COLIFORM GROUP OF BACTERIA (MOST PROBABLE NUMBER METHOD)

PRINCIPLE

Coliform bacteria are estimated by probability statistics after determining the presence of gas production in a selective media.

SCOPE

The method is applicable to starches, syrups and sugars, and most coproducts of the corn wet milling industry. However, if greater sensitivity is required, or low counts are expected in sugars and syrups, use of the membrane filter method is recommended.

SPECIAL APPARATUS

1. Long wavelength UV lamp
2. Test tubes containing gas collector tubes (Durham Tubes)

MEDIA AND REAGENTS

1. Brilliant green lactose bile broth (BGLB), 2%
2. Lauryl sulfate tryptose broth (LST)
3. EC broth
4. Levine eosin methylene blue agar (EMB)
5. Tryptone broth
6. MR-VP broth
7. Koser's citrate broth
8. Plate Count Agar (PCA)

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9. Butterfield's Phosphate Diluent:

   Stock Solution: Dissolve 34 g of potassium dihydrogen phosphate (KH_2PO_4) in 500 mL of purified water, adjust to pH 7.2 with about 175 mL of 1*N* NaOH solution and dilute to 1 L volume. Store under refrigeration.

   Diluent: Dilute 1.25 mL of stock solution to 1 L volume with purified water. Prepare dilution blanks using this solution.

10. Dilution Blanks

    Fill dilution bottles to 90 mL or desired volume with Butterfield's Phosphate Diluent (Note 2).

11. Lauryl sulfate tryptose with MUG (LST-MUG)

    Prepare the above media according to the manufacturer's directions. Cap bottles/tubes and sterilize at 121°C for 15 minutes in the autoclave.

12. Kovac's reagent:

p-Dimethylaminobenzaldehyde	5 g
n-amyl or isoamyl alcohol	75 mL
Hydrochloric acid, conc.	25 mL

    Dissolve the dimethylaminobenzaldehyde in the amyl alcohol, then add the hydrochloric acid. Test on a known sample of indole.

13. Voges-Proskauer reagents:

Vp-1: Alpha-naphthol	5 g
Absolute alcohol	100 mL

    This must be used the same day as prepared.

VP-2: Potassium hydroxide	40 g

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Distilled water	100 mL

14. Gram stain reagents

15. Methyl red solution:

Methyl red	0.1 g
Ethyl alcohol	300 mL
Distilled water	200 mL

16. Sodium Hydroxide Solution (NaOH), 1 *N*

PROCEDURE

A. Quantitative Procedure for Total Coliforms

Using separate sterile pipets, prepare serial decimal dilutions from the 1:10 primary dilution blank (PBD). The number of dilutions depends on individual samples and may be determined by past experience. Shake all dilutions thoroughly. Transfer 1 mL portions from each dilution to 3 LST tubes containing gas collector tubes for 3 consecutive dilutions. Three serial dilutions of 1:10, 1:100 and 1:1000 are commonly used for low numbers of coliforms.

In the case of pregelatinized starches, a MPN sequence may be prepared as follows:

Aseptically weigh 1 g samples of starch and transfer to 3 bottles of 50 mL LST broth. Prepare a 1:50 dilution blank and transfer two serial aliquots (5 and 0.5 mL), corresponding to 0.1 and 0.01 g of starch, to 2 sets of 3 bottles of LST broth. The dilution sequence is 1 g, 0.1 g and 0.01 g per 50 mL of LST broth.

Incubate the tubes at 35-37°C and examine tubes at 24 an 48 hours for gas production. Perform the confirmed test for coliforms on all positive tubes using BGLB broth as described below. Calculate MPN of coliforms based on proportion of confirmed gassing LST tubes for 3 consecutive dilutions. (see attached table).

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B. Coliform Confirmation

Gently agitate each gassing LST tube and transfer a loopful of suspension to tube of BGLB broth containing a gas collector tube. Hold LST tube at angle and insert loop to avoid transfer of pellicle, if present. Incubate BGLB tubes at 35-37°C and examine tubes for gas production at 24 and 48 hours. All gassing tubes are considered to be positive for the presence of coliforms.

C. *E. coli* Confirmation (Note 2)

Gently agitate each gassing LST tube and transfer a loopful of suspension to tube of EC broth. Incubate EC tubes at 45.5°C and examine tubes for gas production at 24 and 48 hours. Streak a loopful of suspension from each gassing tube to EMB plate. Incubate the plates at 35-37°C and examine for suspicious *E. coli* colonies, i.e., dark centered with or without metallic sheen. Pick 2 suspicious colonies from each EMB plate and transfer them to PCA agar slants for morphological and biochemical tests. Incubate PCA slants at 35-37°C for 18-24 hours. If typical colonies are not present, pick 2 colonies most likely to be *E. coli* from each plate.

Perform Gram stain and examine all cultures appearing as Gram-negative short rods or cocco-rods for the following IMViC test (Indole, Methyl Red, Voges-Proskauer, Citrate):

1. Indole test: Inoculate tubes of tryptone broth and incubate at 35-37°C for 22-36 hours. Add 0.2-0.3 mL of Kovac's reagent. A positive test for indole is indicated by the appearance of a deep-red color within 5 minutes in the upper layer.

2. Voges-Proskauer test: Inoculate tubes of 5 mL MR-VP broth and incubate at 35-37°C for 46-50 hours. Transfer 1 mL to 13 x 100 mm tube. Add 0.6 mL of VP-1 solution and 0.2 mL of 40% KOH (VP-2) and shake. Add a few crystal of creatine. Shake and let stand 2 hours. Test is positive if eosin pink color develops, indicating the presence of acetylmethylcarbinol.

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3. Methyl red test: Incubate MR-VP tubes for additional 46-50 hours at 35-37°C after Voges-Proskauer test. Add 5 drops methyl red solution to each tube. A distinct red color indicates the presence of acid and is recorded as positive. Yellow is a negative reaction.

4. Use of citrate: Lightly inoculate tubes of Koser Citrate broth; avoid detectable turbidity. Incubate 94-98 hours at 35-37°C. Development of distinct turbidity is positive reaction.

5. Production of gas from lactose: Inoculate tubes of LST broth and incubate 46-50 hours at 35-37°C. Production of gas is positive reaction.

6. Interpretation: All cultures that (a) ferment lactose with production of gas within 48 hours at 35°C, (b) appear as Gram-negative non-sporeforming rods or cocci, and (c) give IMViC patterns ++-- (biotype 1) or -+-- (biotype 2) are considered to be *E. coli*.

CALCULATION

MPN is derived from the attached table.

NOTES AND PRECAUTIONS

1. When sterilizing dilution blanks, a portion of the diluent may be lost. When this occurs, the sterilized blanks are brought to the proper volume with the sterile diluent. This instruction applies to all methods.

2. The presence of *E. coli* in gassing LST tubes may be detected by the MUG reagent incorporated in LST broth. Gently agitate each gassing LST tube and transfer a loopful of suspension to tube of LST-MUG broth. Incubate 24 hours at 35-37°C and examine LST-MUG tubes for gas production and fluorescence under long wave UV light. All positive tubes indicate the presence of *E. coli*.

   The MUG test is based on the detection of beta glucuronidase, which is present in most *E. coli* (97%). It is also present in some *Salmonella*,

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Shigella and *Yersinia*. However, these are non-lactose fermenting microorganisms, which can easily be differentiated from *E. coli*.

Since the MUG test is not an official method, it should not be used for analysis of regulatory samples. The conventional method should be used for such samples.

Sterile LST broth with MUG is also available commercially. These ready-to-use tubes are equally reliable for the presumptive test for *E. coli*.

REFERENCE

Compendium of Methods for the Microbiological Examination of Foods, Current Edition, American Public Health Association.