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Growth of *Thiobacillus ferrooxidans* on solid media containing heterotrophic bacteria

Barbara J. Butler and Alan G. Kempton

Department of Biology, University of Waterloo, Ontario, Canada

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SUMMARY

The study of the chemolithotrophic bacterium *Thiobacillus ferrooxidans*, an organism important in mining and metallurgical applications, has always been complicated by the inability to cultivate and enumerate many wild strains on solid agar. Here, we have described a plate-count procedure that uses an acidophilic heterotrophic bacterium to scavenge organic material from normal agar, permitting colony formation of the autotroph without visible evidence of growth of the heterotroph. This technique could enable any *T. ferrooxidans* isolate, including those that are agar-sensitive, to grow and develop colonies on solid medium.

INTRODUCTION

The effectiveness of *Thiobacillus ferrooxidans* in mining and metallurgical applications is usually measured by some biochemical activity of this chemolithotroph, such as a change in pH or the release of oxidative products from a specific metal sulfide. Although total activity is the ultimate measure of an industrial process, a reliable procedure for counting these bacteria would be valuable in separating microbial activity from spontaneous oxidation. However, because *T. ferrooxidans* is an obligate autotroph and inhibited by many organic compounds, cells grown in liquid media are usually counted microscopically, while the most probable number (MPN) technique is used to count cells in naturally turbid suspensions. These methods lack the precision of a plate count. The microscopic method enumerates non-viable in addition to viable cells, and the MPN is inefficient and has a large error term. A number of attempts have been made to achieve growth of *T. ferrooxidans* on solid media containing ferrous iron [1,7–9,11], but later studies indicated that many strains remain difficult or impossible to raise on solid media [4,12].

Our procedure was derived from a report by Harrison [4], who noted that point inoculations of acidophilic heterotrophs into ferrous iron agar allowed *T. ferrooxidans* growth in the area surrounding the points of inoculation. This report describes a plate-count procedure in which added heterotrophic bacteria scavenge organic impurities in the medium, allowing *T. ferrooxidans* to develop colonies.

Correspondence: Dr. B.J. Butler, Department of Biology, University of Waterloo, Waterloo, Ontario, Canada N2L 3G1.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The two bacterial strains used were obtained from the Minto, New Brunswick, Canada coal mining area. *T. ferrooxidans* strain NB was purified by repeated dilution to extinction [6] in 9K medium [10]. An acidophilic heterotrophic bacterium designated NB10 was isolated and purified by repeated streaking on trypticase soy (TS)-glucose agar [3]. NB10 was routinely grown in yeast agar-glucose medium (with 0.1 g/l yeast extract instead of TS) at 30°C for 72 h. Cells were harvested by centrifugation, washed twice in cold dilute sulfuric acid (pH 3.5), and then resuspended in dilute acid. Suspensions of 10^9–10^{10} cells/ml (total count with a Petroff-Hausser chamber) were used successfully. *T. ferrooxidans* cultures were grown in 9K medium at 30°C, and were diluted in sterile dilute sulfuric acid (pH 2.3) to obtain cell concentrations suitable for plating.

Soluble iron assay

The soluble iron concentration of liquid *T. ferrooxidans* cultures was measured with a bipyridine assay [5]. Ferrous iron content was determined by omitting the reducing agent from the assay. The amount of ferric iron produced by the growing culture at a time t was calculated as Fe^{3+} (time t) = Fe_{total} (time 0) − Fe^{2+} (time t).

Media preparation

ISP agar was prepared as decribed by Manning [8]. After mixing of the sterile iron, basal salts and agar solutions, the complete medium was tempered at 48°C. NB10 cell suspension was added to some of the tempered ISP medium, prior to pouring plates, in a proportion of 10 ml cell suspension per 325 ml medium. Four types of plates were prepared: (1) normal plates of ISP agar, without heterotrophic cells (NORM); (2) plates of ISP agar with heterotrophic cells (HET); (3) HET plates overlaid with a layer of normal ISP agar (HETLAY); and (4) plates of normal ISP agar which were allowed to solidify, then spread with 0.2 ml of NB10 cell suspension, dried for 12 h, and overlaid with normal ISP agar (SPRLAY). Plates of each type were spread with 0.1 ml of appropriately diluted *T. ferrooxidans* culture. The plates were incubated at 30°C, in plastic bags to avoid excessive drying of the agar.

RESULTS AND DISCUSSION

Preliminary studies with a number of our *T. ferrooxidans* isolates showed that none developed colonies on solid media such as Manning's ISP agar [8] or Mishra and Roy's carrageenan medium [9], nor did incubation of inoculated membrane filters on the surface of ferrous iron agar result in readily detectable colonies using the method of Tuovinen and Kelly [11]. However, ISP agar amended with NB10 supported the growth of not only strain NB, as reported in detail herein, but also growth of nine other *T. ferrooxidans* strains from our collection, including an ATCC culture.

After 7 days, colonies were readily distinguishable on HET, HETLAY and SPRLAY plates, but no *T. ferrooxidans* growth was apparent on the NORM plates. The plate types containing heterotrophic bacteria within the medium were equally effective in detecting viable cells (Table 1). *T. ferrooxidans* colonies were most easily observed on SPRLAY plates, because such colonies were largest, with diameters up to 0.5 mm, and the agar surface had become opaque yellow in colour. Against this background the colonies were rusty red-brown in colour, somewhat darker in the center, and surrounded by a yellow halo. The colonies were circular or slightly irregular in form, and umbonate, with an erose margin. On HET and HETLAY plates, the *T. ferrooxidans* colonies were red-brown dots of only pinpoint size, but they were readily distinguished from the agar background, which remained translucent in these plates.

Incubation for an additional 10 days resulted in only a small increase in the number of colonies detected (Table 1). The principal change was in the size of the colonies, some of which approached 2–3 mm diameter on uncrowded SPRLAY plates. Colonies on HET and HETLAY plates grew larger

Table 1

Enumeration of *T. ferrooxidans* isolate NB

A 96-h culture of *T. ferrooxidans* grown in 9K medium was enumerated. Total count was measured in a Petroff-Hausser counting chamber. Viable counts were measured on the four types of prepared plates of medium. Colonies were enumerated after 7 and 17 days of incubation.

Method	Cell count (cells/ml or CFU/ml)	
	7 days	17 days
Total count	1.4×10^8	–
NORM	0	0
HET	6.6×10^7	7.6×10^7
HETLAY	6.2×10^7	6.7×10^7
SPRLAY	6.6×10^7	7.2×10^7

but remained undifferentiated. No growth was detected on the NORM plates after a total of 17 days of incubation (Fig. 1).

No visible evidence of growth of the heterotroph was detected on the plates, even with low power microscopy. Presumably the amount of organic material available for these cells was very small, precluding colony formation.

Fig. 2 illustrates the growth of a batch culture of *T. ferrooxidans* in liquid 9K medium as measured by total count, viable count and iron oxidation. The viable counts were performed on SPRLAY plates. It is evident that such plates can successfully detect changes in viable cell numbers.

The techniques described in this report offer several potential advantages over the ferrous iron-agarose medium used by Harrison [4]. Plates may be

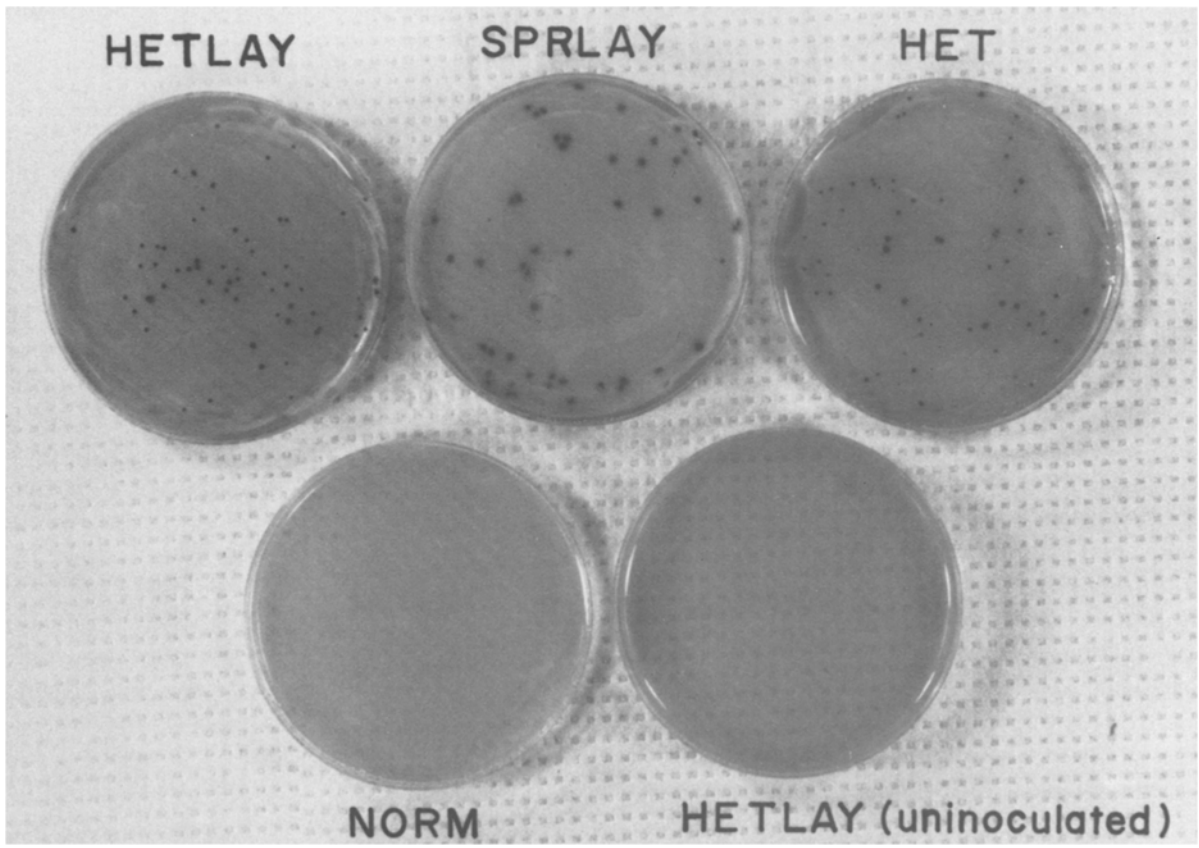

Fig. 1. Colonies of *T. ferrooxidans* on solid medium. Plate types: top row, left to right: HETLAY, SPRLAY, HET; bottom row, left to right: NORM, uninoculated HETLAY. Inoculated plates were spread with 0.1 ml diluted 96-h *T. ferrooxidans* cell suspension. Plates were incubated at 30°C for 17 days.

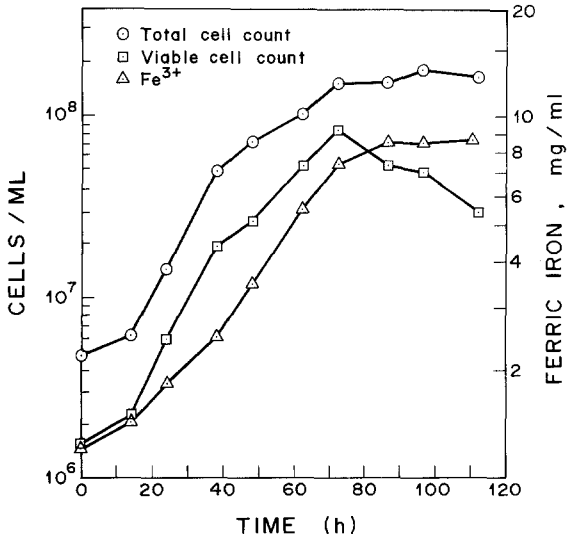

Fig. 2. Growth and ferrous iron oxidation by *T. ferrooxidans* in 9K medium (pH 2.3). ⊙, total count (cells/ml); ⊡, viable count (CFU/ml); △, Fe^{3+} (mg/ml). Plates were incubated at 30°C for 14 days.

completely prepared ahead of time, so the necessity of preparing and working with small volumes of molten medium at the time of plating is avoided. Adequate supplies of oxygen and carbon dioxide are ensured because the chemolithotrophic cells are on the surface, rather than subsurface. For routine enumeration of *T. ferrooxidans* suspensions HET plates could be used. The layered plates, where heterotrophs are remote from the surface, may be more suitable for the isolation of pure *T. ferrooxidans* clones from single colonies, although subsequent liquid cultures must be screened for heterotroph contamination. Use of agar, rather than agarose, as the gelling agent would be cheaper. Purified agar (Oxoid, L28) was most commonly used in this work, although bacteriological agar (Difco, Bacto-agar) was successfully substituted. It should be noted that the ferrous iron in stored plates becomes oxidized, turning the medium brown after several weeks at 4°C. Such plates did not support *T. ferrooxidans* growth.

It is probable that other heterotrophic microorganisms could be substituted for NB10. The acidophilic heterotroph *Acidiphilium cryptum* [3] (which NB10 may resemble), the facultative chemolithotroph *Thiobacillus acidophilus* [2], or a sufficiently acid-tolerant, iron-tolerant yeast species, may be suitable substitutes.

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