Growth and Maintenance of *Thiobacillus ferrooxidans* Cells

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A rapid and sensitive spectrophotometric procedure was developed for monitoring the growth of *Thiobacillus ferrooxidans* in liquid culture. Values determined for the optical densities at 500 nm of washed *T. ferrooxidans* cell suspensions were directly proportional to both total cell number and total cell protein concentration and provided an accurate measurement of culture growth rate. The utility of this procedure was demonstrated by conducting physiological studies on the influence of CO₂ and FeSO₄ availability on the growth of *T. ferrooxidans*. In addition, we describe a procedure for the long-term maintenance of cells *T. ferrooxidans* that ensures culture purity and genetic stability.

*Thiobacillus ferrooxidans* is an obligately aerobic, gram-negative, chemoautotrophic organism that generates its energy and reducing power for CO₂ fixation from the oxidation of inorganic iron and reduced sulfur compounds (12). Since its initial isolation by Colmer et al. (3) in the 1940s and its implication in the problem of acid mine drainage, numerous studies have appeared concerning the unique metabolic properties of this organism and its ability to grow optimally under highly acidic conditions (<pH 2.5). Most recently, with the heightened awareness of the environmental problems attendant with the use of high-sulfur coals and the recovery of precious metals from pyrite-rich ores, mining and coal companies have displayed a renewed interest in the use of *T. ferrooxidans* as a biological solution to coal desulfurization and ore bioleaching processes. However, applied studies in these areas employing *T. ferrooxidans* have generally yielded inconsistent or ambiguous results; consequently, the potential of this organism as the solution to industry problems has not been realized. Unfortunately, it appears that the eagerness of investigators to initiate applied studies with *T. ferrooxidans* has superseded important studies focused upon the growth, adaptation, and maintenance of this organism. As a consequence, fundamental procedures for obtaining and maintaining pure cultures and for adapting, optimizing, and monitoring the growth of cultures of *T. ferrooxidans* either have not appeared in the literature or have not been uniformly adopted. In this regard, in the present report we describe optimal conditions for the growth and maintenance of cells of *T. ferrooxidans* and, most importantly, details of a spectrophotometric procedure for rapid and accurate monitoring of the growth of cultures of this organism. These procedures and growth conditions have greatly augmented our basic studies on the physiology and molecular biology of *T. ferrooxidans* and should facilitate both applied and basic studies by other investigators employing this organism.

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MATERIALS AND METHODS

Organism, medium, and growth conditions. *T. ferrooxidans* ATCC 19859 was obtained from the American Type Culture Collection and was purified by four successive isolations of individual colonies from plates containing solidified minimal medium. Growth was routinely conducted with an FeSO₄-based minimal medium (25) containing the following (grams per liter): (NH₄)₂SO₄, 3.5; KCl, 0.116; K₂HPO₄, 0.058; MgSO₄·7H₂O, 0.058; and Ca(NO₃)₂, 0.00168. The basal salts solution was initially prepared in 420 ml of distilled H₂O, adjusted to pH 1.9 with 10 N H₂SO₄, and sterilized by autoclaving. This solution was then combined with 580 ml of a filter-sterilized solution of FeSO₄ (74.674 g of FeSO₄·heptahydrate) per 580 ml of distilled H₂O [pH 1.9] to yield 1 liter of medium containing a final Fe²⁺ concentration of 15 g/liter. Solidified minimal medium containing agarose as the gelling agent was prepared exactly as described by Yates and Holmes (25), and cells were plated by a modification of the plate overlay procedure of Harrison (8). Cells to be plated were harvested from the logarithmic phase of growth, washed twice, and serially diluted in basal salts (FeSO₄-free minimal medium), and 0.1-ml samples of these dilutions were added to tubes containing 3 ml of top agarose that had been freshly prepared and maintained at 55°C (sterile top agarose should be dispensed into pre-warmed tubes and used immediately). After brief vortexing, the cells were then evenly spread over plates containing 10 to 15 ml of solidified medium (bottom agarose); after the top agarose was allowed to solidify for 5 to 6 h, the plates were inverted and incubated at 30°C under an atmosphere of 5% CO₂–95% air in a NAPCO CO₂ incubator. Under these conditions, colonies were visible in 3 to 4 days.

The growth of *T. ferrooxidans* in liquid medium was routinely conducted in 18- by 150-mm culture tubes and 500-ml serum bottles containing 15 and 300 ml of medium, respectively. Incubations were conducted at 30°C, and cultures were sparged with air or CO₂-air mixtures as described in Results and Discussion. Appropriate gas mixtures were obtained with a Cole-Palmer gas proportioner, and total flow rates were directly measured. Cells employed as inocula for growth studies were extensively adapted (five to eight mass doublings) to the designated growth conditions. Cells of *T. ferrooxidans* to be utilized for growth studies were adapted to logarithmic-phase growth in FeSO₄-based minimal medium and then harvested, washed, and sus-
pended in fresh minimal medium and stored (maximum of 1 to 3 days) at 4°C. Before use, these cells were adapted to growth under the designated conditions. Stock cultures were maintained as agarose slants under sterile mineral oil in 18-ml screw-capped tubes at 4°C and on Prolab Protect-100 beads at −70°C (see Results and Discussion).

Measurement of cell growth. Culture growth was monitored spectrophotometrically by reading optical density measurements at 500 nm (OD500) with a Beckman DU-50 spectrophotometer. Culture samples (1 ml) were removed at timed intervals and transferred to 1.5-ml microfuge tubes, and the cells were harvested by centrifugation at 11,000 × g for 10 min in a Beckman Microfuge 12. The cells were then suspended and washed (twice) in basal salts and finally suspended in either the same volume or 0.5 volume of basal salts, and the OD500 of the suspension was determined by using basal salts as a reference. All determinations were performed in duplicate. An OD500 of 0.2 corresponds to a cell density of 109 cells per ml and a total culture protein concentration of 53 μg/ml.

Total cell numbers were determined with a Petroff-Hausser counting chamber; 400 to 500 cells were counted per determination. Culture samples were removed and transferred to tubes containing an equal volume of 5% (wt/vol) Formalin and stored at 4°C before determinations. Calculations of total cells per milliliter of culture were performed by employing a factor of 2 × 103 (17).

Analytical procedures. Total cell protein determinations were conducted by the method of Peterson (19) on cells of T. ferrooxidans that had been washed (twice) in basal salts and resuspended in the same solution. The procedure included the steps for trichloroacetic acid and deoxycholate precipitation of the experimental samples with bovine serum albumin as a standard.

Chemicals. Beads employed for cell storage were purchased from ProLab Inc., Roundrock, Tex. Type I: Low EE0 agarose utilized for solid medium was purchased from Sigma Chemical Co., St. Louis, Mo. Cylinders of carbon dioxide gas were purchased from Interstate Valweld, Marquette, Mich. Analyzed cylinders of 7.5% CO2-92.5% air mixture were obtained from Alphagaz Liquid Air Corp., Countryside, Ill. through Interstate Valweld. All other reagents were purchased from Sigma and Fisher Scientific Co. (Pittsburgh, Pa.) and were of reagent grade or better.

RESULTS AND DISCUSSION

Maintenance of T. ferrooxidans cells. The ability to easily obtain and maintain pure cultures of a microorganism is a prerequisite fundamental to the use of microorganisms for research purposes. This is especially true for studies employing T. ferrooxidans because of the tendency of cultures of this organism to become contaminated with various acidophilic heterotrophs (1, 7, 8, 13). However, few investigators indicate that they have rigorously ensured the purity of their T. ferrooxidans cultures, and this has prompted much skepticism concerning the published results obtained with this organism. This situation arises primarily from the absence of clearly defined and generally adopted procedures for the handling of T. ferrooxidans cells. In the present study, T. ferrooxidans was obtained in pure culture by the repeated isolation of individual colonies from solidified minimal medium followed by extended growth of the organism in FeSO4-based minimal medium (see Materials and Methods). Colonies that appeared after 2 to 3 days of growth at 30°C on solidified FeSO4-based minimal medium under an atmosphere of 5% CO2-95% air were well isolated; upon transfer of cells to tubes containing 15 ml of FeSO4-based liquid medium, cell growth was rapidly recovered when incubations were conducted at 30°C with constant sparging (5% CO2-95% air). When the presence of elevated CO2 levels (i.e., sparging with air), the recovery of cell growth was unpredictable and the time required for recovery was typically extended.

Surprisingly, having obtained a pure culture of T. ferrooxidans, we found that no generally adopted, reliable procedure for the storage of this organism was available. Although Gupta and Agate (6) reported some success with the storage of cells of T. ferrooxidans on chalcopyrite at 8°C, apparently most investigators maintain T. ferrooxidans cultures via serial transfers of the organism into fresh medium on a monthly or bimonthly basis. In fact, Holmes (10) recently addressed this problem and expressed concern for the lack of progress in the development of a storage procedure for cells of T. ferrooxidans. Indeed, if T. ferrooxidans is to be more widely adopted as an experimental organism, a reliable storage procedure that will ensure culture purity and genetic stability of populations of this organism must be available.

In the present study, numerous attempts were made to preserve cells of T. ferrooxidans by storage of the organism at −70 and −20°C in the presence of various concentrations of either glycerol or dimethyl sulfoxide. Although T. ferrooxidans was not amenable to storage by such conventional procedures, cell growth could be reliably recovered after storage at −70°C if the cells were first mixed with Prolab Protect-100 beads in the presence of complete medium. For these studies, cells were grown in FeSO4-based minimal medium (15 g of Fe2+ per liter) sparged for 15 min in 7.5% CO2-92.5% air and harvested during the logarithmic phase of growth (OD500, 0.075; see below). The cells were then washed twice (see Materials and Methods) and concentrated 30-fold by suspension in minimal medium, and 1 ml of this concentrated cell suspension was directly added to a vial of biobeads from which the original liquid had been removed. The vial contents were then mixed, and the vial was incubated at room temperature for 45 min before freezing at −70°C. Cells stored in this manner were routinely recovered by dispensing the contents of a thawed vial into an 18- by 150-mm culture tube containing 10 ml of FeSO4-based minimal medium (6 g of Fe2+ per liter) and incubating the tube at 30°C with sparging (7.5% CO2-92.5% air). With this procedure, growth of cell stocks that had been stored at −70°C for an extended period was visible in 10 to 12 h. Importantly, the recovery of growth was critically dependent upon the use of fresh, complete growth medium for the suspension of cells to be frozen, the concentration of cells to be frozen, and the temperature of storage. Cells stored in spent medium, in fresh medium lacking FeSO4, or at low cell densities were not reliably recovered.

In addition to the procedure outlined above for the long-term storage of cells of T. ferrooxidans, a procedure for the routine maintenance of working stocks of this organism was developed. In this regard, pure cultures of T. ferrooxidans could be easily maintained as agarose slant cultures in 18-ml screw-capped tubes when stored under sterile mineral oil at 4°C. For this procedure, agarose-solidified FeSO4-based minimal medium (see Materials and Methods) was prepared in the form of slant cultures, and the slants were inoculated with a loopful of logarithmic-phase-adapted cells. The cultures were then incubated at 30°C under an atmosphere of 5% CO2-95% air in a NAPCO CO2 incubator. When sufficient growth had occurred (2 to 4 days), the cultures were
removed, covered with sterile mineral oil, and stored at 4°C. To obtain a working stock of the organism, a loopful of cells may be aseptically removed from the slant, and growth of the cells can be recovered as described above for the recovery of cells from long-term storage. Although we have found that T. ferrooxidans cells remain viable for >9 months when stored in this manner, we recommend that new slant cultures be prepared on a bimonthly basis and that, when questions concerning culture purity or genetic stability arise, new working stocks of the organism should be prepared with cells that have been previously obtained in pure culture and stored at -70°C.

**Measurement of cell growth.** Due to the diverse conditions (i.e., medium composition, initial pH, CO₂ availability, etc.) and procedures employed by investigators for the growth and determination of growth rates of cultures of *T. ferrooxidans*, significant disparities have appeared in the literature concerning growth rates and cell yields obtained with this organism. For instance, specific growth rates ranging from 0.07 to 1.78 h⁻¹ (2, 14, 15, 18, 20) have been reported for *T. ferrooxidans* on FeSO₄-based medium, with the most frequently reported values being in the range of 0.1 to 0.2 h⁻¹. Many investigators rely upon measurements of the rate of FeSO₄ oxidation as a monitor of culture growth; however, the applicability of this procedure presupposes that the specific cellular level of FeSO₄ oxidation has been shown to be independent of the stage of culture growth, that fastidious attention has been devoted to the adaptation of inocula to the logarithmic phase of growth, and that the legitimacy of the procedure has been verified for the particular growth condition being utilized. These requirements are rarely satisfied; as a consequence, values for the specific growth rates of cultures of *T. ferrooxidans* obtained employing this procedure, which, interestingly, are among the highest rates reported for this organism (14), are necessarily suspect. Alternatively, culture growth may be monitored by determining total cell number, which, although time consuming, is subject to less error; however, the use of viable cell numbers as a moniter of culture growth is impractical because of wide variation in the values for plating efficiencies reported for cells of *T. ferrooxidans* (8, 24) as well as the rapid spreading of colonies of *T. ferrooxidans* on solidified medium (21). In the present study, logarithmic-phase-adapted cells of *T. ferrooxidans* occurred predominantly as doublets, and the percentage of doublets within the population became variable as the cells entered the stationary phase of growth. This fact probably explains the variation in plating efficiencies observed with this organism.

In view of the problems discussed above, it is apparent that there is a need for a rapid and sensitive method for monitoring the growth of cultures of *T. ferrooxidans*. With the spectrophotometric procedure described in Materials and Methods, measurements of the OD₅₀₀ values of washed cell suspensions of *T. ferrooxidans* growing in FeSO₄-based minimal medium provided a rapid, accurate, and sensitive method for determining the stage and rate of growth of cultures of this organism. The results obtained with this procedure are presented in Fig. 1A and 2. OD₅₀₀ values were directly proportional to the total number of cells (Fig. 1A), and whole-cell protein (Fig. 1B) per milliliter of culture and growth rates were highly uniform when determined from semilogarithmic plots of OD₅₀₀ versus time. The procedures employed for the determination of total cells per milliliter of culture and whole-cell protein are described in Materials and Methods. The data presented in Fig. 2 are representative of the results obtained when the measured OD₅₀₀ values are converted to values for total cells per milliliter culture, utilizing the data presented in Fig. 1A, and the logarithms of these values are then plotted as a function of time. Unfortunately, because of the interference of ferric complexes and other medium components, the direct measurement of OD₅₀₀ values of culture samples was not possible. The cells contained in samples of the culture must be pelleted by centrifugation (see Materials and Methods), suspended and washed twice in basal salts, pelleted again by centrifugation, and finally suspended in basal salts before the determination of the OD₅₀₀. With this procedure, cell growth may be monitored throughout the logarithmic and stationary phases of culture growth as long as precautions have been taken to ensure the maintenance of culture pH. This procedure provides an easy and rapid method for the virtual real time monitoring of the growth of *T. ferrooxidans* cultures and will hopefully promote the use of this organism by other

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**FIG. 1.** (A) Relationship of OD₅₀₀ to total cell number in cultures of *T. ferrooxidans*. Data were obtained with a washed, concentrated suspension of logarithmic-phase-adapted cells of *T. ferrooxidans*. Absorbance measurements and direct cell counts were conducted as described in Materials and Methods. All determinations were performed in duplicate. (B) Relationship of OD₅₀₀ to total cell protein concentration in cultures of *T. ferrooxidans*. Data were obtained with a washed, concentrated suspension of logarithmic-phase-adapted cells of *T. ferrooxidans*. Absorbance measurements and determinations of total cell protein were conducted as described in Materials and Methods. All determinations were performed in triplicate.
used in the medium of the investigators and facilitate a more rigorous approach to culture adaptation by investigators currently conducting studies with *T. ferrooxidans*. In addition to *T. ferrooxidans*, 19859, the procedure is directly applicable to *T. ferrooxidans* ATCC 13661 (data not shown).

**Optimization of culture growth.** *T. ferrooxidans* is a chemoautotrophic organism and, as such, is an obligate aerobe with a strict requirement for CO2 as its sole source of carbon for growth. Nevertheless, investigators conducting studies with this organism rarely aerate cultures by sparging; in instances where sparging of the culture is included, the sparging is generally conducted with air lacking any enrichment in CO2. In view of the limited solubility of CO2 at the pHs (<2.0) required for the optimum growth of *T. ferrooxidans*, it would be predicted that the growth of cells in the absence of sparging with a gas mixture enriched with CO2 would be limited by CO2 availability. Indeed, in studies where CO2 has been supplied above atmospheric levels for either physiological or bioleaching experiments, improved growth yields, rates of growth, or elevated rates of metal bioleaching have been observed (4, 9, 23). The importance of CO2 availability for achieving optimal growth rates and maximum cell yields of cultures of *T. ferrooxidans* was clearly and most recently demonstrated by Holuigue et al. (11) during their studies on the purification and characterization of the *T. ferrooxidans* ribulose-1,5-bis-phosphate carboxylase. These investigators reported that culture sparging with a level of CO2 that was 5% in air provided maximal stimulation of culture growth and that significantly higher cell yields, although not growth rates, were obtained as the level of CO2 availability was increased. The data presented in Fig. 3A extend and confirm the results previously reported by Holuigue et al. (11) pertaining to the influence of CO2 availability on the growth of *T. ferrooxidans*. For these studies, cells of *T. ferrooxidans* extensively adapted to the logarithmic phase of growth (five to eight mass doublings) in FeSO4-based medium (15 g of ferrous iron per liter) were utilized to inoculate the same medium under identical conditions, and the growth of the culture was monitored by employing the newly developed spectrophotometric procedure (see Materials and Methods). For the data presented in Fig. 3A, culture sparging was conducted at a rate of 4.2 ml/min per ml of culture; however, additional studies (data not shown) have shown that the growth stimulation observed with a specific level of CO2 in air is relatively independent of the rate of culture sparging over a range of 0.06 to 4.2 ml/min per ml of culture. Cultures adapted and grown with only air sparging typically displayed extended growth lags and the slowest rates of growth (µ, h⁻¹, 0.06) upon transfer to fresh medium. In agreement with the data of Holuigue et al. (11), the rate of culture growth increased as the level of CO2 in air was increased to 5%; however, the level of CO2 that supported the maximal rate of growth was found to be in the range of 7 to 8% CO2. Furthermore, increases in the level of CO2 beyond 8% resulted in inhibition of culture growth, with maximal inhibition occurring at 12% CO2. The molecular basis for this inhibition is unclear, although it should be
mentioned that higher cell yields were obtained as the percentage of CO₂ was elevated to inhibitory levels. In these instances, however, the formation of culture precipitates attendant to increased cell yields precluded an accurate evaluation of the influence of the level of CO₂ on total cell mass produced. Importantly, throughout this study, the culture precipitates commonly noted to occur by other investigators (16) were not observed until the cells had entered the stationary phase of growth. Thus, for our studies, experimental cultures and inocula were always completely devoid of precipitates.

Finally, in addition to the influence of CO₂ availability on the growth of T. ferrooxidans, numerous reports have appeared concerning the influence of the concentration of Fe²⁺ and Fe³⁺ on the growth of this organism (14). Although a number of different media have been utilized for the growth of T. ferrooxidans (24, 25), the medium most commonly employed by investigators is 9K medium, which was originally described by Silverman and Lundgren (22) and contains FeSO₄ as an energy source. However, to our knowledge, a thorough study on the effect of FeSO₄ on the rate of growth of T. ferrooxidans employing logarithmic-phase-adapted cells and a reliable technique for monitoring culture growth rate has not appeared in the literature. Thus, an optimum concentration of FeSO₄ for the growth of T. ferrooxidans has not been established.

In the present study, the effect of the concentration of FeSO₄ on the growth of T. ferrooxidans was examined by employing the spectrophotometric procedure for monitoring cell growth (Fig. 3B). Cells extensively adapted to the logarithmic phase of growth were utilized to inoculate medium containing the designated concentration of FeSO₄ (Fig. 3B), and the resulting culture was incubated with sparging (4.2 ml/min per ml of medium) under an atmosphere of 7.5% CO₂-92.5% air. Growth rates were determined from plots of OD₅₀₀ versus time, and these rates were plotted as a function of the FeSO₄ concentration (Fig. 3B). Clearly, the growth of T. ferrooxidans was significantly influenced by the concentration of FeSO₄, with maximal rates of growth in the presence of 2 to 3 g of Fe²⁺ per liter. Increases beyond this level of FeSO₄ resulted in an inhibition of growth, with maximal inhibition at an Fe²⁺ concentration of 20 g/liter, a level approaching the maximum solubility of FeSO₄. In fact, the data presented in Fig. 3B are typical of data from systems in which substrate inhibition of growth occurs (5).

In summary, the data presented in Fig. 3A and B show that the availability of CO₂ and FeSO₄ exerts dramatic effects on the growth of T. ferrooxidans. However, it should be mentioned that the growth condition selected by an investigator will be largely dependent upon the proposed application. If rapid rates of cell growth at low cell densities are required, the optimum condition would be medium containing 2 to 3 g of Fe²⁺ per liter and sparging with air containing 7 to 8% CO₂. Alternatively, if cell yield but not growth rate is important, medium containing higher levels of Fe²⁺ sparged with >10% CO₂ in air might be selected.

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LITERATURE CITED


