13 Microbiological Methods

Coliform – *E. coli*

(American Association of Cereal Chemists – AACC-Method 42-15)
First approved 10-8-76; reviewed 10-27-82

1 Scope

This Standard specifies a method for the enumeration of coliforms present by means of the colony count techniques at 35°C. The method is applicable to all cereals and other food products.

2 Apparatus

2.1 Sterile sampling devices suitable for product; scoop, trier, etc.

2.2 Sterile sample jars or containers with suitable covers or sterile plastic bags .

2.3 Autoclave for sterilising app and media.

2.4 Balance; sensitivity 30 mg, with wts.

2.5 Waring Blendor and blending jars of 1000 ml capacity with covers.

2.6 Sterile serological pipettes capable of delivering 1.0, 10.0, and 11.0 ml.

2.7 Sterile cans for pipettes.

2.8 Sterile petri dishes, 100 x 15 mm, preferably disposable plastic.

2.9 Diln bottles with screw caps with capacity of 150-200 ml.

2.10 Sterile weighing papers. Sterilize for minimum of 1 hr at 121°C in suitable container.

2.11 Holding bath adjusted to 45-48°C for tempering agar.

2.12 Bunsen burner.

2.13 Spencer Quebec colony counter or equiv.

2.14 Sterile culture tubes with suitable covers and racks.

2.15 Incubator adjusted to 35 ± 1.

2.16 Covered water bath adjusted to 45.5 ± 0.05°C.

2.17 Inoculating needles and loops made of platinum or Ni chrome steel.

3 Reagents

3.1 *Lauryl sulfate tryptose (LST) broth:* Dissolve 20.0 g trypticase or tryptose (pancreatic digest of casein), 5.0 g NaCl, 5.0 g lactose, 2.75 g K_2HPO_4, 2.7 g KH_2PO_4, and 0.1 g Na lauryl sulphate in 1 L water with gentle heat if necessary. Dispense 10 ml portions in 16 x 150 mm culture tubes contg 6 x 50 mm inverted fermentation tubes. Autoclave 15 min at 121°C. Final pH, 6.8 ± 0.1.

3.2 *Brilliant green lactose bile (BGLB) broth*: Dissolve 10.0 g peptone and 10.0 g lactose in ca 500 ml water. Add soln (pH 7.0-7.5) of 20 g dehydrated oxgall or oxbile in 200 ml water. Dil to 975 ml and adjust pH to 7.4. Add 13.3 ml 0.1 % soln of brilliant green and dil to 1 L with water. Filter through cotton and dispense 10 ml portions into 20 x 150 mm test tubes contg inverted 10 x 75 mm fermentation tubes. Autoclave 15 min at 121°C . Final pH. 7.2 ± 0.1.

3.3 *E. C. broth:* Dissolve 20.0 g trypticase or tryptose (pancreatic digest of casein), l.5 g Bacto bile salt No.3 or bile salt mixt, 5.0 g lactose,4.0 g K_2HPO_4, 1.5 g KH_2PO_4, and 5 g NaCl in 1 L water. Dispense 15 ml into 16 x 150 mm culture tubes contg inverted 6 x 50 mm fermentation tube. Autoclave 15 min at 121°C. Final p H, 6.9 ± 0.1.

3.4 *Eosin methylene blue (EMB) agar (Levine):* Dissolve 10.0 g peptone, 2.0 g K_2HPO_4 and 20.0 g agar in 1 L water. Boil to dissolve and add water to original vol. Dispense in 100 or 200 ml portions and autoclave 15 min at 121°C. Final pH, 7.1 ± 0.1. Before use, melt and to each 100 ml add 5 ml sterile 20% lactose soln, 2.0 ml 2 % aq Eosin Y soln, and 1.3 ml 0.5 % aq methylene blue soln.

3.5 *Tryptophan broth:* Dissolve by heating, with stirring, 10.0 g tryptone or trypticase in 1 L water. Dispense 5 ml portions into culture tubes and autoclave 15 min at 121°C.

3.6 *Buffered glucose broth (MR-VP medium):* Dissolve 5.0 g proteose peptone, 5.0 g glucose, and 5.0 g K_2HPO_4 in ca 800 ml water with gentle heat and occasional stirring. Filter, cool to 20°C and dil to 1 L. Dispense 10 ml portions into culture tubes and autoclave 12-15 min at 121°C. Maximum exposure to heat should be ≤30 min. Final pH, 6.9 ± 0.1.

3.7 *Koser's citrate broth:* Dissolve 1.5 g $NaNH_4HPO_4 \bullet H_2O$, 1.0 g K_2HPO_4, 0.2 g $MgSO_4 \bullet 7H_2O$, and 3.0 g Na citrate $2H_2O$ in 1 L water. Dispense in 10 ml portions in culture tubes and autoclave 15 min at 121°C. Final pH , 6.7 ± 0.1.

Stock soln. Dissolve 34.0 g KH_2PO_4 in 500 ml water, adjust to pH 7.2 with ca 175 ml 1 N NaOH, and dil to 1 L. Store in refrigerator.

b. Diluent. Dil 1.25 ml stock soln to 1 L with water. Prep diln blanks with this soln, dispensing enough to allow for losses during autoclaving. Autoclave 20 min at 121°C.

3.9 *Plate count agar (tryptone glucose yeast agar):* Suspend 5.0 g peptone tryptone (pancreatic digest of casein), 2.5 g yeast ext, l.0 g glucose, and 15.0 g agar in 1 L water. Heat and boil until all ingredients are dissolved. Autoclave 15 min at 121°C. Final pH, 7.0 ± 0.1.

3.10 *Kovac's reagent:* Dissolve 5.0 g p-dimethylamino-benzaldehyde in 75 ml amyl alc and slowly add 25ÿml HCI.

3.1l *α-Naphthol soln, 5 %:* Dissolve 5.0 g α-naphthol in 100 ml absolute alc.

3.12 *KOH soln, 40 %:* Dissolve 40 g KOH in water and dil to 100 ml.

3.13 *Methyl red indicator:* Dissolve 0.10 g methyl red in 300 ml 95 % alc and dil to 500 ml with water.

Note

For convenience, dehydrated media of any brand equiv to formulation may be used. Test each lot of medium for sterility and growth-promoting qualities, of suitable organism (eg, inoculate media contg lactose with coliform bacteria, Staphylococcus media with Staphylococcus, etc.).

Det pH before autoclaving with pH meter stdzd against std buffers. Adjust pH, when necessary, by adding 1 N NaOH or 1 N HCl so that stated final pH results after autoclaving.

5 Procedure

5.1 Use sample taken and prepared as directed in "Microbiological examination".

Note: Steps in the original method describing the preparation of sample for microbiological examination are deleted.

5.2 Seed 3-tube most probable number (MPN) series into LST broth (reagent 3.1) using 1 ml inoculum of 1:10,1:100, and 1:1000 dilns with triplicate tubes at each diln. Incubate 48 ± 2 hr at 35°C for gas formation as evidenced by displacement of liquid in insert tube or by vigorous effervescence when tubes are shaken gently. Examine tubes for gas formation at 24 and 48 hr intervals. Transfer, using 3 mm loop, from gassing tubes to BGLB broth (reagent 3.2) and E. C. broth (reagent 3.3) at time gas formation is noted.

5.3 Incubate BGLB broth 48 ± 2 hr at 35°C . Using MPN table, compute MPN on basis of no. of tubes of BGLB broth producing gas by end of incubation period. Report as MPN of coliform bacteria/g.

5.4 Incubate E. C. broth 48 ± 2 hr at 45.5 ± 0.05°C in covered water bath. Submerge broth tubes in bath so that water level is above highest level in medium. Examine for gas at 24 or 48 hr intervals. Streak gas positive tubes on Levine's EMB agar plates (reagent 3.4) and incubate plates 24 ± 2 hr at 35°C.

5.5 Pick two or more well-isolated typical colonies from Levine's EMB agar plates and transfer to agar slants prepd from plate count agar (reagent 3.9). Incubate 18-24 hr at 35°C. If typical colonies are not present, pick two or more colonies most likely to be E. coli. Pick ≥2 from every plate. Transfer growth from plate count agar slants into biochemical test broths.

5.6 Tryptophan test broth (reagent 3.5). Incubate 24 ± 2 hr at 35ÿoC and test for indole by adding 0.2-0.3 ml Kovac's reagent (reagent 3.10) to 24 hr culture. Test is positive if upper layer turns red.

5.7 MR-VP medium (reagent 3.6). Incubate 48 ± 2 hr at 35°C. Aseptically transfer 0.7 ml culture to porcelain spot plate to test for acetylmethylcarbinol. Add 0.1 ml 5% alc α-naphthol soln (reagent 3.11), 0.1 ml KOH soln (reagent 3.12), and few crystals of creatine. Let stand 2 hr. Test is positive if erosin pink develops.

Alternatively, aseptically transfer 1 ml of 48 hr culture to culture tube and add 0.6 ml α-naphthol soln (reagent 3.11) and 0.2 ml 40% KOH soln (reagent 3.12). Shake after each addn. To intensify and speed reaction, add few creatine crystals to test medium. Read results 4 hr after adding reagents. Positive VP test is development of eosin pink colour.

Incubate remainder of MR-VP medium for addnl 48 hr and test for methyl red reaction by adding 5 drops methyl red soln (reagent 3.13) to culture. Test is positive if culture turns red; negative if yellow.

5.8 Koser's citrate broth (reagent 3.7): Incubate 96 hr at 35°C and record growth as + or -.

5.12 LST broth (reagent 3.1): Incubate 48 ± 2 hr at 35°C. Examine tubes for gas formation.

5.9 Gram stain: Perform Gram stain on 18 hr agar slant (Standard Methods for the Examination of Water and Waste Waters, 13th ed., 1971). Coliform organisms will stain red (negative). Gram-positive organisms will stain blue-black.

5.10 Classification: Classify biochemical types as follows:

Indole	MR	VP	Citrate	Type
+	+	—	—	Typical *E. coli*
—	+	—	—	Atypical *E. coli*
+	+	—	+	Typical Intermediate
—	+	—	+	Atypical Intermediate
—	—	+	+	Typical A. *aerogenes*
+	—	+	+	Atypical A. *aerogenes*

Other groupings may appear; in such cases, cultures are usually mixed. Restreak to det their purity.

Compute MPN of E. coli/g, considering Gram negative, non spore-forming rods producing gas in lactose and producing + + - - or - + - - IMViC patterns as E. coli.

Reference

Association of Official Analytical Chemists. 1980. Official Methods of Analysis, 13th ed. Sec. 46.016, pp. 825-826.