13 Microbiological Methods

JECFA Tests for Carob Bean Gum

The following microbiological tests are part of the JECFA Specification 1999

Total plate count

Using aseptic technique, disperse 1 g of sample into 99 ml of phosphate buffer and use a Stomacher, shaker or stirrer to fully dissolve. Limit dissolving time to about 10 min and then pipette 1 ml of the solution into separate, duplicate, appropriately marked petri dishes. Pour over the aliquot of sample in each petri dish 12-15 ml of Plate Count Agar previously tempered to 44-46°. Mix well by alternate rotation and back and forth motion of the plates, allow the agar to solidify. Invert the plates and incubate for 48±2 h at 35±1°.

After incubation count the growing colonies visible on each plate and record the number of colonies. Take the average of both plates, and multiply by the sample dilution factor, 100. Where no colonies are visible, express the result as less than 100 CFU/g.

E. coli determination:

The use of cellulase to degrade the gum sample prior to analysis is essential in order to avoid gelling of the gum during its addition to the enrichment broth. Prepare a 1.0% cellulase solution (1 g cellulase to 99 ml water) and sterilize by filtration through a 0.45 µm membrane. (Cellulase solution may be stored at 2-5° for up to two weeks.) Into a sterile tube containing 9 ml of sterile lauryl sulfate tryptose (LST) broth, aseptically add 0.1 ml of the sterile 1% cellulase solution. Add 1g gum sample to the tube and vortex vigorously to disperse the sample. Incubate the tube for 24-48 h at 35±1°. After 24 h, gently agitate the tube and examine for gas production, i.e., effervescence. Reincubate for an additional 24 hours if no gas evolution is observed. Examine a second time for gas. Perform the following confirmation test on the presumptive positive (gassing) result.

Gently agitate the gassing LST tube and transfer one loopful of the suspension to a tube containing 10 ml of EC broth and a fermentation (Durham) vial. Incubate the EC tube for 24-48 h at 45.5±0.2°. After 24 h, examine for gas production; if negative, examine again at 48 h. Streak a loopful of the suspension from the gassing tube onto L-EMB agar. It is essential that one portion of the plate exhibit well-separated colonies. Incubate 18-24 h at 35°. Examine the plates for colonies typical of *E. coli*, i.e., dark-centered with or without metallic sheen. Select two presumptively positive colonies and transfer them to PCA agar slants for morphological and biochemical tests. Incubate PCA slants for 18-24 h at 35±1°, then perform a Gram stain on the culture.

If the culture is Gram-negative (short rods) perform *either* of the following two biochemical test schemes:

Scheme1. A. Tests for IMViC biochemical activity:

a. Indole production: Inoculate a tube of tryptone broth and incubate 24 h at 35°. Test for indole by adding 0.2-0.3 ml Kovacs' reagent. Appearance of a distinct red colour in the upper layer is a positive test.

b. Voges-Proskauer-reactive compounds: Inoculate a tube of MR-VP broth and incubate 48 h at 35°. Transfer 1 ml to a 13 x100 mm tube. Add 0.6 ml alpha-naphthol solution and 0.2 ml 40% KOH, and shake. Add a few crystals of creatine. Shake and let stand 2 h. Test is positive if eosin pink colour develops.

c. Methyl red-reactive compounds: Incubate the MR-VP tube from the Voges-Proskauer test an additional 48 h at 35°. Add 5 drops of methyl red solution to each tube. A distinct red colour is a positive test. A yellow colour is negative.

d. Use of citrate: Lightly inoculate a tube of Koser's citrate broth; avoid detectable turbidity. Incubate at 35° for 96 h. Development of distinct turbidity is positive reaction.

Scheme1.B. Using the growth from the PCA slants, re-inoculate a tube of LST broth containing a Durham vial and incubate at 35° for 48 h to verify that the isolate has the ability to produce acid and gas from the fermentation of lactose.

Interpretation: Cultures that (a) produce gas as a result of the inoculation of LST broth and subsequent incubation for 24-48 h at 35°, (b) appear as Gram-negative non-spore-forming rods, and (c) give IMViC patterns ++-- (biotype 1) or -+-- (biotype 2), are considered to be *E. coli*.

Scheme 2. Disperse any colony growth into a small volume of 0.85% saline. Confirmation of the identity of the bacterial growth by chemical tests is conveniently done using API 20E or Micro ID strips or equivalent systems. After completion of the tests, identify the organism from the Identification Manual of the system used and record the final result.

Salmonella determination:

The use of cellulase to degrade the gum sample prior to analysis is essential in order to avoid gelling of the gum during its addition to the enrichment medium. Prepare a 1.0% cellulase solution (1 g cellulase to 99 ml water) and sterilize by filtration through a 0.45 µm membrane. (The cellulase solution may be stored at 2-5° for up to two weeks.) Aseptically weigh 25 g of sample into a sterile beaker (250 ml) or other appropriate container. Into a sterile, wide mouth, screw-cap jar (500 ml) or other appropriate container, introduce 225 ml of sterile lactose broth and 2.25 ml sterile 1% cellulase solution. While vigorously stirring the cellulase/lactose broth with a magnetic stirrer, quickly transfer the 25 g sample through a sterile glass funnel into the cellulase/lactose broth. Cap jar securely and let stand 60 min at room temperature. Loosen the cap and incubate the container at 35±1° for 24±2 h.

Tighten lid and gently shake incubated sample mixture; transfer 1 ml mixture to 10 ml Selenite cystine (SC) broth and another 1 ml mixture to 10 ml Tetrathionate (TT) broth. Incubate 24±2 h at 35°. Mix (vortex, if tube) and streak a 3 mm loopful incubated TT broth on Bismuth sulfite (BS) agar, xylose lysine desoxycholate (XLD) agar, and Hektoen enteric (HE) agar. (Prepare BS plates the day before streaking and store in dark at room temperature until streaked.) Repeat with a 3-mm loopful of SC broth. Incubate plates 24±2 h at 35°. Continue as indicated on pages 221-226 of the Guide to Specifications, FAO Food and Nutrition Paper 5 Revision 2, Rome 1991, "Examine plates for presence of colonies _".

Yeasts and moulds:

Weigh 25 g of sample and add to 2475 ml of sterile 0.1% peptone water (prepared by adding 1 g of peptone to 1 liter of distilled water, mixing to dissolve peptone, and autoclaving at 121° for 15 min) while vigorously stirring with a magnetic stirrer. Stir until the sample is completely dissolved. This is a 1:100 dilution. Aseptically pipette 0.1 ml to each of 10 pre-poured, solidified CCPDA-D plates (see below). Spread inoculum evenly over the surface of the plates using a sterile, bent glass rod. Incubate plates in the upright position at 25° undisturbed for 5 days.

After incubation, count the growing colonies on each plate using a colony counter and record the total number of colonies present on the 10 plates. Separate the yeasts from the moulds according to their morphology and count them separately. Take the total number of colonies present in all 10 plates and multiply by 100 to obtain the CFU/g of sample. If none of the plates shows growth, express the result as less than 100 CFU/g.

CCPDA-D medium: First, prepare a 2% dichloran (2,6-dichloran-4-nitroaniline) stock solution in 95% ethanol. Then, to PDA, add a quantity of the dichloran stock solution sufficient to give a concentration of 2.5 mg/l. Add chloramphenicol to a concentration of 50 mg/l and autoclave at 121° for 15 min. Cool medium to about 50° and just before pouring the plates, add sufficient filter-sterilized chlortetracycline to give a concentration of 50 mg/l.