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ISOLATION AND CHARACTERIZATION OF SOME ACIDOPHILIC THIOBACILLI FROM SEWAGE WATERS

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By appropriate enrichment, two acidophilic chemolithotrophic bacterial strains resembling *Thiobacillus thiooxidans* and *T. ferrooxidans* have been isolated from sewage waters. These strains possess characteristics which make them suitable in bacterial solubilization of sulphide as well as carbonate bearing ores by sulphur or ferrous iron amendments. Growth behaviour of these strains on sulphur, ferrous iron and thiosulphate is reported.

Key words: Isolation, Thiobacilli, Acidophilic.

INTRODUCTION

Bacterial oxidation of minerals is an economical technique for leaching of copper and uranium in heap, dump and in situ leaching [1, 2]. The principal reactions involved in bacterial leaching are the direct oxidation of sulphide minerals and the indirect dissolution of metal sulphides and uranium oxides by ferric iron, which can be formed from bacterial oxidation of ferrous iron or iron sulphide minerals [3]. In general, sulphur and iron oxidizers are ubiquitous in soil, fresh water and marine enviornments, as well as in sediments. These bacteria have been isolated by enrichment techniques from natural sites such as hydrogen sulphide containing sediments from rivers, canals, estuarines and tidal flats, acid sulphate soils, acid springs, mine drainage effluents [4]. The methods for the isolation, purification, identification and characterization of such microorganisms have been described by a number of authors [5-10].

The results reported here established the ubiquitous distribution of *Thiobacillus thiooxidans* and *T. ferrooxidans.* Their characterization for oxidation of sulphur, ferrous iron and thiosulphate utilization are also reported.

MATERIALS AND METHODS

Media. In this study a medium containing inorganic salts described by Silverman and Lundgren, [9], without ferrous sulphate (9K minus Fe), was used as basal medium. This medium after supplementation with filter sterilized ferrous sulphate (5 % w/v 9K Fe), or after the addition of sterilized sulphur (1 % w/v 9KS) was used for enrichment and growth studies. The pH of these media was adjusted to 2.5. For growth studies of *T. thiooxidans* (NB101) isolated during the course of this work, the 9K minus Fe medium was supplemented with sodium thiosulphate (40 mM) at pH 4.5. For the utilization of thiosulphate by *T. thiooxidans* (NB101), the medium reported by Vishniac and Santer [11] at pH 6.0 was also used. Growth studies were also made using Harrison's mineral salt (MS) medium [7] containing 0.2 % powder sulphur. Sulphur was sterilized by heating at 105° for 30 min on two successive days. All pH adjustments were made with sulphuric acid or ammonium hydroxide. Distilled water at pH 2.5, adjusted with sulphuric acid was used as diluent where ever needed.

Source of bacteria and enrichment. Sewage water from four disposal points in Faisalabad, were collected and pooled to form a composit sample. For enrichment, this sample (5 % w/v was mixed with mineral medium described above and incubated at 30° in shake flasks. Alliquote from flasks with positive growth were used as inoculum for fresh medium, and this type of transfer was successively carried out three times. Drop in pH in case of sulphur grown cultures and production of ferric sulphate in case of iron was taken as positive growth.

Enrichment procedure used, was adopted from Bergey's manual of determinative bacteriology [12] (results shown in Table 1). Media 9KS and 9K Fe, respectively, are used for the enrichment of *T. thiooxidans* and *T. ferrooxidans.* These enriched cultures were separated by centrifuge at 12,000 RPM for 15 min at 5°. The pallet of cells, in each case was suspended in pH 2.5 H_2SO_4 and used for purification.

Purification. Purification of *T. thiooxidans* was carried out by statistical dilution method [7]. Suspension of cell grown on 9KS medium was serially diluted and 0.1 ml of

Table 1. Enrichment procedure adopted (summarized from Vishniac 1974) to isolate *Thiobacillus thiooxidans* and *Thiobacillus ferrooxidans* from sewage water.

Tests	Bacterial to be enriched		Expected enrichment
	Thiobacillus thiooxidans	*Thiobacillus ferrooxidans*	
Original pH of medium	2.5	2.5	Acidophilic bacteria
Growth on sulphur	+++	+	Acidophilic Thiobacilli
Temperature of growth	25-30°C	25-30°C	Mesophilic thiobacilli
Minimum pH attained*	0.87	1.4	*T. thiooxidans* and *T. ferrooxidans*
Growth on Fe^{++} pH 2.5**	–	++	*T. ferrooxidans*
Growth on Fe^{++} pH 1.5**	–	++	*T. ferrooxidans*
Thiosulphate utilization at			
pH 6.0	+	–	*T. thiooxidans*
pH 4.5	+++	–	*T. thiooxidans*
Gram's test	–ve	–ve	
Shape	rods	rods	
Motility	+	+	

*Three successive transfers to fresh medium, to grow for 10 days to pH <1.0 to eliminate *T. ferrooxidans*.

**Three successive transfers to fresh medium, to grow to ferric iron production (10 day), to eliminate *T. thiooxidans*.

each dilution was inoculated into 50 ml fresh medium and incubated under shake flask conditions for upto 3 weeks to make sure that growth has occurred at the highest dilution possible. Cells of separated from the highest dilution were used as a source of inoculum for *T. thiooxidans* (NB101).

For the purification of *T. ferrooxidans*, 9K Fe grown cells were diluted as above and a known volume was passed through sterile sartorius membrane filters, which were boiled in distilled water for 15 min, to remove material inhibitory to *T. ferrooxidans* [10] and then sterilised in distilled water by autoclaving. These membrane filters were then placed on a solid medium [4] and incubated for 14 days at 30°. Single colony appearing on the highest dilution was transferred to 9K Fe medium, pH 1.5 for the cultivation of *T. ferrooxidans* (NB102).

Analysis. At regular intervals two flasks were withdrawn for analysis. Utilization of sulfur was estimated by the drop in pH using Corning 130 digital pH meter. Total acid produced by the culture was estimated by titrating a known volume against decinormal sodium hydroxide, to initial pH. The results are expressed as grams of acid produced per litre of medium.

Ferrous iron in solution was estimated [13] using 1,10 phenanthroline solution as complexing agent, and optical density was measured at 510 nm using Bausch and Lomb spectronic 21.

Thiosulphate in solution was determined by standard Iodometric analysis [13].

RESULTS AND DISCUSSION

Enrichment and purification procedures used in these test resulted in the isolation and purification of *Thiobacillus thiooxidans* (NB101) and *Thiobacillus ferrooxidans* (NB102). Microscopic and biochemical examination showed that the two strains isolated from indigenous sewage resemble those of the type strains of these cultures described in literature [4,12].

Oxidation of sulphur by T. thiooxidans *(NB 101).* Oxidation of sulphur by *T. thiooxidans* (NB101) produced acid, decreasing the pH of the medium (Fig. 1). During incubation of 25 days pH dropped from 2.5 to 0.85, which is equivalent to 25.5 g/l sulphuric acid production. Production of acid from sulphur by *Thiobacillus thiooxidans* resulting in pH <1 has also been reported by many workers [14-16]. Similarly the production of sulphate ions 30 mg/ml in 30 days by *T. thiooxidans* is also reported [17].

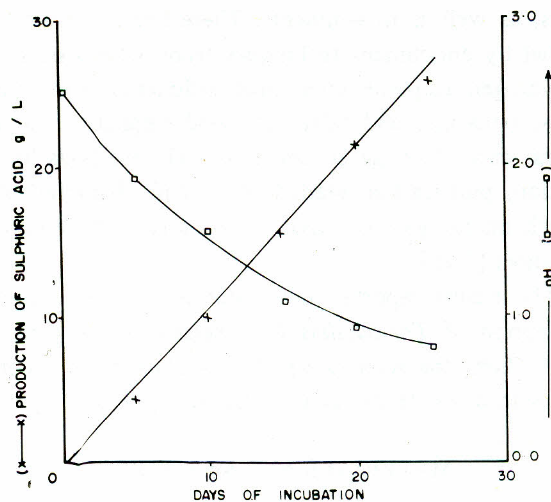

Fig. 1. Production of sulphuric acid and pH drop as a measure of growth of *T. thiooxidans* (NB 101) on MS medium supplied with 0.2 % w/v Sulphur at 30°C and 100 rpm.

T. acidophilus when grown on sulphur reduced the pH from 3.5 to 1.2 [18]. A new acidophilic Thiobacillus species (*T. kabobis*) oxidized sulphur to sulphuric acid during growth as seen by the rapid drop in pH from 3.7 to

2.2 [19]. A decrease in pH from 3.7 to 1.8, when *T. albertis* was grown on sulphur has also been reported [5]. Comparison of 8 different strains of *T. thiooxidans* isolated from mine drainage water and ore lumps of various mines and dumps in Bulgaria was made. Some strains were similar while others differed distinctly from each other [20], with respect to sulphur oxidizing activities (3.85 – 7.30 mg/l/hr). From these studies also, it is confirmed that isolate (NB101) is *T. thiooxidans.*

Growth of T. ferrooxidans *(NB102) on ferrous sulphate.* Utilization of ferrous iron by *T. ferrooxidans* (NB 102) is presented in Fig. 2. As compared to 6000 ppm ferrous iron oxidation in uninoculated (control), the bacterial oxidation is 17,000 and 17,500 ppm (85.5 and 87.5 % of total) in the replicates within 4 days.

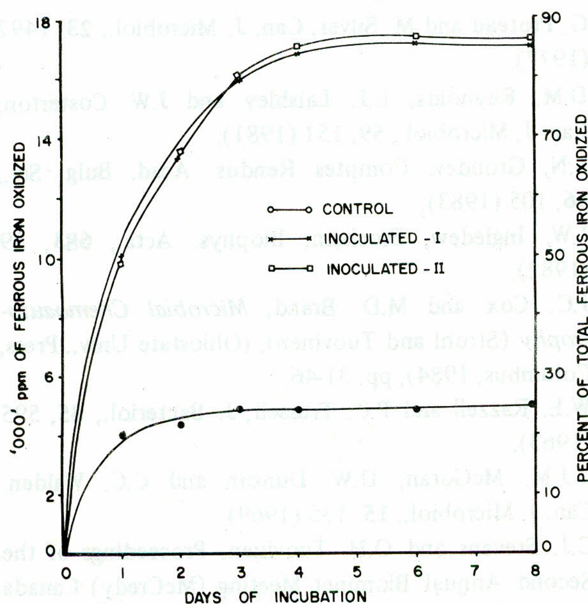

Fig. 2. Oxidation of ferrous iron by *Thiobacillus ferrooxidans* (NB 102) when grown in 9K Fe medium pH 2.5 under shaking condition.

Ferrous iron is sufficiently stable in acid solutions for use as a substrate in microbiological studies. The oxidation of ferrous iron by *T. ferrooxidans* has been recently summarized by different workers [21, 22].

Growth behaviour of *T. ferrooxidans* on ferrous sulphate (120 mM solution) have been studied [10, 23-26]. The role of ferrous iron oxidation in uranium extraction by *T. ferrooxidans* has also been studied [27]. Studies on the effect of finely ground particles on ferrous iron oxidation by these bacteria has also been carried out [28]. Ten different strains of *T. ferrooxidans* for ferrous iron oxidizing activities showed that some strains differed distinctly (177-311 mg/l/hr) from others [20].

Utilization of thiosulphate by T. thiooxidans *(NB 101).* To study the effect of low pH on thiosulphate decomposition and utilization by bacteria, pH 4.5 and 6.0 were tested with uninoculated as control. The same 9K medium of pH 4.5 for thiosulphate utilization by *T. thiooxidans* has already been reported [29]. The results for *Thiobacillus thiboxidans* (NB101) utilization of thiosulphate (initial concentration 40 mM) is given in Fig. 3. In uninoculated samples of pH 4.5 and pH 6.0 media, only 16 % was decomposed within one days and remained constant in further incubation. Thus chemical decomposition (for equilibrium formation) at pH 4.5 and 6.0 is same. In case of inoculated samples at pH 4.5, 75-78 % of thiosulphate disappeared within one day after inoculation. Same inoculum took 7 days for 90 % utilization of thiosulphate at pH 6.0. The reason may be that the acid medium is preferred by *Thiobacillus thiooxidans* and production of acid during growth lowers the pH of the medium.

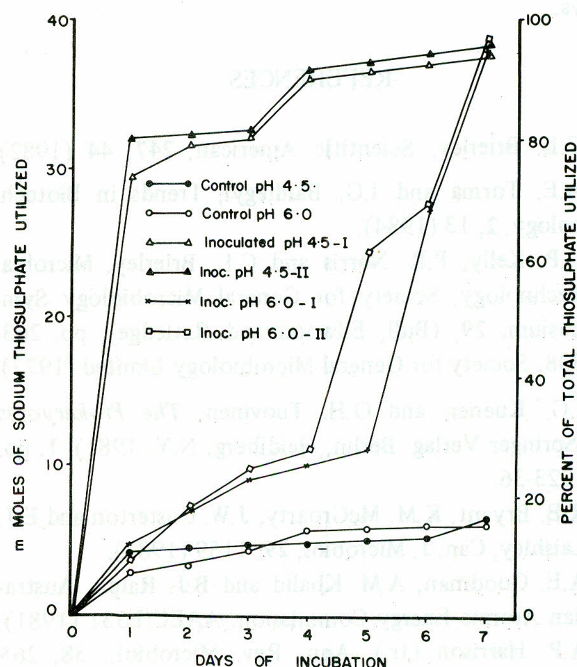

Fig. 3. Utilization of thiosulphate by *Thiobacillus thiooxidans* (NB 101) when grown on 9K medium pH 4.5 and Vishniac and Santer (V.S.) medium pH 6.0 with 40m M sodium thiosulphate in shake flasks.

Thiosulphate has been used as a source of energy for the growth of *Thiobacillus thiooxidans* [4, 7, 11]. However, Harrison [7] has also reported the decomposition of thiosulphate in mild acid (pH above 4). The equilibrium between thiosulphate and its decomposition products (elemental sulphur, H_2S, SO_2 and various polythionic acids) is complex [7].

Thiosulphate utilization by *T. thiooxidans* have been reported by various workers [16, 29, 30]. Vishniac and Santer [11] proposed pH 6.0 for estimating thiosulphate utilization. Medium of pH 3.8 – 4.4 has been mentioned as suitable for thiosulphate oxidation by *Thiobacillus thiooxidans* [4]. Thiosulphate utilization has been studied at pH 4 by *T. kabobis* [19] by *T. albertis* [5].

In conclusion, the microscopic examination and biochemical characterization of the purified bacterial strains isolated from sewage water reveal that NB101 is *Thiobacillus thiooxidans* and NB102 is *T. ferrooxidans* which establishes the ubiquitous distribution of thiobacilli. *T. thiooxidans* (NB101) could oxidize sulphur to produce acid (25.5 g/l) to decrease pH from 2.5 to 0.85. The same strain could utilized thiosulphate (initial conc. 40 mM) at pH 4.5 upto 75-80 % within a day and at pH 6.0 upto 90 % in 7 days. *T. ferrooxidans* (NB102) could oxidize ferrous iron (initial conc. 2 %) upto 87.5 % within 4 days.

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