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ISOLATION AND IDENTIFICATION OF IRON-SULPHUR OXIDIZING THIOBACILLI FROM IRAN ENVIRONMENTS

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Abstract

Two hundred samples were taken from various sites in Iran, including coal storage areas, waste water in the Tabas and Zirab coal mines, and sulphur-containing acid springs in Ramsar and Abask, and were screened for *Thiobacillus ferrooxidans*. During this investigation, seven strains were isolated which could oxidize sulphur, pyrite, thiosulphate and ferrous iron as the sole energy source. Macroscopic, microscopic and biochemical studies showed that the isolates were *T. ferrooxidans*.

Introduction

Biological leaching processes for metal recovery are based on the bacterial oxidation of sulphide minerals, basically involving ferrous iron and various inorganic sulphur compounds as the electron donors [9]. These processes are used in industrial scale for leaching metals [13, 23].

The main microorganisms isolated which catalyze the oxidation of sulphurated minerals are *Thiobacillus ferrooxidans, Thiobacillus thiooxidans, Leptospirillum ferrooxidans, Acidianus brierleyi, Sulfobacillus thermosulphidooxidans, Sulfolbus acidocaldarius* [1]. These microorganisms have an important ecological role in nature [6]. In mining environments, particularly in mine waters where large quantities of heavy metals, low pH and, in some cases, high temperatures are possible, some microorganisms which have adapted to this environment can be found [27]. Among these iron-oxidizing bacteria, *T. ferrooxidans*, which was first isolated and named by Colmer and his co-workers [3], is the microorganism most widely used in industry to leach metals from mineral ores [5,6,14,15,17,21,27,28] and in coal desulphurization [19,20,25]. It has also been used for the bioleaching of metals from sewage sludge [2,28].

The iron-sulphur oxidizing microbial flora from environments in Iran, has, to the best of our knowledge, not been investigated. Reports concerning the isolation of iron-sulphur oxidizing thiobacilli from these environments have not yet been published in the literature. In this paper, the results of our investigations into the presence, isolation and identification of iron-sulphur oxidizing thiobacilli or its application in the biological leaching process are presented.

Keywords: Bioleaching; *Thiobacillus ferrooxidans*

Materials and Methods

Collection of Samples

Two hundred samples for isolation were collected from various sites in Iran, including coal storage areas, waste water in the Tabas and Zirab coal mines, and sulphur-containing acid springs in Ramsar and Abask.

Isolation

Isolation was carried out in a test tube under aerobic and static conditions at 30°C by using 9K medium [24] consisting of 3.0 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4$, $7H_2O$, 0.1 g KCl, 0.5 g K_2HPO_4, 0.01 g $Ca(NO_3)_2$ and 42.2 g $FeSO_4$.,

$7H_2O$ in one litre of distilled water, adjusted to pH 2.00 by 6N H_2SO_4. Fifteen days after enrichment, culture media, whose colour had changed to reddish brown indicating iron oxidation, were transferred to 3.00 ml of the basal solution of solid 2:2 medium [8] for single colony isolation and morphological studies on solid 2:2 medium. Plates were incubated at 30°C and transferred to 9K liquid media when colonies formed.

Growth under Autotrophic Conditions

Isolates were cultivated autotrophically in basal salt 9K medium supplemented with one of the following as the sole energy source: 2% w/v pyrite which was ground to minus 100 µm and saved; sulphur (1%w/v); thiosulphate (0.5% w/v); or ferrous sulphate heptahydrate (4.2% w/v). An uninoculated control was used to assess the extent of chemical oxidation of substrate. Soluble ferric iron concentration was monitored by determining the residual ferrous concentration at various intervals. The ferrous iron concentration was determined by its reaction with *o*-phenanthroline [26]. Total soluble iron was determined by the Colorimetric *o*-Phenantroline Method suggested by the American Society for Testing Materials (ASTM Designation: D516-88). Ferric iron concentration was calculated by subtracting ferrous iron from total soluble iron. The rate of ferric iron production is determined from the linear portion of a curve plotting ferric produced versus time. In fact the exponential rate of iron oxidation was indicative of exponential growth [12].

To adapt the isolates to elemental sulphur as a solid substrate, the strains were subcultured aerobically at 30°C in the 9K basal salt media with 1% w/v elemental sulphur. The initial pH of the media was adjusted to 2.00 by using H_2SO_4. Cultures were transferred at two-week intervals. Ten transfers were necessary to achieve the adaptation of the *T. ferrooxidans* [11]. Seven-day inoculums previously grown in the media were used in the subsequent experimental runs. The liquid samples were analyzed for sulphate [ASTM Designation: D 1068-88].

Pyrite oxidation was monitored by determining the rate of soluble iron production from pyrite which was calculated by plotting iron concentration in solution against time. The rate is obtained by determining the slope of the linear part of the leaching curve and is expressed in mg of iron per litre per hour [18]. No attempt was made to optimize pyrite oxidation. Control and inoculated flasks were incubated in a shaker incubator at 200 rpm at 30°C for 15 days.

Thiosulphate oxidation ability of isolated strains was determined by cultivation in 9K mineral media at pH 4.5 and supplemented by 0.5% thiosulphate. The residual thiosulphate concentration was determined by titration with 0.01N iodine solution [7] after shaking at 200 rpm in an orbital incubator shaker at 30°C for six days.

Heterotrophic and Mixotrophic Properties of Strains

Strains were subcultured from 9K media in heterotrophic media which contained Difco yeast extract 0.01% w/v. Mixotrophic properties of isolates were determined by adding 0.01% w/v Difco yeast extract to 9K media by weight per volume [10].

Electron Microscopy Study

Transmission electron microscopy (Phillips Model R400) was carried out on isolates with negative staining by 3% w/v phosphotangstate. *T. ferrooxidans* (DSM 581) was purchased from Deutshe Summlung Von Microorganism and used in all experiments as standard reference.

Results

Collection of Samples and Isolation

Strains designated M3, M7, M8, M9 and strains M4, M5, M6 were isolated during screening program on samples collected from a sulphur-containing acid spring in Ramsar and Zirab coal mine respectively. All cultures were purified

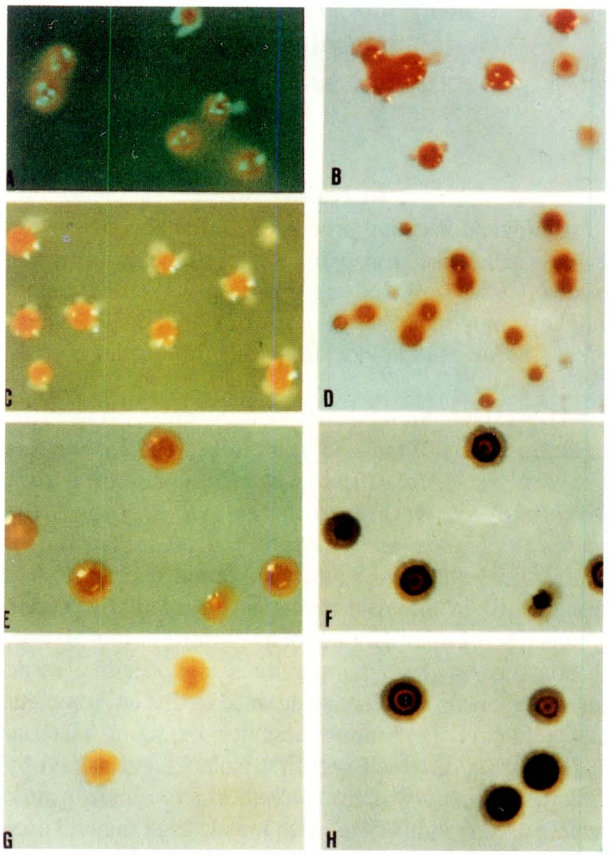

Figure 1. Colony of isolated strains on solid 2:2 medium. (A)M3, (B)M4, (C)M5 (D)M6 (E)M7 (F)M8 (G)M9 (H)*T. ferrooxidans*.

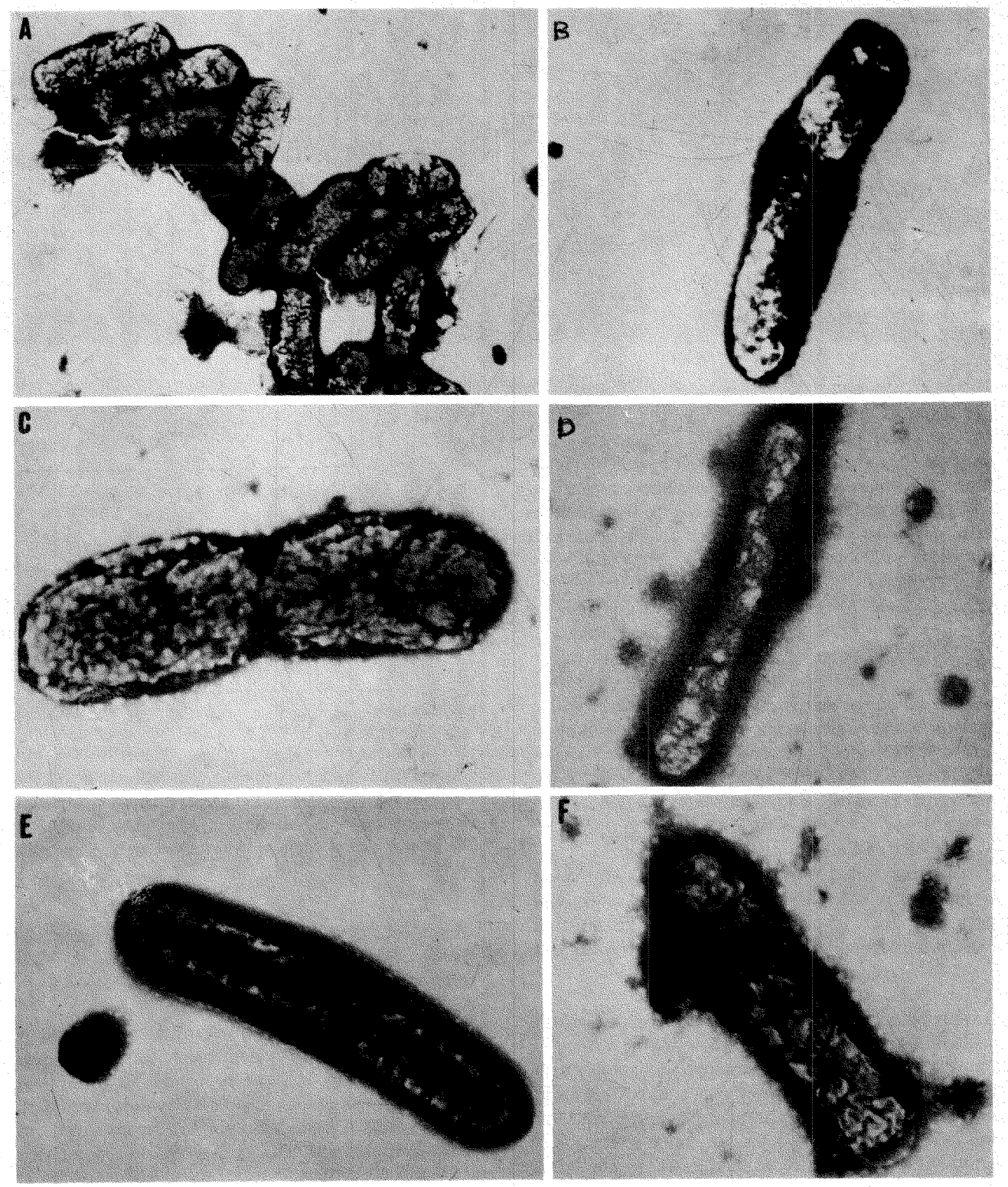

Figure 2. Transmission electron micrograph of isolated strains. (A)M3 ×45000, (B)M4 × 34000, (C)M5 × 34000, (D)M6 × 20000, (E)M7 × 40000, (F)M8 × 60000, (G)M9 × 34000 and (H) *T. ferrooxidans* DSM 581 × 45000

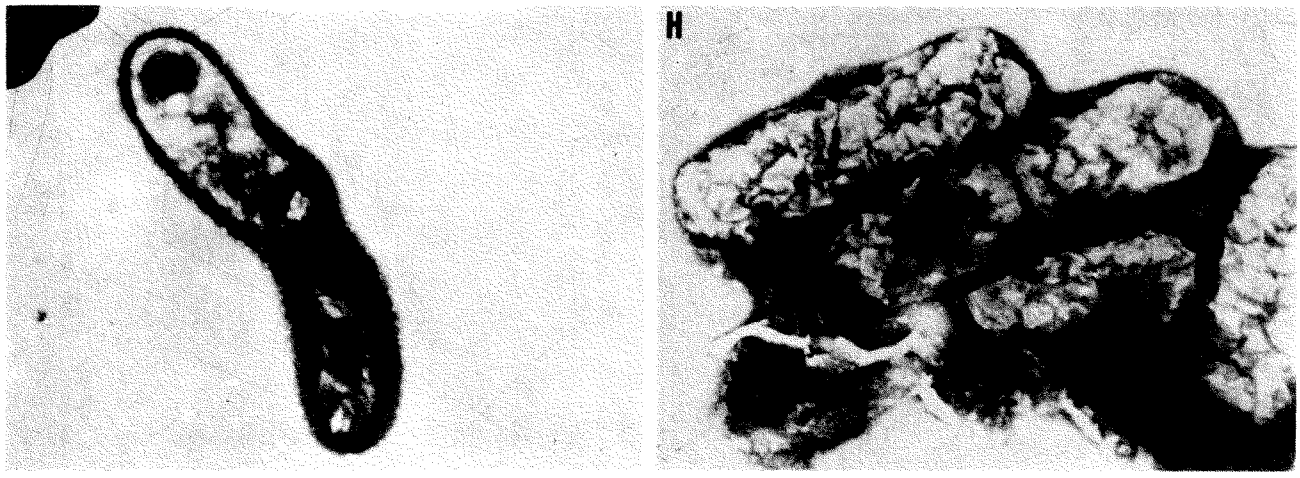

Figure 2. (continued) (G)M9 × 34000 and (H) *T. ferrooxidans* DSM 581 × 45000

by obtaining a single colony on solid 2:2 medium. Figure 1 shows a section of the plates with colonies related to isolates. The most frequently observed type was generally semi-spheroidal with a smooth surface, a round centre with a white or yellow band outside, and a margin with many short projections. Sometimes two opposite projections extended more than others and the colony resembled a tortoise. Growth began with a very small clear colony with an entire round margin. Subsequently, a white or yellow substance was deposited in the centre, and many projections extended from the margin and gradually spread forming many petal-like attachments around the center, resulting in an appearance similar to a sunflower. Colonies of isolates M3, M4, M5, M6, M7, M8 and M9 generally belong to the same type described for *T. ferrooxidans* [8].

Morphology of Cells

All the isolates M3, M4, M5, M6, M7, M8 and M9 were Gram-negative and appeared as rods, usually single or in pairs, 0.5 ×1.0 to 1.5 μm. Figure 2 shows a transmission electron micrograph of isolated strains and *T. ferrooxidans* DSM 581.

Autotrophic, Heterotrophic and Mixotrophic Growth

Liquid media with ferrous sulphate at pH 1.6 changes from clear pale green to an amber to red-brown colour with ferric sulphate. At pH 1.9 and above, considerable ferric sulphate precipitation (jarosite) takes place. Iron oxidation was initiated 17-20 hours after inoculation with isolated strains. They completely converted ferrous iron to ferric iron after 42-45 hours. Figure 3 shows the rate of ferric iron production (mg/l/h) by isolated strains compared with uninoculated flask.

The oxidation of sulphur was initiated by sulphur

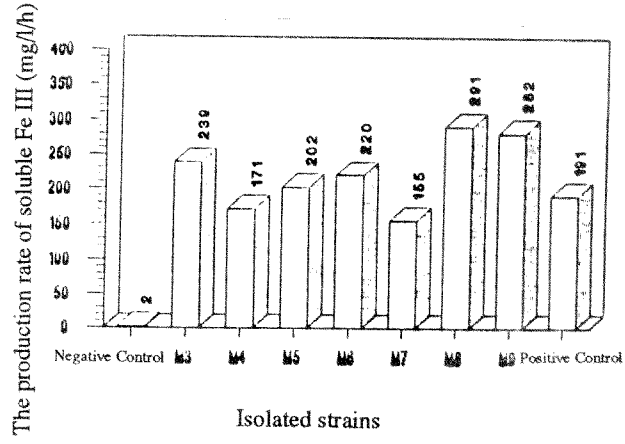

Figure 3. The production rates of soluble FeIII (mg/litre/h) in 9K media inoculated by the isolated strains and *T. ferrooxidans* DSM 581 (positive control) compared with uninoculated flask (negative control)

adapted cells, therefore the pH of sulphur-containing media changed from 4 to about 1. Figure 4 shows the final sulphate concentration of media after incubation for ten days.

All of the isolated bacteria oxidize pyrite as sole energy source. Figure 5 shows soluble iron production rates (mg/l/h) and final pH of the media inoculated with isolates and standard reference and compared with uninoculated flask during fifteen days incubation. Furthermore, Figure 6 shows the percent of residual thiosulphate in inoculated and uninoculated flasks after six days.

The effect of temperature on the growth of isolates was studied in the range of 20°C to 50°C. The effect of pH on growth of isolates was evaluated by direct microscopic examination and generation of low pH during growth on

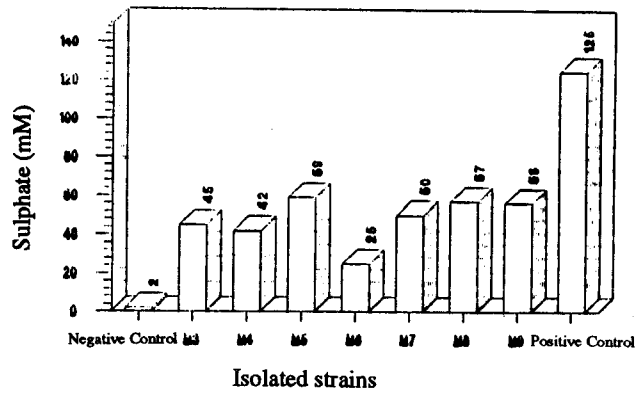

Figure 4. Sulphate production by the isolated strains and *T. ferrooxidans* DSM 581 (positive control) in sulphur-containing media compared with uninoculated flask (negative control)

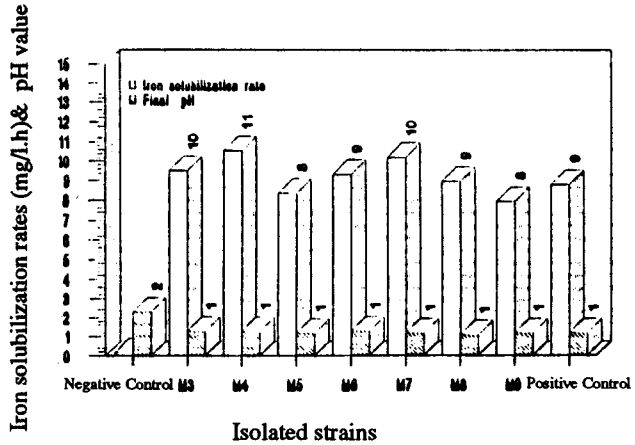

Figure 5. Iron solublization rates (mg/litre/h) and final pH of 9K mineral salts media supplemented with 2% pyrite and inoculated with isolated strains and *T. ferrooxidans* DSM 581 (positive control) compared with uninoculated flask (negative control)

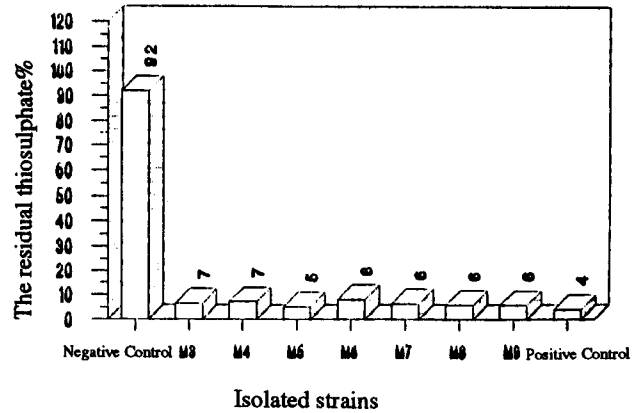

Figure 6. The percent of residual thiosulphate in 9K mineral salts media supplemented with 0.5% thiosulphate and inoculated with isolated strains and *T. ferrooxidans* DSM 581 (positive control) compared with uninoculated flask (negative control)

9K media adjusted pH to 2, and sulphur-containing 9K basal salt media adjusted pH to 4,5,6,7 and 8. It was found that they grew at a pH below 5 and a temperature below 37°C. None of the strains could grow under heterotrophic and mixotrophic conditions.

Discussion

The most important iron-sulphur oxidizing microorganisms were divided into mesophiles of the genera *Thiobacillus* and *Leptospirillum*, moderate thermophiles of the genus *Sulfobacillus*, and extreme thermophiles of the genera *Sulfolobus, Acidianus, Metallophaera* and *Sulfurococcus*. The main criteria for this categorizing is optimum temperature which is usually 30, 50, and 70°C for mesophiles, moderate thermophiles and thermophiles [15]. Isolated strains were obligately chemolithotrophic and grew at a pH below 5 and a temperature below 37°C. *T. ferrooxidans, T. thiooxidans,* and *L. ferrooxidans* are mesophilic, iron and sulphur oxidizing acidophilic bacteria. *T. ferrooxidans* is one of the most important organisms in the oxidation of minerals sulphide in most acidic environments if the optimum temperature is below 40°C. They are Gram-negative rods, rod-shaped bacteria are usually 0.5 by 1 to 1.5 μm, occurring singly or in pairs [10,15]. The optimum temperature for most strains of *T. ferrooxidans* is probably about 30 to 35°C [10]. *T. ferrooxidans* grows on pyrite, sulphur, ferrous sulphate, and thiosulphate. *T. thiooxidans* resembles *T. ferrooxidans* in its acidophily, in its growth on sulphur with an optimum temperature of about 30°C, and in its morphology. In pure cultures, *T. thiooxidans* differs from *T. ferrooxidans* in its inability to oxidize iron. Also *T. thiooxidan* is unable to degrade pyrite effectively. *L. ferrooxidans* occurs as curved rods and the vibrioid cells, which often develop into spiral forms of varying length [22], are readily distinguishable from *T. ferrooxidans* because their cells are slightly thinner. In contrast to *T. ferrooxidans*, no strains of *L. ferrooxidans* have been reported to oxidize sulphur and thiosulphate [15], but *L. ferrooxidans* could efficiently degrade pyrite in pure culture [16].

Isolated strains were Gram-negative, rod-shaped, and occurred singly or in pairs. Strains oxidized and grew on ferrous iron, pyrite, sulphur and thiosulphate as sole energy sources and there was a significant difference between oxidation rates of substrates (Figs. 3,4,5,6) in inoculated and uninoculated flasks. By the above results, these strains are characterized as *T. ferrooxidans*.

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