

Analytical Methods

1. Protein determination (Lowry *et al.*, 1951)

Reagents

1. A: 2% sodium carbonate in 0.1 N NaOH
2. B: 0.5% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in 1% sodium citrate
3. C: 2 N Folin-Ciocalteu's phenol reagent + distilled water (ratio of 1:1)
4. D: 50 ml reagent A + 1 ml reagent B
5. Standard reagent: Bovine serum albumin (BSA) at a concentration of 1 mg/ml

Method

1. To each of eight disposable cuvette, add the following reagents as shown in table.
2. To tube No. 8, 200 μL of protein sample were added and mix well by using the vortex mixer.
3. Add 2 ml reagent D to each of the standards and unknown tube and then vortex immediately.
4. Incubate precisely for 10 min at room temperature.
5. Add 0.2 ml reagent C (previously dilute 1:1 with distilled water) and vortex immediately.
6. Incubate 30 min at room temperature (sample incubated longer than 60 min should be discarded).
7. Read absorbance at 750 nm.
8. Plot standard curves and calculate the unknown.

Table: Experimental set up for the Lowry's assay

Tube number	Water (μL)	1 mg/ml BSA (μL)	Effective BSA Concentration (mg/ml)
1	200	0	0
2	180	20	0.1
3	160	40	0.2
4	140	60	0.3
5	100	100	0.5
6	60	140	0.7
7	0	200	1.0
8	0	0	unknown

2. Determination of total viable count for mesophilic and psychrotrophic microorganisms analysis (Speck, 1976 and Cousin *et al.*, 1992)

Media and reagents

1. Peptone water diluent (2% NaCl)
2. Standard plate count agar (PCA)

Method

1. Weigh 50 g whole samples (without peeling) into a stomacher bag containing 450 ml peptone water (2% NaCl).
2. Blend in a Stomacher for 2 min.
3. Dilute the mixture to 1:100, 1:1,000 and 1:10,000 in 2% NaCl peptone water.
4. Spread 0.1 ml of sample on plate count agar (PCA). Incubate the plates at 35°C for 2 days and 4°C for 7-10 days for mesophilic and psychrotrophic, respectively.
5. Express microbial counts as log colony-forming unit (CFU)/g.

Calculation

$$\text{CFU/g sample} = \text{Average number of colonies} \times \text{dilution factor}$$

3. Determination of lactic acid bacteria count (Ordóñez *et al.*, 1991)

Media and reagents

1. Peptone water diluent (2% NaCl)
2. Man Rogosa Sharpe (MRS) agar

Method

1. Dilute the mixture to 1:100, 1:1,000 and 1:10,000 in 2% NaCl peptone water.
2. Transfer 0.1 ml in double-layer in man Rogosa Sharpe (MRS) agar and incubate at 35°C for 3 days.
3. Express microbial counts as log colony-forming unit (CFU)/g.

Calculation

$$\text{CFU/g sample} = \text{Average number of colonies} \times \text{dilution factor}$$

4. Determination of coliforms bacteria and *E. coli* (Geissler *et al.*, 2000)

Media and reagents

1. Peptone water diluent (2% NaCl)
2. Lauryl tryptose (LST) broth
3. EC broth
4. Levine's eosin-methylene blue (L-EMB) agar

Method

A. Examination for presumptive coliforms

1. Dilute the mixture to 1:100, 1:1,000 and 1:10,000 in 2% NaCl peptone water.
2. Select appropriate dilutions and for every dilution, transfer 1 ml aliquots into each of 3 LST tubes for at least 3 consecutive dilutions.
3. Incubate LST tubes at 35°C for 48 h.
4. Any tube producing gas is considered positive for the presence of coliforms. Perform confirmed test on all presumptive positive (gas) tubes.

B. Confirmation tests for coliforms

1. From each gassing LST tube from the presumptive test, transfer a loopful of each suspension to a tube of EC broth (a sterile wooden applicator stick may also be used for these transfers).

2. Incubate EC tubes 24 ± 2 h at 45.5°C and examine for gas production. If negative, reincubate and examine again at 48 ± 2 h. Use results of this test to calculate fecal coliform MPN. Calculate the MPN of coliforms based on the proportion of confirmed EC tubes (with gas production) for 3 consecutive dilutions.

C. Examination for presumptive *E. coli*

1. Transfer a loopful from each LST tube (with gas production) into a tube of EC tubes.
2. Incubate the EC tubes at 44.5°C .
3. Examine tubes and record reactions at 24 ± 2 h for gas, i.e., displacement of medium in fermentation vial or effervescence when tubes are gently agitated. Re-incubate gas-negative tubes for an additional 24 h and examine and record reactions again at 48 ± 2 h.
4. Any tube showing gas production is considered positive for the presence of presumptive *E. coli*.

D. Confirmation tests for *E. coli*

1. Gently agitate each gassing positive EC tube and streak for isolation, a loopful to a L-EMB agar plate
2. Incubate for 18-24 h at 35°C .
3. Examine plates for suspicious *E. coli* colonies, i.e., dark centered and flat, with or without metallic sheen.

Calculation of MPN

$$\text{Most Probable Number (MPN)} = \frac{\text{Index}}{10} \times (450+W) \times \frac{1}{W}$$

Where W : weight of sample in g

Index : from MPN Tables

5. Determination of *Salmonella* (Hammack, 1999)

Media and reagents

1. Nutrient broth
2. Tetrathionate broth
3. Selenite broth

4. Xylose lysine desoxycholate (XLD) agar

Method

A. Resuscitation (pre-enrichment)

1. Aseptically weigh 50 g sample into sterile plastic bags.
2. Add 450 ml sterile nutrient broth and blend 2 min.
3. Aseptically transfer homogenized mixture to sterile wide-mouth, screw-cap jar (500 ml) and incubate at 35°C for 24 h.

B. Selective enrichment

1. Mix the resuscitated culture gently and add 1 ml each to 10 ml of selenite broth.
2. Incubate at 35°C for 24 h.

C. Plating on selective agar media

1. Each culture of enrichment medium is inoculated onto XLD agar plates.
2. Transfer a loopful of culture and streak to obtain isolate colonies.
3. Incubate at 35°C for 24 h.
4. Examine plates for suspicious *Salmonella* from XLD agar, i.e., pink colonies with or without black centers. Many cultures of *Salmonella* may produce colonies with large, glossy black centers or may appear as almost completely black colonies.

6. Determination of *Staphylococcus aureus* (Bennett *et al.*, 1986).

Media and reagents

1. Peptone water diluent (2% NaCl)
2. Baird-Parker medium

Method

1. Dilute the mixture to 1:100, 1:1,000 and 1:10,000 in 2% NaCl peptone water.
2. Aseptically transfer 0.1 ml sample suspension to 3 plates of Baird-Parker agar. Spread inoculum over surface of agar plate, using sterile bent glass streaking rod.
3. Invert plates and incubate 45-48 h at 35°C.
4. Select plates containing 20-200 colonies, unless only plates at lower dilutions (>200 colonies) have colonies with typical appearance of *S. aureus*. Colonies of *S. aureus* are circular, smooth, convex, moist, 2-3 mm in diameter on uncrowded plates, gray to jet-

black, frequently with light-colored (off-white) margin, surrounded by opaque zone and frequently with an outer clear zone; colonies have buttery to gummy consistency when touched with inoculating needle.

6. Determination of *Vibrio parahaemolyticus* (Hasegawa, 1987)

Media and reagents

1. Thiosulfate citrate bile salts sucrose (TCBS) agar
2. Glucose salt teepol broth (GSTB).

Method

1. Blended whole samples as previously described.
2. Make a series of ten-fold dilutions using the saline solution.
3. Aseptically transfer 1 ml sample suspension to glucose salt teepol broth (GSTB).
4. Incubate at 37°C for 24 h and use as inoculum for the three-tube MPN procedure.
5. Streak a loopful from the top of tubes showing turbidity onto thiosulfate citrate bile salts sucrose (TCBS) agar
6. Incubate TCBS plates at 35°C for 24 h. *V. parahaemolyticus* appear as round, opaque, green or bluish colonies, 2 to 3 mm in diameter on TCBS agar.