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Characterization of arsenopyrite oxidizing *Thiobacillus*. Tolerance to arsenite, arsenate, ferrous and ferric iron

Marie-Noelle Collinet[1,2] & Dominique Morin[1]
[1] *BRGM Avenue de Concyr, BP 6009, 45060 Orléans Cédex, France;* [2] *Orstom Laboratoire de Microbiologie, Université de Provence, Place Victor Hugo, 13331 Marseille Cédex 3, France*

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Abstract

Two strains of *Thiobacillus, T. ferrooxidans* and *T. thiooxidans*, have been isolated from a bacterial inoculum cultivated during a one-year period in a 100 l continuous laboratory pilot for treatment of an arsenopyrite/pyrite concentrate. The optimum pH for the growth of both strains has been found to be between 1.7 and 2.5. Because of the high metal toxicity in bioleach pulps, the tolerance of *T. ferrooxidans* and *T. thiooxidans* with respect to iron and arsenic has been studied. The growth of both strains is inhibited with 10 g/l of ferric ion, 5 g/l of arsenite and 40 g/l of arsenate. 20 g/l of ferrous iron is toxic to *T. ferrooxidans* but 30 g/l is necessary to impede the growth of *T. thiooxidans*.

Introduction

In spite of the high toxicity of arsenic, *Thiobacillus ferrooxidans* can derive its energy from the decomposition of arsenic sulfides (Karavaiko et al. 1977). Arsenic contained in arsenopyrite is solubilized in the form of As^{III} and As^{V} (Panin et al. 1985), and iron in the form of Fe^{2+} and Fe^{3+}. The main reactions enhanced by bacterial metabolism are:

$$2\,FeAsS + 5.5\,O_2 + 3\,H_2O \rightarrow 2\,H_3AsO_3 + 2\,FeSO_4 \quad (1)$$

$$2\,FeAsS + 6.5\,O_2 + 3\,H_2O \rightarrow 2\,H_3AsO_4 + 2\,FeSO_4 \quad (2)$$

$$2\,FeSO_4 + H_2SO_4 + 0.5\,O_2 \rightarrow Fe_2(SO_4)_3 + H_2O \quad (3)$$

Arsenous acid, H_3AsO_3, produced by reaction (1) is also partially oxidized into arsenic acid, H_3AsO_4. Furthermore a large proportion of products precipitate as follows (Livesey-Goldblatt et al. 1983):

$$Fe_2(SO_4)_3 + 2\,H_3AsO_4 \rightarrow 2\,FeAsO_4 + 3\,H_2SO_4 \quad (4)$$

$$Fe_2(SO_4)_3 + 6\,H_2O \rightarrow 2\,Fe(OH)_3 + 3\,H_2SO_4 \quad (5)$$

$$Fe(OH)_3 + 3\,H_2SO_4 \rightarrow Fe(OH)\,(SO_4) + 2\,H_2O \quad (6)$$

Concentrations of arsenic and iron in solution rapidly inhibit the growth of bacteria (Tuovinen et al. 1971, Braddock et al. 1984; Norris & Kelly 1978). Strains more resistant to these metals are selected from among wild bacterial populations by successive cultures in media of increasing arsenopyrite concentration.

In order to interpret and control the parameters

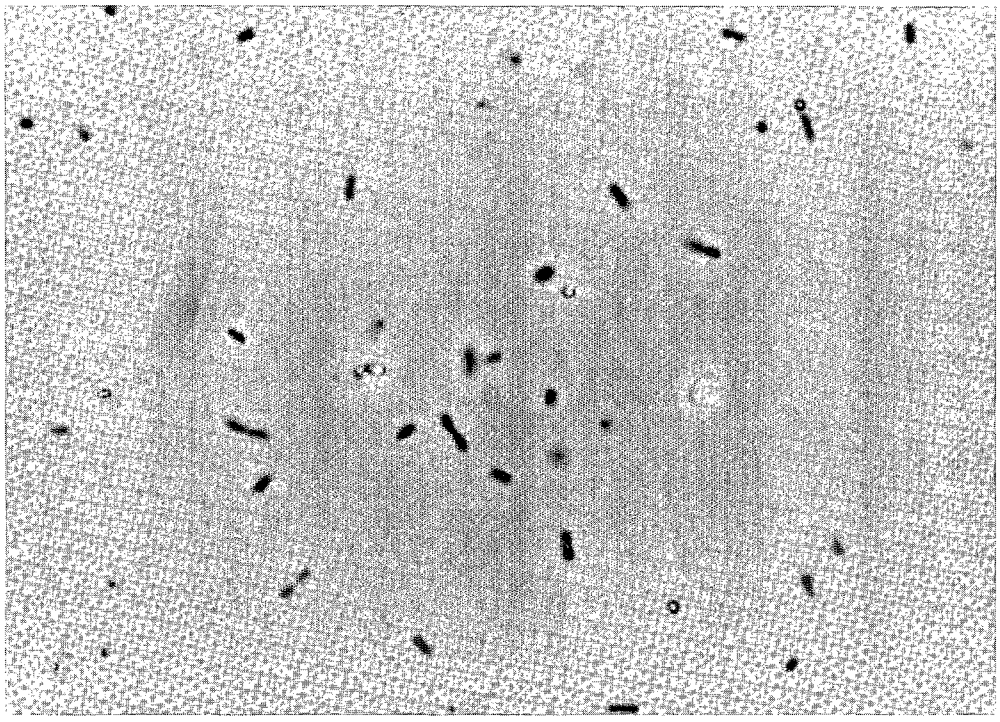

Fig. 1. Observation of the mixed culture by optical microscopy (× 1000).

of the bioleaching process, it is necessary to assess the maximum concentration of metals tolerated by the bacteria involved.

This study deals with the identification of strains contained in adapted mixed culture and with their tolerances of the main elements in solution.

Materials and methods

Inoculum

The inoculum used was obtained by enrichment of cultures from slurries sampled in South African sulfide ore mines. The basal enrichment medium contains per liter 3.7 g $(NH_4)_2SO_4$; 0.8 g H_3PO_4 (80%); 0.5 g KOH; 0.5 g $MgSO_4 \cdot 7H_2O$, 3% sulfide compound and 9 g of ferrous ion as ferrous sulfate.

In order to obtain strains adapted to arsenopyrite treatment, the inoculum was cultivated in successive basal media in which ferrous ions have been progressively replaced by sulfide substrate up to 10% (wt/vol).

The cultures were made in air-lift aerated and agitated tubes. The temperature was maintained at 35° C by a thermostated bath. The inoculum obtained was cultivated first in a 1 litre batch reactor and then in a 100 litre continuous laboratory pilot (agitated and aerated reactors) that has been in operation for three years.

Energy substrate

This is an arsenopyrite flotation concentrate from a French mine which contains 35.5% iron, 19.9% arsenic and 27.7% sulfur as sulfides. These data can be used to calculate the approximate proportions of the principal sulfides, which give, on the basis of their simplest formula:

40% FeAsS, 28% FeS_2 and 14% FeS.

This concentrate was reground to particles $< 80\mu m$. For pure strain culture the ground con-

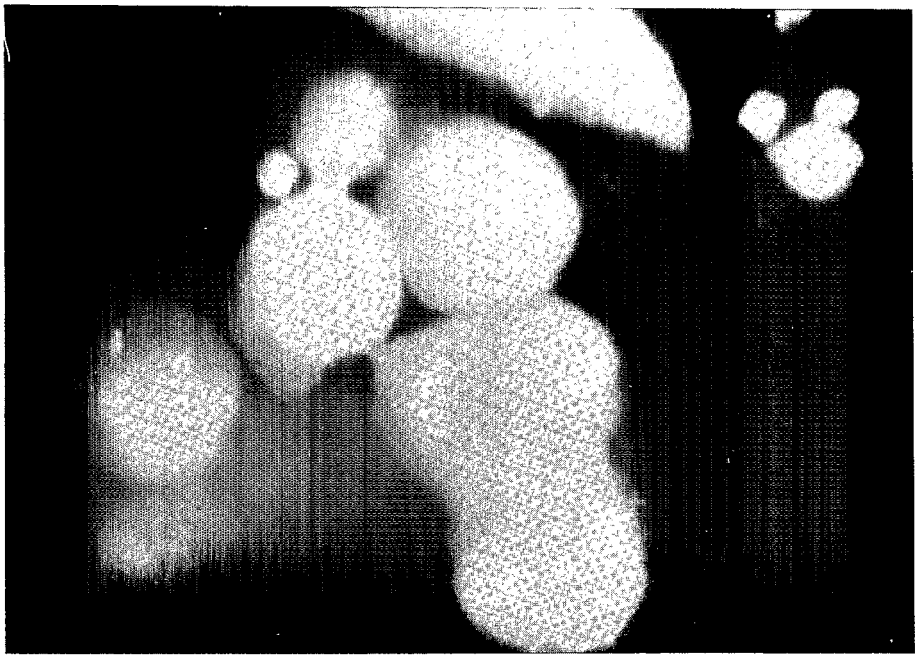

Fig. 2. Colonies obtained on filters covered with elemental sulfur (× 100).

centrate was sterilized at 100° C for one hour a day for three days.

Isolation of microorganisms

The following solid media were tested for this purpose:
- 9 K medium (Silverman & Lundgren 1959) solidified with agar of different qualities; noble agar, bacto agar Difco and agarose Sigma; at various concentrations.
- ISP medium (Manning 1975),
- FeTsB medium (Johnson et al. 1983),
- Elemental sulfur medium (Mouraret & Baldensperger 1977).

The following liquid media were also used:
- 9 K medium (Silverman & Lundgren 1959).
- Waksman medium (Waksman & Joffe 1922), pH initialized at 1.8.
- Glucose medium (Kuenen & Tuovinen 1981).

Energy sources were sterilized separately, the ferrous sulfate by filtration and elemental sulfur by heating at 100° C for one hour a day for three days. The nutrient solution and agar were sterilized separately in an autoclave at 120° C for twenty minutes.

Characterization of strains

The influence of pH between 1 and 5 was studied using the basal medium containing 10% (wt/vol) of sulfide concentrate.

The tolerances of the bacteria to iron and arsenic were studied separately using the basal medium to which 10 g/l of elemental sulfur was added as sole energy source. Iron was introduced at different concentrations as ferrous and ferric sulfate and arsenic as disodic hydro-arsenate and sodium meta-arsenite.

Cultures were performed at 35° C in 500 ml erlenmeyer flasks containing 200 ml of medium and placed on an agitating table.

Analytical methods

Free bacteria in solution were counted directly with a Thoma counting cell under a microscope

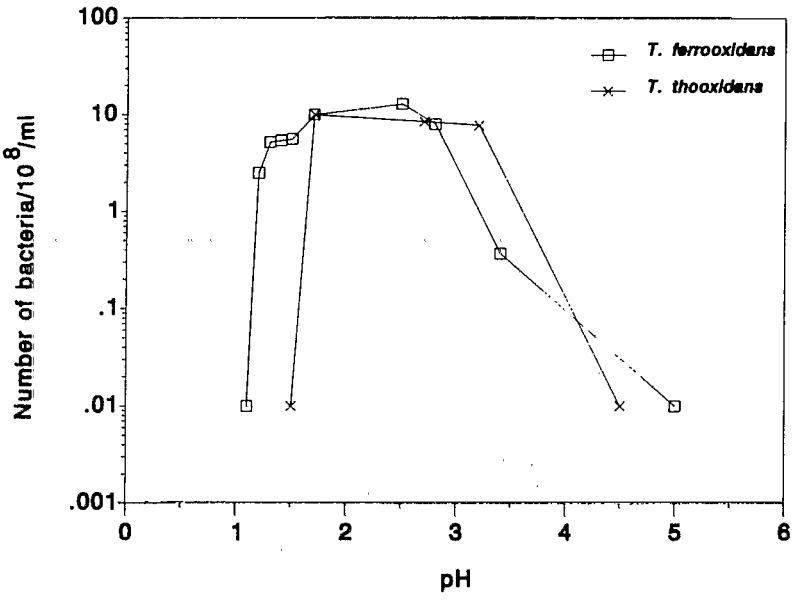

Fig. 3. Effect of pH on the growth of the two strains.

(× 400). Ferrous and ferric iron were titrated by a colorimetric method using ortho-phenantroline (Charlot 1966).

Results and discussion

After one year of continuous bioleaching, a sample of pulp was analysed for its bacterial composition.

Isolation and identification

The sample was observed under a microscope (× 1000). The bacterial population was composed of bacilli, isolated or in pairs, seldom in chains, poorly mobile, asporulated and acapsulated, 0.5 to 2μm in length (Fig. 1). All were Gram negative.

The described culture conditions favor the growth of aerobic, mesophilic, acidophilic, autotrophic bacteria which use sulfides as their energy source. This suggests the existence of *Thiobacillus*-type bacteria. No form of *Leptospirillum* was observed.

Colonies from these bacteria develop only on filters covered with elemental sulfur and laid on solid nutritive medium (medium of Mouraret) and Baldensperger 1977). These colonies, only 0.1 to 0.7 mm in diameter, were yellow, spherical, regular, convex and shiny (Fig. 2). It has been impossible to isolate a pure strain from these colonies because of their mutual contamination through a water layer on the membrane surface.

No colony was obtained on other solid media containing only ferrous iron as energy source, i.e. 9 K solidified, ISP and FeTsB media. This is not surprising, since previous authors (Tuovinen & Kelly 1974; Mishra & Roy 1979) have shown that *Thiobacillus* and especially the extremely acidophilic *Thiobacillus* sampled in mines do not grow easily on solid media. However, trials performed in liquid media enabled two strains of *Thiobacillus* to be isolated.

It can be assumed that 9 K medium with ferric ion as sole energy source and an initial pH of 1.5 is *T. ferrooxidans* selective (if *Leptospirillum ferrooxidans* is not present). A *T. ferrooxidans* strain has been isolated in 9 K medium using the principle of the most probable number (MPN) method. This medium was inoculated with increasing dilutions of the mixed culture, and the MPN method was applied three times. The GC% of this strain, deter-

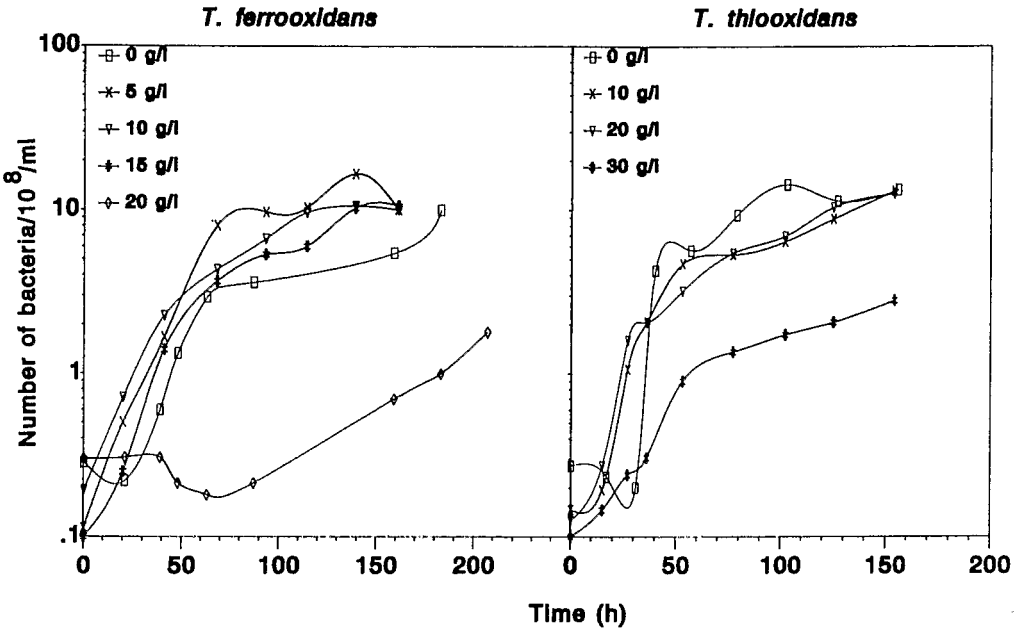

Fig. 4. Effect of ferrous concentration on the growth of the two strains.

mined by the DSM German Collection of Microorganisms and Cell Cultures, is 57.3.

It also appears that the mixed culture contains no heterotrophic bacteria – the culture was negative in the glucose medium. Also any colonies has been developed on the FeTsB medium that contained tryptone soya broth.

Three successive cultures in Waksman medium inoculated, as soon as growth began, gave a culture that did not oxidize ferrous ion in 9 K medium. The

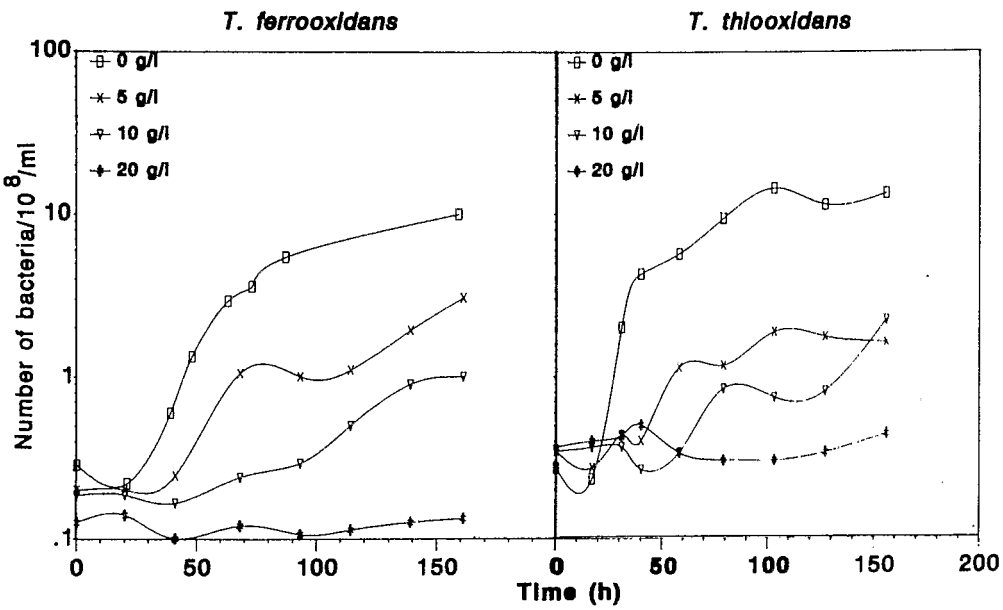

Fig. 5. Effect of ferric concentration on the growth of the two strains.

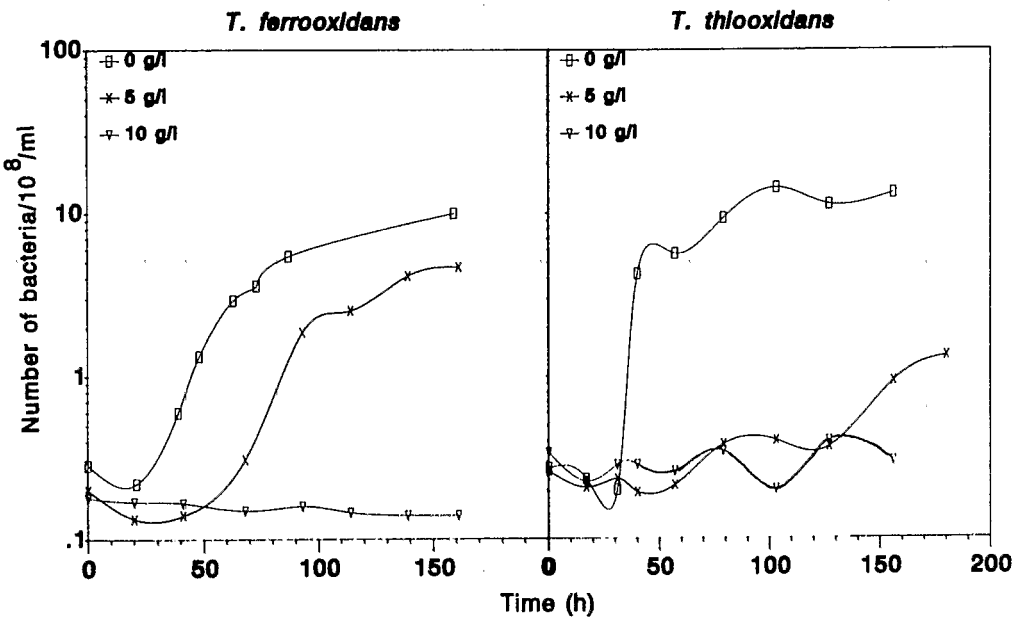

Fig. 6. Effect of arsenite concentration on the growth of the two strains.

extreme acidity of the medium suggests the presence of a *Thiobacillus thiooxidans* strain. The selection towards *T. ferrooxidans* may be the result of a difference in the kinetics of growth of the two species when elemental sulfur is the only source of energy. The GC% of this strain has not yet been determined.

Influence of pH

The influence of pH has been assessed by a series of cultures in erlenmeyer flasks containing the basal medium and 10% of sulfide concentrate at values of pH ranging from 1 to 5. Because the sulfide components tend to buffer the solution to a pH close to 2.5, it was necessary to adjust the pH by daily addition of KOH or H_2SO_4 solution. The pH was no longer regulated once bacterial growth commenced.

The number of bacteria in suspension after 90 h of growth for *T. ferrooxidans* and 150 h for *T. thiooxidans* versus pH is given in Fig. 3. The optimum pH for the two strains is between 1.7 and 2.5.

Influence of iron and arsenic

The influence of iron and arsenic on bacterial growth has been studied in the Waksman medium for both strains. This medium has the advantages that both strains can grow on it and no precipitate is formed either with ferrous and ferric ions or with arsenic III and V.

At concentrations up to 20 g/l, ferrous ion favors the growth of *T. ferrooxidans* (Fig. 4). The latency time of about 20 h when no iron is present vanishes when the medium contains iron. The involvement of the Fe^{II}/Fe^{III} couple in the sulfur metabolism of *T. ferrooxidans* may account for this (Corbett & Ingledew 1987; Sugio et al. 1988). At 20 g/l however *T. ferrooxidans* no longer grows. It must be pointed out the ferrous ion added to the Waksman medium is not oxidized. Cultivated in the 9 K medium, the strain recovers its ability to oxidize ferrous ion but after a latency time during which it is assumed that it synthesizes the appropriate enzymatic metabolic system.

T. thiooxidans is not as sensitive since its growth is slowed down only when the ferrous iron concen-

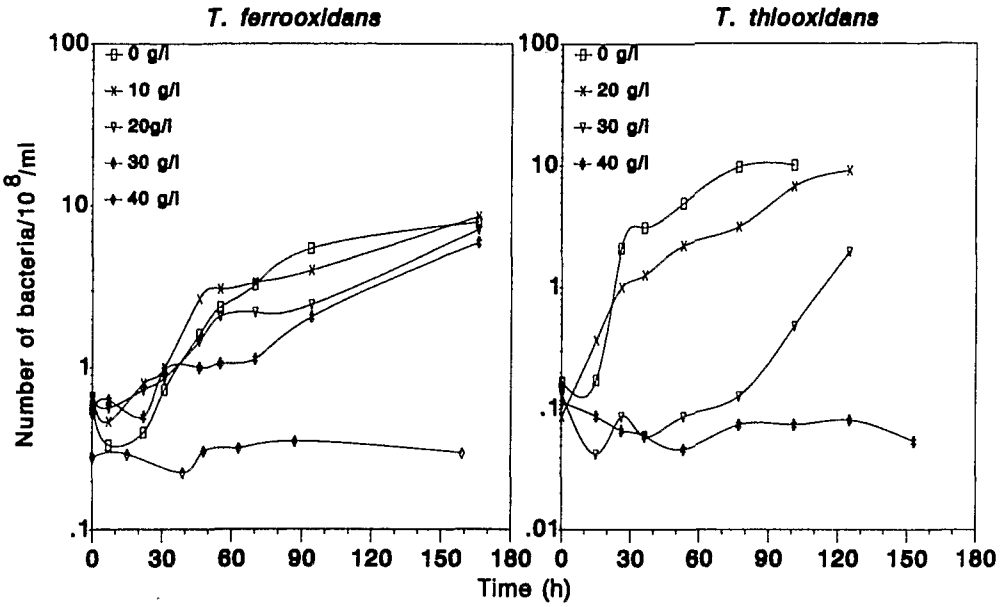

Fig. 7. Effect of arsenous concentration on the growth of the two strains.

tration reaches 30 g/l. Ferric ion is more toxic towards the growth of both strains (Fig. 5). With 5 g/l of ferric ion, the growth rates of *T. ferrooxidans* and *T. thiooxidans* are not strongly affected but the exponential growth phase is much shorter. At 20 g/l growth is completely inhibited.

Arsenite ion has a strong effect on the growth of both strains (Fig. 6). When its concentration reaches 10 g/l, no strain is able to develop. At 5 g/l, the latency phase is increased, much more for *T. thiooxidans* than for *T. ferrooxidans*. As shown in Fig. 7, both *T. ferrooxidans* and *T. thiooxidans* tolerate a high concentration of arsenate, which has the same effect on both strains. Its inhibitory effect is progressive up to 40 g/l.

In accordance with Groudev's observation (personal communication), arsenite is the most toxic element. Its concentration in bioleaching pulps will probably inhibit bacterial growth before the concentrations of other metals reach toxic levels. But, depending on the energy substrate present in the medium, both thiobacilli do not react in the same way, *T. ferrooxidans* being more tolerant of arsenic in a sulfide medium (Tuovinen et al. 1971).

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