Control of Iron and Sulfur Oxidation Activities of *Thiobacillus ferrooxidans* and Bacterial Leaching of Metals From Sulfide Ores

By

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A Thesis

Submitted to the Faculty of Graduate Studies and Research

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FACULTY OF GRADUATE STUDIES

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ABSTRACT

Thiobacillus ferrooxidans is a dominant member of the bioleaching community responsible for mineral solubilization. In order to capitalize on its natural ability to solubilize metal sulfides this study began with a detailed analysis of the bacteria's iron and sulfur oxidation pathways. Ferrous iron oxidation was found to be an inducible trait. Only those cells grown in the presence of high iron concentrations retained the ability to oxidize ferrous iron. Sulfur oxidation was faster in sulfur grown cells but in both the iron and sulfur grown cells it proceeded via a sulfur oxidizing system which used molecular oxygen as the terminal acceptor. The oxidation of sulfite, an intermediate in the sulfur oxidation pathway, in the sulfur grown cells occurred via a sulfite oxidase using molecular oxygen as a terminal electron acceptor. Iron grown *T. ferrooxidans* oxidized sulfite by a free radical mechanism initiated by Fe^{3+} on the cell surface, with cytochrome oxidase involved in the regeneration of Fe^{3+} by the normal Fe^{2+} oxidizing system.

Metal extraction from mineral ore by *Thiobacillus ferrooxidans* is achieved through two reactions: the oxidation of ferrous iron to ferric and that of sulfide/sulfur to sulfuric acid. The oxidation of either ferrous iron or sulfur by *Thiobacillus ferrooxidans* was selectively inhibited or controlled by various anions, inhibitors and osmotic pressure. Iron oxidation was more sensitive than sulfur oxidation to inhibition by chloride, phosphate and nitrate at concentrations below 0.1 M and also to inhibition by azide and cyanide. Sulfur oxidation was more sensitive than iron oxidation to the inhibitory effect of high osmotic pressure. These differences were evident not only between iron oxidation by iron grown cells and the sulfur oxidation by sulfur-grown cells, but also between the iron

and sulfur oxidation activities of the same iron-grown cells. Growth experiments with ferrous iron or sulfur as oxidizable substrates confirmed the higher sensitivity of iron oxidation to inhibition by phosphate, chloride, azide and cyanide. Sulfur oxidation was even stimulated by 50 mM phosphate or chloride.

Bacterial leaching of sulfide ores using *Thiobacillus ferrooxidans*, *Thiobacillus thiooxidans* or a combination of the two was studied under various anionic concentrations. Selective zinc and copper solubilization was obtained by inhibiting iron oxidation without affecting sulfur/sulfide oxidation. Phosphate reduced iron solubilization from a pyrite (FeS_2) - sphalerite (ZnS) mixture without significantly affecting zinc solubilization. Copper leaching from a chalcopyrite ($CuFeS_2$) - sphalerite mixture was stimulated by phosphate, while chloride accelerated zinc extraction. In a complex sulfide ore containing pyrite, chalcopyrite and sphalerite both phosphate and chloride reduced iron solubilization and increased copper extraction, while only chloride stimulated zinc extraction. Maximum leaching obtained was 100 % zinc and 50 % copper. Time course studies of copper and zinc solubilization also suggested the possibility of selective metal recovery following anionic treatment.

Mineral solubilization under natural conditions is a combination of chemical and biological reactions. The chemical or indirect method of mineral solubilization involved the use of bacteria in the regeneration of ferric iron as oxidizing agent. In order to maximize ferric iron production *T. ferrooxidans* was grown on a variety of cell carriers to increase cell concentrations. Of the columns tested the packed-bed reactor containing a mixture of glass beads and nylon mesh oxidized the greatest amount of ferrous iron at 23

grams per liter per day at its operational peak. In general the packed-bed reactors out-competed the fluidized beds providing increased surface area for maximum bacterial attachment. The final test of the columns for iron oxidation in a mine, however, was not carried out due to the adverse effect of the leachate solution on bacterial growth.

ACKNOWLEDGEMENTS

I express my sincere gratitude to Dr. Suzuki for his guidance and financial support during the course of this work. Many thanks to Dr. Maeba, Cr. Lyric, Dr. Sparling and Dr. Van Caeseele for serving as my committee members and providing me with much advice throughout the years.

To my family, both human and otherwise, for all of the love and support one could ask for and then some. Special thanks to my sister Mary, for always pushing me to do my best. Thanks again, but did I really need the chicken pox?

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LIST OF CHEMICALS

All chemicals used were the highest grade commercially available. The potassium salts used in anion studies were obtained from Fisher Scientific (Fairlawn, New Jersey, U.S.A) or Malinkrodt Canada Inc. (Pointe Claire, Quebec, Canada). The three key substrates ferrous sulfate, sulfite and sulfur were obtained from Malinkrodt Canada Inc., Matheson, Coleman and Bell (Norwood, Ohio and East Rutherford, New Jersey, U.S.A), and the British Drug House (BDH) Limited (Toronto, Canada) respectively. The remaining chemicals were primarily obtained from Sigma-Aldrich Canada (Oakville, ON, Canada) with a few noted exceptions.

2,2-dipyridyl.....Sigma

Catalase - from bovine liver (purified powder thymol free).....Sigma

Celite (diatomaceous earth).....Baker

Coarse charcoal (activated charcoal, granular special for gas absorption).....BDH

Diatomaceous earth (acid washed then calcinated).....Sigma

EDTA (Ethylenediaminetetraacetic acid) - disodium salt, dihydrate.....Sigma

Fine charcoal (activated, untreated granular 20-60 mesh).....Sigma

Glass beads (3-5 mm in diameter)

HQNO (2-heptyl-4 hydroxyquinoline-N-oxide).....Sigma

NEM (N-ethyl maleimide).....Sigma

o-phenanthroline (1,10-phenanthroline) - monohydrate.....Sigma

Superoxide dismutase - from bovine blood type I.....Sigma

Tiron (4,5-dihydroxy-1,3-benzene disulfonic acid) - disodium salt.....Sigma

Zeolite (particle diameter less than 10 microns).....Sigma

HISTORICAL

Evolution

Human knowledge is continuously expanding with each new discovery. In order to understand the world around us, life in all of its forms is categorized into six taxonomic groups or kingdoms (Alcamo, 1994). Most bacteria belong to the domain Bacteria (Alcamo, 1994). Within this domain the thiobacilli are located in the phylum Proteobacteria (Lane et al., 1992). The phylum as seen in Figure 1 consists of alpha, beta, gamma and delta subdivisions (based on 5S and 16S rRNA studies) (Lane et al., 1992). The thiobacilli are located in the alpha, beta and gamma subdivisions (Lane et al., 1992). *Thiobacillus thiooxidans* and *Thiobacillus ferrooxidans* are members of the beta subdivision and branch off very near the beta-gamma root (Lane et al., 1992). Based on 720 nucleotides of 16 S rDNA the two species of thiobacilli are intermingled (Goebel and Stackebrandt 1994). A more detailed phylogenetic tree was obtained by Goebel et al. using longer sequences (1320 nucleotides) of 16S rDNA. The detailed map shown in Figure 2 places *T. ferrooxidans* as a distinct cluster separate from its sulfur oxidizing relative. According to this scheme it can be inferred that ferrous iron oxidation evolved in a sulfur-oxidizing ancestor (Goebel and Stackebrandt, 1994).

Physiology

T. ferrooxidans is a Gram-negative, non-sporulating rod occurring singly or in pairs. It is motile by means of a single polar flagellum (Torma, 1977). The cell is composed of 44% protein, 26% lipid, 15% carbohydrate, 10% ash and at least two B-vitamins (Torma, 1977). The cell envelope, typical of gram-negative bacteria consists of three hydrophobic and three hydrophilic layers whose total thickness measures 125-215 Å

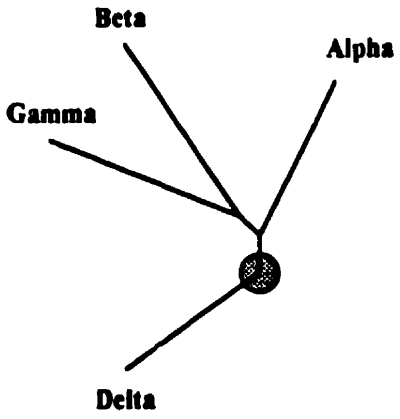


Figure 1. The 16S rRNA relationships among the Proteobacteria. Branch lengths are proportional to calculated evolutionary distances. The structures represent the 184-219 region of 16S rRNA (Lane et al., 1992).

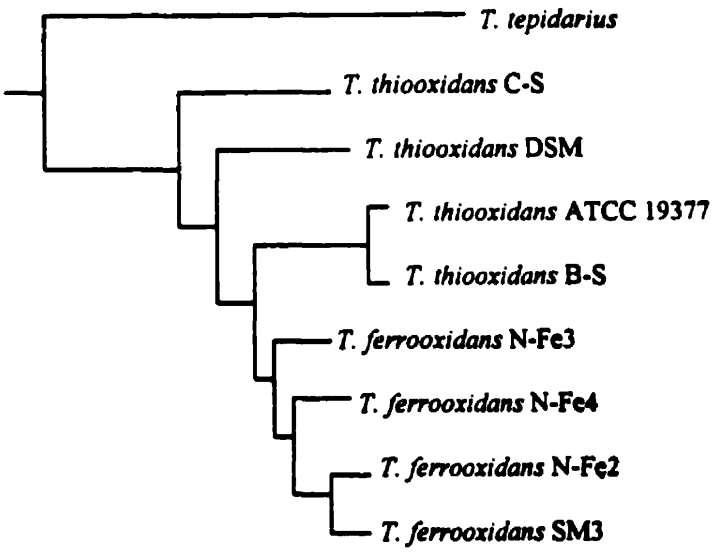


Figure 2. Phylogenetic tree for strains of *T. ferrooxidans* and *T. thiooxidans* based on 1,320 nucleotides of 16S rRNA. The branch lengths are proportional to evolutionary distance. (Goebel and Stackebrandt, 1994).

(Torma, 1977). Beginning at the cell surface there is the lipoprotein, lipopolysaccharide, globular protein and peptidoglycan. The lipopolysaccharide (LPS) layer includes heptose, glucose, galactose, mannose and 2-keto-3-deoxyoctulosonate. (Torma, 1977). Ferric iron is closely associated with the exterior surface of the LPS layer and may serve as a substrate binding site (Torma, 1977). The peptidoglycan layer contains glutamic acid, α-ε-diamino pimelic acid, alanine, glucosamine and muramic acid (Torma, 1977). The cytoplasmic membrane a typical phospholipid bilayer acts as the final barrier before entering the cell cytoplasm.

T. ferrooxidans uses atmospheric carbon dioxide as its cellular carbon source. Carbon dioxide fixation takes place via the Calvin-Benson cycle (Leduc and Ferroni, 1994). Three molecules of ATP and two molecules of NADPH are required to fix one molecule of carbon dioxide. A carbon dioxide fixation mechanism using phosphoenol pyruvate carboxylase is also used to form certain amino acids (Leduc and Ferroni, 1994). *T. ferrooxidans* also has a complete glycolytic pathway and an incomplete citric acid cycle with the absence of α-keto-glutarate dehydrogenase (Leduc and Ferroni, 1994).

Fe oxidation

T. ferrooxidans is a chemolithotroph obtaining its energy for growth and cell maintenance through the oxidation of ferrous iron, sulfur and reduced sulfur compounds. At present there is a great deal of debate surrounding the iron and sulfur oxidation pathways. At one end of the spectrum we have Sugio's theory that the sulfur and iron oxidation pathways are intimately associated with one another (Sugio et al. 1988 and 1989). The opposing theory, suggested by Ingledew (Ingledew et al. 1977) states that the

iron and sulfur oxidation pathways are entirely separate except under anaerobic conditions. In order to understand the complexity of this situation it is important to look at each pathway individually.

The iron oxidation pathway of *T. ferrooxidans* begins on the cell surface. Soluble ferrous iron is oxidized to its ferric state with the subsequent transfer of electrons to the electron transport chain. The actual composition and sequence of this chain is a topic of heated debate between teams of qualified scientists. Three prevailing theories exist. Blake's theory (Figure 3a) begins with cytochrome *c*-containing iron:rusticyanin oxidoreductase as the initial electron acceptor (Blake and Shute, 1994). Electrons then pass to an acid stable rusticyanin (Blake and Shute, 1994). The enzyme is a type I copper protein with a periplasmic location and a molecular mass of 16.5 kDa (Nunzi et al., 1994). Experimental evidence has indicated that cells transferred from sulfur to iron showed increased concentrations of rusticyanin demonstrating its obvious importance in the iron oxidation pathway (Blake and Shute, 1994). The electrons are further transferred to a membrane associated cytochrome-*c* oxidase (Blake and Shute, 1994). The enzyme composed of three subunits has a molecular mass of 150 kDa (Kai et al., 1992). The final reduction of oxygen to water takes place on the inner surface of the cytoplasmic membrane.

Yamanaka's group also begin with ferrous iron oxidation at the cell surface, but the electrons are initially transferred to a periplasmic iron-sulfur protein (Figure 3b). Fe(II)-cytochrome *c* oxidoreductase is a complex enzyme composed of eight molecules with a total molecular weight of 63 kDa (Yamanaka and Fukumori 1995). The electrons

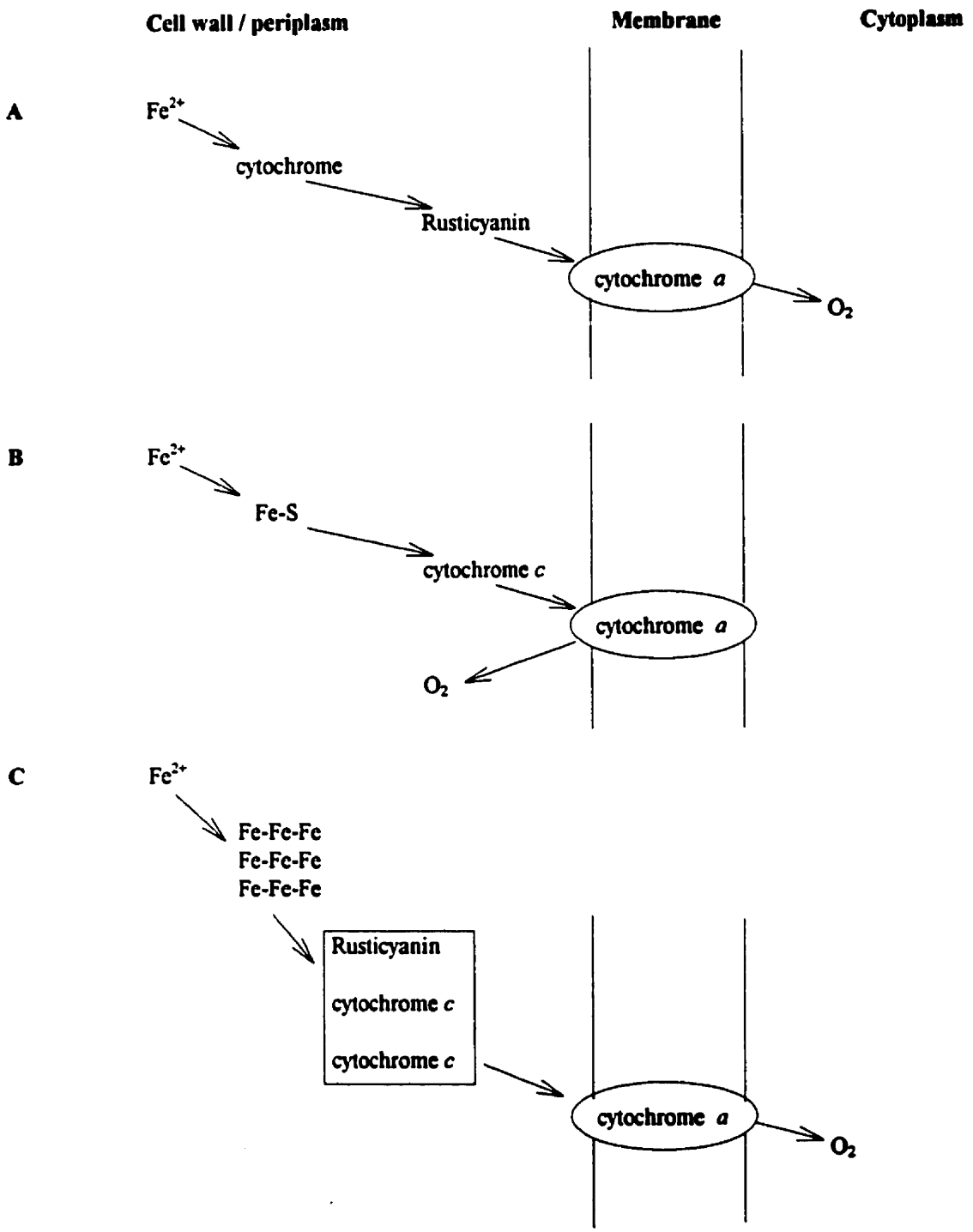
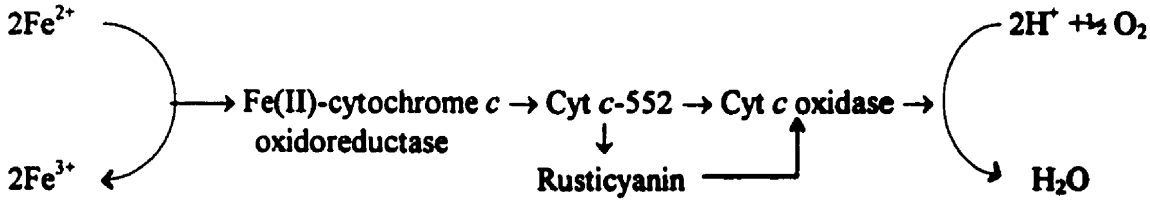


Figure 3. Three schemes that depict the aerobic iron respiratory chain of *Thiobacillus ferrooxidans*. Symbols: Fe-S, iron sulfur protein; Fe. polynuclear iron coat (Blake and Shute, 1994).

move to a second *c*-type cytochrome also located in the periplasmic space (Yamanaka and Fukumori, 1995). Cytochrome *c*-552 with a molecular mass of 13.8 kDa transfers the electrons to cytochrome-*c* oxidase (Yamanaka and Fukumori, 1995). The final reduction of oxygen to water, takes place on the inner surface of the cytoplasmic membrane.

A twist to this otherwise straightforward theory occurs at the point of cytochrome *c*-552. It has been shown experimentally that rusticyanin and membrane associated cytochromes *c*-552 and *c*-550 could be oxidized by cytochrome-*c* oxidase. Equally important, sulfate ions stimulated the transfer of electrons from cytochrome *c*-552 or rusticyanin to cytochrome-c oxidase. The same cannot be said for the membrane associated cytochromes *c*-552 and *c*-550 where electron transfer was inhibited by increased sulfate concentrations (Yamanaka and Fukumori, 1995). Therefore, under natural conditions where sulfate is a dominant anion, rusticyanin and cytochrome *c*-552 (periplasmic) are most likely the electron donors for cytochrome *c* oxidase. Fe(II)-cytochrome-*c* oxidoreductase, however, cannot directly catalyze the reduction of rusticyanin without the addition of catalytic amounts of cytochrome *c*-552 (Yamanaka, 1996). The following pathway is proposed:



(Yamanaka 1996).

Ingledew's theory differs from the rest with the addition of an extracellular polynuclear iron complex (Figure 3c). Electrons from the oxidation of ferrous iron are conducted

through the ferric iron lattice that surrounds the cell (Blake and Shute, 1994). They continue on through the periplasmic space to the membrane associated cytochrome *c* oxidase and oxygen (Blake and Shute, 1994). Within this space they are transferred between rusticyanin and two *c*-type cytochromes. The exact order of this transfer, however, has not been stated (Blake and Shute, 1994).

Sulfur oxidation

The use of sulfur as an energy source is a very complicated mechanism. The first hurdle to overcome is the insoluble nature of elemental sulfur. The initial binding of bacteria to substrate can be viewed using the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory, which regard bacteria as colloids and the changes in free energy between the charged cell surface and solid particle surface are measured as a function of separation distance (Mills et al., 1994). A high energy barrier between the solid surface and the bacterial cell prevents the cell from getting close enough to the solid surface for chemical bond formation.(Ohmura et al., 1996). In order to be used as an energy source, however, the cell must come into contact with its substrate.

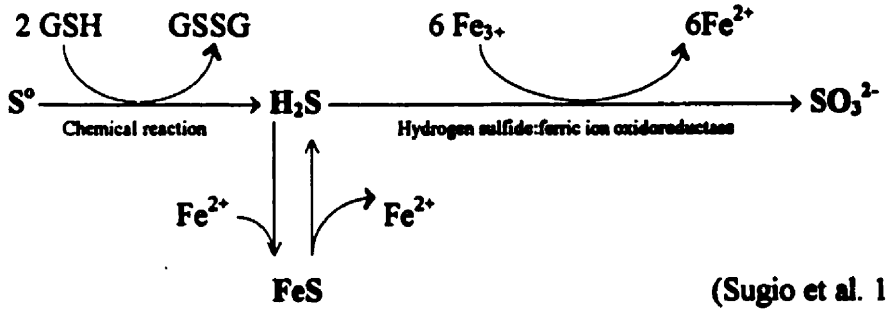
Surface proteins of *T. ferrooxidans* change according to its growth medium, the exact mechanism of regulation has not been determined (Ohmura et al., 1996). Sulfur grown cells contain three unique proteins of 40, 46 and 50 kDa (Ohmura et al., 1996). The 40 kDa protein which is produced as part of the flagella is of particular interest (Ohmura et al., 1996). A disulfide bond is formed between elemental sulfur and the thiol groups of the protein (Ohmura et al., 1996). The anchored cell, now in close contact with its substrate can begin its attack.

Elemental sulfur as a stable eight member ring is chemically reduced by two moles of GSH (reduced glutathione) to hydrogen sulfide and GSSG (oxidized glutathione). Glutathione reductase regenerates GSH in the cytosol. One mole of GSSG and NADPH are converted to two moles of GSH and one mole of $NADP^+$ (Sugio et al., 1989). At this point two diverging theories emerge as to the further oxidation of hydrogen sulfide.

A: Sugio's Group

Since *T. ferrooxidans* exists in an environment with a high soluble iron concentration, it is expected to possess a unique enzyme system which would absolutely require iron for its operation (Sugio et al., 1989). The foundation of Sugio's theory is that sulfur oxidation occurs both aerobically and anaerobically with only ferric iron as an electron acceptor.

Hydrogen sulfide enters the periplasm of the cell where it is oxidized to sulfite by hydrogen sulfide:ferric ion oxidoreductase ($H_2S + 6Fe^{3+} \rightarrow 6Fe^{2+} + SO_3^{2-}$) (Sugio et al., 1989). The enzyme with a total molecular weight of 46 kDa is composed of two identical subunits of 23 kDa each (Sugio et al., 1987). If the hydrogen sulfide concentration in the periplasm rises to a harmful level it chemically reacts with soluble ferrous iron to form FeS (Sugio et al. 1989). The FeS is a stable form of reducing equivalents that provides the cell with continuous low concentrations of H_2S and Fe^{2+} (Sugio et al. 1989).



(Sugio et al. 1988).

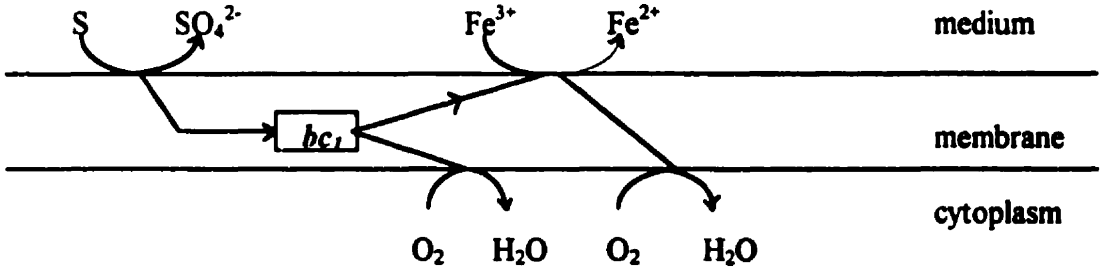
Sulfite moves through the periplasm and is oxidized to sulfate at the cytoplasmic membrane by sulfite:ferric ion oxidoreductase ($SO_3^{2-} + 2Fe^{3+} + H_2O \rightarrow SO_4^{2-} + 2Fe^{2+} + 2H^+$) (Sugio et al., 1988). The ferrous iron that is generated with sulfite and sulfate production is re-oxidized through the iron oxidation pathway (Sugio et al., 1988). The result is a continuous cycling of iron with a steady flow of sulfur oxidation products. Although the sulfur and iron oxidation pathways appear to be linked, the levels of their intermediates must be maintained at relatively low concentrations. Sulfite is a very toxic anion that inhibits hydrogen sulfide:ferric ion oxidoreductase activity at 0.8 mM and iron oxidation at 1 mM (Hirose et al., 1991). Ferrous iron (although present as both substrate and product) inhibits the sulfur oxidation pathway at high concentrations. Hydrogen sulfide:ferric ion oxidoreductase is completely inhibited at 20 mM Fe^{2+}, while sulfite:ferric ion oxidoreductase is inhibited with only 1 mM Fe^{2+} (Hirose et al., 1991).

B: Ingledew's theory

The foundation of the Ingledew theory is that aerobic oxidation of sulfur or reduced sulfur compounds does not require an operating iron oxidation pathway. Sulfur as previously mentioned is chemically reduced to sulfide on the exterior of the cell using reduced glutathione (Sugio et al., 1989). The sulfide enters the periplasm where it is converted to sulfite by sulfide oxidase (Suzuki et al., 1994). The sulfite is further oxidized to sulfate by sulfite oxidase (Suzuki et al., 1994). The electrons generated by these reactions are transferred initially to the cytochrome bc_1 complex and on to cytochrome *c* oxidase with the reduction of oxygen to water occurring within the cytoplasm (Corbett and Ingledew, 1987). Both cytochromes are located within the

cytoplasmic membrane with the cytochrome bc_1 complex envisioned closer to the periplasm and the oxidase closer to the cytosol (Pronk et al., 1991).

The major point of contention with Sugio's theory is that *T. ferrooxidans* has a much higher cell yield on reduced sulfur compounds than on ferrous iron (Pronk et al., 1991). This would not be the case if the electrons from sulfur oxidation enter the electron transport chain at the same level as those of iron oxidation (Pronk et al., 1991). Electrons entering the respiratory chain at cytochrome *b* provide two potential coupling sites for ATP synthesis, while those entering at cytochrome *c* provide only one (Corbett and Ingledew 1987). The generation of twice as much ATP does explain the different cell yields with cells grown on sulfur versus ferrous iron.



(Pronk et al. 1991)

T. ferrooxidans is generally viewed as an aerobe, but anaerobic growth on reduced sulfur compounds is possible (Pronk et al., 1991). Ferric iron can be used as an alternate terminal electron acceptor in lieu of oxygen (Corbett and Ingledew, 1987). Unlike Sugio, Ingledew believes that the S^0/Fe^{3+} pathway is not independent of the S^0/O_2 system (Corbett and Ingledew, 1987). Under anaerobic conditions the oxidation of sulfur to sulfate is coupled to the reduction of ferric to ferrous iron (Pronk et al., 1991). Electrons move through the cytochrome bc_1 complex generating cell yields equivalent to those under

aerobic conditions (Pronk et al., 1991). The theory of a single sulfur pathway is supported by the fact that HQNO, an inhibitor of the cytochrome bc_1 complex is effective with either O_2 or Fe^{3+} as the terminal electron acceptor with sulfur oxidation (Pronk et al., 1991).

The presence of a single sulfur pathway is further supported by Pronk's experiments using formate grown cells. *T. ferrooxidans* is able to use formate under both aerobic and anaerobic conditions (Pronk et al., 1991). Anaerobically the oxidation of formate to carbon dioxide is coupled to the reduction of ferric to ferrous iron ($HCOOH + 2Fe^{3+} \rightarrow CO_2 + 2Fe^{2+} + 2H^+$) (Pronk et al., 1991). The use of formate (along with sulfur) as an electron donor for ferric iron respiration suggests that substrate oxidation and ferric iron reduction occur at different sites of the electron transport chain. It therefore seems unlikely that a single enzyme sulfur:ferric iron oxidoreductase would be involved (Pronk et al., 1991).

The predominant forms of naturally occurring sulfur are sulfate or sulfide in soil or water, and sulfur dioxide in the atmosphere along with small amounts of thiosulfate, polythionates and elemental sulfur (Faou et al., 1990). Thiosulfate and polythionates whose presence in the environment is short lived, are produced by chemical and biochemical processes (Faou et al., 1990). *T. ferrooxidans* hydrolyzes polythionates using a tetrathionate hydrolase enzyme. The enzyme is a dimer with two identical subunits of molecular mass 52 kDa and a pH optimum of around 4 suggesting a periplasmic location. Tetrathionate is broken down to sulfur, thiosulfate and sulfate ($S_4O_6^{2-} + H_2O \rightarrow S + S_2O_3^{2-} + SO_4^{2-} + 2H^+$) (de Jong et al. 1997). The enzyme can also hydrolyze pentathionate

producing similar end products ($S_5O_6^{2-} + H_2O \rightarrow 2S + S_2O_3^{2-} + SO_4^{2-} + 2H^+$) (Jong et al. 1997). Thiosulfate is further oxidized to sulfur and sulfite via a thiosulfate cleaving enzyme ($S_2O_3^{2-} \leftrightarrow S + SO_3^{2-}$) (Suzuki et al., 1994). Rhodanese (molecular mass of 32.9 kDa) is a constitutive enzyme present in almost all bacteria with an aerobic oxidative metabolism (Faou et al., 1990).

Energy generation - Chemiosmotic theory

T. ferrooxidans is an acidophile growing in a pH range of 1 - 4, the optimum being pH 2 - 2.3 (Jensen and Webb. 1995). Although these growth conditions are extreme it maintains a near neutral interior in accordance with most bacteria. The pH differential (ΔpH) and membrane potential ($\Delta\psi$) form the proton motive force (Δp) which reflects the energy status of the cell (Alexander et al. 1987). In a non-respiring cell (Figure 4a) $\Delta p = 0$ with equal yet opposite values of $\Delta\psi$ and ΔpH. The high $\Delta\psi$ value is not an "energy consuming reaction, since its highest value is in inactive cells" (Matin, 1990).

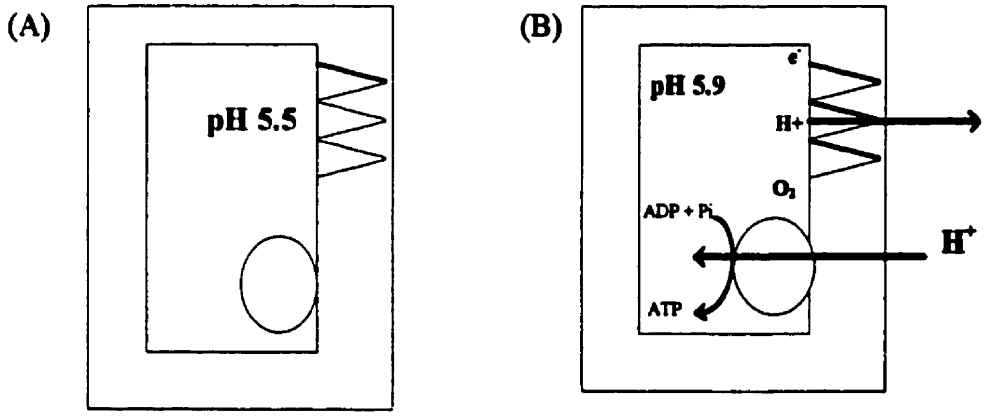


Figure 4. Bioenergetic parameters in acidophilic bacteria with (A) inactive and (B) respiring cells (Matin 1990).

Bacterial cells maintain a remarkable cytoplasmic buffering capacity due to an abundance of titratable groups including the aminoacid sidechains of proteins and phosphorylated groups of nucleic acids and metabolic intermediates (Matin, 1990). Under acidic conditions non viable cells can maintain a positive $\Delta\psi$ due to the protonation of carboxyl groups from aspartate and glutamate and the positively charged amino groups of arginine, tyrosine and lysine (Matin, 1990). The non-diffusible positive charge (Donnan potential) prevents the entry of protons and thus the cell maintains a near neutral internal pH (Matin, 1990).

In *T. ferrooxidans* both iron and sulfur oxidation end at a terminal oxidase with the reduction of oxygen to water. This final reaction consumes four protons for every mole of oxygen reduced. In a respiring cell (Figure 4b) the proton motive force consists solely of the pH differential (ΔpH) (Matin, 1990). Membrane potential ($\Delta\psi$) is negligible due to the movement of anions in and out of the cell (Alexander et al., 1987). A high ΔpH forces the movement of protons into the cell until ΔpH and $\Delta\psi$ are balanced again (Alexander et al., 1987). Protons enter the cell via F_1F_0 ATPases (Brown et al. 1994). These specialized channels allow the cell to couple ATP generation with proton translocation (Brown et al. 1994). The resulting ATP is used for a variety of metabolic functions in addition to the generation of reducing power (Matin 1990).

Bioleaching

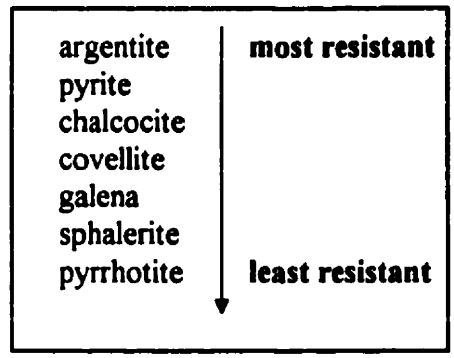
Bioleaching - the use of bacteria in the solubilization of metals from mineral ores is quickly gaining acceptance in the mining industry. The interest in this process is attributed to i) the relative absence of accompanying land and water pollution, ii) the need to use

lower grade ores, which cannot be economically processed by physical/chemical means, iii) the ease with which bioleaching operations can be implemented and iv) the low capital costs necessary for bioleaching operations as compared with conventional processing (Brierly, 1978). Bioleaching proceeds naturally according to a specific series of events. The bacterium must first develop a connection between itself and metal ore. Extracellular polymeric substances (EPS) appear to be a prerequisite for this attachment (Gehrke et al., 1998). EPS consist mainly of sugars (rhamnose, fucose, xylose ,mannose, glucose and glucuronic acid) and lipids with minor amounts of nitrogen, phosphorous and free fatty acids (Gehrke et al., 1998). The substrate used as the bacterial energy source influences the chemical composition of this exopolymer (Gehrke et al., 1998). Iron mostly in the ferric form is closely associated with glucuronic acid, a neutral EPS sugar forming glucuronic acid-iron ion complexes (Gehrke et al. 1998). Electrochemical interactions between the positively charged cell and the negatively charged ore allow the bacteria to initiate its attack (Gehrke et al. 1998).

Solubilization follows the basic principles of electrochemistry. Galvanic interactions determine the order of metal sulfide solubilization. The metal sulfide possessing the highest standard reduction potential is oxidized as the anode. Table 1 summarizes the resistance of some common sulfide minerals. Variations in this order are due to changes in pH, electrolyte chemistry and the presence of certain inhibitory trace metals. (Lawrence et al. 1997).

The presence of bacteria has been experimentally shown to increase the solubilization rate of certain metal sulfides. *T. ferrooxidans* amplifies the current density

Table 1. Level of recalcitrance of common sulfide minerals.



between pyrite and chalcopyrite by a factor of ten, increasing the weathering rate 2-15 times. (Lawrence et al., 1997). The anode (chalcopyrite) rapidly dissolves while pyrite is galvanically protected (Lawrence et al., 1997).

Leaching of metal ores occurs by direct or indirect means. The direct approach involves physical contact between the bacteria and the ore. The insoluble metal sulfide is oxidized to a soluble metal sulfate along with the production of ferric ions and sulfuric acid ($MS + 2O_2 \rightarrow MSO_4$) (Jensen and Webb, 1995). An example, being the oxidation of pyrite by *T. ferrooxidans* to ferric sulfate and sulfuric acid ($4\ FeS_2 + 15\ O_2 + H_2O \rightarrow 2\ Fe_2(SO_4)_3 + 2\ H_2SO_4$) (Rawlings and Silver, 1995). The indirect approach uses ferric iron and sulfuric acid to chemically solubilize the metal sulfide ($MS + Fe_2(SO_4)_3 + O_2 \rightarrow FeSO_4 + MSO_4$) (Jensen and Webb, 1995). An example being the oxidation of uranium oxides with ferric sulfate ($UO_2 + Fe_2(SO_4)_3 \rightarrow UO_2SO_4 + FeSO_4$) (Rawlings and Silver, 1995). "The contribution of the two leaching mechanisms depends on the type of sulfide minerals, the concentration of ferric ions and the operating conditions" (Jensen and Webb, 1995).

The predominant methods of leaching are: dump, heap, vat and in situ leaching (Brierly, 1978). Dump leaching is used to extract various metals from oxide and sulfide ores as well as waste materials removed from open-pit mining operations (Brierly, 1978). The ores in a dump leaching operation are uncrushed and range in size from very fine particles to boulders weighing several tons (Brierly, 1978). The metal concentration in these ores is generally less than 0.4% (Brierly, 1978).

A dump leaching operation as the name implies begins as a mound of ore. A bacterial inoculum is sprinkled on the surface of the mound gradually percolating through (Brierly, 1978). As the bacteria move through the ore a combination of direct and indirect leaching takes place. The extent of metal solubilization is determined by temperature, pH , oxygen availability and the chemical make up of the ore itself (Brierly, 1978). The 'pregnant liquor' containing the soluble metal sulfates is collected at the base of the dump (Brierly, 1978). The dump has to be built on an impermeable base to prevent any loss of this solution (Brierly, 1978). The pregnant liquor is then processed to remove the economically important metals leaving behind a barren solution that is returned to the dump surface for re-application (Brierly, 1978). The incredible size and low permeability of a dump site means that leach cycles are measured in years (Brierly, 1978).

Heap leaching at its basics is very similar to that of dump leaching. The ore, however is of a smaller size and somewhat higher grade (Brierly, 1978). Due to the decreased size of the mound and increased permeability the leach cycle is reduced to months (Brierly, 1978). Vat leaching which is currently used on oxide ores, is the

disintegration of crushed materials using sulfuric acid in an enclosed tank. Leaching is completed in days (Brierly, 1978).

Underground in situ leaching is possible with three types of deposits. Exposed ore bodies such as worked out regions of mines are fractured with the help of explosives to increase their permeability (Brierly, 1978). A bacterial inoculum is sprinkled on the surface and allowed to penetrate the rock (Brierly, 1978). The leachate is collected and processed to allow for metal recovery (Brierly, 1978). The remaining liquid is re-applied to the surface and the process repeats itself (Brierly, 1978).

Type II and type III deposits occur below the water table at depths of under and over 1000 ft respectively (Brierly, 1978). Mining of these deposits requires a great deal of chemical and engineering expertise. The potential for environmental pollution via a contaminated water supply is an obvious concern as is the lack of sufficient oxygen for bacterial or chemical oxidation at these immense depths (Brierly, 1978).

In a bioleaching site, temperature, pH, oxygen availability and toxicity of the leach solution determine bacterial survival (Brierly, 1978). Temperatures of leach solutions can vary significantly depending on the season. Winter temperatures can drop as low as 3 - 4°C, while 35°C can be seen in the summer (Brierly, 1978). With the continually changing conditions that can be seen at mine sites it is obvious that a single species, even *T. ferrooxidans* is not solely responsible for all metal solubilization. Instead there is a consortium of bacteria that work together and under certain conditions specific genera (or species) predominate (Brierly, 1978).

Based on temperature, there are three groups of bacteria located in a bioleaching environment. Mesophiles thrive within a temperature range of 20-40°C (Alcamo, 1994). The chemolithotrophs *T. ferrooxidans*, *T. thiooxidans* and *Leptospirillum ferrooxidans* generally outnumber the heterotrophic *Acidiphilium* species (Rawlings and Silver, 1995). *T. ferrooxidans* is the most adept of this group in that it can oxidize both iron and reduced sulfur compounds. *T. thiooxidans* can oxidize only reduced sulfur compounds, while *L. ferrooxidans* can oxidize only ferrous iron (Rawlings and Silver, 1995). The heterotrophs are unable to oxidize sulfur or iron "and probably grow on the organic carbon excreted by the chemolithotrophs" (Rawlings and Silver, 1995).

The moderate thermophiles grow in a temperature range of 40 - 60°C (Alcamo, 1994). *Thiobacillus* TH strains and *Sulfobacillus thermosufidooxidans* are both able to use ferrous iron, reduced sulfur compounds and organic material as energy sources (Rawlings and Silver, 1995). Active growth, however, is not achieved without aeration with CO_2 enriched air and the addition of yeast extract or some form of organic substrate (Rawlings and Silver, 1995). They are unlikely to play a part in most industrial leaching processes unless a conscious effort is made to encourage their growth (Rawlings and Silver, 1995).

The extreme thermophiles center around the genus *Sulfolobus*, a member of the archaebacteria (Rawlings and Silver 1995). At a temperature range of 60 - 70°C these bacteria are able to metabolize pyrite (FeS_2), chalcopyrite ($CuFeS_2$) and pyrrhotite (FeS) ores faster than *T ferrooxidans* (Rawlings and Silver, 1995). Unfortunately being a member of the achaebacteria, they lack a peptidoglycan layer in their cell wall. This makes

them more sensitive to the abrasive forces found in a typical leaching environment (Rawlings and Silver, 1995). In spite of their rapid leaching ability, this sensitivity may be one reason why no industrial scale bioleaching process using *Sulfolobus* species are currently in operation (Rawlings and Silver, 1995).

Other industrial applications

Thiobacilli, among other bacteria, have been implicated as the primary cause of acid mine drainage (Rawlings and Silver, 1995). Exposed sulfide minerals are solubilized and released as metal sulfates and sulfuric acid contaminating the surrounding soil and water systems. Understanding the interaction between the geochemical, electrochemical and microbial controls of sulfide mineral oxidation is key to controlling and exploiting these processes for environmental and industrial purposes (Lawrence et al., 1997).

The classic technique of treating acid mine drainage is a purely chemical method (Murayama et al. 1987). The effluent is neutralized by the addition of calcium hydroxide which results in precipitation of ferric salts and arsenic compounds (Murayama et al. 1987). The precipitates settle out in the solid-liquid separation tank and are transported by pumping to a sludge storage dam (Murayama et al. 1987). The treated water is then discharged from the surface of the tank to a nearby river (Murayama et al. 1987).

In 1974, a bacterial oxidation system was implemented at the Yanahara pyrite mine of Dowa Mining Co. Ltd. (Murayama et al. 1987). Naturally occurring *T. ferrooxidans* (which is accustomed to the existing environmental conditions and thus required no adaptation) was grown on diatomaceous earth, a cell carrier agent, allowing for the development of high cell concentrations (Murayama et al., 1987). The acid mine water

was distributed to numerous oxidizing tanks containing the bacteria (Murayama et al. 1987). Following a short retention time (in which bacteria oxidized $Fe^{2+} \rightarrow Fe^{3+}$) the slurry was transferred to a settling tank (Murayama et al., 1987). The supernatant continued on to the neutralization tanks while the bacteria were recycled back to the oxidizing tanks (Murayama et al., 1987). Neutralization to pH 4 with calcium carbonate (an inexpensive alternative to calcium hydroxide in the absence of ferrous ions) was sufficient to precipitate ferric and arsenic salts. In financial terms, the bacteria saved the company more than 640,000 Yen in its first year of operation (Murayama et al., 1987).

The use of *T. ferrooxidans* as a ferrous iron oxidizer has not been limited to the treatment of acid mine drainage. New regulations dealing with air pollution control has made it very difficult and expensive to treat a variety of waste gases. Hydrogen sulfide, a particularly pungent gas is injected into a ferric sulfate solution producing ferrous sulfate, sulfuric acid and elemental sulfur ($H_2S + Fe_2(SO_4)_3 \rightarrow 2\ FeSO_4 + H_2SO_4 + S^0$) (Imaizumi, 1986). The sulfur is recovered by solid-liquid separation and the ferrous sulfate is transferred to a microbial oxidation tank for re-oxidation. Following a short retention time the soluble ferric sulfate is recycled to the reactor vessel while the precipitated bacteria are retained for further use (Imaizumi, 1986). The total cost of this process is estimated to be about one-third of the conventional caustic soda absorption process (Imaizumi, 1986).

The desulfurization of waste gas containing sulfur dioxide is also accomplished with a biological loop. The process is based on the wet scrubbing of sulfur dioxide with a ferric sulfate solution ($SO_2 + Fe_2(SO_4)_3 + 2H_2O \rightarrow 2\ FeSO_4 + 2H_2SO_4$) (Imaizumi,

1986). The ferrous sulfate produced is oxidized by the bacteria, a portion of which is recycled (Imaizumi, 1986). The remainder is neutralized with calcium carbonate powder producing gypsum (calcium sulfate) and ferric hydroxide precipitates (Imaizumi, 1986) both of which are later recovered.

T. ferrooxidans, however, is above all a mining bacterium which has proven itself invaluable in the mining of recalcitrant gold ores (Olson, 1994). Gold is often recovered from ore bodies by leaching with cyanide, forming soluble gold-cyanide complexes (Olson, 1994). In the case of recalcitrant ores, however, the gold particles are encapsulated by pyrite and arsenopyrite (Olson, 1994). The cyanide cannot reach the gold and thus it cannot be solubilized by standard techniques (Olson, 1994).

Three methods exist for the recovery of gold from recalcitrant ores. Roasting, involves heating the ore with oxygen to oxidize the sulfide minerals (Olson, 1994). Pressure oxidation, is based on the same concept with autoclaving of aqueous slurries of ore (Olson, 1994). Both of these techniques, however, involve an immense amount of energy and equipment, not to mention a large capital investment. Bioleaching, on the other hand is a relatively inexpensive process which uses sulfide oxidizing bacteria to free the gold for further treatment with cyanide (Olson, 1994). Gold ore bio-oxidation is now commercially applied in South Africa, Australia, Brazil and the USA (Olson, 1994).

Study Objective

With any experiment, or in this case set of experiments is it important to state the purpose behind ones investigation. The question before us, therefore, is why study bacterial leaching? To that end, there are three possible answers. First, there are

environmental reasons - bacterial leaching involves the relative absence of accompanying land and water pollution one would have with conventional physical/chemical extraction methods. Secondly, there are economic reasons - bioleaching is simply cheaper and easier to both implement and operate as compare with conventional means. Finally, there is necessity - the need to use lower grade ores which cannot be economically processed by the conventional physical/ chemical means. The following study looks at the iron and sulfur oxidation pathways of *T. ferrooxidans*, a dominant member of the bioleaching community, in hopes of further understanding the mechanism of mineral extraction.

PART I

POTENTIAL INTERACTION OF SULFUR AND IRON OXIDATION

INTRODUCTION

Thiobacillus ferrooxidans is a Gram-negative acidophilic chemolithotroph obtains its energy for growth from the oxidation of ferrous iron, sulfur and reduced sulfur compounds. Ferrous iron is oxidized on the cell surface (Ingledew et al., 1977). Only electrons enter the periplasm, moving through the electron transport chain (Fe^{2+}-cytochrome *c* oxidoreductase, cytochrome *c*, rusticyanin and cytochrome oxidase) with the final reduction of oxygen to water in the cell cytoplasm ($2Fe^{2+} + ½O_2 + 2H^+ \rightarrow 2Fe^{3+} + H_2O$). Sulfur oxidation also starts on the exterior of the cells with sulfur initially being oxidized to sulfite and then to sulfate ($S^0 + 1½O_2 + H_2O \rightarrow H_2SO_4$). The mechanism of sulfur oxidation, however, is a topic of some debate. Two dominant theories exist. The first proposed by Sugio et al. (1988 and 1989) , stipulates a direct connection between sulfur and iron oxidation. *Thiobacillus ferrooxidans* is naturally found in an environment containing high levels of dissolved iron (e.g. acid mine drainage water). In order to take full advantage of their immediate environment Sugio believes that the bacteria have developed a unique metabolic system which absolutely requires ferric iron to operate. As evidence of this concept he has found two ferric iron requiring enzymes in *T. ferrooxidans* involved in sulfur oxidation. Sulfur:ferric ion oxidoreductase has an optimum pH of 6.5 and a periplasmic location (Sugio et al., 1987). It couples the oxidation of sulfur to ferric iron reduction producing sulfite and ferrous iron ($S^0 + 4Fe^{3+} + 3H_2O \rightarrow H_2SO_3 + 4Fe^{2+} + 4H^+$) (Sugio et al., 1987). Sulfite is then oxidized to sulfate by sulfite:ferric ion oxidoreductase, located in the plasma membrane with an optimum pH of 6 ($H_2SO_3 + 2Fe^{3+} + H_2O \rightarrow H_2SO_4 + 2Fe^{2+} + 2H^+$) (Sugio et al., 1988). This enzyme was considered to be crucial for normal bacterial growth. Elevated sulfite concentrations inhibited both the

sulfur (0.8 mM) and iron (1.5 mM) oxidation pathways resulting in the absence of CO_2 uptake and cell growth (Hirose et al., 1991). An additional mechanism for sulfite removal is its chemical oxidation to sulfate with soluble ferric iron ever present in the environment (Sugio et al., 1987). The ferrous iron produced in each case (bacterial and chemical oxidation reactions) is then re-oxidized by the bacteria to replenish the available ferric iron supply.

An alternative theory proposed by Ingledew et al. states that aerobic sulfur oxidation does not require ferric iron for normal operation (Corbet and Ingledew, 1987). Ferric iron, however, can be used for sulfur oxidation in the absence of molecular oxygen as an alternate electron acceptor (Pronk et al., 1992). Evidence supporting this concept of a ferric iron independent sulfur oxidation system was obtained through bacterial growth studies. Cell yields of *T. ferrooxidans* grown on sulfur were substantially higher than those grown on ferrous iron. This did not agree with Sugio's theory that electrons obtained from both iron and sulfur oxidation enter the electron transport chain at the same level (cytochrome *c*). Further contradictory evidence involved the oxidation of formic acid by *T. ferrooxidans* cells in the presence of ferric iron (Pronk et al., 1991). The fact that ferric iron could be used as an electron acceptor in the oxidation of both sulfur and formic acid suggested that substrate oxidation and ferric iron reduction occurred at different sites in the electron transport chain not at a single sulfur:ferric ion oxidoreductase as presented by Sugio (Sugio et al., 1987).

The controversy, however, rages on. Are the iron and sulfur oxidation pathways connected ? and if so to what extent ? This study took a different approach to answering this question. We began with two specific cell types - *T. ferrooxidans* grown on iron and

those grown on sulfur. (The above theories were based solely on iron grown cells.) Bacterial growth substrate has been shown to significantly affect overall bacterial makeup. Surface proteins and key enzymes were found to vary substantially when cells were moved from iron to sulfur containing medium (Ohmura et al., 1996). Thus in order to properly answer this question it was necessary to test both iron and sulfur grown *T. ferrooxidans*. Secondly we tested the effects of numerous inhibitors and metal chelators on ferrous iron, sulfur and sulfite oxidation. If the iron and sulfur oxidation pathways are connected, inhibiting the former should also inhibit the latter. Our results showed that the oxidation of iron and the oxidation of sulfur are separate and distinct steps, not affected by various inhibitors and conditions the same way. Only the oxidation of externally added sulfite involved a possible connection to iron oxidation.

MATERIAL AND METHODS

Culture procedures for cell suspensions

Iron grown cells. Two strains of *T. ferrooxidans* were used: SM-4 (Lizama and Suzuki, 1988) and Tf-2 (ATCC 19859). Although some data are shown with either SM-4 or Tf-2, the results were comparable whenever both strains were used. *T. ferrooxidans* was grown in modified 9K medium (M9K) : 0.4 g $(NH_4)_2SO_4$, 0.1 g K_2HPO_4, 0.4 g $MgSO_4 \cdot 7H_2O$ and 33.3 g $FeSO_4 \cdot 7H_2O$ per liter and adjusted to pH 2.3 with H_2SO_4. The ferrous sulfate was filter-sterilized separately. The flasks were incubated using a 10 % inoculum (72 hours old) at 25°C and placed on a rotary shaker at 150 rpm for 48 hours. The culture was passed through Whatman No. 1 filter paper to remove the majority of the precipitated ferric iron. The supernatant was centrifuged at 12,000 x *g* for 10 minutes. The cell pellet was resuspended in washing buffer (pH 2.3 β-alanine-H_2SO_4, 0.01 M for sulfite and iron oxidation or 0.1 M for sulfur oxidation unless otherwise stated) and centrifuged at 100 x *g* for 5 minutes to allow for further ferric iron sedimentation. The supernatant was transferred to a second tube and centrifuged at 12,000 x *g* for 10 minutes to collect cells. The washing procedures were repeated and the cells were suspended to a final concentration of 50 mg/ml wet cells.

Sulfur grown cells. Cells grown on sulfur were grouped into three categories based on their exposure to sulfur as a growth substrate. Direct cells were initially grown on ferrous sulfate enriched M9K as described above. A 24 hour culture was then used to inoculate (10 % inoculum) five flasks containing 640 ml M9K supplemented with 0.2 g/l $Fe_2(SO_4)_3 \cdot 5H_2O$ and sprinkled with 10 g/ l precipitated BDH sulfur. The flasks remained

stationary at 28°C for 48 hours prior to their placement on a rotary shaker at 150 rpm for 4 days.

Sulfur adapted cells were similar to the direct cells with the exception of being subcultured three times on precipitated sulfur prior to their collection.

Sulfur grown cells were grown in Starkey No. 1 medium after adaptation on sulfur (Suzuki et al., 1999) : 0.3 g $(NH_4)_2SO_4$, 3.5 g K_2HPO_4 , 0.5 g $MgSO_4 \cdot 7H_2O$, 0.25 g $CaCl_2$ and 18 mg $FeSO_4 \cdot 7H_2O$ per liter and adjusted to pH 2.3 with H_2SO_4. Each 2 l Fernbach flask contained 1 l Starkey No. 1 medium, 2.5 % bacterial inoculum and 10 g/l powdered BDH sulfur spread evenly over the surface. The stationary flasks were incubated at 28°C for four days. The cell collection procedure for all sulfur grown cells was identical to that of iron grown cells.

Determination of iron, sulfur and sulfite oxidation using cell suspensions

The rates of iron and sulfur oxidation, were measured using a Gilson oxygraph equipped with a Clark oxygen electrode at 25°C. The reaction vessel contained 5 µl - 100 µl of the cell suspension (sulfur or iron grown cells), 0.1 ml of sulfur suspension (32 g BDH S^0 in 100 ml 50 ppm Tween 80) (for sulfur oxidation) 1 µmol sulfite (for sulfite oxidation) or 0.5 µmol $FeSO_4 \cdot 7H_2O$ (for iron oxidation) and varying amounts of buffer (various pH's) to make a total volume of 1.2 ml. The effect of inhibitors was studied in β-alanine buffer at pH 3 and pH 6. Cells were incubated with inhibitors for 5 min (NEM, HQNO) or 1 min (other inhibitors) prior to the addition of substrates which initiated the reaction. Cell volumes used were: 100 µl for sulfur, 5 µl for sulfite and 10 µl for Fe^{2+}

assays with iron grown cells and 10 µl for sulfur and 100 µl for sulfite assays with sulfur grown cells.

Determination of iron and sulfur oxidation and carbon dioxide fixation using growing cell cultures. The rates of iron and sulfur oxidation in growing cell cultures were measured using a Micro-oxymax respirometer (Columbus Instruments) at Cominco Research, Trail, British Columbia, Canada. The reaction vessel contained a 5% inoculum, 12 mmoles $FeSO_4 \cdot 7H_2O$ (for iron oxidation) or 1 g BDH sulfur sprinkled on the surface plus 18 mg/l $FeSO_4 \cdot 7H_2O$ (for sulfur oxidation), various concentrations of inhibitors and M9K medium at pH 2.3 making a total volume of 100 ml. The inhibitors were filter sterilized and added to the medium prior to bacterial inoculation. The reaction was stirred with a magnetic stirrer and both the O_2 consumption (oxidation) and CO_2 consumption (autotrophic growth) were measured at 26°C.

Protein determination

Protein concentrations were determined using the Lowry method of protein determination. Bovine serum albumin (1 mg/ml) was used to create a standard curve of 0 - 100 µg protein. Ten microliters of the protein standard or cell suspension was added to 0.9 ml of 0.1 M NaOH and boiled for ten minutes. The sample was centrifuged in a microcentrifuge for 2 minutes removing cellular debris. The supernatant (0.8 ml) containing dissolved protein was added to 4 ml of reagent D (50 ml 2% Na_2CO_3 + 1 ml reagent B (0.5 % $CuSO_4 \cdot 5H_2O$ in 1 % potassium tartrate)). The sample was shaken and incubated at room temperature for ten minutes prior to the addition of 0.4 ml 1 N phenol.

Absorbance was measured in a Hewlett Packard spectrophotometer at 750 nm following an additional 30 minutes of incubation at room temperature.

RESULTS

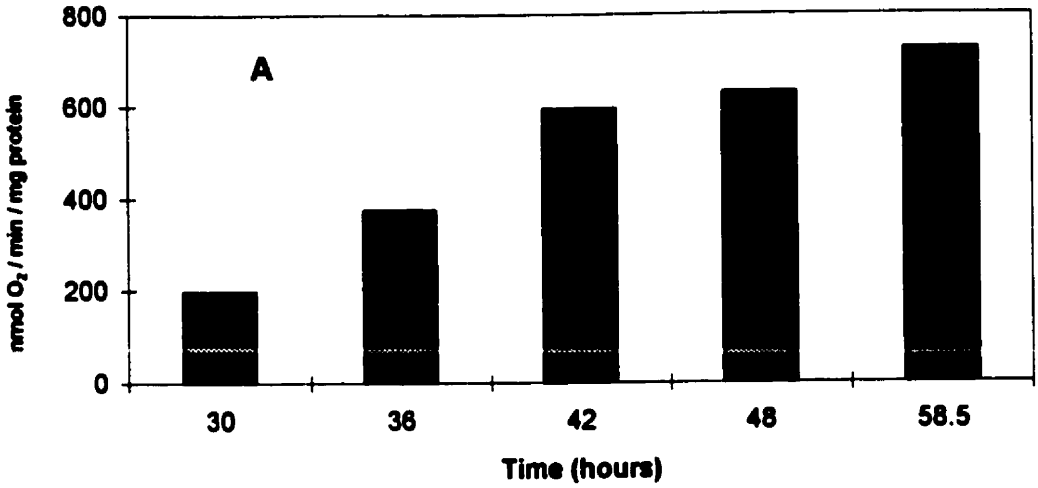
Growth Substrates and Oxidation Activities

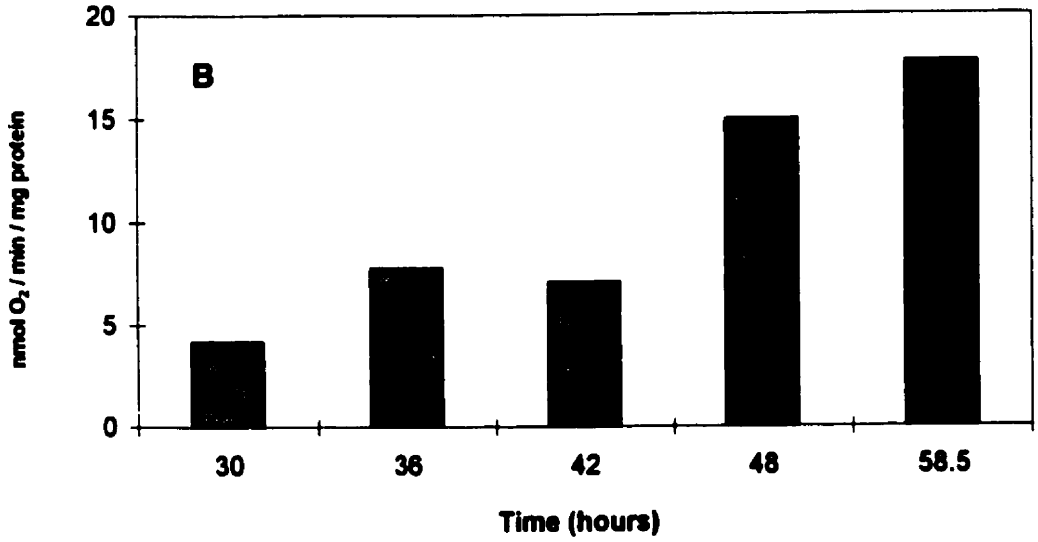
Thiobacillus ferrooxidans derives its energy for growth from the oxidation of ferrous iron, sulfur and reduced sulfur compounds. Prior research has shown bacterial growth substrate to have a significant effect on the overall makeup of the cell (Ohmura et al., 1996). Thus in order to address the question of a potential iron and sulfur pathway interaction it was important to test both iron and sulfur grown cells.

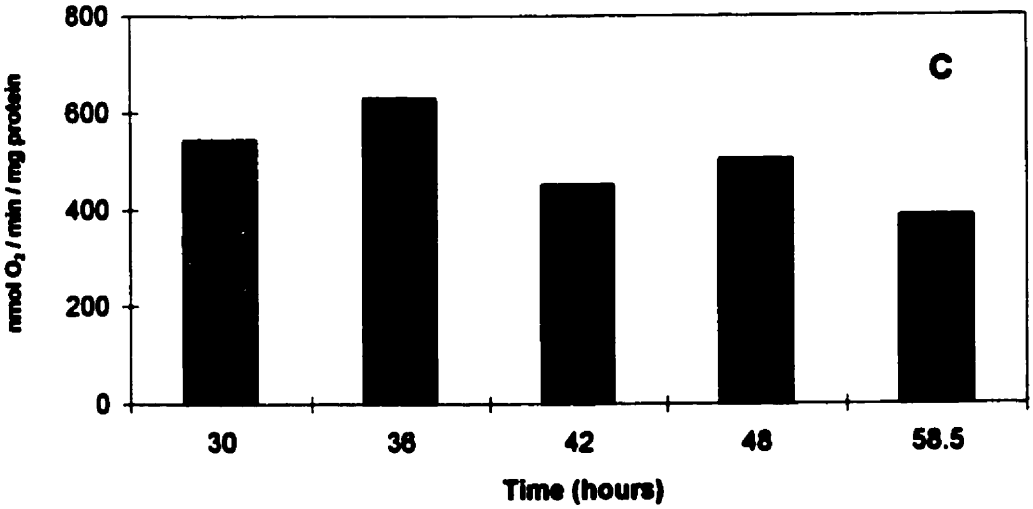
Determination of culture age for optimum activity (iron grown cells)

Five 2-liter flasks containing 800 ml M9K medium with Fe^{2+} were inoculated with *Thiobacillus ferrooxidans* strain SM-4 from a single stock culture and placed at 25°C on a rotary shaker at 150 rpm. Cells from individual flasks were collected as described in materials and methods at 30, 36, 42, 48 and 58.5 hours. The level of ferric iron precipitate increased with the age of the culture which appeared both as a thin layer on the flask walls and a thick pellet in the flask center. The younger cell suspensions contained high levels of dissolved ferric iron giving them a rusty color. Iron, sulfite and sulfur oxidation activities of the iron grown cells were determined using a Gilson Oxygraph. Iron and sulfur oxidation (Fig. 1A and B) both increased in accordance with culture age. Sulfite oxidation was highest at 36 hours followed by a gradual decrease over time. In order to minimize the difference between various batches of cells a standardized collection procedure was developed. The 48 hour culture which provided high levels of iron, sulfite and sulfur oxidation was chosen as the standard culture age. A growth period of 48 hours allowed for the production of large, clean cell (i.e. minimum Fe^{2+} and Fe^{3+} content) yields (34 mg/l wet cells).

Figure 1. Time course comparisons of iron (A), sulfur (B) and sulfite (C) oxidation activities of *T. ferrooxidans* (SM-4) during growth on Fe^{2+}.


A
800
600
400
200
0
nmol O2 / min / mg protein
30
36
42
48
58.5
Time (hours)


B
20
15
10
5
0
nmol O2 / min / mg protein
30
36
42
48
58.5
Time (hours)


C
800
600
400
200
0
nmol O2 / min / mg protein
30
36
42
48
58.5
Time (hours)

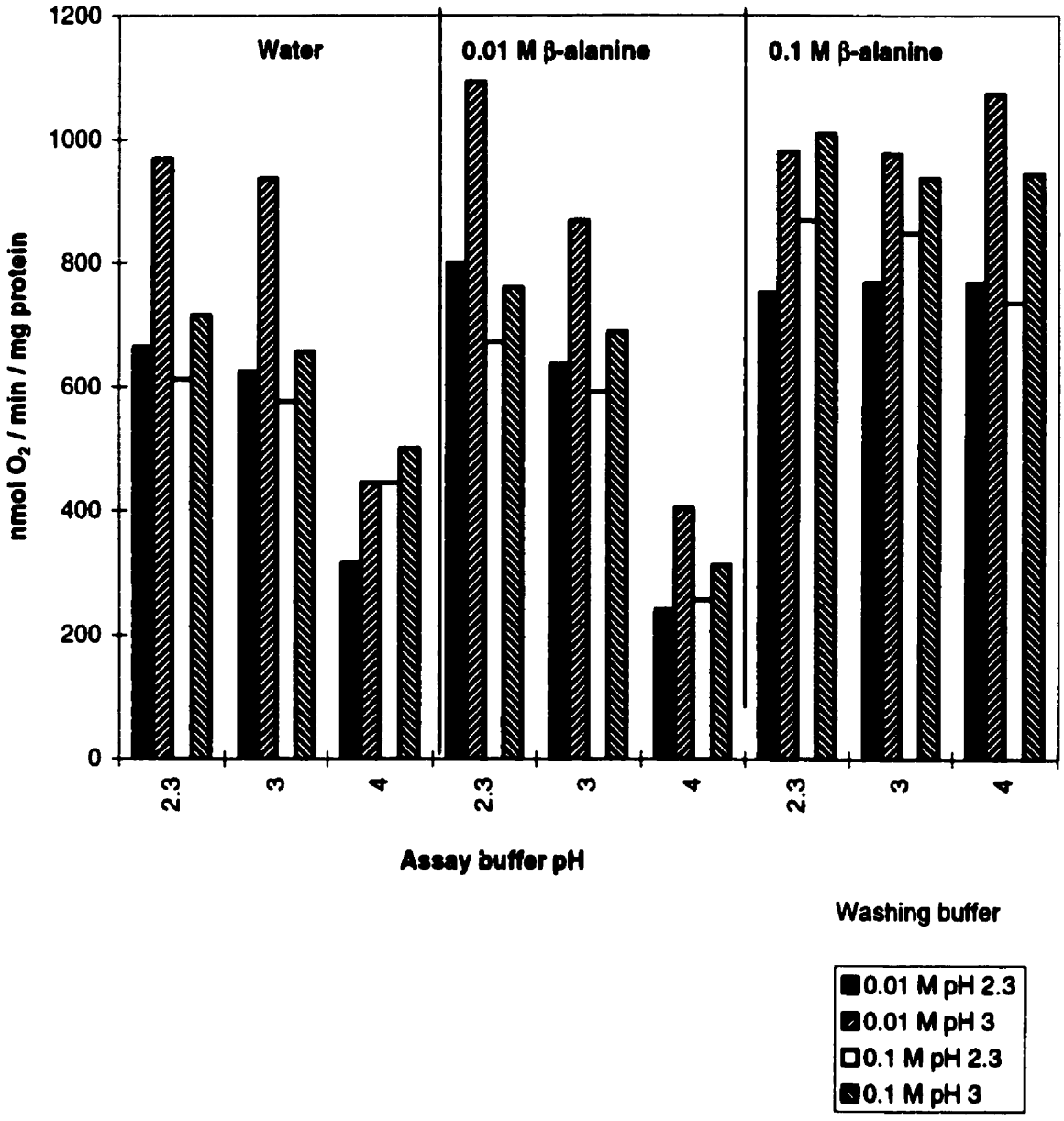
T. ferrooxidans grown once (direct cells) or three times on sulfur (sulfur adapted cells) were collected after six days of growth on a rotary shaker at 150 rpm at 28°C. Cells continuously subcultured on sulfur (sulfur grown cells) were collected after four days of stationary growth at 28°C. The specified growth periods allowed for the development of high cell yields - 37 mg wet cells /l of direct, 42 - 190 mg wet cells / l sulfur adapted and 193 mg wet cells / l sulfur grown cells respectively.

Iron oxidation

Washing buffers. A 48 hour culture of iron grown *T. ferrooxidans* was divided in four and collected as described in Materials and Methods using 0.01 M or 0.1 M β-alanine pH 2.3 or pH 3 as the washing buffer (Figure 2). Iron oxidation activity was then tested in double glass distilled water, 0.01 M and 0.1 M β-alanine buffer (pH 2.3 - 4). Iron oxidation was highest with cells that were washed in 0.01M β-alanine buffer pH 3. Increased acidity or washing buffer concentration had a minor deleterious effect on overall bacterial activity. Other potential washing buffers included 0.01 M K-Pi, KNO_3 and K_2SO_4 (data not shown). Maximum iron oxidation, however, was retained by the pH 3 0.01 M β-alanine washed cells.

Assay buffer. In order to maximize ferrous iron oxidation levels bacterial activity was tested over a large pH range. Figure 2 shows ferrous iron oxidation activity at pH 2.3 - 4. Values above pH 4 were not tested due to the increased chemical reactivity of ferrous iron (i.e. Fe^{2+} is chemically oxidized at increasingly alkaline pH values). Maximum iron oxidation was observed in 0.1 M β-alanine buffer, where washing buffers had only a minor effect on overall activity as can be seen by the high rate of ferrous iron oxidation in all

Figure 2. Effect of washing buffer pH (2.3 or 3) and concentration (0.01 or 0.1 M β-alanine sulfate) on iron oxidation activities of Fe^{2+} grown SM-4 assayed in water, 0.01 M or 0.1 M β-alanine sulfate at pH 2.3-4.


1200
1000
800
600
400
200
0
nmol O2 / min / mg protein
Water
0.01 M β-alanine
0.1 M β-alanine
2.3
3
4
2.3
3
4
2.3
3
4
Assay buffer pH
Washing buffer
0.01 M pH 2.3
0.01 M pH 3
0.1 M pH 2.3
0.1 M pH 3

buffers tested. Iron oxidation was relatively unaffected between pH 2.3 and 3 in double glass distilled water and 0.01 M β-alanine, but was lower at pH 4.

The ferrous iron oxidation data here are limited to the iron grown cells. The three types of sulfur grown cells had essentially lost their ability to oxidize ferrous iron. Cells previously grown on iron and subcultured only once on elemental sulfur had no iron oxidizing activity (data not shown). In an earlier study *T. ferrooxidans* SM-4 retained their ability to oxidize ferrous iron (Suzuki et al., 1990).

Sulfur Oxidation

Washing buffer. A 48 hour culture of iron grown *T. ferrooxidans* was divided in four and washed in 0.01 M or 0.1 M β-alanine buffer (pH 2.3 and 3). Maximum sulfur oxidation (Fig. 3) was obtained with cells washed in 0.1 M β-alanine at pH 2.3.

The preference for a higher washing buffer concentration was further investigated using anionic alternatives to β-alanine. Cells were collected as previously stated and washed in 0.1 M β-alanine, K-Pi, K_2SO_4 or KNO_3 (data not shown). Maximum sulfur oxidation was obtained with cells washed in 0.1 M β-alanine pH 2.3, which was used as the standard washing buffer for the sulfur oxidation assay.

Assay buffer. Iron grown cells (Fig. 4A) washed in 0.1 M β-alanine were tested in 0.01 M and 0.1 M β-alanine pH 2.3 - 8 for sulfur oxidizing activity. In 0.01 M β-alanine, sulfur oxidation showed a gradual increase in activity up to pH 6, followed by a minor drop at pH 7 and 8. In 0.1 M β-alanine sulfur oxidation was inhibited at pH 2.3, while at higher pH values sulfur oxidation remained at a high yet steady rate.

Figure 3. Effect of washing buffer pH (2.3 or 3) and concentration (0.01 or 0.1 M β-alanine sulfate) on sulfur oxidation activities of Fe^{2+} grown Tf-2 assayed in 0.1 M β-alanine sulfate buffer (pH 3-8).

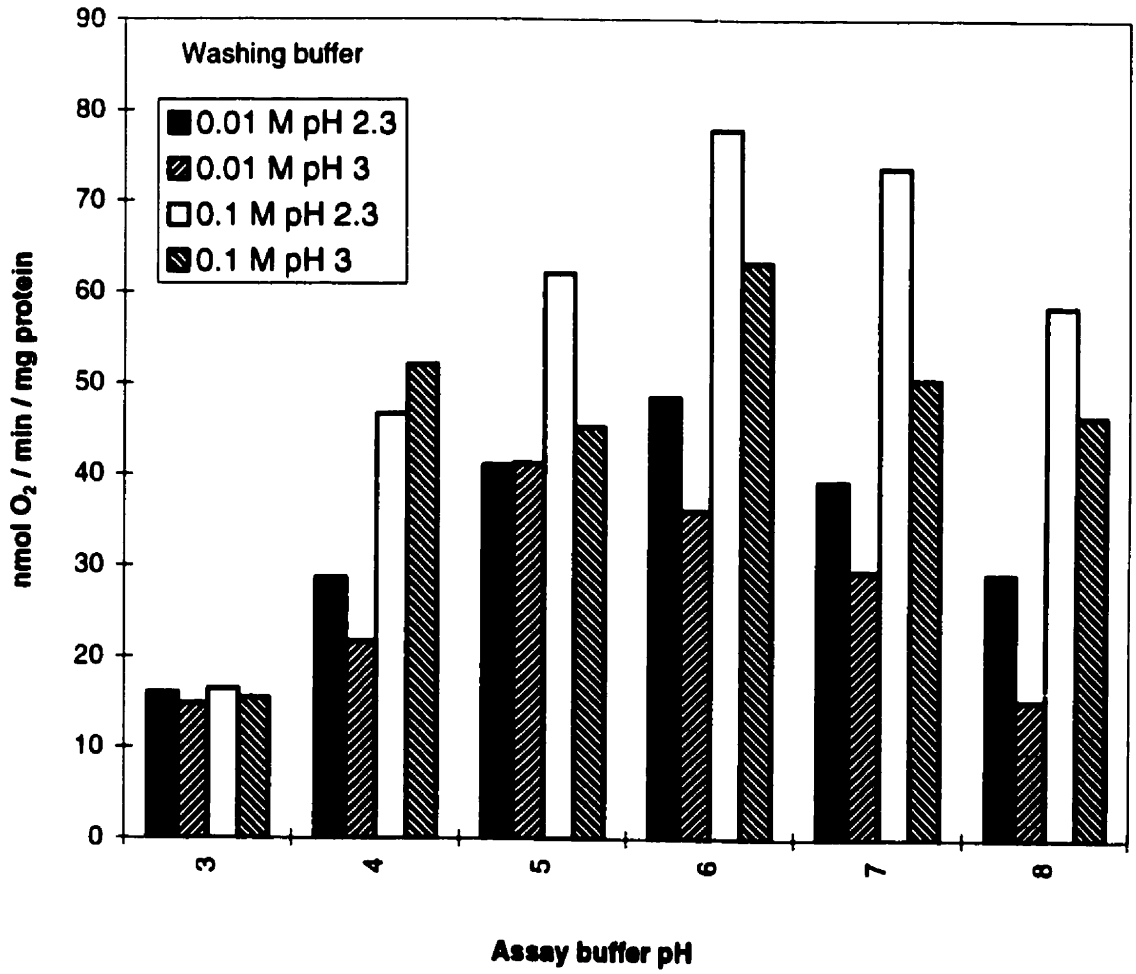
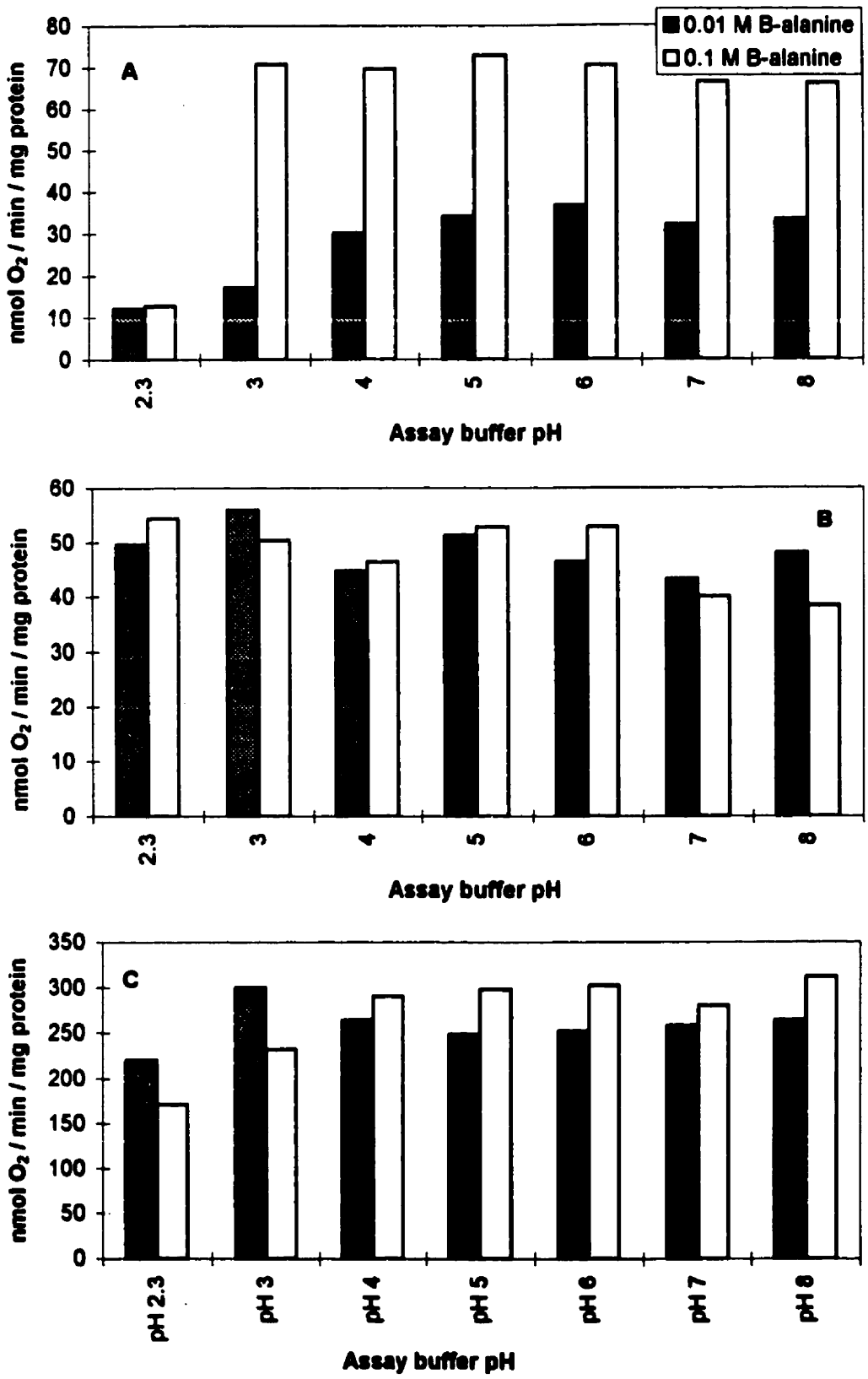

Washing buffer
0.01 M pH 2.3
0.01 M pH 3
0.1 M pH 2.3
0.1 M pH 3
nmol O2 / min / mg protein
0
10
20
30
40
50
60
70
80
90
3
4
5
6
7
8
Assay buffer pH

Figure 4. Effect of pH on sulfur oxidation by Fe^{2+} grown cells (A), Sulfur-direct cells (B) and sulfur grown cells (C) of Tf-2. Cells were washed in 0.1 M β-alanine buffer at pH 2.3.


A
0.01 M B-alanine
0.1 M B-alanine
nmol O2 / min / mg protein
80
70
60
50
40
30
20
10
0
2.3
3
4
5
6
7
8
Assay buffer pH
B
nmol O2 / min / mg protein
60
50
40
30
20
10
0
2.3
3
4
5
6
7
8
Assay buffer pH
C
nmol O2 / min / mg protein
350
300
250
200
150
100
50
0
pH 2.3
pH 3
pH 4
pH 5
pH 6
pH 7
pH 8
Assay buffer pH

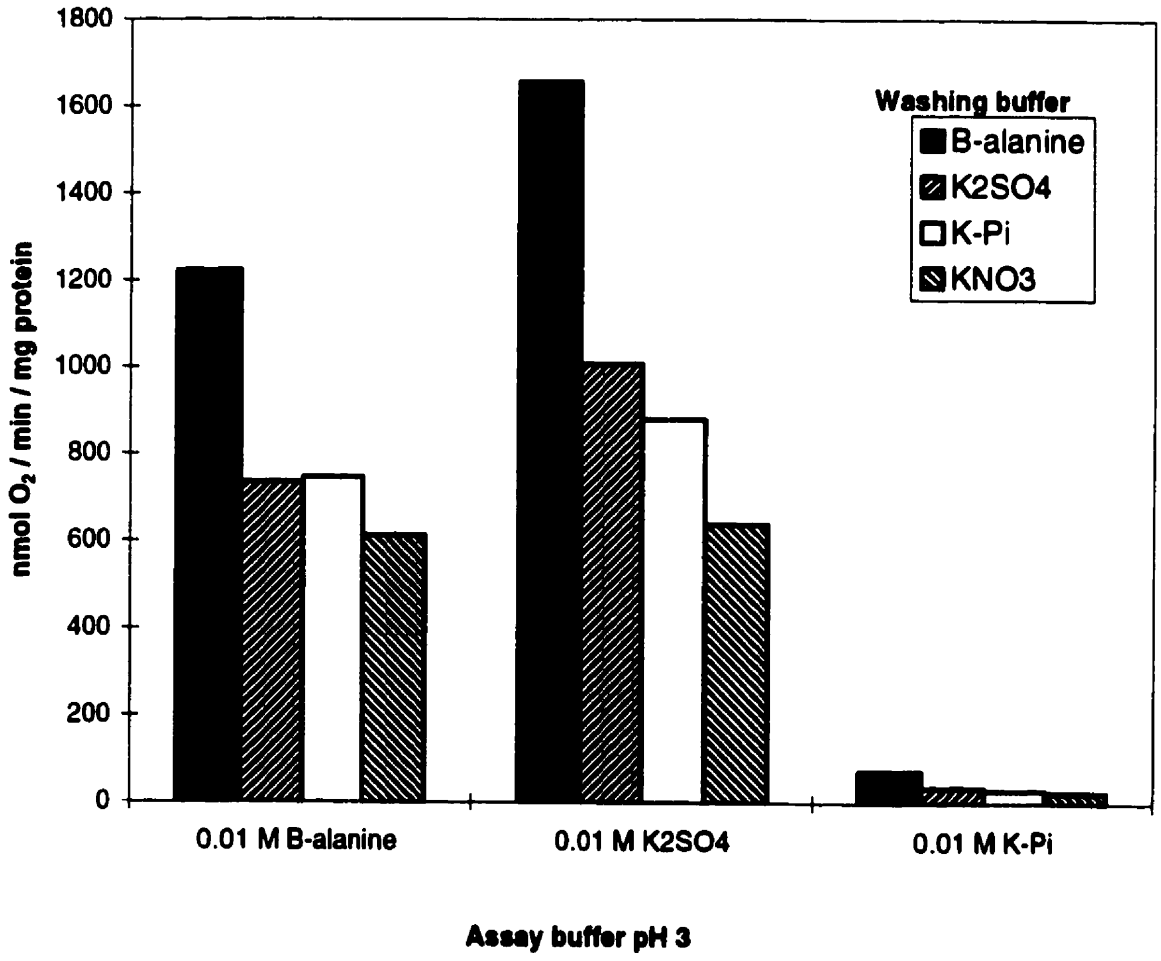
The two types of sulfur grown cells produced sulfur oxidation profiles different from that of their iron grown counterparts. First and foremost the overall rate of sulfur oxidation was substantially higher in cells continuously grown on sulfur. Cells cultured once on sulfur (Fig. 4B) showed oxidation rates similar to those of iron grown cells. They were, however, unaffected by assay buffer pH and concentration showing only minor fluctuations in activity over the values tested.

Cells continuously cultured on sulfur (Fig. 4C) were also unaffected by variation in pH (pH 2.3-8) and buffer concentration (0.01 M - 0.1 M) used. Sulfur oxidation rates approached 300 nmol O_2 / min / mg protein, a value nearly five times that observed with iron grown cells or sulfur direct cells. Thus the rate of sulfur oxidation appears to be greatly influenced by the level of bacterial exposure to substrate sulfur. The standard treatment, therefore, to obtain maximum sulfur oxidation in all cells tested was to wash the cells in 0.1 M β-alanine pH 2.3 and run the experiment at near neutral pH (generally 0.1 M β-alanine pH 6 unless otherwise stated).

Sulfite Oxidation

Washing buffer. A 48 hour culture of iron grown *Thiobacillus ferrooxidans* was collected as described in Materials and Methods and centrifuged at 12,000 x *g* for 10 minutes. The twelve centrifuge bottles were separated into four groups of three. Each group was washed using either 0.01 M β-alanine, K_2SO_4, K-Pi, or KNO_3 at pH 2.3 (a pH comparable to the growth pH). The resulting pellet was resuspended in the same washing buffer to generate a 50 mg/ml wet cell suspension. The four sets of cells were then tested for sulfite oxidizing activity in 0.01 M β-alanine, K_2SO_4 and K-Pi buffer at pH 3.

Figure 5. Effect of different washing buffers on sulfite oxidation by Fe^{2+} grown Tf-2 cells assayed in different buffers at pH 3.


1800
1600
1400
1200
1000
800
600
400
200
0
nmol O2 / min / mg protein
Washing buffer
B-alanine
K2SO4
K-Pi
KNO3
0.01 M B-alanine
0.01 M K2SO4
0.01 M K-Pi
Assay buffer pH 3

Potassium phosphate as assay buffer was strongly inhibitory as shown in Fig. 5. Figure 5 also shows that cells washed in β-alanine-H_2SO_4 provided the highest sulfite oxidizing activities. Potassium sulfate, phosphate and nitrate as washing buffers all slightly inhibited sulfite oxidation as compared to β-alanine-H_2SO_4. M9K medium and double glass distilled water adjusted with sulfuric acid to pH 2.3 were also tested as alternate washing buffers but proved to be inferior to the β-alanine-H_2SO_4 washed cells (data not shown).

Variations of the β-alanine-H_2SO_4 washing buffer were tested. Concentrations ranging from 10 - 100 mM (pH 2.3) were used to wash a single batch of cells. Figure 6 shows decreased sulfite oxidation at the higher washing buffer concentrations. Cells washed in 0.01 M β-alanine-H_2SO_4 (pH 2.3) provided the highest sulfite oxidation activity and thus were chosen as the standard sulfite wash .

Assay buffer. Iron grown *T. ferrooxidans* washed in 0.01 M β-alanine pH 2.3 were tested for sulfite oxidizing activity in 0.01M and 0.05 M β-alanine at pH 2.3 - 6 (Fig. 7). Maximum sulfite oxidation occurred at pH 3 and 4. The activity was very low at pH 2.3 and 6. Buffer concentration also had an inverse effect on sulfite oxidation - the higher the buffer concentration the lower the activity. It should be pointed out that in Figures 6 and 7 the rate of sulfite oxidation increased linearly with increasing sulfite concentrations

Cells grown on sulfur showed a sulfite oxidation profile significantly different from that of iron grown counterparts (Fig. 8). First and foremost the rate of sulfite oxidation was considerably lower in the cells grown on sulfur compared to those on iron. Maximum sulfite oxidation in iron grown cells approached 1,000 nmol O_2 / min / mg protein verses less then 300 nmol O_2 / min / mg protein for the sulfur grown cells. Other differences

Figure 6. Effect of β-alanine washing buffer (pH 2.3) concentration on sulfite oxidation in 0.01 M β-alanine (pH 3) using Fe^{2+} grown Tf-2

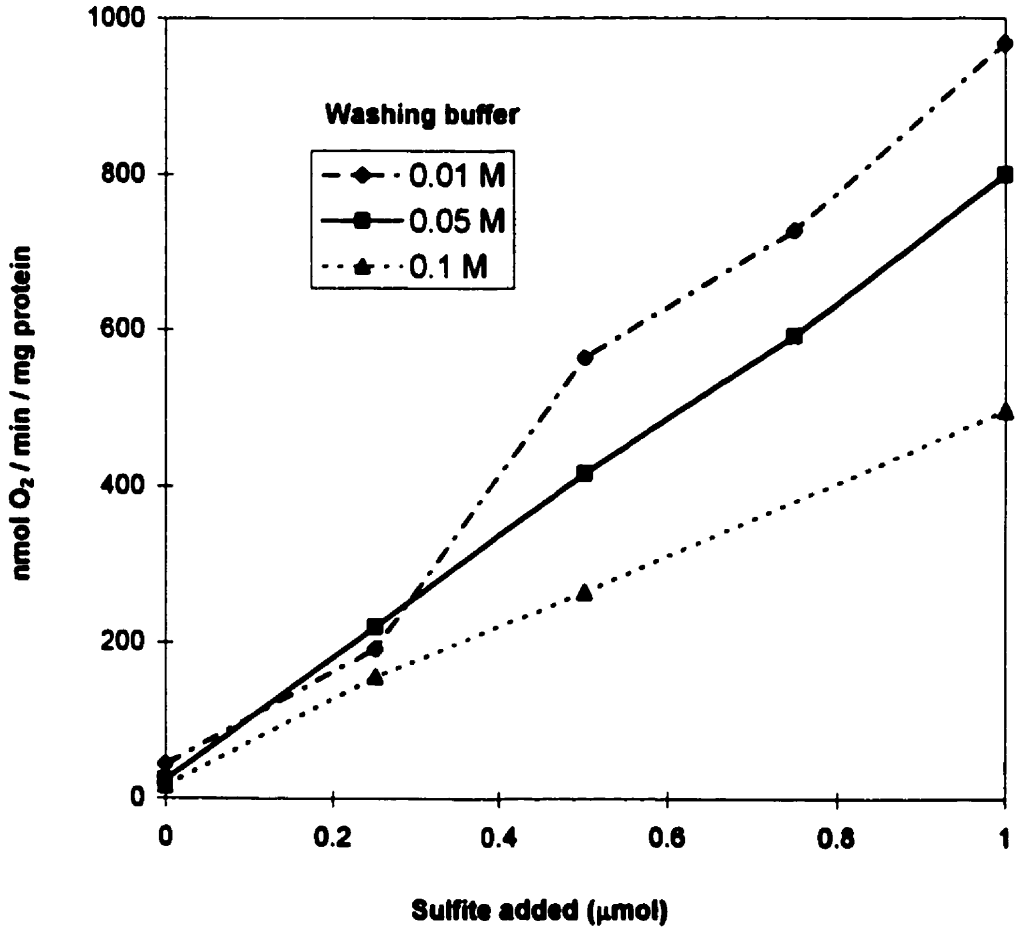

Washing buffer
0.01 M
0.05 M
0.1 M
nmol O2 / min / mg protein
Sulfite added (µmol)
0
200
400
600
800
1000
0
0.2
0.4
0.6
0.8
1

Figure 7. Effect of assay buffer pH on sulfite oxidation using Fe^{2+} grown Tf-2 washed in 0.01 M β-alanine (pH 2.3). A, 0.01 M β-alanine and B, 0.05 M β-alanine.

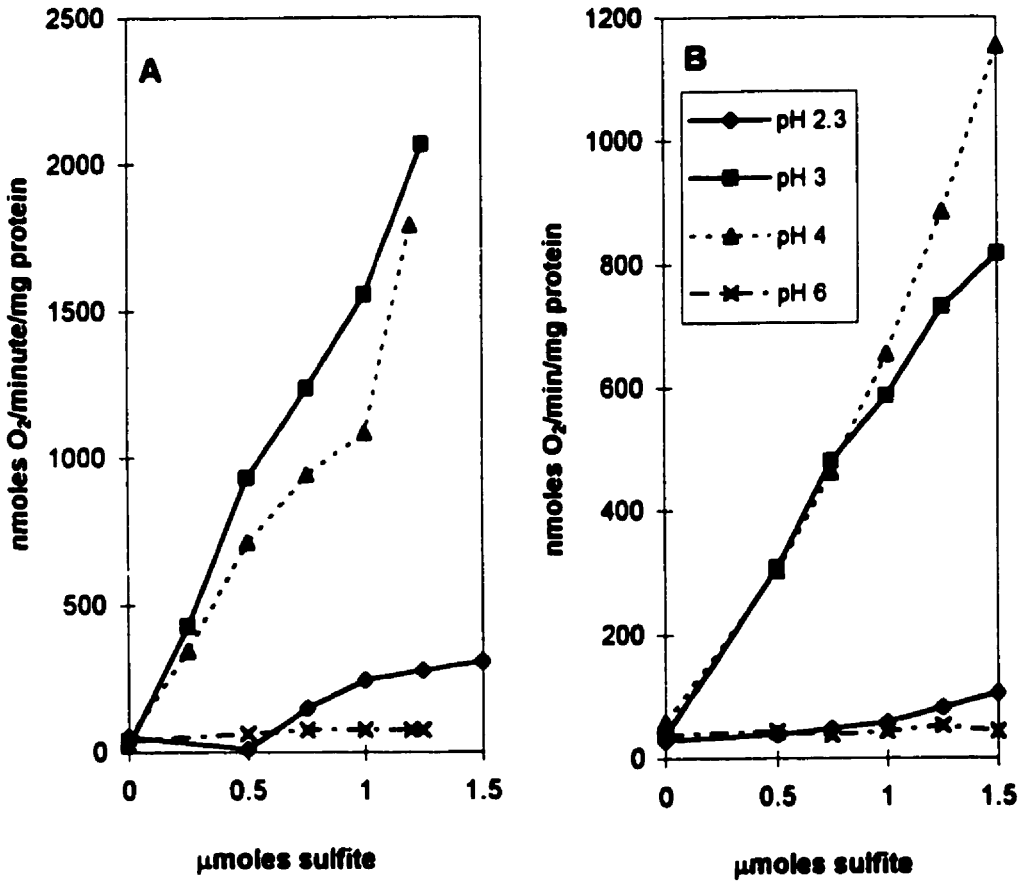
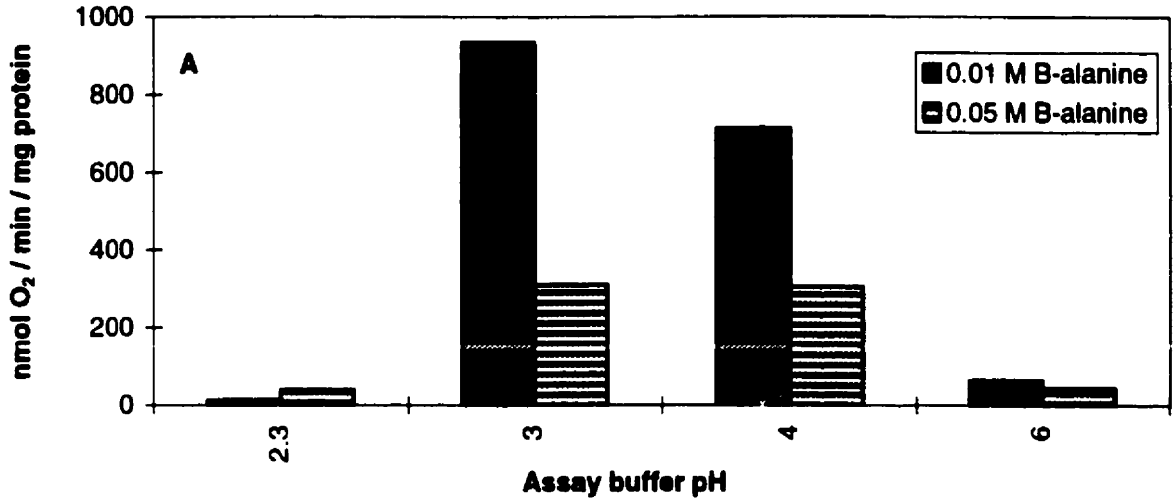
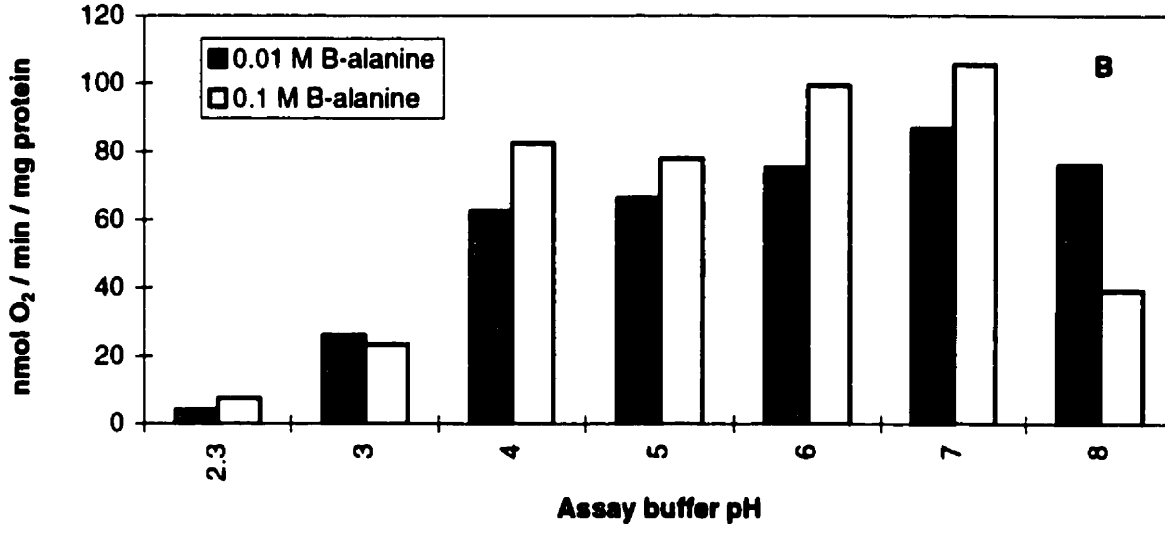
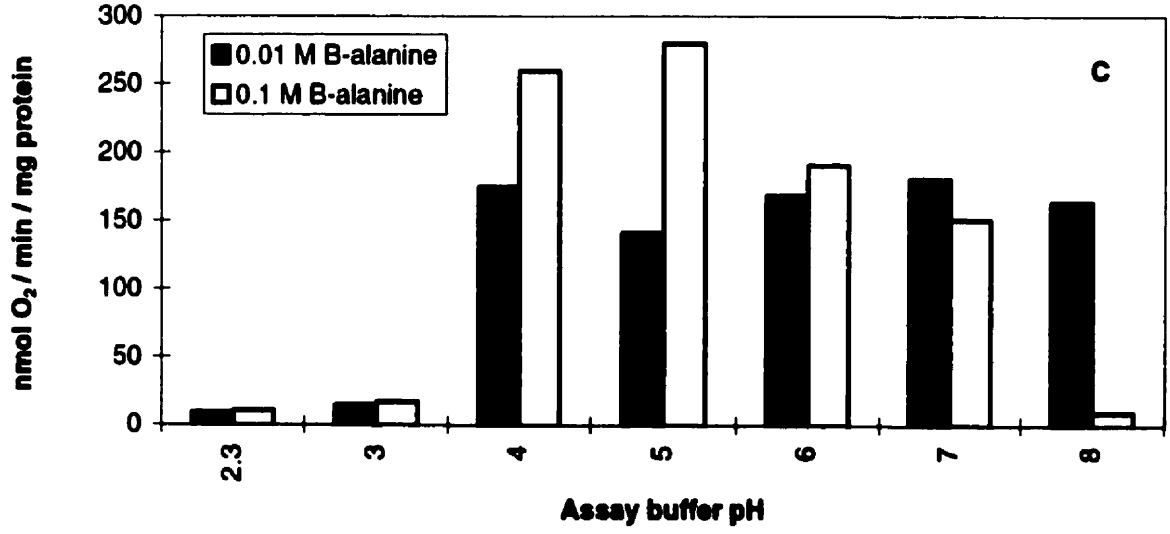

A
2500
2000
1500
1000
500
0
nmoles O2/minute/mg protein
0
0.5
1
1.5
µmoles sulfite
B
1200
1000
800
600
400
200
0
nmoles O2/min/mg protein
pH 2.3
pH 3
pH 4
pH 6
0
0.5
1
1.5
µmoles sulfite

Figure 8. Effect of pH on sulfite oxidation by Fe^{2+} grown cells (A), sulfur-direct cells (B) and sulfur grown cells (C) of Tf-2. Sulfite used was 0.5 μmol in 1.2 ml.


A
0.01 M B-alanine
0.05 M B-alanine
nmol O2 / min / mg protein
1000
800
600
400
200
0
2.3
3
4
6
Assay buffer pH


B
0.01 M B-alanine
0.1 M B-alanine
nmol O2 / min / mg protein
120
100
80
60
40
20
0
2.3
3
4
5
6
7
8
Assay buffer pH


C
0.01 M B-alanine
0.1 M B-alanine
nmol O2 / min / mg protein
300
250
200
150
100
50
0
2.3
3
4
5
6
7
8
Assay buffer pH

include changes in the pH profile. Maximum sulfite oxidation in the sulfur grown cells occurred in the pH range of 4 - 7, while sulfite oxidation in the iron grown cells was maximal at pH 3 and 4 (increased alkalinity inhibited observed activity).

Sulfite oxidation in sulfur grown cells was relatively unaffected with increasing buffer concentrations (0.01 M - 0.1 M). The same could not be said for iron grown cells which showed inhibition with 0.05 M β-alanine. Cells continuously subcultured on sulfur on the other hand, were stimulated at higher assay buffer concentrations.

Effect of Inhibitors on Oxidation Activities

The purpose of this study was two fold. First, to observe the effect, if any, bacterial growth substrate had on cells. This was measured in terms of iron, sulfur and sulfite oxidation. Second, to determine the degree of interaction, if any, between the iron and sulfur oxidation pathways. The basic underlying premise being, if the iron and sulfur oxidation pathways are connected then blocking, one pathway should automatically result in blockage of the second. The 'blockers' to be used consisted of numerous metal chelators and chemical inhibitors.

Ferrous iron oxidation using cell suspensions. The effect of inhibitors on ferrous iron oxidation was measured only with iron grown *T. ferrooxidans*. Cells grown on sulfur once or several times had completely lost the ability to oxidize ferrous iron and therefore will not be discussed. The reaction mixture contained 0.5 mg wet cells (iron grown), 0.5 μmol ferrous sulfate and 0.1 M β-alanine pH 3 to a final volume of 1.2 ml. As shown in Table 1 EDTA and Tiron which chelate ferric iron more strongly than ferrous iron stimulated ferrous iron oxidation presumably by removal of the ferric iron end

Table 1. Inhibition of ferrous iron (0.5 μmol) oxidation using Fe grown SM4 (10 μl). Washing buffer: β-alanine (pH 2.3). Assay buffer 0.1 M β-alanine (pH 3).

Inhibitor	Concentration	Effect
EDTA	4.2 mM	stimulates
Tiron	8.3 mM	stimulates
orthophenanthroline	0.83 mM	33 % inhibition
2,2'-dipyridyl	0.83 mM	20 % inhibition
NaN_3	0.66 μM	50 % inhibition
KCN	3.9 μM	50 % inhibition
Mannitol	83 mM	11 % inhibition

Note: 100 % activity was determined in the absence of inhibitors.

product. The ferrous iron chelator 2,2'-dipyridyl which may compete with the bacteria for substrate ferrous iron resulted in 20 % inhibition at 0.83 mM. Orthophenanthroline which is able to bind both ferrous and ferric iron equally reduced iron oxidation by 33% at the highest concentration used (0.83 mM). Azide and cyanide, kn

own to inhibit the terminal oxidase of the ferrous iron electron transport chain produced a 50 % drop in activity at very low concentrations of 0.66 μM and 3.9 μM respectively.

Sulfur oxidation using cell suspensions. Sulfur oxidation activity was found in both iron and sulfur grown *T. ferrooxidans* although it was much weaker in the iron grown cells. In order to compare the inhibitor effect discussed above on iron oxidation to that on sulfur oxidation the reaction was carried out at pH 3 (growth pH) as well as pH 6 (optimum pH for sulfur oxidation). Table 2 shows the iron chelators, EDTA, Tiron, orthophenanthroline and 2,2'-dipyridyl to be relatively ineffective in inhibiting sulfur oxidation in either the iron or sulfur grown cells.

Azide differentially affected the two cell types. Iron grown cells were strongly inhibited at both pH 3 and 6. The sulfur grown cells, however, showed a similar degree of inhibition only at pH 3; increased pH required increased amounts of inhibitor. This observed variation may be accounted for simply by pH. Hydrazoic acid has a pK value of 4.7 ($HN_3 \rightarrow H^+ + N_3^-$). At pH 3 the undissociated acid freely enters the cell inhibiting cytochrome oxidase. At pH 6, however, the dissociated form predominates (N_3^-) thus explaining the lower sensitivity of the cells to this inhibitor. Iron grown cells, then must have different cell membranes which are equally permeable to HN_3 and N_3^-.

Table 2. Inhibition of sulfur oxidation (washing buffer: 0.1 M β-alanine pH 2.3). Values represent 50 % (or less when indicated) inhibition of sulfur oxidation at the concentrations. Assay buffer: 0.1 M β-alanine.

Inhibitor	Fe^{2+} grown SM4		S^0 grown SM4	
EDTA	4.2 mM (9.2%)	4.2 mM (16%)	4.2 mM (19%)	4.2 mM (0%)
Tiron	8.3 mM (8%)	8.3 mM (10%)	8.3 mM (12%)	8.3 mM (0%)
orthophenanthroline	0.83 mM (19%)	0.83 mM (0%)	0.83 mM (15%)	0.83 mM (0%)
2,2'-dipyridyl	0.83 mM (5%)	0.83 mM (32%)	0.83 mM (3%)	0.83 mM (0%)
NaN_3	35 μM	35 μM	0.17 mM	19 μM
KCN	0.32 mM	32 μM	60 μM	77 μM
$HgCl_2$	3.5 μM	4 μM	14 μM	1.46 μM
Mannitol	83 mM (0%)	83 mM (14%)	58 mM (12%)	58 mM (10%)
NEM	57 μM	43 μM	46 μM	25 μM
HQNO	6.1 μM	4.7 μM	0.3-322 μM (33%)	5 μM
Assay buffer →	**pH 6**	**pH 3**	**pH 6**	**pH 3**

Percentage figures in brackets indicates the inhibition below 50 %.

Cyanide, also a terminal oxidase inhibitor blocked sulfur oxidation at 60 and 70 μM in the sulfur grown cells at pH 3 and 6 respectively. The pK value of hydrocyanic acid is 9.2 ($HCN \rightarrow H^+ + CN^-$). Thus the pH values tested had little to no effect on the ability of the acid to enter the cell. The observed inhibition, however, was time dependent with oxidation rates gradually leveling off with time, suggesting a possibility of cytochrome oxidase reacting with slowly cyanide. The iron grown cells on the other hand, were strongly inhibited only at pH 3. At pH 6, 0.32 mM KCN was required for 50 % inhibition, a value nearly ten times that of pH 3. Thus the cytochrome oxidase was more reactive to cyanide binding at pH 3. Based on the cytochrome oxidase inhibitors alone it is likely that the sulfur oxidation mechanism in iron grown cells is different from that of sulfur grown cells (i.e. the mechanism is determined by the bacterial growth substrate).

Mercuric chloride had a strong inhibitory effect on the sulfur oxidation by both cell types. Mannitol, a free radical scavenger, was ineffective producing a maximum at 14 % inhibition at 83 mM. The sulfhydryl binding agent N-ethylmaleimide (NEM) which has been shown to specifically block the oxidation of sulfur to sulfite in *T. thiooxidans* (Chan and Suzuki, 1994) was equally effective in *T. ferrooxidans*. NEM was shown to inhibit 50 % of the sulfur oxidizing activity at 25-57 μM in both iron and sulfur grown cells.

2-Heptyl-4-hydroxyquinoline-N-oxide (HQNO) has been shown to inhibit the oxidation of sulfite to sulfate in *T. thiooxidans* through interaction with the bc_1 complex (Chan and Suzuki, 1994). The sulfur grown *T. ferrooxidans* showed 50 % inhibition at pH 3 with 5 μM HQNO. This value however, is deceptive for HQNO does not inhibit sulfur oxidation (Chan and Suzuki, 1994). The observed inhibition was possibly due to sulfite

accumulation. Sulfite can enter the cell more easily at pH 3 than at pH 6 ($HSO_3^- + H^+ \leftrightarrow H_2SO_3$, pKa = 1.89), acidifying the cytoplasm and thus indirectly inhibiting sulfur oxidation.

At pH 6 HQNO inhibited only 33 % of the sulfur oxidizing activity (in the sulfur grown cells). Although the mechanism of inhibition had not changed the increased pH prevented the accumulated sulfite from entering the cells. Added HQNO (0.3 μM to 322 μM) did not increase this inhibition suggesting that sulfur oxidation ended at sulfite. The oxidation of sulfur to sulfate requires one and a half moles of oxygen ($S^0 + O_2 + H_2O \rightarrow H_2SO_3$; $H_2SO_3 + ½O_2 \rightarrow H_2SO_4$). If sulfite oxidation is blocked then only one mole of oxygen will be used thus a maximum of 66 % sulfur oxidation activity can be obtained (i.e. a maximum of 33 % inhibition). This is exactly what was observed.

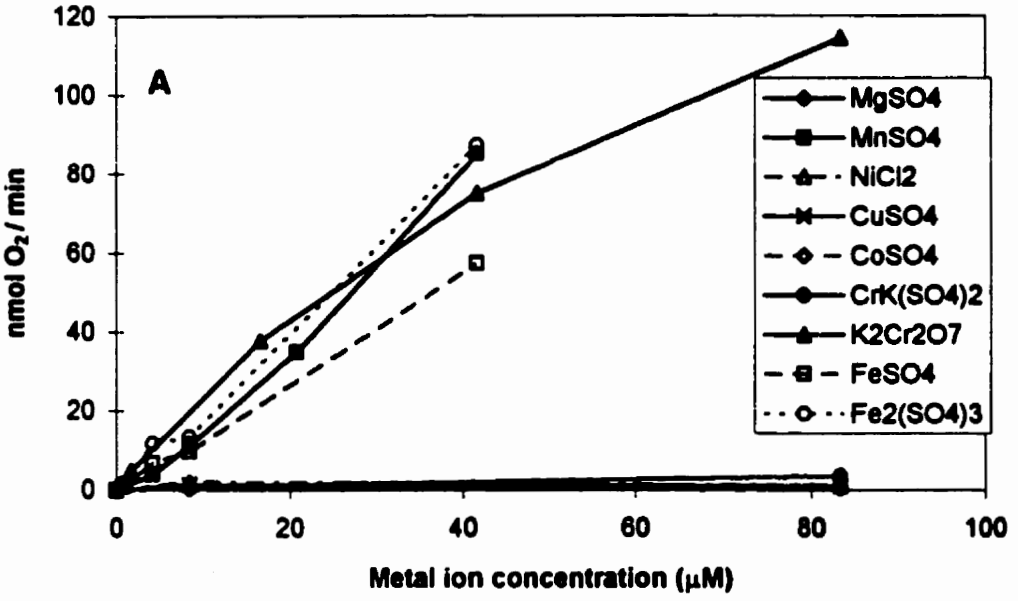
Iron grown *T. ferrooxidans* showed 50 % inhibition at 5-6 μM HQNO at both pH values tested. Since sulfite oxidation by iron grown cells is not supposed to be inhibited by HQNO (Sugio et al., 1987), the inhibition site could be the endogenous metabolism essential for sulfur oxidation. Perhaps the energy reducing power of endogenous metabolism is required to open the sulfur octet ring.

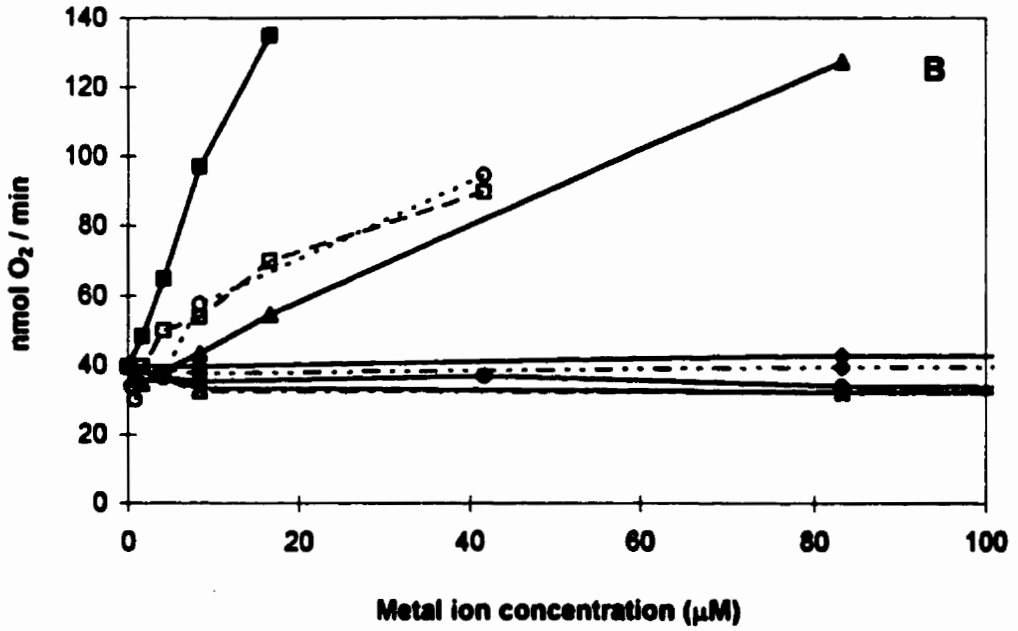
Sulfite oxidation. As previously stated biological sulfite oxidation according to Sugio's theory (Sugio et al., 1987) is coupled to ferric iron reduction using sulfite:ferric ion oxidoreductase ($H_2SO_3 + 2Fe^{3+} + H_2O \rightarrow H_2SO_4 + 2Fe^{2+} + 2H^+$). The alternative theory (Ingledew et al., 1977) states that under aerobic conditions sulfite is oxidized with molecular oxygen using sulfite oxidase to catalyze the reaction: $H_2SO_3 + ½O_2 \rightarrow H_2SO_4$. Sulfite, however, is a highly reactive substance known to be oxidized in the presence of

metal ions. Initial studies have revealed the rapid autooxidation of sulfite solutions to be the result of a metal catalyzed free radical chain reaction. Figure 9A shows chemical sulfite oxidation rates produced using several known oxidizing metals at pH 3. Of the metal ions tested only Fe^{3+} (as $Fe_2(SO_4)_3$), Mn^{2+} (as $MnSO_4$), Cr^{6+} (as $K_2Cr_2O_7$) and Fe^{2+} (as $FeSO_4$) were able to stimulate chemical sulfite oxidation. The potential of added metal ions was then tested in the presence of cells. Figure 9B shows the metal ions that stimulated chemical sulfite oxidation also stimulated sulfite oxidation in the presence of cells. The degree of stimulation with and without cells, however, was not the same. In order to increase the biological sulfite oxidation from 40 to 80 nmol O_2 / min the bacterial system required only 5 μM Mn^{2+} while the chemical system required 23 μM Mn^{2+} to achieve the oxidation rate of 40 nmol O_2 / min, suggesting that the doubling of sulfite oxidation by Mn^{2+} in the presence of cells is not just due to a chemical reaction, the cells are somehow involved. Fe^{3+} and Cr^{6+} were required in higher concentrations to stimulate sulfite oxidation in the presence of cells than without. Ferrous iron, on the other hand, stimulated both reactions (with and without cells) equally. Of the metal ions tested, however, only iron was a potential candidate for sulfite oxidation in our experiment. It is naturally present in the bacterial environment as soluble ferrous and ferric iron and surrounds the bacteria in the form of an iron lattice. Sulfite oxidation, therefore in the presence of cells may be the result of an iron catalyzed sulfite oxidation reaction (i.e. a chemical reaction using the iron surrounding the cell).

In order to determine the exact nature of sulfite oxidation in the presence of cells (i.e. is it a biological or chemical reaction?) the bacterial iron content was measured. Iron

Figure 9. Effect of metal ions on sulfite oxidation. **A**, without cells. **B**, with cells. The reaction mixture contained 1 μmol sulfite, the listed ions and 0.5 mg wet cells in 0.01 M β-alanine buffer pH 3 (total volume of 1.2 ml).


A
MgSO4
MnSO4
NiCl2
CuSO4
CoSO4
CrK(SO4)2
K2Cr2O7
FeSO4
Fe2(SO4)3
nmol O2 / min
Metal ion concentration (μM)
0
20
40
60
80
100
120


B
nmol O2 / min
Metal ion concentration (μM)
0
20
40
60
80
100
120
140

grown *T. ferrooxidans* were found to contain 8.3 nmoles of total iron in 0.25 mg wet cells. The rate of sulfite oxidation using 0.25 mg wet cells corresponded to the chemical oxidation rate using 7.5 nmoles ferric iron. Thus 7.5 nmoles of ferric iron were used as a chemical control corresponding to 0.25 mg wet cells. (Note: Ferric iron was used as the chemical control because its oxygen consumption profile for sulfite oxidation was identical to that found in the presence of cells (Fig. 10). Ferrous iron on the other hand, had an initial lag period prior to active sulfite oxidation - suggesting that ferrous iron had to be oxidized to ferric iron prior to chemical sulfite oxidation.).

Figure 11 shows the effects of numerous chemical inhibitors on sulfite oxidation in the biological (0.25 mg wet cells) and chemical systems (7.5 nmoles of ferric iron). EDTA and Tiron are metal chelators that bind ferric iron more strongly than ferrous iron. Figure 11 A and C shows a substantial difference between the inhibition of the chemical and biological systems using these chelators. EDTA inhibited the chemical system almost 50 % at 2.5 μM without affecting the biological system. The chemical system is almost entirely inhibited at 12.5 μM Tiron, while nearly 7 times as much is required to inhibit the biological reaction. This difference may be due to the different forms of iron present in the two systems. In the chemical system iron is found primarily as ferric iron in the form of added ferric sulfate. The biological system, however, contains both ferrous and ferric iron in a lattice structure surrounding the cell. Orthophenanthroline is able to bind both ferrous and ferric forms equally well. According to Fig. 11 B it inhibited sulfite oxidation in both the biological and chemical systems to a similar degree.

Figure 10. Chemical sulfite oxidation using ferrous and ferric iron. The reaction mixture contained 1 μmol sulfite and 0.05 μmol iron in 0.01 M β-alanine sulfate buffer pH 3.0.

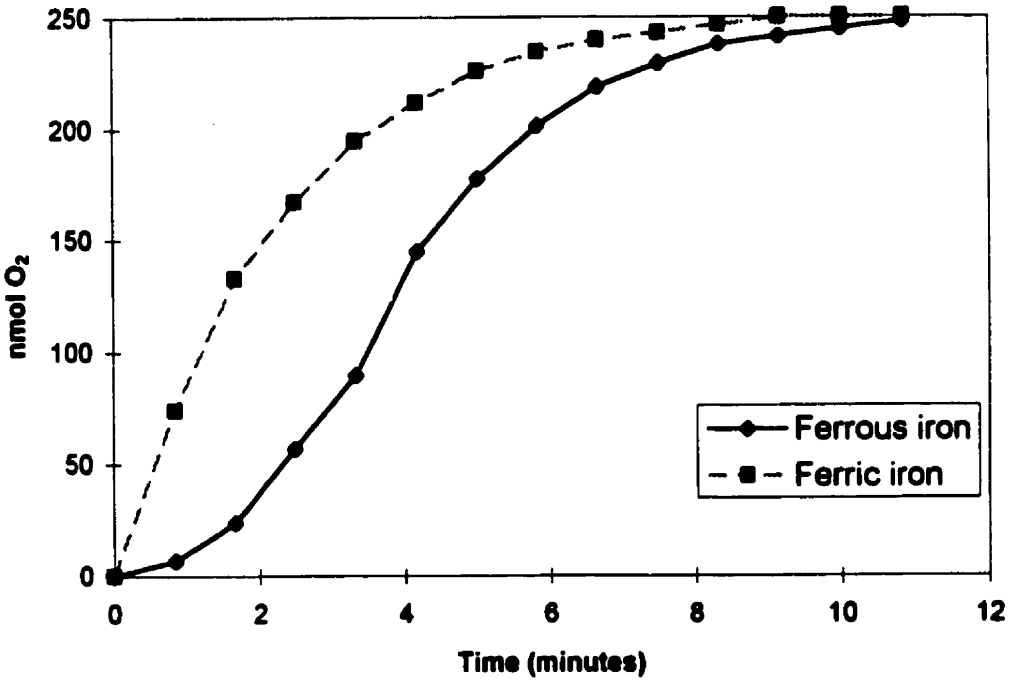
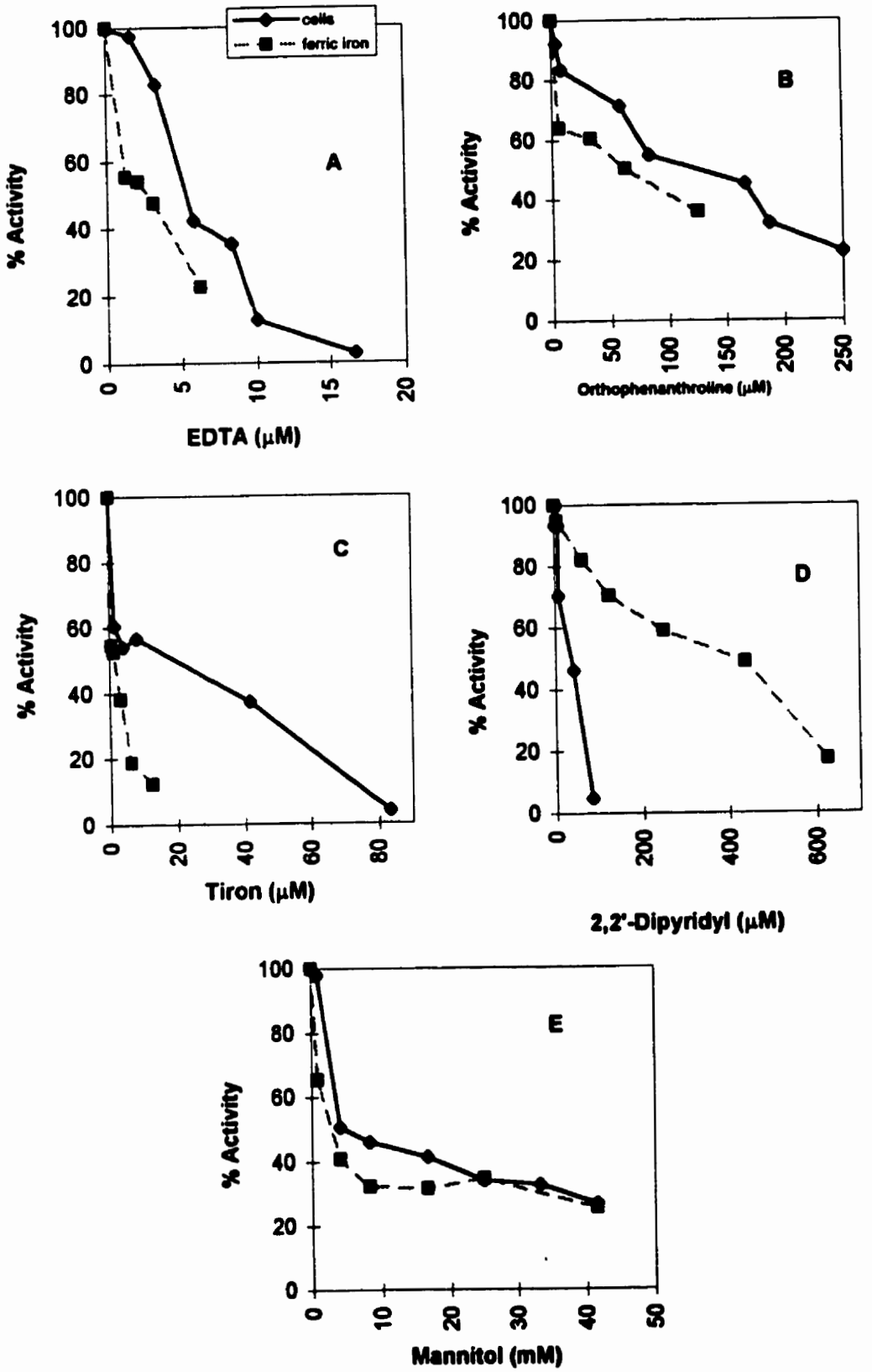

250
200
150
100
50
0
nmol O_2
0
2
4
6
8
10
12
Time (minutes)
Ferrous iron
Ferric iron

Figure 11. Inhibition of chemical and biological sulfite oxidation. The reaction mixture contained 1 μmol sulfite, 7.5 nmol Fe^{3+} (chemical oxidation), 0.5 mg wet cells (biological oxidation) and various inhibitors in 0.01 M β-alanine sulfate buffer pH 3.0 to a total volume of 1.2 ml.


cells
ferric iron
A
% Activity
EDTA (μM)
B
Orthophenanthroline (μM)
C
Tiron (μM)
D
2,2'-Dipyridyl (μM)
E
Mannitol (mM)

The potential importance of ferrous iron in sulfite oxidation was tested using 2,2'-dipyridyl, a metal chelator with a higher affinity for ferrous iron. Figure 11D shows 2,2'-dipyridyl to preferentially inhibit sulfite oxidation in the biological system at 83 μM while greater than seven times this concentration was required to inhibit the chemical system. Thus ferrous iron was shown to be an important constituent in the biological system of sulfite oxidation.

Sulfite oxidation may also be the result of free radical chain reaction. Antioxidants or chain breakers, however, can stop these reactions. Mannitol has been shown to effectively block the free radicals involved in the metal catalyzed autooxidation of sulfite (McCord and Fridovich, 1969). Figure 11E shows mannitol to be equally effective in both the chemical and biological systems indicating the presence of free radicals in sulfite oxidation in both systems.

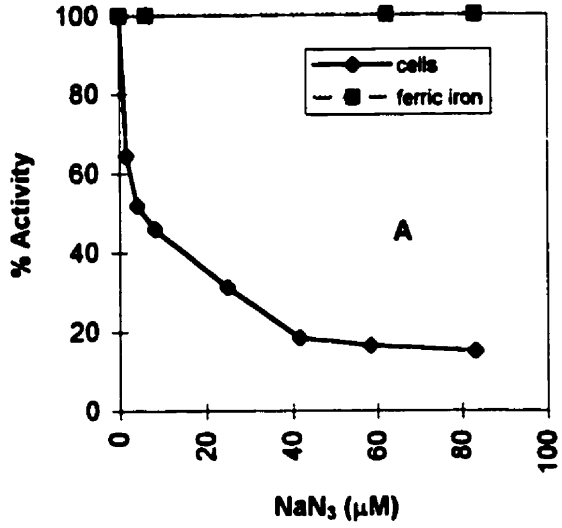
The potential importance of free radicals in sulfite oxidation was further investigated using superoxide dismutase and catalase. Superoxide dismutase found in virtually all aerobic organisms functions in protecting the cell from the superoxide radical ($O^{\bullet}_2^-$). The enzyme removes $O^{\bullet}_2^-$ by converting it to the less reactive hydrogen peroxide ($O^{\bullet}_2^- + O^{\bullet}_2^- + 2H^+ \rightarrow H_2O_2 + O_2$). The hydrogen peroxide is then removed by catalase producing water and oxygen ($2H_2O_2 \rightarrow 2H_2O + O_2$). Sulfite oxidation in both the chemical and biological systems were relatively unaffected by either enzyme (**data not shown**). Thus it is unlikely that either the superoxide radical or hydrogen peroxide are involved in sulfite oxidation.

From the above information sulfite oxidation in iron grown *T. ferrooxidans* appears to be dependent on the presence of iron and free radicals. The potential role of cells, however, was yet to be tested. Azide and cyanide are potential metal chelators and specific inhibitors of the terminal cytochrome oxidase of the electron transport system. Figure 12A and B show that azide and cyanide almost completely inhibited sulfite oxidation with cells at 42 μM and 130 μM respectively. The chemical system, however, was totally unaffected at the concentrations shown. Thus sulfite oxidation in the presence of cells is not just a chemical reaction.

Sulfite oxidation in sulfur grown *T. ferrooxidans*. As previously stated growth substrate has a significant effect on the overall make up of the intact cell. The total iron content was also found to differ between the iron and sulfur grown cells. Iron grown cells (50 mg/ml) contained 0.83 nmol/μl iron while sulfur grown cells (50 mg/ml) contained only 0.53 nmol/μl iron. Previous data (Figs. 5-8) have shown a significant difference in sulfite oxidation between the iron and sulfur grown cells. Maximum sulfite oxidation occurred at pH 3 using iron grown cells, while sulfur grown cells produced a peak at pH 6. This variation in optimum activity was the first sign that a potential difference in sulfite oxidation may exist between the two cell types.

In order to examine this possibility the previously mentioned inhibitors were tested for their effect on sulfur grown cells. The reaction mixture contained 100 μl of sulfur grown SM-4 (50 mg/ml), 1 μmol sulfite and 0.1 M β-alanine pH 6 for a total volume of 1.2 ml. (Acidic pH could not be used, due to weak, unreliable sulfite oxidizing activity with sulfur grown cells at pH 3). Table 3 presents 50 % inhibition values for sulfite

Figure 12. Inhibition of chemical and biological sulfite oxidation. The reaction mixture contained 1 μmol sulfite, 7.5 nmol Fe^{3+} (chemical oxidation), 0.5 mg wet cells (biological oxidation) and various inhibitors in 0.01 M β-alanine sulfate buffer pH 3.0 to a total volume of 1.2 ml.


100
80
60
40
20
0
% Activity
cells
ferric iron
A
0
20
40
60
80
100
NaN3 (μM)

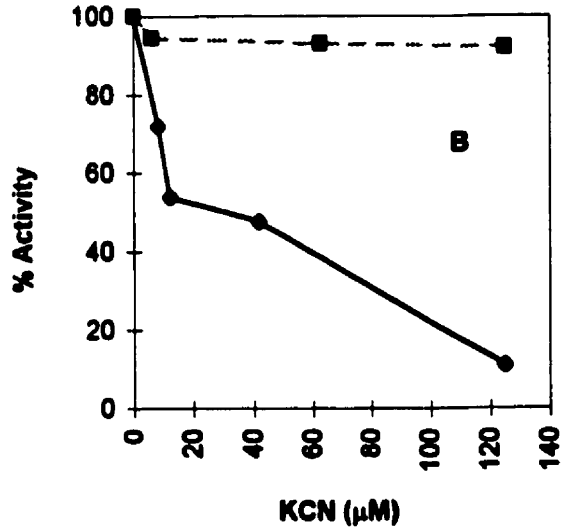

100
80
60
40
20
0
% Activity
B
0
20
40
60
80
100
120
140
KCN (μM)

Table 3. Inhibition of sulfite oxidation by SM-4 cells grown on Fe^{2+} and S°, washed in 0.01 M β-alanine pH 2.3. Values represent 50 % inhibition of sulfite oxidation. Assay buffer: β-alanine.

Inhibitor	**Fe^{2+} grown SM4**	**S^{0} grown SM4**
EDTA	5.2 mM	4.2 mM (0%)
Tiron	20 mM	8.3 mM (6%)
orthophenanthroline	130 mM	0.83 mM (40%)
2,2'-dipyridyl	25 mM	0.83 mM (19%)
NaN_3	5.6 μM	0.11 mM
KCN	84 μM	27 μM
Mannitol	4.8 mM	0.83 mM (0%)
NEM	8.3 mM (46%)	0.86 mM
HQNO	0.23 mM (43%)	0.032 mM
Assay buffer →	**0.01M BA pH 3**	**0.1 M BA pH 6**

Percentage figures in brackets indicates the inhibition below 50 %.

oxidation in both iron and sulfur grown cells so as to allow a direct comparison between the two cell types. The iron chelators EDTA, Tiron, orthophenanthroline and 2,2'-dipyridyl although very effective at inhibiting sulfite oxidation in iron grown cells had little effect on sulfur grown cells even at substantially higher concentrations. Thus iron in either its oxidized or reduced forms is probably not involved in sulfite oxidation using sulfur grown cells.

Azide an inhibitor of the terminal oxidase in the iron electron transport chain inhibited sulfite oxidation in iron grown cells at 5.6 μM while nearly twenty times as much azide was required to inhibit sulfite oxidation in sulfur grown cells. This large variation may in part be a pH effect. As previously mentioned hydrazoic acid (HN_3) has a pK value of 4.7. At pH 3 (used for the iron grown cells) it can freely enter the cells in its undissociated form. At pH 6 (used for the sulfur grown cells), however, the dissociated form (N_3^-) predominates which is not freely permeable. Thus a higher concentration of azide would be required to produce the same degree of inhibition.

Cyanide, another inhibitor of the terminal oxidase, is also reported to specifically inhibit the transfer of electrons from sulfite oxidase to cytochrome *c* (Fridovich and Handler, 1960). It preferentially inhibited sulfite oxidation in the sulfur grown cells at 27 μM while nearly three times as much was required to inhibit the iron grown cells. The pK value of hydrocyanic acid is 9.2, thus the difference in pH (between the two bacterial assays) likely plays only a minor role in the observed inhibition.

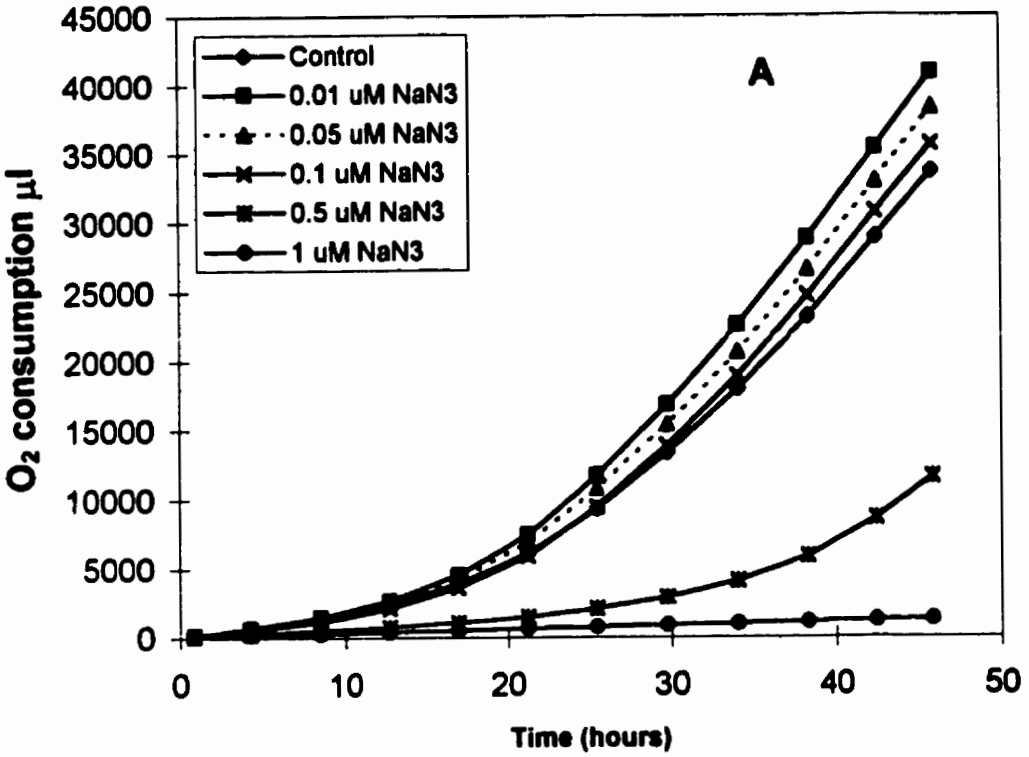
NEM (N-ethyl maleamide) a sulfhydryl binding agent is used to specifically block sulfur oxidation in *T. thiooxidans* (Suzuki et al., 1993) and *T. ferrooxidans* (Table 2).

NEM inhibited 50 % sulfite oxidation in sulfur grown cells at a high concentration of 0.86 mM while nearly ten times this concentration was required to inhibit the iron grown cells. HQNO a specific inhibitor of the bc_1 complex has been shown to block the oxidation of sulfite to sulfate in *T. thiooxidans*. The effect of HQNO is time dependent and thus required an incubation period of approximately 5 minutes with the bacteria prior to the addition of substrate. During this incubation period (which was monitored using the Gilson oxygraph), the initial endogenous activity of sulfur grown cells was observed to be inhibited with increasing HQNO concentrations. The endogenous activity of the iron grown cells was too low for the measurement (0.25 mg wet cells verses 5 mg wet sulfur grown cells). HQNO inhibited 50 % sulfite oxidation in sulfur grown cells at only 0.032 μM while ten thousand times higher concentration (0.23 mM) was required to inhibit iron grown cells only by 43%. Thus the sulfite oxidizing systems of the cells studied are totally different.

Iron and sulfur oxidation in growing cell cultures. The above inhibition data using cell suspensions provided a basis for a general understanding of the sulfur and iron oxidation mechanisms. Based on these data it was observed that iron and sulfur oxidation can be differentially controlled using specific inhibitors. Azide and cyanide both preferentially inhibited iron oxidation in cell suspensions at 0.66 μM and 3.9 μM respectively. In order to verify that the above process of selective inhibition was not limited to cell suspensions it was repeated with growing cell cultures.

Bacterial growth was measured in a Micro-oxymax respirometer as described in Materials and Methods. Each test flask contained M9K medium pH 2.3, 12 mmoles

Figure 13. Azide inhibition of oxidation and growth on iron using *T. ferrooxidans.* **A,** inhibition of iron oxidation. **B,** inhibition of bacterial growth on iron. The reaction was monitored in a Mico-oxymax as described in Materials and Methods.


A
Control
0.01 uM NaN3
0.05 uM NaN3
0.1 uM NaN3
0.5 uM NaN3
1 uM NaN3
O2 consumption µl
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
0
10
20
30
40
50
Time (hours)

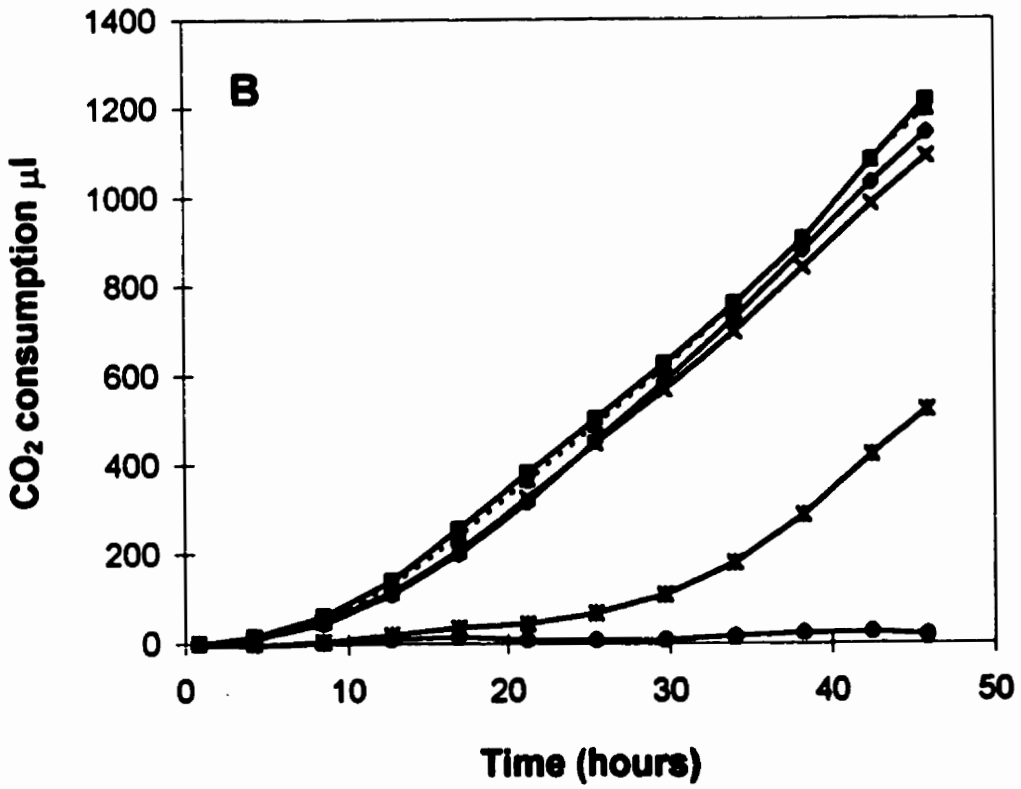
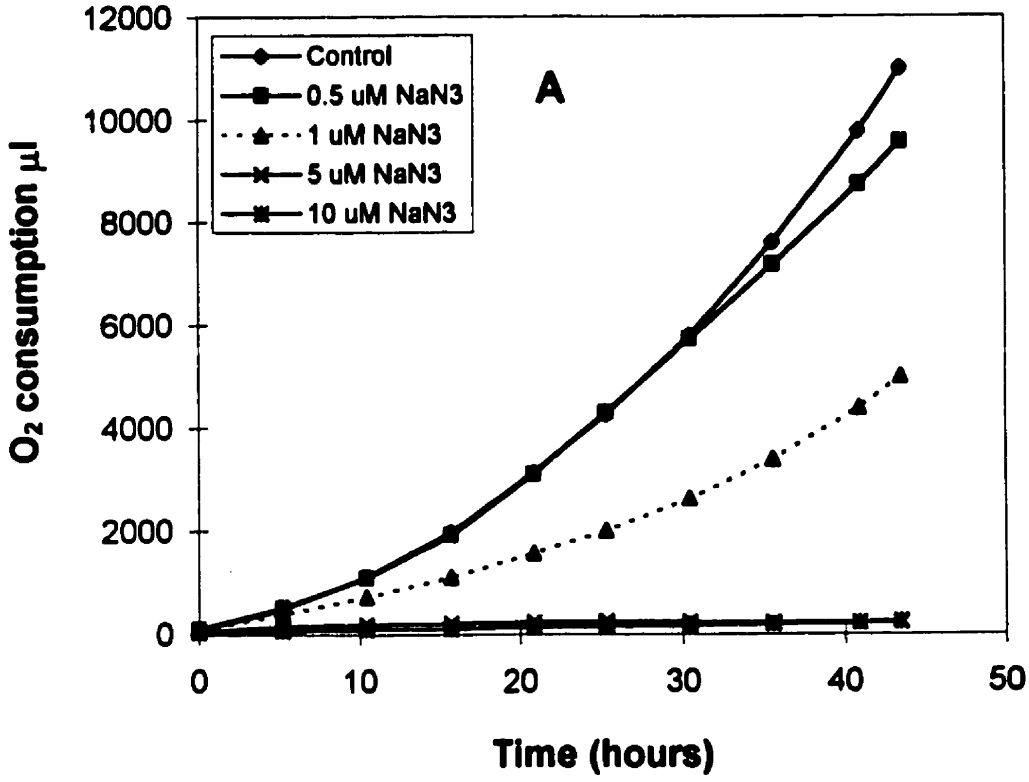
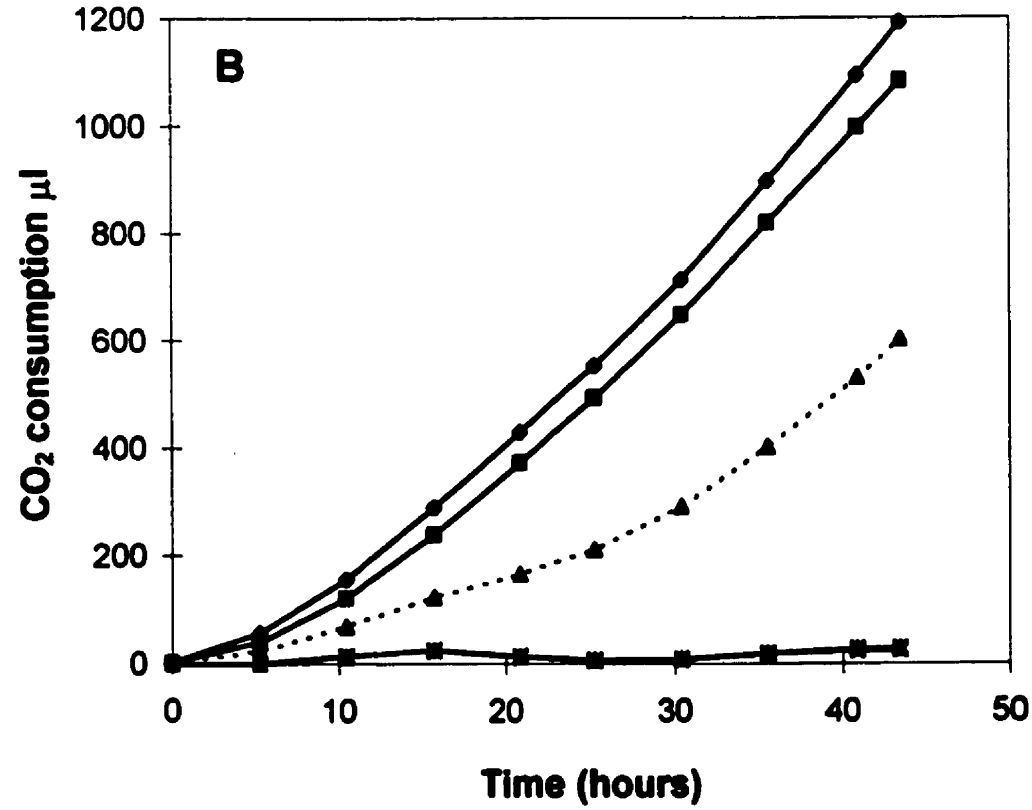

B
CO2 consumption µl
1400
1200
1000
800
600
400
200
0
0
10
20
30
40
50
Time (hours)

Figure 14. Azide inhibition of oxidation and growth on iron using *T. thiooxidans*. **A,** inhibition of sulfur oxidation. **B,** inhibition of bacterial growth on sulfur. The reaction was monitored in a Mico-oxymax as described in Materials and Methods.


12000
10000
8000
6000
4000
2000
0
O_2 consumption µl
Control
0.5 uM NaN3
1 uM NaN3
5 uM NaN3
10 uM NaN3
A
0
10
20
30
40
50
Time (hours)

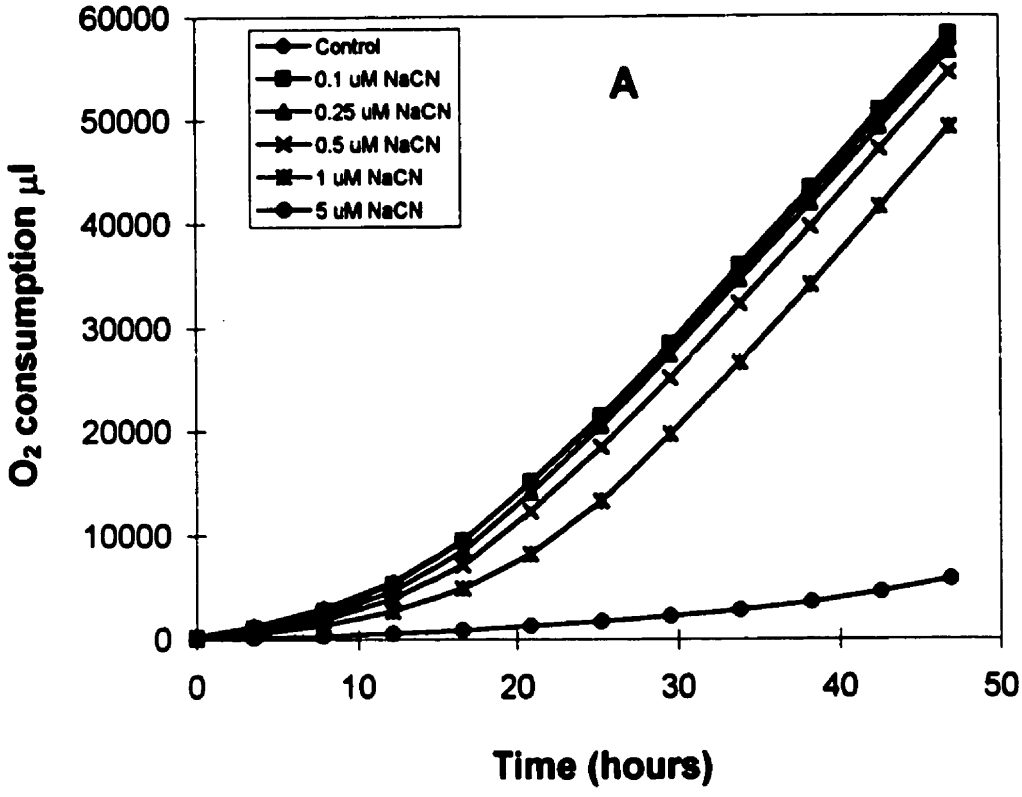

1200
1000
800
600
400
200
0
CO_2 consumption µl
B
0
10
20
30
40
50
Time (hours)

$FeSO_4 \cdot 7H_2O$, *T. ferrooxidans* (10 % inoculum, vol./vol.) and various concentrations of inhibitors to a final volume of 100 ml. Figure 13 shows the effect of increased azide concentrations on bacterial growth (seen as CO_2 consumption) and activity (seen as O_2 consumption) over a 48 hour period. Fifty percent inhibition was obtained with 0.4 μM azide with cells grown on ferrous iron.

Azide inhibition of oxidation and growth on sulfur could not be tested using *T. ferrooxidans* (iron or sulfur grown) due to limited access to the Micro-oxymax. It was, however, investigated using sulfur grown *T. thiooxidans* whose inhibition patterns (using cell suspensions) were very similar to those of sulfur grown *T. ferrooxidans*. Figure 14 shows 50 % inhibition at 0.9-1 μM azide after 48 hours of incubation. This value although less than that observed with the cell suspensions, was higher than that required to inhibit iron oxidation. Thus, it would appear that azide preferentially inhibited iron oxidation in growing cell cultures.

Cyanide, the other inhibitor tested inhibited iron oxidation by 50 % at 3.9 μM using bacterial cell suspensions. Figure 15 shows iron grown *T. ferrooxidans* was inhibited to a similar degree at 2.8-3 μM cyanide. Sulfur grown *T. thiooxidans* (Fig. 16) showed 50 % inhibition at 8-11 μM cyanide. Although this value is substantially less than the 32-77 μM required to inhibit sulfur oxidation in the cell suspensions the overall trend remains - preferential inhibition of iron oxidation.

Figure 15. Cyanide inhibition of oxidation and growth on iron using *T. ferrooxidans*. **A,** inhibition of iron oxidation. **B,** inhibition of bacterial growth on iron. The reaction was monitored in a Mico-oxymax as described in Materials and Methods.


60000
50000
40000
30000
20000
10000
0
O_2 consumption µl
Control
0.1 uM NaCN
0.25 uM NaCN
0.5 uM NaCN
1 uM NaCN
5 uM NaCN
A
0
10
20
30
40
50
Time (hours)

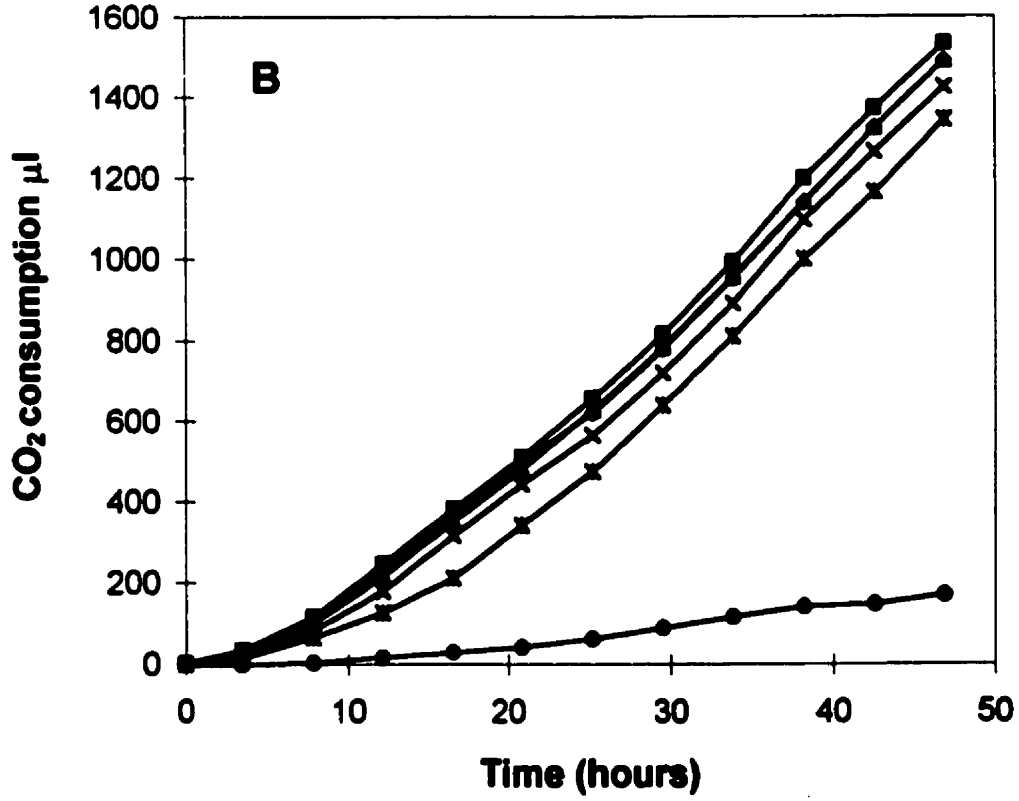
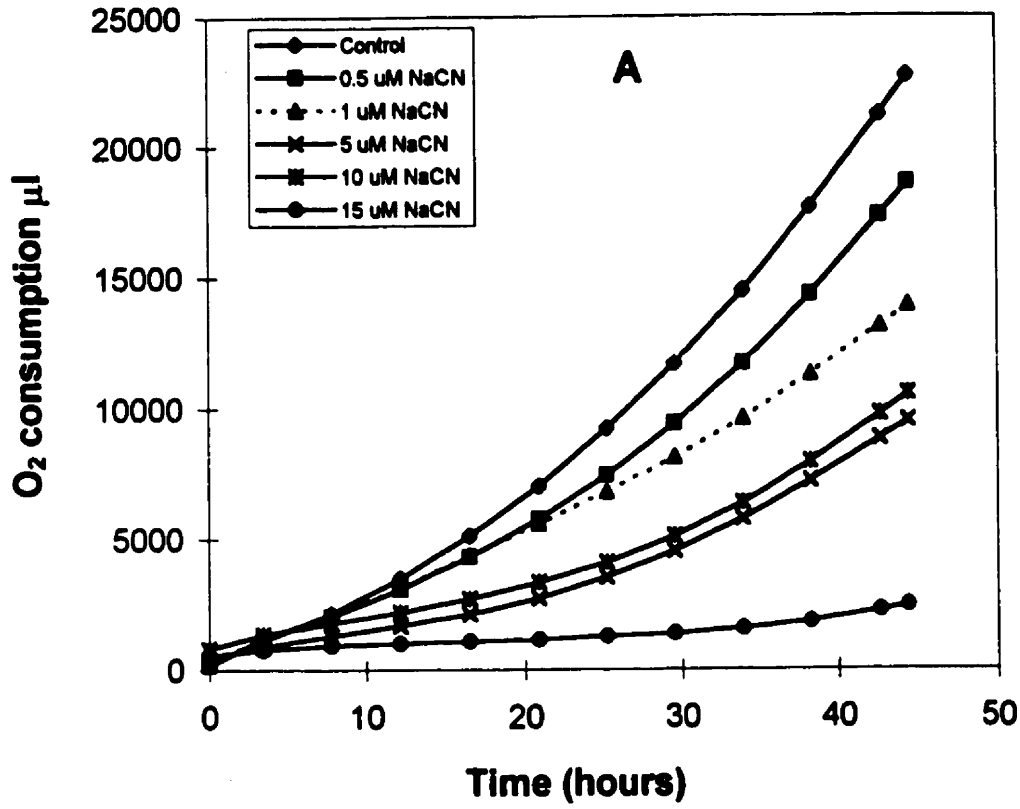
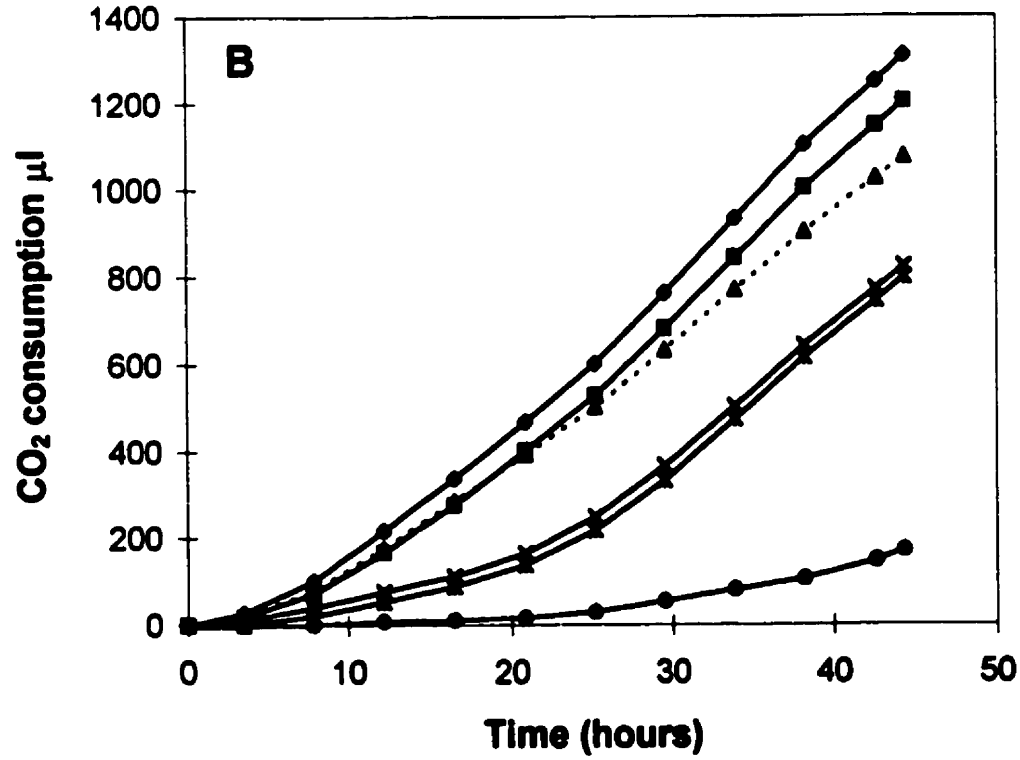

1600
1400
1200
1000
800
600
400
200
0
CO_2 consumption µl
B
0
10
20
30
40
50
Time (hours)

Figure 16. Cyanide inhibition of oxidation and growth on iron using *T. thiooxidans*. **A,** inhibition of sulfur oxidation. **B,** inhibition of bacterial growth on sulfur. The reaction was monitored in a Mico-oxymax as described in Materials and Methods.


A
Control
0.5 uM NaCN
1 uM NaCN
5 uM NaCN
10 uM NaCN
15 uM NaCN
O2 consumption µl
25000
20000
15000
10000
5000
0
0
10
20
30
40
50
Time (hours)


B
CO2 consumption µl
1400
1200
1000
800
600
400
200
0
0
10
20
30
40
50
Time (hours)

DISCUSSION

The purpose of this study was to investigate the effect of growth substrate on bacterial activity and secondly to determine the degree of interaction, if any, between the iron and sulfur oxidation pathways of *T. ferrooxidans*. The bacteria was cultured on ferrous iron or sulfur (once or several times), collected according to standardized techniques (see materials and methods) and tested for iron, sulfite and sulfur oxidation activities.

Ferrous iron oxidation was found to be an inducible trait. Only those cells grown in the presence of high iron concentrations retained the ability to oxidize ferrous iron. Cells previously grown on iron and subcultured only once on sulfur lost their iron oxidizing activity. These results are in contrast to the original observation that SM-4 and Tf-2 retained the iron oxidizing activity in the sulfur medium with 0.2 g $Fe_2(SO_4)_3$ (Lizama and Suzuki, 1988). The stock cultures may have changed.

Sulfur oxidation was found to be constitutive, present in both iron and sulfur grown cells. The rate of sulfur oxidation, however, was directly proportional to the length of exposure to substrate sulfur. These results are in accordance with substrate mediated protein expression (Onhumra et al., 1996). Cells grown in the presence of sulfur would naturally contain higher concentrations of sulfur oxidizing enzymes than their iron grown counterparts. Maximum sulfur oxidation occurred at near neutral pH for both cell types.

Sulfite oxidation in iron and sulfur grown cells varied with respect to rate, pH and buffer concentration. Iron grown cells oxidized sulfite significantly faster than their sulfur grown counterparts reaching 932 nmol O_2 / min / mg protein verses 100 - 300 nmol O_2 / min / mg protein. Maximum sulfite oxidation in iron grown cells was observed in 0.01 M

β-alanine pH 3. Increased pH and or buffer concentration substantially decreased sulfite oxidation. Sulfur grown cells (cultured once or several times on sulfur) on the other hand, showed maximum sulfite oxidation at pH 4 - 7. Acidic pH (although more pronounced in the cells continuously cultured on sulfur) inhibited sulfite oxidation similar to *T. thiooxidans* (Takeuchi and Suzuki, 1993). Increased buffer concentration, unlike with the iron grown cells, did not inhibit sulfite oxidation in the sulfur grown cells. Higher buffer concentrations actually stimulated sulfite oxidation in cells continuously subcultured on sulfur. These oxidation profiles clearly suggest a difference in the process of sulfite oxidation between the two cell types - iron grown cells requiring low levels of dissolved salts and low pH; sulfur grown cells requiring higher pH and salt concentrations for maximum activity.

Finally the potential interaction between iron and sulfur oxidation was investigated using a variety of chemical inhibitors. The metal chelators EDTA, Tiron, orthophenanthroline and 2,2'-dipyridyl had little to no effect on sulfur oxidation (in either cell type), suggesting that sulfur oxidation (under the conditions tested) did not require iron in either its oxidized or reduced forms. The potential use of free radicals was also eliminated as a requirement for sulfur oxidation in the absence of marked inhibition using mannitol, superoxide dismutase and catalase. At this point the mechanism of sulfur oxidation in the iron grown cells appeared similar to that of the sulfur grown cells. Further investigation, however, showed some difference in the response of these cells to azide and HQNO.

Azide differentially affected the two cell types. Hydrazoic acid with a pK value of 4.7 was expected to freely enter the cells only at pH 3. At pH 6 the dissociated form of the acid predominates thus more azide would be required to produce a similar degree of inhibition. The expected results, however, were seen only with the sulfur grown cells which required nine times as much azide to inhibit sulfur oxidation at pH 6 as compared to pH 3. The iron grown cells were equally inhibited at both acidic and near neutral pH. Cellular makeup has been shown to vary with respect to growth substrate. Thus it is possible that this observed variation was due to a difference in the permeability of iron and sulfur grown cells to the inhibitor azide.

Further variation between the sulfur and iron grown cells was observed with HQNO, a specific inhibitor of the bc_1 complex. HQNO does not directly inhibit sulfur oxidation. It blocks the oxidation of sulfite to sulfate, causing sulfite accumulation. The sulfite enters the cell, acidifying the cytoplasm and indirectly inhibiting sulfur oxidation. HQNO strongly inhibited the sulfur grown cells only at pH 3. At pH 6 a maximum of 33 % inhibition was observed at 0.3 μM to 322 μM, suggesting sulfur oxidation was stopped at sulfite, but the sulfite could not enter the cell. These results are expected for sulfite has a pK_2 value of 7.2 (i.e. the dissociated form cannot enter the cell). The iron grown cells, on the other hand, were equally inhibited at both pH 3 and 6, suggesting a difference in the permeability between the two cell types rather than difference in the actual mechanism of sulfur oxidation.

Based on the above information it was concluded that sulfur oxidation in both the iron and sulfur grown cells proceeded via a sulfur oxidizing system which used molecular

oxygen as a terminal electron acceptor. The exact response of the two cell types to the individual inhibitors, however, could be affected by the difference in cell membrane properties of the two cell types.

Looking at the inhibition data (Tables 1 and 2) regarding iron grown *T. ferrooxidans* it was observed that both azide and cyanide inhibited iron oxidation more strongly than they inhibited sulfur oxidation at pH 3. The tests were repeated using growing cell cultures in a Micro-oxymax respirometer which measured bacterial growth and activity over time. Although slightly diminished the overall pattern remained - i.e. preferential inhibition of iron oxidation at low azide or cyanide concentrations.

The mechanism of sulfite oxidation was found to be influenced by the bacterial growth substrate. Cells grown on sulfur (once or several times) oxidized sulfite in the presence of both metal chelators and free radical scavenger, mannitol. Thus neither iron nor free radicals are involved in this sulfite oxidation reaction. Sulfite oxidation, however, was inhibited by azide and cyanide, specific inhibitors of cytochrome oxidase and HQNO, an inhibitor of the bc_1 complex. Thus sulfite oxidation in sulfur grown cells occurs via a sulfite oxidase using molecular oxygen and not ferric iron as a terminal electron acceptor.

Sulfite oxidation in the iron grown cells was difficult to study due to the presence of contaminating iron surrounding the individual cells and in the bacterial suspension. The soluble and precipitated ferric iron was removed through successive centrifugation steps. The iron surrounding the cells, however, was tightly bound. In order to eliminate the surrounding iron as the sole means of 'biological' sulfite oxidation a chemical control was developed using 7.5 nmol Fe^{3+}. Orthophenanthroline (an iron chelator) inhibited the

chemical and biological systems to a similar degree. EDTA and Tiron (Fe^{3+} chelators) preferentially inhibited chemical sulfite oxidation. The ferrous iron chelator, 2,2'-dipyridyl, however, preferentially inhibited biological sulfite oxidation. Thus 'biological' sulfite oxidation involved the use of both Fe^{2+} and Fe^{3+} (likely in the form of the Fe^{2+} / Fe^{3+} lattice).

Chemical sulfite oxidation was known to involve the use of free radicals. Mannitol inhibited chemical sulfite oxidation by 50 % at 1.6 mM but it also inhibited biological sulfite oxidation in iron grown cells by 50 % at 4.8 mM. Thus biological sulfite oxidation also involved the use of free radicals. Azide and cyanide, specific inhibitors of cytochrome oxidase inhibited biological sulfite oxidation with no effect on the chemical system. Thus sulfite oxidation in the iron grown cells was not just a metal catalyzed free radical chain reaction for it involved the use of cytochrome oxidase.

Based on the above information it was concluded that the mechanism of sulfite oxidation in iron grown *T. ferrooxidans* was a mixture of chemical and biological reactions. The following hypothesis is based on the possible formation of a sulfur trioxide free radical as an intermediate (Mottley et al., 1982). Sulfite is chemically oxidized on the cell surface to the sulfur trioxide radical by the ferric iron surrounding the cell:

$$SO_3^{2-} + Fe^{3+} \rightarrow {}^{\bullet}SO_3^{-} + Fe^{2+} \qquad [1]$$

The sulfur trioxide radical further reacts with oxygen to form the peroxyl free radical:

$$\cdot SO_3^{-} + O_2 \rightarrow {}^{\cdot}O_3SOO^{\bullet} \qquad [2]$$

The peroxyl free radical then reacts with surrounding sulfite forming peroxylsulfite and regenerating the sulfur trioxide radical:

$$^{\cdot}O_3SOO^{\bullet} + SO_3^{2-} \rightarrow O_3SOO^{2-} + {}^{\bullet}SO_3^{-} \quad [3]$$

The peroxylsulfite finally reacts with the remaining sulfite forming sulfate:

$$O_3SOO^{2-} + SO_3^{2-} \rightarrow 2SO_4^{2-} \quad [4].$$

The sole purpose of the bacteria in this scheme appeared to be in the regeneration of ferric iron for step 1. Thus sulfite oxidation in iron grown cells was directly connected to the iron oxidation pathway. The relationship of this system and the sulfite:ferric ion oxidoreductase system of Sugio (Sugio et al. 1987) remains unresolved, since the latter reaction is favored at pH 6, while the former system is optimal at pH 3.

PART II

EFFECT OF ANIONS ON IRON AND SULFUR OXIDATION

INTRODUCTION

Thiobacillus ferrooxidans is a Gram negative acidophilic chemolithoautotroph, using CO_2 as a carbon source and obtaining its energy for growth from the oxidation of ferrous iron, sulfur and reduced sulfur compounds (Razzel and Trussell, 1963). *T. ferrooxidans* was initially isolated from acidic copper-leaching waters and believed to be the dominant bacterium responsible for metal sulfide solubilization (Leduc and Ferroni, 1994)). Iron oxidation has been extensively studied in response to pH (Amaro et al., 1991; Sand, 1989), organic acids (Tuttle and Dugan, 1976), anions (Beck and Shafia, 1964; Lazaroff, 1977) and cations (Magnin et al., 1998; Torma, 1977). Sulfur oxidation has received substantially less attention with limited references to certain anions (Razzel and Trussell, 1963).

Thiobacilli have considerable economic importance in the treatment of acid mine drainage (11,23) and desulfurization of waste gases (SO_2, H_2S) (Imaizumi, 1986; Jensen and Webb, 1995; Satoh et al., 1988). The use of bacteria in the mining industry is a growing field of interest (Brierley, 1978; Rawlings and Silver, 1995). Tolerance levels of key metals by *T. ferrooxidans* growth on Fe^{2+} are given as follows: Cd^{2+} 0.75 M, Ni^{2+} 1 M, Zn^{2+} 1 M, Cu^{2+} 0.6 M, Co^{2+} 0.15 M, Cr^{3+} 0.075 M, Pb^{2+} 1 mM, Hg^{+} 0.1 mM, Hg^{2+} 10 mM, Ag^{+} 1 mM (Magnin et al., 1998). The normal counter-ion, sulfate is not inhibitory at 0.14 M (Razzel and Trussell, 1963; Tuovinen and Kelly, 1972) and the concentration may reach as high as 1.25 M during bacterial leaching of sulfide minerals (Espejo and Romero, 1997).

Metal extraction from mineral ore by *T. ferrooxidans* is achieved through two reactions: the oxidation of ferrous to ferric iron ($2Fe^{2+} + \frac{1}{2}O_2 + 2H^{+} \rightarrow 2Fe^{3+} + H_2O$) and

that of sulfide/sulfur to sulfuric acid ($H_2S + 2O_2 \rightarrow H_2SO_4$ or $S^0 + 1½O_2 + H_2O \rightarrow H_2SO_4$). Uranium solubilization from uraninite, for example, requires only iron oxidation ($UO_2 + 2Fe^{3+} \rightarrow 2Fe^{2+} + UO_2^{2+}$, $2Fe^{2+} + ½O_2 + H^+ \rightarrow 2Fe^{3+} + H_2O$), while zinc solubilization from sphalerite necessitates sulfide oxidation ($ZnS + 2O_2 \rightarrow ZnSO_4$). Metal extraction becomes complicated when ores contain mineral combinations (Le Roux and Mehta, 1978; Sakaguchi et al., 1976). In a pyrite-sphalerite mixture *T. ferrooxidans* will oxidize both sulfide ($ZnS + 2O_2 \rightarrow ZnSO_4$) and iron plus sulfide ($4FeS_2 + 15O_2 + 2H_2O \rightarrow 2Fe_2(SO_4)_3 + 2H_2SO_4$) creating difficulty in further zinc recovery from the leachate. Low concentrations of ferric sulfate are beneficial in their use in the indirect leaching of mineral ores. Higher concentrations, however, result in the production of jarosite, a ferric iron precipitate which can cover the ore surface thus preventing further leaching from occurring. Higher jarosite levels also produce an additional disposal problem.

We propose to show that the iron and sulfur oxidation activities of *T. ferrooxidans* can be differentially controlled through the use of specific anions. Under certain conditions iron oxidation can be blocked with little to no effect on sulfur oxidation and vice versa. Through this type of manipulation we hope to achieve specific metal extraction from an ore sample, with the absence or at least reduction of any contaminating metals.

MATERIALS AND METHODS

Media. *T. ferrooxidans* strain SM-4 (Lizama and Suzuki, 1988) was grown in modified 9K medium (M9K) : 0.4 g $(NH_4)_2SO_4$, 0.1 g K_2HPO_4, 0.4 g $MgSO_4 \cdot 7H_2O$ and 33.3 g $FeSO_4 \cdot 7H_2O$ per liter adjusted to pH 2.3 with H_2SO_4. Cells used for sulfur oxidation were grown in Starkey No. 1 medium (Starkey, 1925) after adaptation on sulfur (35): 0.3 g $(NH_4)_2SO_4$, 3.5 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.25 g $CaCl_2$ and 18 mg $FeSO_4 \cdot 7H_2O$ per liter and adjusted to pH 2.3 with H_2SO_4. Powdered sulfur (10 g/l BDH sulfur) was spread evenly over the surface after inoculation. *Thiobacillus thiooxidans* strain SM-6 grown on sulfur was used for most of the growth experiments on sulfur, since sulfur adapted *T. ferrooxidans* was not available, but some important results were later confirmed with sulfur adapted *T. ferrooxidans* strain SM-4.

Culture procedures. Iron grown cells were cultured in M9K using a 10 % inoculum. The flasks were incubated at 25°C and placed on a rotary shaker at 150 rpm for 48 hours. The culture was passed through a Whatman No. 1 filter paper to remove the majority of the precipitated ferric iron. The supernatant was centrifuged at 8,000 × *g* for 10 min. The cell pellet was resuspended in 0.1 M β-alanine-sulfate buffer (pH 2.3) and centrifuged at 1,000 × *g* for 5 min to allow for further ferric iron sedimentation. The supernatant was transferred to a second tube and centrifuged at 10,000 × *g* for 10 min. The cells were centrifuged a fourth time generating a final suspension of 50 mg wet cells per ml in the same buffer. Protein concentration was determined using bovine serum albumin as the standard (Suzuki et al., 1990).

Sulfur-grown cells were cultured in Starkey No. 1 medium using a 2.5 % inoculum. The stationary flasks were incubated at 28°C for four days. The cell collection procedure was identical to that of iron grown cells.

Determination of iron and sulfur oxidation using cell suspensions. The rates of iron and sulfur oxidation were measured using a Gilson oxygraph equipped with a Clark oxygen electrode at 25°C. The reaction vessel contained 10 µl of the cell suspension (sulfur or iron grown cells), 0.1 ml of sulfur suspension (32 g BDH S^0 in 100 ml 500 ppm Tween 80) (for sulfur oxidation) or 0.5 µmol $FeSO_4 \cdot 7H_2O$ (for iron oxidation) and varying concentrations of potassium salts of anions, sucrose or β-alanine buffer (all at pH 2.3 unless otherwise stated) to make a total volume of 1.2 ml. The sulfur oxidation by iron grown cells was the slowest and required 100 µl of the cell suspension for accurate rate determinations. It should be noted that all of the experiments of iron and sulfur oxidation were repeated with *T. ferrooxidans* ATCC 19859 with essentially similar results, except sulfur oxidation activities of either iron or sulfur grown cells were twice those of strain SM-4.

Determination of iron and sulfur oxidation and carbon dioxide fixation using growing cell cultures. The rates of iron and sulfur oxidation in growing cell cultures were measured using a Micro-oxymax respirometer (Columbus Instruments) at Cominco Research. The reaction vessel contained a 5% inoculum, 12 mmoles $FeSO_4 \cdot 7H_2O$ (for iron oxidation) or 1 g BDH sulfur sprinkled on the surface plus 18 mg/l $FeSO_4 \cdot 7H_2O$ (for sulfur oxidation), various concentrations of anionic salts or inhibitors and M9K medium at pH 2.3 making a total volume of 100 ml. The reaction was stirred with a magnetic stirrer and both the O_2 consumption (oxidation) and CO_2 consumption (autotrophic growth) were measured at 26°C.

RESULTS

Effect of Anions on Iron Oxidation

A 48 hour culture of iron grown *T. ferrooxidans* was initially used to determine the effect, if any, anions had on bacterial iron oxidation. The experiment was carried out over a period of 4 days using a single batch of cells, thus eliminating any minor variations which may have arisen through the use of multiple cell cultures. Iron oxidation in *T. ferrooxidans* is known to be stable for several weeks following cell collection. Thus the observed results should be a direct reflection of the anionic effect of ferrous iron oxidation.

The experiment was carried out at 25°C in a Gilson oxygraph which measured oxygen consumption over time. The reaction mixture contained (in order of addition): various assay buffers (pH 2.3), 0.5 mg wet cells and ferrous sulfate to a final volume of 1.2 ml. Ferrous sulfate concentrations were varied to study the effects of various anions on the iron oxidation activities at different concentrations of the substrate, ferrous iron. Sulfate is a naturally occurring anion present in the bacterial environment. It has been found in combination with iron as a lattice surrounding the cell envelope (Dugan and Lundgren, 1965). It has been shown to be involved in ferrous iron oxidation through depolarization of the positively charged active sites on the bacterial surface thus allowing the positively charged Fe^{2+} to approach (Schnaitman et al., 1969). It is the primary anion known to stabilize the hexaaquated substrate Fe^{2+} for *T. ferrooxidans* (Lazaroff. 1962). Most recently it has been shown to play a role in the transfer of electrons from the iron sulfur cluster to the copper(II) anion of rusticyanin in the oxygen dependent iron oxidation electron transport chain (Fry et al., 1986).

Due to the known sulfate requirement for iron oxidation in *T. ferrooxidans* potassium sulfate (K_2SO_4) was used as a control to show maximum ferrous iron oxidation at pH 3. Figure 1 shows iron oxidation to be activated at low, increasing potassium sulfate concentrations. Maximum iron oxidation was obtained with 0.05 M and 0.2 M sulfate, the former at low (below 1.7 mM) and the latter at high (above 1.7 mM) ferrous sulfate concentrations. Sulfate at 0.3 M and above slightly inhibited the oxidation at all of the iron concentrations tested. Replotting these data into a Lineweaver-Burk double-reciprocal plot (Appendix 1) showed also two distinct phases: the first between 0.42 mM - 1.7 mM and the second at 2.5 - 8.3 mM ferrous sulfate. In each case the Vmax increased with increasing sulfate concentrations up to 0.2-0.3 M then declined. Km values for ferrous iron increased with increasing K_2SO_4 concentrations up to 0.3 M sulfate (Table 1).

Table 1. Effect of potassium sulfate on iron oxidation.

Buffer		Range 0.42 - 1.7 mM		Range 2.5 - 8.3 mM	
Concentration (M)		Vmax	Km	Vmax	Km
K_2SO_4	0.01	1203	0.05	1623	0.17
	0.05	1850	0.24	1756	0.06
	0.1	1733	0.30	1895	0.34
	0.2	2135	0.48	1986	0.28
	0.3	2080	0.78	2105	0.82
	0.4	1776	0.78	1984	1.00
	0.5	1421	0.65	1525	0.63

Figure 2 shows the effect of β-alanine-sulfate buffer on bacterial iron oxidation. The cells were washed and suspended in 0.1 M β-alanine-sulfate at pH 2.3. Thus it was

Figure 1. Effect of increasing sulfate concentrations on iron oxidation. The reaction mixture contained 0.5 mg wet cells (SM-4) and Fe^{2+} in K_2SO_4 pH 2.3.

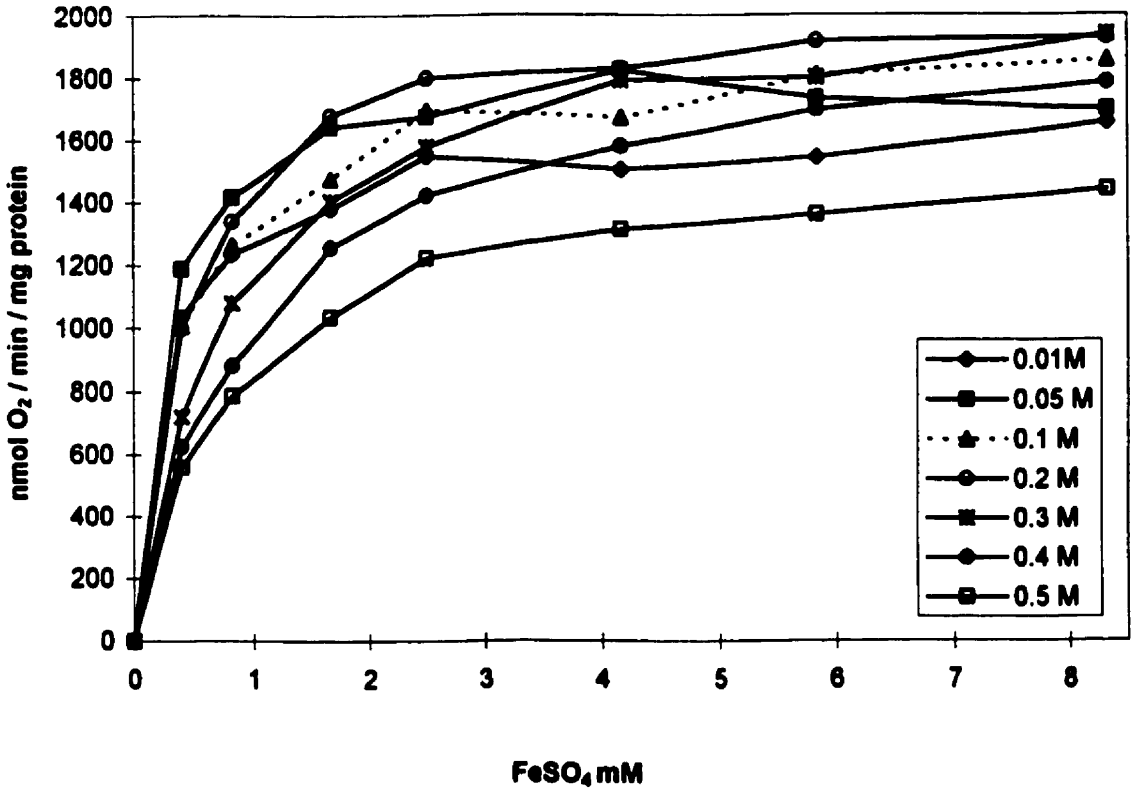
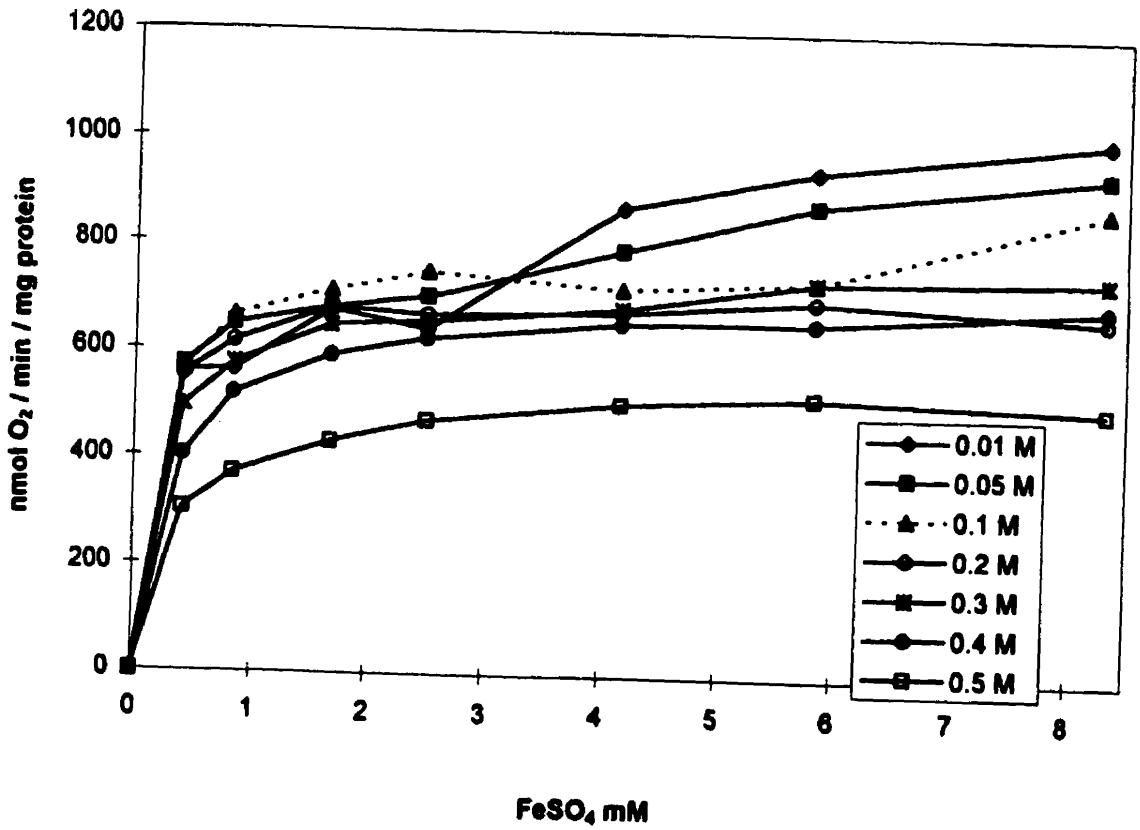

2000
1800
1600
1400
1200
1000
800
600
400
200
0
nmol O2 / min / mg protein
0
1
2
3
4
5
6
7
8
FeSO4 mM
0.01M
0.05 M
0.1 M
0.2 M
0.3 M
0.4 M
0.5 M

Figure 2. Effect of increasing β-alanine sulfate concentrations on iron oxidation. The reaction mixture contained 0.5 mg wet cells (SM-4) and Fe^{2+} in β-alanine sulfate pH 2.3.


1200
1000
800
600
400
200
0
nmol O2 / min / mg protein
0
1
2
3
4
5
6
7
8
FeSO4 mM
0.01 M
0.05 M
0.1 M
0.2 M
0.3 M
0.4 M
0.5 M

expected that β-alanine-sulfate would not inhibit iron oxidation. According to Fig. 2, however, β-alanine even at the lowest concentration used produced almost a 50 % drop in activity compared to that observed with 0.2 M potassium sulfate. No clear-cut activation by increasing β-alanine-sulfate concentrations was observed except that at low $FeSO_4$ (concentrations below 3 mM) β-alanine-sulfate (0.01-0.1 M) slightly activated the iron oxidation rate. In general the buffer inhibited the oxidation. According to Table 2, Vmax remained relatively unchanged between 0.42-1.7 mM ferrous sulfate, while the Km increased slightly with increasing buffer concentration. At higher ferrous sulfate concentrations (2.5-8.3 mM) increasing β-alanine-sulfate decreased both Vmax and Km, but the decrease occurred mainly below 0.1 M β-alanine.

Table 2. Effect of β-alanine buffer on iron oxidation.

Buffer		Range 0.42 - 1.7 mM		Range 2.5 - 8.3 mM	
Concentration (M)		Vmax	Km	Vmax	Km
β-alanine	0.01	679	0.10	1402	2.87
	0.05	731	0.11	1084	1.39
	0.1	787	0.16	836	0.40
	0.2	725	0.13	691	0.08
	0.3	713	0.18	791	0.53
	0.4	708	0.31	712	0.36
	0.5	496	0.27	600	0.33

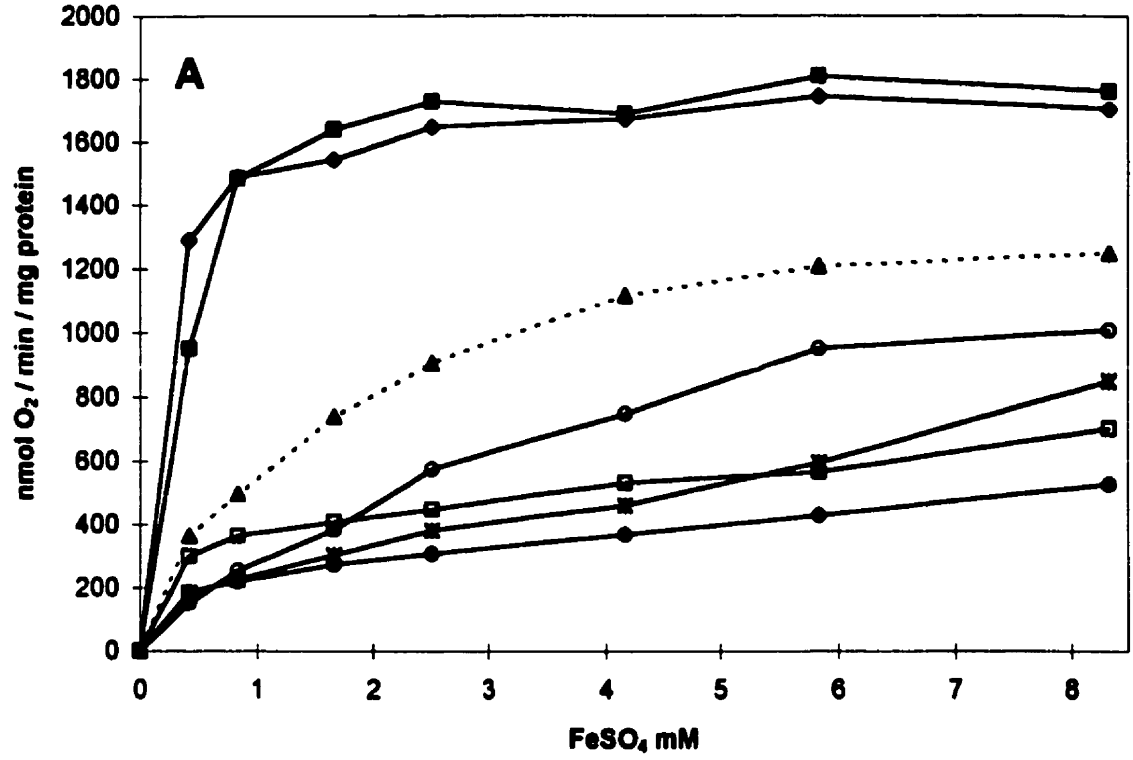
Phosphate is required for normal bacterial function (Beck and Shafia, 1964). Cells grown under phosphate limitation present a filamentous morphology due to lack of cell division (Seeger and Jerez, 1992 and 1993). Phosphate starvation studies show changes in

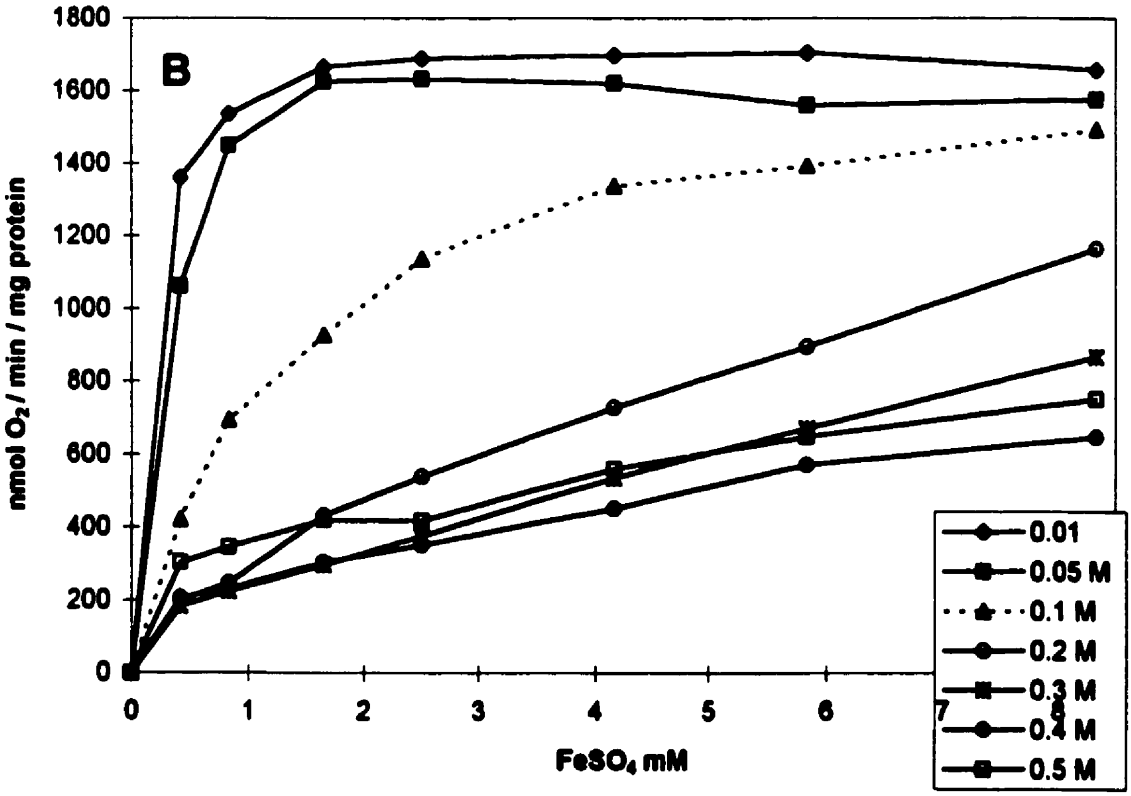
in the degree of synthesis of at least 25 proteins, some of which are exclusively synthesized under starvation conditions (Satoh et al., 1988; Seeger and Jerez, 1992 and 1993a). A number of these proteins have been linked to the bacterial surface suggesting the existence of a phosphate scavenging system in *T. ferrooxidans* (Jensen and Webb, 1995; Satoh et al., 1988). Phosphate concentrations used in this study were not limiting but rather in excess. Iron oxidation activity was high at low (0.01-0.05M) phosphate buffer with rates approaching 1800 nmol O_2 / min / mg protein (Fig. 3A). Increased phosphate concentrations, however, were much more inhibitory than sulfate resulting in decreased iron oxidation rate. Phosphate at 0.5 M, however, stimulated the iron oxidation rate over that in 0.2-0.4 M phosphate at low substrate concentrations (up to 1.7 mM $FeSO_4$). A possible explanation for this may lie in the ability of phosphates to chelate metals. Phosphate may be removing the ferric iron from the system as ferric phosphate, thus removing end-product inhibition and driving the reaction forward. According to Table 3 increased phosphate concentrations produced a decrease in Vmax values. Km values, however, increased up to 0.2 M (for the $FeSO_4$ range of 0.83 - 2.5 mM) and 0.3 M

Table 3. Effect of potassium phosphate on iron oxidation. Cells are incubated with the phosphate for 5 minutes prior to the addition of substrate ferrous iron.

		Range		Range	
Buffer		0.83 - 2.5 mM		2.5 -8.3 mM	
Concentration (M)		Vmax	Km	Vmax	Km
K-Pi	0.01	1697	0.12	1757	0.17
	0.05	1867	0.22	1792	0.12
	0.1	1522	1.73	1540	1.70
	0.2	1203	3.16	1577	4.37
	0.3	541	1.18	1295	6.33
	0.4	378	0.60	677	3.12
	0.5	493	0.30	827	2.19

Figure 3. Effect of increasing phosphate concentrations on iron oxidation. The reaction mixture contained 0.5 mg wet cells (SM-4) and Fe^{2+} in potassium phosphate pH 2.3. **A**, cells were pre-incubated in the phosphate buffer for 5 minutes prior to the addition of substrate ferrous iron. **B**, no pre-incubation of cells.


A
nmol O2 / min / mg protein
FeSO4 mM
0
200
400
600
800
1000
1200
1400
1600
1800
2000
0
1
2
3
4
5
6
7
8


B
nmol O2 / min / mg protein
FeSO4 mM
0
200
400
600
800
1000
1200
1400
1600
1800
0
1
2
3
4
5
6
7
8
0.01
0.05 M
0.1 M
0.2 M
0.3 M
0.4 M
0.5 M

(for the $FeSO_4$ range of 2.5-8.3 mM) phosphate. A further rise in phosphate concentrations produced a decrease in Km. As previously suggested this increased affinity may be due to ferric phosphate formation thus relieving feed-back inhibition.

Phosphate was unique among the anions tested thus far, in that prolonged incubation of the bacteria with the phosphate buffer increased the observed degree of inhibition. In order to test the effect of the phosphate anion on ferrous iron oxidation in the absence of bacterial incubation the experiment was repeated with the cells starting the reaction (i.e. order of addition changed to: buffer, ferrous sulfate, cells). The results as seen in Fig. 3B showed a general decrease in the inhibition of ferrous iron oxidation at all concentrations tested, but particularly at 0.1 M phosphate. The overall patterns, however, remained the same. Increased phosphate concentrations inhibited bacterial activity yet, 0.5 M phosphate at low substrate concentrations (i.e. up to 1.7 mM added ferrous iron) stimulated iron oxidation. Table 4 shows a rise in Km and a relatively stable

Table 4. Effect of phosphate on iron oxidation. Cells start the reaction (i.e. no preincubation).

Buffer		Range 0.83 - 2.5 mM		Range 2.5 -8.3 mM	
Concentration (M)		Vmax	Km	Vmax	Km
K-Pi	0.01	1788	0.14	1671	<0.1
	0.05	1774	0.18	1543	<0.1
	0.1	1568	1.06	1715	1.26
	0.2	1406	3.88	2019	6.98
	0.3	520	1.12	1805	9.59
	0.4	464	0.84	1008	4.74
	0.5	485	0.33	1133	4.28

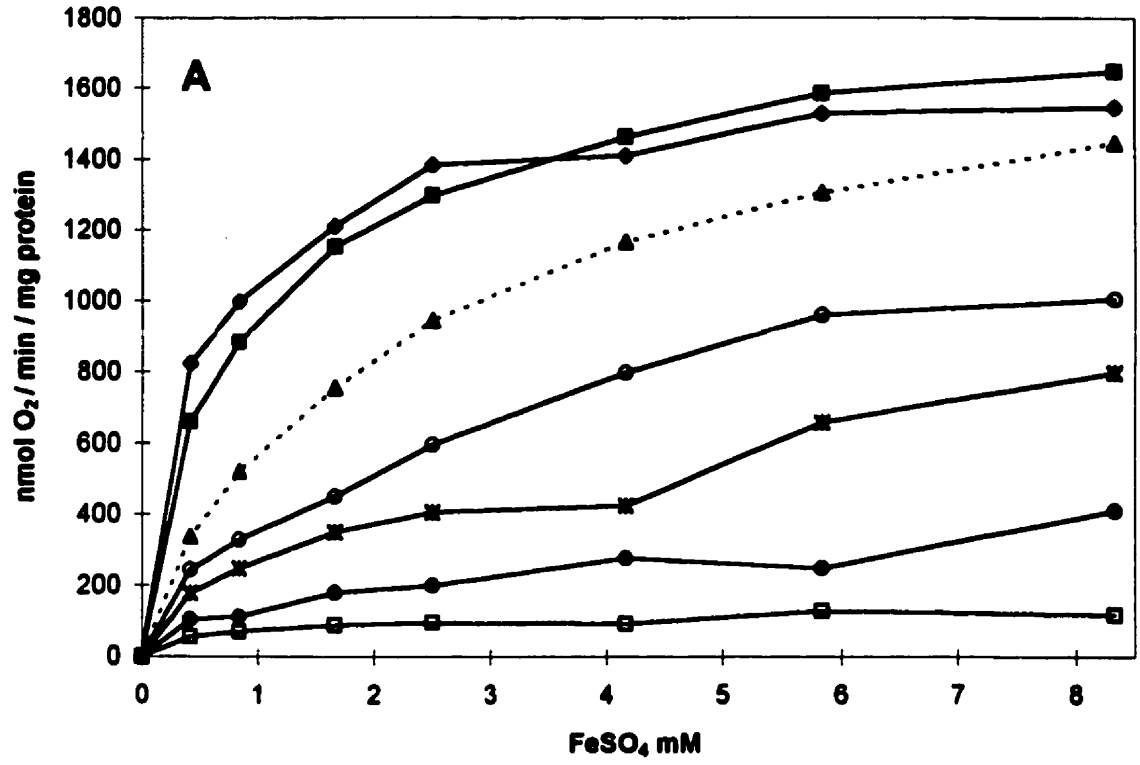
Vmax up to 0.2 M phosphate (for the 0.83-2.5 mM $FeSO_4$ range) and 0.3 M phosphate (for the 2.5-8.3 mM $FeSO_4$ range), a characteristic of competitive inhibition. Beyond these points the Km values drop suggesting ferric phosphate formation and the removal of feed-back inhibition.

Chloride is a known inhibitor of cell growth and iron oxidation (Lazaroff, 1962; Rawlings and Silver, 1995). It is an inhibitor of the cell free iron cytochrome *c* reductase (Corbett and Ingledew, 1987). It can also enter the cell resulting in acidification of the cytoplasm and reduced enzymatic activity. A concentration of 0.14 M was reported as being toxic to the bacteria on its initial description in 1963 (Razzel and Trussel, 1963). Figure 4A shows increased chloride concentrations produced increased inhibition of iron oxidation. At KCl concentrations between 0.01 and 0.1 M Vmax remained constant and Km increased indicating competitive inhibition (Table 5). At higher concentrations of chloride Vmax dropped, suggesting noncompetitive type inhibition.

Table 5. Effect of potassium chloride on iron oxidation. Cells are incubated with the chloride for 5 minutes prior to the addition of substrate ferrous iron.

Buffer		Range 0.83 - 2.5 mM		Range 2.5 - 8.3 mM	
Concentration (M)		**Vmax**	**Km**	**Vmax**	**Km**
KCl	0.01	1659	0.56	1620	0.46
	0.05	1682	0.75	1869	1.11
	0.1	1523	1.62	1847	2.41
	0.2	896	1.47	1532	3.89
	0.3	595	1.17	1124	4.88
	0.4	361	1.83	500	3.80
	0.5	118	0.57	133	1.09

Figure 4. Effect of increasing chloride concentrations on iron oxidation. The reaction mixture contained 0.5 mg wet cells (SM-4) and Fe^{2+} in potassium chloride pH 2.3. **A**, cells were pre-incubated in the KCl for 5 minutes prior to the addition of substrate ferrous iron. **B**, no pre-incubation of cells.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nmol O_2 / min / mg protein
1800
1600
1400
1200
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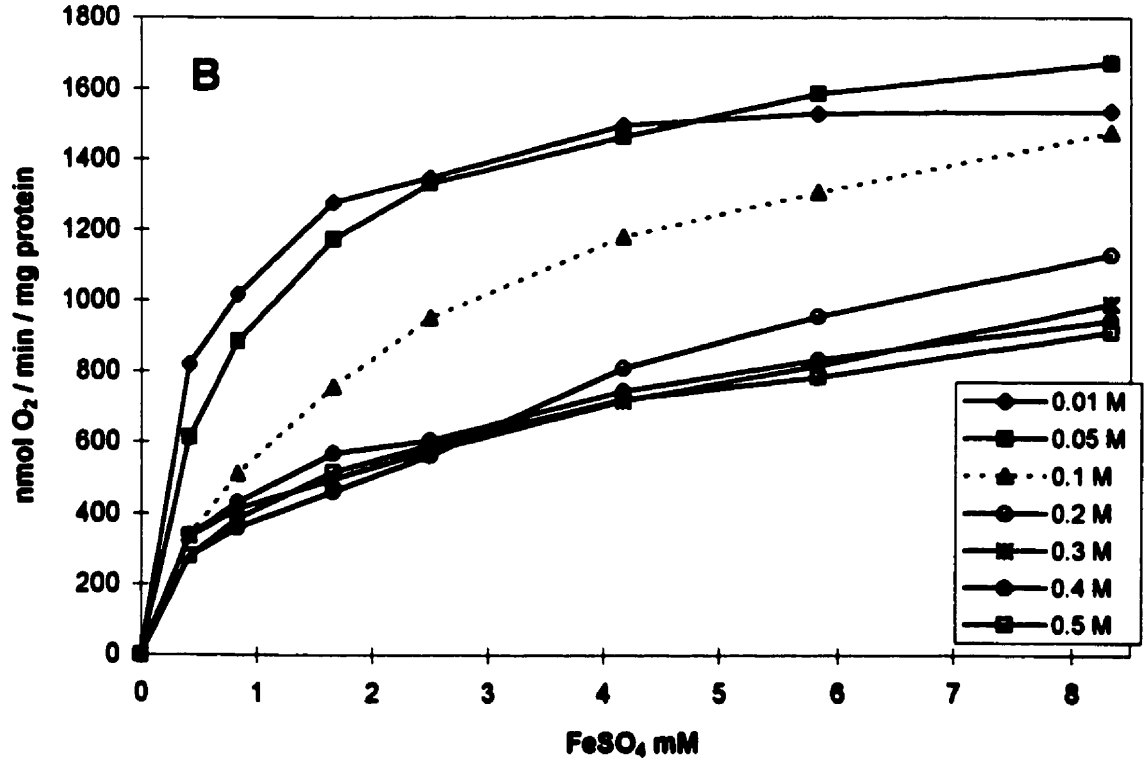
800
600
400
200
0
0
1
2
3
4
5
6
7
8
$FeSO_4$ mM


B
nmol O_2 / min / mg protein
1800
1600
1400
1200
1000
800
600
400
200
0
0
1
2
3
4
5
6
7
8
$FeSO_4$ mM
0.01 M
0.05 M
0.1 M
0.2 M
0.3 M
0.4 M
0.5 M

During the course of this experiment it was noted that prolonged exposure of the bacteria to the chloride buffer resulted in decreased activity on addition of substrate, similar to the situation with phosphate. In order to observe the effect of chloride on iron oxidation in the absence of this incubation the experiment was repeated (as previously done with phosphate) with the cells starting the reaction (i.e. order of addition changed to: buffer, $FeSO_4$, cells). Figure 4B shows only a minor difference from Fig. 4A in the overall activity with 0.01-0.2 M KCl. Higher chloride concentrations, however, showed iron oxidizing activities higher than those observed with the incubated cells (Fig. 4A). In fact the overall oxidation rate between 0.3-0.5 M KCl appeared relatively the same and similar to that in 0.2 M KCl. Table 6 shows an increasing Km and a relatively stable Vmax between 0.01 and 0.1 M KCl (for the $FeSO_4$ range of 0.83-2.5 mM) and 0.2 M (for the $FeSO_4$ range of 2.5-8.3) KCl. Further increases in KCl concentrations produced a drop in Vmax and Km values to constant and similar values, indicating a limiting partial inhibition by KCl.

Table 6. Effect of potassium chloride on iron oxidation. Cells start the reaction (i.e. no preincubation).

Buffer		Range 0.83 - 2.5 mM		Range 2.5 - 8.3 mM	
Concentration (M)		Vmax	Km	Vmax	Km
KCl	0.01	1644	0.51	1667	0.57
	0.05	1770	0.84	1853	1.00
	0.1	1593	1.77	1883	2.46
	0.2	733	0.88	2022	6.45
	0.3	687	0.56	1316	3.28
	0.4	780	0.67	1206	2.52
	0.5	798	0.89	1123	2.27

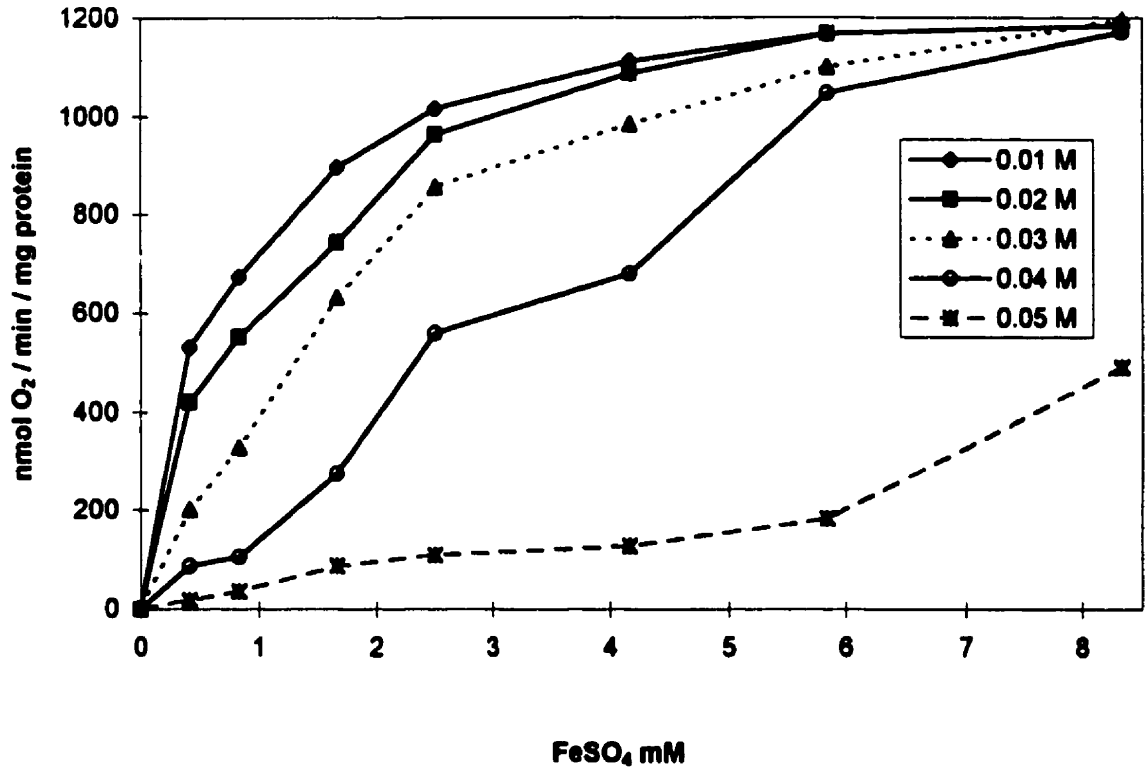
Nitrate is an inorganic anion known to inhibit the oxidation and growth of *T. ferrooxidans* on ferrous iron (Lazaroff, 1962). It transverses the membrane barriers, entering the cells interior, resulting in gradual acidification of the cytoplasm. Nitrate at a concentration of 0.01 M inhibited 33% of the iron oxidizing activity in *T. ferrooxidans* strain SM4. Prolonged exposure to this anion produced complete inhibition (data not shown), thus the observed results are in the absence of bacterial incubation. Figure 5 shows increased inhibition at increased nitrate concentrations, with 0.04 M and 0.05 M inhibition patterns appearing sigmoidal. Table 7 shows that Vmax values were relatively unchanged, but Km values increased when the KNO_3 concentration increased, a characteristic of competitive inhibition.

Table 7. Effect of potassium nitrate on iron oxidation.

Buffer		Range 0.83 - 2.5 mM		Range 2.5 - 8.3 mM	
Concentration (M)		Vmax	Km	Vmax	Km
KNO_3	0.01	1354	0.84	1289	0.67
	0.02	1389	1.28	1339	0.96
	0.03	5170	12.26	1411	1.66
	0.04			2175	7.53
	0.05			1255	28.55

All of the anions tested thus far were potassium salts. Sulfur oxidation using *Thiobacillus thiooxidans* has shown potassium salts to be less inhibitory than their sodium or lithium counterparts (Suzuki et al., 1999). In order to differentiate the anionic from the cationic effects on iron oxidation the experiment was repeated using H_2SO_4, HCl and H_3PO_4. Nitric acid was not tested due to the strong degree of inhibition seen with its

Figure 5. Effect of increasing nitrate concentrations on iron oxidation. The reaction mixture contained 0.5 mg wet cells (SM-4) and Fe^{2+} in potassium nitrate pH 2.3.

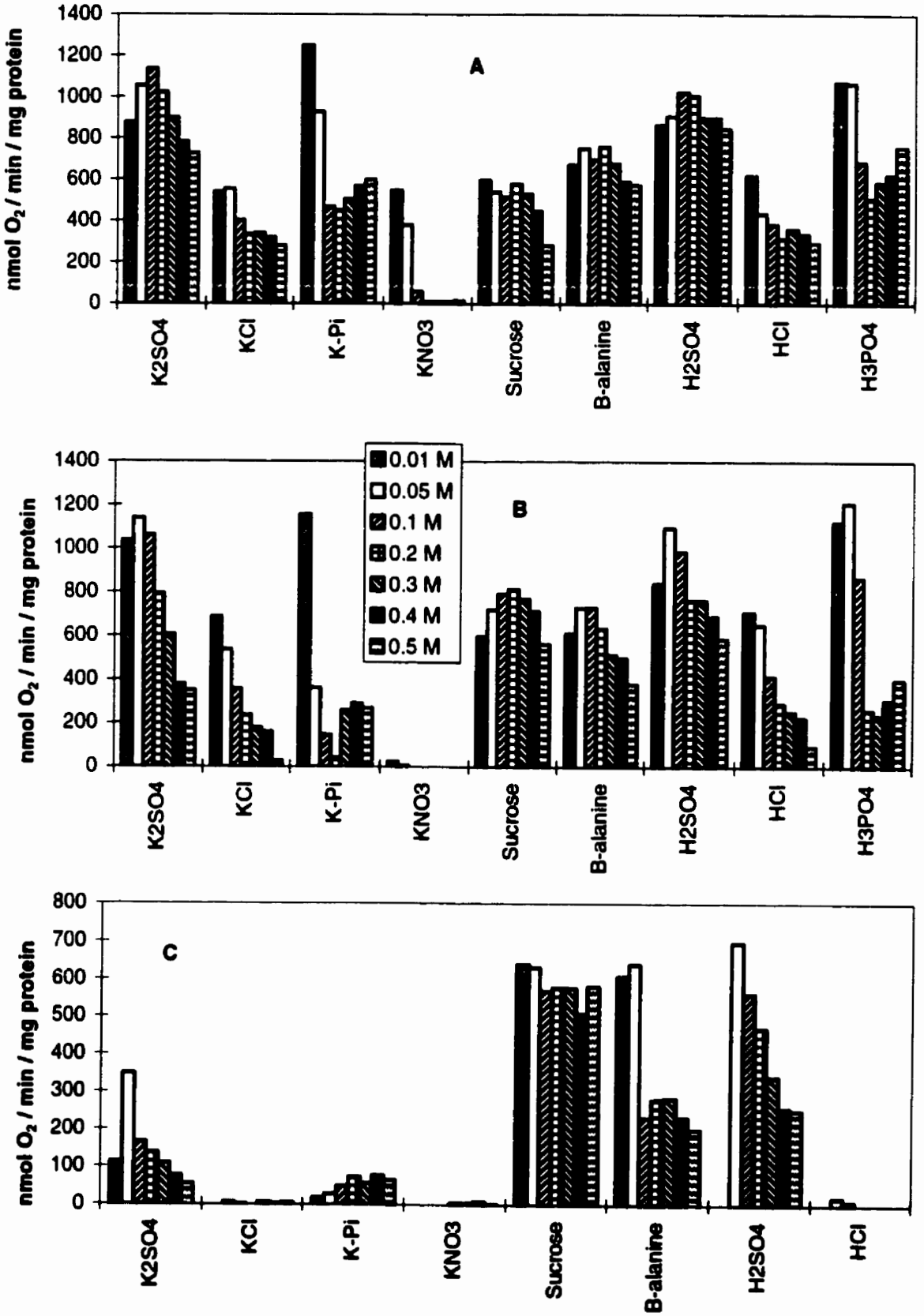

1200
1000
800
600
400
200
0
nmol O_2 / min / mg protein
0
1
2
3
4
5
6
7
8
$FeSO_4$ mM
0.01 M
0.02 M
0.03 M
0.04 M
0.05 M

potassium salt. The potassium ion, however, could not be totally eliminated from the acid assay, because KOH was used to raise the pH of the acid buffers to pH 1.8, 2.3 and 3. The concentration of the cation, however, was generally less than that of the anion which is reflected in the displayed concentration gradient. Thus the observed results using the acid buffers are likely due to the anion and not the cation.

Figure 6 A shows the iron oxidation rates using 0.5 mg wet cells and 0.42 mM $FeSO_4$ at pH 3. Iron oxidation in the potassium salts appeared to be on average 10 % higher than that found in their acid counterparts. The pattern of inhibition, however, remained relatively the same. Iron oxidation gradually increased in the sulfate solution (potassium salts and acid) up to 0.1 M followed by a gradual decline in activity. Chloride (KCl and HCl) was inhibitory (compared to sulfate) even at the lowest concentration used, 0.01 M, while increased chloride concentrations resulted in a marginal added decrease in specific activity. Phosphate (K-Pi and H_3PO_4) at 0.01 M allowed for maximum bacterial oxidation. Increased phosphate concentration produced a decrease in iron oxidation up to 0.2 M followed by a moderate return of activity at higher phosphate concentrations.

The above experiments have been run at pH 3, the sole pH used for the comparison of iron, sulfur and sulfite oxidation. Although pH 3 is an acidic pH, comparable to growth pH the potential effect of anions was also tested at pH 2.3 and 1.8 (Fig. 6 B and C). Figure 6 B shows the general pattern of inhibition caused by increased anion concentrations at pH 2.3 to be similar to that at pH 3 although with a greater degree of inhibition. Iron oxidation, however, with 0.01-0.05 M sulfate and chloride (both potassium salts and acids) showed slight stimulation over that observed at pH 3.

Figure 6. Effect of anions on iron oxidation using iron grown *T. ferrooxidans* strain SM-4. The reaction mixture contained 0.5 mg wet cells (suspended in 0.1 M β-alanine pH 2.3), 0.5 μmol $FeSO_4$ and various assay buffers to make a final volume of 1.2 ml. **A**, assay pH 3; **B**, assay pH 2.3; **C**, assay pH 1.8.


A
nmol O2 / min / mg protein
1400
1200
1000
800
600
400
200
0
K2SO4
KCl
K-Pi
KNO3
Sucrose
B-alanine
H2SO4
HCl
H3PO4
B
0.01 M
0.05 M
0.1 M
0.2 M
0.3 M
0.4 M
0.5 M
C
800
700
600
500
400
300
200
100

A further decrease in pH to 1.8 (pH used in column data), produced a significant amount of inhibition in all of the potassium salts tested. KCl, HCl and KNO_3 all essentially inhibited iron oxidation even at the lowest concentration used. Potassium sulfate produced a peak of activity at 0.05 M. This peak, however, was only half of that observed using 0.05 M sulfuric acid, suggesting an inhibitory effect of potassium on iron oxidation under extremely acidic conditions.

The sucrose and β-alanine buffers proved unique among the solutions tested having little effect on iron oxidation in the pH range of 1.8-3. Iron oxidation was actually stimulated at all sucrose concentrations at pH 2.3 (compared to pH 3), possibly due to the added sulfate content. The pH's of both β-alanine and sucrose were adjusted with concentrated sulfuric acid. A further drop in pH to 1.8 had little effect on iron oxidation in the sucrose solutions. The β-alanine buffers, on the other hand, showed this kind of similarity only between pH 2.3 and 3. The final drop in pH to 1.8 showed significant inhibition at 0.1-0.5 M β-alanine. The inhibition observed at higher β-alanine concentrations was likely due to added sulfate. β-alanine unlike sucrose is a strong buffer with pK values of 3.6 and 10.19. Thus it would take substantially more sulfuric acid to adjust it to this lower pH and high sulfate (H_2SO_4) concentrations are inhibitory at pH 1.8.

Effect of Anions on Sulfur Oxidation

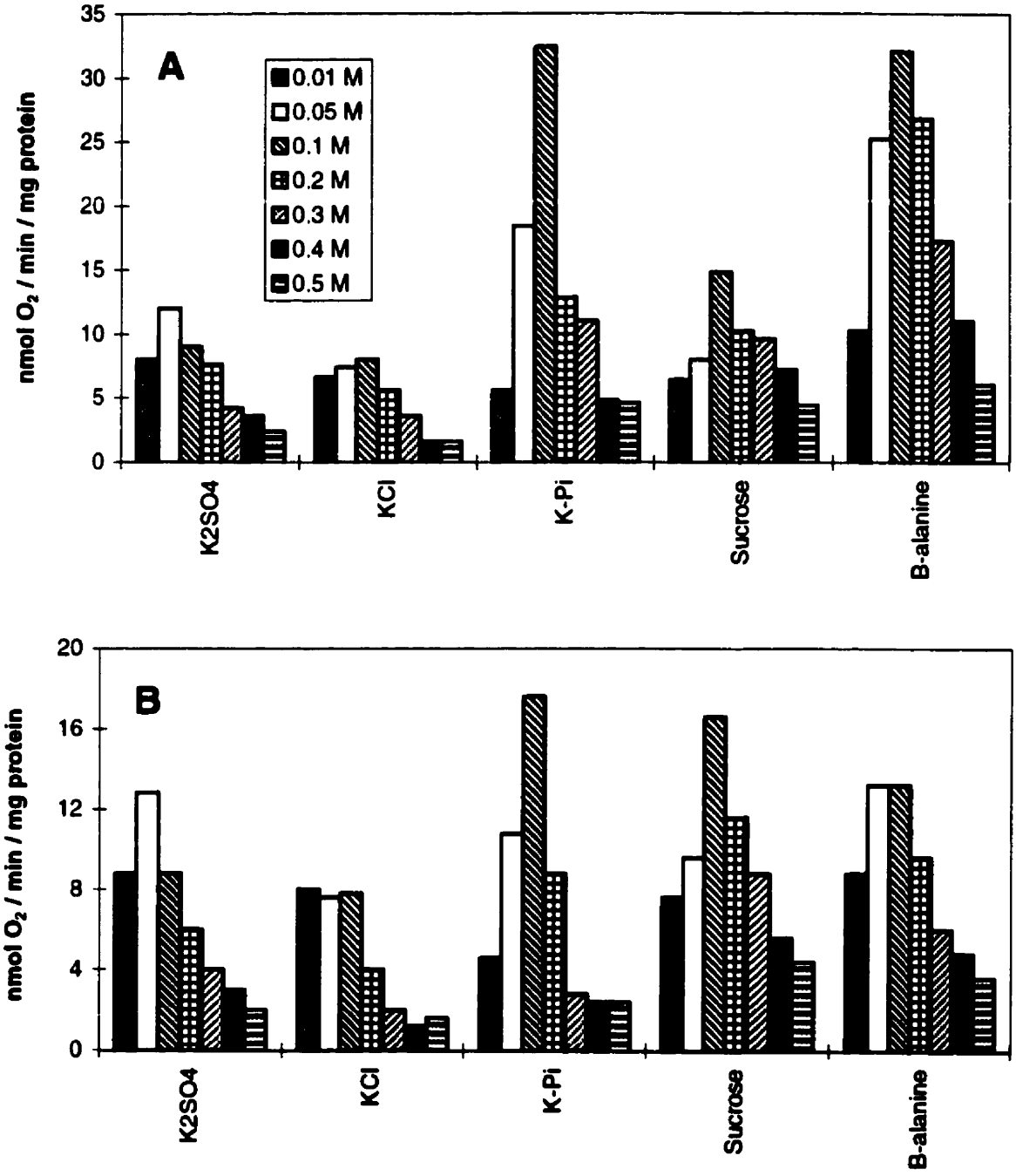
Previous chapters have noted a difference in the mechanism of sulfur oxidation in the two cell types: iron and sulfur grown *T. ferrooxidans*. Further differences involve the sulfur oxidation pH profiles, rates of activity and their susceptibility to various inhibitors. Thus both iron and sulfur grown *T. ferrooxidans* were tested for a potential anionic effect

on sulfur oxidation. The reaction was carried out both at pH 3 (comparable to growth pH) and pH 6 (optimum pH). As previously observed the first notable difference between the two cell types was a variation in the overall rate of sulfur oxidation. The sulfur grown cells oxidized sulfur nearly ten times as fast as the iron grown cells. This difference likely being due to substrate mediated protein expression (i.e. the sulfur grown cells would have higher amounts of the sulfur oxidizing enzymes than would their iron grown counterparts).

Iron grown cells. Sulfur oxidation using iron grown *T. ferrooxidans* showed a similar pattern of activity at both pH 3 and 6 (Fig. 7). Sulfur oxidation in potassium sulfate and sucrose gradually increased up to 0.05 M and 0.1 M respectively. Higher sulfate concentrations inhibited bacterial oxidation. Potassium chloride had little effect on sulfur oxidation up to 0.1 M. Beyond this point, however, KCl proved inhibitory to bacterial oxidation. Potassium phosphate and β-alanine were unique among the assay solutions tested, showing very high sulfur oxidation at 0.1 M at pH 6. Higher concentrations inhibited the activity as other buffers. The pattern of bacterial sulfur oxidation was similar at acidic and near neutral pH.

Potassium phosphate and β-alanine are both excellent buffers. Potassium phosphate has pK values of 2.12, 7.21 and 12.67, while β-alanine has pK values of 3.6 and 10.19. It is possible that the increased sulfur oxidizing activity seen at pH 6 is due to the buffering capacity of K-Pi and β-alanine buffers. Iron grown *T. ferrooxidans* was washed and suspended in 0.1 M β-alanine pH 2.3, a very stable buffer. The reaction mixture contained 100 µl of the cell suspension (50 mg/ml) in a total volume of 1.2 ml. It was previously noted that sulfur oxidation in the iron grown cells was optimal at near neutral

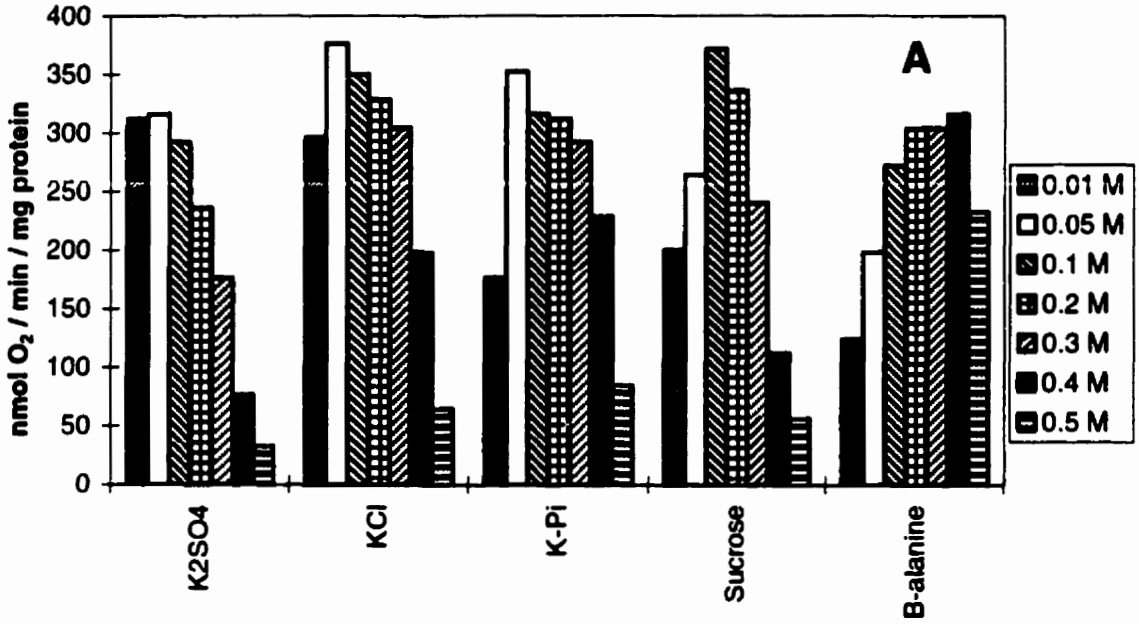
Figure 7. Effect of increasing buffer concentrations on sulfur oxidation using iron grown *T. ferrooxidans*. **A,** assay pH 6; **B,** assay pH 3. The reaction mixture contained 5 mg wet cells (suspended in 0.1 M β-alanine pH 2.3), 0.1 ml S (Tween 80) and various buffers to a total volume of 1.2 ml.

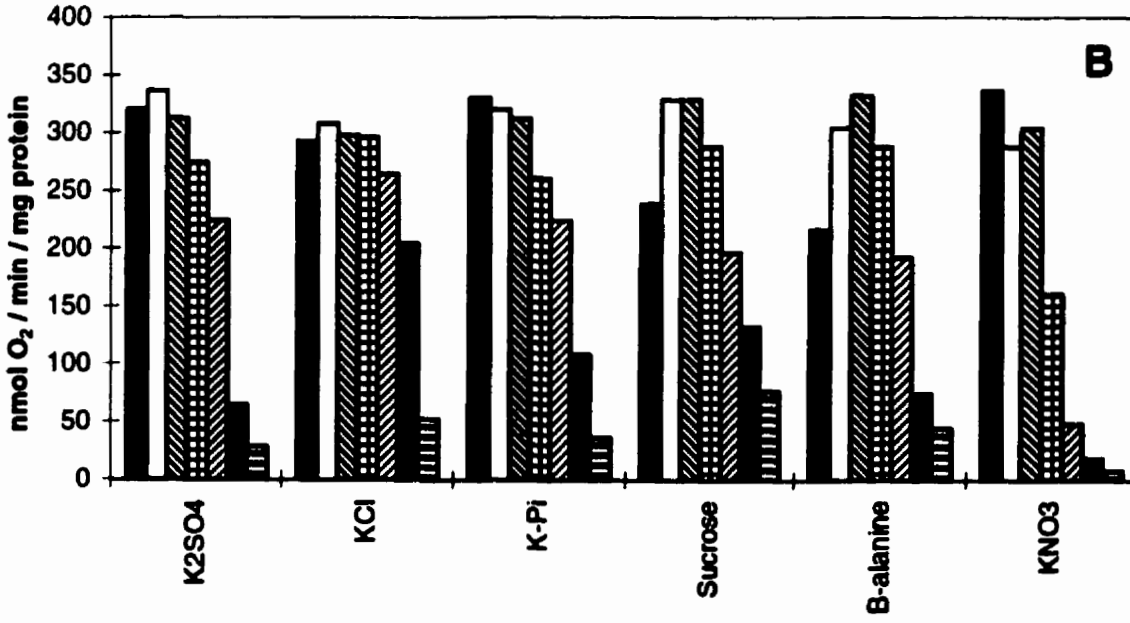

A
0.01 M
0.05 M
0.1 M
0.2 M
0.3 M
0.4 M
0.5 M
nmol O2 / min / mg protein
35
30
25
20
15
10
5
0
K2SO4
KCl
K-Pi
Sucrose
B-alanine
B
nmol O2 / min / mg protein
20
16
12
8
4
0
K2SO4
KCl
K-Pi
Sucrose
B-alanine

pH. If the cells were suspended in 0.1 M β-alanine pH 2.3, a strong buffer and assayed in K_2SO_4, KCl or sucrose all of which have no buffering capacity (K_2SO_4 has a pK value of only 1.92) then the area surrounding the cells remains acidic, thus inhibiting sulfur oxidation. K-Pi and β-alanine (pH 6), however, at the appropriate pK values would raise the pH surrounding the cells and possibly the periplasmic space resulting in increased sulfur oxidizing activity. A similar pattern of inhibition was observed with sulfite oxidation in *T. thiooxidans* (Takeuchi and Suzuki, 1993). The observed results, however, are complicated by the fact that increased phosphate and β-alanine concentrations not only potentially neutralize the area surrounding the bacteria they also increase the osmotic pressure exerted on the bacteria. In fact the overall decrease in sulfur oxidation observed with all anion and buffer solutions suggests a generalized inhibition due to increased osmotic pressure. Sulfur unlike ferrous iron is an insoluble substrate. At high osmotic pressure the cells would lose water and the membranes would shrink making contact with the sulfur particles and their subsequent oxidation difficult.

Sulfur grown cells. Sulfur grown *T. ferrooxidans* as previously noted oxidized sulfur at a substantially higher rate than their iron grown counterparts. Sulfur oxidation was also shown to be relatively unaffected by variations in assay buffer pH. Figure 8 shows sulfur oxidation activities of the sulfur grown cells at pH 3 and 6 in anion and buffer concentrations ranging from 0.01-0.5 M. Bacterial sulfur oxidation showed only minor variations in the activity patterns observed in K_2SO_4, KCl and sucrose in the shift from acidic to near neutral pH. The rate of bacterial sulfur oxidation showed a minor increase from 0.01-0.05 M sulfate, followed by a gradual drop in activity up to 0.5 M.

Figure 8. Effect of increasing buffer concentrations on sulfur oxidation using sulfur grown *T. ferrooxidans*. **A**, assay pH 6; **B**, assay pH 3. The reaction mixture contained 0.5 mg wet cells (suspended in 0.1 M β-alanine pH 2.3), 0.1 ml S (Tween 80) and various buffers to a total volume of 1.2 ml.


A
nmol O2 / min / mg protein
400
350
300
250
200
150
100
50
0
K2SO4
KCl
K-Pi
Sucrose
B-alanine
0.01 M
0.05 M
0.1 M
0.2 M
0.3 M
0.4 M
0.5 M


B
nmol O2 / min / mg protein
400
350
300
250
200
150
100
50
0
K2SO4
KCl
K-Pi
Sucrose
B-alanine
KNO3

KCl showed a similar pattern of activity, although sulfur oxidation at 0.05 M and 0.1 M was slightly higher at pH 6 than pH 3. Sulfur oxidation showed a gradual increase in activity up to 0.1 M sucrose followed by a concomitant decline with increased sucrose concentrations. As in KCl, sulfur oxidation, in 0.1-0.3 M sucrose showed slightly higher activity at pH 6. Finally, potassium phosphate and β-alanine (the true assay buffers) showed a significantly higher bacterial sulfur oxidation in 0.3-0.5 M buffer at pH 6 than at pH 3. This increased activity may be due to the solutions buffering capacity. The cells used for this experiment were suspended in 0.1 M β-alanine pH 2.3. Potassium phosphate and β-alanine likely increased the pH surrounding the bacteria (i.e. above pH 2.3) thus increasing overall sulfur oxidation. The increase, however, was not as visible as with the iron grown cells, which may be due to the difference in cell types. In terms of neutralizing acids surrounding cells the sulfur grown cells should have been affected more by the buffer since only 10 µl of the cell suspension (50 mg/ml) rather than 100 µl of the iron grown cells were used in the 1.2 ml reaction mixture. The increasing activity at pH 6 compared to that at pH 3 in most cases was relatively small for as seen in chapter 1 sulfur oxidation in the sulfur grown cells was relatively stable between pH 2.3-8 showing only minor inhibition at pH 2.3 and 3.

Effect of Anions on Iron and Sulfur Oxidation Using Growing Cell Cultures

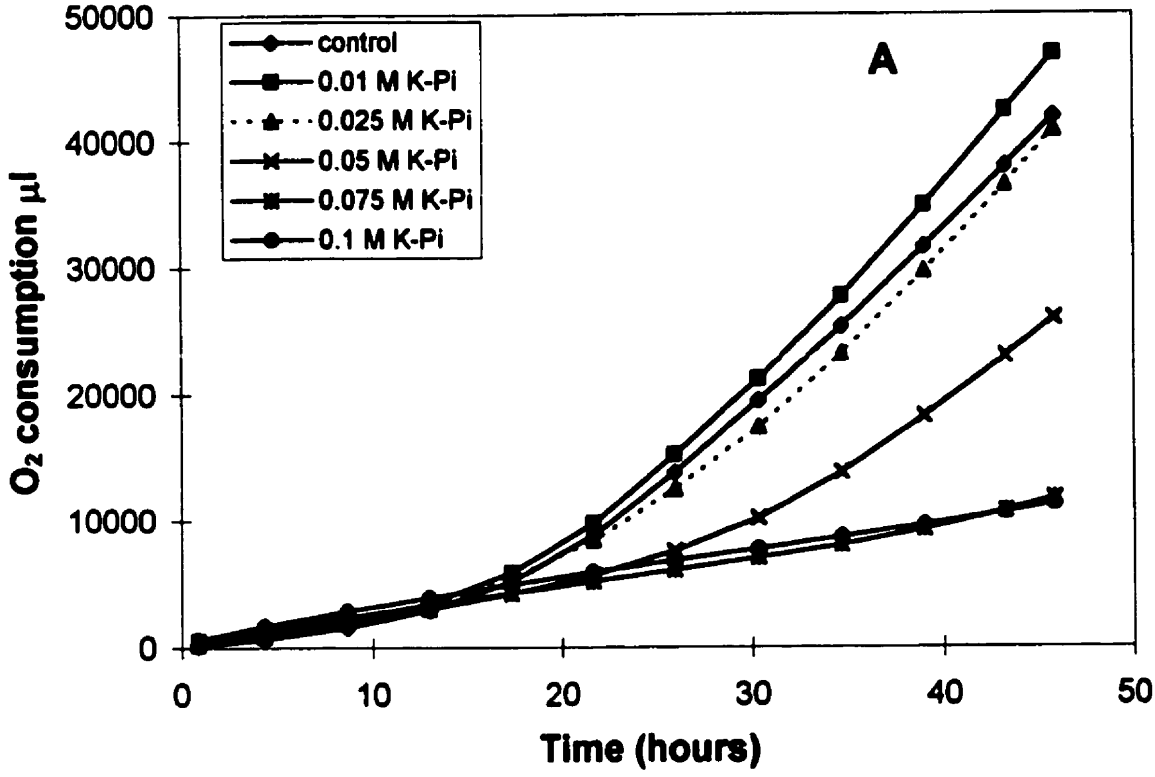
The effect of anions on iron and sulfur oxidation in *T. ferrooxidans* has up to this point been focused on cell suspensions. These data although providing valuable information with respect to anion-cell interaction is regarded as only the first step towards the potential use of anions in selective mineral solubilization. Prior to the development of

a pilot plant the key anions (K-Pi, KCl and KNO_3) had to be tested on growing cell cultures. Cells growing on single substrates were monitored using a Micro-oxymax respirometer. Iron and sulfur oxidation were measured in terms of cumulative oxygen consumption. Since sulfur-adapted SM-4 was not available for these experiments, sulfur-grown *T. thiooxidans* SM-6 was used for the sulfur growth experiments. Figure 9 shows the effects of increasing concentrations of phosphate on iron and sulfur oxidation by growing cells. The control in each case contained a 5 % inoculum along with ferrous sulfate or precipitated sulfur as the substrate. Cumulative oxygen consumption was plotted as a function of time for each of the reaction vessels. Iron oxidation (Fig. 9A) was decreased by half in the presence of 50 mM phosphate. An equivalent concentration actually stimulated sulfur oxidation (Fig. 9B). Nearly three times as much phosphate was required to cause a 50 % drop in sulfur oxidizing activity.

Similar results were found with both chloride and nitrate. Chloride at 50 mM inhibited iron oxidation by 12 % (Fig. 10A) yet stimulated sulfur oxidation by 34 % (Fig. 10B). Nitrate at 25 mM inhibited 17 % iron oxidation (Fig. 11A) while stimulating sulfur oxidation to nearly twice that of the control (Fig. 11B).

Cumulative carbon dioxide consumption was used to measure cellular growth rate. Figure 12 shows the effects of phosphate ions on growth using either ferrous iron or elemental sulfur as substrate. A phosphate concentration of 0.05 M which caused a 50 % drop in iron oxidation was equally effective in inhibiting growth on ferrous iron (Fig. 12A). Growth on elemental sulfur (Fig 12B), however, required more than twice as much phosphate for 50 % inhibition. At 0.05 M, phosphate stimulated growth on sulfur (Fig

Figure 9. Effect of phosphate on iron oxidation and sulfur oxidation. **A,** iron grown *T. ferrooxidans*. **B,** sulfur grown *T. thiooxidans*. Oxidation activities were observed using a Micro-oxymax respirometer at 26°C. The reaction vessel contained a 5 % bacterial inoculum, 12 μmoles $FeSO_4$ (for iron oxidation), or 1 g BDH sulfur sprinkled on the surface plus 18 mg/l $FeSO_4$ (for sulfur oxidation), various K-Pi concentrations and M9K medium at pH 2.3 making a total volume of 100 ml.


A
control
0.01 M K-Pi
0.025 M K-Pi
0.05 M K-Pi
0.075 M K-Pi
0.1 M K-Pi
O2 consumption µl
Time (hours)
0
10000
20000
30000
40000
50000
0
10
20
30
40
50

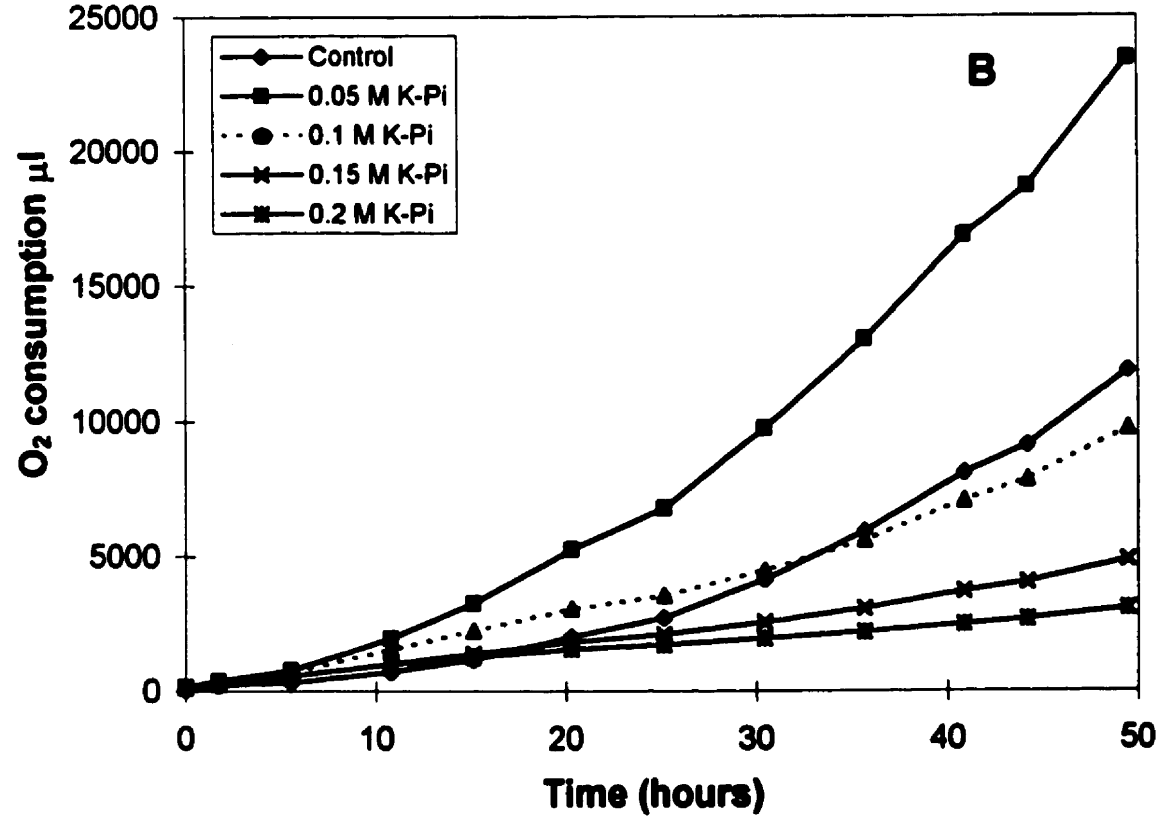
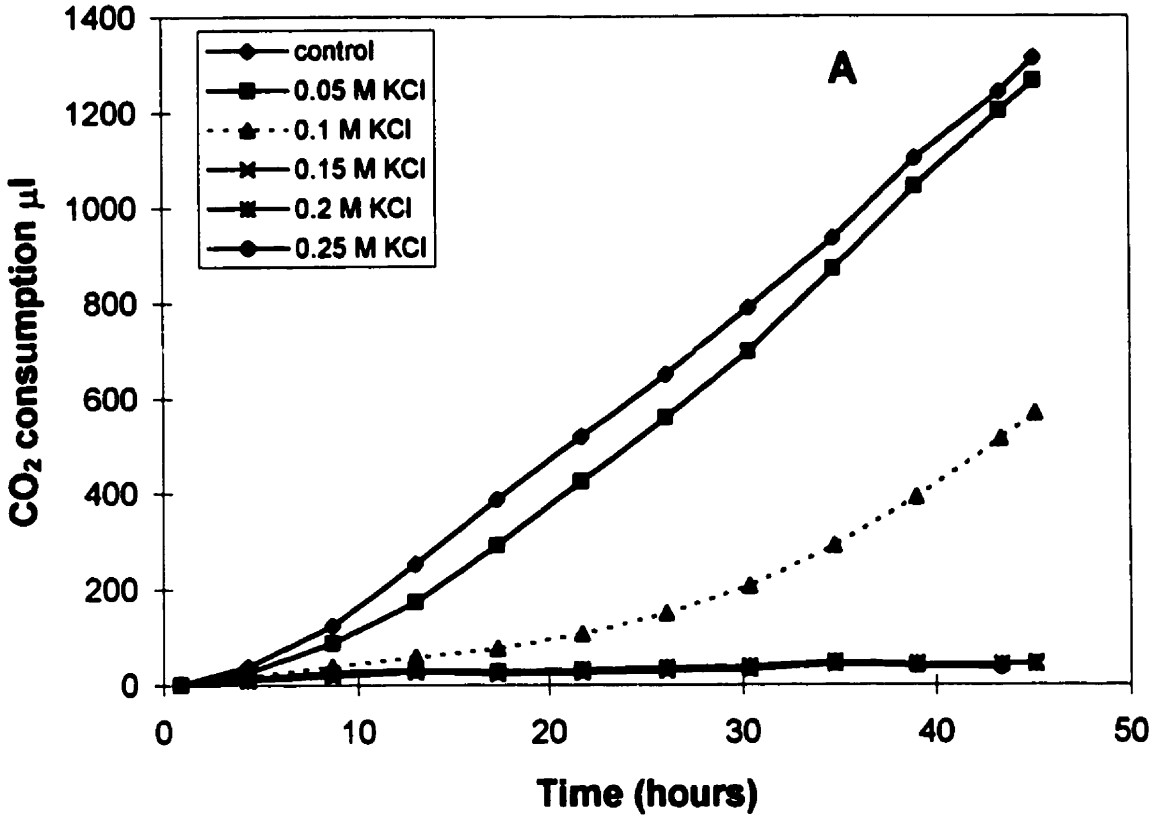

B
Control
0.05 M K-Pi
0.1 M K-Pi
0.15 M K-Pi
0.2 M K-Pi
O2 consumption µl
Time (hours)
0
5000
10000
15000
20000
25000
0
10
20
30
40
50

Figure 10. Effect of chloride on iron oxidation and sulfur oxidation. **A**, iron grown *T. ferrooxidans*. **B**, sulfur grown *T. thiooxidans*. Oxidation activities were observed using a Micro-oxymax respirometer at 26°C. The reaction vessel contained a 5 % bacterial inoculum, 12 μmoles $FeSO_4$ (for iron oxidation), or 1 g BDH sulfur sprinkled on the surface plus 18 mg/l $FeSO_4$ (for sulfur oxidation), various KCl concentrations and M9K medium at pH 2.3 making a total volume of 100 ml.


A
control
0.05 M KCl
0.1 M KCl
0.15 M KCl
0.2 M KCl
0.25 M KCl
CO_2 consumption µl
Time (hours)
0
200
400
600
800
1000
1200
1400
0
10
20
30
40
50

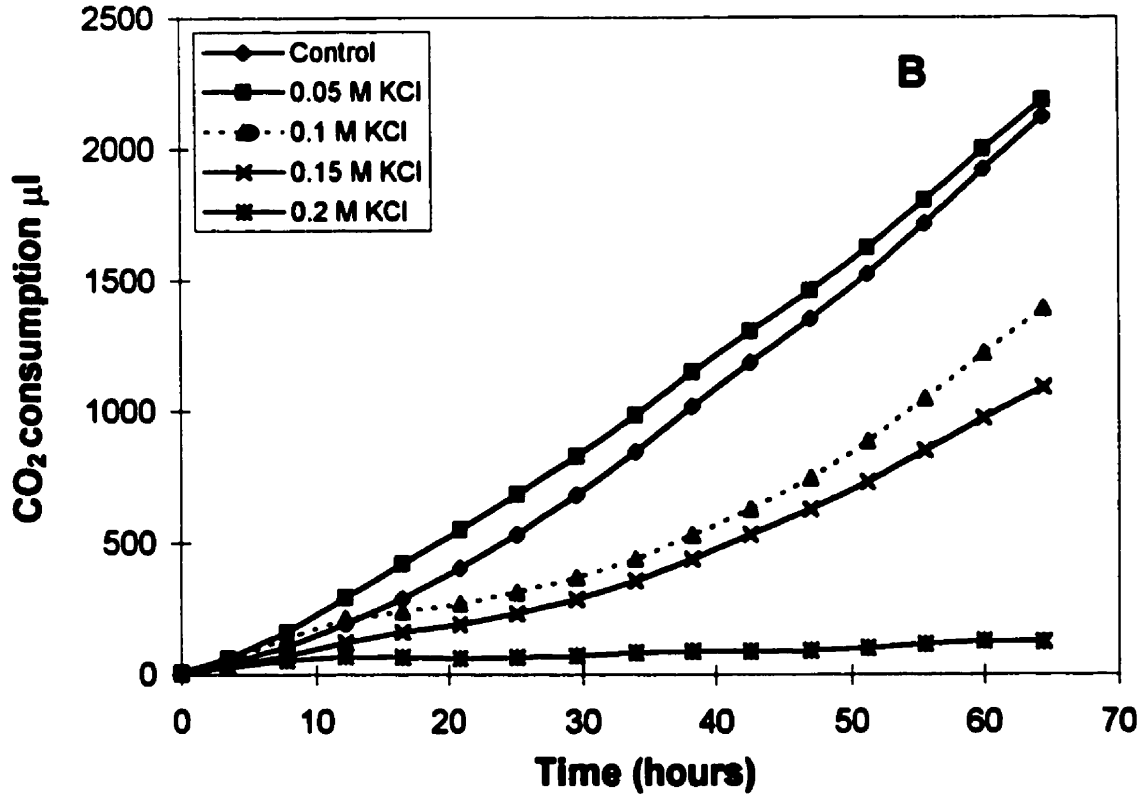
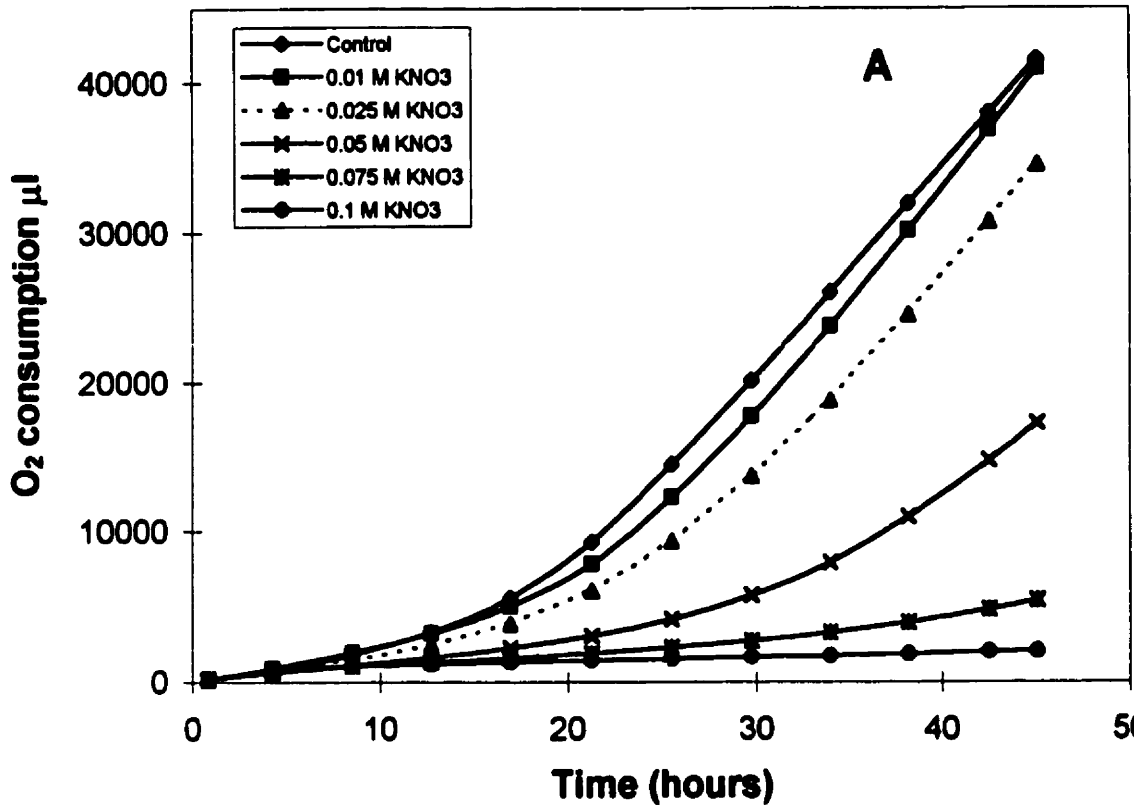

B
Control
0.05 M KCl
0.1 M KCl
0.15 M KCl
0.2 M KCl
CO_2 consumption µl
Time (hours)
0
500
1000
1500
2000
2500
0
10
20
30
40
50
60
70

Figure 11. Effect of nitrate on iron oxidation and sulfur oxidation. **A,** iron grown *T. ferrooxidans*. **B,** sulfur grown *T. thiooxidans*. Oxidation activities were observed using a Micro-oxymax respirometer at 26°C. The reaction vessel contained a 5 % bacterial inoculum, 12 μmoles $FeSO_4$ (for iron oxidation), or 1 g BDH sulfur sprinkled on the surface plus 18 mg/l $FeSO_4$ (for sulfur oxidation), various KNO_3 concentrations and M9K medium at pH 2.3 making a total volume of 100 ml.


A
Control
0.01 M KNO3
0.025 M KNO3
0.05 M KNO3
0.075 M KNO3
0.1 M KNO3
O2 consumption µl
40000
30000
20000
10000
0
0
10
20
30
40
50
Time (hours)

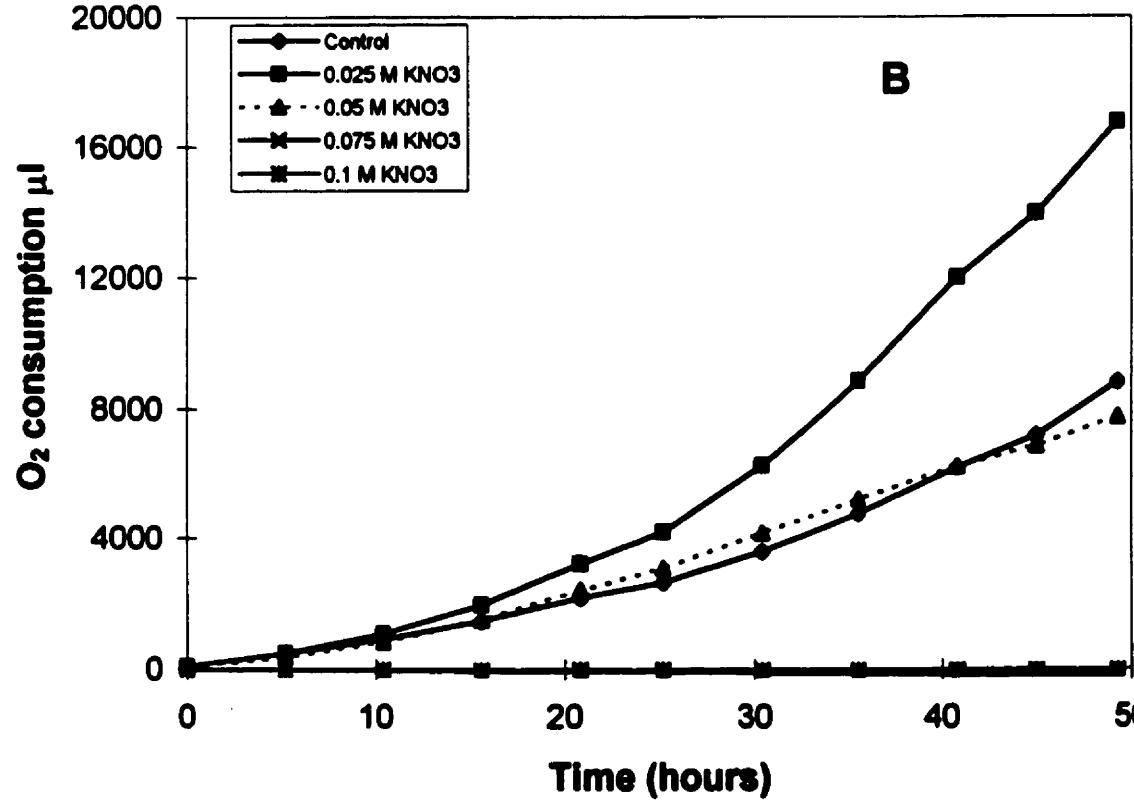
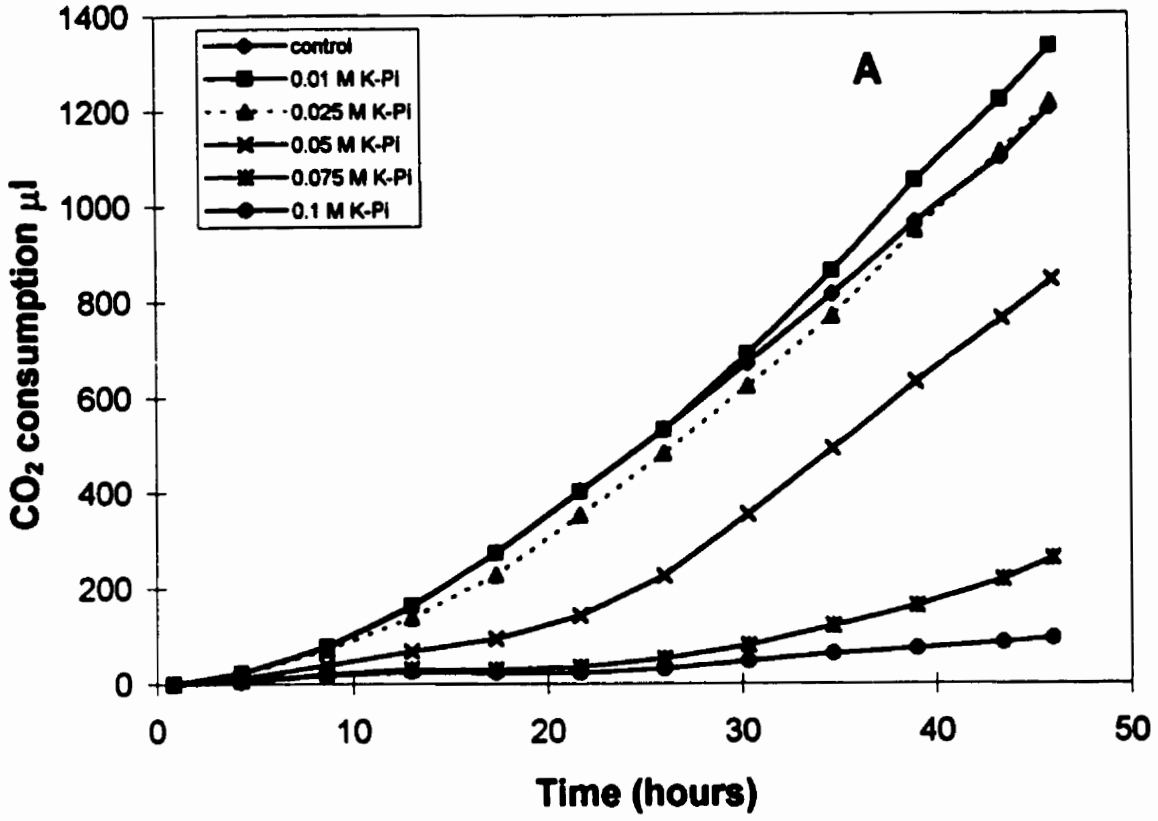
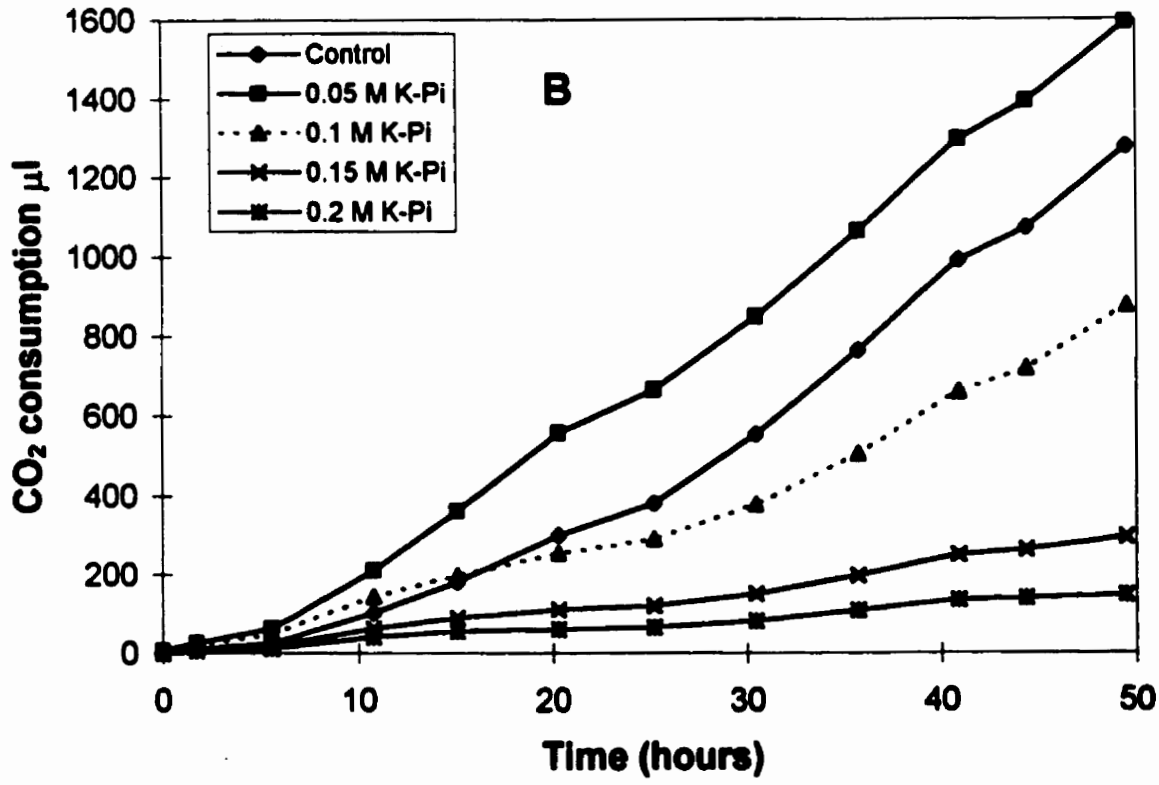

B
Control
0.025 M KNO3
0.05 M KNO3
0.075 M KNO3
0.1 M KNO3
O2 consumption µl
20000
16000
12000
8000
4000
0
0
10
20
30
40
50
Time (hours)

Figure 12. Effect of phosphate on bacterial growth. **A**, iron grown *T. ferrooxidans*. **B**, sulfur grown *T. thiooxidans*. Oxidation activities were observed using a Micro-oxymax respirometer at 26°C. The reaction vessel contained a 5 % bacterial inoculum, 12 μmoles $FeSO_4$ (for iron oxidation), or 1 g BDH sulfur sprinkled on the surface plus 18 mg/l $FeSO_4$ (for sulfur oxidation), various K-Pi concentrations and M9K medium at pH 2.3 making a total volume of 100 ml.


A
control
0.01 M K-Pi
0.025 M K-Pi
0.05 M K-Pi
0.075 M K-Pi
0.1 M K-Pi
CO2 consumption µl
Time (hours)
0
200
400
600
800
1000
1200
1400
0
10
20
30
40
50

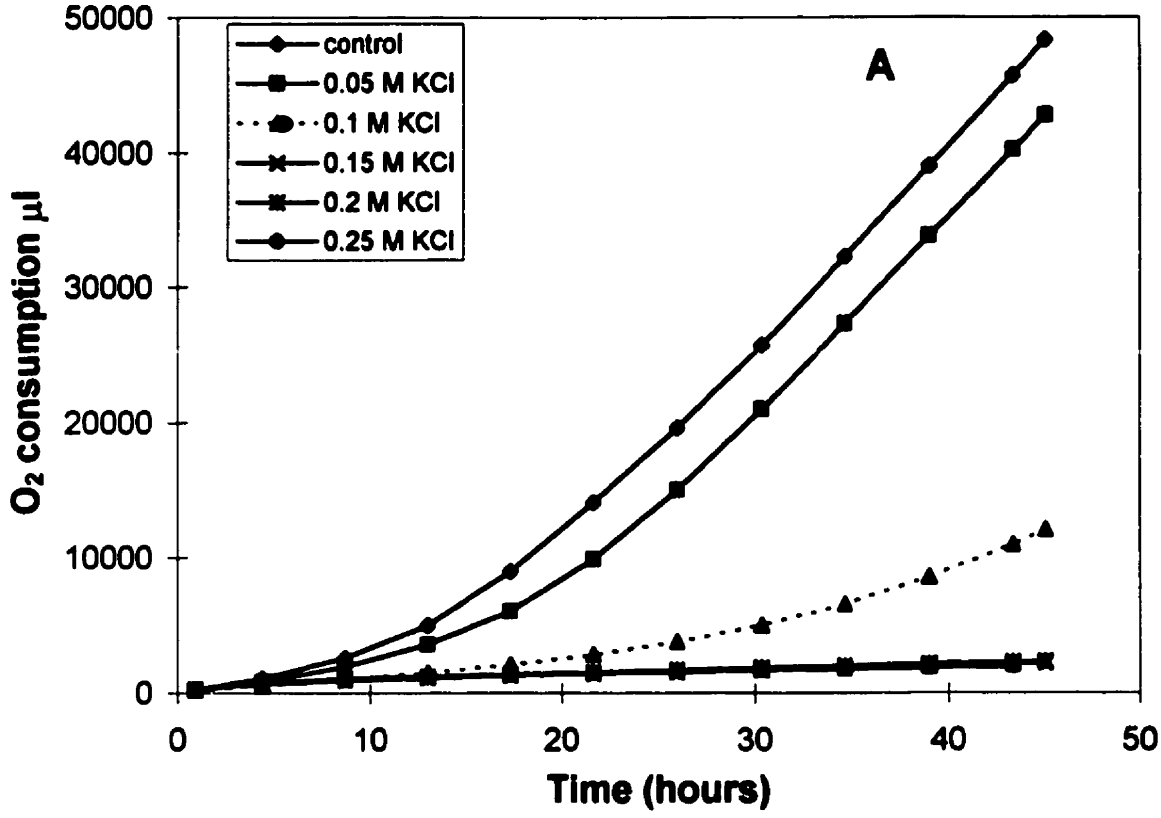

B
Control
0.05 M K-Pi
0.1 M K-Pi
0.15 M K-Pi
0.2 M K-Pi
CO2 consumption µl
Time (hours)
0
200
400
600
800
1000
1200
1400
1600
0
10
20
30
40
50

12B) as well as sulfur oxidation (Fig 9B). The stimulatory effect of phosphate was confirmed with *T. ferrooxidans* adapted on sulfur, where 0.075 M potassium phosphate increased the growth on sulfur by 20 % and sulfur oxidation by 30 %. Chloride although having a substantial effect on substrate oxidation showed only a minor effect on bacterial growth (Fig. 13). At 0.05 M KCl, growth on iron was inhibited by only 4 % while growth on sulfur was stimulated by a similar percentage. Finally, nitrate at 0.025 M inhibited growth on both iron and elemental sulfur at 4 % and 7 % respectively (Fig. 14 A and B). Thus showing stimulation of sulfur oxidation does not necessarily coincide with increased bacterial growth.

Effect of Anions on Sulfite Oxidation

The effect of anions on sulfite oxidation was tested using both iron and sulfur grown *T. ferrooxidans*. As previously noted the mechanism of sulfite oxidation is largely dependent on the bacterial growth substrate. Sulfite oxidation using iron grown *T. ferrooxidans* was tested only at pH 3 (optimum pH for sulfite oxidation and comparable to growth pH). Sulfite oxidation was maximal in 0.01 M KCl showing an oxidation rate approaching 1700 nmol O_2/min/mg protein (Fig. 15A). An increase in the chloride concentration to 0.05 M dropped this rate to 600 nmol O_2/min/mg protein. Sulfite oxidation in 0.01 M K_2SO_4, β-alanine, KNO_3 and sucrose fell within the range of 650-1100 nmol O_2/min/mg protein. A further increase to 0.05 M resulted in a concomitant drop in activity. Of the anions and buffers tested, K-Pi proved unique, almost completely inhibiting sulfite oxidation even at the lowest concentration used. As previously noted in Chapter 1, sulfite oxidation using iron grown *T. ferrooxidans* is believed to involve a

Figure 13. Effect of chloride on bacterial growth. **A**, iron grown *T. ferrooxidans*. **B**, sulfur grown *T. thiooxidans*. Oxidation activities were observed using a Micro-oxymax respirometer at 26°C. The reaction vessel contained a 5 % bacterial inoculum, 12 μmoles $FeSO_4$ (for iron oxidation), or 1 g BDH sulfur sprinkled on the surface plus 18 mg/l $FeSO_4$ (for sulfur oxidation), various KCl concentrations and M9K medium at pH 2.3 making a total volume of 100 ml.


A
control
0.05 M KCl
0.1 M KCl
0.15 M KCl
0.2 M KCl
0.25 M KCl
O2 consumption µl
Time (hours)
0
10000
20000
30000
40000
50000
0
10
20
30
40
50

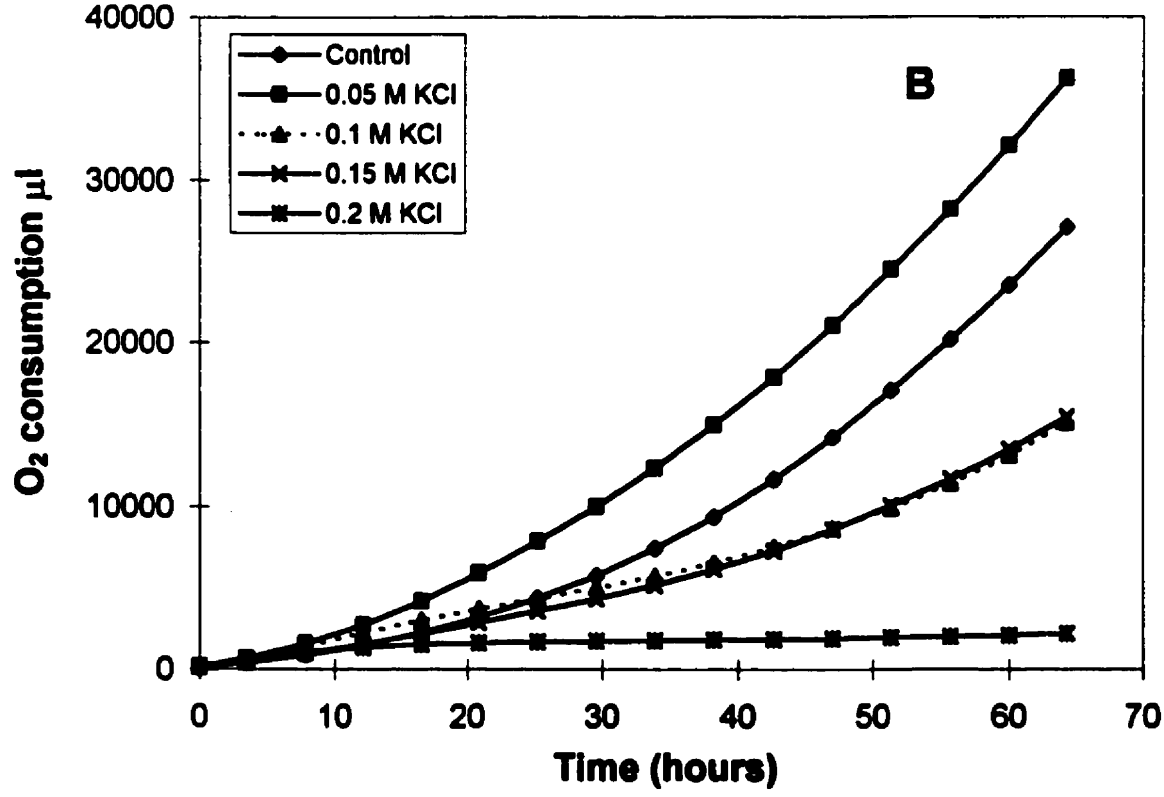
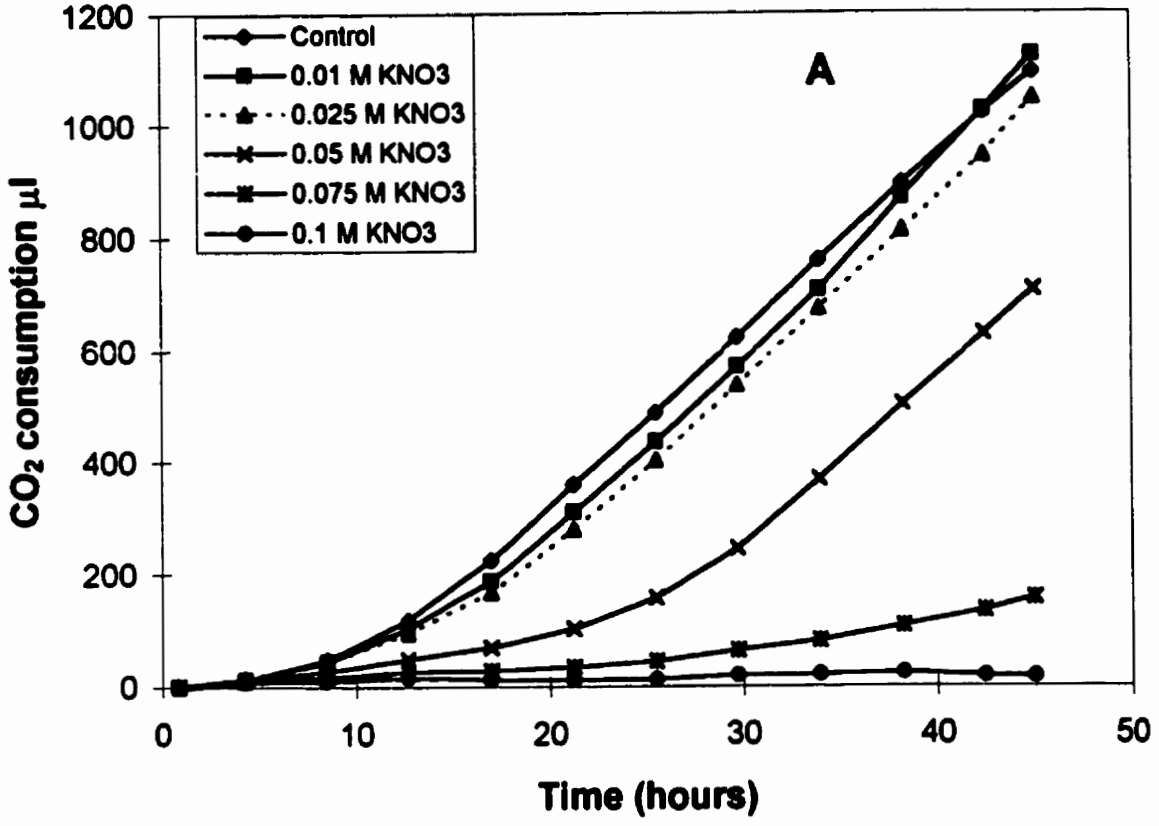

B
Control
0.05 M KCl
0.1 M KCl
0.15 M KCl
0.2 M KCl
O2 consumption µl
Time (hours)
0
10000
20000
30000
40000
0
10
20
30
40
50
60
70

Figure 14. Effect of nitrate on bacterial growth. **A**, iron grown *T. ferrooxidans*. **B**, sulfur grown *T. thiooxidans*. Oxidation activities were observed using a Micro-oxymax respirometer at 26°C. The reaction vessel contained a 5 % bacterial inoculum, 12 μmoles $FeSO_4$ (for iron oxidation), or 1 g BDH sulfur sprinkled on the surface plus 18 mg/l $FeSO_4$ (for sulfur oxidation), various KNO_3 concentrations and M9K medium at pH 2.3 making a total volume of 100 ml.


Control
0.01 M KNO3
0.025 M KNO3
0.05 M KNO3
0.075 M KNO3
0.1 M KNO3
A
CO2 consumption µl
Time (hours)
0
200
400
600
800
1000
1200
0
10
20
30
40
50

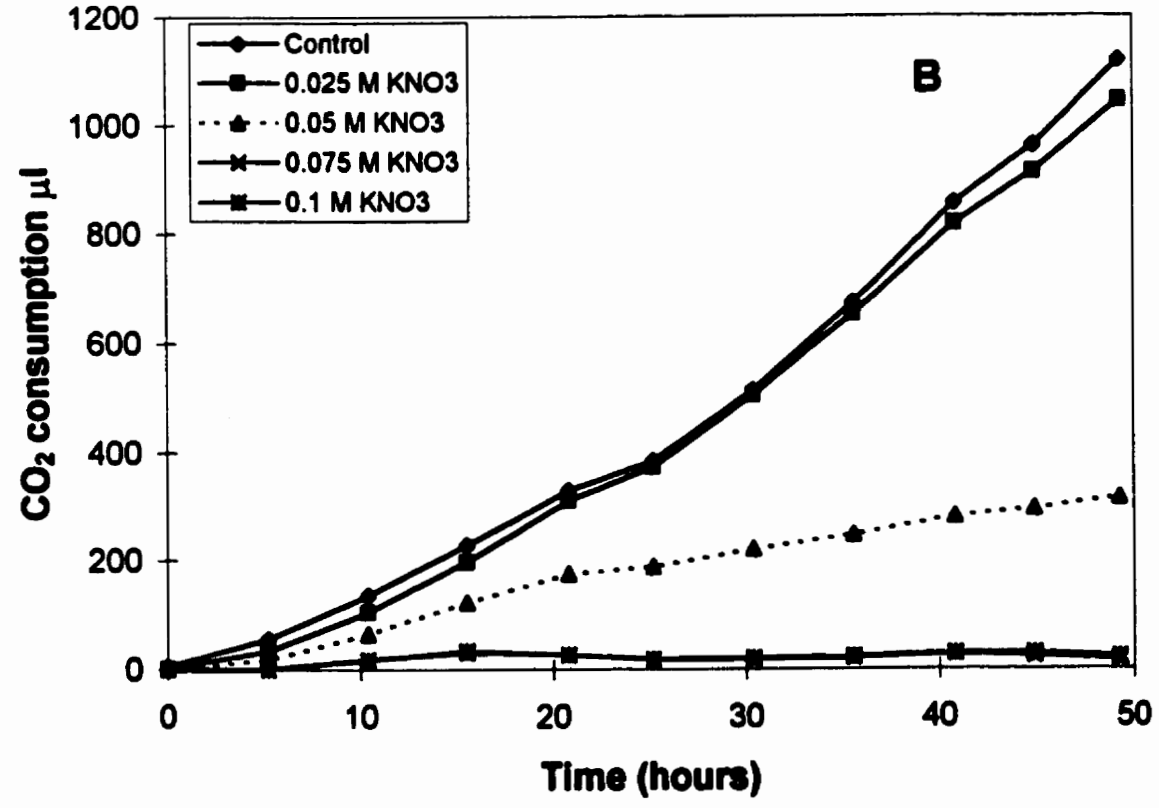
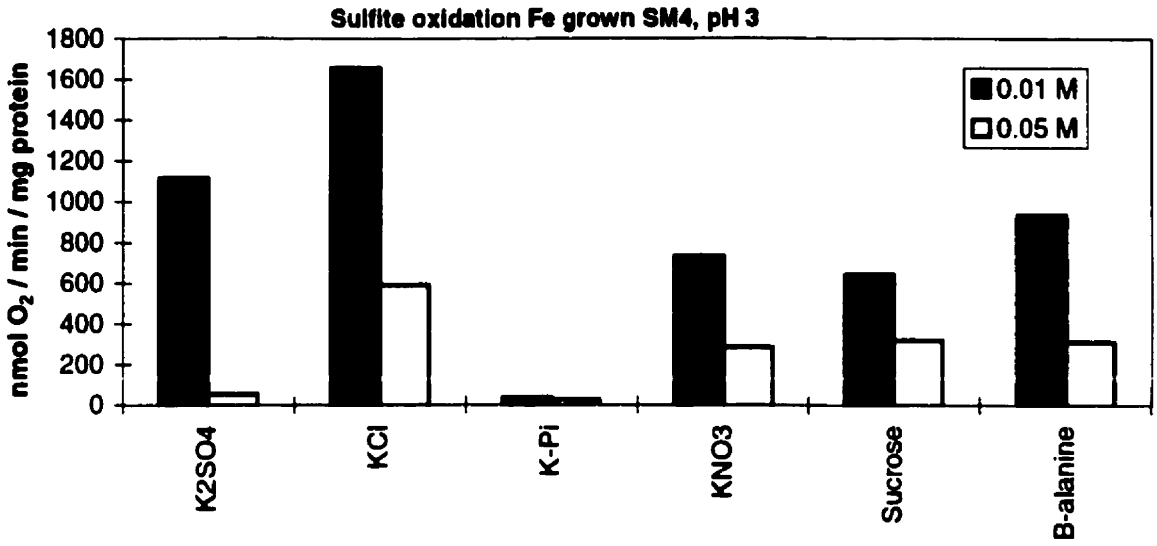
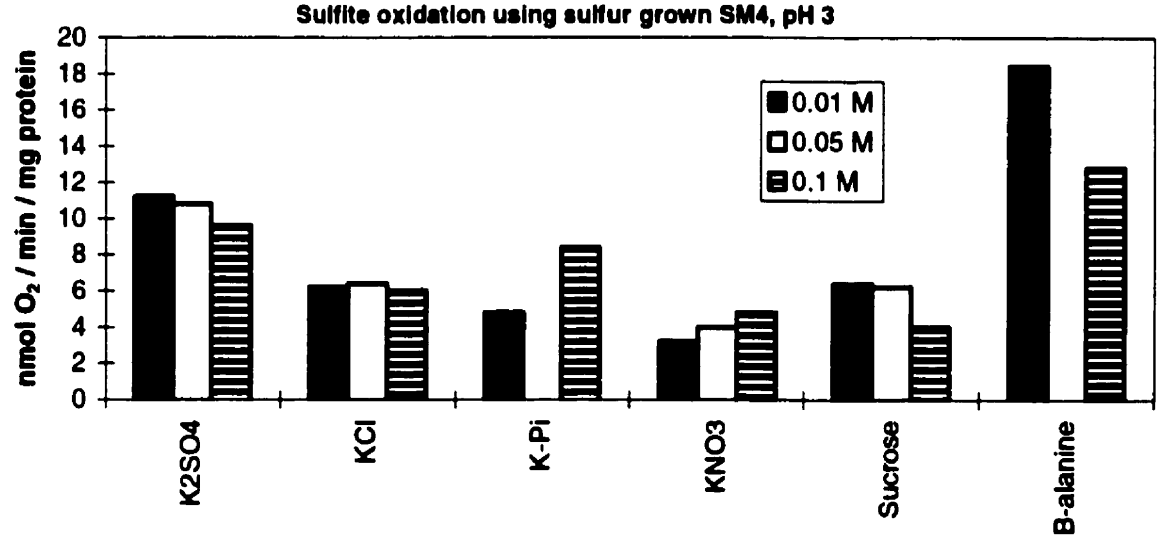
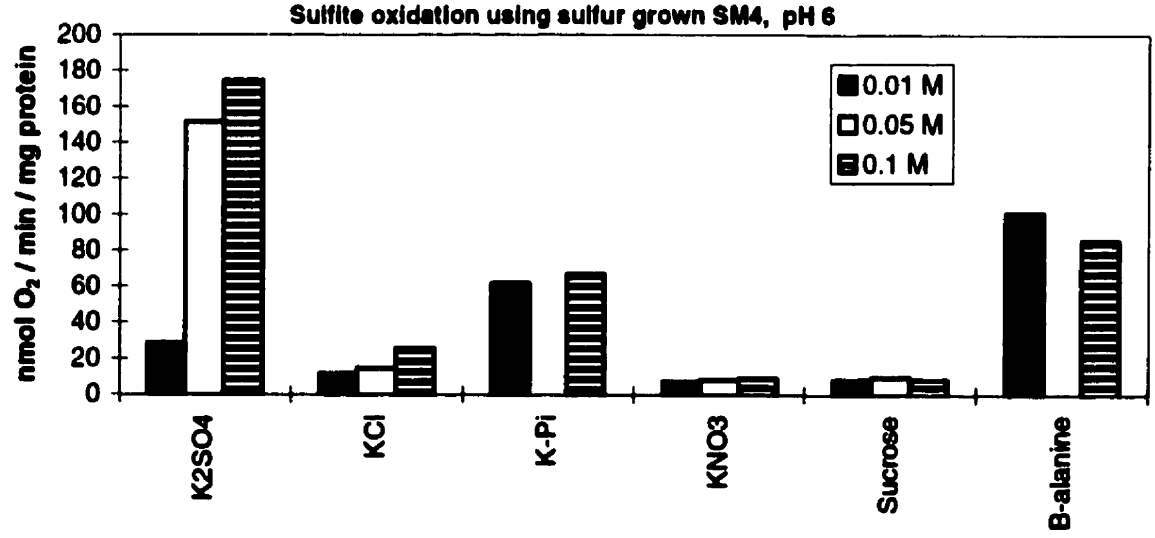

Control
0.025 M KNO3
0.05 M KNO3
0.075 M KNO3
0.1 M KNO3
B
CO2 consumption µl
Time (hours)
0
200
400
600
800
1000
1200
0
10
20
30
40
50

Figure 15. Effect of increasing anion concentrations on sulfite oxidation. The reaction mixture contained 0.5 mg (Fe grown SM-4) to 5 mg (sulfur grown SM-4) wet cells and 1 μmol sulfite with various buffers in a total volume of 1.2 ml.


Sulfite oxidation Fe grown SM4, pH 3
nmol O2 / min / mg protein
1800
1600
1400
1200
1000
800
600
400
200
0
0.01 M
0.05 M
K2SO4
KCl
K-Pi
KNO3
Sucrose
B-alanine


Sulfite oxidation using sulfur grown SM4, pH 3
nmol O2 / min / mg protein
20
18
16
14
12
10
8
6
4
2
0
0.01 M
0.05 M
0.1 M
K2SO4
KCl
K-Pi
KNO3
Sucrose
B-alanine


Sulfite oxidation using sulfur grown SM4, pH 6
nmol O2 / min / mg protein
200
180
160
140
120
100
80
60
40
20
0
0.01 M
0.05 M
0.1 M
K2SO4
KCl
K-Pi
KNO3
Sucrose
B-alanine

metal catalyzed free radical chain reaction. It is therefore possible that phosphate is chelating the iron surrounding the cells thus inhibiting sulfite oxidation.

Sulfite oxidation in the sulfur grown cells was optimal at near neutral pH, thus the potential effect of anions on sulfite oxidation was tested at both pH 3 (comparable to growth pH) and pH 6. Figure 15 B shows maximum sulfite oxidation in 0.01 M β-alanine pH 3. Increased buffer concentrations resulted in increased inhibition. Sulfite oxidation in the remaining solutions fell below 12 nmol O_2/min/mg protein. Sulfite oxidation in K_2SO_4 and sucrose as with β-alanine showed decreased activity with increased solution concentration (the decrease however was minor). Sulfite oxidation in K-Pi and KNO_3 increased with increased solution concentrations. Sulfite oxidation in KCl remained relatively stable over the concentration range used.

At pH 6 maximum sulfite oxidation was observed in 0.1 M K_2SO_4 (Fig. 15C). The rate, approaching 180 nmol O_2/min/mg protein was greater than ten times that observed at pH 3. Decreased sulfate concentrations coincided with decreased activity. A similar pattern was observed with KCl and K-Pi. β-alanine, on the other hand, showed a peak of activity at 0.01 M while activities in KNO_3 and sucrose remained relatively unchanged and low over the range of 0.01-0.1 M. Overall sulfite oxidation was generally higher at pH 6, the exception being KNO_3 and sucrose in which bacterial sulfite oxidation remained relatively unaffected by the rise in pH.

Table 8. Inhibition of sulfur and iron oxidation using *T. ferrooxidans*. Percent inhibition was calculated using the activity in 0.05 M K_2SO_4-H_2SO_4 as 100 %. Assay buffer pH 3. Values in brackets refer to maximum inhibition obtained.

	Concentration required for 50 % inhibition		
Assay	Ferrous iron grown cells		S^0 grown cells
Buffer	Fe^{2+} oxidation	S^0 Oxidation	S^0 Oxidation
K_2SO_4-H_2SO_4	500 mM (31 %)	186 mM	335 mM
KPO_4 - H_3PO_4	93 mM	240 mM	350 mM
KCl - HCl	60 mM	137 mM	420 mM
KNO_3 - HNO_3	14 mM	NA	190 mM
β-alanine-H_2SO_4	500 mM (46 %)	289 mM	320 mM
Sucrose-H_2SO_4	300 mM	375 mM	340 mM

Table 9. Inhibition of oxidation and growth on ferrous iron and sulfur as substrate. Micro-oxymax data on O_2 (oxidation) and CO_2 (growth) consumption after 42 hours. Control (100%) flask contained M9K medium without additions.

	Concentration required for 50 % inhibition			
Assay	Ferrous iron grown cells		Sulfur grown cells	
Buffer	O_2	CO_2	O_2	CO_2
KPO_4 - H_3PO_4	58 mM	59 mM	140 mM	120 mM
KCl - HCl	79 mM	90 mM	160 mM	120 mM
KNO_3 - HNO_3	43 mM	56 mM	63 mM	42 mM

DISCUSSION

Bioleaching has become an increasingly important process due to the growing need to use lower grade ores, the relative ease of implementation and the low start up costs as compared to a conventional mining operation (Brierly, 1978). Under natural conditions an ore body may show a tendency for selective solubilization of certain metals. The mineral that is extensively oxidized is i) the most hydrophobic, ii) the lowest of an electrochemical series or iii) behaving as the anode of a galvanic cell (Lawrence et al., 1997). The purpose of this chapter was to show that the bacterial activities responsible for metal leaching, the oxidation of ferrous iron and sulfur, can be selectively controlled by manipulation of the media.

In order to account for the cellular variations created by the bacterial growth substrate, oxidation activities were tested using both iron and sulfur grown *T. ferrooxidans*. The effect of selected anions and buffers on the iron and sulfur oxidation activities are summarized in Table 8, calculated from the data in Figs. 6 to 8. Potassium sulfate inhibited sulfur oxidation by 50 % at 186 mM and 335 mM in the iron and sulfur grown cells respectively. Iron oxidation, however, at the highest sulfate concentration used, 0.5 M was inhibited by only 31 %. Sulfate is essential for iron oxidation in *T. ferrooxidans* and thus was not expected to be inhibitory. From these data, therefore, sulfate can be listed as preferentially inhibiting sulfur oxidation.

Both β-alanine and sucrose were adjusted with sulfuric acid to pH 3, however, the β-alanine buffers had much higher sulfate concentrations, due to their natural buffering abilities. β-Alanine inhibited only 46 % of the iron oxidation at 0.5 M, while sulfur oxidation was inhibited by 50 % at 289-320 mM β-alanine. Thus, as with the above

potassium sulfate, it showed preferential inhibition of sulfur oxidation. Sucrose, on the other hand, inhibited both iron and sulfur oxidation at a similar concentration 300-375 mM. The remaining anionic salts, K-Pi, KCl and KNO_3 all preferentially inhibited iron oxidation at a concentration 2-14 times less than that required to inhibit sulfur oxidation.

As previously noted metal extraction from mineral ore is achieved though iron and or sulfur/sulfide oxidation - the mineral involved determining which reaction is required. At Cominco's Red Dog Mine the dominant minerals are represented by sphalerite (ZnS) and pyrite (FeS_2), the solubilization of which require sulfur and both iron and sulfur oxidation respectively. Iron oxidation, however, generates ferric sulfate which at high concentrations produces jarosite, a ferric iron precipitate which can cover the ore surface preventing further leaching from occurring. The key to maximum mineral solubilization, therefore, lies in controlling iron oxidation thus preventing jarosite formation. From the above data only K-Pi, KCl and KNO_3 preferentially inhibited iron oxidation in *T. ferrooxidans*. The general trend observed with cell suspensions was found to be applicable to growing cell cultures (Table 9, calculated from Figs. 9 to 14). Growth studies, however, revealed the additional stimulatory effect of 50 mM phosphate on the oxidation of and growth on elemental sulfur.

PART III

SELECTIVE MINERAL SOLUBILIZATION

INTRODUCTION

The use of bacteria in the solubilization of mineral ores has revolutionized the mining industry. The bacterial consortium that makes up a bioleaching community varies with respect to growth pH and temperature, heavy metal resistance and energy and oxygen requirements (Bacelar-Nicolau and Johnson, 1988; Brierley, 1978; Ehrlich, 1998; Espejo and Romero, 1997; Johnson, 1998; Rawlings and Silver, 1995; Rawlings et al., 1999; Rossi, 1990; Sand et al., 1989; Torma, 1977). The ever changing environment in which these bacteria exist necessitates their ability to survive in a wide range of growth conditions. The primary species found in mineral solubilization at ambient temperature is *Thiobacillus ferrooxidans*, a chemolithotroph able to oxidize ferrous iron, sulfur and reduced sulfur compounds. *Thiobacillus thiooxidans*, a close relative, is also found in association with mine waters. Unlike *T. ferrooxidans*, however, it is able to oxidize only sulfur and reduced sulfur compounds. *Leptospirillum ferrooxidans* oxidizes only ferrous iron and is found in some mineral leaching environments (Sand et al., 1992), and according to recent reports predominates over *T. ferrooxidans* at higher temperatures (e.g. 35 - 40°C) and lower pH (e.g. pH 1.6 or lower) conditions of some commercial bioreactors (Rawlings et al., 1999) or pyrite weathering (Edwards et al., 1999). In this study, however, we have used only thiobacilli whose physiological and biochemical characteristics are better known due to extensive studies in the past.

Metal extraction from mineral ore by bacteria is achieved through a combination of iron oxidation ($2Fe^{2+} + ½O_2 + 2H^+ \rightarrow 2Fe^{3+} + H_2O$) and sulfide/sulfur oxidation ($H_2S + 2O_2 \rightarrow H_2SO_4$ or $S^0 + 1½O_2 + H_2O \rightarrow H_2SO_4$). The chemical structure of the ore itself determines which reaction, and therefore, which bacteria are involved. For example,

uranium solubilization from uraninite is based on an indirect mechanism (Silverman and Ehrlich, 1964) and requires only iron oxidation ($UO_2 + 2Fe^{3+} \rightarrow 2Fe^{2+} + UO_2^{2+}$; $2Fe^{2+} + \frac{1}{2}O_2 + 2H^+ \rightarrow 2Fe^{3+} + H_2O$), thus only *T. ferrooxidans* would be involved. Zinc solubilization from sphalerite requires only sulfide oxidation ($ZnS + 2O_2 \rightarrow ZnSO_4$), thus either *T. ferrooxidans* and *T. thiooxidans* could be involved. Specific control of these reactions, therefore, should influence the bacterial leaching of select metals.

In an ore with a mixture of minerals, the mineral that is preferentially oxidized is i) the most hydrophobic, ii) the lowest of an electrochemical series or iii) behaves as the anode of a galvanic cell (Lawrence et al., 1997). We can, theoretically, further specify mineral oxidation through the manipulation of bacterial activities. As we have shown in Part II iron and sulfur oxidation in *T. ferrooxidans* can be differentially controlled with specific anions and inhibitors (Harahuc et al., submitted for publication). In this chapter we want to show that these anions can influence the differential solubilization of metals from mineral ores.

Selective mineral solubilization would prove invaluable to the mining industry not only in the generation of preferred end products but also during the leaching process itself. Ore deposits are often contaminated with iron as pyrite or pyrrhotite. In a pyrite-sphalerite mixture for example, the bacteria will oxidize both sulfide ($ZnS + 2O_2 \rightarrow ZnSO_4$) and iron plus sulfide ($4FeS_2 + 15O_2 + 2H_2O \rightarrow 2Fe_2(SO_4)_3 + 2H_2SO_4$). At low concentrations the ferric sulfate produced assists in solubilizing the sphalerite by an indirect mechanism. Higher ferric iron concentrations, however, result in the production of jarosite ($MFe_3(SO_4)_2(OH)_6$) or ferric hydroxide ($Fe(OH)_3$), ferric iron precipitates which cover the

surface of the ore preventing further oxidation from occurring (Le Roux and Mehta, 1978; Sakaguchi et al., 1976).

We propose to show that iron and sulfur oxidation by *T. ferrooxidans* can be differentially manipulated to allow for selective mineral solubilization. Our goal, using the above example of a pyrite-sphalerite mixture is to manipulate the bacteria to preferentially oxidize sphalerite with little to no pyrite solubilization. The result should be a high zinc, low iron solution which can be used in the production of high grade zinc. As previously stated, preliminary studies using resting cell suspensions showed phosphate, nitrate, chloride, azide and cyanide as specific inhibitors of ferrous iron oxidation with little to no effect on sulfur oxidation. Growth studies produced similar results with the exception of nitrate (which inhibited growth on both iron and sulfur) in that these inhibitors selectively inhibited iron oxidation. In addition sulfur oxidation was stimulated by phosphate, chloride and nitrate at low concentrations (Part II, also Harahuc et al., submitted for publication). The differential control of iron or sulfur oxidation by anions has been applied in our preliminary work of selective solubilization of zinc from a mixture of pyrite and sphalerite (Suzuki and Harahuc, Canadian patent pending, 1998). The present study expands the principle of selective mineral solubilization to the extraction of copper, zinc and iron from chalcopyrite, sphalerite and pyrite and presents a complete analysis of the effect of anions on the bacterial leaching of sulfide minerals.

MATERIALS AND METHODS

Media and cell culturing

T. ferrooxidans strain SM-4 (Lizama and Suzuki, 1988) was grown in modified 9K medium (M9K) : 0.4 g $(NH_4)_2SO_4$, 0.1 g K_2HPO_4, 0.4 g $MgSO_4 \cdot 7H_2O$ and 33.3 g $FeSO_4 \cdot 7H_2O$ per liter and adjusted to pH 2.3 with H_2SO_4. Cells were grown using a 10% inoculum in a 100 ml volume. The flask was incubated at 25°C on a rotary shaker at 150 rpm for 48 hours.

T. thiooxidans strain SM-6 or SM-7 (Lizama and Suzuki, 1988) was grown in Starkey No. 1 medium : 0.3 g $(NH_4)_2SO_4$, 3.5 g K_2HPO_4 , 0.5 g $MgSO_4 \cdot 7H_2O$, 0.25 g $CaCl_2$ and 18 mg $FeSO_4 \cdot 7H_2O$ per liter and adjusted to pH 2.3 with H_2SO_4. Powdered sulfur (10 g/l BDH sulfur) was spread evenly over the surface after inoculation (10%). Cells were grown stationary at 28°C in a 100 ml volume for a total of 4 days.

Mineral samples

The flotation tailings were kindly provided by Cominco Limited, Trail, BC. The tailings contained 3.3 % Zn as sphalerite and 5.5 % Fe as pyrite and were present as a finely ground powder and stored at -20°C until required. The Flin Flon ore samples (-200 mesh) were obtained from the Hudson Bay Mining and Smelting Co. Ltd., Flin Flon, Manitoba, Canada. They consisted of chalcopyrite ($CuFeS_2$), sphalerite and pyrite with small amounts of pyrrhotite and carbonate. The ore sample contained 4.9 % Cu, 12.5 % Zn, 30 % Fe and 37.5 % S. The metal sulfide minerals used were ground to -140 mesh and had a maximum particle size of 100 μm, determined by direct microscopic observations (Lizama and Suzuki, 1990). The pyrite and sphalerite were provided by Dr. M. J. Osbourne, Department of Geological Sciences, University of Manitoba. The pyrite sample contained 45.0 % Fe, 42.3 % S and 12.7 % impurities. The sphalerite sample

contained 63.5 % Zn, 27.2 % S and 9.3 % impurities (Lizama and Suzuki, 1990). Chalcopyrite obtained from the Carolina Biological Supply Company contained 22.1 % Cu, 19.4 % Fe, 27.6 % S and 30.9 % impurities.

Shake flask leaching experiments

Each 250 ml Erlenmeyer flask contained a total of 100 ml of M9K (without $FeSO_4 \cdot 7H_2O$) with varying concentrations of potassium salts (KH_2PO_4, KCl, and KNO_3) and inhibitors (NaN_3 and NaCN). The flotation tailings and Flin Flon ore experiments each contained 5 grams of sample, while the remainder of the tests maintained the 0.165 g Zn and 0.275 g Fe concentrations (0.26 g sphalerite and 0.61 g pyrite or 1.42 g chalcopyrite) in order to mimic the iron and zinc content in the tailings sample. A 5 % (5 ml) bacterial inoculum of *T. ferrooxidans* (SM-4) or *T. thiooxidans* (SM-6 or SM-7) was used unless otherwise stated. The flasks remained stationary at 25°C for 24 hours to allow for initial growth development. They were then transferred to a rotary shaker at 180 rpm for the remainder of the experiment (13-20 days).

Sample analysis

Within the course of the experiment intermittent samples were taken to test for bacterial activity (i.e. level of mineral solubilization). Prior to sampling each culture was vigorously shaken to allow for even mineral distribution. Each 5 ml sample was passed through Whatman No. 1 filter paper to remove the majority of the insoluble metal sulfides. The leachate was then tested using atomic absorption spectrophotometry (Varian SpectAA-20) to measure dissolved iron, zinc and copper. The level of metal extraction was calculated by comparing the amount of solubilized metal to that found in the original mineral sample.

RESULTS

Bacterial Mineral Extraction

Flotation Tailings

Initial experiments were performed in conjunction with Cominco to develop a system of selective zinc solubilization. Tailings contained 3.3 % zinc and 5.5 % iron. Addition of bacteria to the tailings resulted in the rapid solubilization of both sphalerite (ZnS) and pyrite (FeS_2). The experiment was divided in three based on the type of bacteria used - *T. ferrooxidans*, *T. thiooxidans* or a combination of the two. Table 1 shows the percent extraction of zinc and iron in each of the three experiments. Overall, *T. ferrooxidans* leached up to 100 % of the zinc and 50 % of the iron; *T. thiooxidans* solubilized less than 70 % zinc and 25 % iron; while the mixed bacterial inoculum managed up to 95 % zinc and 65 % iron leaching. Among the inhibitors of iron oxidation phosphate was the most effective in reducing iron solubilization using either *T. ferrooxidans*, *T. thiooxidans* or a mixture of the two.

Phosphate is required for normal bacterial function (Beck and Shafia, 1964). Cells grown under phosphate limitation develop a filamentous morphology due to a lack of cell division (Seeger and Jerez, 1993). Phosphate starvation studies show changes in the synthesis of a number of proteins, some of which have been linked to the bacterial surface suggesting the existence of a phosphate scavenging system in *T. ferrooxidans* (Seeger and Jerez, 1992; Jerez et al., 1992). Phosphate concentrations used in this paper were not limiting but rather in excess. Iron solubilization with *T. ferrooxidans* alone was inhibited by 25 mM phosphate, while only 10 mM phosphate was sufficient with a mixture of *T. ferrooxidans* and *T. thiooxidans*. Zinc leaching was also reduced by phosphate but to a much lesser extent.

Table 1. Effect of anions and inhibitors on bacterial leaching of flotation tailings[a]

	T. ferrooxidans		*T. thiooxidans*		Both	
Growth medium	% Fe	% Zn	% Fe	% Zn	% Fe	% Zn
M9K	55	93	18	57	58	87
10 mM K-Pi	55	93	< 1	42	2	71
25 mM K-Pi	4	60	< 1	33	3	53
50 mM K-Pi	2	52	1	37	2	45
75 mM K-Pi	2	49	1	35	3	42
100 mM K-Pi	2	52	1	31	3	43
10 mM KCl	33	99	15	60	59	92
25 mM KCl	36	97	16	58	54	77
50 mM KCl	36	95	11	60	45	78
75 mM KCl	30	95	9	55	43	80
100 mM KCl	20	78	8	54	40	79
10 mM KNO_3	24	104	19	61	37	84
25 mM KNO_3	20	95	10	54	39	87
50 mM KNO_3	8	78	2	39	19	58
75 mM KNO_3	3	51	1	34	12	42
100 mM KNO_3	< 1	37	< 1	28	11	36
0.1 μM NaN_3	54	100	22	61	59	88
0.5 μM NaN_3	49	100	15	64	60	90
1 μM NaN_3	47	100	17	61	59	90
5 μM NaN_3	53	90	20	67	58	90
0.1 μM NaCN	51	97	15	66	63	93
0.5 μM NaCN	55	100	20	61	63	93
1 μM NaCN	54	100	17	65	60	92
5 μM NaCN	55	100	19	58	59	88

[a]Flotation tailings (5g) containing 5.5 % Fe (0.275 g) and 3.3 % Zn (0.165 g) were leached in shake flask experiments at 25°C for 14 days in 100 ml M9K medium with and without additional salts. Bacterial cultures used were 5 % inoculum of *T. ferrooxidans* (SM-4) or *T. thiooxidans* (SM-6) or both. Extent of leaching is shown as percent extraction for each metal.

Chloride is a known inhibitor of ferrous iron oxidation and growth of *T. ferrooxidans* (Razzel and Trussel, 1963; Lazaroff, 1977). According to Table 1 the addition of chloride resulted in less than 60 % inhibition of iron solubilization with only a minor effect on zinc solubilization.

Nitrate is an inorganic anion known to inhibit oxidation and growth of *T. ferrooxidans* on ferrous iron (Lazaroff, 1977). It transverses the membrane barriers, entering the cell interior, resulting in a gradual acidification of the cytoplasm. Growth on either ferrous iron or sulfur was inhibited by 50 % at around 50 mM nitrate (Part II and Harahuc et al., submitted for publication). The inhibitory effect of nitrate can be seen in both cell types (Table 1). Nitrate concentrations higher than 50 mM showed a substantial drop in both zinc and iron solubilization.

Azide and cyanide are specific inhibitors of the terminal oxidase in ferrous iron oxidation (Pronk et al., 1991). Using cell suspensions, they specifically inhibited iron oxidation at low concentrations with little to no effect on sulfur oxidation (Part II and Harahuc et al., submitted for publication). According to Table 1, however, they had no effect on the solubilization of either zinc or iron after 14 days. The extended incubation period used for the shake flasks must have resulted in the loss of inhibitors due to evaporation of the protonated forms at acidic pH ($N_3^- + H^+ \leftrightarrow HN_3$, pKa = 4.7 and $CN^- + H^+ \leftrightarrow HCN$, pKa = 9.2).

Pyrite-Sphalerite Mixture

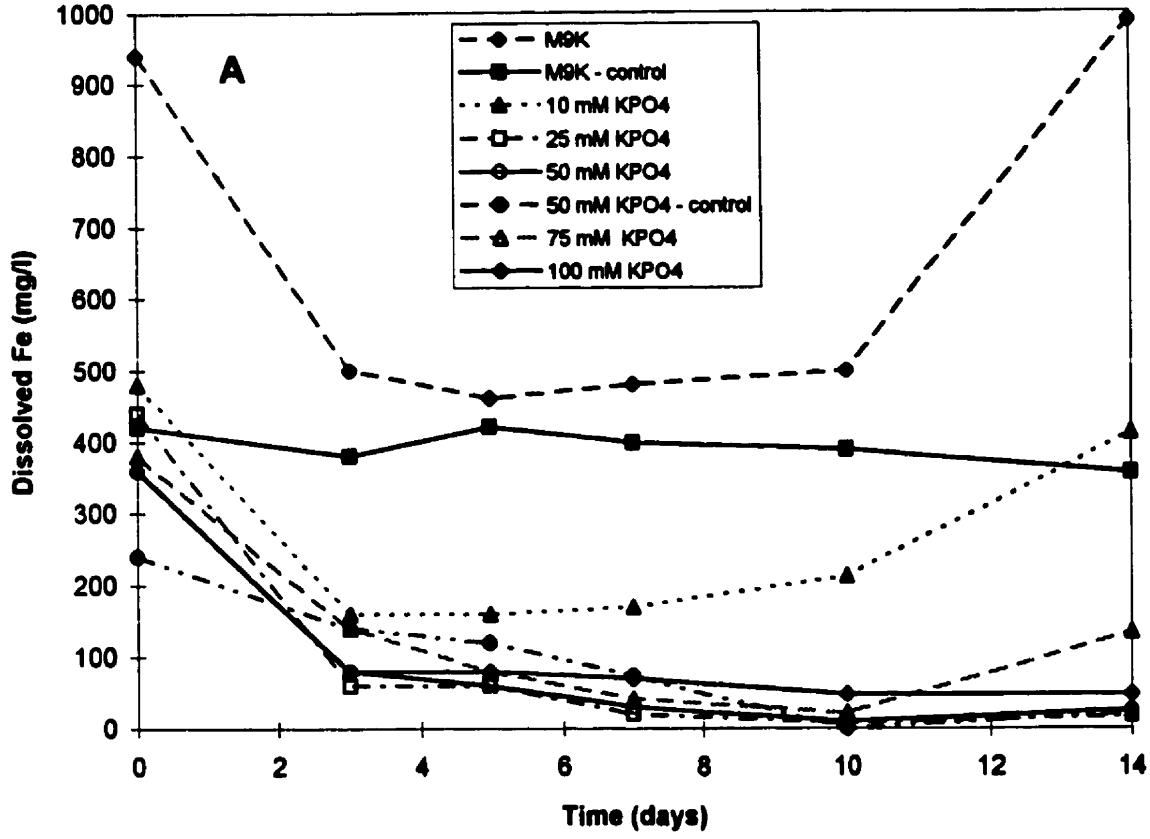
The second part of this chapter deals with the selective solubilization of metals from a mixture of known minerals. Experimental conditions were identical to those used for tailings with the exception of azide and cyanide, which were no longer used. In order

to account for the possibility of chemical mineral solubilization, uninoculated controls were run simultaneously for selected flasks. In the absence of cells (Fig. 1A) the level of dissolved iron remained relatively unchanged over the 14 day incubation period showing a lack of significant chemical pyrite solubilization. The addition of cells to the M9K medium increased initial dissolved iron concentrations to 925 mg/l - the apparent rise due to the addition of soluble iron which accompanied the 5 % bacterial inoculum. Over a period of three days the dissolved iron concentration dropped to the level of the chemical control. (This coincided with a ferric iron precipitate observed on the flask interior.) At 14 days the dissolved iron concentration jumped to 1000 mg/l, indicating active bacterial pyrite solubilization. The M9K medium provided *T. ferrooxidans* with an optimum growth environment in which to utilize the mineral substrates and thus represented a bacterial control for the experiments shown in Figures 1-3.

The addition of 10-100 mM K-Pi to the M9K medium significantly inhibited iron extraction. On inoculation the initially clear medium took on a cloudy appearance with the formation of a ferric phosphate precipitate. Initial levels of dissolved iron were therefore substantially lower than that found in the M9K medium. Over time these iron concentrations continued to drop with the exception of 10 mM K-Pi in which iron concentrations rebounded at 10 days indicating the presence of pyrite solubilization. The continued drop in dissolved iron at the higher phosphate concentrations was likely due to ferric phosphate precipitation as a white precipitate was observed.

Sphalerite undergoes solubilization via sulfide oxidation ($ZnS + 2O_2 \rightarrow ZnSO_4$). In the absence of cells (M9K control) only 25 % of the zinc was extracted at the end of the 14 day experiment (Fig. 1B, Table 2). The addition of cells raised this final value to 74

Figure 1. Effect of potassium phosphate on the solubilization of Fe (A) and Zn (B) from a mixture of pyrite and sphalerite.


A
M9K
M9K - control
10 mM KPO4
25 mM KPO4
50 mM KPO4
50 mM KPO4 - control
75 mM KPO4
100 mM KPO4
Dissolved Fe (mg/l)
Time (days)
0
100
200
300
400
500
600
700
800
900
1000
0
2
4
6
8
10
12
14

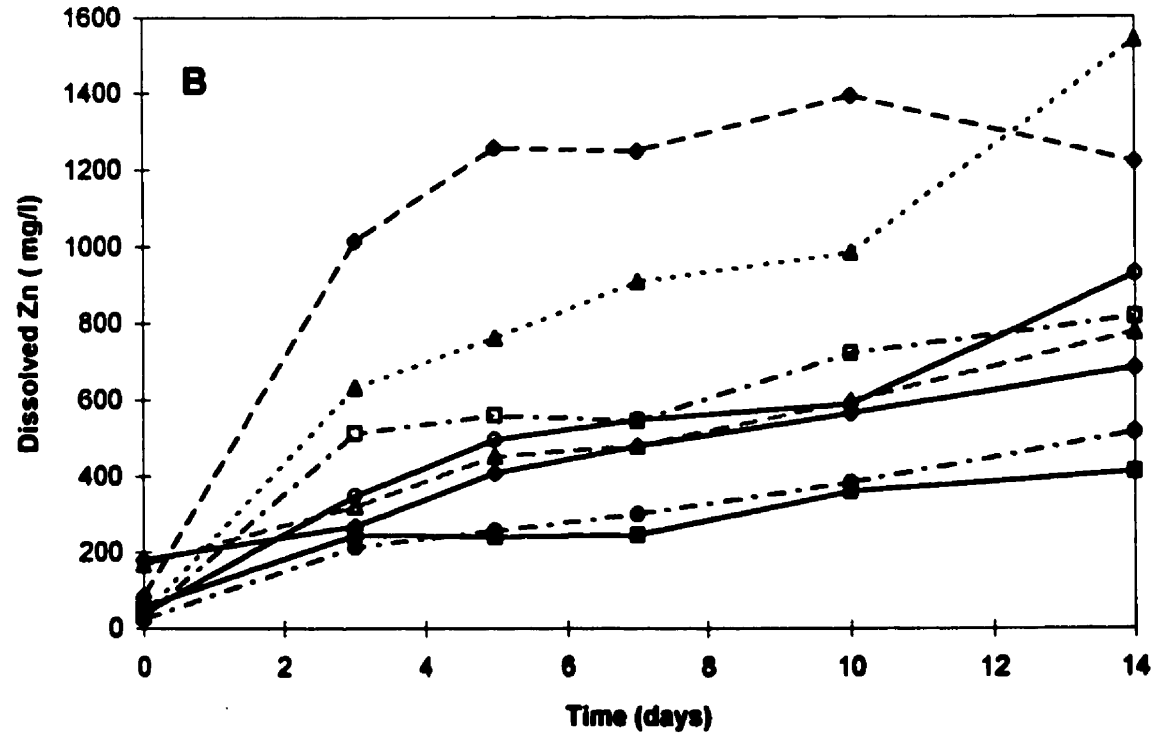

B
Dissolved Zn (mg/l)
Time (days)
0
200
400
600
800
1000
1200
1400
1600
0
2
4
6
8
10
12
14

Table 2. Effect of anions on bacterial leaching of pyrite and sphalerite mixtures[a]

	T. ferrooxidans		Both	
Growth medium	% Fe	% Zn	% Fe	% Zn
M9K	36	74	40	64
M9K - control[b]	13	25	16	30
10 mM K-Pi	15	93	17	53
25 mM K-Pi	1	49	5	49
50 mM K-Pi	1	56	5	43
50 mM K-Pi-control[b]	1	31	3	36
75 mM K-Pi	5	47	4	41
100 mM K-Pi	2	41	6	40
10 mM KCl	17	87	42	100
25 mM KCl	13	82	38	98
50 mM KCl	15	93	44	89
50 mM KCl-control[b]	14	29	16	33
75 mM KCl	14	98	40	100
100 mM KCl	13	94	38	100
10 mM KNO_3	18	94	43	100
25 mM KNO_3	13	88	36	77
50 mM KNO_3	25	40	27	31
50 mM KNO_3-control[b]	13	30	15	30
75 mM KNO_3	31	35	26	33
100 mM KNO_3	26	34	26	31

[a]Pyrite (0.61 g) and shalerite (0.26 g) powder below 140 mesh were leached in shake flask experiments at 25°C for 14 days in 100 ml M9K medium with and without additional salts after 5 % inoculum of *T. ferrooxidans* (SM-4) culture or a 10 % mixture of *T. ferrooxidans* and *T. thiooxidans* (5 % each) cultures.

[b]Control: medium leaching without bacterial inoculation.

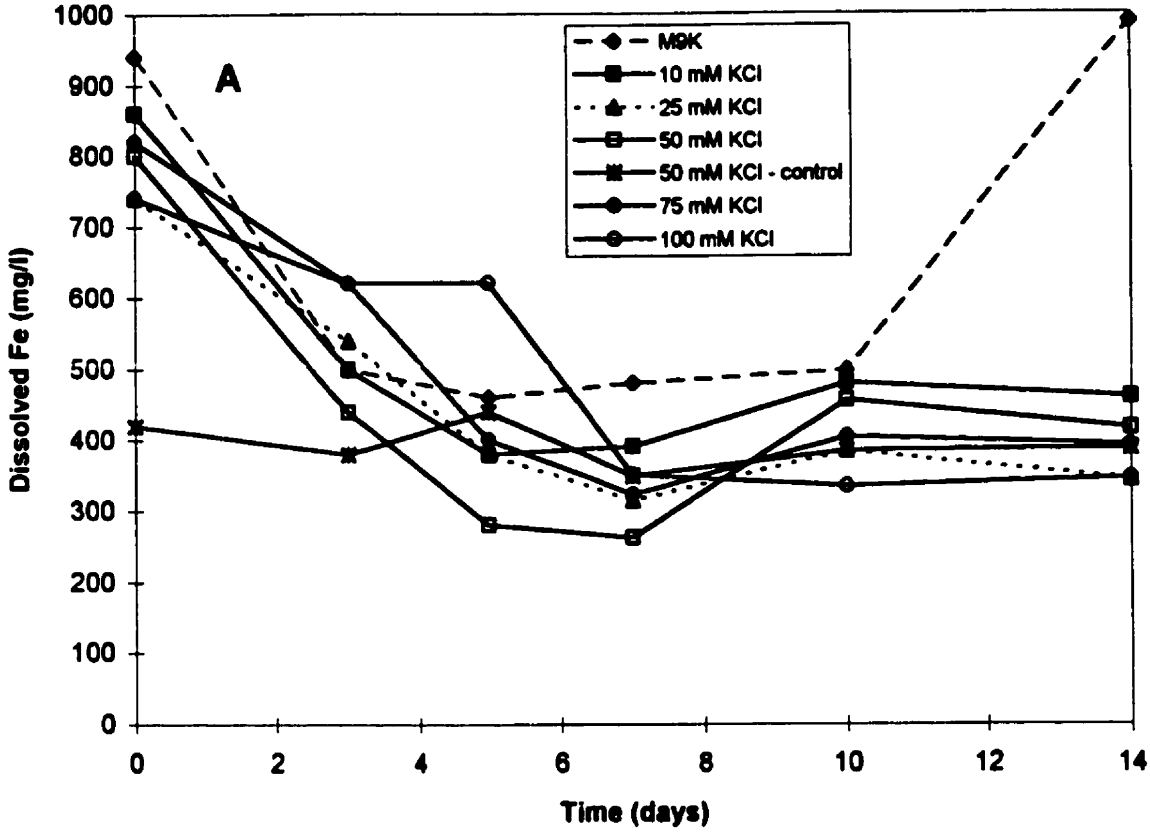
74%, with the majority of the activity taking place within the first 5 days. Phosphate at 10 mM showed a gradual increase in zinc extraction peaking at 14 days with 93 % Zn extracted. Higher phosphate concentrations, although showing similar patterns of activity inhibited overall zinc extraction with final percentages of only 41-49 % (Table 2).

Chloride (Fig. 2A) markedly inhibited pyrite solubilization at all of the concentrations tested. Initial levels of dissolved iron were around 800 mg/l - half of which came from the pyrite (as can be seen with the 50 mM KCl uninoculated control) and half from the bacterial inoculum. Over time the dissolved iron concentrations dropped to 400-500 mg/l, which coincided with the appearance of an orange-brown precipitate (jarosite) lining the flask interior. Zinc solubilization (Fig. 2B) in the chloride containing flasks was noticeably inhibited (as compared to the M9K flask) up to 7 days. Beyond this point chloride appeared to enhance zinc solubilization reaching nearly 100 % (Table 2).

Nitrate, the final anion tested showed two distinct patterns of activity with regard to iron and zinc solubilization (Fig. 3). At 10 and 25 mM nitrate dissolved iron concentrations decreased over time to that of the chemical control. Higher nitrate concentrations retained their initial dissolved iron concentrations showing only minor fluctuations over time. Nitrate is a known anion able to bind ferric iron as ferric nitrate - a highly soluble compound. It is likely that the ferric iron present after inoculation was chelated by nitrate (above 50 mM) forming ferric nitrate thus maintaining high dissolved iron levels. Initial iron concentration of 800 mg/l would have required a minimum of 43 mM KNO_3 to chelate all of the iron.

Zinc solubilization (Fig. 3B) also showed two distinct patterns of activity based on nitrate content. The lower nitrate concentration (10-25 mM) inhibited initial zinc

Figure 2. Effect of potassium chloride on the solubilization of Fe (A) and Zn (B) from a mixture of pyrite and sphalerite.


A
M9K
10 mM KCl
25 mM KCl
50 mM KCl
50 mM KCl - control
75 mM KCl
100 mM KCl
Dissolved Fe (mg/l)
Time (days)
0
100
200
300
400
500
600
700
800
900
1000
0
2
4
6
8
10
12
14

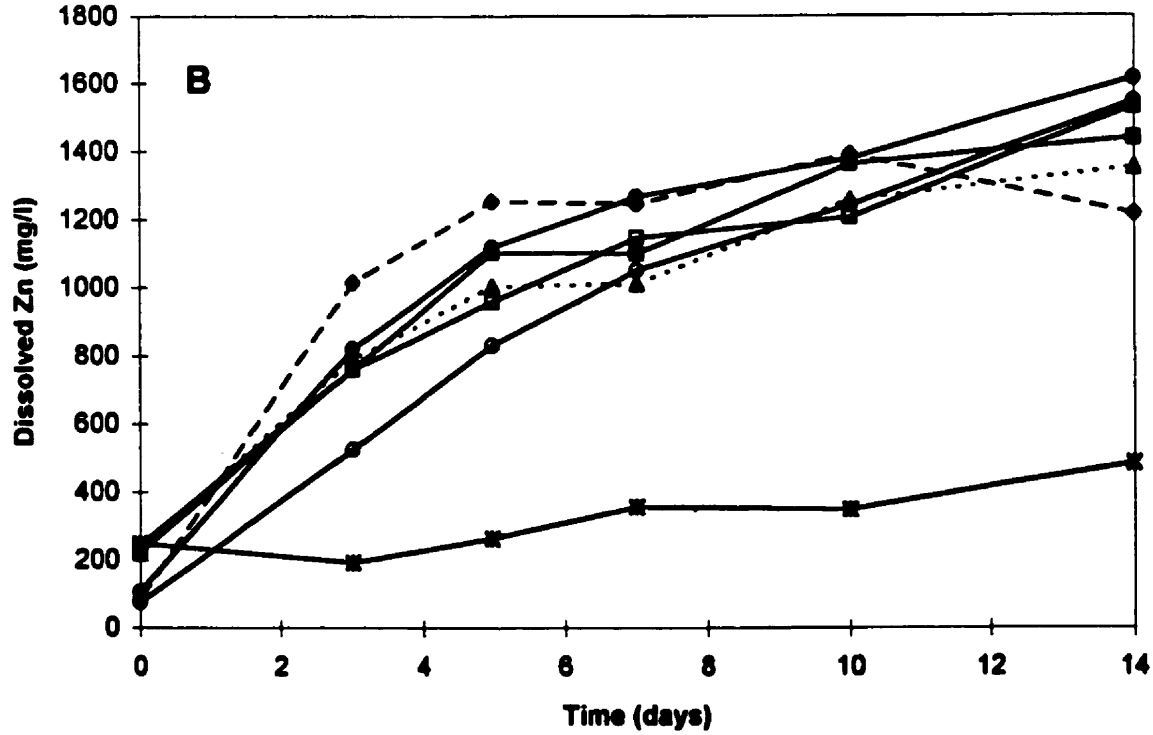
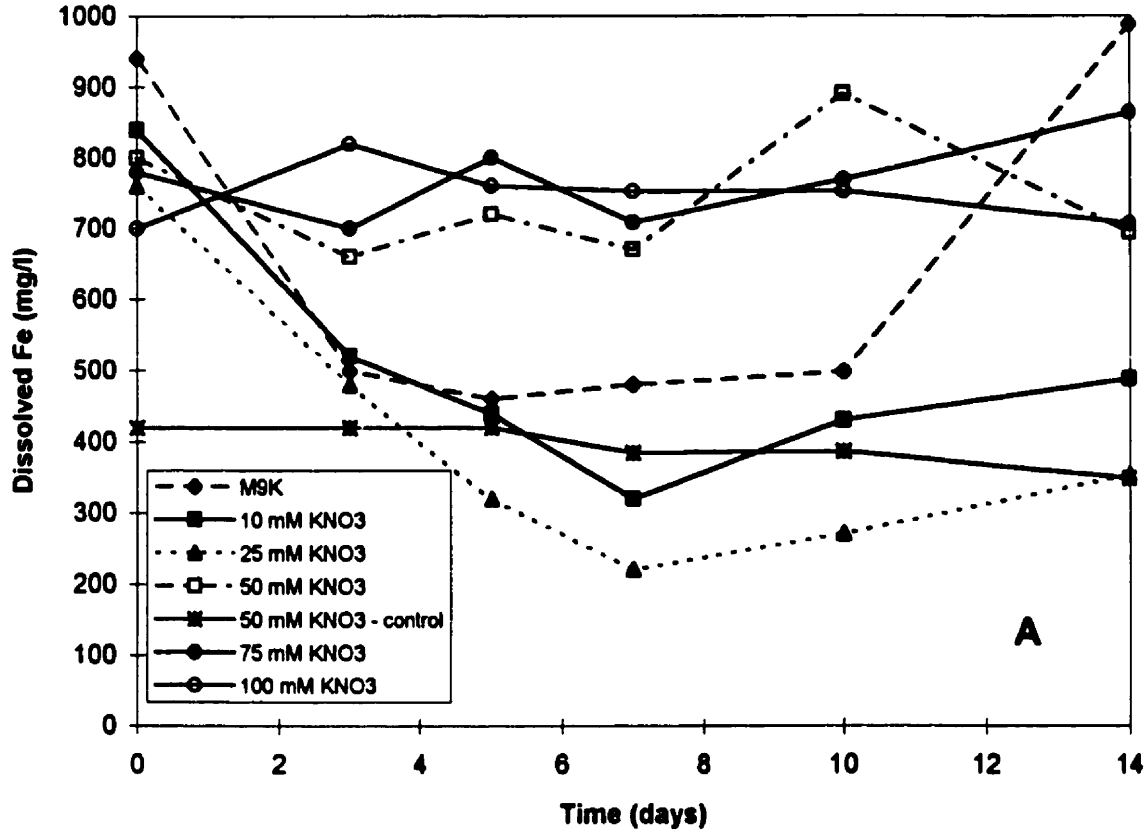
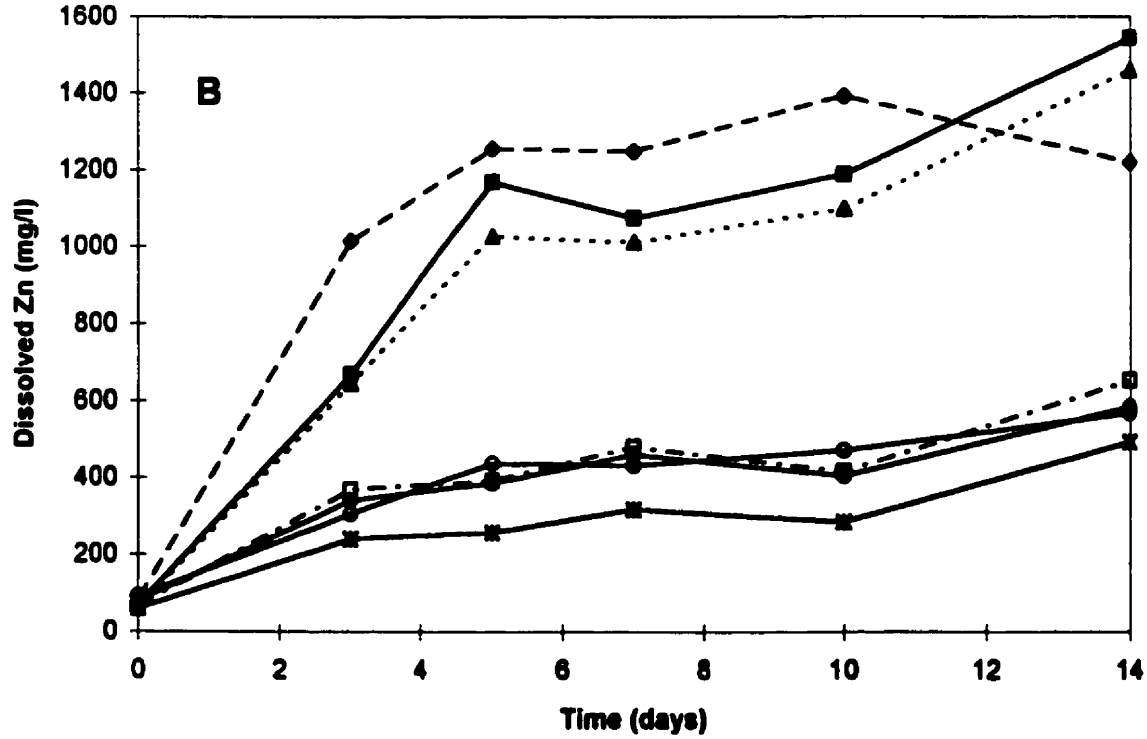

B
Dissolved Zn (mg/l)
Time (days)
0
200
400
600
800
1000
1200
1400
1600
1800
0
2
4
6
8
10
12
14

Figure 3. Effect of potassium nitrate on the solubilization of Fe (A) and Zn (B) from a mixture of pyrite and sphalerite.


Dissolved Fe (mg/l)
Time (days)
M9K
10 mM KNO3
25 mM KNO3
50 mM KNO3
50 mM KNO3 - control
75 mM KNO3
100 mM KNO3
A


Dissolved Zn (mg/l)
Time (days)
B

solubilization (when compared to the M9K flask). At 14 days, however, zinc extraction in these nitrate flasks surpassed that of the bacterial control (M9K flask) with 88-94 % zinc extraction. Higher nitrate concentrations (50-100 mM), on the other hand, inhibited zinc solubilization throughout the entire growth period with final levels of zinc extraction similar to that of the uninoculated control (50 mM KNO_3-control), thus suggesting inhibition of both bacterial growth and activity. A summary of the iron and zinc extraction levels for all of the anions tested could be found in Table 2. The anionic effects observed with the pyrite-sphalerite mixture are in agreement with those found in Table 1 using the flotation tailings.

In order to test the potential of other bacteria in selective mineral solubilization the above experiment was repeated using 5 % *T. thiooxidans* strain SM-7 and a 10 % mixture of *T. ferrooxidans* (5%) and *T. thiooxidans* (5%) (which would mimic a natural environment in which both bacteria are present). *T. thiooxidans* proved ineffective over the 14 day experiment showing zinc and iron extraction levels similar to that of the chemical control (data not shown). The bacterial combination, however, showed zinc extraction approaching 100 % and iron extraction up to 44 % (Table 2). The higher levels of both zinc and iron extraction in the bacterial combination may be due to the increased acidity created by the *T. thiooxidans* - added production of sulfuric acid would drop the pH possibly stimulating initial zinc solubilization and allowing more iron to remain in solution (rather than precipitate as jarosite).

The most promising results in selective leaching of zinc up to this point were achieved only with phosphate and chloride. Phosphate inhibited iron solubilization, but at higher concentrations it also inhibited zinc solubilization. Chloride, on the other hand,

stimulated zinc leaching while maintaining iron at the level of the chemical control. Due to these unique characteristics, a phosphate-chloride mixture was tested for its effects on mineral solubilization (Table 3). Phosphate maintained low levels of iron solubilization, but as with previous tests higher concentrations inhibited zinc solubilization. We had hoped the addition of chloride might relieve this inhibition, but it did not.

Pyrite or Sphalerite

In an attempt to show the importance of galvanic interactions between minerals in metal solubilization *T. ferrooxidans* was grown on pyrite (FeS_2) or sphalerite (ZnS) alone. Zinc solubilization in sphalerite as previously mentioned requires only sulfide oxidation ($ZnS + 2O_2 \rightarrow ZnSO_4$). Table 4 shows in the absence of cells only 9 % Zn extraction. The addition of cells raised this number up to 50 %. This value, however, was substantially lower than the nearly 100 % zinc extraction observed in the pyrite-sphalerite mixture. In the mineral mixture the sphalerite acts as the anode, preferentially being oxidized to zinc sulfate while the pyrite acts as the cathode showing little to no solubilization.

Pyrite solubilization requires both iron and sulfide oxidation ($4FeS_2 + 15O_2 + 2H_2O \rightarrow 2Fe_2(SO_4)_3 + 2H_2SO_4$). In the absence of cells 16 % iron was solubilized. The addition of cells raised this value to 48 %. The presence of phosphate significantly inhibited iron extraction. Phosphate, is a metal chelator that can bind ferric iron producing ferric phosphate precipitate. Based on the observed data, however, it was difficult to determine if pyrite solubilization was inhibited or if the phosphate was simply precipitating the ferric iron that was produced (as ferric phosphate). Chloride and nitrate both inhibited iron oxidation 10-20 % (as compared to the M9K flask). They have the potential to

Table 3. Effect of chloride-phosphate combinations on bacterial leaching of pyrite and sphalerite mixtures[a]

	5 % *T. ferrooxidans*	
Growth medium	% Fe	% Zn
M9K	44	89
M9K - control[b]	15	25
10 mM KCl + 10 mM K-Pi	25	85
10 mM KCl + 25 mM K-Pi	4	44
10 mM KCl + 50 mM K-Pi-control[b]	6	32
10 mM KCl + 50 mM K-Pi	4	34
25 mM KCl + 10 mM K-Pi	19	80
25 mM KCl + 25 mM K-Pi	5	40
25 mM KCl + 50 mM K-Pi-control[b]	4	32
25 mM KCl + 50 mM K-Pi	3	35
50 mM KCl + 10 mM K-Pi	16	78
50 mM KCl + 25 mM K-Pi	2	38
50 mM KCl + 50 mM K-Pi-control[b]	3	33
50 mM KCl + 50 mM K-Pi	2	33
75 mM KCl + 10 mM K-Pi	11	76
75 mM KCl + 25 mM K-Pi	1	30
75 mM KCl + 50 mM K-Pi-control[b]	2	34
75 mM KCl + 50 mM K-Pi	2	32
100 mM KCl + 10 mM K-Pi	9	65
100 mM KCl + 25 mM K-Pi	2	37
100 mM KCl + 50 mM K-Pi-contol[b]	3	33
100 mM KCl + 50 mM K-Pi-control[b]	2	27

[a]Pyrite (0.61 g) and sphalerite (0.26 g) powder below 140 mesh were leached in shake flask experiments at 25°C for 14 days in 100 ml M9K medium with and without additional salts after 5 % inoculum of *T. ferrooxidans* (SM-4) culture. Extent of leaching is shown as percent extraction for each metal.

[b]Control: medium leaching without bacterial inoculation.

Table 4. Effect of anions on bacterial leaching of pyrite or sphalerite alone[a]

	FeS_2	ZnS
Growth medium	% Fe	% Zn
M9K	48	50
M9K - control[b]	16	9
10 mM K-Pi	17	18
25 mM K-Pi	6	17
50 mM K-Pi	5	18
50 mM K-Pi-control[b]	4	9
75 mM K-Pi	5	16
100 mM K-Pi	5	16
10 mM KCl	41	51
25 mM KCl	32	54
50 mM KCl	32	54
50 mM KCl-control[b]	17	9
75 mM KCl	26	55
100 mM KCl	26	55
10 mM KNO_3	39	56
25 mM KNO_3	27	53
50 mM KNO_3	32	17
50 mM KNO_3-control[b]	17	8
75 mM KNO_3	27	17
100 mM KNO_3	30	18

[a]Pyrite (0.61 g) and sphalerite (0.26 g) powder below 140 mesh were leached in shake flask experiments at 25°C for 14 days in 100 ml M9K medium with and without additional salts after 5 % inoculum of *T. ferrooxidans* (SM-4) culture. Extent of leaching is shown as percent extraction for each metal.

[b]Control: medium leaching without bacterial inoculation.

chelate ferric iron forming ferric chloride or ferric nitrate. Unlike ferric phosphate, however, ferric chloride and ferric nitrate are quite soluble in water. Thus the observed inhibition was likely due to the blockage of iron oxidation. Zinc extraction from sphalerite was inhibited by phosphate and to a lesser extent by nitrate. Chloride showed a slight stimulation.

Chalcopyrite-Sphalerite Mixture

We next tested a more complicated mixture of minerals containing iron, zinc and copper. The chemical solubilization level was 9 % iron, 16 % zinc and 2 % copper (Table 5). The addition of bacteria increased solubilization levels of all metals to 11 %, 62 % and 9 % respectively. The presence of phosphate reduced iron solubilization keeping it at or below that of the chemical control. Copper solubilization rose with increasing phosphate concentrations up to 50 mM. Zinc leaching was stimulated at 10 mM but inhibited at higher phosphate concentrations. The addition of chloride did not relieve this inhibition (data not shown). Chloride itself produced high levels of zinc solubilization while maintaining the copper and iron at low levels. Nitrate was inhibitory at concentrations above 10 mM with metal extraction rates similar to those of the chemical control.

Complex sulfide ore

Flin Flon ore, which is a complex sulfide ore containing 4.9 % Cu, 12.5 % Zn, 30 % Fe and 37.5 % S, includes pyrite, chalcopyrite and sphalerite with small amounts of carbonates and pyrrhotite (Lizama and Suzuki, 1988). In the absence of cells in M9K medium, 3 % Fe, 27 % Zn and 10 % Cu were extracted after 21 days (Table 6). In the presence of *T. ferrooxidans*, these values increased to 23 % Fe, 75 % Zn and 14 % Cu. Low concentrations (< 50 mM) of phosphate increased copper extraction while those of

Table 5. Effect of anions on bacterial leaching of a chalcopyrite and sphalerite mixture[a]

Growth medium	Metal solubilized (%)		
	Fe	Zn	Cu
M9K	11	62	9
M9K - control[b]	9	16	2
10 mM K-Pi	9	73	12
25 mM K-Pi	5	49	14
50 mM K-Pi	2	33	20
50 mM K-Pi-control[b]	2	27	3
75 mM K-Pi	8	39	16
100 mM K-Pi	3	33	20
10 mM KCl	4	100	7
25 mM KCl	4	94	8
50 mM KCl	4	100	8
50 mM KCl-control[b]	8	24	< 1
75 mM KCl	5	100	8
100 mM KCl	1	41	5
10 mM KNO_3	10	88	9
25 mM KNO_3	19	26	3
50 mM KNO_3	21	22	3
50 mM KNO_3-control[b]	7	19	2
75 mM KNO_3	20	23	4
100 mM KNO_3	20	24	3

[a]Chalcopyrite (1.42 g) and sphalerite (0.26 g), -140 mesh, were leached as described in Table 3.

[b]Control: medium leaching without bacterial inoculation.

Table 6. Effect of anions on bacterial leaching of a complex sulfide ore[a]

	Metal solubilized (%)					
	T. ferrooxidans			*T. ferrooxidans*+ *T. thiooxidans*		
Growth medium	% Fe	% Zn	% Cu	% Fe	% Zn	% Cu
M9K	23	75	14	23	84	19
M9K - control[b]	3	27	10	3	29	11
10 mM K-Pi	21	67	30	28	95	25
25 mM K-Pi	25	85	36	11	42	19
50 mM K-Pi	2	59	27	10	35	15
50 mM K-Pi-control[b]	1	35	16	< 1	29	12
75 mM K-Pi	< 1	39	16	10	28	14
100 mM K-Pi	1	32	13	10	28	15
10 mM KCl	22	97	20	22	100	53
25 mM KCl	8	100	44	16	100	46
50 mM KCl	3	87	37	14	100	32
50 mM KCl-control[b]	1	32	10	1	36	< 1
75 mM KCl	< 1	40	16	14	100	29
100 mM KCl	1	39	3	9	93	23
10 mM KNO_3	< 1	37	14	14	100	15
25 mM KNO_3	1	32	12	7	76	13
50 mM KNO_3	1	32	11	11	31	17
50 mM KNO_3-control[b]	1	34	< 1	1	29	11
75 mM KNO_3	1	30	12	6	33	13
100 mM KNO_3	2	32	12	5	33	12
10 mM KCl + 10 mM K-Pi	31	100	53	36	100	23
10 mM KCl + 25 mM K-Pi	30	97	24	27	68	23
10 mM KCl + 25 mM K-Pi-control[b]	< 1	32	7	1	35	10
10 mM KCl + 50 mM K-Pi	10	83	54	27	44	43

[a]A complex sulfide ore from Flin Flon (5 g) was leached as described in Table 3 with 5 % inoculum of *T. ferrooxidans* (SM-4) with and without additional 5 % *T. thiooxidans* (SM-7) inoculum for 21 days.

[b]Control: medium leaching without bacterial inoculation.

chloride stimulated both zinc and copper solubilization. Higher concentrations produced values similar to those of the chemical control. Iron extraction was inhibited by both phosphate and chloride. In the presence of nitrate, iron, zinc and copper extraction levels were identical to those of the chemical control.

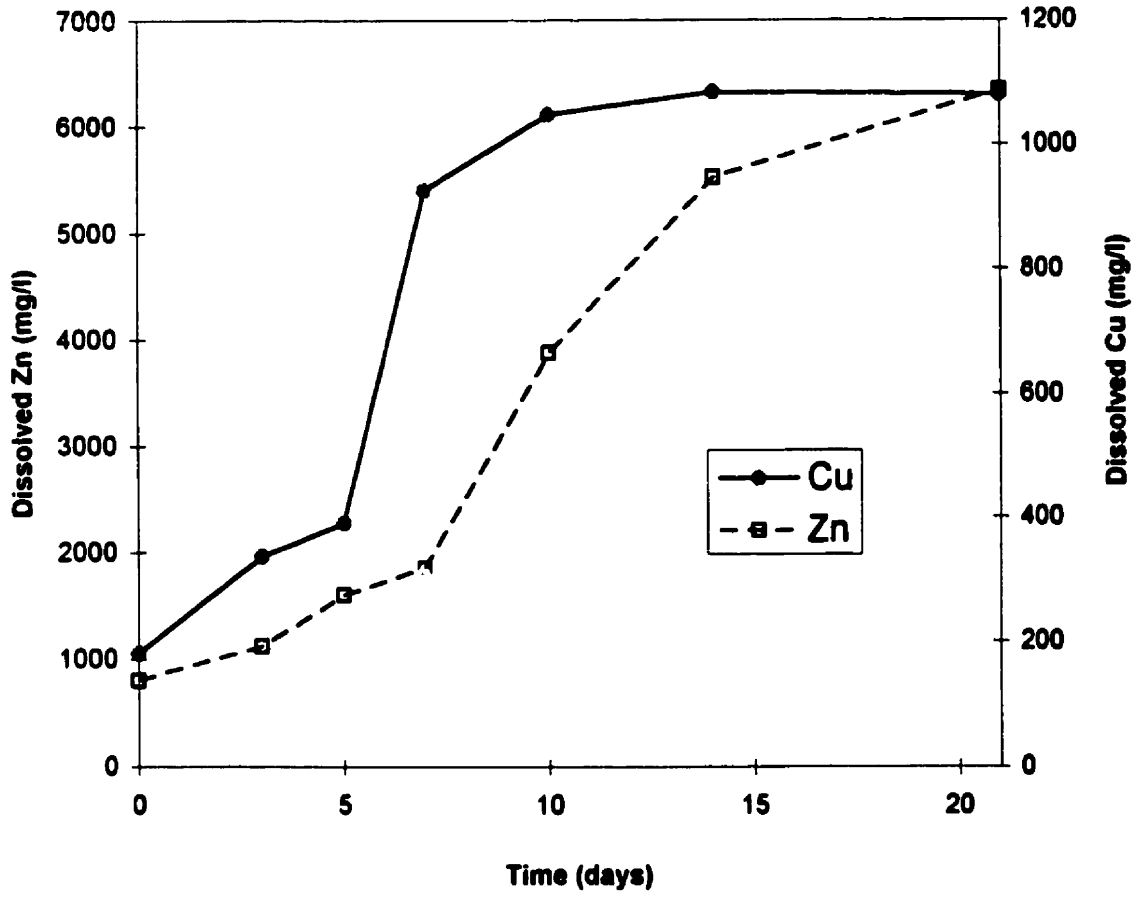
T. thiooxidans (strain SM-7) was not as effective as *T. ferrooxidans* (strain SM-4) at leaching Flin Flon ore. Iron, zinc and copper concentrations showed only a slight improvement over the chemical control (data not shown). The combination of the two organisms (Table 6), however, surpassed *T. ferrooxidans* alone particularly with Zn extraction in KCl. The addition of *T. thiooxidans* introduces a small amount of sulfur (which could be used as an alternate energy source) but also a drop in pH. Sulfur grown *T. thiooxidans* produces vast quantities of sulfuric acid, such that the pH after 4 days of growth is pH 1.0 - 1.3. It is likely that this initial drop in pH as well as the addition of sulfate allowed the bacterial mixture to generate the high concentrations of zinc and copper even at elevated chloride concentrations. Phosphate, chloride and nitrate all inhibited iron solubilization at concentrations greater than 10 mM in the presence of these two organisms. Zinc extraction was complete at all chloride concentrations. Phosphate and nitrate both inhibited zinc solubilization at concentrations greater than 10 mM. Copper solubilization was stimulated by chloride, reaching greater than 50 % extraction at 10-25 mM chloride.

Since chloride and phosphate suppressed iron solubilization and enhanced zinc and copper solubilization, it was hoped that a chloride-phosphate mixture would improve overall extraction levels (Table 6). With *T. ferrooxidans*, 10 mM chloride counteracted the phosphate inhibition of zinc leaching up to 50 mM phosphate, achieving nearly 100 %

zinc extraction and over 50 % copper solubilization. Overall metal extraction of a mixture of *T. ferrooxidans* and *T. thiooxidans* was not improved using the chloride-phosphate combination.

In most of the experiments in Table 6 the final pH of the ore suspension medium was pH 1.5-2.0 when the leaching of zinc and copper was high, indicating high levels of sulfuric acid production from sulfur oxidation. Control flasks without bacteria had a final pH of pH 3.5-3.7. The time course elution profiles of zinc and copper also proved interesting for the complex sulfide ore. According to figure 4, *T. ferrooxidans* extracted copper prior to zinc with 25 mM KCl added to the M9K medium. Similar results were observed with 50 mM KCl and 25 and 50 mM K-Pi. The bacterial mixture (i.e. *T. ferrooxidans* and *T. thiooxidans*), however, showed comparable results only with 25-50 mM KCl. In either case, these data show the incredible potential of not only selective mineral solubilization but also the possibility of initial copper recovery prior to the leaching and recovery of zinc.

Figure 4. Time course comparison of Cu and Zn extraction from Flin Flon ore in M9K plus 25 mM KCl using *Thiobacillus ferrooxidans.*


7000
6000
5000
4000
3000
2000
1000
0
Dissolved Zn (mg/l)
1200
1000
800
600
400
200
0
Dissolved Cu (mg/l)
0
5
10
15
20
Time (days)
Cu
Zn

DISCUSSION

This study began with a detailed look at sulfur and iron oxidation in *T. ferrooxidans* and their selective controls. Iron and sulfur oxidation use different electron transport systems with the former more sensitive to inhibition by azide (Fukumori et al., 1988; Ingledew et al., 1977; Pronk et al., 1991). We also found sulfur and iron oxidation to be differentially affected by select anions and inhibitors. Phosphate, chloride, nitrate, azide and cyanide all inhibited ferrous iron oxidation more strongly than sulfur oxidation in cell suspensions (Part II and Harahuc et al., submitted for publication). Growth on ferrous iron or sulfur was affected in a similar manner, with phosphate, chloride, azide and cyanide preferentially inhibiting growth on and oxidation of ferrous iron (Part II and Harahuc et al., submitted for publication).

The focus of this work was to apply the selective control of iron and sulfur oxidation to selective mineral solubilization. Initial experiments were performed on flotation tailings containing sphalerite and pyrite with the intention of creating a selective zinc solubilization system. In *T. ferrooxidans*, *T. thiooxidans* and the mixed bacterial cultures, added phosphate lowered iron solubilization but had only minor effects on zinc solubilization (Table 1). McCready estimated the minimum phosphate concentration required for bacterial leaching to be 0.1 - 0.2 mM (McCready, 1986). Our M9K medium had a phosphate concentration of 0.6 mM, substantially higher than the minimum required for growth. Phosphate itself is a metal chelator able to bind to iron in its ferrous and ferric form. On the exterior surface of the cell, phosphate may bind to the ferric iron lattice (Ingledew et al., 1977) surrounding the ferrous iron receptors, thus inhibiting iron oxidation. Phosphate may also bind soluble ferric iron forming a ferric phosphate

precipitate. Iron leaching would then be incorrectly assessed when only the soluble leachate is analyzed.

Experiments in Table 2 using a mixture of sphalerite and pyrite confirmed the results obtained with the tailings, i.e. the phosphate inhibited iron solubilization with minor reduction in zinc solubilization. Chloride partially inhibited iron oxidation but clearly stimulated zinc solubilization. Nitrate at a concentration below 25 mM acted similar to chloride. These results are in agreement with the selective inhibition of iron oxidation by anions favoring the solubilization of sphalerite over pyrite. This is beyond the normal galvanic effect of pyrite on shalerite solubilization reported earlier (Lizama and Suzuki, 1990).

The replacement of pyrite with chalcopyrite as the iron containing mineral generated more complex results (Table 5). Iron solubilization was reduced by both phosphate and chloride, while zinc solubilization was stimulated by chloride as in previous experiments. Copper leaching, however, was unexpectedly stimulated by the addition of phosphate. Further experiments with complex sulfide ore containing pyrite, sphalerite and chalcopyrite (Table 6) confirmed i) the inhibitory effect of phosphate and chloride on iron solubilization, ii) the stimulatory effect of chloride on zinc solubilization and iii) the stimulatory effect of phosphate on copper solubilization. In addition chloride was also effective in stimulating copper solubilization. In some experiments as much as 50 % of the copper was solubilized by *T. ferrooxidans* when both phosphate and chloride were present. Copper solubilization was also enhanced by a mixture of *T. ferrooxidans* and *T. thiooxidans* when chloride was present (Table 6). This extraction was several fold higher

than in an earlier report using the same ore sample (Lizama and Suzuki, 1988). The role of chloride in the stimulation of zinc and copper leaching is likely biological. Chloride is not a good metal chelator and the chemical controls were unaffected by its presence. An earlier study showed that *T. ferrooxidans* SM-4 grown on this ore in the M9K medium could efficiently oxidize both ferrous iron and elemental sulfur (Suzuki et al., 1990). Although these oxidation activities were not measured in the present work, the enhanced leaching of copper and zinc, depressed solubilization of iron and increased production of sulfuric acid (lowering pH) are in agreement with the effect of phosphate and chloride of not only inhibiting iron oxidation, but also stimulating sulfur oxidation. This is supported by recent growth experiments of the organism where 50 mM phosphate or chloride stimulated the oxidation of sulfur but inhibited that of iron (Part II and Harahuc et al., submitted for publication). If indeed the effect by chloride or phosphate is due to its inhibition of iron oxidation and stimulation of sulfur oxidation, the application of this method to other minerals becomes potentially important. Furthermore, the time course differences between copper and zinc leaching (Fig. 4) may be manipulated to facilitate easier recovery of individual metals.

PART IV

DEVELOPMENT OF A CONTINUOUS FERROUS IRON OXIDIZING SYSTEM

INTRODUCTION

The process of mineral extraction is a combination of biological and chemical reactions. The biological or direct mechanism of extraction requires intimate contact between the bacteria and the ore. The insoluble metal sulfide is oxidized by the bacteria to a soluble metal sulfate according to the following reaction: $MS + 2O_2 \rightarrow MSO_4$. An example of this would be the oxidation of pyrite using *Thiobacillus ferrooxidans* ($4\ FeS_2 + 15\ O_2 + 2\ H_2O \rightarrow 2\ Fe_2(SO_4)_3 + 2\ H_2SO_4$). The ferric sulfate produced can then be used in the chemical or indirect mechanism of mineral extraction. In this case the insoluble metal sulfide chemically reacts with the soluble ferric iron forming metal sulfate, ferrous iron and elemental sulfur ($MS + Fe_2(SO_4)_3 \rightarrow MSO_4 + 2\ FeSO_4 + S^0$). The ferrous sulfate and elemental sulfur are used by the surrounding bacteria (*T. ferrooxidans)* as an energy source replenishing the ferric iron supply ($2\ FeSO_4 + ½\ O_2 + H_2SO_4 \rightarrow Fe_2(SO_4)_3 + H_2O$) and maintaining the surrounding environment at an acidic pH ($S^0 + 1½\ O_2 + H_2O \rightarrow H_2SO_4$).

Ferric iron is a powerful oxidizing agent that has been used in numerous industrial applications. The Barite Industries Co. in Kosaka Japan for example uses *T. ferrooxidans* in its treatment of hydrogen sulfide gas (Imaizumi, 1986). The gas is injected into a ferric sulfate solution producing ferrous sulfate, sulfuric acid and elemental sulfur ($H_2S + Fe_2(SO_4)_3 \rightarrow 2\ FeSO_4 + H_2SO_4 + S^0$). The elemental sulfur is removed by solid-liquid separation while the ferrous sulfate is sent to the bacterial oxidation tank. *T. ferrooxidans* oxidizes ferrous to ferric iron and the process is repeated (i.e. the chemical oxidation of hydrogen sulfide).

The use of this single bacterial loop in an otherwise standard chemical treatment process has saved the company 66 % of its normal operating costs. Other testimonials involving such a bacterial loop include acid mine drainage neutralization plants, the treatment of copper process solutions, the desulfurization of waste SO_2 gas etc. (Imaizumi, 1986). Based on the above studies we had hoped to develop a simple ferrous iron oxidizing system that can be used to enhance indirect mineral solubilization at the Quebrada Blanca (QB) copper mine in Chile.

Two reactor types were investigated - fluidized bed and packed bed. Each of which used a cell carrier material that allowed for the development of maximum cell concentrations yet restricted bacterial movement out of the column (through cell carrier attachment). The ferrous iron containing feed would enter at the base of the column and be oxidized by the bacteria prior to leaving as effluent. At the QB mine, however, the column feed would contain not only ferrous iron but also significant concentrations of other metals. Thus the second part of this study will deal with the effect of metal ions on cell growth and ferrous iron oxidation.

MATERIALS AND METHODS

Media. *Thiobacillus ferrooxidans* strain SM-4 was grown in modified 9K medium (M9K): 0.4 g $(NH_4)_2SO_4$, 0.1 g K_2HPO_4, 0.4 g $MgSO_4 \cdot 7H_2O$ and 33.3 g $FeSO_4 \cdot 7H_2O$ per liter adjusted to pH 2.3 with concentrated H_2SO_4.

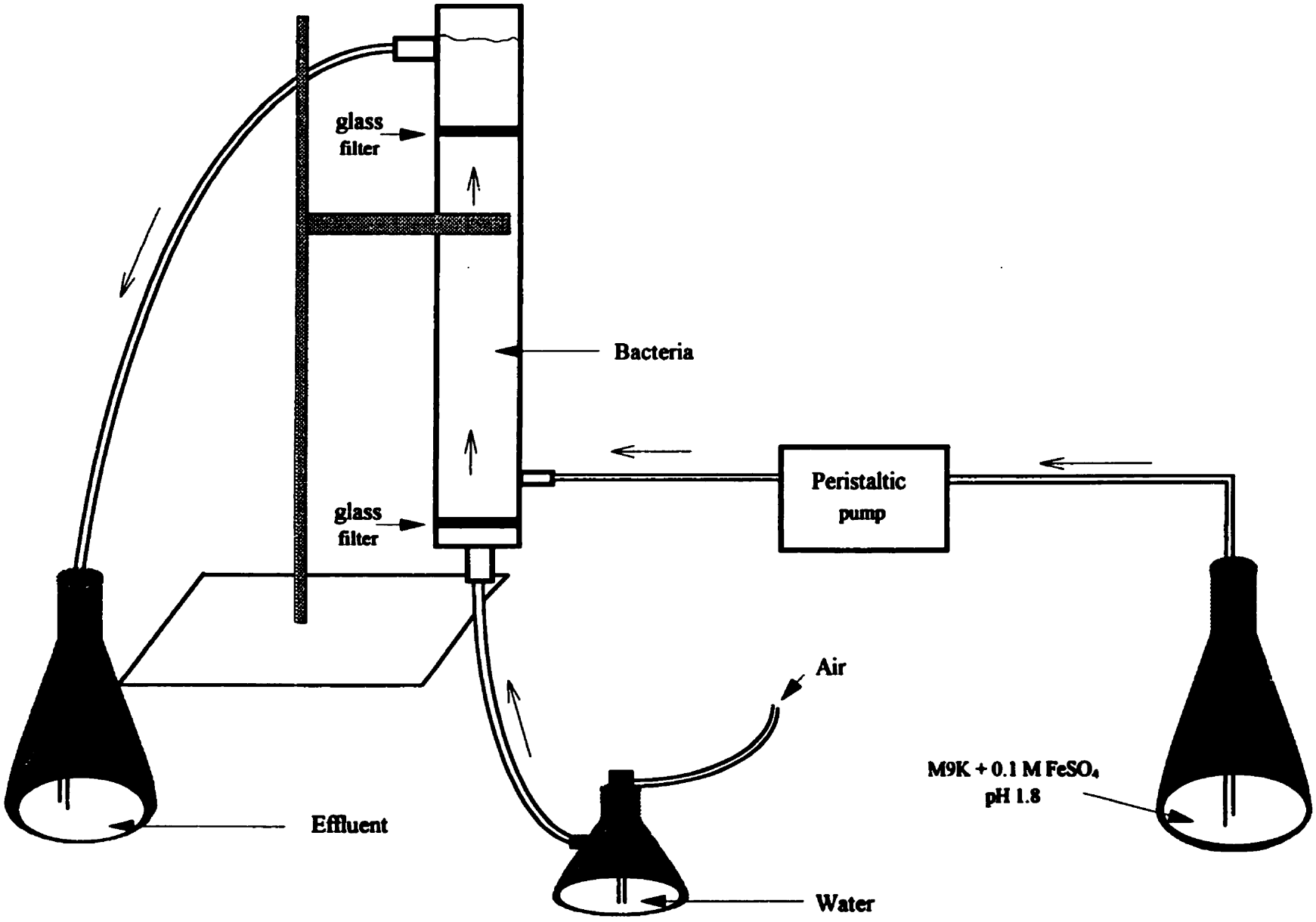
Ore sample. The sulfide ore (-200 mesh) was obtained from the Hudson Bay Mining and Smelting Company Ltd., Flin Flon, Manitoba, Canada. It contained 3.1 % Cu, 9.1 % Zn, 33.4 % Fe and 37.8 % S as pyrite (FeS_2), chalcopyrite ($CuFeS_2$) and sphalerite (ZnS) with small amounts of carbonates and pyrrhotite,

Bacterial adaptation to acidic pH (pH 1.8). A stock culture of *T. ferrooxidans* strain SM-4 was grown on iron enriched M9K using a 10 % inoculum (vol/vol). It was left stationary for 48 hours at 25°C prior to being transferred to an orbital shaker at 150 rpm for an additional 48 hours. This process was repeated six times with progressive drops in pH of 0.1 pH units to pH 1.8. The final flask contained a bacterial culture that actively oxidized ferrous iron at pH 1.8. This culture was used as an inoculum for columns 8 - 14.

Bacterial adaptation to growth on ore at acidic pH (pH 1.8). The above culture of *T. ferrooxidans* (actively oxidizing Fe^{2+} at pH 1.8) was gradually adapted to grow solely on ore through successive subculturing. The substrate ferrous iron in the medium was gradually replaced by increasing amounts of powdered ore (up to 2.5 g). Each flask remained stationary for two to three days followed by shaking at 150 rpm for up to five days. The final flask contained a bacterial culture that actively oxidized ore at pH 1.8. This culture was used as an inoculum for flasks 1 - 2 and columns 1 - 4.

Column setup. The standard column setup can be seen in Figure 1. Each glass column was attached to an upright metal stand using clamps to ensure proper vertical

Figure 1. Schematic representation of the ferrous iron oxidizing bacterial loop. Two column sizes were used: 34 cm x 3 cm and 20 cm x 2 cm. The air was initially filtered through a cotton filter prior to entering the water trap. Moistened air was pushed through the bottom filter. The ferrous sulfate containing M9K (pH 1.8) was pumped into the column at varying speeds using a peristaltic pump.


glass
filter
Bacteria
glass
filter
Peristaltic
pump
Air
M9K + 0.1 M FeSO4
pH 1.8
Effluent
Water

alignment. Aeration was provided using a compressed air system with an adjustable air valve. The air was filtered through a cotton filter tube to remove the majority of dust particles and moistened by passage through a water trap prior to entering the bottom of the column.

Each column was initially grown as a closed system to increase cell numbers and allow for attachment to the cell carrier material. Once bacterial growth was established within the column ferrous iron enriched M9K feed (33.3g $FeSO_4 \cdot 7H_2O$ per liter M9K pH 1.8) was pumped in below the lower filter. The rate of feed was controlled using a peristaltic pump. The overflow left the column above the upper filter and was collected in an Erlenmeyer flask for ferrous iron determination.

Ferrous iron determination. The reaction mixture contained 3 ml Milli Q water, 0.99 ml of 0.1 % orthophenanthroline and 10 µl of sample. The absorbance was measured after 10 minutes of incubation at room temperature at 500 nm. Values were compared to a standard curve using a known concentration of ferrous sulfate to determine the amount of unoxidized iron leaving the system, thus providing direct information regarding bacterial activity.

Solvent extraction - growth studies. The media in the two flasks containing the metal chelators and solvent was prepared using a separatory funnel. The solvent reaction mixture contained 100 ml of M9K (pH 1.8), 90 ml exxsol D-80, 5 ml lix 84 and 5 ml lix 860. Following vigorous mixing for 5 minutes the mixture was allowed to separate into organic and aqueous layers. The solvent flask contained 80 ml of the aqueous layer, 10 ml of the ferrous sulfate solution (1.2 M), and 10 ml of the bacterial inoculum (24 hour

culture of iron grown *T. ferrooxidans* pH 1.8). The flask containing all of the chemicals plus the solvent was prepared in a similar manner.

RESULTS

Ferrous Iron Oxidation in Growing Cell Cultures - Flask Study

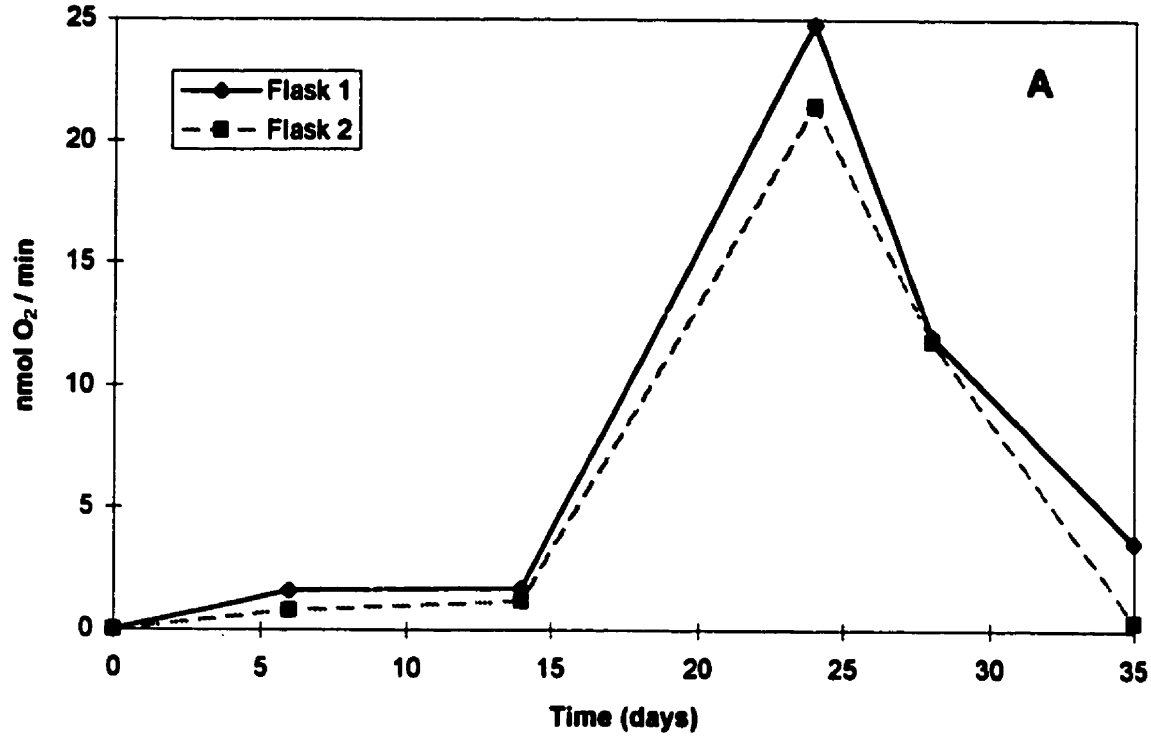
T. ferrooxidans oxidizes iron according to the following reaction: $2Fe^{2+} + ½O_2 + 2H^+ \rightarrow 2Fe^{3+} + H_2O$. Two one liter Erlenmeyer flasks containing 360 ml of M9K pH 1.8, 10 g ore and 40 ml of an ore adapted bacterial inoculum (also pH 1.8) were left stationary in a 25°C incubator for 24 hours. Two forms of aeration were tested to provide maximum bacterial growth and activity. Flask 1 was aerated using an orbital shaker at 150 rpm, while flask 2 was aerated using a magnetic stirrer with a 7.5 cm stir bar. Test samples were periodically removed to monitor bacterial growth and activity over time. Iron oxidation activity was measured using a Gilson oxygraph. The reaction mixture contained 1.19 ml of sample and 1 μmol of ferrous sulfate (10 μl of a 0.1 M solution). Ferrous iron oxidation activity progressively increased in both flasks (Fig. 2A) up to day 25, followed by a drop to day 35. Bacterial cell numbers, as determined by protein content (Fig. 2B) were a reflection of the oxidation profile shown in Figure 2A. Cell numbers increased to peak at 25 days while older cultures showed decreasing cell numbers. The mechanism of aeration had little to no effect on the iron oxidation rate. Figure 2A showed both cultures oxidized ferrous iron at a similar rate. Bacterial cell numbers, however, were slightly higher in the shaken flask.

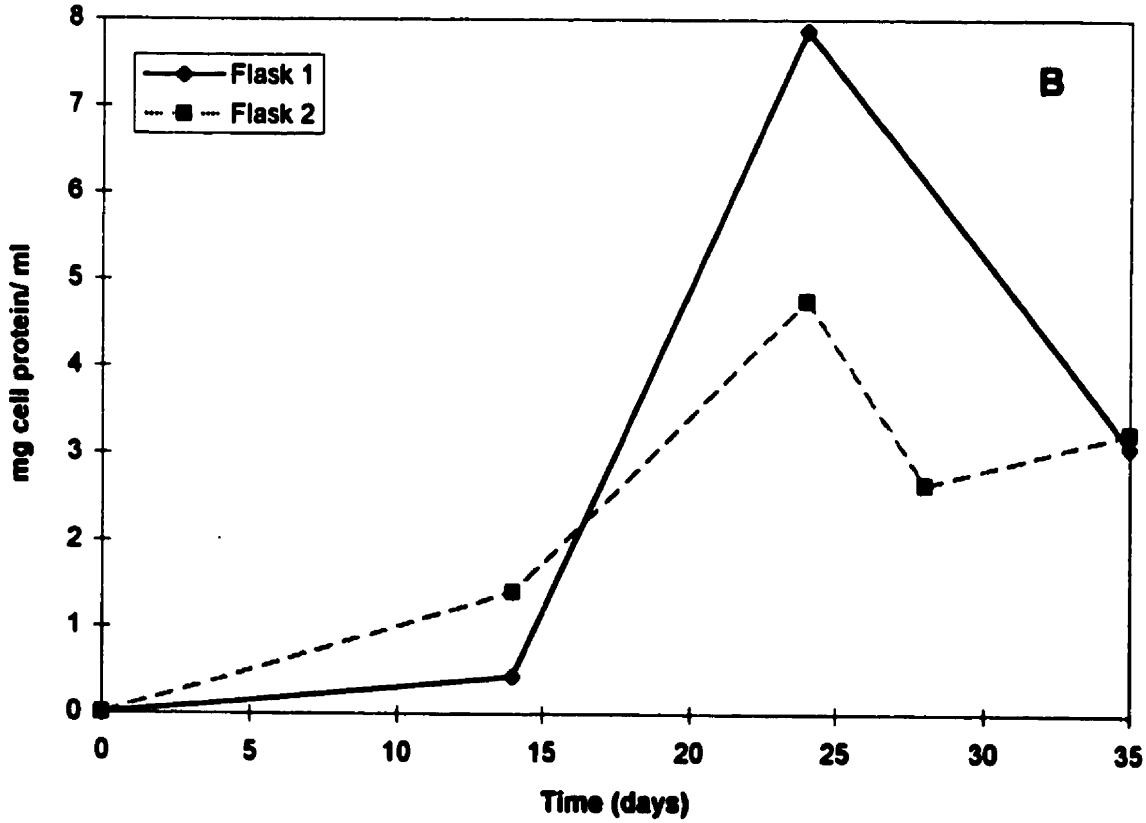
Optimizing Column Conditions

Batch culture operation

Batch culture experiments are important starting points in any experimental design. Batch cultures themselves, however, are rather limited with respect to practical applications. The potential use of *T. ferrooxidans* at the QB mine required the development of a continuous ferrous iron oxidizing system. A typical column setup can be

Figure 2. Effect of aeration methods on bacterial growth. Flask 1, aerated using an orbital shaker. Flask 2, aerated using a stir bar. **A:** Fe^{2+} oxidation activity by the culture (1.19 ml). **B:** cell growth in the culture. Cell concentrations were estimated by the Lowry method for insoluble protein (boiling in 1 N alkali) as described (in Methods in Enzymology vol. 3) by Layne (1957) and by multiplying the amount of protein by 10.


Flask 1
Flask 2
A
nmol O_2 / min
Time (days)
0
5
10
15
20
25
30
35

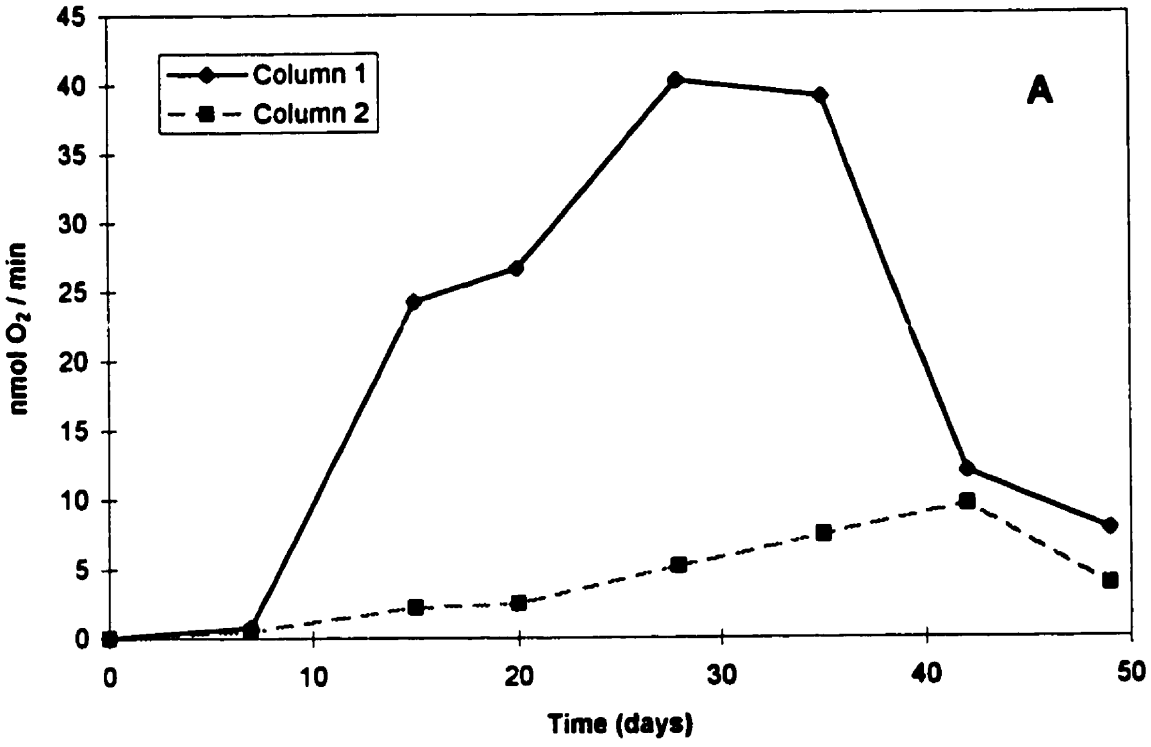

Flask 1
Flask 2
B
mg cell protein/ ml
Time (days)
0
1
2
3
4
5
6
7
8
0
5
10
15
20
25
30
35

seen in Figure 1 (The batch culture, however, was not fed fresh media using the peristalitic pump). All glassware and connective Tygon tubing was autoclaved for 20 minutes and allowed to cool to room temperature prior to use.

Inoculum Age. The first step in developing a ferrous iron oxidizing system was to determine the optimum age of the inoculating culture. In the flask studies, maximum cell number and iron oxidizing activity was observed at 25 days. Generally, however, the optimum age for an inoculum is during its logarithmic growth phase, thus 7 and 14 day old cultures were used to inoculate two identical columns. Each column was inoculated with 50 ml of ore grown SM-4 and immediately aerated. Figure 3B shows column 1 inoculated with the one week old culture produced greater than twice as many cells as column 2 (inoculated with a two week old culture) throughout the 35 days. Iron oxidation was also substantially higher in column 1, but the increased activity could not be accounted for by mere cell numbers. At 28 days column 1 had twice as many cells as column 2, yet iron oxidation was 8 times that of column 2. Thus a younger bacterial inoculum (column 1) produced not only a greater number of cells but a greater number of more active cells.

Filter choice. In our particular column design air passes through a glass filter prior to reaching the bacterial culture. The filter pore size then determines the size of the air bubble that enters the column. Two columns - one with a fine glass filter and the other with a coarse one were set up to determine what effect if any bubble size had on bacterial growth and activity. Each column was inoculated with 100 ml of ore grown SM-4 and immediately aerated. Up to 7 days (Figure 4) both columns responded similarly with respect to cell number and iron oxidizing activity. Beyond this point, however, column 4 containing the coarse glass filter surpassed column 3 in both categories. Although this

Figure 3. Effect of inoculum age on column growth. Column 1, one week old inoculum. Column 2, two week old inoculum. Conditions for assays were described in Fig. 2.


Column 1
Column 2
A
nmol O_2 / min
Time (days)
0
5
10
15
20
25
30
35
40
45
0
10
20
30
40
50

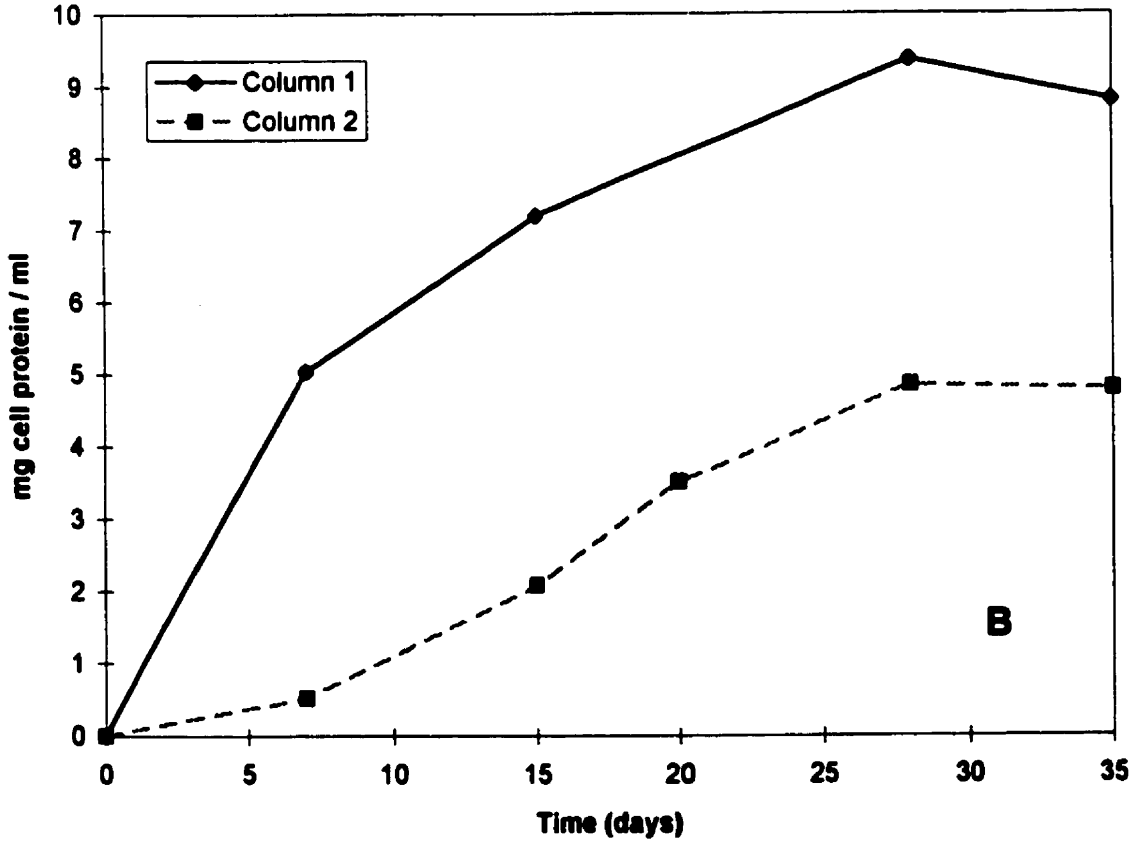
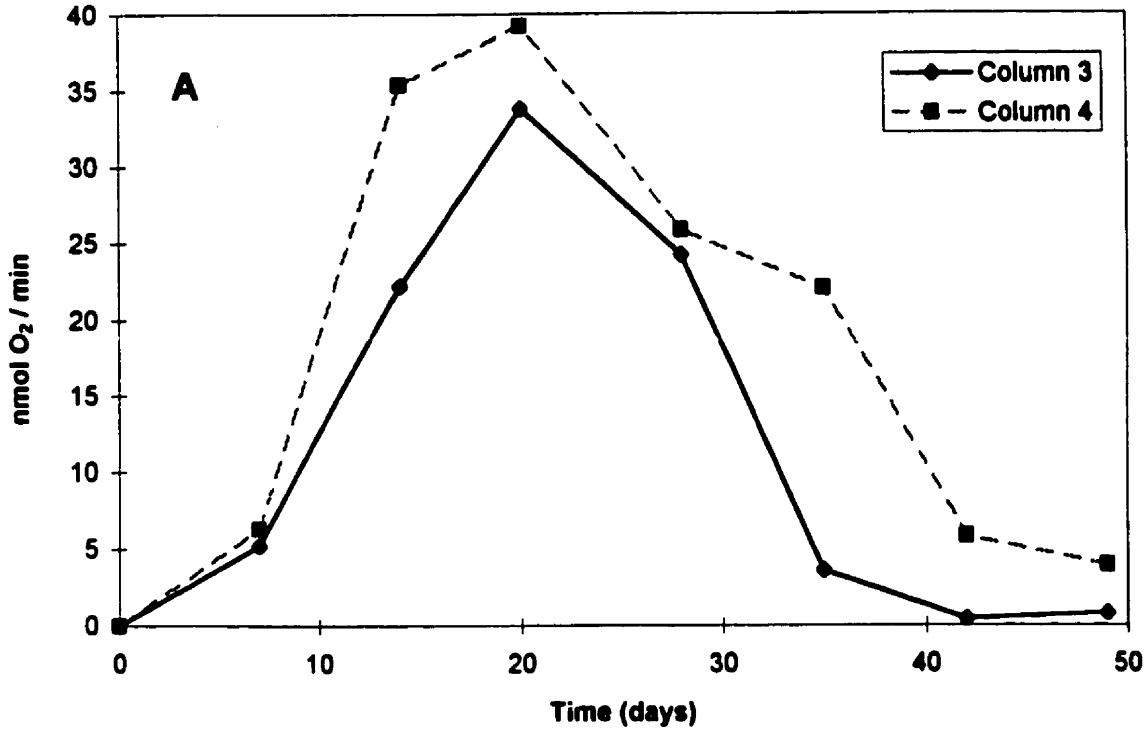
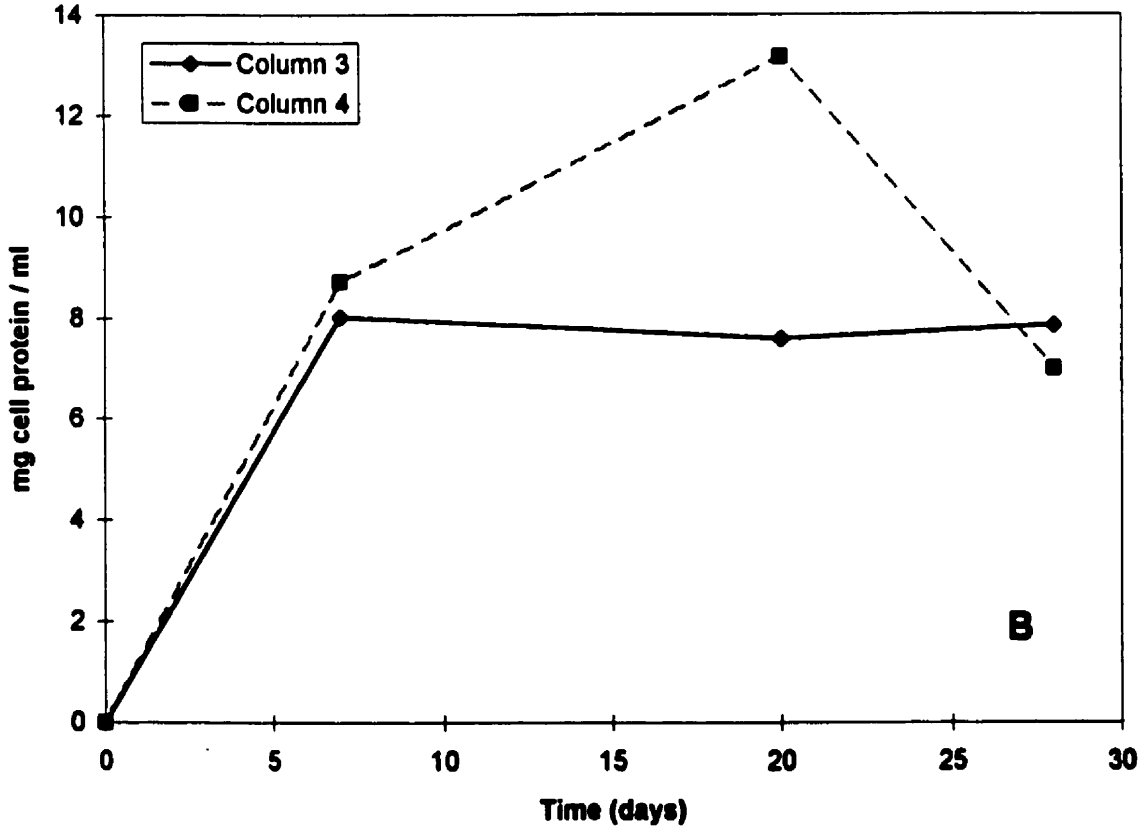

Column 1
Column 2
B
mg cell protein / ml
Time (days)
0
1
2
3
4
5
6
7
8
9
10
0
5
10
15
20
25
30
35

Figure 4. Effect of filter pore size on column growth. Column 3, fine filter. Column 4, coarse filter. Velocity on addition of 1 mmol Fe^{2+}. Conditions for assays were described in Fig. 2.


A
Column 3
Column 4
nmol O_2 / min
Time (days)
0
5
10
15
20
25
30
35
40
0
10
20
30
40
50


B
Column 3
Column 4
mg cell protein / ml
Time (days)
0
2
4
6
8
10
12
14
0
5
10
15
20
25
30

difference in some cases was rather minor, the subsequent columns were all made with coarse glass filters.

Continuously fed columns

The above columns were all maintained as batch cultures in the desire to optimize inoculum and growth conditions. The practical use of a ferrous iron oxidizing column, however, required its conversion to a continuous system. A feed solution containing ferrous iron enriched M9K (33.3 g $FeSO_4 \cdot 7H_2O$ per liter M9K pH 1.8) was pumped into each column below the lower filter using a peristaltic pump. The bacteria remained in the column attached to the cell carrier material trapped between the two filters. The incoming ferrous iron was oxidized by the bacteria within the column prior to its removal as effluent. This overflow was periodically checked for its ferrous iron content in an attempt to measure the bacterial iron oxidizing activity.

Fluidized bed reactor

Ore - cell carrier. Tables 1 and 2 show the iron oxidizing results for columns 3 and 4 both of which were converted to continuous systems. The ferrous iron feed was started at the low initial rate of 48 ml/day which corresponded to a residence time of nearly 5 days. Low levels of ferrous iron leaving the column indicated the rate of incoming feed could be increased. At 55 ml/day column 3 oxidized greater than 99 % of the incoming ferrous iron. At 86 ml/day, however, this value dropped to 72 %. The rate of feed was either too fast for the bacterial growth or the change from 55 to 86 ml/day was too great of an immediate increase, thus the speed was reduced to 74 ml/day. After several days the ferrous iron oxidation rate rose to 99 %. Unfortunately column 3 was stopped shortly afterwards due to blockage of the lower filter. A combination of ore

Table 1. **Column # 3** - 100 ml SM4 (1 week old on ore # 3, pH 1.8), fine frit plus air
Started - July 25,1996. Feed started Aug. 22, 1996.
(1- x), fraction ferrous iron remaining in effluent.
Note: all columns were allowed to equilibrate for 24-36 hours following changes to substrate influx.

Speed (ml / day)	(1 - x)	Fe^{2+}_{ox} (g / day)	g Fe^{2+}_{ox} g/ reactor vol. (l) / day	g Fe^{2+}_{ox} g/ liquid vol. (l) / day	Residence time (days)
48	0.0013	0.32	1.36	1.39	4.79
55	0.0039	0.37	1.57	1.61	4.18
85.7	0.2744	0.42	1.78	1.83	2.68
74	0.2817	0.36	1.53	1.57	3.11
74	0.0021	0.49	2.10	2.15	3.11

Table 2. **Column # 4** - 100 ml SM4 (1 week old on ore # 3, pH 1.8), coarse frit plus air
Started - July 25,1996. Feed started Sept. 9, 1996.
(1- x), fraction ferrous iron remaining in effluent.
Note: all columns were allowed to equilibrate for 24-36 hours following changes to substrate influx.

Speed (ml / day)	(1 - x)	Fe^{2+}_{ox} (g / day)	g Fe^{2+}_{ox} g/ reactor vol. (l) / day	g Fe^{2+}_{ox} g/ liquid vol. (l) / day	Residence time (days)
48	0.0065	0.319	1.36	1.39	4.79
55	0.0050	0.366	1.56	1.59	4.18
74	0.0057	0.492	2.09	2.14	3.11
85.7	0.0068	0.569	2.42	2.48	2.68
94	0.0001	0.629	2.68	2.73	2.45
111.86	0.0318	0.725	3.08	3.15	2.06
115	0.0039	0.763	3.25	3.32	2.00
141.5	0.0000	0.947	4.03	4.12	1.63
163.7	0.0014	1.094	4.65	4.75	1.41
180	0.0081	1.194	5.08	5.19	1.28
185	0.8221	0.220	0.94	0.96	1.24

particles and ferric iron precipitate formed a crusty layer on the filter surface inhibiting the flow of air and media entering the base of the column.

A similar problem was observed with column 4 (Table 2) which up to a speed of 164 ml/day oxidized greater than 99 % of the incoming ferrous iron. At this point, however, column 4 developed several air leaks. As with the previous column the lower filter became clogged with ore and ferric iron deposits preventing the incoming flow of media and air. In an attempt to save this column its contents were emptied into a sterile flask and then it was soaked in concentrated HCl to solublilize the precipitated ferric iron clogging the filter. The column was then rinsed several times with Milli Q water and the bacterial culture was reintroduced. All tubes were reconnected and the system was again started. The rate of ferrous iron oxidation remained above 99 % up to 180 ml/day. At 185 ml/day fine ore particles were observed leaving the column as effluent, the iron oxidation dropped to less then 20 % and the column operation was stopped. Although column 4 reached 5 g Fe^{2+} oxidized per liter per day, ore proved to be a difficult cell carrier to work with - clogging both the upper and lower filters and eventually leaving as effluent.

Celite (diatomaceous earth) - cell carrier. Celite has been used in numerous industrial applications as a cell carrier. The abandoned Matsuo sulfur-pyrite mine for example uses *T. ferrooxidans* in its acid mine drainage neutralization plant (Imaizumi, 1986). Liquids entering the plant are distributed among four parallel oxidation tanks in which the bacteria oxidize the majority of the available ferrous iron. The celite allows for the development of cell concentrations of 10^8 cells per ml. From here the material moves to a settling tank in which the cell covered celite precipitates out and is recycled back to

the oxidation tank. The soluble ferric sulfate continues on to the neutralization tank where it is removed from the system as a ferric salt precipitate at pH 4 (via addition of calcium carbonate).

Column 12 contained 100 ml of iron grown SM4 (pH 1.8), 90 ml iron enriched M9K and 2.3 g of powdered celite. It was grown as a batch culture for three days prior to its conversion to a continuous system. Table 3 shows celite up to a speed of 110 ml/day oxidized greater than 99% of the incoming ferrous iron. At 116 ml/day, however, the column was gradually losing its turbidity. Fine celite particles were leaving as effluent along with 23 % of the incoming iron. The speed was dropped to 110 ml/day, yet 81 % of the iron along with significant quantities of celite continued to be lost and thus the column operation was stopped. Celite, therefore, proved an inadequate cell carrier in our simple one step column design.

Zeolite - cell carrier. Zeolite was another potential cell carrier similar to that of celite. Column 13 contained 100 ml of iron grown SM4 (pH 1.8), 90 ml iron enriched M9K and 2.3 g zeolite. Table 4 shows the bacteria oxidized greater than 99 % of the incoming ferrous iron up to a speed of 141 ml/day. Unfortunately, zeolite as with above columns gradually clogged the upper filter creating leaks and the column was lost.

Fine charcoal - cell carrier. Activated charcoal is known to chemically oxidize ferrous iron (Kai et al, 1992). Its shape (irregular with cracks and crevices) also allows it to be used as a potential cell carrier. Column 11 contained 100 ml of iron grown SM4 pH 1.8, 90 ml of ferrous iron enriched M9K and 25 ml of fine charcoal. It operated as a batch culture for three days to allow for cell growth and attachment prior to its conversion to a continuous iron oxidizing system. The fine charcoal remained suspended within the

Table 3. **Column # 12** - 100 ml SM4 (Fe^{2+} grown, pH 1.8) + 80 ml M9K pH 1.8 + 10 ml $FeSO_4$ (stock) pH 1.8 + 2.3 g celite (diatomaceous earth) + air. Started - Oct. 18,1996. Feed started Oct. 21, 1996. Stopped Dec. 6, 1996. (1- x), fraction ferrous iron remaining in effluent.
Note: all columns were allowed to equilibrate for 48-60 hours following changes to substrate influx.

Speed (ml / day)	(1 - x)	Fe^{2+}_{ox} (g / day)	g Fe^{2+}_{ox} g/ reactor vol. (l) / day	g Fe^{2+}_{ox} g/ liquid vol. (l) / day	Residence time (days)
51	0.0024	0.34	1.45	1.48	4.53
81.9	0.0028	0.55	2.32	2.38	2.82
91.2	0.0004	0.61	2.59	2.65	2.53
110	0.0003	0.74	3.13	3.20	2.10
116	0.2334	0.59	2.53	2.59	1.99
110	0.8110	0.14	0.59	0.60	2.10

Table 4. **Column # 13** - 80 ml M9K pH 1.8 + 10 ml $FeSO_4$ pH 1.8 + 2.3 g Zeolite + 100 ml SM4 (Fe^{2+} grown, pH 1.8) + air
Started - Oct. 28,1996. Feed started Dec. 17, 1996.
(1- x), fraction ferrous iron remaining in effluent.
Note: all columns were allowed to equilibrate for 48-60 hours following changes to substrate influx.

Speed (ml / day)	(1 - x)	Fe^{2+}_{ox} (g / day)	g Fe^{2+}_{ox} g/ reactor vol. (l) / day	g Fe^{2+}_{ox} g/ liquid vol. (l) / day	Residence time (days)
48	0.0000	0.32	1.37	1.40	4.79
55	0.0012	0.37	1.56	1.60	4.18
74	0.0018	0.49	2.10	2.15	3.11
85.7	0.0010	0.57	2.44	2.49	2.68
94	0.0034	0.63	2.67	2.72	2.45
111.9	0.0020	0.75	3.18	3.25	2.06
123	0.0002	0.82	3.50	3.58	1.87
141.3	0.0066	0.94	4.00	4.08	1.63

Table 5. **Column # 11** - 100 ml SM4 (Fe^{2+} grown, pH 1.8) + 80 ml M9K pH 1.8 + 10 ml $FeSO_4$ pH 1.8 + 25 ml fine charcoal (acid washed) + air. Started - Sept. 6,1996. Feed started Sept. 9, 1996. Stopped: Oct.17, 1996. (1- x), fraction ferrous iron remaining in effluent.
Note: all columns were allowed to equilibrate for 36-48 hours following changes to substrate influx.

Speed (ml / day)	(1 - x)	Fe^{2+}_{ox} (g / day)	g Fe^{2+}_{ox} g/ reactor vol. (l) / day	g Fe^{2+}_{ox} g/ liquid vol. (l) / day	Residence time (days)
48	0.0020	0.32	1.36	1.54	4.33
55	0.0000	0.37	1.57	1.77	3.78
85.7	0.0176	0.56	2.40	2.71	2.43
94	0.0462	0.60	2.55	2.88	2.21
112	0.6840	0.24	1.01	1.14	1.86

column oxidizing greater than 95 % of the incoming ferrous iron up to a speed of 94 ml/day (Table 5). As with the above columns, however, fine particles of charcoal slowly blocked the filters causing leaks and the column was shut down.

Packed bed reactors

The above fluidized bed reactors failed to provide adequate rates of ferrous iron oxidation with only 1.8, 5.2,3.2, 4.1 and 2.9 g of ferrous iron oxidized per liter per day in columns 3, 4,12,13 and 11 respectively. For this reason an alternate reactor type was investigated. A packed bed reactor as the name implies is packed with a cell carrier material. It, therefore, has the potential of producing higher cell yields (than a fluidized bed reactor) due to the added available surface area onto which the bacteria could attach. Increased cells numbers mean increased iron oxidizing activity and thus an increase in the overall efficiency of the column.

Charcoal - cell carrier. A preliminary study showed a packed bed reactor filled with charcoal required an upper filter to remain within the column - in its absence the charcoal escaped as effluent. An upper filter, however, as seen with the fluidized bed reactors had the potential of getting clogged up with fine particles. In order to avoid this problem the charcoal was weighed down using glass beads (in a layered effect) and the upper filter was removed. Under the weight of the glass beads the majority of the charcoal remained within the column and thus available for bacterial attachment..

Columns 8, 9 and 14 each contained 20 ml charcoal and 20 ml glass beads in an alternating layered arrangement prior to being autoclaved for 20 minutes at 121 psi. Once cooled to room temperature columns 8 and 9 each received 20 ml of an iron grown *T. ferrooxidans* culture at pH 1.8 while column 14 which was used as a chemical control was

filled with 20 ml of iron enriched M9K. The columns (8 and 9) were aerated and allowed to grow as batch cultures for 3 days. This allowed the bacteria time to grow in number and attach themselves to the charcoal. At the end of this incubation period the ferrous iron containing M9K feed was started.

Column 8 containing fine charcoal oxidized nearly 99 % of the iron up to a speed of 41.5 ml/day (Table 6). During this time, however, the charcoal did not remain in its original position. It gradually moved up the column, above the glass beads. The resulting configuration consisted of a single top layer of glass beads followed by a thick layer of charcoal and a bottom layer of glass beads. The central packed layer of charcoal created a problem with respect to media and air flow. At a speed of 41.5 ml/day 48 % of the ferrous iron was leaving the column untouched. A slight increase in speed to 42 ml/day produced a bubbling in the charcoal layer and a concomitant drop in the effluent ferrous iron concentration (4 %). Thus it is likely that the charcoal layer was gradually becoming more compact forcing air and media to flow around it as opposed to through it. At the elevated speed the observed bubbling loosened this layer allowing air and media to contact the central cells thus increasing total iron oxidation. Increased speeds continued to show this bubbling effect with the continued movement of charcoal up the column. At 71.5 ml/day significant amounts of charcoal were being lost as effluent producing a 29 % loss of incoming ferrous iron. The media speed was dropped to 50 ml/day and the ferrous iron oxidation rate rose to 98 %. At a return to 70 ml/day, however, nearly 50 % of the ferrous iron was being lost as effluent along with significant quantities of fine charcoal and column 8 was stopped.

Table 6. **Column # 8** - 20 ml fine charcoal + 20 ml glass beads + 21 ml SM4 (1 day old, pH 1.8) plus air. Started - Aug. 23,1996. Feed started Aug. 26, 1996.
(1- x), fraction ferrous iron remaining in effluent.
Note: all columns were allowed to equilibrate for 36-48 hours following changes to substrate influx

Speed (ml / day)	(1 - x)	Fe^{2+}_{ox} (g / day)	g Fe^{2+}_{ox} g/ reactor vol. (l) / day	g Fe^{2+}_{ox} g/ liquid vol. (l) / day	Residence time (days)
6.24	0.0034	0.042	0.83	1.98	3.37
14.5	0.0068	0.096	1.93	4.59	1.45
19.5	0.0065	0.130	2.59	6.17	1.08
20.5	0.0068	0.136	2.72	6.49	1.02
25	0.0102	0.166	3.31	7.88	0.84
27.5	0.0128	0.182	3.63	8.65	0.76
34	0.0128	0.225	4.49	10.69	0.62
41.5	0.4780	0.145	2.90	6.90	0.51
41.8	0.0400	0.268	5.37	12.78	0.50
46.5	0.0102	0.308	6.16	14.66	0.45
53.5	0.0118	0.354	7.07	16.84	0.39
65.35	0.0383	0.420	8.41	20.02	0.32
71.5	0.2883	0.340	6.81	16.20	0.29
50	0.0126	0.330	6.61	15.73	0.42
70	0.4597	0.253	5.06	12.05	0.30

Column 9 was packed with 20 ml of coarse charcoal and 20 ml glass beads. Unlike the fine charcoal the coarse charcoal showed very little movement upon the introduction of air and media. Table 7 shows column 9 oxidized greater than 98 % of the incoming ferrous iron up to a speed of 53 ml/day. At 68.3 ml/day. however, greater than 19 % of the ferrous iron was being lost. The pump speed was immediately dropped to 42 ml/day yet ferrous iron losses continued to rise (46 %). After several days of adjustment column 9 was again oxidizing nearly all of the incoming ferrous iron. The pump speed was raised to 50.4 and 53 ml/day without significant ferrous iron losses. A return to 68 ml/day, however, resulted in a 50 % loss of incoming ferrous iron. Thus the working limit for this column was determined to be less then 68 ml/day and the column was stopped.

As previously stated charcoal can chemically oxidize ferrous iron. In order to determine the degree of bacterial involvement seen in columns 8 and 9 a chemical control was developed. Column 14 contained 20 ml glass beads in alternating layers with 20 ml of coarse charcoal. (Note: fine charcoal was not tested due to inherent problems seen in column 8). The column and all of its connection was autoclaved for 20 min at 121 psi and allowed to cool prior to inoculation with 21 ml of iron enriched M9K. The air and media feed were started immediately and the resulting oxidation activities are listed in Table 8. At the lowest speed of 14.5 ml/day nearly 18 % of the incoming ferrous iron was being lost as effluent. Further increases to media flow coincided with increased ferrous iron losses. At a rate of 30 ml/day 40 % of the ferrous iron was being lost as effluent and the column was shut down. Based on this control data ferrous iron oxidation using coarse charcoal was determined to be a combination of bacterial and chemical ferrous iron oxidation.

Table 7. **Column # 9** - 20 ml coarse charcoal + 20 ml glass beads + 21 ml SM4 (1 day old, pH 1.8) plus air. Started - Aug. 23,1996. Feed started Aug. 26, 1996.
(1- x), fraction ferrous iron remaining in effluent.
Note: all columns were allowed to equilibrate for 36-48 hours following changes to substrate influx.

Speed (ml / day)	(1 - x)	Fe^{2+}_{ox} (g / day)	g Fe^{2+}_{ox} g/ reactor vol. (l) / day	g Fe^{2+}_{ox} g/ liquid vol. (l) / day	Residence time (days)
6.24	0.0000	0.04	0.83	1.99	3.37
14.5	0.0002	0.10	1.94	4.62	1.45
19.5	0.0001	0.13	2.61	6.21	1.08
20.5	0.0189	0.13	2.69	6.40	1.02
25	0.0017	0.17	3.34	7.95	0.84
27.5	0.0018	0.18	3.67	8.74	0.76
34	0.0013	0.23	4.54	10.82	0.62
41.5	0.0011	0.28	5.55	13.20	0.51
41.8	0.0007	0.28	5.59	13.30	0.50
42	0.0014	0.28	5.61	13.36	0.50
53	0.0036	0.35	7.07	16.82	0.40
68.3	0.1934	0.37	7.37	17.55	0.31
42	0.3381	0.19	3.72	8.86	0.50
50.4	0.0201	0.33	6.61	15.73	0.42
53	0.0252	0.35	6.91	16.46	0.40
68	0.1767	0.37	7.49	17.83	0.31
68	0.4925	0.23	4.62	10.99	0.31

Table 8. **Column # 14** - 21 ml glass beads + 21 ml coarse charcoal + 21 ml (90 ml M9K + 10 ml $FeSO_4$, pH 1.8) + air. Started - Nov. 5,1996. Feed started Nov. 5, 1996.

(1- x), fraction ferrous iron remaining in effluent.

Note: all columns were allowed to equilibrate for 36-48 hours following changes to substrate influx.

Speed (ml / day)	(1 - x)	Fe^{2+}_{ox} (g / day)	g Fe^{2+}_{ox} g/ reactor vol. (l) / day	g Fe^{2+}_{ox} g/ liquid vol. (l) / day	Residence time (days)
14.5	0.1740	0.08	1.60	3.82	1.45
19.5	0.2981	0.09	1.83	4.36	1.08
24.5	0.3393	0.11	2.17	5.16	0.86
30	0.3973	0.12	2.42	5.76	0.70

Glass beads and nylon mesh - cell carriers. During the operation of columns 8 and 9 bacterial growth was observed on the surface of both the glass beads and nylon mesh. Column 10 was developed to test the suitability of glass beads and nylon mesh as cell carriers. Column 10 was packed with alternating layers of glass beads and nylon mesh, autoclaved for 20 min at 121 psi and allowed to cool to room temperature. It was then inoculated with 45 ml of SM4 (iron grown at pH 1.8), 5 ml of 1.2 M $FeSO_4 \cdot 7H_2O$ and immediately aerated. After three days of batch growth the ferrous iron feed was started. Oxidation activities were above 95 % up to a speed of 170 ml/day which corresponded to nearly 23 g of Fe^{2+} being oxidized per day (Table 9). At 185 ml/day up to 84 % of the ferrous iron was being lost as effluent. A drop in speed to 180 ml/day showed a slight improvement in iron oxidation but the column did not fully recover and was shut down.

Bacterial Growth in Mine Leachate

Up to this point in the above columns iron enriched M9K medium was used as the ferrous iron containing feed. In a mine site, however, the feed would contain not only high concentrations of ferrous iron but also numerous other metals. An analysis of the leachate solution from the Quebrada Blanca mine is found in Table 10. Of the chemicals present only Al, As, Ca, Fe, Mg and SiO_2 were found in high enough concentrations to pose a potential problem with respect to bacterial growth and activity. Although not listed in Table 10 the leachate also contained significant amounts of two metal chelators lix 84 and lix 860 along with the solvent exxsol D-80 (a type of kerosene) which were added to the leachate during the process of copper extraction.

Table 9. **Column # 10** - 45 ml SM4 (Fe^{2+} grown pH 1.8) + 5 ml FeSO4 (stock, pH 1.8) + glass beads plus nylon mesh + air. Started - Sept. 6,1996. Feed started Sept. 9, 1996. (1- x), fraction ferrous iron remaining in effluent.
Note: all columns were allowed to equilibrate for 48-60 hours following changes to substrate influx.

Speed (ml / day)	(1 - x)	Fe^{2+}_{ox} (g / day)	g Fe^{2+}_{ox} g/ reactor vol. (l) / day	g Fe^{2+}_{ox} g/ liquid vol. (l) / day	Residence time (days)
48	0.0003	0.32	3.21	6.42	1.04
55	0.0032	0.37	3.67	7.33	0.91
85.7	0.0002	0.57	5.73	11.46	0.58
94	0.0000	0.63	6.29	12.58	0.53
107.5	0.0015	0.72	7.18	14.36	0.47
126.3	0.0386	0.81	8.12	16.25	0.40
165.1	0.0000	1.10	11.04	22.09	0.30
170	0.0009	1.14	11.36	22.72	0.29
185	0.3582	0.79	7.94	15.89	0.27
185	0.6692	0.41	4.09	8.19	0.27
185	0.8389	0.20	1.99	3.99	0.27
107.5	0.8016	0.14	1.43	2.85	0.47

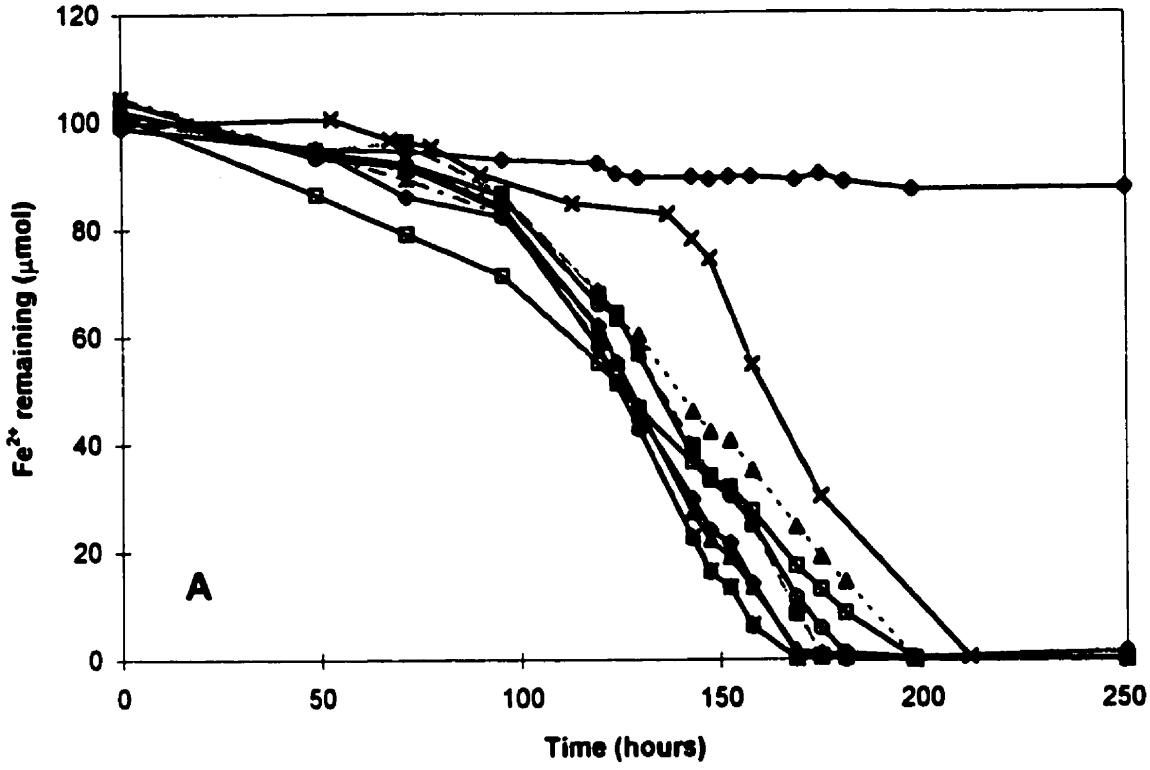
Table 10. Chemical analysis of leachate from the Quebrada Blanca mine.

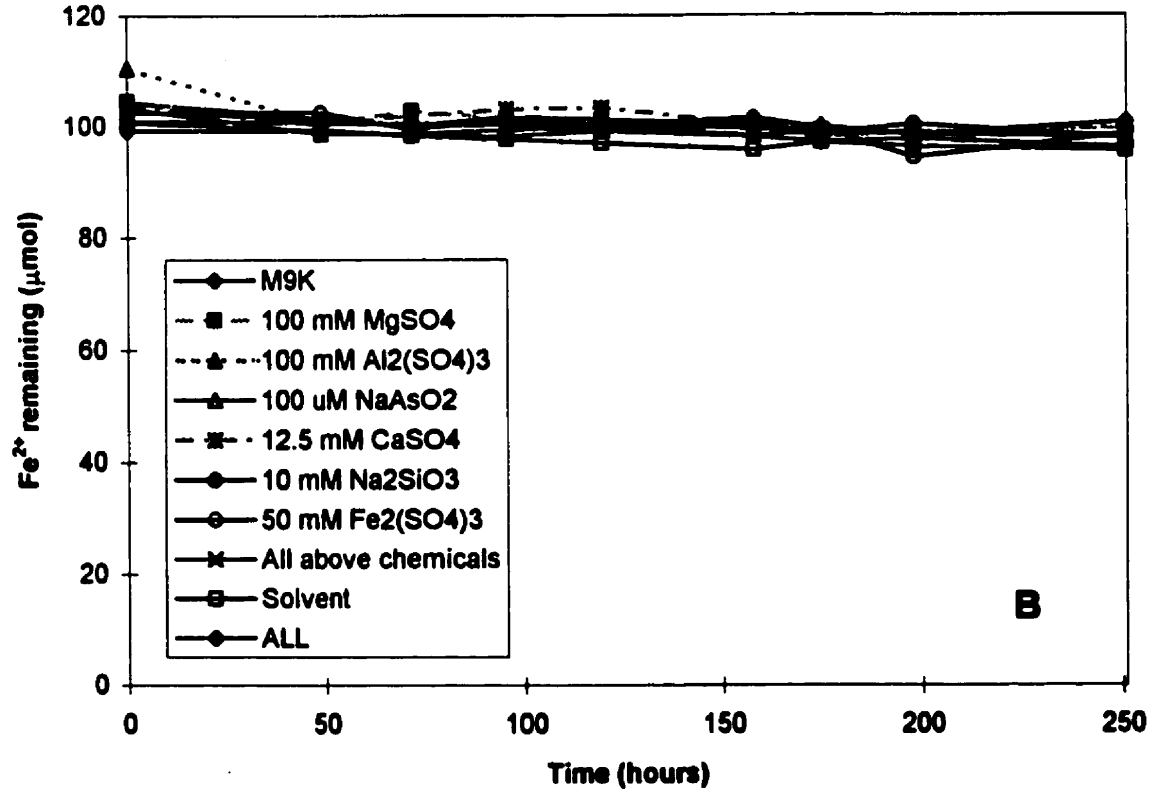
Chemical concentration (mg/l)	
Ag	< 0.5
Al	2900
As	25
Ba	< 0.5
Bi	< 0.5
Ca	500
Cd	< 0.5
Co	8
Cr	2
Cu	75
Fe	4700
Ga	< 0.5
In	< 0.5
K	600
Li	1
Mg	2400
Mn	55
Mo	< 0.5
Na	165
Ni	1
P	175
Pb	5
Sb	< 0.5
SiO2	415
Sn	< 0.5
Te	1
Tl	< 0.5
Zn	14

Prior to exposing any of our columns to the actual leachate the individual effects of the above chemicals were tested on batch cultures of iron grown *T. ferrooxidans*. Each flask contained 80 ml of M9K medium at pH 1.8, 10 ml of 1.2 M $FeSO_4 \cdot 7H_2O$ (pH 1.8) and 10 ml of pH 1.8 culture. Ten media variations were tested - M9K (bacterial control), M9K + 100 mM $MgSO_4$, M9K + 100 mM $Al_2(SO_4)_3$, M9K + 100 μM $NaAsO_2$, M9K + 12.5 mM $CaSO_4$, M9K + 10 mM Na_2SiO_3, M9K + 50 mM $Fe_2(SO_4)_3$, M9K + all of the above chemicals, M9K + metal chelators and kerosene and (solvent extraction) with and without all of the above chemicals. Subsequent to inoculation the flasks remained stationary for 24 hours at 25°C followed by 10 days of shaking on an orbital shaker at 150 rpm. Bacterial activity was measured in terms of ferrous iron oxidation. During the course of the experiment intermittent samples were taken to measure the amount of ferrous iron remaining in the flask. In order to exclude the possible chemical oxidation of ferrous iron by the above chemicals, uninoculated controls were run simultaneously with each test flask.

According to Figure 5A bacterial iron oxidation was initially stimulated slightly in the flask containing the kerosene and metal chelators. The flasks containing Na_2SiO_3 and $NaAsO_2$ stimulated iron oxidation slightly beyond 100 hours, while $MgSO_4$, $Fe_2(SO_4)_3$ and $Al_2(SO_4)_3$ slightly inhibited iron oxidation during the same time period. In the flask containing all of the chemicals save the solvent and metal chelators iron oxidation was significantly inhibited up to 150 hours. Beyond this point, iron oxidation proceeded at an otherwise normal rate. The combination of all of the chemicals including solvent and chelators inhibited iron oxidation nearly completely. All observed oxidation results were

Figure 5. Effect of recycling mine leachate components on the growth of *T. ferrooxidans*. **A**: with inoculum. **B**: without inoculum.


120
100
80
60
40
20
0
Fe2+ remaining (µmol)
A
0
50
100
150
200
250
Time (hours)


120
100
80
60
40
20
0
Fe2+ remaining (µmol)
M9K
100 mM MgSO4
100 mM Al2(SO4)3
100 uM NaAsO2
12.5 mM CaSO4
10 mM Na2SiO3
50 mM Fe2(SO4)3
All above chemicals
Solvent
ALL
B
0
50
100
150
200
250
Time (hours)

directly linked to bacterial activity. Figure 5B containing the uninoculated controls showed no change in total ferrous iron content in 251 hours.

DISCUSSION

T. ferrooxidans is a Gram negative bacterium that obtains its energy for growth and CO_2 fixation from the oxidation of ferrous iron, sulfur and reduced sulfur compounds. This unique ability has made it an invaluable asset to the mining industry for it is involved in both direct and indirect mineral solubilization. This chapter deals solely with the indirect mechanism of mineral solubilization. Our goal was to develop a simple bacterial iron oxidizing loop that would maintain high levels of ferric iron in the leachate solution, thus maximizing chemical or indirect mineral solubilization.

The bacteria were initially adjusted to growth at pH 1.8, the operating pH at the Quebrada Blanca mine. This highly acidic pH allowed for the presence of elevated ferric iron concentrations in the leachate solution. A simple glass column (Fig. 1) was designed to house the ferrous iron oxidizing bacteria. The cells remained within the column attached to the cell carrier material. The carriers chosen were based on several publications that investigated the potential of multiple carrier materials. This study, however, used only ore, celite, zeolite, charcoal, glass beads and nylon mesh.

Of the columns tested the packed bed reactors far out competed the fluidized beds with respect to the total amount of ferrous iron oxidized. The packed bed reactors as the name implies were packed with cell carrier material, while the fluidized beds possessed only a fraction of this amount suspended, allowing for a fluidized operation. Thus the observed difference in iron oxidizing activity may simply be linked to bacterial cell numbers which were higher in the packed bed reactors due to the increased amount of available cell carrier material.

Although surface area was obviously very important it alone did not determine the productivity of a column. The carrier material also had to be compatible to our particular column design. Fine carrier materials such as ore, celite and zeolite used for the fluidized bed reactors created havoc during column operation clogging the upper filters or simply leaving as effluent. Maximum rates of iron oxidation were only 5.2, 3.2 and 4.1 grams of ferrous iron oxidized per liter per day respectively.

Increased carrier size alleviated this problem to a certain extent. Fine charcoal, for example, proved very effective oxidizing up to 20 g of ferrous iron per liter per day yet it caused other problems. It was difficult to work with, easily compacting at low speeds within the column preventing media and air inflow or in the absence of the upper filter with increased media flow simply leaving the column as effluent. Course charcoal proved to be a far superior choice with respect to column management (i.e. remaining within the column in its original arrangement), yet it oxidized only 18 g of ferrous iron per liter per day at its peak; slightly less than that of the fine charcoal. Charcoal alone, however, was unique among the cell carriers chosen in that it could chemically oxidize ferrous iron. Although the rate of this chemical iron oxidation (as seen in column 11) only reached a peak of less than 3 grams of ferrous iron oxidized per liter per day. Thus the dominant force responsible for iron oxidation using charcoal as a cell carrier was the bacteria, *T. ferrooxidans*.

The final cell carrier material tested proved to be by far the best. Column 10 containing a mixture of glass beads and nylon mesh oxidized nearly 23 grams of ferrous iron per liter per day at its peak. The column was simple to maintain. The beads and

mesh remained in their original places held down by their own weight. The sole problem occurred during washout.; a drop in feeding speed, an attempt to save the column proved futile.

The final portion of this study dealt with the effects of key chemical present in the QB leachate. Prior to exposure of any of the columns to the actual mine leachate a series of naturally occurring chemicals were tested as to their effect on bacterial growth and iron oxidation. Figure 5 shows $MgSO_4$, $Al_2(SO_4)_3$, $NaAsO_2$, $CaSO_4$, Na_2SiO_3, $Fe_2(SO_4)_3$ or solvent (the metal chelators and kerosene) did not individually cause any significant amount of inhibition with respect to bacterial growth and ferrous iron oxidation. The combination of all of the chemicals except the solvent and chelators prolonged the initial lag period prior to active bacterial iron oxidation. The flask containing all of the chemicals (including the solvent and chelators), however, showed only a minor drop in total ferrous iron over 251 hours. Thus actual QB leachate as a feed solution would likely be prohibitive to bacterial growth and oxidation. In order to be used as potential feed solution this leachate must be diluted or chemically treated prior to contacting the bacterial cells.

APPENDIX

Figure 1. Effect of increasing β-alanine concentrations on iron oxidation. The reaction mixture contained 0.5 mg wet cells (SM-4) and Fe^{2+} in β-alanine sulfate pH 2.3.

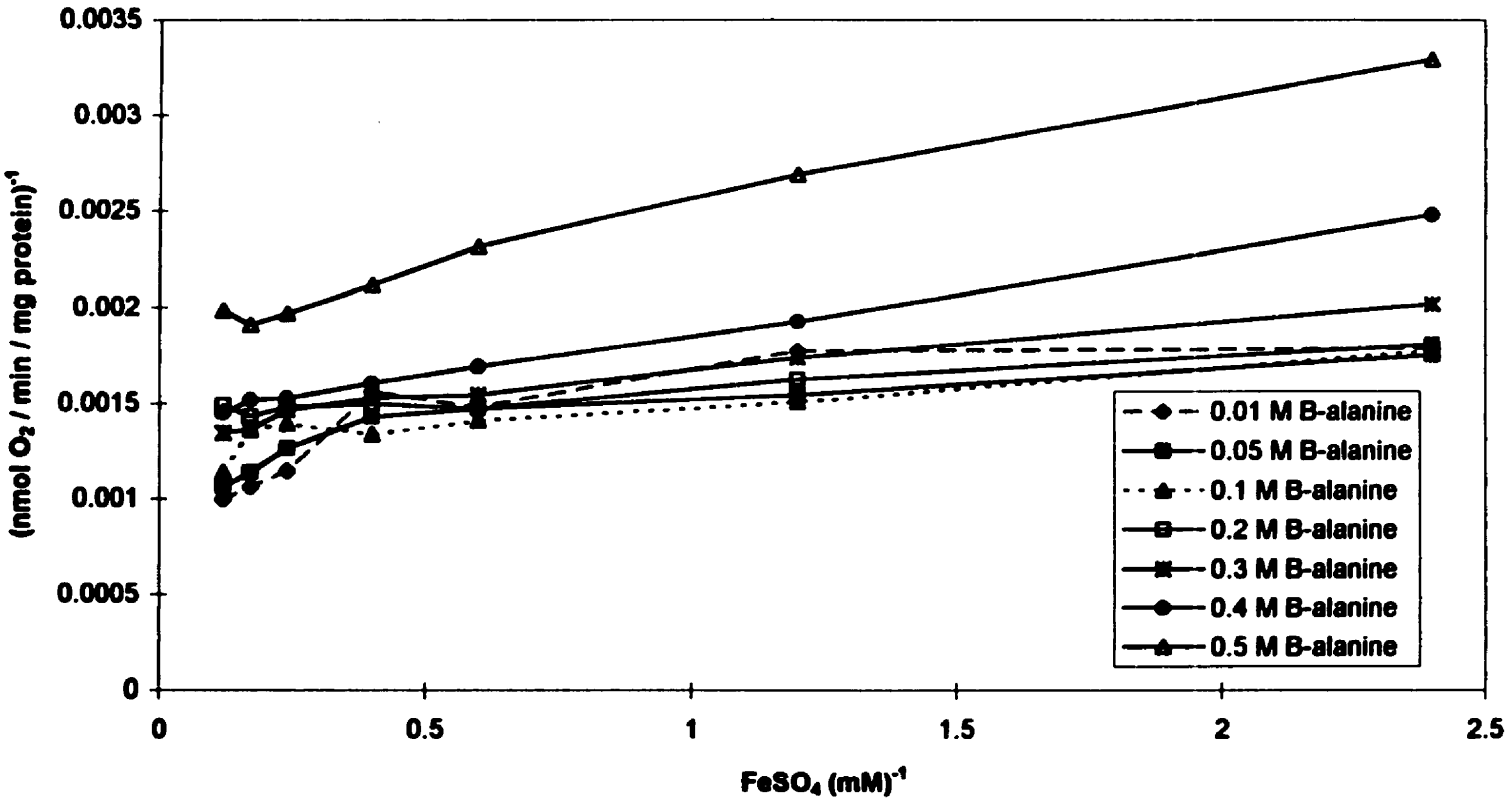

0.0035
0.003
0.0025
0.002
0.0015
0.001
0.0005
0
0
0.5
1
1.5
2
2.5
(nmol O2 / min / mg protein)-1
FeSO4 (mM)-1
0.01 M B-alanine
0.05 M B-alanine
0.1 M B-alanine
0.2 M B-alanine
0.3 M B-alanine
0.4 M B-alanine
0.5 M B-alanine

Figure 2. Effect of increasing potassium sulfate concentrations on iron oxidation. The reaction mixture contained 0.5 mg wet cells (SM-4) and Fe^{2+} in potassium sulfate pH 2.3.

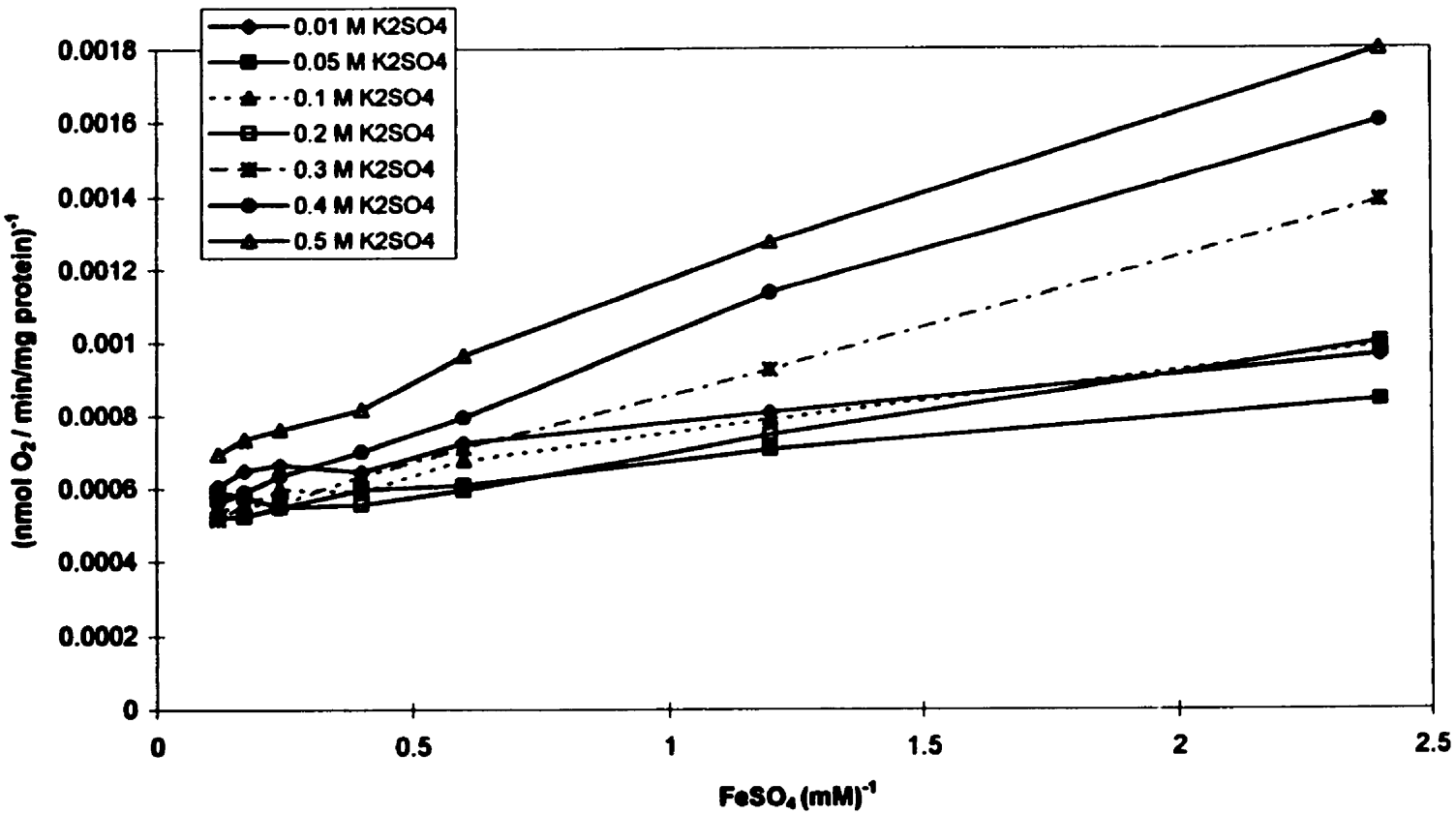
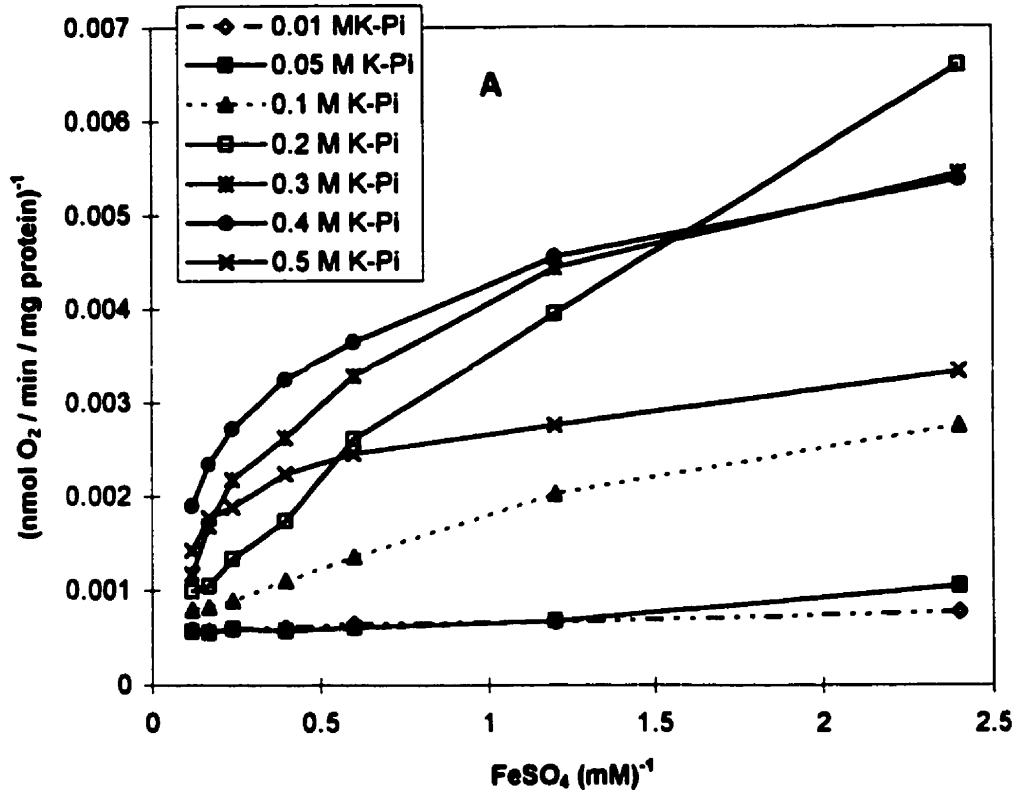

0.01 M K2SO4
0.05 M K2SO4
0.1 M K2SO4
0.2 M K2SO4
0.3 M K2SO4
0.4 M K2SO4
0.5 M K2SO4
0.0018
0.0016
0.0014
0.0012
0.001
0.0008
0.0006
0.0004
0.0002
0
(nmol O2 / min/mg protein)-1
0
0.5
1
1.5
2
2.5
FeSO4 (mM)-1

Figure 3. Effect of increasing potassium phosphate concentrations on iron oxidation. The reaction mixture contained 0.5 mg wet cells (SM-4) and Fe^{2+} in potassium phosphate pH 2.3. **A,** cells were pre-incubated in the phosphate buffer for 5 minutes prior to the addition of substrate ferrous iron. **B,** no pre-incubation of cells.


0.01 MK-Pi
0.05 M K-Pi
0.1 M K-Pi
0.2 M K-Pi
0.3 M K-Pi
0.4 M K-Pi
0.5 M K-Pi
A
(nmol O2 / min / mg protein)-1
FeSO4 (mM)-1
0
0.001
0.002
0.003
0.004
0.005
0.006
0.007
0.5
1
1.5
2
2.5

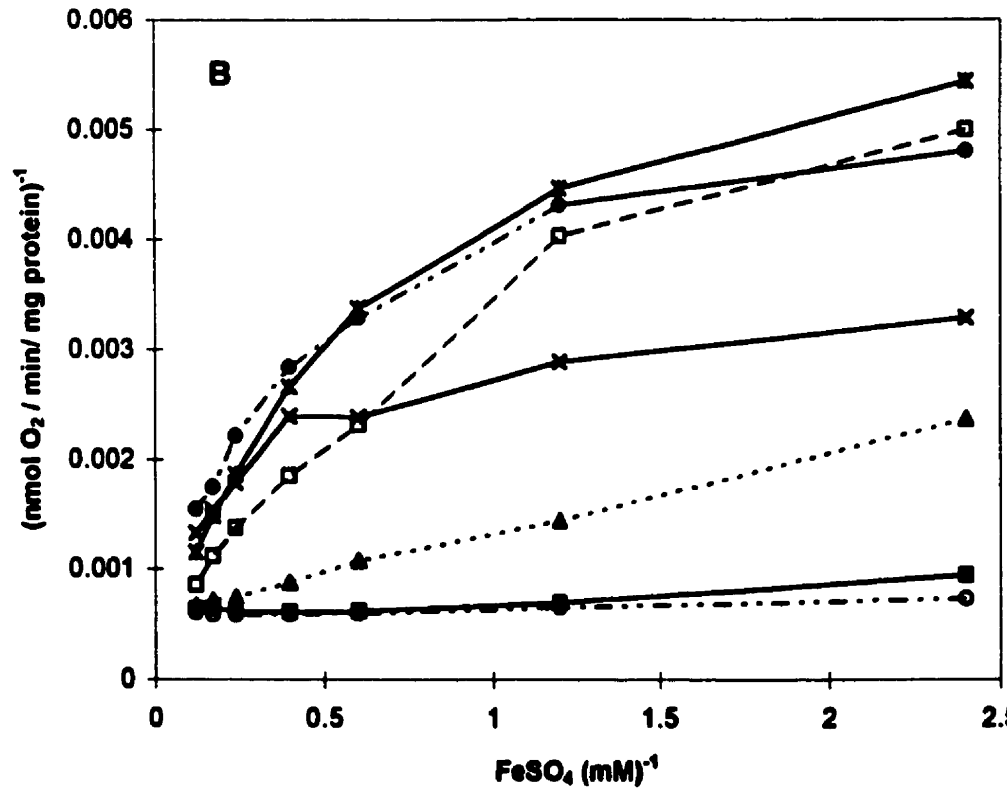
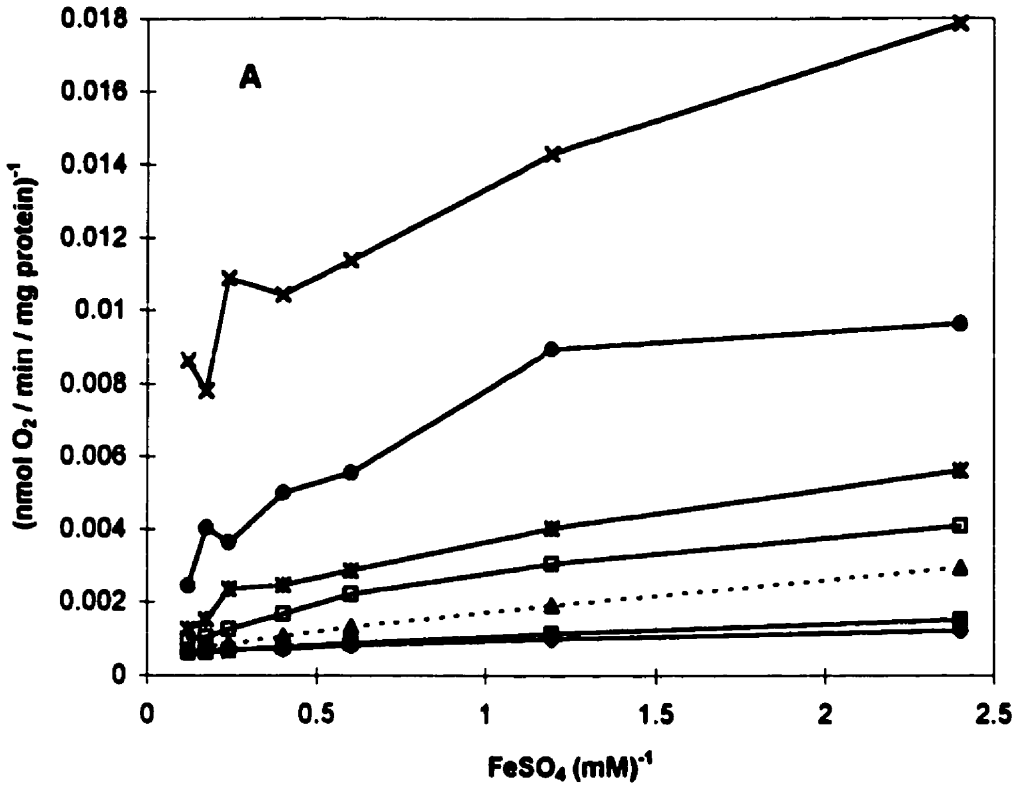

B
(nmol O2 / min/ mg protein)-1
FeSO4 (mM)-1
0
0.001
0.002
0.003
0.004
0.005
0.006
0.5
1
1.5
2
2.5

Figure 4. Effect of increasing potassium chloride concentrations on iron oxidation. The reaction mixture contained 0.5 mg wet cells (SM-4) and Fe^{2+} in potassium chloride pH 2.3. **A,** cells were pre-incubated in the phosphate buffer for 5 minutes prior to the addition of substrate ferrous iron. **B,** no pre-incubation of cells.


A
0.018
0.016
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
0
0.5
1
1.5
2
2.5
(nmol O2 / min / mg protein)-1
FeSO4 (mM)-1

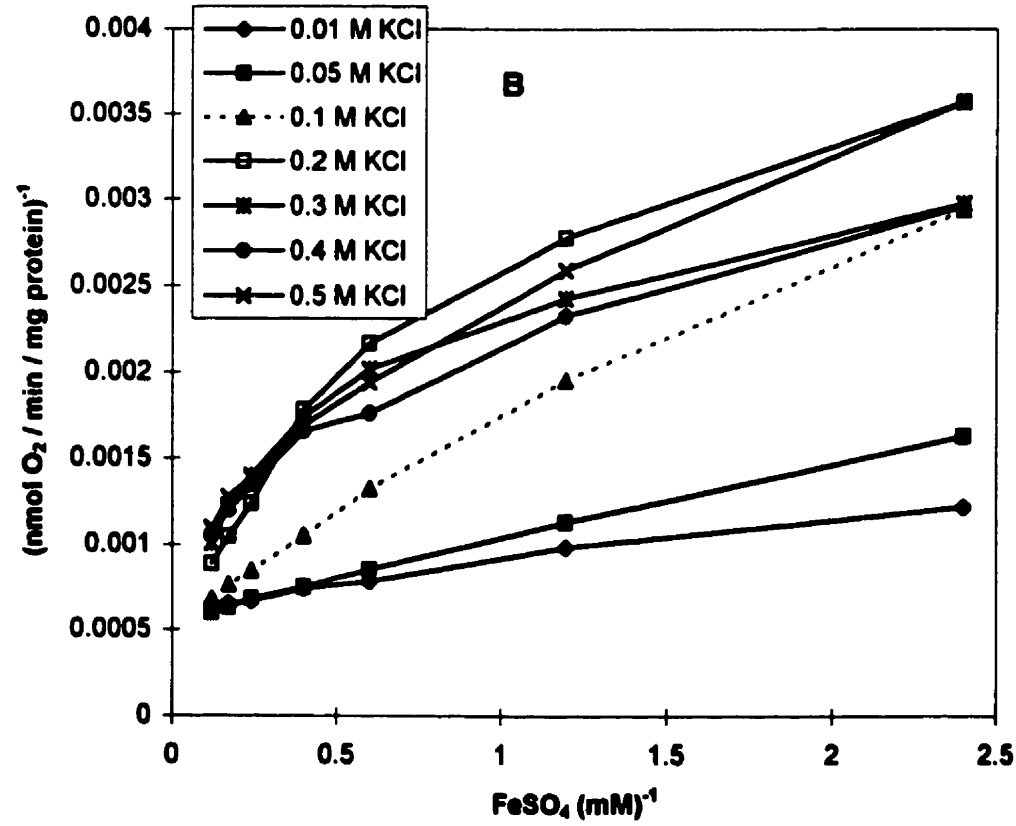
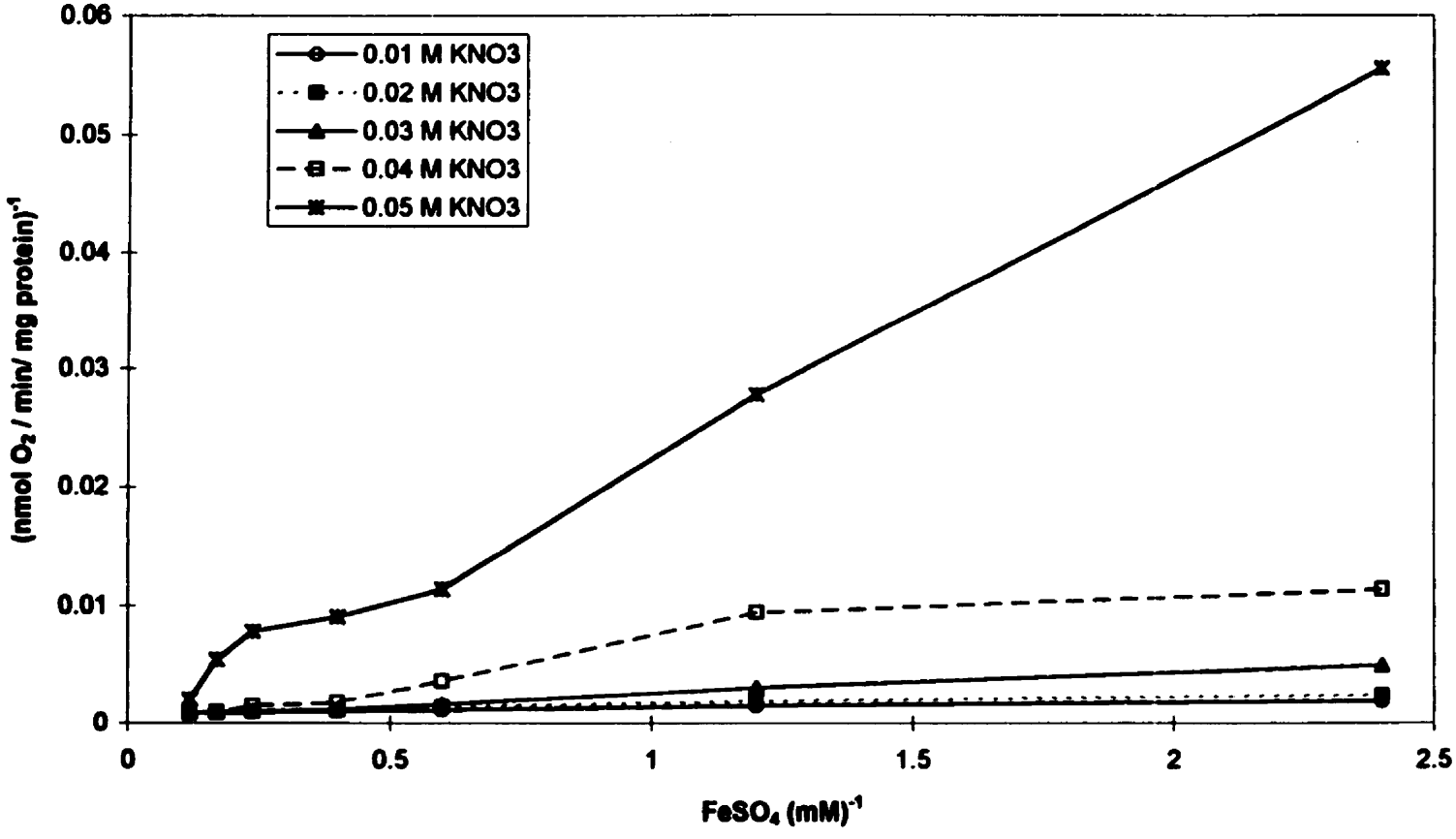

B
0.01 M KCl
0.05 M KCl
0.1 M KCl
0.2 M KCl
0.3 M KCl
0.4 M KCl
0.5 M KCl
0.004
0.0035
0.003
0.0025
0.002
0.0015
0.001
0.0005
0
0
0.5
1
1.5
2
2.5
(nmol O2 / min / mg protein)-1
FeSO4 (mM)-1

Figure 5. Effect of increasing potassium nitrate concentrations on iron oxidation. The reaction mixture contained 0.5 mg wet cells (SM-4) and Fe^{2+} in potassium nitrate pH 2.3.


0.06
0.05
0.04
0.03
0.02
0.01
0
0.01 M KNO3
0.02 M KNO3
0.03 M KNO3
0.04 M KNO3
0.05 M KNO3
(nmol O2 / min/ mg protein)-1
0
0.5
1
1.5
2
2.5
FeSO4 (mM)-1

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