A simple medium modification for isolation, growth and enumeration of Acidithiobacillus thiooxidans (syn. Thiobacillus thiooxidans) from water samples

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A B S T R A C T

High concentrations of H2S in groundwater are commonly removed using Biological Trickling Filter (BTF) that contains high numbers of biofilm immobilized sulfur oxidizing bacteria (mainly Thiobacillus thiooxidans). BTF performance requires continuous monitoring of these bacteria at several sampling points. The Most Probable Number (MPN) technique is at the moment the method of choice to enumerate viable Thiobacillus thiooxidans cells under the above conditions. However, this method is extremely time-consuming (7–10 days) and not always suitable for environmental monitoring. In the present study, Thiobacillus agar recommended for isolation and cultivation of Thiobacillus species by Spread plate method was modified by addition of bromocresol green (BCG) in order obtain a clear-cut resolution of the growing colonies resulting in similar or higher numbers compared to other methods. Visual emergence of bacterial colonies on the 3rd and 4th days, from the initial plating, was associated with sulfuric acid production, resulting in an unambiguous color change from blue to yellow, around each colony. This study revealed that BCG modified Thiobacillus agar is substantially time saving and much easier to infer compared to MPN technique.

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1. Introduction

As a result of potable water scarcity in southern part of Israel, groundwater containing high concentrations of H2S (~20 ppm) is used as a water supply source for drinking water. H2S removal process combines an air degasifying tower releasing around 600 ppm H2S/m2 conveyed to a cooling tower. The processed air is conveyed by the ministry of environmental protection and according to current regulations for ambient air a daily average of 5 ppm/m2 is demanded. As much as 98% of the produced H2S is removed from the air stream by this method. Optimization of the BTF performance involves monitoring of SOB at several sampling points. The main goal of the present study was to develop the most appropriate growth medium to enumerate viable Thiobacillus thiooxidans cells, in order to support rapid and effective operational data of the BTF. Thiobacillaceae, effectively oxidize reduced forms of sulfur (H2S, metal sulfides, thiosulphates and elemental sulfur – S0) in order to obtain energy and fix atmospheric CO2 for growth requirements (Ehrlich, 1996). Typical members of this group are Thiobacillus thiooxidans, Thiobacillus albertsi, Thiobacillus ferrooxidans (all strict autotrophs) that oxidize reduced forms of sulfur to sulfuric acid (terminologically acidophilic) to drop pH close to 0 value. In order to estimate Thiobacillus species, the MPN technique with liquid media is commonly recommended and widely applied (Andrew et al., 2005; Southam and Beveridge, 1992; Shinabe et al., 1995, 2000; Knickerbocker et al., 2000). It is still the most precise method allowing detection of viable cells with the accuracy of 1 colony forming units (CFU)/100 ml is MPN. Regrettably, this technique is time consuming both for medium preparation (sterilization by intermittent steam for each of the three consecutive days) and growth detection (measurement of pH or optical density of each tube) therefore not suitable for extensive monitoring use. Occasionally monitoring of Thiobacillus cells is performed by measuring optical density of growth culture or bacterial suspension that allows easy and adequately fast tracking of bacterial growth dynamics or biofilm development (Crescenzi et al., 2006; Kurosawa et al., 1991; Lee et al., 2005, 2006). However the precision of the method does not extend over one to two orders of magnitude. Occasionally, hemocytometer — a device originally designed for blood cell count that is used for quantification of Thiobacillus cells (Chen et al., 2002). Nevertheless this method has two shortcomings: 1) it is impossible to differentiate between viable and dead cells and 2) at very low initial concentration the count is highly inaccurate. The use of Spread Plate method should be the most appropriate technique for CFU detection, but it requires a solid nutrient medium (with agar). Culture of acidophilic microorganisms was extensively performed in liquid media (Johnson, 1995), but...
presently new solid media formulations have been developed (Atlas, 2005a; Ramirez et al., 2009). For example, Spread Plate method was applied for T. thiooxidans bacteria culturing and counting of ATCC290 on S65-agar plates (agar 1.5% w/v) (Ramirez et al., 2009). In the present study, the sensitivity of MPN and Spread Plate methods was compared in order to evaluate the possible use of Spread Plate methods for T. thiooxidans monitoring along with BTF performance optimization. Two of the simplest defined media for Thiothrix spp. isolation were selected and compared to a modified medium (comprising brom cresol green — BCG as a pH indicator) to facilitate environmental monitoring of Thiothrix spp.

2. Materials and methods

2.1. Experimental strains

Experiments were carried out with pure cultures of T. thiooxidans (ATCC 8085), purchased from American Type Culture Collection — (ATCC). T. ferrooxidans was obtained from the German Collection of Microorganisms and Cell Cultures (DSM 14882) and environmental T. thiooxidans originated from a BTF system (Mekorot Water Company, Israel). Final identification of environmental T. thiooxidans was performed as further described.

2.2. Environmental T. thiooxidans identification

Water samples were subjected to DNA extraction employing Promega kit (Cat # A1120, Wizard Genomic, DNA purification Kit). The DNA samples were sent to a commercial lab (Research and Test Laboratory, 4321 Marsha Sharp Fwy, Lubbock, TX 79407, USA) for identification. Following Pyrosequencing, all failed sequence reads, low quality sequence ends and tags and primers were removed and sequence collections depleted of any non-bacterial ribosome sequences and chimeras using B2C2 (Gontcharova et al., 2010) as has been described previously (Dowd et al., 2008). Based upon the above BLASTn derived sequence identity (percent of total length query sequence which aligns with a given database sequence) and validated using taxonomic distance methods the bacteria were classified at the appropriate taxonomic levels based upon the following criteria. Sequences with identity scores, to known or well characterized 16S sequences, greater than 97% identity (<3% divergence) were resolved at the species level, between 95% and 97% at the genus level, between 90% and 95% at the family and between 85% and 90% at the order level, 80 and 85% at the class and 77% to 80% at phyla. After resolving based upon these parameters, the percentage of each bacterial and fungal ID was individually analyzed for each sample providing relative abundance information within and among the individual samples based upon relative numbers of reads within each sample (Dowd et al., 2008). T. thiooxidans were the most predominant species (over 70%).

2.3. Experimental media

In MPN method, the Thiobacillus medium (ATCC medium #125) (Anonymous, 2011a) contained the following ingredients (g/l): 0.2 g — (NH₄)₂SO₄, 0.5 g — MgSO₄·7H₂O, 0.25 g — CaCl₂, 3 g — K₂HPO₄, 0.005 g — FeSO₄, 10 g-sulfur in 1 l of tap water. MPN set-up consisted of 5 tubes/row and up to 7 dilutions. Previous to medium addition, 0.1 g of sulfur was placed in each dry tube. Afterward, aliquots of 10 ml were carefully poured down the side of the tube without wetting of sulfur, in order to allow the sulfur to float. Tubes with growth medium were sterilized under flowing steam for 3 consecutive days, 30 min each day. For Spread Plate method two media were used: Thiobacillus agar (0.4 g — (NH₄)₂SO₄, 0.5 g — MgSO₄·7H₂O, 0.25 g — CaCl₂, 4 g — K₂HPO₄, 0.01 g — FeSO₄, 5 g — Na₂S₂O₃, 12.5 g — agar, 11 — distilled water; autoclaved at 121 °C for 15 min) and Thiobacillus acidophilus agar (3 g — (NH₄)₂SO₄, 1 g — MgSO₄·7H₂O, 0.1 g — KCl, 0.5 g — K₂HPO₄, 0.01 g — FeSO₄, 18 mg — Ca(NO₃)₂, 4H₂O, 15 g — agar, 20 ml — of 10% glucose solution and 980 ml — distilled water, autoclaved at 121 °C for 15 min) (Atlas, 2005a, 2005b).

Some of the experiments were carried out on Thiobacillus agar with addition of pH indicators such as phenol red (PHR) (Fluka, Germany) and brom cresol green (BCG) (Sigma, Israel) at a final concentration of 0.07 and 0.4% in distilled water, respectively. Identification of experimental bacteria was conducted on Thiobacillus agar containing 1 and 2 ml of Phr solution and 0.5, 1, 2, 4, 5, 6, 7, 8, 10 ml of BCG solution in 1 l of the medium (at concentrations mentioned above). The effect of agar pH on bacteria isolation was studied on Thiobacillus agar containing 2 ml/l of BCG solution at pH-s 3.7, 4.1, 4.5, 5.0, and 5.5. Subsequent to inoculation, Petri dishes containing solid medium were incubated for 3 to10 days at 31 ± 0.5 °C. Saline (0.85% NaCl) was used for serial dilutions and preparation of bacterial suspension. In each experiment, samples were diluted up to 10⁻³ and were plated on three agar plates, every dilution, and average colony number were calculated. Each experiment was repeated twice. T. ferrooxidans bacteria were grown under intensive aeration (bubbling) in a 1-liter Erlenmeyer flask within a medium containing: 0.4 g l⁻¹ (NH₄)₂O, 0.4 g l⁻¹ MgSO₄·7H₂O, 0.1 g l⁻¹ K₂HPO₄, and 12 g l⁻¹ FeSO₄·7H₂O (48). Initial pH of the growth medium was adjusted to 1.7 by the addition of 2 N H₂SO₄.

3. Results and discussion

Isolation and enumeration of T. thiooxidans bacteria were performed in water probes sampled at different points of Mekorot Water Company BTF plant on Thiobacillus and T. acidophilus agars (Atlas, 2005a, 2005b). Experimental results given in Table 1 show that examined T. thiooxidans bacteria did not produce colonies (no growth) on T. acidophilus agar, while on Thiobacillus agar different colony counts were observed, probably expressing sampling variability.

According to HiMediaLabs (Anonymous, 2012) colony description of Thiobacillus spp. on their solid medium is described as: “Thiobacillus forms small sulfur impregnated colonies with clear zones, indicating acid formation from thiosulfate oxidation”. In fact, during the growth process the bacterium forms minuscule white colonies which are poorly detectable, especially in the early growth phase (on the 3rd and 4th days) (Fig. 1A-left). Facilitated detection and enumeration of T. thiooxidans colonies was obtained by addition of pH indicators such as PhR or BCG to Thiobacillus agar. The indicator PhR gradually transits from yellow to red color over the pH range of 6.8 to 8.2, and

<table>
<thead>
<tr>
<th>Media</th>
<th>Sample number</th>
<th>Thiobacillus acidophilus agar [CFU/100 ml]</th>
<th>Thiobacillus agar [CFU/100 ml]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>1</td>
<td>n.d.</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>2</td>
<td>n.d.</td>
<td>3.1 × 10⁻¹ ± 0.02 × 10⁴</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>n.d.</td>
<td>&lt;10</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>n.d.</td>
<td>&lt;10</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>n.d.</td>
<td>1.5 × 10⁻² ± 0.02 × 10⁷</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>n.d.</td>
<td>2.1 × 10⁻³ ± 0.05 × 10³</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>n.d.</td>
<td>&lt;10</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>n.d.</td>
<td>1.2 × 10⁻⁴ ± 0.03 × 10⁴</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>n.d.</td>
<td>9.0 × 10⁻⁴ ± 0.04 × 10⁴</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>n.d.</td>
<td>4.0 × 10⁻⁵ ± 0.07 × 10⁷</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>n.d.</td>
<td>2.4 × 10⁻⁴ ± 0.08 × 10⁷</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>n.d.</td>
<td>2.0 × 10⁻⁴ ± 0.03 × 10⁴</td>
<td></td>
</tr>
<tr>
<td>13 (b)</td>
<td>n.d.</td>
<td>3.3 × 10⁻⁵ ± 0.06 × 10⁶</td>
<td></td>
</tr>
</tbody>
</table>

a n.d. — not detected.
b Bacterial suspension washed off sponge filter.
BCG indicator from yellow to blue over the pH range of 3.8 to 5.4 (Anonymous, 2011a, 2011b). At this stage of the present study, these indicators were added to *Thiobacillus* agar and the pHs were adjusted to 7.5 (for PhR) and to 4.0 (for BCG) values with NaOH (1 M) followed by boiling and autoclave. In these experiments, *T. thiooxidans* culture was grown from environmental isolates obtained from well water samples (Mekorot Water Company, Israel). *T. thiooxidans* isolation and enumeration on the modified media are presented in Table 2.

It can be seen that BCG addition to *Thiobacillus* agar does not affect both the growth and detection of the experimental *Thiobacillus* bacteria. On the other extreme, no bacterial growth was observed on the modified agar containing PhR. This observed fact may be explained by the initial high pH of −7.5 (while initial optimal pH for acidophilic *Thiobacillus* species is between the interval of 2.5–4.5 values) or simply by an inhibitory effect of PhR towards *Thiobacillus*. Obviously, the pH of PhR-containing agar was adjusted to 7.5 in order to obtain an initial red color since below the pH value of 6.8, the indicator color shifts to yellow. With regard to PhR toxicity, no further experiments were performed; however it seems that medium containing PhR allows isolation and growth of some *Thiobacillus* spp. under specific conditions (Atlas, 2005a, 2005b).

*T. thiooxidans* growth on *Thiobacillus* modified agar supplemented with BCG revealed very good results. Fig. 1A is a photograph of the two media, in which colonies grown on standard *Thiobacillus* agar (left) are faint and very difficult to count while on BCG *Thiobacillus* modified agar (right) the colonies are yellow bright and entirely countable. Furthermore, addition of BCG to standard nutrient agar had made possible to roughly detect the experimental bacteria from environmental samples with an accuracy of one order of magnitude, without the need of a direct colony count. According to Fig. 1B (right side) it can be easily estimated that bacterial concentration in the experimental water sample is not less than 10^6 CFU/ml (according to last dilution in which modified agar changed its color from blue to yellow). Empirically, it was observed that medium containing BCG changed entirely its color from blue to yellow, when at least 7±2 colonies per plate were present. At enhancing the incubation period (6–8 days), it may be occurred even when only 2–3 colonies have grown on the agar plate. (Fig. 2). The shortest time for visual observation of *T. thiooxidans* cell growth was after 3 incubation days.

Additionally, optimal conditions (BCG concentration and pH) were also determined for the modified *Thiobacillus* agar. The value of the initial pH of a standard *Thiobacillus* agar is ~3.63. At this pH addition of 2 ml of BCG (0.4% w/v) resulted in a yellow–green color

Table 2

<table>
<thead>
<tr>
<th>Growth medium</th>
<th>*T. thiooxidans [CFU/100 ml]</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Thiobacillus</em> agar (control)</td>
<td>8.5 x 10^6 ± 0.12 x 10^6</td>
</tr>
<tr>
<td><em>Thiobacillus</em> agar + 1 ml PhR</td>
<td>n.d.*</td>
</tr>
<tr>
<td><em>Thiobacillus</em> agar + 2 ml PhR</td>
<td>n.d.*</td>
</tr>
<tr>
<td><em>Thiobacillus</em> agar + 1 ml BCG</td>
<td>3.0 x 10^7 ± 0.3 x 10^6</td>
</tr>
<tr>
<td><em>Thiobacillus</em> agar + 2 ml BCG</td>
<td>1.5 x 10^7 ± 0.8 x 10^6</td>
</tr>
<tr>
<td><em>Thiobacillus</em> broth medium</td>
<td>1.0 x 10^7 ± 0.6 x 10^6</td>
</tr>
</tbody>
</table>

* n.d. — not detected.
* Isolation and enumeration of *T. thiooxidans* by MPN method.
* X ± S.D. (average ± standard deviation) of three plates.
development in the medium, which is very close to BCG isosbestic point. Shifting the medium pH up to more basic conditions (from 3.8 to 5.0) a more intensive blue color was obtained, resulting in a better identification of the developing colonies. Performing a continuous set of experiments, it was found that successful isolation and identification of *T. thiooxidans* isolates were achievable at a medium pH interval from 4.0 to 5.0 and at 1 to 10 ml of BCG (0.4% water solution) as supplement. At higher BCG concentrations and pH values>5.0, bacterial growth was retarded (data not shown). These experiments showed that optimal conditions to obtain the highest bacterial count were pH 4.0–4.2 and 2 ml/l BCG supplement (0.4%). From the laboratory point of view, it is important to mention that BCG solution (0.4%) may be added to medium both before autoclave or after (following filtration).

Following the optimization experiments, a comparison between the MPN and Spread plate methods with standard *Thiobacillus* broth and the modified BCG medium, was performed. The results of this comparison are shown in Table 3 with pure culture of *T. thiooxidans* ATCC 8085 and with environmental isolates from the BTF reactor (Mekorot Water Co., Israel). According to Table 3 results, bacterial cell count was in the same order of magnitude revealing no significant difference between the three methods.

*Thiobacillus* agar procedure used in the investigation is recommended for the isolation of different *Thiobacillus* species (*Atlas, 2005a, 2005b*). In this connection the culture of *T. ferrooxidans* was used in order to determine the possibility of using both standard and modified *Thiobacillus* agar for the isolation of other species of *Thiobacillus* genera. Unfortunately no growth of *T. ferrooxidans* was observed on both tested agars.

### 4. Conclusions

Comparison of MPN and Spread Plate method (with and without BCG) revealed equal results. BCG did not affect bacterial growth but facilitated observation and monitoring process along the 5 days of incubation, starting on the 3rd day. It should be noted that the standard *Thiobacillus* agar procedure, generally recommended for isolation of *Thiobacillus* species, did not showed growth of *T. ferrooxidans* on both media (Standard and BCG supplemented) when inoculated with this species.

In conclusion, the present study showed that supplementation of *Thiobacillus* agar with BCG at pH 4.0–4.5 simplifies and accelerates the enumeration of *T. thiooxidans* spp. from environmental water samples and can be used as a presence/absence test, a feature very much needed for field examination.

### Acknowledgments

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### References


