

Appendix U

DETECTION OF *ACTINOMYCETES* IN WATER

Overview:

The following is a method for isolation of Actinomycetes from water. The plating method used is the double agar layer (DAL) method, adopted from APHA, (2000).

Media and reagents:

Cycloheximide

- Antifungal antibiotic solution
 - 1 mg cycloheximide (Sigma, St. Louis, MO, C7698-1G) into 1 mL reagent water
 - Autoclave for 15 minutes
 - *Store at 4 °C for up to six months*

ISP Medium 1 (ISP#1) broth

- Used for reviving freeze-dried *Streptomyces albus* and growing the organism for plating the positive controls
- Make according to the directions on the bottle.
 - 8 g ISP#1 (VWR, Pittsburgh, PA, DF0769-17) into 1 L reagent water
 - Heat to dissolve
 - Autoclave for 15 minutes
 - *Store at 4 °C for up to 6 months*

ISP Medium 2 (ISP#2) agar

- Used for obtaining pure colonies of *S. albus* from the broth culture
- Make according to the directions on the bottle
 - 38 g ISP#2 (VWR, Pittsburgh, PA, DF0770-17) into 1 L reagent water
 - Heat to dissolve
 - Autoclave for 15 min
 - Agar can be stored in dilution bottles for up to 6 months in the refrigerator
- Preparing plates
 - Molten, tempered ISP#2 agar is prepared by autoclaving a 100-mL dilution bottle briefly, then tempering in a 48°C water bath
 - Swirl until well mixed and dispense 17-20 mL per 100-mm plate
 - *Plates can be stored inverted at 4 °C for up to 2 weeks*

Actinomycete Isolation Agar (AIA)

- Used for making top agar and bottom agar plates for the samples and controls
- Make according to the directions on the bottle
 - 22 g AIA (VWR, Pittsburgh, PA, DF0957-17) into 1 L reagent water

- Heat to boiling to dissolve completely
- Add 5 g Glycerol and mix well
- Dispense 100 mL into dilution bottles
- Autoclave for 15 minutes
- *Agar can be stored in dilution bottles for up to 6 months in the refrigerator.*

Sample analysis:

- For each sample, four plates are included - two duplicate plates for each dilution, undiluted and 10^{-2} , using the DAL method.

Double agar layer method

1. For the water sample to be tested, make dilutions as followed:
 - Undiluted: Aliquot from sample bottle
 - 10^{-2} : 1 mL of undiluted sample into 99 mL MI buffer

2. Make enough AIA for bottom agar plates and top agar (approximately 37 mL per plate, or about 230 mL total for four plates and a positive and negative control).
 - Bottom agar is melted from the dilution bottles containing AIA.
 - Melt a bottle of agar in the autoclave for 2 to 3 minutes.
 - Before pouring plates, let agar cool in 48°C water bath.
 - Pour plates, about 17 to 20 mL per plate, and set aside to allow the agar to harden.
 - If poured plates were stored in the fridge, pre-warm them in the 36°C incubator before plating the samples.

 - Top agar is melted the day the samples are run.
 - The agar is put into a 50°C water bath to cool.
 - *Do not allow liquefied top agar to solidify before plating.*

2. Add 17 ml top agar into a sterile 50- mL sterile centrifuge tube.
3. Add 2 ml of undiluted sample.
4. Add 1 ml cycloheximide.
5. Gently mix.
6. Pipet 5 ml over a bottom agar plate. In order to spread mixture evenly before the agar hardens, swirl the plate while dispensing the mixture.
7. Incubate at 28°C until no new colonies appear (usually 6 to 7 days).
8. Repeat steps 2 through 6 for each sample dilution and duplicate.

Counting colonies:

- Ideal count is between 30-300 colonies.
- Identify actinomycetes by gross colony appearance. If necessary, verify by microscopic examination at a magnification of 50 to 100x.
- Characteristics to look for in an actinomycetes colony when compared to a nontarget typical colony:
 - Actinomycetes: An earthy, soil smell, opaque, sometimes chalky appearance in mature colonies, darker in the center and lighter farther from the center, irregular, fuzzy edge, of hyphal appearance, strong adherence to medium, and strong and leathery texture.
 - Typical nontarget colony: Shiny appearance, regular, smooth edge, uniform look throughout colony, weak adherence to medium, and soft texture.

Quality control:

Positive control

- A positive control is plated every time samples are analyzed.
- A dilution is made so it can produce a countable range of colonies. A count of 30 to 300 colonies on a plate is suitable.
 - 10^{-2} dilution: 1 mL broth containing *S. albus* into 99 mL MI buffer. (*Use S. albus that has been grown in ISP#1 broth. Make sure it is not more than two weeks old.*)
 - Plate the dilution following the directions for the DAL method. Add 2 mL of the 10^{-2} dilution in place of the sample.

Negative control

- A negative control is plated every time samples are analyzed.
- A negative control is plated to ensure the media is not contaminated.
- Follow the directions for the DAL method, except add 2 mL of sterile water instead of sample.

Maintaining organism

- *Streptomyces albus* is stored at 4°C on an ISP#2 slant.
- Transfer the organism to a new ISP#2 slant and every two months.

Preparing organism- Rehydration

- Done only when a new ATCC strain is ordered.
- Add 5-6 mL of ISP#1 broth to a test tube.
- Transfer 0.3 to 0.4 ml broth to vial containing *Streptomyces albus* (ATCC, Manassas, Virginia) pellet.
- Mix thoroughly.

- Transfer mixture back to test tube containing broth.
- With a loopful of the broth containing *S. albus*, inoculate a second tube containing broth, and using the 3-phase streak technique, a plate with ISP#2 agar as a medium, and another plate with AIA as a medium.
- Incubate tubes and plates at 26°C for 48 hours.

- If it grows on both plates, transfer a colony from the broth containing *S. albus*, to a slant with ISP#2 agar as a medium for storage.
- Store slant at 4°C.
- If the organism grows on the slant, the original ISP#1 broth containing *S. albus* can be autoclaved and discarded.

Documentation

- Records of the date and results of all QC samples and environmental samples must be maintained.
- In addition, records of media sterility and a stock control log must be maintained in a separate log book.

Reference: American Public Health Association, American Water Works Association, and Water Environment Federation, 1998, Standard methods for the examination of water and wastewater (20th ed.): American Public Health Association, part 9250, p. 9-88 to 9-90.

ODML # _____

Actinomycetes Results Sheet

Sample Date _____

Time _____

Station _____

ID# _____

District _____

Contact _____

Bench Calculations

Date tested _____

Date plates out of incubator _____

Date read results _____

Double agar layer (DAL)

Undilute

10⁻² dilution

Plate 1 _____ colonies/ 0.5 ml

Plate 1 _____ colonies/ 0.005 ml

Plate 2 _____ colonies/ 0.5 ml

Plate 2 _____ colonies/ 0.005 ml

Positive control

10⁻² dilution _____ colonies/ 0.005 ml

Negative control _____ colonies/ 0.5 ml

Results

Sample DAL

Undilute

Average _____ colonies/ mL

Plate 1 _____ colonies/ ml

Plate 2 _____ colonies/ ml

(+) and (-) controls _____

10⁻² dilution

Plate 1 _____ colonies/ ml

Date results sent _____

Plate 2 _____ colonies/ ml

Circle one: Phone E-mail

