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Short contribution

An improved solid medium for isolation, enumeration and genetic investigations of autotrophic iron- and sulphur-oxidizing bacteria

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Abstract. An improved solid medium using Gelrite as a supporting gel for isolation and enumeration of acidophilic chemolithotrophic *Thiobacillus* strains was tested. Dark-brown circular colonies, which were robust and well-differentiated, developed on this medium within 72–96 h. The plating efficiency of *T. ferrooxidans* strains was 92.1 ± 5.8%. Linear correlations with an optical density (OD) at 400 nm of 0.5, corresponding to $7 \cdot 10^6$ and $5 \cdot 10^7$ cells · ml^{-1} for strains ATCC 13661 and TMB, respectively, were obtained. Linear correlations between OD, total bacterial protein, dry biomass and cell number were also determined. These data can be used for modelling and scale-up design of microbial leaching operations. With this plating technique, one prerequisite for selection of different mutants of *T. ferrooxidans* has been fulfilled.

Introduction

Precise determination of bacterial cell counts and cell mass remains an onerous problem in microbial research. This dilemma becomes more evident in the case of certain bacteria, viz., acidophilic thiobacilli (*Thiobacillus ferrooxidans* and *T. thiooxidans*), where the lack of a suitable solid medium has hindered some investigations of a fundamental nature (Bärtels et al. 1989; Holmes et al. 1983; Rawlings and Woods 1988; Tuövinen and Kelly 1973). The extreme conditions of pH (< 2.0), at which these bacteria grow, preclude the use of common gelling agents such as agar-agar, for making solid media plates. When agar is sterilized at such low pH values it does not form a stable gel and in some instances no solidification occurs at all due to its hydrolysis. To overcome these problems, media have been designed that contain inert supports such as silica gels or membrane filters, which have been placed on refined agar or agarose plates (Goodman et al. 1980; Manning 1975; Mishra and Pradosh 1979; Tuövinen and Kelly 1973; Visca et al. 1989). All these methods are not only cumbersome and tedious, but also time consuming: 2–3 weeks of incubation are usually required to produce visible bacterial colonies. These procedures are further marred by non-reproducibility of the results.

Specific immunological methods for the determination of these thiobacilli have been developed (Apel et al. 1976; Azzendondo and Jerez 1989). The use of fluorescent antibodies to estimate pyrite-oxidizing microorganisms in acid mine drainage waters has been reported (Bakers and Mills 1982). Gates and Pham (1979) have developed a specific and very sensitive immunobinding assay for the detection and enumeration of *T. ferrooxidans*. Although these methods have been claimed to be very sensitive, their major drawback is their inability to distinguish between dead and living cells.

Recently a bacterial polysaccharide, "Gellan Gum" or "Gelrite" has been reported as a gelling agent for bacterial cultures and has been claimed to be a better support than agar (Kang et al. 1982; Lin and Casida 1984). Gelrite has been used successfully for plating thermophilic archaebacteria such as *Sulfolobus acidocaldarius*, which is capable of growing at temperatures of 55–120° C (Deming and Baross 1986; Lindström and Sehlin 1989). However, at present no significant study has used Gelrite for culturing mesophilic autotrophic bacteria belonging to the genus *Thiobacillus*. In this report we have employed Gelrite as a solidifying agent for the solid culturing of acidophilic thiobacilli and have attempted to devise a method for estimating the growth of these bacteria.

Materials and methods

Bacterial cultures used. Axenic as well as naturally isolated thiobacilli were used during these investigations. Pure cultures of *T. ferrooxidans* [ATCC no. 13661: (Lundgren: *Ferrobacillus ferrooxidans*)] and *T. thiooxidans* (ATCC no. 8085), were from the American Type Culture Collection (Rockville, Md., USA). Strain TMB was isolated from the outflow of leaching columns containing uranium ore and was found to resemble closely *T. ferrooxidans*.

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Growth conditions. T. ferrooxidans strains were grown on medium prepared as follows. Solution A was prepared by dissolving 50.0 g $FeSO_4 \cdot 7H_2O$ in 200 ml distilled water, the pH of which was already adjusted to 2.2 with 5 M H_2SO_4. This solution was sterilized by passing through a 0.45-μm sterilized membrane filter (Schleicher and Schuell). Solution B was a mineral basal salts medium, which was prepared in a modified form according to 9K medium (Mishra and Pradosh 1979; Silverman and Lundgren 1959). It contained (g/l): $(NH_4)_2SO_4$, 3.00; K_2HPO_4, 0.1; $MgSO_4 \cdot 7H_2O$, 0.5; KCl, 0.1; $Ca(NO_3)_2 \cdot 2H_2O$, 0.02. All ingredients were dissolved in 500 ml distilled water and the final pH was adjusted to 2.3 with 5 M H_2SO_4. This solution was autoclaved for 20 min at 4 kPa and 120° C.

Solutions A and B were mixed to give a final concentration of $FeSO_4$ of 5% (w/v) and 200-ml aliquots were aseptically added to pre-sterilized erlenmeyer flasks. These flasks were inoculated with actively growing cultures of *T. ferrooxidans* (final cell density $5 \cdot 10^5$ cells $\cdot ml^{-1}$) and inocubated on a shaker (rpm 150) at 30° C. When ferrous sulphate was completely exhausted the culture fluids were centrifuged at 10000 *g* in a refrigerated centrifuge (Kokusan Model H.251; Kokusan Ensinki Co., Japan) for 15 min. The pellets, containing cells and ferric salts, were resuspended in sterile H_2SO_4, pH 2.5, transferred into a separating funnel and allowed to stand overnight at 4° C. The upper milky layer, which contained cells, was removed aseptically and centrifuged at 10000 *g* for 10 min. The pellet containing pure, active cells was resuspended in 20 ml H_2SO_4 (pH 2.5) and was used for further experiments. These cells were found to retain their biological activity for at least 3 months.

T. thiooxidans strains were grown on medium containing (g/l): KH_2PO_4, 3.0; $(NH_4)_2SO_4$, 0.40; $MgSO_4 \cdot 7H_2O$, 0.5; $CaCl_2 \cdot 2H_2O$, 0.25; $FeSO_4 \cdot 7H_2O$, 0.01. The final pH was adjusted to 4.0 with 0.5 M: H_2SO_4. 200 ml of this medium was added to erlenmeyer flasks (500-ml capacity) and autoclaved for 20 min at 4.0 kPa. Flowers of sulphur were sterilized by tyndalization and 20 g of this sterilized sulphur was added aseptically to each flask. Actively growing cultures of *T. thiooxidans* were used to inoculate each flask. Incubation was performed on a shaker at 30° C for 96 h. Then, unused sulphur was removed by filtration through Whatman No: 1 paper and the filtrate containing the cells was centrifuged at 10000 *g* for 10 min. The pellet was resuspended in 20 ml H_2SO_4 (pH 2.5) and stored for further experimentation.

Preparation of Gelrite plates. Four grams of Gelrite (Kelco Division of Merck, San Diego, Calif., USA) or Gelan-Gum (Gel-Gro) (ICN Biochemicals, Costa Mesa, Calif., USA) was soaked in 300 ml distilled water, for 20 min and autoclaved at 4 kPa for 20 min.

Plates for testing *T. ferrooxidans* were prepared by mixing solution A, and solution B with sterilized Gelrite solution to a final concentration of Gelrite of 0.4%. These solutions were kept constantly stirred during mixing. The final pH of the solution should be 2.3–2.5 and the temperature near 70° C. Approximately 25 ml of this mixture was poured into pre-sterilized petri dishes, which were used for further investigations. Hardness of the poured gel was monitored by an apparatus designed by Khalid et al. (1992) and was maintained within a range of 420–490 Pa.

When preparing Gelrite plates for growing *T. thiooxidans,* sulphur was replaced by 10.0 g $Na_2S_2O_3 \cdot 5H_2O$. The medium containing 10 g/l of $Na_2S_2O_3 \cdot 5H_2O$ was sterilized by passing through a 0.45-μm sterilized membrane filter (Schleicher and Schuell). The pH of the basal salts solution, as described above for *T. thiooxidans,* was adjusted to 4.5.

Replica plating. The suitability of Gelrite plates for carrying out genetic studies on *Thiobacillus* spp. was determined by replica plating using a standard procedure (Lederberg and Lederberg 1952).

Determination of plating efficiency. In order to determine the plating efficiency, a Petroff-Hausser counting chamber and a phase contrast microscope (Zeiss, MC63) with a magnification of 400 were used to determine total cell counts of the strains: 10–15 squares were counted and dilutions were prepared so that 10–50 cells per square were observed.

Viable cells were counted as the number of colonies developed on the Gelrite plates. Samples were diluted with a sterile 9K basal salt medium and 1 ml of different dilutions was spread onto the hard Gelrite plates containing ferrous sulphate or sodium thiosulphate, which served as the sole energy source for *T. ferrooxidans* and *T. thiooxidans* respectively. These plates were incubated at 30 ± 1° C for 48–96 h. Plating efficiency (PE) was computed as the percentage ratio between the viable and total counts.

Determination of protein, dry mass and ferrous ions. Total protein of bacterial cells was determined by the method of Goa (1953). The dry mass of bacterial cells was estimated by drying the cells at 70° C, to a constant weight. The ferrous ion concentration was determined spectrophotometrically using 1:10 *ortho*-phenanthraline as described by Marczenko (1976).

Results

After 48-h incubation tiny transparent colonies appeared on the solid plates. In a further 2 days these colonies gradually turned dark-brown and were bigger in size (1–3 mm). Colonies of *T. ferrooxidans* (ATCC 13661) grown on two Gelrites obtained from different commercial sources (Gelrite from Kelco, Merck, and Gelan-Gum from ICN Biochemicals) are shown in Fig. 1.

To evaluate the PE of Manning medium (Manning 1975), standardized bacterial suspensions of ATCC 13661 and TMB strains were plated on Manning medium and Gelrite respectively. Plates containing Gelrite as the supporting medium were found to produce crisp, clear colonies within 48–72 h (ATCC 13661: $6.97 \cdot 10^8 \cdot ml^{-1}$, TMB: $5.6 \cdot 10^8 \cdot ml^{-1}$). The total colonies of ATCC 13661 on Manning medium varied from 0 to $1.3 \cdot 10^8$ colonies $\cdot ml^{-1}$ (20% recovery or PE), whereas an average of $2.8 \cdot 10^8$ colonies $\cdot ml^{-1}$ of strain TMB was obtained on these plates (about 50% PE).

When colonies developed on Gelrite were transferred from one plate to another by the replica-plating technique, it was found that young colonies (72–144 h) were easily transferable by the velvet bristles from one plate to another, forming an excellent replica plate. However,

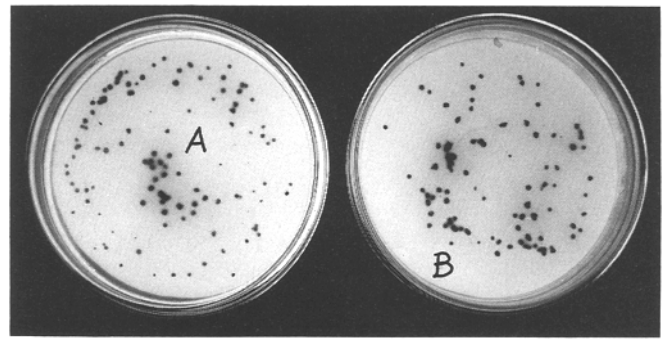

Fig. 1. Colonies of *Thiobacillus ferrooxidans* after 4 days of incubation at 30 ± 1° C on 0.4% Gelrite (**A**) and Gelan Gum (**B**) plates

Table 1. Plating efficiency of Gelrite for *Thiobacillus ferrooxidans*

OD_{400}	Total bacterial counts (ml)	Colonies on Gelrite (ml)[a]	Plating efficiency (%)
ATCC 13661			
0.962	$8.8 \cdot 10^8$	$7.5 \cdot 10^8$	85.2
0.745	$5.0 \cdot 10^7$	$4.8 \cdot 10^7$	96.0
0.273	$6.8 \cdot 10^5$	$6.6 \cdot 10^5$	97.1
	Average plating efficiency = 92.8 ± 6.6		
TMB strain			
0.680	$6.0 \cdot 10^8$	$5.8 \cdot 10^8$	96.7
0.516	$8.0 \cdot 10^7$	$7.0 \cdot 10^7$	87.5
0.358	$4.0 \cdot 10^6$	$3.6 \cdot 10^6$	90.0
	Average plating efficiency = 91.4 ± 4.8		
	Mean plating efficiency = 92.1 ± 5.2		

OD_{400}, optical density at 400 nm
[a] Plates were incubated at 30° C and colonies developed after 48–96 h were counted

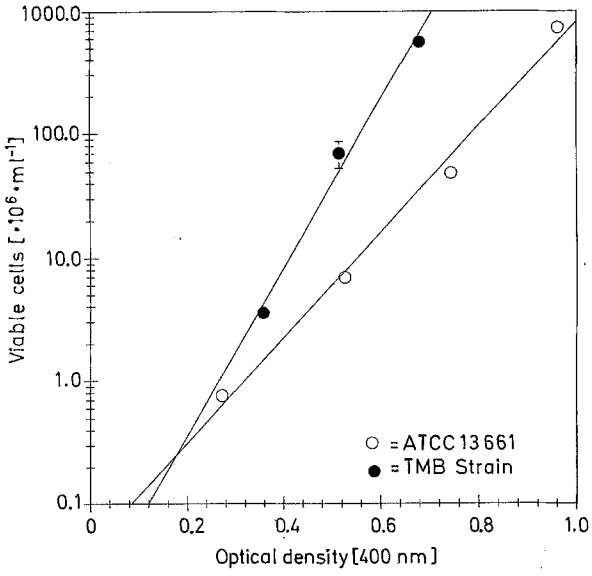

Fig. 2. Growth of *T. ferrooxidans* strains ATCC 13661 and TMB in liquid culture and on solid Gelrite plates. Cells in liquid culture were grown in erlenmeyer flasks by incubating on a shaker (rpm 150) at 30° C. Gelrite plates were also incubated at 30° C. Numbers of colonies are plotted against the corresponding optical density values during growth at 400 nm. At least ten petri dishes were counted from three consecutive dilutions. Plates with 5–300 colonies were used. *Error bars* represent the standard deviation

with older colonies (more than 144 h) a hard ferric crust was found to hinder the transfer.

The PE for the axenic strain ATCC 13661 (Table 1), was found to vary between 85 and 97%, giving a mean average value of 92.8% with a standard deviation of 6.6. Similarly the wild-type strain, TMB, yielded PE values of 87–97%, which also rendered a mean average value of 91.4% with a standard deviation of 4.8. However, the overall PE value was found to be 92.1 ± 5.2%, a value very close to the individual PEs.

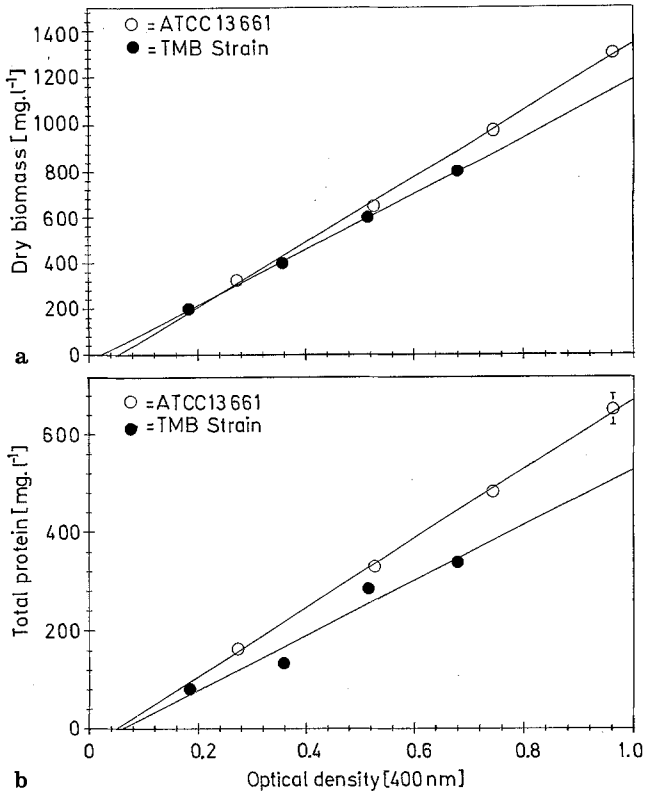

Fig. 3a, b. Relationship between optical density and dry biomass (**a**) and total protein (**b**) contents of *T. ferrooxidans* strains ATCC 13661 and TMB during growth in a liquid medium in erlenmeyer flasks incubated on a shaker (150 rpm) at 30° C. At least ten observations were recorded from three consecutive dilutions. *Error bars* represent the standard deviation

During growth of these microorganisms, samples were withdrawn aseptically from the flasks and optical density (OD), dry mass and total protein contents were determined as described previously. When the OD measured at 400 nm (OD_{400}) was plotted against the viable counts, a linear correlation was obtained (Fig. 2). For an axenic culture of *T. ferrooxidans* (ATCC 13661), an OD_{400} of 0.5 corresponded to $7 \cdot 10^6$ cells $\cdot$ ml^{-1}, whereas for a natural isolate strain (TMB), the OD_{400} was $5 \cdot 10^7$ cells $\cdot$ ml^{-1}.

When dry biomass of bacterial suspensions was plotted against OD_{400}, linear correlations were obtained (Fig. 3A). An OD_{400} of 0.5 corresponded to a dry biomass of 580 mg $\cdot$ l^{-1} for strain TMB whereas for the ATCC 13661 strain the OD_{400} was equivalent to 650 mg $\cdot$ l^{-1} of dry biomass. Total bacterial protein, when plotted against OD_{400}, was found to yield a linear correlation (Fig. 3B) and 0.5 OD_{400} was found to be equivalent to 245 and 320 mg total protein $\cdot$ l^{-1} for the TMB and ATCC 13661 strains, respectively. The relationship between dry biomass and viable counts of these *T. ferrooxidans* strains is depicted in Fig. 4. The relationship was also found to be linear giving a value where 0.5 g dry mass $\cdot$ l^{-1} was equivalent to $2.8 \cdot 10^6$ and $2 \cdot 10^7$ cells $\cdot$ ml^{-1} of the ATCC 13661 and TMB strains, respectively.

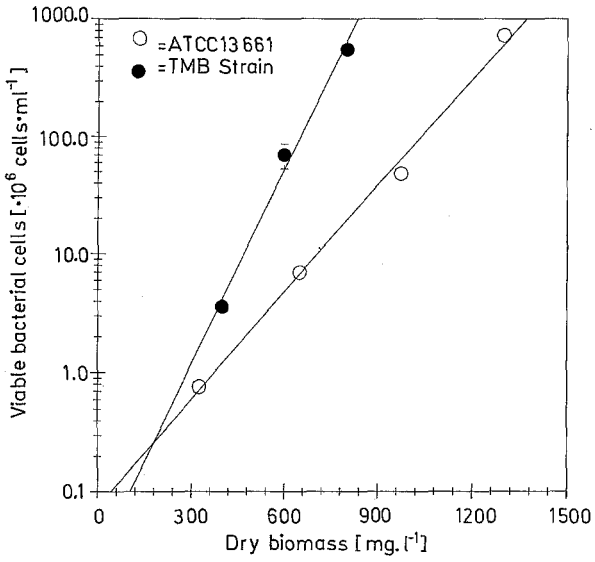

Fig. 4. Relationship between the number of colonies and dry biomass of *T. ferrooxidans* strains ATCC 13661 and TMB during their growth on liquid and on solid medium by incubation at 30° C. At least ten petri-dishes were counted from three consecutive dilutions. Plates containing 5–300 colonies were used

Discussion

In contrast to the silica gel plates used earlier by Goodman et al. (1980) or the membrane techniques employed by Tuövinen and Kelly (1973), colonies on Gelrite were found to be well-differentiated and easily manageable. These colonies could be readily transferred from one plate to another. Two commercially available Gelrites were found to be comparable in supporting the growth of *T. ferrooxidans*. The same bacterial suspension produced $1.3 \cdot 10^9$ colonies $\cdot ml^{-1}$ on the Gelrite, while $1.1 \cdot 10^9$ colonies $\cdot ml^{-1}$ were counted on the plates containing "Gelan Gum" as the supporting medium.

Development of colonies on plates containing Manning medium started after 9–10 days and even then a very erratic pattern of colony formation was noticed. Holmes et al. (1983) also reported poor PEs on Manning medium and obtained colony yields that were non-quantitative.

The above results indicate that Gelrite could be a suitable support medium for the growth of autotrophic *T. ferrooxidans*. This medium was later employed to successfully purify cultures of *T. ferrooxidans* strains. Therefore, it is recommended that this medium should be employed to purify axenic cultures of *T. ferrooxidans* strains, which have frequently been reported to have been associated with some satellite strains (Harrison et al. 1980; Barros et al. 1984). It can also be used to purify these cultures from a single cell colony. However, *T. thiooxidans* strain ATCC no. 8058 was unable to give quantitative colony yields on the Gelrite medium containing thiosulphate at pH 4.0, and is being investigated further. High PE obtained with this plating technique, one prerequisite for the selection of mutants, resistant to toxic metals and for classical genetic investigations of *T. ferrooxidans* has been successfully demonstrated.

Values of viable counts, dry biomass and total bacterial protein were found to bear a linear relationship with OD. Although the correlation between OD and viable counts for both strains of *T. ferrooxidans* was straight, the slope was different for each strain. This can, however, be explained since strains of the same species differ slightly in their optical properties from one another. All these data indicate a definite congruous attitude and yield within reasonable limits of errors, invariably consistent and equable values for cell numbers, dry biomass and cellular protein. Thus this method offers an easy technique for measuring and monitoring viable counts of *T. ferrooxidans* in natural as well as in controlled milieux. Therefore, biomass/cell number/bacterial protein can be determined with precision in commercial leaching operations as well as in laboratory experiments. Data thus collected can be employed in modelling and scale-up the leaching processes.

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