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Floating Filters, a Novel Technique for Isolation and Enumeration of Fastidious, Acidophilic, Iron-Oxidizing, Autotrophic Bacteria

JOHANNA C. DE BRUYN,* FRED C. BOOGERD, PIETER BOS, AND J. GIJS KUENEN

Department of Microbiology and Enzymology, Kluyver Laboratory for Biotechnology, Delft University of Technology, Julianalaan 67, 2628 BC Delft, The Netherlands

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Nuclepore polycarbonate filters floating on a liquid, $FeSO_4$-containing medium (pH 1.6) were used to isolate a moderately thermophilic bacterium from a pyrite-oxidizing enrichment culture. The isolate failed to grow on any of the conventional solid media tried. To test the general applicability of the method, the enumeration of a fastidious acidophilic organism, *Thiobacillus ferrooxidans*, was carried out and the results compared with those obtained with other filters, solid media, and the most probable number technique. *T. ferrooxidans* showed better viability on the floating polycarbonate filters and grew in a much shorter time (4 to 5 days) than with the other techniques.

It is common practice in microbiology to use agar media for the isolation and enumeration of bacteria. However, it is well known that culture media solidified with this natural polysaccharide are not always suitable for particular bacteria. In some cases, for no obvious reason, the organisms involved will not grow on solid media. In other cases, the problems encountered are related to the chemical composition of agar-agar. This solidifying agent is, as a natural product, not well defined chemically. It often contains, in addition to high-molecular-weight carbohydrates, considerable amounts of low-molecular-weight compounds which can prove inhibitory, especially to oligotrophic organisms. The concentration of these low-molecular-weight compounds might even increase drastically during the incubation of the acidophilic cultures, due to the increased hydrolysis of the carbohydrates, especially the sulfated polysaccharides (9).

A survey of the literature on isolation and enumeration techniques for sulfur-oxidizing, acidophilic bacteria, especially *Thiobacillus ferrooxidans*, provides illustrations of these problems and attempted solutions. Colmer et al. (3) were able to isolate *T. ferrooxidans* on a medium solidified with agar-agar, and Mackintosh (6) and Mishra and Roy (7) suggested improvements in the composition of the medium and the use of agarose instead of agar-agar. Tuovinen and Kelly (9) suggested the use of membrane filters placed on a ferrous iron-containing mineral medium solidified with agar-agar. Harrison (4) proposed the use of a two-layered gel prepared with agarose. In order to minimize the formation of hydrolyzation products from the polysaccharides, the author used a procedure in which the upper layer containing the bacterial cells was solidified as rapidly as possible. The efficiency of plating was reported to be in the range of 50 to 80%. The observation of Harrison (4) that the presence of oligotrophic acidophilic heterotrophs such as *Acidiphilium cryptum* stimulated the growth of *T. ferrooxidans* has led to the idea of including *A. cryptum* in the solid medium for the enumeration of *T. ferrooxidans* (2). *A. cryptum* will consume the compounds present in the gel which are inhibitory to *T. ferrooxidans*, thus creating a "clean" environment for the latter organism.

* Corresponding author.

During our studies on the microbial desulfurization of coal, we studied also the potentials of moderate thermophiles, with an optimum temperature around 50°C. In order to be able to study the physiology of moderately thermophilic, pyrite-oxidizing bacteria, we attempted to isolate the dominant organism from a pyrite-oxidizing enrichment culture obtained from high-pyrite-content coal reject material from a coal mine in Zambia (Maamba mine). Various methods and solid media suggested by different investigators to isolate and enumerate *T. ferrooxidans* were used, but none of them were effective. It seems likely that the polysaccharides hydrolyzed even more rapidly with the combined acidity and higher temperatures (45 to 50°C). Alternatively, the bacteria might be more sensitive to the hydrolysis products and impurities of the gelling agent. It proved possible to isolate the organism only by using a novel technique. The aim of this paper is to give a detailed description of this technique, in which polycarbonate membranes floating on well-defined liquid media are used. Besides its use in the isolation of fastidious organisms, its application in the enumeration of bacteria will also be discussed.

MATERIALS AND METHODS

Bacterial cultures. *T. ferrooxidans* 3G (LMD 81.68.C), a single-cell isolate from LMD 81.68 (ATCC 19859), was prepared with the aid of microslides, as described by Mackintosh (6).

A moderately thermophilic, pyrite-oxidizing enrichment culture was obtained from a high-pyrite-content coal sample from the Maamba coal mine (Zambia). This "Maamba culture" was dominated by a rod-shaped bacterium.

Culture media. *T. ferrooxidans* was grown in 100 ml of the F2 base medium described by Mackintosh (6) but containing 0.05 μM rather than 0.04 mM Na_2MoO_4. Ferrous sulfate (180 mM) was added as an energy source. The pH was adjusted to 1.6. Cells were grown in round-bottomed flasks (500 ml) at 30°C in a rotary shaking machine at 150 rpm.

The Maamba culture was grown at 45°C in a rotary shaking machine, also at 150 rpm, in 250-ml infusion flasks containing 50 ml of the F2 base medium, supplemented with 0.2% pyrite and 0.01% yeast extract.

For the isolation of the rod-shaped bacterium from the Maamba culture, the F2 base medium was used, completed with 180 mM $FeSO_4$ and 0.01% yeast extract. The agarose media with an overlay were prepared by the method of Harrison (4). A filter was sometimes used instead of an overlay.

Filters. The following 25-mm filters with a pore size of 0.2 μm were tested: (i) standard (polyvinyl pyrrolidone [PVP]-coated) Nuclepore polycarbonate membranes, either unstained or Irgalan black stained, with and without grid marks (PVP is a wetting agent); (ii) PVP-free Nuclepore polycarbonate membranes; (iii) Nuclepore polyester membranes; (iv) MF-Millipore GSWP membranes (mixed esters of cellulose).

Floating-filter technique. Before use, the polycarbonate and polyester filters were autoclaved for 20 min at 110°C on a piece of filter paper in a petri dish and the Millipore filters were boiled for 10 min in demineralized water, as described by Tuovinen and Kelly (9). The sterile filters were mounted in glass filter holders, which had been previously sterilized by autoclaving at 120°C or, alternatively, rinsed with 70% (vol/vol) ethanol and sterile demineralized water. Control experiments showed that the latter method did not lead to (detectable) contamination of the samples. The filter was rinsed with approximately 10 ml of demineralized water and 10 ml of acid water (dilute H_2SO_4, pH 1.6). With the hydrophobic filters (PVP free and polyester), the procedure was started by filtering 2 ml of 70% (vol/vol) ethanol, followed by water and acid water. At least 2 ml of a suitable dilution of the (enrichment) culture was then filtered. The filter was then carefully placed on top of the liquid medium in a 6-well cell culture cluster (Costar Europe Ltd., Badhoevedorp, The Netherlands). This is a sterilized, optically clear polystyrene plate, 130 by 85 mm, consisting of 6 wells with diameters of 35 mm and depths of 20 mm. Other vessels or petri dishes could also be used. Because the Millipore filters did not float, they were supported by sterilized sintered glass. The incubation temperature depended on the cultures involved (30 or 45°C). At 45°C the clusters were wrapped in Parafilm to limit evaporation if extended incubation times were needed. However, long incubation times should be avoided with Fe^{2+}-containing media, because, due to bacterial activity, the filters will become covered with Fe^{3+}-containing precipitates and will eventually sink.

Isolation. After serial dilutions of the FeS_2-enrichment culture in F2 medium with $FeSO_4$ and yeast extract, a sample of the highest dilution showing growth was used as inoculum for the floating-filter technique. After an incubation time of 3 to 5 days, the filters were placed on sterile, wet (pH 1.6) filter paper in a sterile petri dish under a dissecting microscope. With the illumination (Olympus fibre optic illuminator highlight 2000) almost parallel to the filter, the microcolonies could be discerned and taken from the filter with a thin glass needle. For the observation of colorless colonies, the illumination is especially important. Different positions of the light should be tried. The cells from a selected colony were suspended in water, and this suspension was used to repeat the filtering technique twice in order to obtain pure cultures.

Enumeration. In order to be able to determine the viability of the different bacterial cultures on the floating filters, cell counts were made with a Bürker-Türk counting chamber. For colony counts, the dry, stained filters (see below) were fixed on a microscope slide with a small drop of immersion oil or Hydromount at the edge of the filter and counted directly under the dissecting microscope. For comparison, other isolation and enumeration techniques were also used. These included the agarose overlay plating method described by Harrison (4) and the most probable number technique.

Staining. For easy counting, the filters were stained by the following procedure. After incubation, the filters were washed by successively floating them three times on acid water (pH 1.6) and three times on demineralized water in a six-well culture cluster. Effective washing was achieved by gently stirring the fluid around the filter with a glass rod. Staining was accomplished by floating the filter on the staining solution for 30 min. For normal light microscopy, several dyes such as methylene blue (5 ml of saturated solution in ethanol and 195 ml of water), malachite green (0.2% aqueous solution), and Irgalan black (0.2% in 2% [vol/vol] acetic acid) appeared to be effective. Excess stain could be removed by floating the filters on demineralized water in a well culture cluster. Spreading of cells over the filter was checked with fluorescence microscopy (after staining with 0.01% acridine orange in 5% formaldehyde in water) with black and standard polycarbonate filters by using a phase-contrast epifluorescence microscope. In spite of the higher background fluorescence of the unstained filters, even single cells could be observed. All liquids used in the fluorescence technique were filtered through a 0.2 μm filter prior to use. Immunofluorescence staining with specific antiserum against *T. ferrooxidans* was carried out as described by Muyzer et al. (8).

RESULTS AND DISCUSSION

Isolation of the dominating organism from the moderately thermophilic, pyrite-oxidizing enrichment culture. After unsuccessful attempts to isolate the dominant rod from the Maamba culture by using different solidified media, either in combination with membrane filters or without, growth was obtained on unstained standard Nuclepore polycarbonate filters which floated on the F2 medium supplemented with $FeSO_4$ and yeast extract. After an incubation period of 4 days at 45°C, yellowish colonies (0.6 to 1.2 mm) could be discerned. These became dark brown after longer incubation, due to the formation of iron-containing precipitates. In the liquid medium underneath the filter, no cells could be detected. By repeating the procedure with cell material from a single colony, a pure culture could be obtained. Details of the physiology of this gram-positive, rod-shaped (2 to 8 μm by 0.7 to 1 μm), facultatively chemolithoautotrophic, occasionally motile organism are reported elsewhere (1). Attempts to obtain growth on Irgalan black-stained Nuclepore polycarbonate filters or on Millipore GSWP filters were unsuccessful.

Enumeration. In order to test the utility of the floating-filter technique in the enumeration of fastidious bacteria, *T. ferrooxidans* was used as a model organism. After 2 days of incubation, tiny colonies of *T. ferrooxidans* could already be observed under the dissecting microscope. After 4 to 5 days, the maximal number of (colorless or slightly yellow) colonies was obtained. The shape of the colonies on the Nuclepore filters was variable, i.e., round and relatively high to flat and slightly spreading (Fig. 1). This may be due to irregularities in the hydrophilicity of the filters (5). Cells from both colony types gave positive results with the anti-*T. ferrooxidans* serum, and after replating on the floating filters, both colony forms were again observed.

Polyester filters were more difficult to handle, quickly

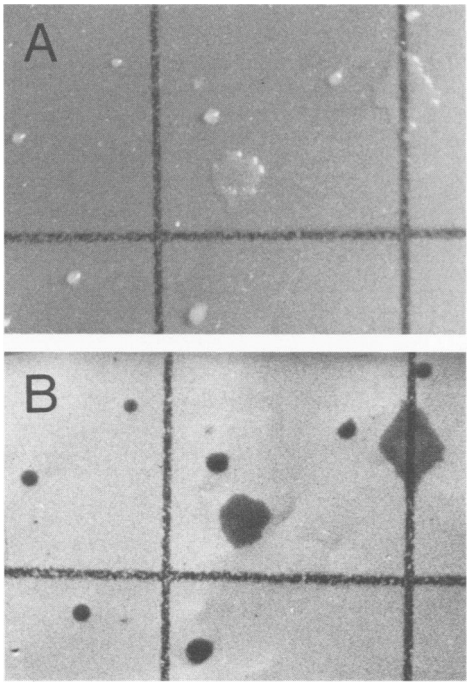

FIG. 1. (A) Micrograph of colonies of *T. ferrooxidans* on a floating standard Nuclepore polycarbonate membrane after 5 days of incubation. (B) The same filter after staining with malachite green. Magnification, ×10.

folding and sometimes sinking after a few days of incubation. The viability percentages, calculated for growth on both types of filter, are indicated in Table 1. The counting chamber gave significantly (95% confidence level) higher counts than the other method, probably because in this case both viable and nonviable cells are counted, whereas on the filters only the number of CFU is obtained. No significant (95% confidence level) differences in the growth on different filters or with different pretreatment were observed, except for the non-prerinsed polycarbonate filters without PVP and the prerinsed polyester filters without PVP. Incubation on black-stained filters appeared to be effective in increasing the visibility of the colonies but also resulted in a lower viability.

The results obtained with Millipore GSWP filters were not satisfactory. They did not float, and after growth on supported filters, they did not yield well-defined colonies. Moreover, the liquid culture medium underneath those filters contained bacterial cells. These features made the Millipore GSWP filters unsuitable for use with these enumeration and isolation techniques.

TABLE 1. Growth of *T. ferrooxidans* and percent viability in different floating filters

Filter	PVP[a]	Rinsed[b]	Mean no. of cells or CFU (10^7/ml), ±SD (*n*)	% Viability
Counting chamber			5.92 ± 0.89 (7)	100
Polycarbonate	+	+	3.35 ± 0.34 (5)	57
Polycarbonate	+	−	2.50 ± 0.49 (5)	42
Polycarbonate	−	+	3.05 ± 0.85 (5)	52
Polycarbonate	−	−	0.85 ± 0.57 (6)	14
Polyester	−	+	1.55 ± 0.22 (4)	26
Polyester	−	−	2.20 ± 0.26 (4)	37

[a] +, PVP present; − PVP absent.
[b] +, Rinsed as described in Materials and Methods; −, not rinsed.

TABLE 2. Comparison of different counting methods with *T. ferrooxidans*

Method	% Viability[a]	Incubation time (days)
Counting chamber	100	
Most-probable-number technique	24–114	21
Agarose overlay method	N.C.	21
Floating standard polycarbonate filters	31–78	5
Standard polycarbonate filters on agarose	N.C.	21
Millipore filters on agarose	N.C.	21

[a] The observed variations in percentages are caused by a varying vitality of the cells. Nevertheless, the most-probable-number technique and the floating filter technique yielded comparable viability percentages. N.C., No colonies developed from any culture dilution.

The incubation time of 4 to 5 days on floating standard polycarbonate filters is much shorter than the time required for the formation of maximal number of colonies on solid media. Only Mishra and Roy (7) found colony formation after 4 days, but an agar-tolerant strain was used. Much longer incubation times are generally reported: 7 to 17 days on agar media (2, 3) and 7 to 28 days on agarose media (4, 7). Indeed, it has been reported that growth does not occur (2). The enumeration technique suggested by Butler and Kempton (2) requires incubation periods of over 14 days to reach viabilities over 50%, but this technique is not suitable for the isolation of organisms. In Table 2, the results of different counting experiments with *T. ferrooxidans* 3G are presented. The figures from the floating filters were obtained after 5 days of incubation, and those from the other methods after 3 weeks.

It can be concluded that the floating-filter technique using polycarbonate filters is a successful tool in the isolation and enumeration of fastidious, acidophilic bacteria. Recently, fastidious, neutrophilic autotrophs have also been isolated by using the same technique (G. C. Stefess, R. de Schrijver, J. C. de Bruyn, and J. G. Kuenen, unpublished results). The use of this technique results in growth after comparatively short incubation periods and allows the use of well-defined liquid media, without the need for solidifying agents, which possibly contain growth-inhibiting substances.

LITERATURE CITED

1. **Boogerd, F. C., M. M. Q. van Alphen, W. J. van Anrooij, J. C. de Bruyn, P. Bos, and J. G. Kuenen.** 1990. The role of growth and maintenance in the oxidation of pyrite in batch culture by a moderately thermophilic, facultative chemolithautotroph, p. 735–751. *In* J. Salley, R. G. L. McCready, and P. L. Wichlacz (ed.), Proceedings of the 1989 International Symposium on Biohydrometallurgy. Canada Centre for Mineral and Energy Technology, Ottawa, Ontario, Canada.
2. **Butler, B. J., and A. G. Kempton.** 1987. Growth of *Thiobacillus ferrooxidans* on solid media containing heterotrophic bacteria. J. Ind. Microbiol. **2**:41–45.
3. **Colmer, A. R., K. L. Temple, and M. E. Hinkle.** 1950. An iron-oxidizing bacterium from the acid drainage of some bituminous coal mines. J. Bacteriol. **59**:317–328.
4. **Harrison, A. P., Jr.** 1984. The acidophilic *Thiobacilli* and other acidophilic bacteria that share their habitat. Annu. Rev. Microbiol. **38**:265–292.
5. **Hobbie, J. E., R. J. Daley, and S. Jasper.** 1977. Use of Nuclepore

filters for counting bacteria by fluorescence microscopy. Appl. Environ. Microbiol. **33**:1225–1228.
6. **Mackintosh, M. E.** 1978. Nitrogen fixation by *Thiobacillus ferrooxidans*. J. Gen. Microbiol. **105**:215–218.
7. **Mishra, A. K., and P. Roy.** 1979. A note on the growth of *Thiobacillus ferrooxidans* on solid medium. J. Appl. Bacteriol. **47**:289–292.
8. **Muyzer, G., A. C. de Bruyn, D. J. M. Schmedding, P. Bos, P. Westbroek, and G. J. Kuenen.** 1987. A combined immunofluorescence-DNA-fluorescence staining technique for enumeration of *Thiobacillus ferrooxidans* in a population of acidophilic bacteria. Appl. Environ. Microbiol. **53**:660–664.
9. **Tuovinen, O. H., and D. P. Kelly.** 1973. Studies on the growth of *Thiobacillus ferrooxidans*. Arch. Mikrobiol. **88**:285–298.