Journal of Microbiological Methods 92 (2013) 178–182

Contents lists available at SciVerse ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

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

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ARTICLE INFO

Article history:
Received 14 November 2012
Accepted 14 November 2012
Available online 23 November 2012

Keywords:
Acidithiobacillus thiooxidans
Thiobacillus agar
Bromocresol green
Phenol red
Spread Plate method
MPN technique

ABSTRACT

High concentrations of H_2S 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 *T. thiooxidan* 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 H_2S (~20 ppm) is used as a water supply source for drinking water. H_2S removal process combines an air degasifying tower releasing around 600 ppm H_2S/m^2 conveyed to a cooling tower. The processed air is conveyed to a BTF through several polyurethan foam layers as media for sulfate oxidizing bacteria (SOB) (mainly *Acidithiobacillus thiooxidans* syn. *Thiobacillus thiooxidans*). H_2S emissions are controlled and regulated by the ministry of environmental protection and according to current regulation for ambient air a daily average of <5 ppm/m^2 is demanded. As much as 98% of the produced H_2S 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 *T. thiooxidan* cells, in order to support rapid and effective operational data of the BTF. Thiobacilliaceae, effectively oxidize reduced forms of sulfur (H_2S, metal sulfides, thiosulates and elemental sulfur — S^0) in order to obtain energy and fix atmospheric CO_2 for growth requirements (Ehrlich, 1996). Typical members of this group are *T. thiooxidans*, *Thiobacillus albertis*, *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 dynamic 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 *T. thiooxidan* 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

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0167-7012/$ – see front matter. Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.mimet.2012.11.009

presently new solid media formulations have been developed (Atlas, 2005a; Ramirez et al., 2009). For example, Spread Plate method was applied for *T. thiooxidan* 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 *Thiobacillus* spp. isolation were selected and compared to a modified medium (comprising bromcresol green — BCG as a pH indicator) to facilitate environmental monitoring of *Thiobacillus* 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. thiooxidan 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 Testing 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 — $(NH_4)_2SO_4$, 0.5 g — $MgSO_4.7H_2O$, 0.25 g — $CaCl_2$, 3 g — KH_2PO_4, 0.005 g — $FeSO_4$, 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 — $(NH_4)_2SO_4$, 0.5 g — $MgSO_4.7H_2O$, 0.25 g — $CaCl_2$, 4 g — KH_2PO_4, 0.01 g — $FeSO_4$, 5 g — $Na_2S_2O_3$, 12.5 g — agar, 1 l — distilled water; autoclaved at 121 °C for 15 min) and *Thiobacillus acidophilus* agar (3 g –$(NH_4)_2SO_4$, 1 g — $MgSO_4.7H_2O$, 0.1 g — KCl, 0.5 g — KH_2PO_4, 0.01 g — $FeSO_4$, 18 mg — $Ca(NO_3)_2$. $4H_2O$, 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 bromcresol 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^{-7} 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} $(NH_4)_2$, 0.4 g l^{-1} $MgSO_{40}\cdot 7H2O$, 0.1 g l^{-1} K_2HPO_4, and 12 g l^{-1} $FeSO_4\cdot 7H_2O$ (48). Initial pH of the growth medium was adjusted to 1.7 by the addition of 2 N H_2SO_4.

3. Results and discussion

Isolation and enumeration of *T. thiooxidan* 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. thiooxidan* 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 (Anonymus, 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. thiooxidan* 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 1
Enumeration of environmental *T. thiooxidans* bacteria from water samples (BTF of Mekorot Water Company) on two media.

Media		
Sample number	*Thiobacillus acidophilus* agar [CFU/100 ml]	*Thiobacillus* agar [CFU/100 ml]
1	n.d.[a]	<10
2	n.d.	$3.1\times10^3\pm0.02\times10^3$
3	n.d.	<10
4	n.d.	n.d.
5	n.d.	$1.5\times10^7\pm0.02\times10^7$
6	n.d.	$2.1\times10^3\pm0.05.4\times10^3$
7	n.d.	<10
8	n.d.	$1.2\times10^4\pm0.03\times10^4$
9	n.d.	$9.0\times10^7\pm0.04\times10^7$
10	n.d.	$4.0\times10^7\pm0.07\times10^7$
11	n.d.	$2.4\times10^7\pm0.08\times10^7$
12	n.d.	$2.0\times10^4\pm0.03\times10^4$
13[b]	n.d.	$3.3\times10^6\pm0.06\times10^6$

[a] n.d. — not detected.
[b] Bacterial suspension washed off sponge filter.