Supplement to MFLP-44
September 1999

Supplement to the Method MFLP-44

1. Application

The following information is offered as a supplement to the method, MFLP-44, dated April 1998, and should be used with this method. The purpose of the attached is to provide additional information (such as: source(s) of materials, critical steps, confirmation steps, or helpful hints etc.).

This information will become incorporated into the method when it has undergone review and editing (following Health Canada policy).

5. Materials and Special Equipment

Cooked Meat Media (CMM) (see MFHPB-16)
Tripticase (BBL) or Special Peptone (Oxoid)
Peptone (Difco)
McClung Toabe /Egg yolk/Yeast Extract agar plates ((MTEYE) (or *C. botulinum* Isolation Medium see MFHPB-16)
SM Media
Fortified Tryptone Glucose Extract Agar (FTGE).

8. Verification of the Method MFLP-44

The following methods may be used to obtain aerobic and anaerobic spore suspensions, which can be used to verify the ability of this method to enumerate both aerobic and anaerobic sporeformers.

8.1. Aerobic Sporeformers

A spore suspension of *B. subtilis* can be prepared as follows:

Use an ATCC strain or equivalent of *B. subtilis*. An 18 - 24 h culture of *B. subtilis* grown in nutrient broth in a water bath shaker at 37°C is used to inoculate Fortified Tryptone Glucose Extract Agar (FTGE). FTGE is incubated at 37°C for 48 h. Collect surface growth in sterile distilled water (~20 mL). The spore suspension is cleaned by repeated washing and differential centrifugation starting at 1,500 g for 20 min. and increasing the speed by 500 g up to 10,500 g. Before each centrifugation, pellets are observed for stratification. Strata is separated into individual centrifuge bottles and any stratum containing predominantly vegetative cells (as detected by phase contrast microscopy) is discarded. The final suspension should be made up a pellet showing a homogenous stratum and a high percentage of refractile spores. Final pellets are stored in distilled water at 4°C until needed.

Alternately, spores can be frozen @-80°C.

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8.2 Anaerobic Sporeformers

A spore suspension of *C. sporogene*s can be prepared as follows:

Use an ATCC strain or equivalent of *C. sporogenes.* Inoculate 20 ml of deaerated Cooked Meat Media (CMM) medium with 0.1 ml of thawed *C. sporogenes* . Heat shock for 10 min. @ 100°C or 20 min. @ 80°C; cool and incubate anaerobically @ 35°C overnight or for 2 days @ 30°C. Inoculate 200 ml of deaerated SM medium with CMM culture and incubate anaerobically for 8 hr. @ 30°C. Inoculate 1800 ml of deaerated SM medium (2 L flask) with 200 ml of 8 hr. culture and incubate anaerobically for 4 days @ 30°C. Harvest when maximum spore yield is obtained; stir culture before taking sample. Growth should be good (visibly) and spore production should be ~90% when observed under phase contrast. Centrifuge the culture @ 10,000 rpm for 10 min. and then wash the spores 3X with sterile cold distilled water (~20 ml) centrifuging @ 7,000 rpm for 10 min. between each wash. Resuspend the washed spores in ~5 ml of sterile distilled water and dipense in small volumes (0.1 ml) to sterile microcentrifuge tubes.

Freeze spore suspensions @-80°C.

Check spore suspension concentration by preparing dilutions and spread-plating 0.1 ml aliquots on McClung Toabe /Egg yolk/Yeast Extract agar plates ((MTEYE) (5g/L of Yeast extract)) in duplicate. Dilutions should be heat shocked @ 75°C for 20 min. before aliquots are spread on MTEYE agar. Incubate anaerobically as in MFLP-44 (or MFHPB-16).

*C. sporogene*s colonies should appear within 48 h and should have a shiny zone (lipase reaction) around each colony.

8.3 Media

8.3.1. Fortified Tryptone Glucose Extract Agar (FTGE).

beef extract	3.0 g/L
tryptone	5.0 g/L
glucose	1.0 g/L
agar	15g/L

50 mL solution of

$MnSO_4 \cdot 1H_2O$	1.0 g/L
$ZnSO_4 \cdot 7 H_2O$	0.1 g/L
$FeSO_4 \cdot 5 H_2O$	0.1 g/L

distilled water 950 mL

Adjust pH to 7.0
Autoclave, temper and then dispense into sterile petri dishes.

8.3.2. SM Medium

5% Tripticase or Special Peptone	50g/L
1% Peptone	10g/L

Dissolve in hot, distilled water, cool and adjust pH 7.2, autoclave.

After autoclaving and cooling to 50°C add 1 ml of a 10% Na-thioglycolate filter-sterilized solution to 100 ml of medium.