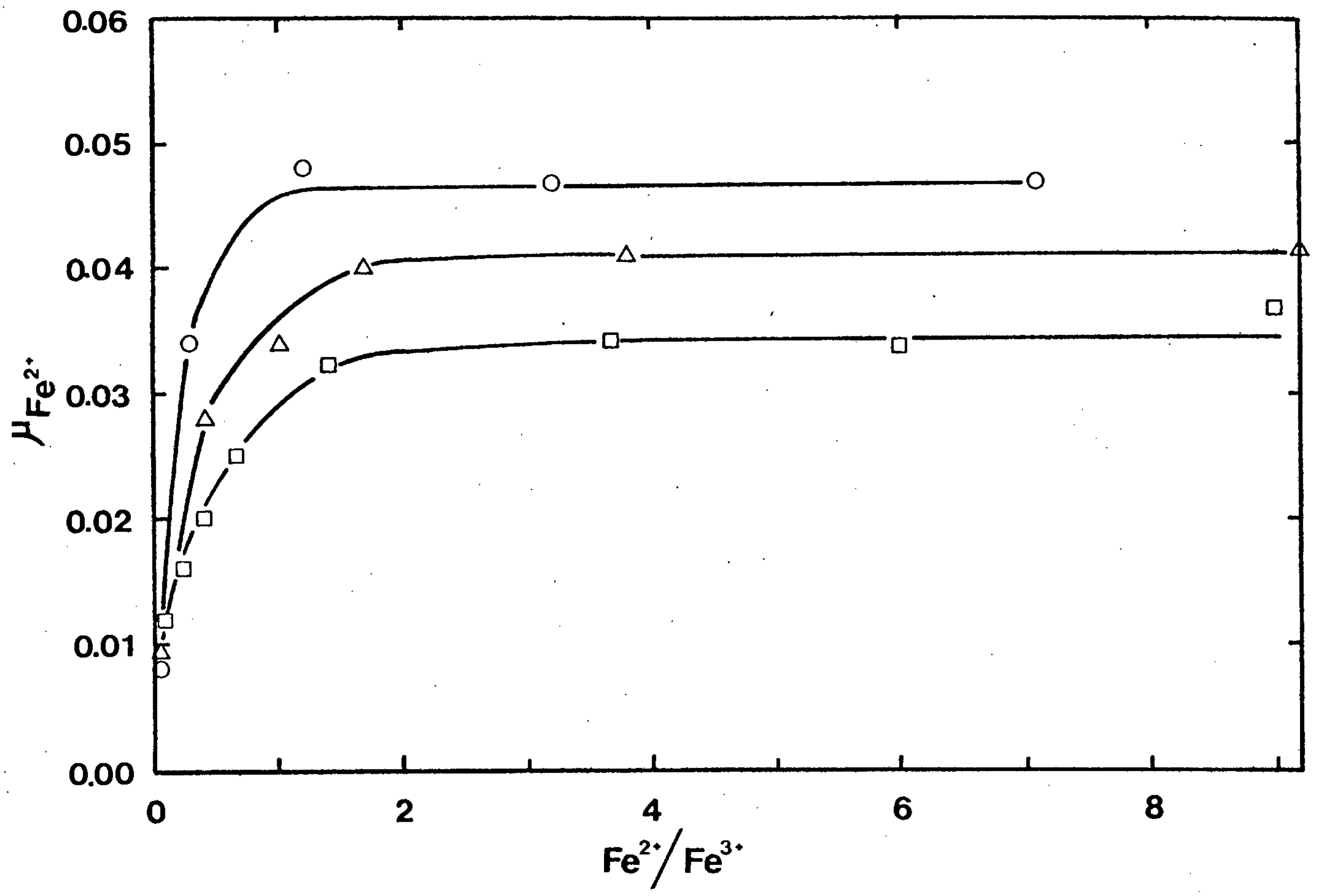


FIGURE 3.4: The relationship between Fe^{2+}/Fe^{3+} ratio and the exponential growth rate (μFe^{2+}) of T. ferrooxidans FW1 at different initial ferrous iron concentrations. Concentrations of $FeSO_4$; ○ 70 mM; △ 139 mM; □ 268 mM.

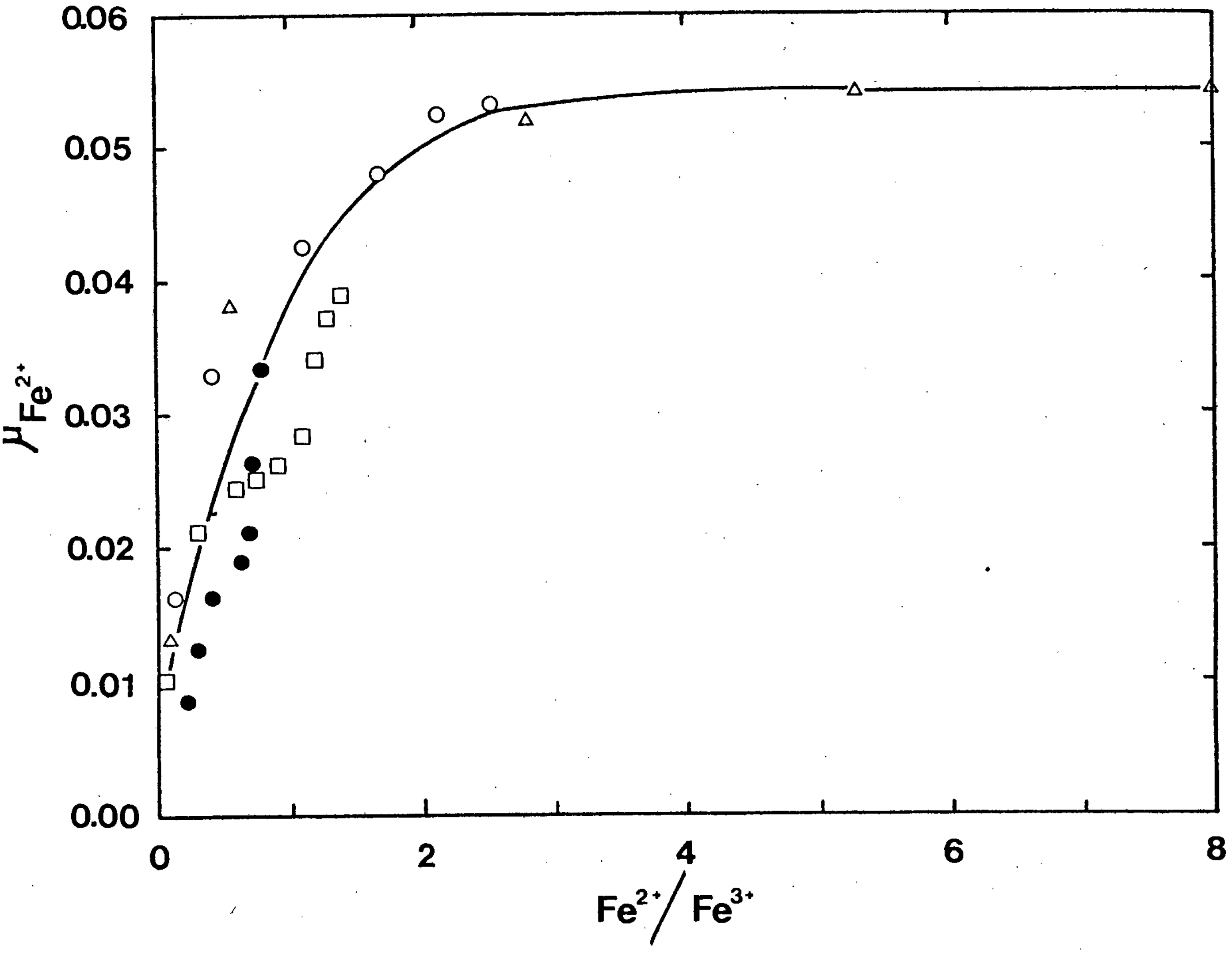


FIGURE 3.5: The relationship between the Fe^{2+}/Fe^{3+} ratio and the exponential growth rate (μFe^{2+}) of T. ferrooxidans FW1 in the presence of different concentrations of added ferric iron. Initial $FeSO_4$ concentration; 110 - 118 mM. Initial ferric iron concentrations; △ 8 mM; ○ 44 mM; □ 80 mM; ● 149 mM.

The observation that the exponential growth rate remained constant for the ratio of ferrous to ferric greater than 1 and decreased when this ratio was lower than 1 indicated that the ferrous-ferric ratio was the determining factor in the rate of iron oxidation. This was confirmed when the growth medium was supplied with different concentrations of ferric iron. The effect of ferric inhibition was mainly due to the lowering of the ferrous-ferric ratio. These results are consistent with the competitive inhibition of ferric iron on ferrous iron oxidation shown in the manometric studies of Kelly & Jones (1978). This competitive inhibition can be explained by the model of Silver (1978) in which it is suggested that ferric ions can combine with the enzyme responsible for iron oxidation in such a way that it competes with ferrous ions for binding at the active site. A competitive inhibition by ferric iron was then suggested for ferrous-ferric ratios lower than 1. Kelly & Jones (1978) found that the specific growth rate increased at initial ferrous sulphate concentrations ranging from 10-60 mM and remained constant at concentrations of 77-165 mM. In the present study maximum rates of iron oxidation were obtained at ferrous concentrations between 60-80 mM. This confirmed previous reports that ferrous iron could be an inhibitory substrate at high concentrations (Kelly & Jones, 1978).

CHAPTER 4

CHARACTERISATION OF BACTERIA IN MINING LEACH LIQUOR

Summary: After a stepwise adaptation of T. ferrooxidans strains to grow on glucose media, heterotrophic bateria were isolated from LT2 and FD1 cultures - Het LT2 and Het FD1 respectively. ATCC 19859 and TF1-35 could not be adapted to grow on glucose media and were then considered to be pure cultures.

The growth of T. ferrooxidans on iron medium supplemented with different levels of glucose and yeast extract was investigated. LT2, FD1 and ATCC 19859 were able to utilize both organic compounds up to a concentration of 0.1% but 0.5% of either glucose or yeast extract inhibited growth.

The relationship between T. ferrooxidans strains and the heterotrophic bacteria was studied. The heterotrophic bacteria benefitted from T. ferrooxidans by utilizing organic by-products released during the growth of Thiobacillus. These acidophilic heterotrophs did not seem to affect the growth of T. ferrooxidans cultures in iron media.

The DNA base composition of the iron-oxidising Thiobacillus LT2, FD1, ATCC 19859 and TF1-35 was found to vary from 59,80 to 60,54 mol% GC. A GC content of 60,54 was observed for the FD1 strain grown in iron and iron-glucose

medium (*FD1). A high degree of DNA homology (85%) was obtained between FD1 and *FD1 suggesting that these two bacteria are the same organism. The T. ferrooxidans FD1 strain was able to utilize glucose in the presence of iron suggesting that it is a facultative autotrophic iron-oxidising bacterium. This was confirmed by the higher bacterial yield obtained in iron-glucose medium in contrast to iron medium. When grown in mixotrophic medium the ratio of iron to glucose determined the ability of the T. ferrooxidans FD1 strain to use either of the substrates. For the same glucose concentration (0.5%) when the ferrous iron in the medium was 4g/ℓ, iron and glucose were utilized simultaneously. However, when the ferrous iron concentration was 7g/ℓ the iron was used first followed by glucose utilization.

The cell densities of different T. ferrooxidans strains were determined by centrifugation in a Percoll gradient. Cells from the facultative autotroph T. ferrooxidans FD1 strain had a much lower density (1,023 mg/mℓ) than the cells from the obligate autotrophic strains. This centrifugation method has the potential of separating these two groups of T. ferrooxidans strains.

4.1 INTRODUCTION

Mixed populations of bacteria are known to exist in natural leaching systems. In order to understand bacterial leaching it is of fundamental importance to know the microorganisms involved in the leaching process and their interactions.

The types of organisms reported to be involved in leaching besides *T. ferrooxidans*, include *T. thiooxidans*, *Thiobacillus acidophilus*, *Leptospirillum ferrooxidans*, *Sulfolobus* and *Sulfolobus*-like bacteria, heterotrophic bacteria and fungi (Kelly, Norris & Brierley, 1979). *T. thiooxidans* cannot oxidise iron but it was suggested that mineral leaching by *T. ferrooxidans* could be enhanced by this organism's ability to oxidise sulphur generated in leaching systems (Brierley, 1982). A possible mutualistic action in ore leaching exists between *L. ferrooxidans* and *T. organoparus*. *L. ferrooxidans* in pure culture can only grow on soluble ferrous iron and *T. organoparus* can only oxidise sulphur. However mixed cultures of these two bacteria grow and degrade pyrite and chalcopyrite which neither of them can metabolize alone (Balashova *et al*, 1974; Kelly, Norris & Brierley, 1979). A *Beijerinckia* species has been reported to accelerate the leaching of copper-nickel sulphide by *T. ferrooxidans* possibly by either fixing atmospheric nitrogen or removing the autoinhibitory organic secretions (Tsuchiya, Trivedi & Schuler, 1974; Kelly, Norris & Brierley, 1979). This suggests that heterotrophic bacteria may also have a stimulatory effect in the leaching of minerals.

In contrast to early reports that organic materials inhibited *T. ferrooxidans* growth more recent data indicate that in some strains of this bacterium, when autotrophically grown cells are adapted to grow on glucose they can use it as the sole source of energy (Shafia & Wilkinson,jr., 1969). The prolonged incubation on organic matter resulted in the organism becoming an obligate heterotroph. However, not all iron-oxidising bacteria can be adapted to grow on glucose suggesting that this is a stable feature among certain species which could be considered as a basis for species differentiation (Shafia & Wilkinson, jr., 1969; Tabita & Lundgren, 1971 - a). A study of the metabolic alterations occurring in T. ferrooxidans when it switches from an autotrophic existence to a heterotrophic one, showed an induction of the enzymes glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrase and 2-keto-3-deoxy-6-phosphogluconate alolase (Tabita & Lundgren, 1971 - b). The increase in the levels of these enzymes which are involved in glucose metabolism occurred when the organism was grown on an iron-glucose-supplemented medium or on glucose alone. In contrast, low levels are present in autotrophic cells (Tabita & Lundgren, 1971 - b).

Zavarzin (1972) reported the isolation of a heterotrophic bacterium after supplementing the growth medium of a T. ferrooxidans culture with yeast extract. The addition of an excess of organic matter rendered the cells incapable of growth on ferrous iron. The characteristics of this heterotrophic satellite of T. ferrooxidans are identical to the so-called glucose adapted cells of Shafia and Wilkinson, jr.(1969) and Tabita & Lundgren (1971 - a).

Research has been carried out on the transition of chemolithotrophic <u>T. ferrooxidans</u> to obligate organotrophy by Shafia <u>et al</u> (1972). Conversion of cellular nutrition to organotrophy resulted in a gradual loss of Fe^{2+} oxidation and cessation of CO_2 fixation with an increase in the ability to utilize a variety of organic compounds. Furthermore, the above investigators observed that although the bacterium became obligately heterotrophic it still retained a characteristic of the orginal autotrophic isolate involving a requirement for high concentrations of hydrogen ions. A role for this hydrogen iron requirement was suggested to be associated with adsorption and transport of nutrient because incorporation of glucose by the cell was not observed in slightly acidic or neutral media. Shafia <u>et al</u> (1972) did some serological studies on the authotrophic <u>T. ferrooxidans</u> cells and the heterotrophic convertant and showed the existence of a definite antigenic relationship between the two cell types.

Although intrigued with the inability of the heterotrophically grown <u>T. ferrooxidans</u> to revert to its autotrophic mode of nutrition, workers tended to assume that the adapted bacterium was in fact <u>T. ferrooxidans</u>. It was only in 1975 that Guay & Silver, in order to test the validity of this assumption, examined the DNA base composition of the ferrous iron grown organism with the strain obtained through this autotrophic-heterotrophic transition. Based on the results obtained in the analysis of DNA, the authors proposed that this facultative heterotrophic bacteria derived from <u>T. ferrooxidans</u> cultures, which grow on either organic compounds or reduced sulphur, but not on ferrous iron, be included in a new species - T. acidophilus. As yet, it is not known what exactly allows the maintenance of the autotrophic population in the glucose grown culture nor the nature of the close interdependence between T. ferrooxidans and T. acidophilus. These findings suggest the possibility of a heterogenous culture of iron-oxidising bacteria (Guay & Silver, 1975). In addition, it has been demonstrated that it is possible to grow T. ferrooxidans on glucose providing that the toxic material produced by glucose was constantly removed (Borichewski, 1967). Apparently, it was the inhibitory accumulation of this organic material, identified as pyruvic acid, that limited the growth of this autotroph (Borichewski, 1967).

Mackintosch (1978) observed that when T. ferrooxidans from a liquid culture was plated out, two types of colonies were produced. She suggested that the colony that produces a brown precipitate is T. ferrooxidans and the one that does not have this precipitate of iron is T. acidophilus. Once again, mixed cultures of acidophilic bacteria were proposed. This hypothesis was later put forward by Tuovinen et al (1978) who postulated that some strains of T. ferrooxidans are mixed cultures. According to these workers the heterotrophic variants can survive on organic materials liberated by the autotrophic organism in a medium containing ferrous iron or inorganic sulphur compounds as the energy source. Similarly, in heterotrophic cultures the autotrophic bacteria may be able to survive at low concentrations. The loss of iron oxidation by the mixed population could indicate that the autotrophic iron-oxidisers are gradually eliminated during the series of subcultures on glucose (Tuovinen et al, 1978).

Recently, a yellow-pigmented heterotroph was reported to be a contaminant in T. ferrooxidans and other Thiobacillus cultures (Harrison, jr., Jarvis & Johnson, 1980). This bacterium differs from the previously reported obligately acidophilic heterotrophs in that it grows better at pH 6.7 and the colonies formed on glucose agar are white or pinkish in colour. In addition, it does not utilize sulphur or ferrous iron as energy sources. The GC content of its DNA was later found to be 69,8 mol% and its name was proposed to be Acidophilium cryptum sp.nov. (Harrison, jr., 1981). The high GC value of this organism differentiates it from both T. acidophilus, which has a GC content of 62,9 to 63,2 mol% and T. ferrooxidans with a GC value of 57,2 mol% (Harrison, jr., 1981). Harrison, jr., Jarvis & Johnson (1980) suggested that all obligate heterotrophs in T. ferrooxidans cultures may probably be contaminants that seem to play some rolé with this organism in its natural habitat.

A search for heterotrophic bacteria existing in acidic mine drainages was undertaken by Wichlacz & Unz (1981). These results indicate that a variety of bacteria with a requirement of an acid pH for growth exist in various parts of natural mine streams, with a high proportion found in association with ferric hydrates. However, neither a taxonomic position nor a function could be established for these acidophilic heterotrophic bacteria. Co-existence of two different strains of the same species in a presumably pure culture of T. ferrooxidans was reported by Harrison, jr. (1982). A comparison of the GC content of the DNA obtained when this culture was grown on ferrous sulphate with the DNA from the same culture grown on sulphur showed different values and yielded different DNA homology results. Apparently, the individual strains attained different relative populations in the two media. Similar results were recorded earlier by Torma (1977) and by Silver (1978). They reported that different DNA base compositions were obtained when the iron oxidising bacteria were grown on different substrates.

Recently, Sugio et al (1982) isolated two kinds of iron-oxidising bacteria from a T. ferrooxidans strain. One was a strict autotroph able to utilize ferrous iron or sulphur as the energy source and unable to utilize organic substances. The other was a facultative iron-oxidising bacterium able to utilize ferrous iron or sulphur in addition to organic substances. A colony of the facultative iron-oxidising bacterium strain obtained from a ferrous-glucose-salts agar plate managed to grow on ferrous sulphate-glucose-salts liquid medium. However, it failed to do so on glucose-salts liquid medium alone. This suggests the requirement of some growth factors in the absence of iron (Sugio et al, 1982).

Brierley (1982) reported the isolation of heterotrophic microorganisms in acidic environments. However, the role of these microorganisms in the leaching process is still unknown. It is therefore important to establish the genetic relationship between these obligate heterotrophs and T. ferrooxidans as well as to determine if the heterotrophs are just contaminants or contribute in some way to the growth of leaching bacteria.

Separation of soil microorganisms have been successfully carried out by density gradient centrifugation in Percoll (Martin & Macdonald, 1981). Percoll is composed of coloidal silica particles which have been coated with polyvinylpyrrolidone to render them non-toxic to cells. The heterogeneity of its particles' sizes results in the spontaneous formation of a gradient upon centrifugation due to the differential sedimentation in a gravitational field (Martin & Macdonald, 1981). In an attempt to isolate possible different species of acidophilic Thiobacillus present in our cultures, samples were mixed with a suspension of Percoll and the cells separated on the basis of their relative densities.

In the present study the isolation of a heterotrophically grown bacterium from South African isolates of T. ferrooxidans using the method by Shafia & Wilkinson, jr. (1969) is described. The function of this heterotrophic bacterium in T. ferrooxidans cultures was investigated.

Silver, Margalith & Lundgren (1967) reported that glucose had an inhibitory effect on ferrous iron oxidation by T. ferrooxidans. This was confirmed later when

Tuovinen, Niemela & Gyllenberg (1971) tested the addition of some common organic compounds to the ferrous sulphate medium. In the present investigation the influence of organic matter on the oxidation of ferrous iron by _T. ferrooxidans_ in pure culture and in the presence of the heterotroph was compared.

Finally, the differentiation of iron grown and glucose grown _Thiobacillus_ strains was determined based on the guanine-cytosine mole ratio of DNA. This was followed by DNA homology studies between the different strains in order to determine to what extent they are genetically related.

4.2 MATERIALS AND METHODS

Details of the media are listed in Appendix A, buffers and solutions in Appendix B.

4.2.1 Isolation of Heterotrophic Bacteria from _T. ferrooxidans_ Cultures

The iron-oxidising bacterial strains used in these experiments were LT2, FD1, ATCC 19859 and TF1-35.

Stock cultures of the organisms were maintained in 9k liquid medium. Sixty millilitres of 0.5% glucose medium supplemented with 2% $FeSO_4.7H_2O$ (FeG medium) were placed in 100 mℓ conical flasks. An inoculum (0,5%) of each _T. ferrooxidans_ stock culture was given to each tube respectively. After being inoculated the flasks were incubated at 30°C on a gyratory shaker. Once all the iron was oxidised the cultures were tested for heterotrophic growth in 100 mℓ flasks containing 60 mℓ of 0,5% glucose medium (GYE medium) and a 5% inoculum from the FeG cultures. GYE cultures were incubated at 30°C on a gyratory shaker. Growth was tested as total cell count, with the use of a haemocytometer. Cells grown in GYE medium were inculated back into fresh FeG medium to test for iron oxidation.

Plates were prepared for FeG and GYE media with 2% Difco Noble Agar. Once the cultures were grown on liquid media they were streaked on the corresponding solid media and incubated at 30°C until colony growth was visible.

4.2.2 Effect of Glucose and Yeast Extract on the Oxidation of Iron by *T. ferrooxidans* Strains in Pure Culture and in the Presence of the Heterotroph

The iron-oxidising bacterial strains LT2, FD1, ATCC 19859 and the heterotrophic bacteria isolated from LT2 strain were used.

Sterile 9K medium (150 mℓ) was placed in seven sterile 250 mℓ conical flasks. Three of the flasks were supplemented with glucose to give a final concentration of 0,1, 1,0 and 5,0 g/ℓ. To another three flasks yeast extract was added to give final concentrations of 0,1, 1,0 and 5,0 g/ℓ. The remaining flask was used as a control.

Four sets of seven flasks were used. Three sets of flasks were given a 5% inoculum from a fresh culture of LT2, ATCC 19859 and FD1, respectively. The fourth set of flasks were given a 5% inoculum from the ATCC 19859 culture together with 1,5% inoculum of the Het LT2 culture. The inoculated cultures were incubated at 100 rev/min at 30°C in a GallenKamp Orbital Shaker.

Samples (5 mℓ) of each culture were withdrawn at daily intervals and the ferrous iron concentration determined by volumetric titration with standard potassium dichromate (Vogel, 1961).

4.2.3 Relationship between T. ferrooxidans FD1 Strain and Het FD1

Sterile 9K medium (150 mℓ) was placed in four sterile 250 mℓ flasks. The first flask was given a 5% inoculum of a FD1 culture, the second a 5% inoculum of FD1 together with 1,5% inoculum of Het FD1 and the third a 1,5% inculum of Het FD1. The fourth flask was used as a control. Samples were withdrawn daily and assayed for ferrous iron concentration and direct bacterial counts. Viable counts were obtained by plating the cells onto FeG and GYE plates. The plates were incubated at 30°C for 7 to 28 days.

4.2.4 Comparative Study of T. ferrooxidans FD1 Strain Grown on Iron Supplemented with Glucose

Sterile 9K medium (1 200 mℓ) and, sterile FeG medium

(1 200 mℓ) containing 0,5% glucose and 2% $FeSO_4.7H_2O$ was placed in two sterile 2 ℓ conical flasks. A 5% inoculum of a FD1 culture grown on 9K medium was given to each flask. The flasks were incubated at 30°C in a gyratory shaker.

Samples of each culture were withdrawn at daily intervals and assayed for ferrous iron concentration, direct bacterial count, protein content by the Coomassie Blue protein-binding assay and glucose concentration. To determine glucose 10 mℓ samples of the FeG culture were centrifuged at 3 000 rpm for 10 minutes and 10 µℓ of the supernatent tested for glucose using the Beckman Glucose Analyzer 2. Viable counts were attempted by plating the cells onto FeG and GYE plates. Once all the iron was oxidised the yield of both cultures was determined by measuring dry cell weight. Samples (200 mℓ) of each culture were centrifugued at 7 000 rpm for 10 minutes and the pellet resuspended in 10 mℓ of 9K Basal Salts pH 1.6. Each sample was filtered through a preweighed 0,45 µm membrane filter. The membrane filters and cells were dried in an oven at 100°C until constant weight. The filters were weighed again and the yield calculated according to the previous weight of the membranes.

4.2.5 Utilization of Glucose by T. ferrooxidans FD1 Strain at Different Concentrations of Iron

Sterile double strength 9K Basal Salts (75 mℓ) supplemented with 5 g/ℓ sterile glucose were dispensed into six sterile

250 mℓ conical flasks. Double strength ferrous sulphate solutions (75 mℓ) containing 4,7 and 9 g/ℓ of ferrous iron were prepared in duplicate. The pH of the iron solutions was adjusted to 1.8 with H_2SO_4 and sterilised through a membrane filter (0,45 µm pore size) and added to the rest of the medium when cool. The final pH of the culture medium was adjusted to 1.7 and kept constant throughout the experiment. One set of flasks was inoculated with 5% FD1 culture and the other set was used as control. Samples (10 mℓ) were withdrawn daily, centrifuged at 3 000 rpm for 10 minutes and 10 µℓ of the supernatent tested for glucose using a Beckman Glucose Analyzer 2. In addition samples were assayed daily for ferrous iron concentration and direct bacterial count.

4.2.6.1 Extraction of DNA

The bacteria used are listed in Table 4.1

TABLE 4.1: Strains of iron-oxidising and heterotrophic bacteria as well as the energy source of which they were grown for DNA isolation

Bacteria	Strain	Energy Source
T. ferrooxidans	LT2	Iron
T. ferrooxidans	ATCC 19859	Iron
T. ferrooxidans	FD1	Iron
T. ferrooxidans	TF1-35	Iron
Heterotrophic bacteria	Het LT2	Glucose
Heterotrophic bacteria	Het FD1	Glucose
T. ferrooxidans	*FD1	Iron and Glucose
E. coli	*E. coli*	Beef Extract, Yeast Extract, Peptone

*FD1 - grown on FeG medium

LT2, ATCC 19859, FD1 and TF1-35 cultures were grown in 2 000 mℓ conical flasks on a Gallenkamp Orbital Shaker at 30°C. The flasks contained 1 200 mℓ of 9K medium (7 g/ℓ Fe^{2+}), pH 1.7 and were given a 5% inoculum of the above iron-oxidising Thiobacillus. The cultures were incubated until all the iron was oxidised. Het LT2 and Het FD1 were grown in 1 000 mℓ conical flasks on a Gallenkamp Orbital Shaker at 30°C. The flasks contained 600 mℓ of GYE medium and were given a 5% inoculum of the abvoe heterotrophic bacteria. The flasks were incubated for 2 days. In addition, another 5% inoculum of FD1 was used to inoculate 1 200 mℓ of FeG medium. The flask was incubated until all the iron was oxidised. An overnight culture of E.coli was diluted 1:10 in Nutrient Broth (NB) and incubated at 37°C with constant aeration until a log phase culture was reached.

The bacterial cultures were harvested by centrifugation. The collected cells were suspended in a total volume of 10 mℓ of single strength acidified Basal Salts (pH 1.6). The mixture was centrifuged using a Sorvall Centrifuge and SS-34 Rotor at 7000 rpm for 10 min. The pellets were resuspended in 3 to 5 mℓ of SET buffer. The suspension was kept at -20°C for at least 1 hour. Predigested (1h at 37°C) pronase (20mg/mℓ stock) was added to give a final concentration of 2 mg/mℓ. Once the mixture had melted 1% SDS was added to give a final concentration of 10%. The suspension was kept at 4°C for 10 min. RNase was added to a final concentration of 50 µg/mℓ and the mixture incubated at 37°C for 10-15 min. The solution was placed in a dialysis bag and dialysed overnight at 50°C against 0.1X SSC. After dialysis the sample was cooled to ±4°C and an equal volume of cold chloroform: isoamyl alcohol (24:1 v/v) was added. The two layers were gently mixed by rotation and then centrifuged at 3000 rpm for 10 min. The top layer was removed and deproteinized repeatedly with cold chloroform: isoamyl alcohol until the interface was clear. The final layer was cooled to ±4°C and sodium acetate added to a final concetration of 0.3 M. This mixture was kept in an ice bath for approximately 30 min. The nucleic acids were precipitated by gently layering 2 volumes of cold 95% alcohol on the previous mixture. The nucleic acids were spooled in a glass rod and dissolved in 0.1X SSC. The small pieces of DNA present in the mixture were collected by centrifugation at 3000 rpm for 10 min. The pellet was dissolved in 0.1X SSC. The solution was then placed in a dialysis bag and dialysed overnight at 4°C against 0.1 SSC. The DNA was stored at 4°C over chloroform.

4.2.6.2 Determination of Guanine plus Cytosine Content of DNA

The DNA base composition was calculated from the thermal denaturation profiles of the DNA determined in 0.1X SSC on a Beckman DU-8 Spectrophotometer equipped with a Tm Analysis System. The DNA solution was degassed to prevent the formation of bubbles during heating. One sample was analysed at a time. The rate of change in absorbance per degree change in temperature was printed out by the instrument as well as the first derivative. The temperature at which the first derivative was at a maximum was considered to be the Tm value.

E. coli DNA was used as a reference DNA which has a GC ratio of 51± 0,25 mol%. The formula provided by Mandel & Marmur (1968) was corrected using this value and the experimental Tm value for E. coli DNA. The GC content of the samples was calculated from the equation GC = (Tm - 54,49) 2,44.

4.2.6.3 Determination of DNA Homology

The DNA samples were diluted with 0.1X SSC to give a concentration of 20 µg/mℓ. The absorbance reading was determined at 260 nm using a Beckman Du-8 Spectrophotometer. Each DNA solution (2 mℓ) was denatured by heating at 90°C for 10 min followed by a rapid cooling on ice. The absorbance reading at 260 nm was determined again. Each DNA sample was pipetted into a siliconised scintillation vial of 25 mm in diameter. A nitrocellulose disc was placed into the scintillation vial and left for 1h at 4°C with intermittent shaking to allow DNA to bind to the filter. The filter was then baked for 2 hours at 80°C. The remaining DNA in the vial was heated for 10 min. at 90°C and the absorbance reading determined at 260 nm in the Beckman Du-8 Spectrophotometer. A prehybridization mixture was prepared as follows: 5 mg of crude salmon sperm DNA contained in 10 mℓ of 6X SSC was sonicated twice for 15 sec. each time. Dernhart's solution (20 µℓ) was added containing 10% Ficol, 10% Polyvinylpyrolidine and 10% Bovine Serum Albumin. The prehybridization mixture (2 mℓ) was added to 25 mm vials. Each of the nitrocellulose discs was placed into the vial and incubated at 65°C for 2h. The prehybridization mixture was then removed with a pasteur pipette and 2 mℓ of hybridization mixture were added followed by incubation with shaking at 65°C overnight. The prehybridization mixture was prepared as follows: to 1 mℓ of nick translated DNA (reference DNA) in TE buffer was mixed with 0,2 mℓ of 1N NaOH and left at room temperature for 10 min. An aliquot of 1M Tris-HCl buffer (0,2 mℓ) pH 8.4 and 0,2 mℓ 1M HCl were added and mixed. A solution containing 3 mℓ of 20X SSC and 3,9 mℓ H_2O containing 25 mg crude sonicated DNA was added. Denhart's solution (100 µℓ) and 0,5 mℓ 10% SDS were added and the volume made up to 10 mℓ with H_2O. Labelled DNA was separated from the unreacted nucleotides by washing through a Sephadex G50 column with 10 mM TE buffer. Fractions containing labelled DNA (0,5 mℓ) were collected and the radioactivity counted in a scintillation counter.

The two reference DNA strains used, namely, FD1 and Het FD1 were prepared for hybridization by mixing each DNA sample (1 µg) with nucleotide/buffer solution (10 µℓ), labelled (α ^{32}P)d CTP(5µℓ), an enzyme solution (5 µℓ) and water to 50 µℓ . This reaction mixture was incubated at 15°C for 2 h. Specific activities of 5×10^6 cpm/µg and 8×10^6 cpm/µg were obtained for FD1 and Het FD1 respectively.

4.2.7 Separation of Thiobacillus Cultures Using Density Gradient Centrifugation in a Percoll Solution

The bacterial strains used are listed in Table 4.2.

TABLE 4.2: Bacterial strains used in the characterisation of bacteria in iron liquors.

Bacteria	Strain	Source
T. ferrooxidans	LT2	BACFOX unit at the Buffelsfontein Mines - General Mining - SA
T. ferrooxidans	FD1	Florence Devonian Ore - General Mining - SA
T. ferrooxidans	TF1-35	Claude Nicolau - Centre de Biophysique Moleculaire - Orleáns
T. ferrooxidans	ATCC 19859	American Type Culture Collection

The four *T. ferrooxidans* strains were grown in 250 mℓ flasks containing 150 mℓ of ferrous sulphate medium at pH 1.7. The cultures were incubated at 37°C until the ferrous iron in the medium was completely oxidised. All cultures were harvested by centrifugation, washed once, and resuspended in 2 mℓ single strength basal salts medium at pH 1.6.

Percoll was purchased from Pharmacia Fine Chemicals. Self-generating gradients (8 mℓ) of 50% Percoll were prepared with physiological saline (G. Carr, personal communication). The gradient solution containing 4 mℓ of Percoll, 0,8 mℓ of

8,5% saline and 2,7 mℓ distilled water was thoroughly mixed with 0,5 mℓ of sample. A suspension (10 µℓ) of density gradient marker beads (Pharmacia Fine Chemicals, Uppsala, Sweden) were added to the gradient tubes. The tubes were centrifuged at 21000 rpm in a Beckman L8-70 Ultracentrifuge with an angle-head rotor (Ti-50) for 25 min. at 15°C. The beads are coloured derivatives of Sephadex of known density and cover the range 1,017 - 1,143 g/mℓ for Percoll/0,15 M NaCl mixtures (Percoll Density Marker Beads, 1978). The cell densities were estimated by correlating the position of the cell band with the positions of the Coloured Marker Beads.

After centrifugation bacterial cells contained in each fraction were inoculated into ferrous sulphate medium to test the ability of viable bacteria to oxidise iron.

4.3 RESULTS

4.3.1 Adaptation of T. ferrooxidans to Grow on Glucose

Following a short adaptation period to glucose, two of the T. ferrooxidans cultures were able to grow on glucose media in the absence of any iron (Table 4.3) overleaf.

Upon prolonged incubation on GYE media a heterotrophic bacterium was isolated from strains LT2 and FD1. These two bacteria when transferred back to FeG media failed to oxidise iron suggesting the presence of an iron-oxidising

TABLE 4.3: Growth of four strains of *T. ferrooxidans* on two different liquid media - iron medium supplemented with glucose and glucose medium.

Bacterial strain	Medium	
	FeG	GYE
LT2	G	G
FD1	G	G
ATCC 19859	G	NG
TF1-35	G	NG

G - Growth
NG - No growth

bacterium in the above cultures. No heterotrophic growth was obtained when strains ATCC 19859 and TF1-35 were inoculated into GYE liquid medium.

LT2 and FD1 colonies were formed on GYE plates after three to five days of incubation. The colonies were slightly pinkish and became darker pink upon ageing. No growth was observed on the FeG plates.

4.3.2 Effect of Glucose and Yeast Extract on the Rate of Iron Oxidation

The inhibitory effect of two sources of organic matter on the ability of ATCC 19859, LT2 and FD1 strains of *T. ferrooxidans* to oxidise iron was established for increasing doses of glucose and yeast extract (Figures 4.1, 4.2 and 4.3). The iron-oxidising bacteria seemed to

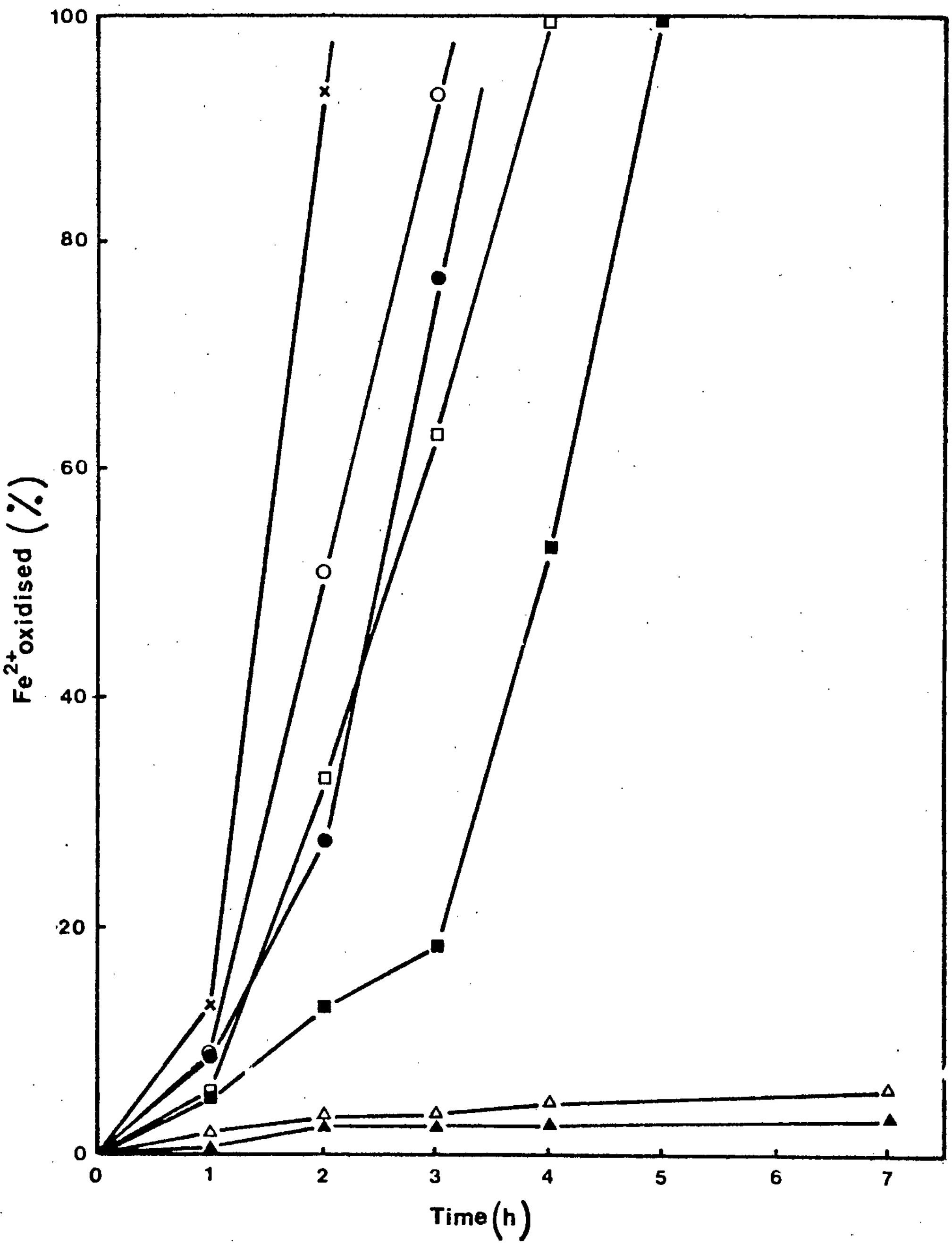


FIGURE 4.1: Effect of increasing concentrations of glucose and yeast extract on iron oxidation by *T. ferrooxidans* ATCC 19859. Control (X) Glucose concentrations; 0,5 g/ℓ (○); 1,0 g/ℓ (□); 5,0 g/ℓ (△). Yeast extract concentrations; 0,5 g/ℓ (●); 1,0 g/ℓ (■); 5,0 g/ℓ (▲).

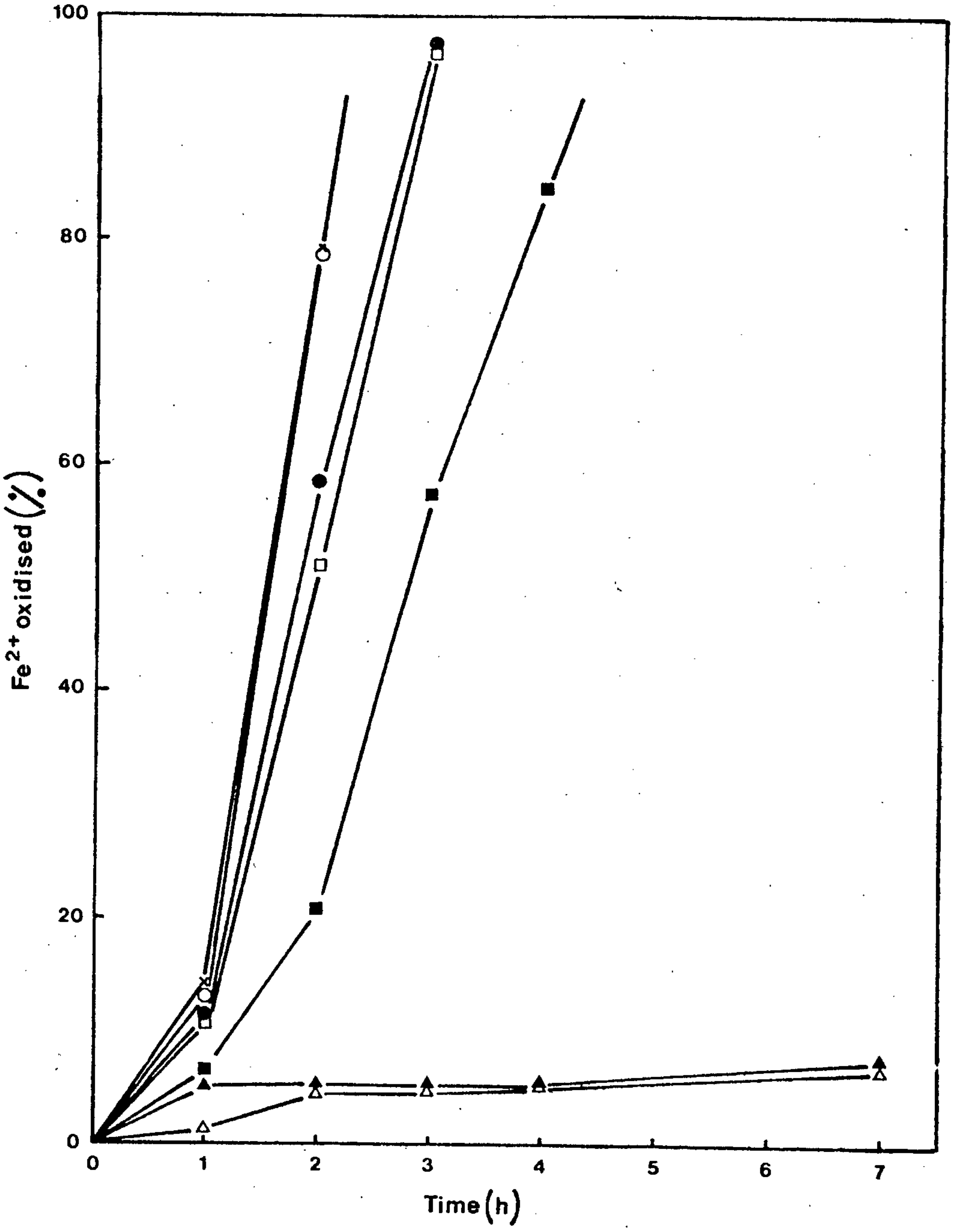


FIGURE 4.2: Effect of increasing concentrations of glucose and yeast extract on iron oxidation by *T. ferrooxidans* LT2. Control (X). Glucose concentrations 0,5 g/ℓ (O); 1,0 g/ℓ (□); 5,0 g/ℓ (△). Yeast extract concentrations; 0,5 g/ℓ (●); 1,0 g/ℓ (■); 5,0 g/ℓ (▲).

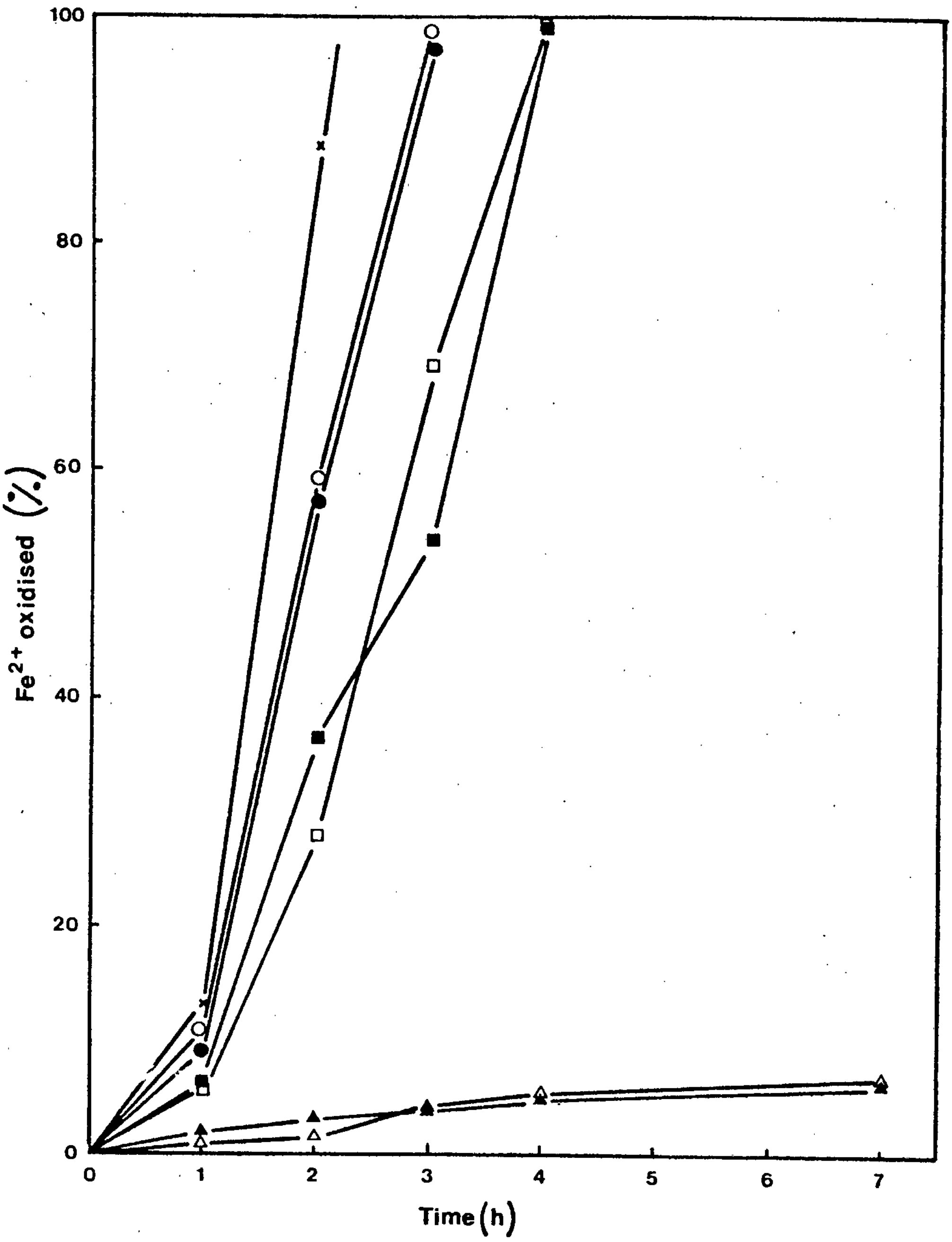


FIGURE 4.3: Effect of increasing concentrations of glucose and yeast extract on iron oxidation by *T. ferrooxidans* FD1. Control (X). Glucose concentrations; 0,5 g/ℓ (○); 1,0 g/ℓ (□); 5,0 g/ℓ (△). Yeast extract concentrations; 0,5 g/ℓ (●); 1,0 g/ℓ (■); 5,0 g/ℓ (▲).

tolerate glucose and yeast extract up to a concentration of 1 g/ℓ. A concentration of 5 g/ℓ of either of the above carbon sources was found to be inhibitory in that no growth was obtained after 7d of incubation. Yeast extract appeared to be more inhibitory than glucose at the same concentration, especially for strains LT2 and ATCC 19859. In order to establish whether the heterotrophic organism is able to assist T. ferrooxidans to tolerate greater concentrations of organic matter, the rate of iron oxidation in iron medium supplemented with different concentrations of glucose and yeast extract was also compared for ATCC 19859 in pure culture and in the presence of Het LT2. Although the ATCC 19859 - Het LT2 culture showed only a slight increase in the rate of iron oxidation when compared with the ATCC 19859 strain alone, this difference was more marked at higher concentrations of both carbon sources (Figures 4.4 and 4.5). A concentration of 5 g/ℓ of glucose caused complete inhibition of iron oxidation by ATCC 19859 alone in contrast to 15% iron oxidation observed for ATCC 19859 - Het LT2. The same concentration of yeast extract was inhibitory in both cases.

4.3.3 Relationship between T. ferrooxidans FD1 Strain and Het FD1

Previously it was shown (Section 4.3.2) that the heterotrophic bacteria slightly improved the rate of iron oxidation by T. ferrooxidans in 9K medium containing organic matter. However, the heterotrophic bacteria might benefit from the autotrophic Thiobacillus. In order to determine if the

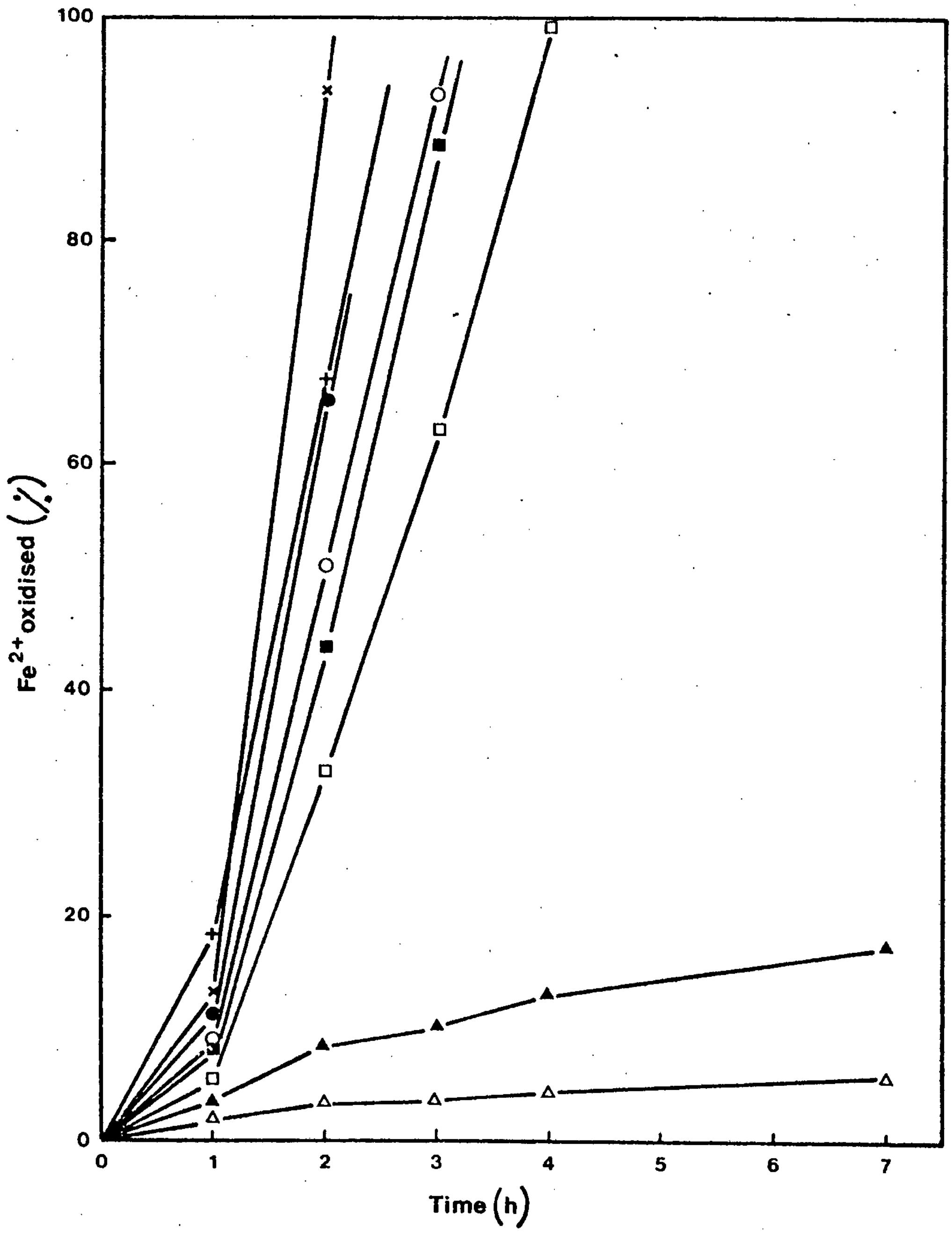


FIGURE 4.4: Effect of increasing concentrations of glucose on iron oxidation by *T. ferrooxidans* ATCC 19859 (open symbols) and *T. ferrooxidans* ATCC 19859 - Het LT2 mixed culture (closed symbols). Control ATCC 19859 (X). Control ATCC 19859 - Het LT2 (+). Glucose concentrations; 0,5 g/ℓ (○,●); 1,0 g/ℓ (□, ■); 5,0 g/ℓ (△, ▲).

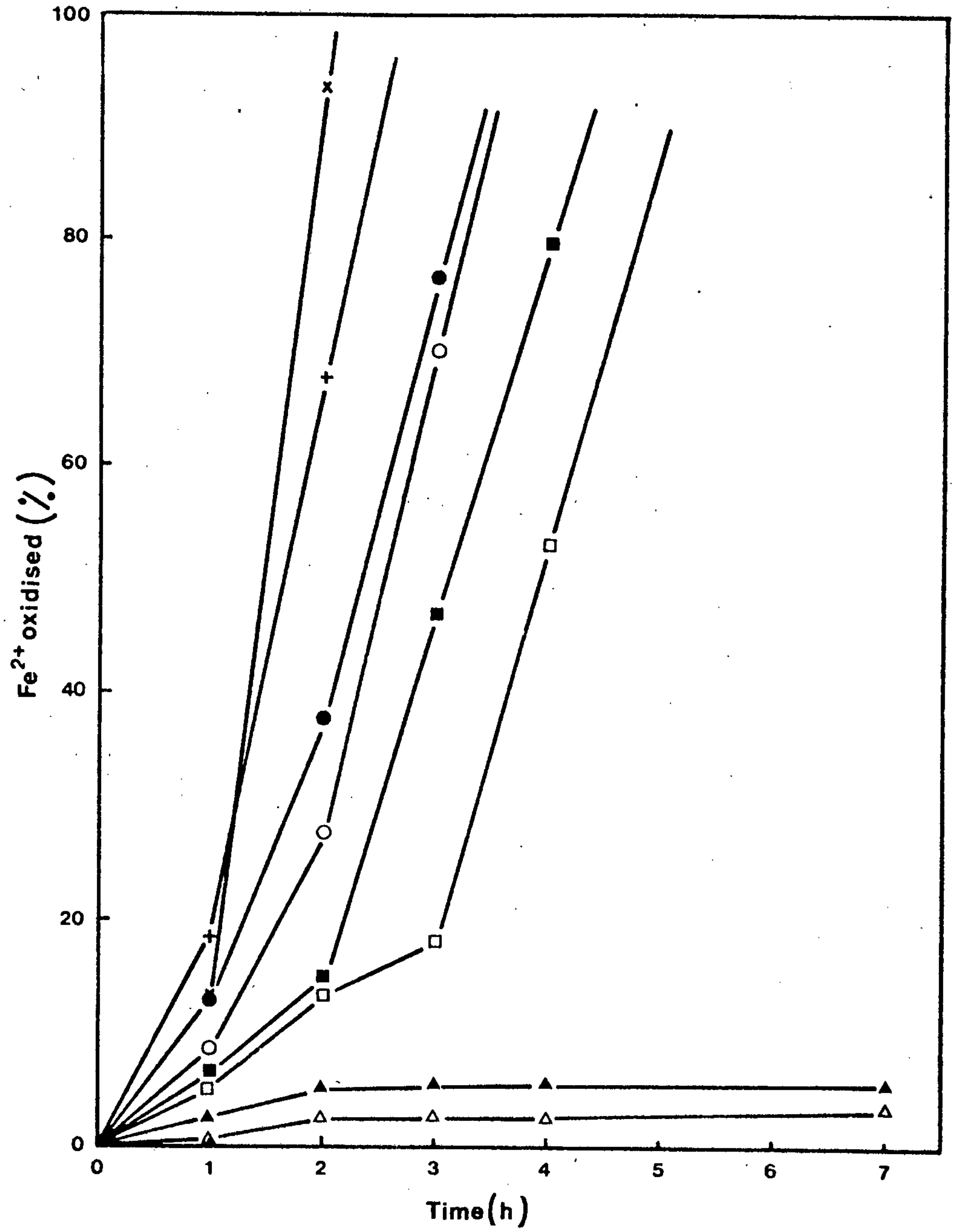


FIGURE 4.5: Effect of increasing concentrations of yeast extract on iron oxidation by *T. ferrooxidans* ATCC 19859 (open symbols) and *T. ferrooxidans* ATCC 19859 - Het LT2 mixed culture (closed symbols). Control ATCC 19859 (X). Control ATCC 19859 - HET. LT2 (+). Yeast extract concentrations; 0,5 g/ℓ (○,●); 1,0 g/ℓ (□,■); 5,0 g/ℓ (△,▲).

growth of the heterotrophic bacterium is influenced by T. ferrooxidans, a culture containing FD1 and Het FD1 was compared with two other cultures, one containing FD1 alone and the other Het FD1 alone (Figure 4.6). The three cultures were in 9K medium with no organic matter present. FD1 culture oxidised iron completely after 3d of incubation with a proportional increase in bacterial number as determined by direct count. No plate counts were obtained on GYE solid medium since this medium contains no iron. Although this strain of T. ferrooxidans managed to grow on iron solid media (Section 2.4.1) no growth was observed on FeG plates containing iron and glucose probably because of the extra amount of organic matter in addition to the agar. No iron oxidation or growth detected by direct count was observed for Het FD1 since no organic matter was present in the medium. No colonies were detected on FeG plates and a drop in the number of colonies was observed on GYE plates. The rate of iron oxidation of the FD1 - Het FD1 mixed culture was similar to the rate observed for the FD1 culture alone. Growth was observed on GYE plates but not on FeG plates. A marked increase in viable counts on GYE solid media occurred after the second day of incubation when approximately 62% of the iron had been oxidised indicating that Het FD1 was growing in the mixed culture and presumably living on the by-products released during the growth of FD1.

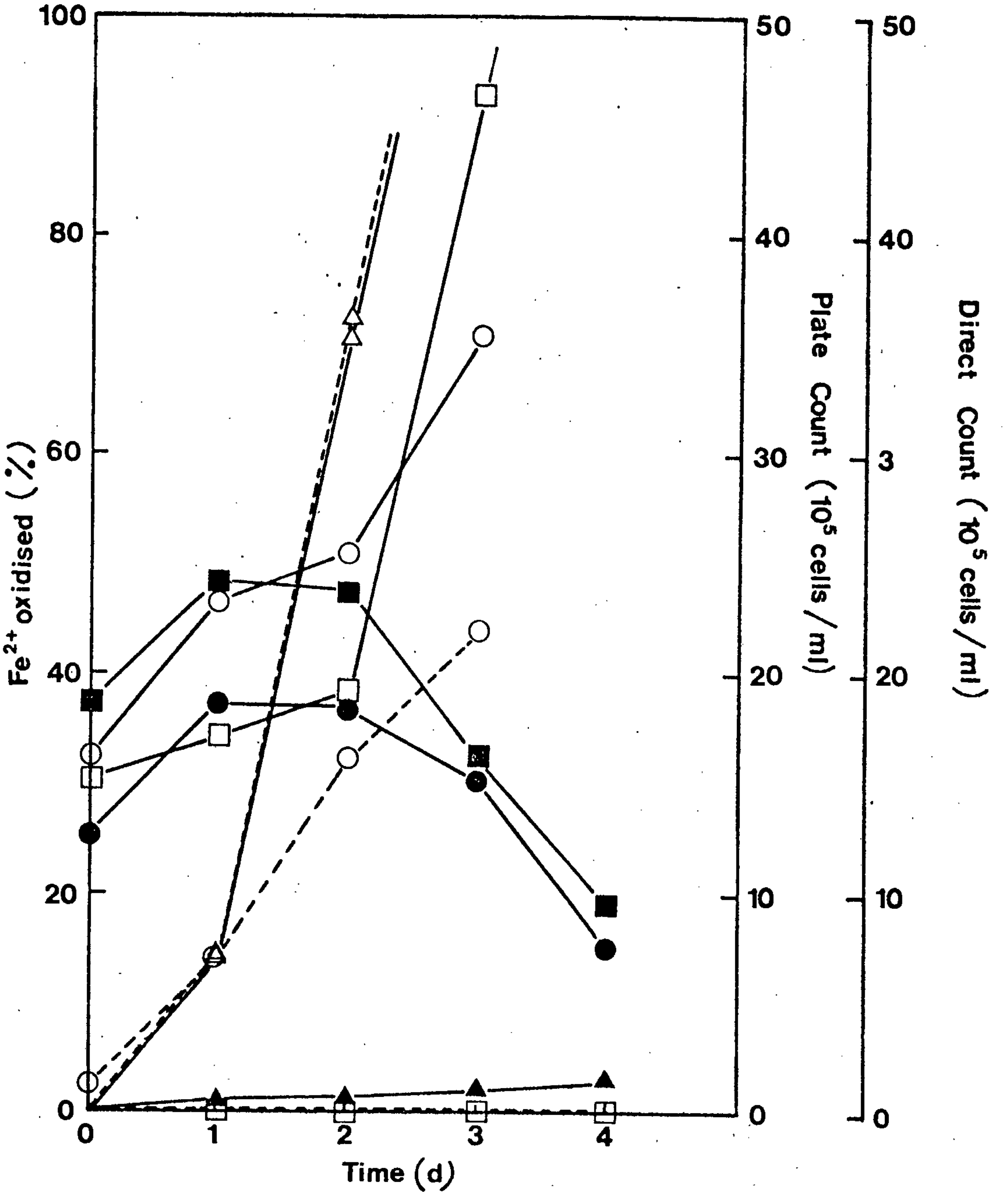


FIGURE 4.6: Relationship between the growth of *T. ferrooxidans* FD1 (-- △ --), Het FD1 (- ▲ -) and *T. ferrooxidans* FD1 - Het FD1 mixed culture (- △ -) on ferrous iron media. Growth was determined by ferrous iron oxidation (-- △ --, -▲-, -△-), direct bacterial count (--○--, -●-, -○-) and plate count (--□--, -■-, -□-

4.3.4 Utilization of Glucose by Iron Grown FD1 Strain of *T. ferrooxidans*

The growth of *T. ferrooxidans* FD1 strain in iron medium and in iron medium supplemented with glucose was determined using all the different methods that have been followed in this study to estimate the growth of *T. ferrooxidans*. Strain FD1 grew equally well in both types of media (Figure 4.7). The rate of iron oxidation was faster in 9K medium containing 9 g/ℓ of ferrous iron than in FeG medium which had only 4 g/ℓ of ferrous iron. However, FeG medium contained 0.5% glucose which could have slowed down the rate of iron oxidation. The amount of glucose present in FeG medium decreased from 5 g/ℓ to 2,5 g/ℓ as the iron was oxidised. This decrease was not due to acid hydrolysis of glucose as no glucose was degraded in an uninoculated control. *T. ferrooxidans* cells failed to grow on FeG and GYE plates. This indicated that some component of the agar together with the glucose present in the media was inhibitory to growth. A higher bacterial yield (4,3096 g/mole Fe^{2+} and 0,388 g/mole glucose) was obtained when *T. ferrooxidans* was grown in FeG medium as compared to 9K medium (1,5291 g/mole Fe^{2+}). The yield obtained in FeG medium was due to both iron and glucose in the proportion of 0,7071 g/mole Fe^{2+} to 3,6025 g/mole glucose. Both the increase in yield and disappearance of glucose from FeG medium suggested that *T. ferrooxidans* FD1 strain was able to use the glucose present in FeG medium as a carbon and energy source. There was good correlation between the three methods used to estimate *T. ferrooxidans* growth, namely, iron oxidation, direct counts and protein.

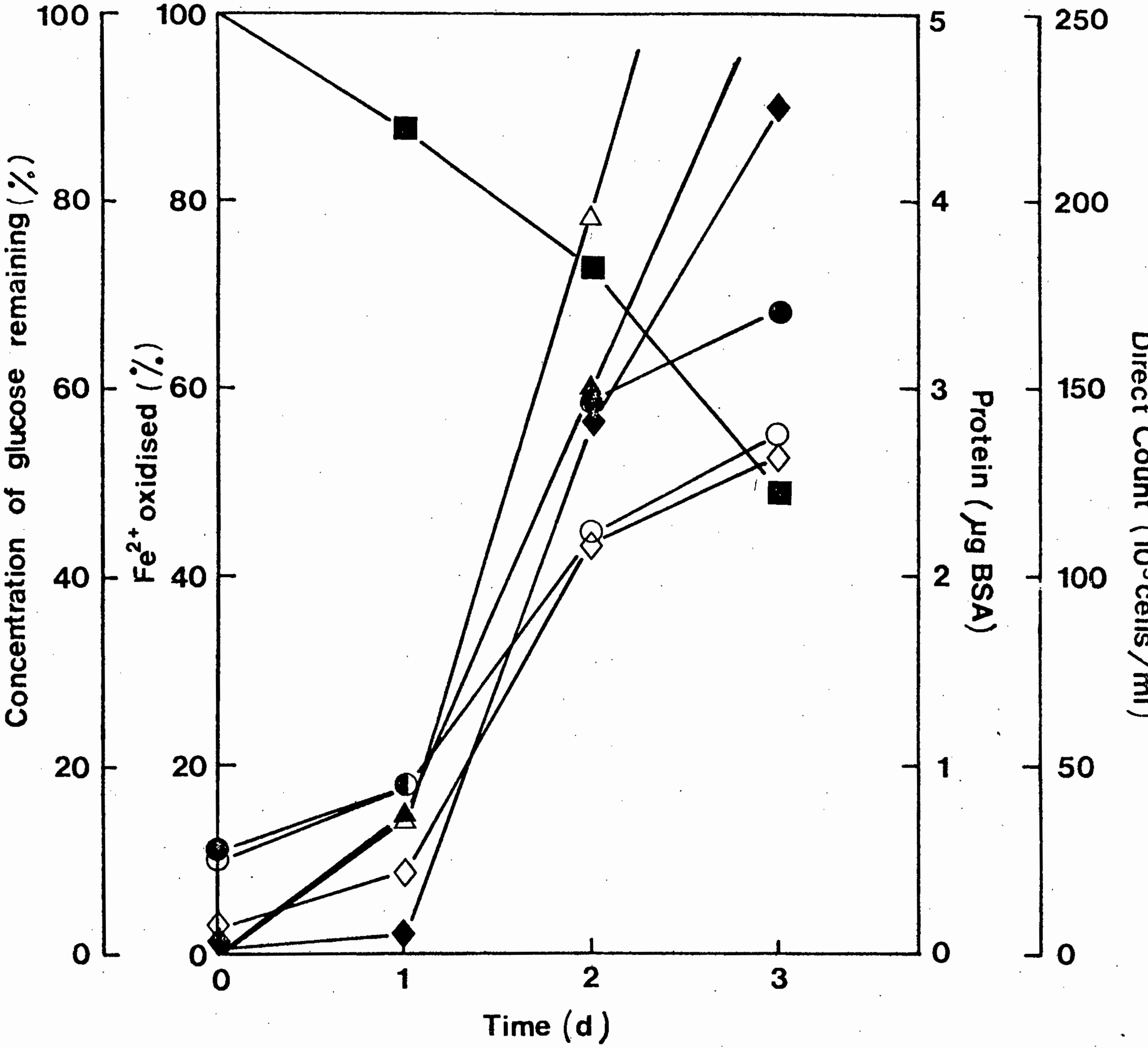


FIGURE 4.7: Comparison between the growth of *T. ferrooxidans* FD1 strain in iron medium (open symbols) and in iron-glucose medium (closed symbols). The growth was determined by ferrous iron oxidation (△,▲), direct bacterial count (○,●), protein estimation (◇,◆) and glucose utilization (■) in iron-glucose medium.

4.3.5 Utilization of Glucose by T. ferrooxidans FD1 strain at Different Concentrations of Iron

To determine whether FD1 strain of T. ferrooxidans when grown in mixotrophic medium utilizes ferrous iron preferentially or, ferrous iron and glucose simultaneously, its growth was determined for different concentrations of ferrous sulphate at a constant amount (0,5%) of glucose (Figure 4.8) At a concentration of 4 g/ℓ of ferrous iron FD1 utilized both iron and glucose concurrently although iron was exhausted first. At 7 g/ℓ of ferrous iron there was a slight lag phase where ferrous iron was utilized alone. Ferrous iron was therefore utilized preferentially although some glucose was also consumed, but when the concentration of ferrous iron dropped to approximately 4 g/ℓ both iron and glucose were utilized simultaneously with iron being exhausted first. At 9 g/ℓ of iron there was complete inhibition of iron oxidation and glucose utilization suggesting that at this concentration of iron, glucose becomes inhibitory. Furthermore it seems that glucose per se is not inhibitory in mixotrophic medium but rather the ratio of iron to glucose affected the ability of the organism to use either of the substrates.

4.3.6 DNA Base Composition

The base compositions of the DNA of the iron-oxidising T. ferrooxidans were found to be identical (Table 4.4). However, the GC content of LT2 and FD1 differed signifi-

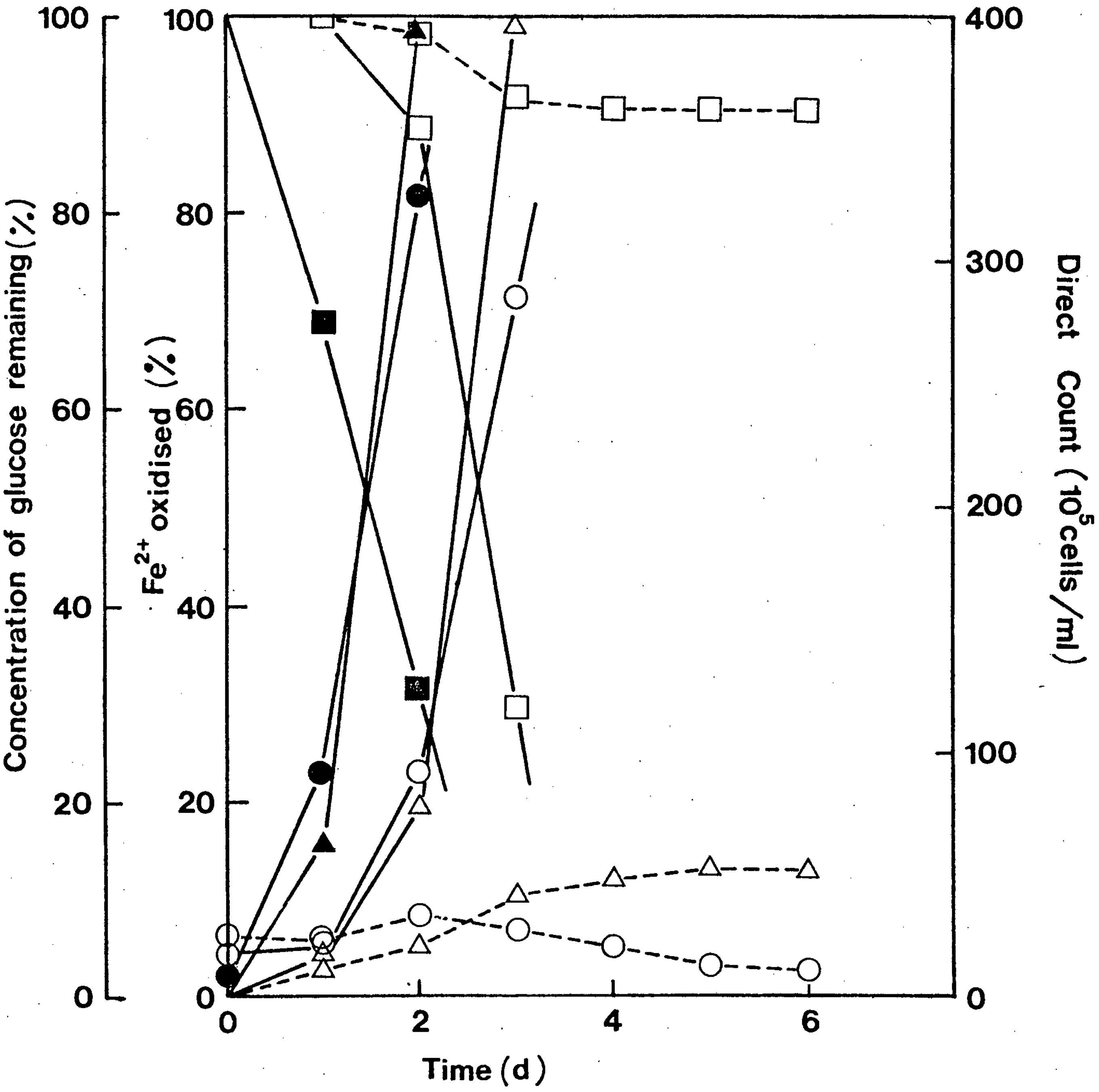


FIGURE 4.8: Utilization of glucose by T. ferrooxidans FD1 in iron medium supplemented with glucose, at different ferrous iron concentrations. Initial glucose concentrations; 5 g/ℓ. Initial ferrous iron concentrations; 4 g/ℓ (-▲-); 7 g/ℓ (-△-); 9 g/ℓ (--△--). Glucose utilization (-■-, -□-, --□--). Iron oxidation (-▲-, -△-, --△--). Direct bacterial count (-●-, -○-, --○--.)

cantly from their respective heterotrophs suggesting that they are two completely different bacteria.

TABLE 4.4: Moles % GC content of DNA of different bacterial strains.

Bacterial Strain	Tm	% GC
LT2	79,0	59,80
FD1	79,3	60,54
ATCC 19859	79,3	60,54
TF1-35	79,3	60,54
Het LT2	82,3	67,86
Het FD1	82,3	67,86
*FD1	79,3	60,54
E. coli	75,4	51,02

DNA homology studies for the iron-oxidising Thiobacillus strains and the two heterotrophic isolates were determined using FD1 and Het FD1 as probes. Homology values lower than 5% were obtained between Het FD1 and the iron-oxidising strains showing that these bacteria are not related (Table 4.5). Zero homology was observed between FD1 and the two heterotrophs confirming that both heterotrophs are not related to the iron oxidising T. ferrooxidans strains. Different degrees of homology occurred between FD1 and the other T. ferrooxidans strains (Table 4.5). FD1 and *FD1 seem to be closely related since a high degree of homology was obtained between them. Similarly, the two heterotrophs proved to be the same organism.

TABLE 4.5: DNA homologies among strains of *T. ferrooxidans* and heterotrophic bacteria.

Strain	Energy source	Percent homology to	
		FD1	Het FD1
LT2	Iron	27	0
Het LT2	Glucose	0	100
FD1	Iron	100	3,1
Het FD1	Glucose	0	100
*FD1	Iron and Glucose	85	4,8
ATCC 19859	Iron	39	2,2

4.3.7 Differentiation of Thiobacillus Cultures by Cell Density on Percoll Gradients

The four *T. ferrooxidans* strains formed a single band after centrifugation on Percoll gradients (Figure 4.9). The band produced by the FD1 strain was at much lower density (1,023 mg/mℓ) than the bands produced by the other three *Thiobacillus* strains. The cell densities for *T. ferrooxidans* strains LT2, ATCC 19859 and TF1-35 were 1.066, 1.071 and 1.078 mg/mℓ respectively (Figure 4.10). The growth in the ferrous iron medium of *T. ferrooxidans* cells recovered from the Percoll gradient fraction indicated that Percoll has no effect on the ability of *Thiobacillus* to oxidise iron.

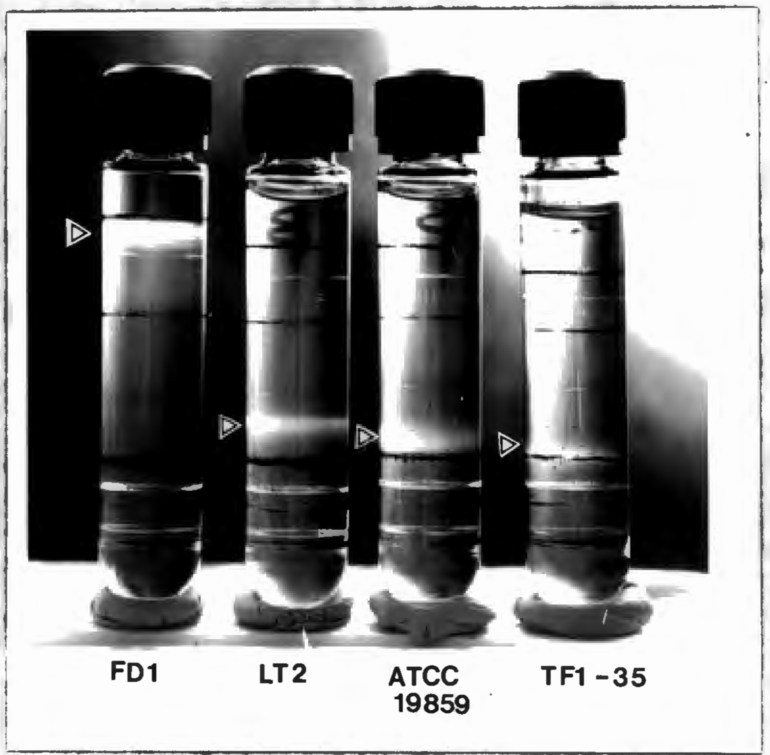


FIGURE 4.9: Percoll density gradients of four T. ferrooxidans cultures; FD1, LT2, ATCC 19859, TF1-35. The arrows indicate the bands containing the organisms.

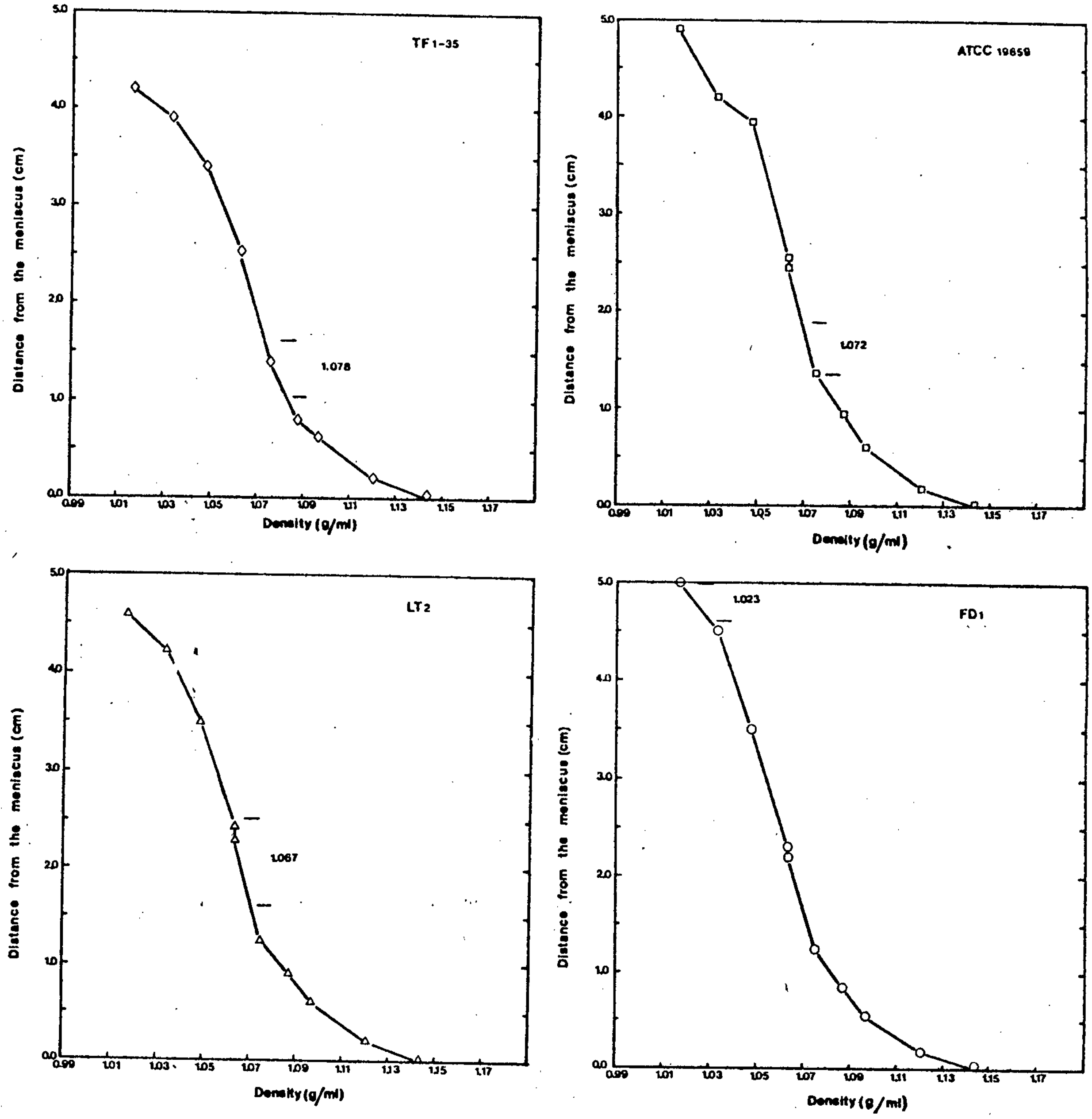


FIGURE 4.10: Density distribution of Percoll gradients and position of cell bands using density marker beads for four *T. ferrooxidans* strains. TF1-35 (◇); ATCC 19859 (□); LT2 (△); FD1 (○).

4.4 DISCUSSION

The heterotrophic bacteria isolated from LT2 and FD1 cultures after prolonged incubation on organic matter had similar characteristics to the glucose-grown cells reported by Shafia & Wilkinson, jr.(1969): glucose adapted cells became obligately heterotrophic after a couple of transfers in a medium lacking oxidisable iron and, on agar medium the colonies were circular, smooth and slightly pinkish. However, only two of the Thiobacillus cultures tested were able to adapt to grow on glucose alone. TF1-35 and ATCC 19859 could tolerate 0,5 g/ℓ glucose in the presence of iron but did not grow on glucose alone. One possible explanation for the difference in growth characteristics among the four strains of Thiobacillus tested could be that LT2 and FD1 cultures existed as a mixed culture of T. ferrooxidans and a closely associated heterotrophic acidophilic bacterium, while ATCC 19859 and TF1-35 were pure T. ferrooxidans cultures. An alternative explanation for this phenomenon, based on reports that there are different strains of T. ferrooxidans with different metabolic activities, could be that, LT2 and FD1 belonged to a different group of T. ferrooxidans capable of using organic substrates. However, the obligate heterotrophy of Het LT2 and Het FD1 ruled out this second possibility and it was apparent that LT2 and FD1 were mixed cultures. Attempts were made to get LT2 free from the heterotrophic bacteria by subculturing in iron medium but the heterotrophic bacteria were not completely eliminated from the iron grown culture. As a result, upon prolonged adaptation of this culture to glucose the heterotrophic bacteria were isolated again. A more efficient way of purifying T. ferrooxidans LT2 from this apparent mixed culture would be in the same way that T. ferrooxidans FD1 was purified, through repeated transfers on solid iron media. However, as reported in Chapter 2, this is not possible due to the inability of T. ferrooxidans LT2 to form colonies on purified agar media. It was however possible to obtain pure cultures of Het FD1 and Het LT2 by repeated subculturing on media lacking iron.

The study of the effect of different levels of organic matter in the rate of iron oxidation for three strains of iron-oxidising Thiobacillus demonstrated that these autotrophic bacteria are in fact able to oxidise iron in the presence of glucose and yeast extract up to a concentration of 1 g/ℓ. Glucose was more easily utilised than yeast extract which was expected since yeast extract is a more complex organic mixture. Tuovinen, Niemela & Gyllenberg (1971) observed a similar type of response when 5 g/ℓ of glucose and yeast extract were used in ferrous sulphate medium containing 2 g/ℓ of ferrous iron. A lag of three days was observed for glucose and a complete inhibition up to fourteen days was found for yeast extract. However, in the present study a concentration of 5 g/ℓ of either of the two organic compounds caused complete inhibition of iron oxidation in 9K medium possibly because the concentration of ferrous iron in the medium was 9 g/ℓ.

The close association of the heterotrophic bacteria with

T. ferrooxidans illustrated by the difficulty in isolating pure cultures of LT2 led to the investigation of a possible role for the heterotrophic bacteria. As suggested by Harrison, Jarvis and Johnson (1980) the heterotrophic bacteria might play a role in the natural habitat of *T. ferrooxidans* by utilizing the organic matter harmful to *T. ferrooxidans*. In an attempt to find some kind of function for the heterotrophic bacteria a pure culture of *T. ferrooxidans* ATCC 19859 was mixed with Het LT2. A marked increase in the rate of iron oxidation, in the presence of organic matter, was expected for the ATCC 19859 - Het LT2 mixed culture if the heterotroph had a positive effect in *T. ferrooxidans* growth. Although only a slight improvement in the rate of iron oxidation was observed at all concentrations of glucose and yeast extract the ATCC 19859 - Het LT2 mixed culture oxidised iron at a greater rate than the ATCC 19859 culture alone. If the role for this heterotrophic bacterium is in the removal of potentially inhibitory organic matter from the *T. ferrooxidans* environment, this role would appear to be a minor one. This is not an unexpected result since if some organic matter is present in a mining environment, its level is unlikely to be as high as 0,5%. Furthermore, when *T. ferrooxidans* FD1 was mixed with the Het FD1 in a mixotrophic medium the acidophilic bacteria did not contribute in any way to the development of *T. ferrooxidans*. On the other hand the reason for the close existence between *T. ferrooxidans* and the acidophilic heterotrophic bacteria may be that the heterotrophs might obtain their organic carbon from the autotrophic Thiobacillus without necessarily benefitting the Thiobacillus. In order to test this possibility the growth pattern as well as the rate of iron oxidation, in 9K medium with no organic matter, was determined for the T. ferrooxidans FD1 strain mixed with Het FD1 and was compared with FD1 and Het FD1 cultures. Being a heterotrophic bacterium, Het FD1 alone did not grow in 9K medium which had no carbon source, but it managed to do so when mixed with T. ferrooxidans FD1. This suggested that FD1 contributed to the growth of Het FD1 which is able to grow on the organic by-products of the T. ferrooxidans strain. Brierley (1982) suggested that the heterotrophic bacteria found in leaching environments live either, on the small amounts of organic matter present in those environments or, on the organic excreta produced by the iron-oxidising Thiobacillus. The present work shows that the latter is able to take place. T. ferrooxidans plays then a role in supporting the growth of heterotrophic microorganisms found in acidic environments and the dependence of these bacteria on T. ferrooxidans explains the tenacious association of these bacteria.

Analysis of the DNA base composition of the iron-oxidising and the heterotrophic bacterial strains demonstrated that these two groups of bacteria are not related. The GC content of the DNA within each group, was similar for all bacteria belonging to that particular group. An interesting observation was the fact that the same GC value was obtained for FD1 when grown in iron medium and when grown in iron medium supplemented with glucose (*FD1′). If FD1 was a mixed culture the heterotroph would be expected to have outgrown the autotroph in the presence of glucose due to the high energy cost of autotrophic growth versus heterotrophic growth. The GC value would then be the same as the one obtained for the heterotroph. However, it was not similar to the GC content of the Het FD1 but was identical to the iron grown cultures of T. ferrooxidans. The high DNA homology between the two FD1 strains and the low DNA homology between these strains and the obligate autotrophic T. ferrooxidans LT2 and ATCC 19859 suggested that there are two distinct groups of iron-oxidising Thiobacillus. The higher bacterial cell yield obtained when the FD1 strain was grown in iron-glucose medium compared to iron medium alone indicated that this Thiobacillus strain used glucose as an energy source as well as a carbon source. FD1 is therefore a facultative autotrophic T. ferrooxidans similar to the one reproted by Sugio, Tano & Imai (1982) while ATCC 19859 and LT2 seem to be strict autotrophs. Sugio, Tano & Imai (1982) reported the isolation of a facultative iron-oxidising strain of T. ferrooxidans based on the uptake of $[^{14}C]$ glucose into the cells and the ability to metabolize it to CO_2 and H_2O in a medium containing both iron and glucose. However, they did not show that the genetic composition of the facultative autotropic isolate grown in iron medium, is the same to the one grown in iron-glucose medium. The above authors found that at a concentration of 10 g/ℓ ferrous iron and 0,5% glucose, ferrous was utilized immediately and rapidly followed by uptake of glucose into the cells. The *T. ferrooxidans* FD1 strain used in the present study also utilized iron first until it dropped to approximately 4 g/ℓ after which glucose was also utilized. However, 9 g/ℓ of ferrous iron strongly inhibited the growth of *T. ferrooxidans* FD1 in a mixotrophic medium containing 0,5% glucose. As Sugio, Tano & Imai (1982) suggested the concentration of ferrous iron seems to determine the growth of *T. ferrooxidans* in the presence of glucose although the reason is still unclear. DNA homology studies between Het FD1 and Het LT2 and between these bacteria and the *T. ferrooxidans* strains confirmed that Het FD1 and Het LT2 are not iron-oxidising bacteria but acidophilic heterotrophs. This supported the hypothesis proposed by Tuovinen *et al* (1978) that, *T. ferrooxidans* strains capable of growing on organic matter with subsequent loss in their ability to oxidise iron after a series of transfers in glucose, were in fact mixed cultures. From these results it appears that some *T. ferrooxidans* strains are obligate autotrophs, some are facultative autotrophs able to use glucose in the presence of iron and that often *T. ferrooxidans* cultures are very closely associated with a heterotrophic bacterium.

The cells densities of *T. ferrooxidans* strains, grown in the same substrate, were determined by centrifugation in a Percoll gradient. *T. ferrooxidans* LT2 ATCC 19859 and TF1-35 had similar densities with a maximum difference of 1,15%. However the cells obtained from the facultative autotrophic *T. ferrooxidans* FD1 had a much lower density.

This suggested that Percoll gradients could be used to separate the obligate autotrophic T. ferrooxidans strains found in mining environments from the facultative autotrophic ones.

CONCLUSION

The aim of this study was:

(i) to develop a method for monitoring the growth of *T. ferrooxidans* that did not depend on iron oxidation alone;

(ii) to investigate the effect of ferrous and ferric iron concentrations on the rate of iron oxidation;

(iii) to isolate other bacteria besides *T. ferrooxidans* that might be present in mining leach liquors.

An indirect method of determining bacterial growth was developed. This method involved the measurement of protein by the Coomassie Blue protein-binding assay. It was found to be a sensitive and reproducible method completely free of interference from ions found in acid mine leach liquors. Other protein assays tested, namely, Ninhydrin and Folin Coicalteu reagent were subject to interference by inorganic ions and were not sensitive to very low levels of protein. The inability to obtain isolated colonies of *T. ferrooxidans* on different solid media supported previous findings that some component of the agar inhibits colony development on ferrous iron medium. However isolated colonies were obtained for the *T. ferrooxidans* FD1 strain suggesting that it is an agar tolerant strain.

Iron oxidation rates in batch culture were found to be exponential and a function of the initial ferrous iron concentration. The exponential rates were not affected by the ferric iron concentration *per se* but rather by the ferrous-ferric ratio. High concentrations of ferrous iron were found to inhibit the rate of iron oxidation.

The heterotrophic bacteria isolated from *T. ferrooxidans* LT2 and FD1 strains after a stepwise addition of glucose to the medium indicated that these two cultures were mixed cultures while ATCC 19859 and TF1-35 were pure. The DNA base composition determined for the iron-oxidising *Thiobacillus* and the heterotrophic bacteria, showed that these two groups of bacteria were not related to each other. In addition, the heterotrophic bacteria isolated from the *T. ferrooxidans* cultures are the same organism since a high degree of homology was observed between their DNA. All *T. ferrooxidans* cultures had identical DNA GC content including a *T. ferrooxidans* FD1 culture grown in iron-glucose medium. The high degree of homology observed between the DNA obtained from the FD1 culture grown in iron and the one grown in iron-glucose medium confirmed that they are the same organism. The ability of this *T. ferrooxidans* strain to utilize either iron or glucose but not glucose alone proved that it is a facultative autotrophic iron-oxidising bacterium. This was further confirmed by the higher bacterial yields obtained in the iron-glucose medium compared to iron medium.

The cell densities of the facultative autotrophic

T. ferrooxidans FD1 and the obligate autotrophic LT2, ATCC 19859 and TF1-35 strains were determined by centrifugation in a Percoll gradient. Cells from the facultative autotrophic strain had a much lower density than the other strains suggesting the potential of this centrifugation method in separating these two groups of T. ferrooxidans strains.

A commensal relationship exists between T. ferrooxidans and the heterotrophic bacteria in that the acidophilic heterotrophs live on organic by-products released by T. ferrooxidans. T. ferrooxidans appears to be unaffected by this close association.

The present study shows that some of the T. ferrooxidans strains isolated from the mining environment are obligate autotrophs and some are facultative autotrophs which are able to utilize glucose in the presence of iron. Furthermore many cultures of T. ferrooxidans are closely associated with acidophilic heterotrophs.

APPENDIX A

MEDIA

9k Liquid Medium

Solution A:	$(NH_4)_2SO_4$	3,0 g
	kCl	0,1 g
	K_2HPO_4	0,5 g
	$MgSO_4.7H_2O$	0,5 g
	$Ca(NO_3)_2$	0,01 g
	Distilled water	500 mℓ
Solution B:	$FeSO_4.7H_2O$	45 g unless otherwise specified
	Distilled water	500 mℓ
	pH adjusted to 1.8 with H_2SO_4	

Solution A was sterilized by autoclaving and solution B was filter sterilized. The two solutions were mixed together when cool and the pH adjusted to 1.7 with H_2SO_4.

9k Solid Medium

Solution A: As in 9k liquid medium

Solution B:	$FeSO_4.7H_2O$	45 g unless otherwise specified
	Distilled water	300 mℓ
	pH adjusted to 1.8 with H_2SO_4	

Solution C:	Agar	16 g
	Distilled water	200 mℓ

Solutions A and C were sterilized by autoclaving. Solution B was filter sterilized. Solutions A and C were removed from the autoclave, allowed to cool for 10 min and then mixed. Solution B was then added and mixed well. The pH was adjusted with H_2SO_4 and the mixtured poured into petri dishes.

Ferrous Iron Solid Medium (Tuovinen & Kelly, 1973)

Solution A:	K_2HPO_4	0,8 g
	$MgSO_4.7H_2O$	0,8 g
	$(NH_4)_2SO_4$	0,8 g
	Distilled water	400 mℓ
Solution B:	$FeSO_4.7H_2O$	66,6 g
	Distilled water	300 mℓ
	pH adjusted to 1.8 with H_2SO_4	
Solution C:	Agar	7,0 g
	Distilled water	300 mℓ

Solutions A and C were sterilized by autoclaving and mixed 10 min after being removed from the autoclave. Solution B was then added and mixed well.

FeG (Iron-glucose) Liquid Medium

Solution A:	$(NH_4)_2SO_4$	2 g
	$MgSO_4.7H_2O$	0,04 g
	KH_2PO_4	0,01 g
	Distilled water	400 mℓ
Solution B:	Glucose	5 g
	Yeast extract	0,1 g
	Distilled water	300 mℓ
Solution C:	$FeSO_4.7H_2O$	20 g
	Distilled water	300 mℓ

Solutions A and B were autoclaved separately. They were allowed to cool for 10 min at room temperature and then mixed well. Solution C was filter sterilized and then added to the previous mixture. The pH was adjusted to 1.7 with H_2SO_4.

FeG (Iron-Glucose) Solid Medium

Solution A:	As in FeG liquid medium	
Solution B:	As in FeG liquid medium	
	Agar	20 g
Solution C:	As in FeG liquid medium	

Solutions A, B and C were sterilized and mixed as in FeG liquid medium and the mixture poured into petri dishes.

GYE (Glucose) Liquid Medium

Solution A:	$(NH_4)SO_4$	2 g
	$MgSO_4.7H_2O$	0,04 g
	KH_2PO_4	0,01 g
	Distilled water	700 mℓ

Solution B:	Glucose	5 g
	Yeast extract	0,1 g
	Distilled water	300 mℓ

Solutions A and B were autoclaved separately. They were allowed to cool for 10 min at room temperature and then mixed well. The pH was adjusted to 2.0 with H_2SO_4.

GYE (Glucose) Solid Medium

Solution A:	As in GYE liquid medium	
Solution B:	As in GYE liquid medium	
	Agar	20 g

Solutions A and B were autoclaved separately then mixed and plates poured.

ISP (Iron-salts-purified agar) Medium

Solution A:	$(NH_4)SO_4$	6,0 g
	KCl	0,2 g
	$MgSO_4.7H_2O$	1,0 g

	$Ca(NO_3)_2$	0,02 g
	Distilled water	550 mℓ

The pH was adjusted to 3.0 and the solution autoclaved at 121°C for 15 min.

Solution B:	$FeSO_4.7H_2O$	33,4 g
	Distilled water	300 mℓ

The pH was adjusted to 2.5 with H_2SO_4 and the solution was filter sterilized.

Solution C:	Agar	7,0 g
	Distilled water	150 mℓ

The mixture was soaked for 15 min and autoclaved at 121°C for 15 min.

Solutions A and C were allowed to cool for 10 min at room temperature after autoclaving. Solution A was then added to solution C with gentle mixing. Solution B was then added and mixed well. The mixture was poured into petri dishes.

Tryptone-Glucose-Yeast Extract Liquid Medium

Tryptone	5,0 g
Glucose	1,0 g
Yeast extract	2,5 g
Distilled water	1 000 mℓ

APPENDIX B

BUFFERS AND SOLUTIONS

Phosphate Buffer (0.5M)

Solution A:	$NaH_2PO_4.7H_20$	15,601 g
	Distilled water	200 mℓ
Solution B:	Na_2HPO_4	14,196 g
	Distilled water	100 mℓ

Solution A (200 mℓ) and solution B (100 mℓ) were mixed to give a final pH of 5.70.

SET Buffer

Tris (50 mM)	0,6057 g
EDTA (2mM)	0,0745 g
Sucrose	25 g
Distilled water	100 mℓ

pH adjusted to 8.0 with HCl

Tris-HCl Buffer (0.2 M)

Solution A:	Tris (0.2 M)	0.6057 g
	Distilled water	25 mℓ
Solution B:	HCl (0.1 N)	30 mℓ

Solution A and solution B were mixed and diluted to 100 mℓ to give a final pH of 7.9.

Tris-HCl Buffer (1M)

Tris (1M)	12,114 g
Distilled water	100 mℓ

pH adjusted to 8.4 with concentrated HCl

TE Buffer (10 mM)

Tris (10 mM)	0,1211 g
EDTA (1 mM)	0,0372 g
Distilled water	100 mℓ

pH adjusted to 8.0 with concentrated HCl

SSC (0.1X)

NaCl (0.015 M)	0,8766 g
Trisodium citrate	0,4412 g
Distilled water	1000 mℓ

pH adjusted to 7.0

Cuprous Iodide Catalyst

$CuSO_4$ (1N) in Distilled water	1 mℓ
KI (10% w/v) in Distilled water	3 mℓ
H_2SO_4 (5% v/v) in Distilled water	1 mℓ
Starch (1% w/v) in Distilled water	2 drops

This solution was titrated with $Na_2S_2O_3$ solution (as below) until blue colour disappears. When the precipitate has settled the supernatent is poured off and 20 mℓ H_2O added and the solution well mixed. The clear solution is then decanted off and 20 mℓ H_2O added. The solution is shaken before use.

Sodium Diphenylamine Sulphonate Indicator

Barium diphenylamine sulphonate	0,32 g
Concentrated H_2SO_4	1 mℓ
Distilled water	100 mℓ

First the water was added to the sulphonate, then the acid. When the barium sulphonate had settled the clear reagent was decanted off and used.

Standard Potassium Dichromate solution

$K_2CR_2O_7$	4,39 g
Distilled water	1000 mℓ

Starch Indicator

Starch (1% w/v) in distilled water

H_2SO_4-H_3PO_4-H_2O Mixture

Distilled water	700 mℓ
H_3PO_4 (85% v/v)	150 mℓ
Concentrated H_2SO_4	150 mℓ

$Na_2S_2O_3$

$Na_2S_2O_3.5H_2O$ (0.05 N)	12,409 g
Distilled water	1000 mℓ

Ferrous (Fe^{2+}) Iron Titration (Vogel, 1961, p.309)

To 5 mℓ of sample, 15 mℓ of $H_2SO_4.H_3PO_4.H_2O$ mixture was added as well as 6-8 drops of sodium diphenylamine sulphonate indicator. The solution was titrated with standard potassium dichromate solution to purple end point.

Ferric (Fe^{3+}) Iron Titration (Vogel, 1961, p.372)

To 5 mℓ of sample were added 1 mℓ 5% H_2SO_4, 1 mℓ Cuprous iodide catalyst suspension and a few crystals of potassium iodide. The mixture was titrated with 0.05 N $Na_2S_2O_3$ until a pale straw yellow colour. Starch indicator was added (2-3 drops) and the solution titrated further until clear colour for at least 30-45 secs.

APPENDIX C

CHEMICALS

Bovine serum albumin (BSA) (5 x crystallized) - Miles Laboratories, Goodwood, SA.

Coomassie Brilliant Blue G-250-Sigma Chemical Co.

Density Gradient Marker Beads - Pharmacia Fine Chemicals, Uppsala, Sweden.

Percoll - Pharmacia Fine Chemicals, Uppsala, Sweden.

Pronase E - Merck, Darmstadt

Ribonuclease A - Sigma Chemical Co.

Salmon Sperm DNA - Sigma Chemical Co.

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