Isolation of *Thiobacillus ferrooxidans* from Various Habitats and Their Growth Pattern on Solid Medium

Ajit K. Mishra,* Pampa Roy, and S. S. Roy Mahapatra

Molecular Genetics Unit, Department of Microbiology, Bose Institute, Calcutta, India

**Abstract.** Different strains of iron-oxidizing *Thiobacillus ferrooxidans* were grown and purified on solid medium containing Bapco agar, agarose, and carrageenan (Type 1). These strains produced easily countable isolated colonies that could be transferred after 7 days of incubation at 30°C. Increase in viable cell number in relation to growth and iron oxidation was studied by both microscopic count and direct plating method. Colony morphology of different strains growing on solid medium helped in differentiating the colony types.

Ever increasing demand for metals and energy has made a tremendous impact on the research activity on *Thiobacillus ferrooxidans* throughout the world. Very little work has yet been done on the genetic improvement and selection of an efficient strain for making practical leaching systems commercially feasible. Several problems are actually responsible for the complete lack of genetical studies of this bacterium [2]. Studies on genetic manipulation essentially require the growth of the bacterium on solid medium; this, however, is still not satisfactory since agar inhibits growth on ferrous iron medium [3,7]. Attempts had been made to grow *Thiobacillus ferrooxidans* on solid agar medium by the membrane filter technique, which shows doubtful application for the process [7]. Manning [3] has reported the isolation of iron-oxidizing strains from acid mine drainage on solid agar medium but was unable to determine the percentage of recovery. A standard strain of *Thiobacillus ferrooxidans* has been reported to grow on solid agar medium with good viability and high recovery [4].

We report here our findings that different strains of *Thiobacillus ferrooxidans* can be grown directly on solid ferrous iron medium by using low concentrations of Bapco agar, agarose, or carrageenan. Brief morphological studies of the colonies of different strains developed on solid ferrous iron medium were also undertaken.

**Materials and Methods**

Cultures of *Thiobacillus ferrooxidans* were isolated from acid mine water, ore bodies, and coal samples and were grown in liquid medium containing ferrous sulfate [7]. Standard and identified strains of *Thiobacillus ferrooxidans* were also incorporated in these studies. Solid medium was prepared according to Manning [3] with some modifications: ammonium sulfate, 0.3% instead of 0.6%; magnesium sulfate, 0.05% instead of 0.1%; and potassium chloride, 0.01% instead of 0.02% were used; calcium nitrate was omitted; and ferrous sulfate (10 g/l) was sterilized through membrane filtration. A low concentration of Bapco agar (Japan), agarose, and carrageenan (Sigma, U.S.A.) was chosen as the solidifying agent. Purification of each culture was made by dilution plating and single-colony isolation on solid medium. These single-colony cultures were enriched and maintained by weekly transfers of 5% (v/v) inocula to 100 ml of liquid medium (pH 1.6) and incubation at 30°C under rotary shaking condition (150 rev/min). The iron oxidation of each strain in liquid medium was estimated by titrating the residual ferrous iron against standard potassium permanganate solution. The viable cells were estimated by plating and spreading appropriate dilutions on solid medium after growth at 30°C for 7–10 days. In order to ascertain the extent of recovery of viable cells on solid medium, viable counts (by both microscopic and plating methods) were followed after inoculating 5 ml of a 40-h-old culture into 95 ml of liquid medium contained in a 500-ml Erlenmeyer flask and incubation at 30°C under shaking condition at 150 rev/min. The cell concentrate was prepared by filtering a 40-h-old culture on a Millipore membrane filter (0.45 μm pore size), washing several times with 0.01 N H₂SO₄, and resuspending in a requisite volume of 0.001 N H₂SO₄ to obtain a 10- to 50-fold concentration of the original culture.

After 15 days, the isolated colonies on solid medium were photographed directly with MIKAS microattachment for Leica

* To whom reprint requests should be addressed.
Camera M III loaded with ORWO NP 22, 35-mm pan film fitted on a Ortholux Microscope (Ernst Leitz Wetzlar) with objective 10 and eye piece × 1. Close-up views of different plates and colonies were taken with transmitted light by the same camera and a lens Elmar f = 5 cm 1:3.5, fitted with an extension tube.

Results

The thick cell concentrate containing 0.1 ml of each enriched culture (5 × 10⁹ cells/ml, by microscopic count) when spread on solid iron agar medium, showed an iron-oxidized zone, orange red in color, after 3–4 days of incubation at 30°C. Strain BITH 256 showed an unusual pale yellow zone of iron oxidation. When these were streaked onto another plate containing the same medium with Bapco agar (1%), agarose (1%), or carrageenan (1.5%), no isolated colonies appeared except the strain NCIB 8455, which showed development of isolated colonies after 10 days of incubation. The medium solidified with a low concentration of Bapco agar (0.6%), agarose (0.6%), and carrageenan (0.8%), however, showed successful single-colony isolation of each culture after 10 days of incubation. In each case, a group of colonies was selected, and growth was maintained on solid medium having low concentration of agar. After four successive transfers, each strain produced easily countable, isolated colonies on solid medium containing Bapco agar (1%), agarose (1%), or carrageenan (1.5%). These agar-tolerant strains were used in subsequent experiments.

Growth, i.e., increase in cell number and iron oxidation in liquid medium containing 6.31 gm Fe³⁺ l⁻¹ at pH 1.6 showed that in all the strains the oxidation is complete within 40 h. It was observed that the plate counts were always nearly equivalent to the direct microscopic cell count in all the strains (Table 1). This showed that there was always 100% recovery of viable cells on solid medium. Generation time calculated for each strain in liquid medium was about 6.5 h, in agreement with previous reports [4,6,7]. Agarose supported better growth and larger colony formation at pH 3.0.

The different types of colonies observed here could be grouped into two broad categories, viz., circular type and lobe type. Circular type colonies were smooth with entire edges and had little elevation at the center (Fig. 1). This type of colony did not usually form any protrusion of growth, but sometimes the aged colonies were found to form proliferations when some knotch-like appearances occurred at one edge of the colony (Fig. 7a and 7b, arrow). Another type of circular colony was flat with a reddish-brown center (Fig. 2). This type of colony had no complete edges and usually formed concentric, wave-like structures migrating from the center towards the periphery; these resembled the growth rings of a tree (Fig. 8). Lobed colonies were of various types. One of them was star-shaped with variable symmetric or asymmetric extending arms; Fig. 3a (arrow) shows such star-shaped colonies, which could be confirmed under close-up view (Fig. 3b). The extending lobes of these types of colonies might vary from 5 to 12, with almost symmetric or asymmetric assembly (Fig. 9a and 9b). Lobed colonies, as shown in Fig. 4a, sometimes were found to be highly symmetrical, and more often they formed radiating colonies (Fig. 4b, 10a, and 10b). Usually all strains that formed lobed colonies were found not to be identical. Peculiar types of lobed colonies were formed by strain BIDM 582 (Fig. 5). Their morphological appearance could account for what might be called spindle-shaped colonies (Fig. 11a and 11b). In the radiating lobe and spindle-shaped colonies, different types of cap-like covering over the apex of each extending arm were seen (Fig. 10a–11b).

Discussion

Any strain of Thiobacillus ferrooxidans can be adapted to grow on solid medium under the conditions specified here. Strains may have natural agar tolerance, or agar-tolerant strains may be obtained by adaptation. These agar-tolerant strains have the capacity to grow easily on solid medium even if they are maintained in liquid medium. Agar is inhibitory for growth of this bacteria, as stated earlier [3,7] and also confirmed here. The earlier unsuccessful attempts to grow strains of Thiobacillus ferrooxidans on solid agar medium [1,3,5–8] were mainly the result of the quality and amount of agar used as well as the addition of phosphate. Strain variation as one of the factors, as suggested earlier [4], can be nullified by these findings. The addition of phosphate is not required to produce isolated transferable and countable colonies on solid medium; Manning [3] has suggested that the amount of phosphate present in agar is sufficient to support good growth, and this has been confirmed here for all of the isolated and standard strains. Thus the solid medium preparation, as suggested by Manning [3] and modified in our laboratory, can be universally accepted for producing isolated colonies.