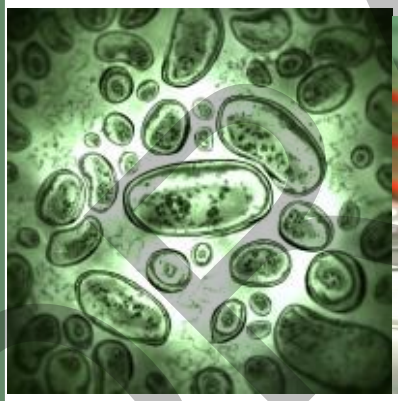
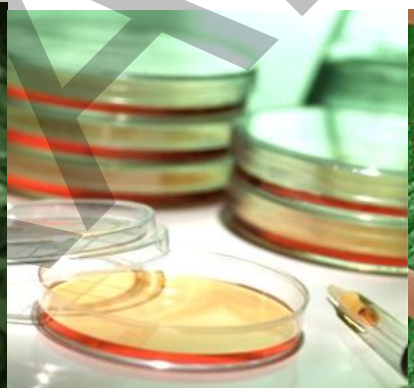
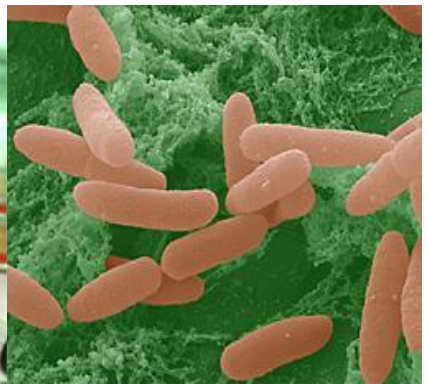
LAB. MANUAL 14

MANUAL OF METHODS

OF

ANALYSIS OF FOODS

MICROBIOLOGICAL TESTING

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FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA
MINISTRY OF HEALTH AND FAMILY WELFARE
GOVERNMENT OF INDIA
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MANUAL ON METHOD OF MICROBIOLOGICAL TESTING

TABLE OF CONTENTS

Microbiological Methods for Analysis of Foods, Water, Beverages and Adjuncts

Chapter 1

1. Aerobic Mesophilic Plate count

Indicates microbial counts for quality assessment of foods

1.2 Equipment:

Refer to Chapter 3 (Equipment, Materials & Glassware).

1.3 Medium:

- Plate count agar;
- Peptone water 0.1%,
- (Chapter 2 for composition of medium)

1.4 Procedure:

1.4.1 Preparation of food homogenate

Make a 1:10 dilution of the well mixed sample, by aseptically transferring sample to the desired volume of diluent.

Measure non-viscous liquid samples (i.e., viscosity not greater than milk) volumetrically and mix thoroughly with the appropriate volume of diluent (11 ml into 99 ml, or 10 ml into 90 ml or 50ml into 450 ml).

Weigh viscous liquid sample and mix thoroughly with the appropriate volume of diluent (11 $\pm$ 0.1g into 99ml; 10$\pm$ 0.1g into 90ml or 50$\pm$0.1g into 450ml).

Weigh 50$\pm$0.1g of solid or semi-solid sample into a sterile blender jar or into a stomacher bag. Add 450 ml of diluent. Blend for 2 minutes at low speed (approximately 8000 rpm) or mix in the stomacher for 30-60 seconds.

Powdered samples may be weighed and directly mixed with the diluent. Shake vigorously (50 times through 30 cm arc).

In most of the food samples particulate matter floats in the dilution water. In such cases allow the particles to settle for two to three minutes and then draw the diluent from that portion of dilution where food particles are minimum and proceed.

1.4.2 Dilution:

If the count is expected to be more than 2.5 $x10^3$ per ml or g, prepare decimal dilutions as follows. Shake each dilution 25 times in 30 cm arc. For each dilution use fresh sterile pipette. Alternately use auto pipette. Pipette 1 ml of food homogenate into a tube containing 9 ml of the diluent. From the first dilution transfer 1ml to second dilution tube containing 9ml of the diluent.

Repeat using a third, fourth or more tubes until the desired dilution is obtained.

1.4.3 Pour plating:

Label all petriplates with the sample number, dilution, date and any other desired information. Pipette 1ml of the food homogenate and of such dilutions which have been selected for plating into a petri dish in duplicate. Pour into each petri dish 10 to 12ml of the molten PCA (cooled to 42-45^oC) within 15 min from the time of preparation of original dilution. Mix the media and dilutions by swirling gently clockwise, anti-clockwise, to and fro thrice and taking care that the contents do not touch the lid. Allow to set.

1.5 Incubation:

Incubate the prepared dishes, inverted at 35^{o}C for 48$\pm$2 hours. (Or the desired temperature as per food regulation e.g. in case of packaged drinking water).

1.6 Counting Colonies:

Following incubation count all colonies on dishes containing 30-300 colonies and record the results per dilution counted.

1.7 Calculation

In dishes which contain 30-300 colonies count the actual number in both plates of a dilution and as per the formula given below:

$$N = \frac{\sum C}{(N1 + 0.1N2)\, D}$$

$\sum C$ is the sum of colonies counted on all the dishes retained

N1 is the no. of dishes retained in the first dilution

N2 is the no of dishes retained in the second dilution

D is the dilution factor corresponding to first dilution

E.g.

At the first dilution retained (10^{-2}):165 & 218 colonies

At the second dilution retained (10^{-3}) 15 & 24

$$N = \frac{165+218+15+24}{[2 + (0.1x2) \times 10x\text{-}2]} = \frac{422}{0.022} = 19182$$

Rounding the result to first two digits gives 19000 CFU.

1.8 Expression of Result

Aerobic (Mesophilic) Plate Count = 19000 CFU/g or $1.9x10^4$ CFU/g

or

If plates from all dilutions have no colonies and inhibitory substances have not been detected, the result is expressed as less than 1×10^1 CFU per g or ml.

If plates from the lowest dilutions contain less than 30 colonies, record the actual number and calculate as above but express results as CFU per g or ml.

Note:- *This method, as all other methods, has some limitations. Microbial cells often occur as clumps, clusters, chains or pairs in foods, and may not be well distributed irrespective of the mixing and dilution of the sample. Moreover the single agar medium used, the conditions of incubation, aeration etc., are not conducive to the growth of various populations of bacteria that may be present in a food sample.*

For statistical reasons alone, in 95% of cases the confidence limits of this test vary from ± 12% to ± 37%. In practice even greater variation may be found specially among results obtained by different microbiologists. (Corvell and Morsettle, J. Sci. Fd. Agric., 1969, vol. 20 p 573)

References:

1. Official Methods of Analysis of AOAC International (1995). 16th Edition. Edited by Patricia Cuniff. Published by AOAC International. Virginia. USA. Test 17.2.01 p.3-4.

2. Compendium of Methods for the Microbiological Examination of Foods. (1992) Carl Vanderzant and Don F. Splittstoesser Eds. Washington D.C. p. 75-87
3. Bacteriological Analytical Manual (1992) 6th Edn. Arlington, V.A. Association of Official Analytical Chemists for FDA, Washington, D.C. p. 17-21.
4. Microbiology- General guidance for the enumeration of Microorganisms-Colony count technique at 35°C (first revision) IS5402-2002, ISO4833:1991. Bureau of Indian Standards, Manak Bhavan, 9 Bhadur Shah Zafar Marg, New Delhi110002.

2. To Determine and Confirm Aciduric Flat Sour Spore Formers in Foods.

The organism of this group is *Bacillus coagulans.* It is responsible for spoilage of canned products.

2.1Equipment:

Refer to Chapter 3 (Equipment, Material and Glassware)

2.2 Culture Media:

Dextrose tryptone agar (with bromocresol purple)

2.3 Procedure:

Weighed samples or dilutions of the sample are taken in a test tube and heat shocked at 88°C for 5 min. The sample tubes are immediately cooled and one ml of the heat shocked sample or decimal volume is transferred to petri plates. 18 to 20 ml of melted bromocresol purple agar is added. After mixing the plates are incubated at 55°C for 48h.

Surface colonies on dextrose tryptone agar will appear slightly moist, usually slightly convex and pale yellow. Subsurface colonies on this medium are compact with fluffy edges. Colonies are surrounded by a yellow zone. Suspected colonies are counted and expressed as number per g of the sample.

2.4 Calculation

Average plate count x dilution factor = X

2.5 Expression of Result

Aciduric flat sour spore formers = X/g

References:

1. Official Methods of Analysis of AOAC International (1995). 16th Edition. Edited by Patricia Cuniff. Published by AOAC International. Virginia. USA. Test. 17.6.03., p.44.
2. Compendium of Methods for the Microbiological Examination of Foods. (1992) Carl Vanderzant and Don F. Splittstoesser Eds. Washington.D.C.p.291-295.

3. Detection and Determination of *Bacillus cereus* in Foods, and Beverages.

3.1 Equipment:

Refer to Chapter 3

3.2 Culture media and reagents

- Mannitol-egg yolk-polymyxin (MYP) agar
- Trypticase-soy-polymyxin broth
- Phenol red dextrose broth

- Nitrate broth
- Nutrient agar slants and plates
- Nutrient agar with L-tyrosine
- Nutrient broth with lysozyme
- Modified Voges- Proskauer medium (VP)
- Motility medium
- Nitrate test reagents
- Voges- Proskauer test reagents

3.3 Procedure

3.4 Preparation of food homogenate

Prepare as directed in 1.4.1

3.4.1 Dilution

Prepare decimal dilutions by pouring 1ml in 9 ml of dilution water.

3.4.A Most Probable Number Method

This procedure is suitable for the examination of foods which are expected to contain fewer than 1000 *B.cereus* per g.

i. Inoculate each of three tubes of trypticase-soy-polymyxin broth with 1 ml food homogenate and its dilutions.
ii. Incubate at 30^{o}C for 48 hours.
iii. Examine for dense growth typical of *B.cereus*
iv. Vortex-mix and using a 3mm loop transfer one loopful from each growth positive tube to dried MYP medium plates. Streak to obtain isolated colonies.
v. Incubate at 30^{o}C for 48 hours

vi. Pick one or more eosin pink (mannitol fermentation positive) colonies surrounded by precipitate zone (due to lecithinase activity) from each plate and transfer to nutrient agar slants for confirmation tests.

vii. The confirmed *B.cereus* count is determined using the MPN Table 4 of Test No. 10 for coliform count. On the basis of the number of tubes at each dilution in which *B.cereus* was detected and reported as MPN of *B.cereus* per gram.

3.4.B Plate Count Techniques

This procedure is suitable for the examination of foods expected to contain more than 1000 *B. cereus* per gram.

Inoculate duplicate MYP agar plates with the homogenate and each dilution of homogenate by spreading 0.1 ml evenly on to each plate in duplicate with sterile bent glass streaking rods (hockey sticks). Incubate plates 24 hours at 30^{o}C.

3.4.B.I Counting Colonies

The number of eosin pink colonies surrounded by lecithinase zone are counted. If reactions are not clear, incubate plates for added 24 hours before counting. Plates must ideally have 15-150 colonies.

Five or more colonies of presumptive *B.cereus* are picked from plates and transferred to nutrient agar slants for confirmation (3.5).

3.5 Confirmation Techniques

Gram Stain

Incubate the streaked nutrient agar slant either from for confirmation for 24 hours at 30°C. Make Gram stain and examine under microscope. *B. cereus* will appear as large Gram positive bacilli in short to long chains; spores are ellipsoidal, central to sub-terminal and do not swell sporangium.

Biochemical tests

Transfer 3 mm loopful of this culture to a tube containing 0.5ml sterile diluent. Vortex mix. Inoculate (or streak) the suspended culture into the following media and read the biochemical reaction.

Table 3: Biochemical tests

Media	Incubation at 35°C	Typical Reaction
Phenol red dextrose broth	Incubate anaerobically for 24 hours	Acid produced (color changes from red to yellow).
Nitrate broth	For 24 hours	Reduces nitrates to nitrites
Modified VP Medium	For 48 hours	Positive
Nutrient agar with tyrosine	For 48 hours	Positive
Nutrient broth with lysozyme	For 24 hours	Growth positive

3.6 Calculations:

As per 1.6

3.7 Reporting:

After confirmation, the number of *B.cereus* colonies is multiplied by the reciprocal of the dilution that the countable plate represents (It should be noted that the dilution factor is 10-fold higher than the sample dilution since only 0.1 ml was plated) and report as *B.cereus*/gram.

3.8 Expression of Results:

Bacillus cereus= Present/Absent

References:

1. Official Methods of Analysis of AOAC International (1995). 16th Edition. Edited by Patricia Cuniff. Published by AOAC International. Virginia. USA Test 17.8.01 p.52-54.
2. Compendium of Methods for the Microbiological Examination of Foods. (1992) Carl Vanderzant and Don F. Splittstoesser Eds. Washington. D.C. p.593 – 603.
3. Bacteriological Analytical Manual (1992) 6th Edn. Arlingon. V.A. Published by Association of Official Analytical Chemists for FDA, Washington.D.C.p.191-198.

4. Detection and Determination of Anaerobic Mesophilic Spore formers *(Clostridium perfringens)*.

4.1 Equipment and media

4.2 Equipment: Refer to Chapter 3. (Equipment, Materials and Glassware)

4.3 Media: Tryptone sulfite cycloserine agar (TSC),
Cooked meat medium

4.4 Procedure

Inoculate 2 g of food sample into 15 to 20 ml of cooked meat medium in duplicates. Incubate at 35^{o} for 24 h.

Positive tubes showing turbidity and gas production are streaked on to TSC agar plates. Overlay with TSC agar. Incubate plates upright, anaerobically for 18 to 20 h at 35^{o}C.

Count all colonies that are black in color surrounded by a zone of precipitate.

4.5 Confirmation

Inoculate a portion of the selected black colony from TSC agar on to motility nitrate agar and lactose gelatin agar by stabbing. Also inoculate a tube of fluid thioglycollate medium. Incubate at 35^{o} C for 24 h.

Observe microscopically the culture growing in thioglycollate media for the presence of large gram-positive rods. The culture is non-motile and growth therefore occurs only along the line of inoculum in mobility nitrate

agar, and they are positive for reduction of nitrate to nitrite which is indicated by the development of red or orange color of the medium. On lactose gelatin medium, the culture shows positive reaction for fermentation of lactose as indicated by gas bubbles and change in color of medium from red to yellow. Gelatin is liquified by *C.perfringens.*

4.6 Calculation

NA

4.7 Expression of Result

Clostridium perfringens = present/absent

Reference:

1. Official Methods of Analysis of AOAC International (1995). 16th Edition. Edited by Patricia Cuniff. Published by AOAC International. Virginia. USA. Test No. 17.7.02 p. 48 – 50.
2. Compendium of Methods for the Microbiological Examination of Foods. (1992) Carl Vanderzant and Don F. Splittstoesser. Eds. Washington D.C. p. 623 – 635.
3. Bacteriological Analytical Manual (1992) 6th Edn. Arlington.V.A. Association of Official Analytical Chemists for FDA, WashingtonDC.p.209–214.

5. Detection, Determination and Confirmation of *Coliforms, Faecal coliforms* and *Escherichia coli* in Foods and Beverages.

5.1 Equipment:

Refer to Chapter 3. (Equipment, Media and Glassware)

5.2 Culture media and reagents

Refer to test 5

5.3 Procedure

5.3.1 Test for Coliforms

Coliforms in foods may be enumerated by the solid medium method or by the Most Probable Number (MPN) method.

5.3.1.A Solid medium method

Preparation of food homogenate

Prepare as directed under 1.4.1

5.3.1.A.1 Dilutions

Prepare as directed under 1.4.2

5.3.1.A.2 Pour Plating

Pipette 1ml of the food homogenate (prepared sample) and of each dilution into each of the appropriately marked duplicate petri dishes.

Pour into each petri-dish 10-12 ml of VRBA (tempered to 48^{o}C) and swirl plates to mix. Allow to solidify. Overlay with 3 to 5 ml VRBA and allow to solidify.

Incubate the dishes, inverted at 35^{o}C for 18 to 24 hours.

5.3.1.A.3 Counting the colonies

Following incubation, count all colonies that are purple red in colour, 0.5 mm in diameter or larger and are surrounded by a zone of precipitated bile acids. Optimally the plates should have 30 to 100 colonies.

5.3.1.A.4 Calculation

Multiply the total number of colonies per plate with the reciprocal of the dilution used and report as coliforms per g or ml.

5.3.1.B Most Probable Number method

This method is valuable in those samples where coliform density is low because higher quantity of sample can be used for examination. It is based on probability statistics wherein aliquots of decimal volumes/dilutions of the sample are transferred to several (1 to 5) tubes of specific medium. Positive tubes are scored and the MPN estimate is directly made using the Table 5.

5.3.1.B.1 Preparation of food homogenate

Prepare as directed under 1.4.1

5.3.B.1.2 Dilutions:

Prepare as directed under 1.4.2

5. 3.B.1.3 Inoculation

Inoculate each of 3 tubes of LST broth (containing inverted Durham tubes) with 1ml of food homogenate (1:10).

Carry out the same operation from the first (1 in 100) and the second (1 in 1000) dilution tubes. Using a fresh sterile pipette for each dilution.

5. 3.B.1.4 Incubation

Incubate the LST tubes at 35±0.5°C for 24 and 48 hours.

5.4 Presumptive test for coliforms

Record tubes showing gas production after 24 hours and reincubate negative tubes for further 24 hours. Then record tubes showing gas production.

5.5 Confirmed test for coliforms

Transfer a loopful from each gas positive tube of LST to a separate tube of BGLB broth.

Incubate the BGLB broth tubes at 35±0.5° C for 48±2h.

The formation of gas confirms the presence of coliform bacteria. Record the number of positive tubes that were confirmed as positive for coliform.

5.6 Calculation

Note the MPN appropriate to the number of positive tubes from the table 5.4.

For example:

3 in 1:10; 1 in 1:100 and 0 in 1:1000. The table shows that MPN=43 coliforms per g or ml.

Coliforms= present/absent per g

Table 5. Most Probable Number (MPN) per 1 g of sample, using 3 tubes with each of 0.1, 0.01, and 0.001 g portions.

Positive tubes				Positive Tubes				Positive tubes				Positive tubes			
0.1	**0.01**	**0.001**	**MPN**	**0.1**	**0.01**	**0.001**	**MPN**	**0.1**	**0.01**	**0.001**	**MPN**	**0.1**	**0.01**	**0.001**	**MPN**
0	0	0	<3	1	0	0	3.6	2	0	0	9.1	3	0	0	23
0	0	1	3	1	0	1	7.2	2	0	1	14	3	0	1	39
0	0	2	6	1	0	2	11	2	0	2	20	3	0	2	64
0	0	3	9	1	0	3	15	2	0	3	26	3	0	3	95
0	1	0	3	1	1	0	7.3	2	1	0	15	3	1	0	43
0	1	1	6.1	1	1	1	11	2	1	1	20	3	1	1	75
0	1	2	9.2	1	1	2	15	2	1	2	27	3	1	2	120
0	1	3	12	1	1	3	19	2	1	3	34	3	1	3	160
0	2	0	6.2	1	2	0	11	2	2	0	21	3	2	0	93
0	2	1	9.3	1	2	1	15	2	2	1	28	3	2	1	150
0	2	2	12	1	2	2	20	2	2	2	35	3	2	2	210
0	2	3	16	1	2	3	24	2	2	3	42	3	2	3	290
0	3	0	9.4	1	3	0	16	2	3	0	29	3	3	0	240
0	3	1	13	1	3	1	20	2	3	1	36	3	3	1	460
0	3	2	16	1	3	2	24	2	3	2	44	3	3	2	1100
0	3	3	19	1	3	3	29	2	3	3	53	3	3	3	>1100

5.7.C Test for Faecal Coliforms

Proceed as directed from 5.5

Transfer a loopful from each gas positive tube of LST to a separate tube of EC broth.

Incubate the EC tubes at 45.5±0.2°C in water bath for 24±2 hours. Submerge broth tubes so that water level is above highest level of medium.

Record tubes showing gas production.

5.7.C.1 Calculations:

As directed under the test for coliforms.

5.8.D Test for *Escherichia coli*

(i) Proceed as directed under test for faecal coliforms.

Streak one plate L-EMB from each positive BGLB tube in a way to obtain discrete colonies.

Incubate inverted plates at 35 ±0.5° C for 24±2 hours.

Examine plates for typical nucleated dark centered colonies with or without sheen. If typical colonies are present pick two from each EMB plate by touching needle to the center of the colony and transfer to a PCA slant.

Incubate slants at 35±0.5° C for 18 to 24 hours

Transfer growth from PCA slants to the following broth for biochemical tests (vide Chapter 4 under Biochemical Tests).

Tryptone broth: Incubate 24±2 hours at 35±0.5° C and test for indole.

MR-VP Medium: Incubate 48±2 hours at 35±0.5° C. Aseptically transfer 1ml of culture to a 13x100 mm tube and perform the Voges Proskauer test. Incubate the remainder of MR-VP culture an additional 48h and test for methyl red reaction.

Koser citrate broth: Incubate 96 hours at 35±0.5° C and record as + or – for growth.

LST broth: Incubate 48±2 hours at 35±0.5° C and examine for gas formation.

Gram stain: Perform the Gram stain in a smear prepared from 18 hours PCA slant. Presence of small red coloured rods confirms *Escherichia coli*.

Compute MPN of E.coli per g or ml considering gram negative, nonspore forming rods producing gas in lactose and classify biochemical types as follows (IMViC)(Table 5.1).

Table 5.1: Micro-organism & IMViC

Indole	MR	VP	Citrate	Type
+	+	-	-	Typical *E. coli*.
-	+	-	-	Atypical *E. coli*.
+	+	-	+	**Typical intermediate**
-	+	-	+	Atypical Intermediate
-	-	+	+	Typical *Enterobacter aerogenes*
+	-	+	+	Atypical *Enterobacter aerogenes*

5.8.D.1 Calculations

As per MPN table

5.8.D.2 Interpretation

Escherichia coli= x MPN/g

Reference:

1. Official Methods of Analysis of AOAC International (1995). 16th Edition. Edited by Patricia Cuniff. Published by AOAC International. Virginia. USA. Test No. 17.2.02, p. 4-5.
2. Compendium of Methods for the Microbiological Examination of Foods. (1992) Carl Vanderzant and Don F. Splittstoesser. Eds. Washington D.C. p.325-341.
3. Bacteriological Analytical Manual (1992) 6th Edn. Arlington. V.A. Published by Association of Official Analytical Chemists for FDA, WashingtonD.C.p.27–31.

6. Direct Microscopic Count in Tomato Puree, Sauce, Paste, Chutney.

1. Howard Mold Count
2. Bacterial Count
3. Yeast and Bacterial Spore Count

6.1.1 Equipment:

Refer to Chapter 3.

6.1.2 Special Equipment

i) Howard mould counting slide

(ii) Haemocytometer

The Howard mould counting slide is a thick glass slide with a flat plane of rectangle of 20x15 mm in the middle of the slide, surrounded by a moat flanked on each side by shoulders 0.1mm higher than flat plane surface. The cover glass when placed is supported on the shoulders and leaves a depth of 0.1mm between underside of cover glass and plane surface. In the case of Haemocytometer the flat plane surface is ruled in the form of a square with sides measuring 1mm each. This square is divided into 25 medium size squares and 400 small size squares.

6.2 Procedure:

6.2.A Mould Count

Preparation of sample

Tomato juice: Use juice as it comes from container

Catsup (Ketchup) or sauce: Place 50ml stabilizer solution in 100 ml graduated cylinder, add 50ml well mixed sample by displacement and mix thoroughly.

Stabilizer solution: 0.5% Sodium Carboxy Methyl Cellulose (NaCMC) – place 500ml boiling water in high speed blender. With blender running add 2.5gms NaCMC and 10ml formalin and blend for 1 minute. Keep in a stoppered bottle (handle the blender carefully because hot materials in the blender create pressure on closure with blender lid).

Puree and Paste: Dilute the sample with stabilizer solution and mix thoroughly so that the refractive index of 1.3448 to 1.3454 at 20^{o}C (or 1.3442 to 1.3448 at 25^{o}C) is obtained.

6.2.A.1 Preparation of slide

Clean Howard slide so that Newton's rings are produced between slide and cover glass. Remove cover and with knife, blade or scalpel, place portion of well mixed sample upon central disk. Spread evenly over disk and cover with cover glass to give uniform distribution. Discard any mount showing uneven distribution or absence of Newton's ring or spillage of liquid into moat.

6.2.A.2 Mould count

Place slide under microscope and examine with such adjustments that each field of view covers 1.5 sq.mm obtained by so adjusting draw-tube that diameter of field becomes 1.382 mm^2. When such adjustment is not possible make use of accessory drop in ocular diaphragm with aperture accurately cut to necessary size. Diameter of area of field of view can be determined by use

of stage micrometer. When instrument is properly adjusted, volume of liquid examined per field is 0.15 mm^3. Use magnification of 90-125X. Use approximately 200X magnification to confirm identity of mould filament.

Prepare two mounts and count only 25 fields from each, observing in such a manner as to be representative of all sections of mount. Observe each field noting presence or absence of mould filament and recording results as positive when aggregate length of not less than 3 filaments present exceeds 1/6 of diameter of field. In case a single filament is traversing several fields of microscope it is counted as one positive field. For calculations refer 6.3.

6.2.B Bacterial count

Preparation of sample for bacterial count in the case of a homogeneous food sample such as catsup and sauce involves proper dilutions to facilitate easy identification and counting. Normally a 1:5 dilution would serve the purpose. To 20ml of distilled water in a 25 ml graduated cylinder add 5 ml of sample by displacement and shake thoroughly.

Place the haemcytometer slide under microscope and using 400 to 500 magnification count four small size squares from each corner of ruled chamber and the central medium square (total 20 small squares). For calculations refer 6.3.

6.2.C Yeast and Bacterial spore count

The same slide prepared for bacterial counts is used and a total of 200 squares comprising of 80, 40 and 80 from the top, middle and base of ruled chamber respectively is counted. For calculations refer 6.3.

6.2 Calculations

Calculation 6.2.A for Mould Count

Calculate proportions of positive fields from results of examination of all observed field and report as percent fields containing mould filaments.

$$\text{Percent positive fields} = \frac{\text{No. of positive fields}}{\text{No. of fields observed}} \times 100$$

Calculation 6.2.B for Bacterial count

No. of bacteria in 20 small squares = B

No. of bacteria in 400 small square= (B x 400) / 20

That is - 20 B bacteria

1.0 mm^3 contains - 20 x 10B

1.0 cc contains - 20 x 10 x 10^3 B or 2 x 10^5 B

If the material is diluted five times then the number of bacteria per ml of sample is

=5 x 2 x 10^5B or 10 x 10^5B or 10^6B or B million/cc.

e.g. If B = 20 then the count will be 20 million per cc.

Calculation 6.2.C for yeast and spores

Calculate number of yeasts/bacterial spores per 1/60 mm^3 as follows:

No. of yeasts/spores in 200 small squares = Y

No. of yeast/spores in 400 small squares = (400 x Y)/200 or = 2Y

Or 0.1 mm^3 contains = 2Y yeast

1.0 mm^3 contains = 2 x 10 Y yeasts

1/60 mm3 = (2 x 10 x 1 Y yeast)/60

Or 1/3 Y yeasts

If diluted 5 times then 5 x 1/3Y; or

5/3 Y yeasts/bacterial spores per 1/60 mm^3 of the sample.

6.4 Expression of Results

Mould Hyphae positive fields = %

Microscopic Bacterial Count = 10^6 per cc

Yeast and Bacterial spores = 1/60 mm^3

Reference:

1. Official Methods of Analysis of AOAC International (1995). 16th Edition. Edited by Patricia Cuniff. Published by AOAC International. Virginia. USA.

2. Compendium of Methods for the Microbiological Examination of Foods. (1992) Carl Vanderzant and Don F. Splittstoesser. Eds. Washington D.C. p. 97-104.

7. Fermentation Test (Incubation test).

To determine commercial sterility of processed canned foods.

7.1 Equipment:

Refer to Chapter 3. (Equipment, Materials and Glassware)

7.2 Media:

- Tryptone broth
- Cooked meat medium
- Orange serum broth
- Potato dextrose agar
- APT broth

7.3 Procedure

The most reliable test for determining commercial sterility of a container of a product is to incubate that container in an appropriate temperature, long enough to allow any significant microorganisms contained therein to grow and to manifest their presence. This is the incubation or fermentation test.

7.4 Routine Production Monitoring

For low acid products destined for storage at temperatures above 40^{o}C, containers from each sampling period or retort load should be incubated at 55^{o}C for 5 to 7 days.

For all other low-acid products incubate at 30^{o}C to 35^{o}C for 10 days.

For acid or acidified foods incubate at 25^{o} to 30^{o}C for 10 days.

7.5 Examination

Containers may be removed from the incubator whenever outward manifestations of microbial growth appear (e.g., swells or with transparent containers, noticeable product change). At the end of the incubation period,

some containers should be opened to detect possible flat sour spoilage by measure of reduced pH as compared to good packs.

Weigh each suspect container to the nearest gram. Subtract the average tare weight of the empty container and determine net weight.

Before opening, the container must be cleaned with detergent and water, rinsed and wiped dry with clean paper towels.

Containers are opened employing aseptic techniques with extra precautions. Note abnormal odour, consistency changes, and frothiness. Measure pH electrometrically or colorimetrically.

7.6 Sub culturing of Product Samples

Transfer about 2g of product from each container to media mentioned below. Tubes for anaerobes should be exhausted in flowing steam for an exposure of 20 min and cooled to 55^{o}C prior to inoculation if not freshly prepared and autoclaved. For detection of molds in high acid foods, potato dextrose agar pour plates are prepared. Measure pH of the product and observe product odor and appearance.

7.6.A Low acid foods (pH > 4.5 or 4.5)

Medium	Incubation temperature and time	Organism
Tryptone broth	30 to 35^{o}C for 5 days	Mesophilic aerobes
Tryptone broth	55^{o}C for 5 days	Thermophilic aerobes
Cooked meat medium	30 to 35^{o}C for 5 days	Mesophilic anaerobes
Cooked meat medium	55^{o}C for 5 days	Thermophilic anaerobes

7.6.B High acid Foods (pH 4.5 or <4.5)

Medium	Incubation temperature and time	Organism
Orange serum broth	25 to 30°C for 5 days	For bacteria and yeasts
Potato dextrose agar	30°C for 5 days	For molds
APT broth	35°C for 5 days	For Lactobacilli, *B.coagulans* and other acid tolerant bacteria

7.7 Interpretation of Data

The development of swelled containers may indicate microbial activity. Growth must be confirmed by demonstrating excessive microorganisms by direct smear or by subculturing or abnormal product(pH, texture, odor, discolouration, evolution of gas).

Swelling may also be due to overfilling; low filling temperatures, improper vacuum closing procedures, incipient spoilage and chemical swells.

7.8 Expression of Results

Incubation test Negative/positive when incubated at 30°C/35°C for a period of 10 days.

References

1. Compendium of Methods for the Microbiological Examination of Foods. (1992) Carl Vanderzant and Don F. Splittstoesser. Eds. Washington D.C. p. 1037 – 1092.

8. Rope Producing Spores in Flours.

8.1 Requirements of the procedure

8.2 Equipment and media

8.3 Equipment:

Refer to Chapter 3. (Equipment, Materials and Glassware)

8.4 Culture Media:

Dextrose tryptone agar

8.5 Procedure:

Fifty grams of the sample is weighed and transferred to 450 ml of sterile 0.1% peptone water in a blender jar for mixing. Alternately a stomacher may also be used for mixing.

Ten and one ml volumes of the peptone water suspension are pipetted into separate 100ml portions of melted dextrose tryptone agar contained in 250 ml flasks and held at 45^{o}C. The flasks and a control flask is submerged in a boiling water bath for 15 mins.

After heating, the flask contents are cooled to about 45^{o}C and contents of each flask is poured into 5 sterile plates in approximately equal volumes. When the agar has solidified, the plates are inverted and incubated at 35^{o}C for 48h.

8.6 Calculation:

Count as rope producing organisms, the surface colonies that are grey-white, vesicle like, becoming at first drier and finally wrinkled. Add to this count any subsurface colony that displays stringiness when tested. The total colonies on the set of 5 plates from the flask that received 10ml suspension are considered as rope spores per gm of sample.

8.7 Expression of Results

Rope spores = __ /g

References:

1. Compendium of Methods for the Microbiological Examination of Foods. (1992) Carl Vanderzant and Don F. Splittstoesser. Eds.WashingtonD.C.p.265-274.

9. Detection and Confirmation of *Salmonella species* in foods.

9.1 Equipment: Refer to Chapter 3. (Equipment, Materials and Glassware)

9.2 Culture Media:

- Lactose broth
- Trypticase Soy Broth
- Trypticase Soy Broth Containing Potassium Sulfite at a final concentration of 0.5%.
- Reconstituted Non-Fat Dry Milk
- 1% aqueous Brilliant Green Dye Solution.
- Selenite Cystine Broth

- Tetrathionate Broth
- Xylose Lysine Deoxycholate (XLD) Agar
- Hektoen Enteric Agar (HEA)
- Bismuth Sulphite Agar (BSA)
- Triple Sugar Iron (TSI) Agar
- Lysine Iron Agar (LIA)
- Urea Broth
- Phenol Red Dulcitol Broth
- Phenol Red Lactose Broth
- Tryptone Broth
- KCN Broth
- Malonate Broth
- Buffered Glucose (MR-VP) Medium
- Brain Heart Infusion (BHI) Broth
- Buffered Peptone Water

9.3 Procedure:

9.4 Preparation of sample and pre-enrichment

Aseptically open the sample container and weigh 25g sample into a sterile empty wide mouth container with screw cap or suitable closure.

Add 225ml of sterile lactose broth to the sample. Buffered peptone water, Trypticase soy broth, and nutrient broth can also be used for pre-enrichment. Make a uniform suspension by blending if necessary. Cap container and let stand at room temperature for 60 min. Instead of lactose broth the recommended pre-enrichment broth for the following food samples is as follows :

Non fat dry milk and dry whole milk – Sterile distilled water. Add 0.45 ml of 1% aqueous briliant green dye before incubation.

Dried active yeast – Trypticase soy broth

Onion-garlic powder – Trypticase soy broth containing potassium sulfite at a final concentration of 0.5%

Milk Chocolate – Reconstituted non fat dry milk.

Shake and adjust pH (if necessary) to 6.8±0.2 with sterile 1N NaOH or 1N HCl.

Incubate at 35°C for 24±2 hours

9.5 Selective enrichment

Gently shake incubated sample mixture and transfer 1 ml to 10 ml of selenite cystine broth and an additional 1 ml to tetrathionate broth. Incubate 24±2 hours at 35°C.

9.6 Selective media plating

Vortex – mix and streak 3 mm loopful of incubated selenite cystine broth on selective media plates of XLD, HEA and BSA. Repeat with 3mm loopful of incubated tetrathionate broth.

Incubate plates at 35°C for 24±2 hours and 48±2 hours.

Observe plates for typical Salmonella colonies

On XLD (after 24h) - Pink colonies with or without black centres.

On HEA (after 24h) - Blue green to blue colonies with or without black centers.

On BSA (after 24 to 48h) – Brown, grey or black colonies sometimes with metallic sheen. Surrounding medium is usually brown at first, turning black with increasing incubation time.

9.7 Treatment of typical or suspicious colonies

Pick with needle typical or suspicious colonies (if present) from each XLD, HEA and BSA plates. Inoculate portion of each colony first into a TSI agar slant, streaking slant and stabbing butt and then do the same into an LIA slant.

Incubate TSI and LIA slants at 35^{o}C for 24$\pm$2 hours and 48$\pm$2h respectively. Cap tubes loosely to prevent excessive H_2S production.

Table 9.7.A Typical Salmonella reactions are :

	TSI	**LIA**
Slant	Alkaline (red)	Alkaline (Purple)
Butt	Acid (Yellow)	Alkaline (Purple)
H_2S production (blackening in butt)	+ or -	+

A culture is treated as presumptive postive if the reactions are typical on either or both TSI and LIA slants.

9.8 Biochemical tests

Using sterile needle inoculate a portion of the presumptive positive culture on TSI slant into the following broths. Incubate at 35^{o}C for the specified period of days and read for Salmonella typical reactions.

Table 9A: Biochemical tests

Broth/ Media	Time of incubation	Results
Urea broth	24±2h	Negative (no change in yellow colour of medium)
Phenol red lactose broth	48±2h	*Negative for gas and/or acid reaction
Phenol red sucrose broth	48±2h	*Negative for gas and/or acid reaction
Phenol red dulcitol broth	48±2h	*Postive for gas and/or acid reaction
Tryptone broth	24±2h	Negative for indole test
KCN broth	48±2h	Negative (no turbidity)
Malonate broth	48±2h	*Negative (green colour unchanged)
MR-VP medium	48±2h	Negative for VP test but positive for MR test.

***(Note : Majority of *S. arizonae* are atypical for these reactions).**

Table 9B: Criteria for discarding Non-Salmonella Cutlures

Test(s) or Substrate(s)	Results
Urease test	Postive (purple-red)
Indole test	Positive (red)
Flagellar test (Polyvalent or spicer-Edwards	Negative (no agglutination)
Lysine decarboxylase test	Negative (yellow)
KCN broth	Positive (growth)
Phenol red lactose broth*	Positive (acid and/or gas)**
Lysine decarboxylase test	Negative (yellow)
Phenol red sucrose broth	Positive (acid and/or gas)**
Lysine decarboxylase test	Negative (yellow)
KCN broth	Positive (growth)
Voges-Proskauer test	Positive (red)
Methyl red test	Negative (yellow)

* Malonate broth positive cultures are tested further to determine if they are *Salmonella arizonae*

** Do not discard positive broth cultures if corresponding LI agar cultures give typical Salmonella reactions; test further to determine if they are Salmonella sp. (vide 9).

9.9 Serological Tests

To reduce number of presumptive positive cultures (TSI positive and urease negative) carried through biochemical identification tests, the following serological flagellar (H) screening test may be carried out.

Transfer 3mm loopful of culture into 5ml of BHI broth and incubate at 35^{o}C until visible growth occurs (About 4-6 hours).

Add about 2.5ml formalized physiological saline solution.

Test with Salmonella flagellar (H) antisera. Positive cultures show visible agglutination.

Further confirmation can be made by using Salmonella Polyvalent (O) antiserum.

9.10 Calculation:

NA

9.11 Expression of Result:

Salmonella = Present/Absent per 25 g

References:

1. Official Methods of Analysis of AOAC International (1995). 16th Edition. Edited by Patricia Cuniff. Published by AOAC International. Virginia. USA. Test. 17.9.01 p. 55 – 62.
2. Compendium of Methods for the Microbiological Examination of Foods. (1992) Carl Vanderzant and Don F. Splittstoesser. Eds. Washington D.C. p.371-422.
3. Bacteriological Analytical Manual (1992) 6th Edn. Arlington. V.A. Association of Official Analytical Chemists for FDA, Washington, D.C.p.51–69.

10 Detection and Confirmation of *Shigella* species

10.1 Equipment:

Refer to Chapter 3. (Equipment, Materials and Glassware)

10.2 Culture media:

- Gram Negative (GN) Broth
- MacConkey agar
- Xylose Lysine Deoxycholate (XLD) Agar
- Triple Sugar Iron (TSI) Agar slants
- Urea Broth
- Acetate Agar Slants
- Carbohydrate Fermentation Media
- Tryptone Broth (for Indole test)
- Buffered Glucose (MR-VP) Medium
- Koser's Citrate Broth
- Decarboxylase Test Media with Lysine or Ornithine
- Motility Test Medium
- Thornley's Semi-Solid Arginine Medium.

10.3 Procedure:

10.4 Enrichment:

Using aseptic techniques mix or blend if necessary 25 g sample with 225 ml of gram negative broth. Transfer to a sterile 500 ml bottle.

Adjust pH (if necessary) to 6.0 - 7.0 with sterile 1N NaOH or 1N HCl. Incubate at 35-37^{o}C for 18 hours.

10.5 Selective streaking:

Transfer a 5mm loopful of the enrichment broth culture to the surface of MacConkey agar and XLD agar plates and streak to obtain isolated colonies.

Invert and incubate plates at 35-37^{o}C for 24±2h. Typical Shigella colonies on XLD agar appear as red or pink colonies usually about 1mm in diameter and on Mac Conkey agar as opaque or transparent colonies.

Inoculate each suspected colony into TSI agar slant by streaking the slant and stabbing the butt. After overnight incubation at 35-37^{o}C, typical Shigella reaction is alkaline (red) slant and acid (yellow) butt with no H_2S or gas production:

10.6 Other Biochemical tests to confirm *Shigella*

Perform the following biochemical tests on a portion of the suspected culture on the TSI slant noted in 9.7 & 9.8

Table 10

Test	Reaction
Urease	-
Motility	-
Acetate utilization	-
Gas from glucose	-
IMVIC Reaction	+ + - - or - + - -
Lysine decarboxylase	-
Arginine dihydroloase	- or +
Ornithine decarboxylase	+ or -

10.7 Expression of Results

Shigella = Present / Absent per 25g of sample

References:

1. Compendium of Methods for the Microbiological Examination of Foods. (1992) Carl Vanderzant and Don F. Splittstoesser. Eds. Washington D.C. p. 423 – 431.
2. Bacteriological Analytical Manual (1992) 6th Edn. Arlingon. V.A. Association of Official Analytical Chemists for FDA, Washington D.C. p. 71 – 76.

11. Detection, Determination and Confirmation of *Staphylococcus aureus*.

11.1 Equipment:

Refer to Chapter 3. (Equipment, Materials and Glassware).

11.2 Culture media:

- Tripticase (tryptic) soy broth with 10% sodium chloride and 1% sodium pyruvate.
- Baird Parker (BP) Medium
- Brain Heart Infusion (BHI) Broth
- Desiccated Coagulase Plasma (rabbit) with EDTA
- Butterfields Buffered Phosphate Diluent
- Plate Count Agar (PCA)

11.3 Procedure:

11.3.1 Preparation of food homogenate:

Aseptically weigh 50 g food sample into the sterile blender jar. Add 450ml of diluent (1:10) and homogenize 2 min at high speed (16000-18000 rpm).

Alternately use stomacher for sample preparation

11.3.2 Dilution:

Pipette 10ml of the food homogenate into 90ml of diluent (or 1ml to 9ml) to make a 1:100 dilution. Mix well using a vortex-mixer.

Transfer 1ml from this dilution to a fresh tube of 9ml to give a 1:1000 dilution. Repeat until the desired dilution is obtained.

11.3.A Most probable number method:

This procedure is recommended for testing processed foods likely to contain a small number of *S. aureus.*

11.3.A.I Inoculation:

Inoculate each of 3 tubes of tryptose soy broth (with 10% sodium chloride and 1% sodium pyruvate) with 1ml of food homogenate.

Carry out the same operation from the first and subsequent dilutions using a fresh sterile pipette each time.

Maximum dilution of sample must be high enough to yield negative end point. Incubate at 35^{o}C for 48h.

11.3.A.II Surface Streaking:

Vortex mix the tubes from .A.1 and then using 3mm loop transfer one loopful from each growth positive tube to dried BP medium plates. Streak so as to obtain isolated colonies. Incubate at 35-37^{o}C for 48 hours.

11.3.A.III Interpretation:

Colonies of *S. aureus* are typically grey black to jet black, circular, smooth, convex, moist and 2-3 mm diameter on uncrowded plates. Frequently there is a light colored (off-white) margin, surrounded by opaque zone (precipitate) and frequently with outer opaque zone (precipitate) and frequently with outer clear zone; colonies have buttery to gummy consistency when touched with the inoculating needle.

11.3.A.IV Confirmation techniques:

Using a sterile needle, transfer (noting the dilution) at least one suspected colony from each plate to tubes containing 5ml BHI and to PCA slants.

Incubate BHI cultures and slants at 35^{o}C for 18-24h.

Perform coagulase test on the BHI cultures. Retain slant cultures for repeat tests.

11.3.A.V Reporting:

Coagulase positive cultures are considered to be *S. aureus*. Now record number of positive tubes (and the respective dilutions) of *S. aureus*. Report most probable number (MPN) of S. aureus per gram from Table 4 of MPN values.

11.3.B Surface Plating method :

This method is applicable for general purpose use in testing foods expected to contain > 10 cells of *S. aureus* per g.

Transfer 1ml of the food homogenate (1:10 dilution) and other dilutions to triplicate plates of BP medium and equitably distribute 1ml

inoculum over the triplicate plates. Spread inoculum over agar surface using sterile bent glass streaking rods (hockey sticks).

Incubate plates in upright position in the 35-37^{o}C incubator for about 1 hour or until inoculum is absorbed by medium. Then invert plates and incubate 45-48 hours.

11.3.B.1 Counting colonies:

Count colonies of typical *S. aureus* appearance (as described in 19.5.3.3). Test for coagulase production on suspected colonies. Add number of colonies on triplicate plates represented by colonies giving positive coagulate test. Multiply the count obtained by inverse of corresponding sample dilution. Report as *S. aureus* per gm or ml of the sample.

11.3.B.2 Expression of result:

Staphylococcus aureus = x/g

References:

1. Official Methods of Analysis of AOAC International (1995). 16th Edition. Edited by Patricia Cuniff. Published by AOAC International. Virginia. USA Test. 17.5.01 p.32 – 34.
2. Compendium of Methods for the Microbiological Examination of Foods. (1992) Carl Vanderzant and Don F. Splittstoesser. Eds. Washington D.C. p. 533 – 550.
3. Bacteriological Analytical Manual (1992) 6th Edn. Arlingon. V.A. Association of Official Analytical Chemists for FDA, Washington D.C. p. 161 – 165.

12. Detection and Confirmation of Sulfide Spoilage Spore formers in Processed Foods.

12.1 Equipment:

Refer to Chapter 3. (Equipment, Materials and Glassware)

12.2 Cuture Media:

Sulfite agar

12.3 Procedure :

For sample preparation and heat treatment follow the steps mentioned for anaerobic thermophilic spore formers. (Test No. 4)

Inoculations of the prepared sample are placed into the sulfite agar medium with a nail. Incubate at 55^{o}C for 24 to 48h in anaerobic jar.

D. nigrificans will appear as jet-black spherical areas, the color due to the formation of iron sulfide. No gas is produced. Colonies can be counted and reported as spores/g of sample.

12.4 Calculation:

Number of colonies x dilution factor

12.5 Expression of Result

Spores of sulfide spoilage/g

References:

Compendium of Methods for the Microbiological Examination of Foods. (1992) Carl Vanderzant and Don F. Splittstoesser. Eds. WashingtonD.C.p.317-323.

13. Detection and Determination of Thermophilic Flat Sour Sporeformers.

13.1 Equipment:

Refer to Chapter 3. (Equipment, Materials and Glassware)

13.2 Culture Media:

Dextrose Tryptone Agar.

13.3 Procedure:

Weighed samples or dilution are heat treated at 100^{o}C for 30 min followed by rapid cooling. Aliquots of these solutions are then transferred to petri plates. Dextrose tryptone agar is added and swirled gently to distribute the inoculum. Allowed to solidify. The inverted plates are incubated at 55^{o}C for 48 to 72 hr.

In case of starch samples the dilutions are added directly to sterile dextrose tryptone agar (100 ml) contained in flasks. The flasks are autoclaved at 5lb for 10min. The flasks are then gently agitated while cooling as rapidly as possible. The entire mixture is distributed equally into 5 plates and allowed to harden. It is then layered with a thin layer of sterile plain 2% agar in water and allowed to harden. The inverted plates are incubated at 50 ^{o}C to 55^{o}C for 48 to 72 hr.

Flat sour colonies are round, are 2 to 5mm in diameter, show a dark, opaque center and usually are surrounded by a yellow halo in a field of purple.

The colonies are counted and expressed in terms of number of spores per g of the sample.

13.4 Calculation:

=Number of colonies x dilution factor

13.5 Expression of Result:

Thermophilic flat sour bacteria = x/g

References:

1. Official Methods of Analysis of AOAC International (1995). 16th Edition. Edited by Patricia Cuniff. Published by AOAC International. Virginia. USA

2. Compendium of Methods for the Microbiological Examination of Foods. (1992) Carl Vanderzant and Don F. Splittstoesser. Eds. Washington D.C. p. 299 – 307

14. Detection and Determination of Pathogenic *Vibrios* in Foods.

14.1 Equipment:

Refer to Chapter 3. (Equipment, Materials and Glassware)

14.2 Culture Medium:

- Thiosulphate Citrate Bile Salts Sucrose Agar
- Gelatin Phosphate Salt Broth and Agar.

- Kligler Iron Agar.
- T1 N1 Agar.

14.3 Procedure:

14.4 Enrichment

Weigh 25g sample and transfer to 225ml of GPS broth. Incubate at 35°C for 6 to 8 h.

14.5 Plating:

Prepare dried plates of TCBS and GPS agar medium. Transfer a loopful of the surface growth of the broth culture to the surface of the two plating medium and streak in a manner that will yield isolated colonies.

Incubate plating medium for 18 to 24 h at 35°C.

14.6 Interpretation:

Typical colonies of *V.cholerae* on TCBS agar are large (2 to 3 mm in diameter) smooth, yellow (occasional slow sucrose fermentors are green), and slightly flattened with opaque centers and translucent peripheries. On GPS agar the colonies have a cloudy zone around them that becomes more definite after a few minutes of refrigeration. In oblique light, the colonies appear iridescent green to bronze colored and finely granular.

Typical colonies of *V. parahaemolyticus* on TCBS agar appear round, opaque, green or bluish colonies, 2 to 3 mm in diameter.

14.7 Confirmation:

Subculture all suspect colonies of *V. cholerae* on to T_1N_1 agar and incubate at 35°C for 24h. Stab streak a KIA slant with the culture and incubate the KIA slant overnight at 35°C. *V. cholerae* cultures have an alkaline (red) slant and an acid (yellow) butt, no gas and no blackening in the butt. Also perform the string test on suspect cultures as follows. Emulsify a large inoculum from the T_1 N_1 agar culture in a large drop of 0.5% sodium desoxycholate in 0.85% saline solution. Within 60 seconds, a mucoid mass forms and this material strings when a loopful is lifted (up to 2 to 3cm) from the slide. Further confirmation is by serological reactions.

Stab streak suspect colonies of *Vibrio* on the TSI slant and incubate overnight at 35°C. Typical reaction of *V. parahaemolyticus* is an alkaline slant and an acid butt but no gas or H_2S production

14.8 Results:

Test for pathogenic *Vibrios* = Positive/ Negative

Reference:

1. Official Methods of Analysis of AOAC International (1995). 16th Edition. Edited by Patricia Cuniff. Published by AOAC International. Virginia. USA, Test No. 17.11.01 p. 108 – 110.
2. Compendium of Methods for the Microbiological Examination of Foods. (1992) Carl Vanderzant and Don F. Splittstoesser. Eds. Washington D.C. p. 451 – 473.
3. Bacteriological Analytical Manual (1992) 6th Edn. Arlington. V.A. Association of Official Analytical Chemists for FDA, Washington D.C.p.111–121.

15. Estimation of Yeasts and Molds in Foods and Beverages.

15.1 Equipment:

Refer to Chapter 3. (Equipment, Materials and Glassware)

15.2 Media:

- Potato Dextrose Agar
- Mycophilic Agar
- Antibiotic Solution

15.3 Procedure:

Prepare food homogenate and decimal dilutions as directed under 1.4.1 and 1.4.2 respectively.

15.4 Pour plating:

Label all petri plates with the sample number, dilution, date and any other described information.

Pipette 1ml of the food homogenate of such dilutions which have been selected for plating into a petri dish in duplicate.

Acidify PDA or malt agar with sterile 10% tartaric acid to pH 3.5$\pm$0.1. Do not reheat medium once acid has been added. Pour 10-12 ml of the agar medium (tempered to 45^{o}C). Mix by swirling and allow to solidify.

(OR)

Add 2ml antibiotic solution to 100ml of plate count, mycophil or malt agar. Mix and pour 10-12ml of the agar medium tempered to 45^{o}C. Mix by swirling and allow to solidify.

15.5 Incubation:

Invert plates and incubate at 20 or 25^{o}C for 2 to 5 to 7 days. Discard plates after seven days of if growth is not observed, observe plates every day and mark the colonies because some time fungal growth spreads to entire plate and mask the colonies. Do not open the plates which are showing fungal sporangia.

15.6 Counting colonies:

Count colonies, multiply by the inverse of the corresponding dilution and report as yeast or mould count per g or ml.

15.7 Reporting:

Yeast and Mould count = x/g

Reference:

1. Compendium of Methods for the Microbiological Examination of Foods. (1992) Carl Vanderzant and Don F. Splittstoesser. Eds. Washington D.C. p. 239 – 249.
2. Bacteriological Analytical Manual (1992) 6th Edn. Arlington. V.A. Association of Official Analytical Chemists for FDA, Washington D.C. p.227-230.

16. Detection and confirmation of *Listeria monocytogenes* in Food

Warning: while testing *L. monocytogene*s it is recommended that a properly equipped laboratory under supervision of skilled Microbiologist is done. The material used during testing is carefully disposed off after sterilization.

Pregnant personnel may be asked to avoid handling of *L. moncytogens* cultures and undertaking the tests.

16.1 Equiupments:

Refer chapter 3

16.2 Culture media and reagents:

- Phosphate buffered peptone water
- Half Frazer broth
- Frazer broth
- Modified Oxford Agar
- PALCAM Agar
- Tryptone Soya Yeast Extract Agar
- Tryptone Soya Yeast Extract Broth
- Sheep Blood Agar
- Carbohydrate utilization broth (Rhamnose and Xylose)
- Motility Agar
- CAMP Medium and test organisms
- Hydrogen peroxide solution

16.3 Preparation of test sample:

Take 25 g of a well mixed sample in stomacher bag and use 225 ml of Half Frazer broth. Stomach the sample for two minutes. Pour aseptically the contents in to a wide mouth bottle and incubate at 30°C for 24±2h (a black coloration may develop).

Take one ml of the above culture and transfer to 9ml of Frazer broth. Incubate the inoculated tube at 37°C for 48± 2h at 35-37°C.

From 24 h culture of Half Frazer broth and 48h Frazer broth streak out culture on Modified Oxford Agar and PALCAM agar so that well separated colonies are obtained.

Invert the plates and incubate at 35 or 37°C for 24 h and if required an additional 18 h if growth is slight or no colonies appear. Examine the plates for colonies presumed to be *L. monocytogenes*.

16.4 Appearance of colonies:

On M Ox agar the colonies are small (1mm) greyish surrounded by a black halo.

After 48 h the colonies turn darker with a possible green luster and are about 2 mm in diameter with black halos and sunken centres.

On PALCAM agar after 24 h the colonies appear1.5 to 2 mm in diameter greyish green or olive green some times with black centre and always surrounded by a black halo and depressed centre.

16.5 Confirmation of Listeria species:

Select five typical colonies from one plate of each medium. If presumed colonies are less than five on a plate, take all of them.

Streak the selected colonies from each plate on to the surface of a well dried TSYEA for obtaining well separated colonies. Invert the plates and incubate at 35°C or 37°C for 18 to 24 h or until the growth is satisfactory.

Typical colonies are 1 mm to 2 mm in diameter, convex, colorless and opaque with an entire edge. Carry out the following tests from colonies of a pure culture on the TSYEA.

Catalase reaction:

With the help of loop pick up an isolated colony and place it in H_2O_2 solution on a glass slide. Immediate production of gas bubbles indicates catalase positive reaction.

Gram staining:

Perform Gram staining on a colony Listeria are Gram positive slim short rods.

Motility Test:

Take colony from TSYEA plate and suspend it TSYE broth. Incubate at 25°C for 8 to 24 h until cloudy medium is observed. Take a drop of culture and place it on a glass slide. Cover the top with a cover slip and observe under a microscope. *Listeria* is seen as slim rods with a tumbling motility (cultures grown above 25°C fail to show this motion. Compare them with a known culture – *cocci* or large rods with rapid motility are not *Listeria*.

As an alternative stab motility agar tube with an isolated colony from TSYEA and incubate at 25°C for 48 h. typical umbrella like appearance around the stab indicate motility positive culture. If growth in not positive incubate up to five days and observe for the stab again.

16.6 Confirmation of *Listeria monocytogenes:*

Heamolysis test:

Take a colony from TSYEA and stab it on a well dried surface of sheep blood agar plate. Simultaneously stab positive (*L. monocytogenes*) and

negative (*L. innocua*) control cultures. Inver the plates and incubate at 35°C or 37°C for 24±2 h. examine the plates.

L. monocytogenes show clear light zones of beta haemolysis. *L. innocua* does not show any haemolysis. Examine the plates in a bright light to compare test cultures with the controls.

Carbohydrate utilization:

Inoculate each of the carbohydrate utilization broths (rhamnose and xylose) with a culture from TSYE broth and incubate at 35 °C or 37v for upto 5 days. Appearance of yellow color indicates a positive reaction within24 to 48 h.

CAMP test

On a well dried surface of sheep blood agar streak each of the *Staphylococcus aureus* and *Rhodococcus equi* cultures in single lines and parallel to each other and diametrically opposite, a thin even innoculum is required.

Streak the test strain separated in a similar manner at right angles to these cultures as that the test strain and *S. aureus* and *R.equi* cultures do not touch but their closest are about 1 mm or 2 mm apart. Several test strains can be streaked on the same plate. Simultaneously streak control cultures of L *monocytogene*s, L innocua and *L. ivanovii.* Incubate plates at 35 to 37 ^{o}C for 18 to 24 h.

Observe plates against bright light. In *L. monocytogenes* case there is enhanced zone of beta haemolysis at the intersectiono of *S. aureus*.

L. innocua does not show any enhanced zone of haemolysis with *S. aureus* or *R. equi.*

In case of *L. ivanovii* enhamced beta zone of haemolysis is seen on *R. equi* side.

16.7 Interpretation of results:

All Listeria species are small, Gram positive rods that demonstrate motility and catalase positive reaction. *L monocytogenes* are distinguished from other species by the characteristics listed in table given below.

Table 16

Species	**Haemolysis**	**Production of acid with Rhamnose**	**Production of acid with Xylose**	**CAMP Test**	
				S. aureus	***R equi***
L.monocytogenes	+	+	-	+	-
L innocua	-	V	-	-	-
L. ivanovii	+	_	+	-	+
L. seeligeri	(+)	-	+	(+)	-
L welshmeri	-	V	+	-	-
L grayi sub species *grayi*	-	-	-	-	-
L..grayi subspecies *murrayi*	-	V	-	-	-

16.8 Expression of results:

Based on the observations and interpretation of the results report presence or absence of *L. monocytogenes* in test portion specifying the mass in grams or mililitres of the sample taken.

L. monocytogenes =present or absent/ g or ml.

17.Bacteriological Examination of Water for Coliforms

17.1 Equipment:

Refer to Chapter 3.

17.2 Culture Media:

Lauryl Sulphate Tryptose (LST) broth tubes of single and double strength with inverted Durham tubes (10 ml quantities in tubes and 50 ml in bottles).

17.3 Procedure:

Dechlorination:

Samples collected in pre-sterilised bottles are mixed well. To every 100ml portion of chlorinated samples 0.1 ml of a 10% sterile solution of sodium thiosulphate is added.

Various combinations of sample volume are taken depending on the probable load of coliform in the sample. To 10 ml or 50 ml volume of double strength broths, equal volumes of sample is added. To 10ml of single strength broths 1 ml or 0.1 ml is added.

Tubes/bottles are incubated at 35^{o}C for 24 and 48 hrs.

17.4 Interpretation

Record tubes showing gas production after 48 hrs. The MPN index per 100ml sample is determined using the following statistical tables.

Table 17A: MPN index and 95% Confidence limits for various combinations of positive and negative results when five 10 ML portions are used.

No. of tubes giving positive reaction out of 5 of 10 ml each	MPN Index/100 ml	95% confidence Limits (Approximate)	
		Lower	Upper
0	< 2.2	0	6.0
1	2.2	0.1	12.6
2	5.1	0.5	19.2
3	9.2	1.6	29.4
4	16.0	3.3	52.9
5	> 16.0	8.0	Infinite

Table 17B: MPN index and 95% Confidence limits for various combinations of positive and negative results when Ten 10 ML portions are used

No. of tubes giving positive reaction out of 10 of 10 ml each	MPN Index/100 ml	95% Confidence Limits (Approximate)	
		Lower	Upper
0	< 1.1	0	3.0
1	1.1	0.03	5.9
2	2.2	0.26	8.1
3	3.6	0.69	10.6
4	5.1	1.3	13.4
5	6.9	2.1	16.8
6	9.2	3.1	21.1
7	12.0	4.3	27.1
8	16.1	5.9	36.8
9	23.0	8.1	59.5
10	> 23.0	13.5	Infinite

Table 17C: MPN Index and 95% Confidence Limits for Various combinations of positive results when five tubes are used per dilution (10 ml, 1.0 ml, 0.1 ml)

Combination of Positives	MPN Index/100 ml	95% Confidence Limits (Approximate)		Combination of Positives	MPN Index/100 ml	95% Confidence Limits (Approximate)	
		Lower	Upper			Lower	Upper
0-0-0	< 2	-	-	4-2-0	22	9.0	56
0-0-1	2	1.0	10	4-2-1	26	12	65
0-1-0	2	1.0	10	4-3-0	27	12	67
0-2-0	4	1.0	13	4-3-1	33	15	77
				4-4-0	34	16	80
1-0-0	2	1.0	11	5-0-0	23	9.0	86
1-0-1	4	1.0	15	5-0-1	30	10	110
1-1-0	4	1.0	15	5-0-2	40	20	140
1-1-1	6	2.0	18	5-1-0	30	10	120
1-2-0	6	2.0	18	5-1-1	50	20	150
				5-1-2	60	30	180
2-0-0	4	1.0	17	5-2-0	50	20	170
2-0-1	7	2.0	20	5-2-1	70	30	210
2-1-0	7	2.0	21	5-2-2	90	40	250
2-1-1	9	3.0	24	5-3-0	80	30	250
2-2-0	9	3.0	25	5-3-1	110	40	300
2-3-0	12	5.0	29	5-3-2	140	60	360
3-0-0	8	3.0	24	5-3-3	170	80	410
3-0-1	11	4.0	29	5-4-0	130	50	390
3-1-0	11	4.0	29	5-4-1	170	70	480
3-1-1	14	6.0	35	5-4-2	220	100	580
3-2-0	14	6.0	35	5-4-3	280	120	690
3-2-1	17	7.0	40	5-4-4	350	160	820
4-0-0	13	5.0	38	5-5-0	240	100	940
4-0-1	17	7.0	45	5-5-1	300	100	1300
4-1-0	17	7.0	46	5-5-2	500	200	2000
4-1-1	21	9.0	55	5-5-3	900	300	3900
4-1-2	26	12	63	5-5-4	1600	600	5300
				5-5-5	$\geq$ 1600	---	---

17.5. Expression of Results:

Coliforms = x MPN/250 ml or 100ml

17.5.1 Plating Technique:

Alternately take desired volume of water (250 ml or 100 ml) and pass it through a micropore filter of 0.2U. Take the filter disk and place on a well dried surface of LST Agar. Incubate at 35°C for 24 to 48 hrs. Count the typical colonies and express the result as given below.

17.5.2 Expression of Result:

Coliform count = x cfu/g

References:

1. Standard Methods for the Examination of water and waste water. (1989). 17th Edition. Edited by Lenore. S. Clesceru; Arnold. E. Greenberg and R. Rhodes Trussell. Test 9221. P. 66 - 76
2. Bacteriological Analytical Manual (1992) 6th Edn. Arlington. V.A. Published by Association of Official Analytical Chemists for FDA, Washington D.C. p. 28 – 31

18. Bacteriological Examination of Water for Detection, Determination and Confirmation of *Escherichia coli.*

18.1 Equipment:

Refer to Chapter 3.

18.2 Culture Media:

Refer to test No. 5.

18.3 Procedure:

Take 250ml or 100 ml (as per the requirement of standard). Pour 50 ml of sample in 50 ml of double strength LST broth in five bottles containing inverted tubes, or 20 ml of sample in 20 ml of double strength LST broth in sugar tubes containing inverted Durham tubes. Incubate the tubes at 35°C for 24 to 48 hrs. Note the number of bottles/tubes showing gas formation. Refer MPN tables from test No. 17 for calculation of number of presumptive +ve *E. coli* .Proceed further as per test No. 5 for confirmation of *E. coli*

18.4 Calculation:

As per test No. 17

18.5 Expression of Result:

Escherichia coli = present/ absent in 250ml or 100 ml.

18.6 Filteration Technique:

Alternately filter the required volume through a 0.2U micropore filter and place it on VRBA Mc Conkey agar plate and incubate at 35°C for 24 to 48 hrs. Count typical colonies and select five such colonies for confirmation as per test No. 5.

References:

1. Standard Methods for the Examination of water and wastewater. (1989). 17th Edition. Edited by Lenore. S. Clesceru; Arnold. E. Green berg and R. Rhodes Trussell. Test 9221. P. 66 - 76
2. Compendium of Methods for the Microbiological Examination of Foods. (1992) Carl Vanderzant and Don F. Splittstoesser. Eds. Washington D.C. p.325 – 369.

19. Bacteriological examination of water for presence of *Salmonella and Shigella*

19.1 Equipment:

Refer to Chapter 3.

19.2 Culture Media:

Refer to Test No. 9 and 10.

19.3 Procedure:

Refer to test no. 9 and 10.

Expression of Result:

Test for Salmonella = present or absent/250 ml

Test for Shigella = present or absent/ 250 ml

References:

1. Official Methods of Analysis of AOAC International (1995). 16th Edition. Edited by Patricia Cuniff. Published by AOAC International. Virginia. USA. Test 17.9.01 p. 55-62.

2. Compendium of Methods for the Microbiological Examination of Foods. (1992) Carl Vanderzant and Don F. Splittstoesser. Eds. Washington D.C. p.371 – 431 .
3. Bacteriological Analytical Manual (1992) 6th Edn. Arlington. V.A. Association of Official Analytical Chemists for FDA, Washington, D.C. p. 51 – 69.

20. Bacteriological examination of water for Detection and Confirmation of *Clostridium perfringens*

20.1 Equipment:

Refer to Chapter 3. (Equipment, Materials and Glassware)

20.2 Culture Medium:

- Cooked Meat Medium
- Tryptone sulfite Cycloserine Medium

20.3 Procedure:

Take 50 ml of sample water and pour in to five tubes of Cooked Meat medium (de-chlorinate the sample if required. Put the tubes in water bath maintained at 80 °C for 15 minutes. Plug the tubes with vespar and incubate at 35 °C for 18 to 24 hrs. Formation of gas bubble below the wax seal indicates anaerobic growth.

From each positive tube take a loopfull of culture and streak on to a TSC medium. Overlay a thin layer of TSC agar. Incubate the inverted plates at 35 °C 18 to 24 hrs. Appearances of black colonies surrounded by a black zone are Clostridium perfringens colonies.

20.4 .Expression of Results:

Clostridium perfringens = present or absent/50 ml

References

1. Official Methods of Analysis of AOAC International (1995). 16th Edition. Edited by Patricia Cuniff. Published by AOAC International. Virginia. USA. Test. 17.7.02 p. 48 – 50.
2. Compendium of Methods for the Microbiological Examination of Foods. (1992) Carl Vanderzant and Don F. Splittstoesser. Eds. Washington D.C.p. 623 – 635.
3. Bacteriological Analytical Manual (1992) 6th Edn. Arlington. V.A. Association of Official Analytical Chemists for FDA, Washington, D.C. p. 209 – 214.

21. Bacteriological Examination of water - *Bacillus cereus*.

21.1 Equipment:

Refer to Chapter 3. (Equipment, Materials and Glassware)

21.2 Culture Medium:

Refer to Test No. 6

21.3 Procedure:

Take 250 ml of water sample and pour 50 m in five bottles containing 50ml of double strength Trypticase Soy Polymixin broth. Incubate at 30 °C for 48 hrs and proceed as per test No.3.3.

21.4. Calculations:

If desired estimate MPN as per table No. 17.1.

21.5 Expression of Result:

Bacillus cereus = present absent/250 ml

References:

1. Official Methods of Analysis of AOAC International (1995). 16th Edition. Edited by Patricia Cuniff. Published by AOAC International. Virginia. USA. Test 17.8.01 p. 52-54.
2. Compendium of Methods for the Microbiological Examination of Foods. (1992) Carl Vanderzant and Don F. Splittstoesser. Eds. Washington D.C. p. 593 – 603.
3. Bacteriological Analytical Manual (1992) 6th Edn. Arlington. V.A. Association of Official Analytical Chemists for FDA, Washington, D.C.p191–198.

22. Bacteriological analysis of water for Detection and determination of *Pseudomonas aeruginosa* in water

22.1 Equipment:

Refer chapter 3

22.2 Culture media:

- Pseudomaonas presumptive test broth-single strength and Concentrated.
- Milk agar

- Gelatin medium
- Starch agar

22.3 Presumptive test:

Add 250 ml of water sample in to five bottles (50 ml in each) containing 50 ml of concentrated Pseudomonas presumptive test broth.

Incubate the bottles at 37±1 °C for 48 h. examine for growth and blue green fluorescence under an ultra violet lamp in a darkened room or UV light chamber.

22.4 Confirmation:

Take a loopful of culture above growth from each container and streak onto a milk agar plate. Invert the plate and incubate at 42±0.5°C for 24h. Examine the plate for growth, pigment production and casein hydrolysis (zone of clearance around colonies).

22.5 Enumeration:

All containers showing either growth or fluorescence which yield typical colonies (after subculture on milk agar plates) shall be considered positive for *Pseudomonas aeruginosa*.

Use table no. 4 for calculation of MPN count.

22.6 Non pigmented strains:

If growth is observed in the Pseudomonas presumptive test broth and casein is digested in the Milk agar then inoculate a loopful from the broth into gelatin medium and incubate at 37±1°C.

Oxidative reaction can be confirmed by Hough and Liefson test.

Nitrate is reduced to ammonia from the breakdown of acetamide.

From the presumptive test inoculate Milk agar and incubate the inverted plate at 42±1°C for 24h . Positive reaction for Pseudomonas confirms atypical *Pseudomonas aeruginosa* in the sample.

Table 22: For reactions of *Pseudomans aeruginosa.*

S.No.	Mode of reaction	
	Typical	Atypical
Casein hydrolysis	+	+
Growth at 42°C	+	+
Blue green fluorescence	+	-
Gelatine liquefaction	+	+
Nitrate reduction to ammonia	+	+
Oxidative reaction in Hough and Liefson medium	+	+

22.7 Expression of Result:

Reference:

Packaged Natural Mineral Water Specifications (second revision) IS13428-2005. Manak Bhavan, 8 Bahadur Shah Zafar Marg, New Delhi-110002.

Culture Media

Chapter 2

Acetate agar

Sodium chloride.....	5.0 g
Magnesium sulfate	0.1 g
Monoammonium phosphate	1.0 g
Dipotassium phosphate	1.0 g
Sodium acetate	2.0 g
Bromothymol blue	0.08 g
Agar	20.0 g
Distilled water.....	1.0 liter

Mix ingredients in distilled water and heat gently to dissolve. Dispense 7 ml portions into 16 x 150 mm tubes.

Sterilize at 121^{o}C for 15 minutes, and slant the tubes to obtain a 1 inch butt and a 1.5 inch slant, pH 6.8$\pm$0.2.

Baird-Parker medium

Basal medium

Tryptone	10.0 g
Beef extract.....	5.0 g
Yeast extract	1.0 g
Glycine	12.0 g
Lithium chloride $6H_2O$	5.0 g
Agar	20.0 g

Suspend ingredients in 950 ml distilled water.

Boil to dissolve completely. Dispense 95.0 ml portions in screw capped bottles. Autoclave 15 minutes at 121^{o}C. Final pH 6.8-7.2 at 25^{o}C.

Bismuth Sulfite Agar

Peptone	10.0 g
Beef extract	5.0 g
Dextrose	5.0 g
Disodium phosphate	4.0 g
Ferrous sulfate	0.3 g
Bismuth ammonium citrate	1.85 g
Sodium sulfite	6.15 g
Agar	20.0 g
Brilliant green	0.025 g
Distilled water	1.0 liter

Dissolve ingredients in distilled water by boiling approximately 1 minute. Adjust to pH 7.7±0.2, cool to 45 to 50°C, suspending precipitate with gentle agitation, and pour plates without sterilizing medium. Let plates dry with covers partially open. Caution: Plates lose selectivity after 72 hours.

Brain Heart Infusion Broth

Calf brain, infusion from	200.0 g
Beef heart, infusion from	250.0 g
Proteose peptone or polypeptone	10.0 g
Dextrose	2.0 g
Sodium chloride	5.0 g
Disodium phosphate	2.5 g
Distilled water	1.0 liter

Dissolve ingredients in distilled water by bringing to a boil. Dispense into tubes and autoclave for 15 minutes at 121°C. Final reaction should be pH 7.4±0.2.

Brilliant – Green Lactose Bile Broth 2%

Peptone	10.0 g
Lactose	10.0 g
Oxbile	20.0 g
Brilliant-green	0.0133 g
Distilled water	1.0 liter

Dissolve the peptone and lactose in 500ml of distilled water, add the ox bile dissolved in 200ml of water, mix and make up to 975 ml, and adjust pH to 7.4±0.1. Add 13.3 ml of 0.1% aqueous solution of brilliant green. Add distilled water to bring the total volume to 1 liter. Dispense in 10ml portions into 20 x 50 mm test tubes containing inverted Durham tubes. Sterilize for 15 minutes at 121^{o}C.

Bromocresol Purple Carbohydrate Broth

Basal Medium

Peptone	10.0 g
Beef extract (optional)	3.0 g
Sodium chloride	5.0 g
Bromocresol purple	0.04 g
Distilled water	1.0 liter

Dissolve the desired carbohydrate (5.0 g or 10.0 g glucose, 5.0 g adonitol, 5.0 g arabinose, 5.0 g mannitol 5.0 g maltose, 5.0 g sucrose, 5.0 g lactose, 5.0 g sorbitol, 5.0 g cellobiose, 5.0 g salicin, 5.0 g trehalose or raffinose) per liter of basal medium. Adjust pH to 7.0±0.2. Dispense 8 ml aliquots to 16 x 150 mm tubes containing inverted 12 x 75 mm tubes. Autoclave 10 minutes at 121^{o}C. Allow autoclave temperature to drop slowly.

Buffered Peptone Water

Peptone	10.0 g
Sodium chloride	5.0 g
Disodium hydrogen phosphate	9.0 g
Potassium dihydrogen phosphate	1.5 g
Distilled water	1.0 liter

Adjust pH to 7.0, dispense in portions of 225ml into bottles of 500ml capacity and of 9ml in tubes. Sterilize for 20 min at 121^{o}C.

Butterfield's Buffered Phosphate Diluent

Stock solution :

Monopotassium hydrogen phosphate	34.0 g
Distilled water	500.0 ml

Adjust to pH 7.2 with about 175 ml sodium hydroxide solution dilute to one liter. Sterilize at 121^{o}C for 15 minutes and store in refrigerator.

Diluent

Dilute 1.25 ml stock solution to 1.0 liter with distilled water. Prepare dilution blanks in suitable containers. Sterilize at 121^{o}C for 15 minutes.

Cooked Meat Medium

Beef heart.....	454.0 g
Proteose peptone.....	20.0 g
NaCl.....	5.0 g
Glucose	2.0 g

Finely chop beef heart. Add approximately 1.5 g of heart particles to test tubes. Add remaining components to distilled water and bring volume to 1.0 L. Mix thoroughly. Distribute into tubes in 10 ml volumes. Autoclave for 15 min at 121^{o}C.

Czapek Yeast Autolysate (CYA) Agar

Sucrose	30.0 g
Agar	15.0g
Yeast extract	5.0 g
$NaNO_3$	5.0 g
K_2HPO_4	1.0 g
KCL	0.5 g
$MgSO_4.7H_2O$	0.5 g
$FeSO_4.7H_2O$	0.01 g

pH 7.3$\pm$0.2 at 25^{o}C

Add sucrose to 100ml distilled water and autoclave for 15 mins at 121^{o}C. Cool to 50^{o}C. Add the other components to 900 ml distilled water. Autoclave for 15 mins at 121^{o}C. Aseptically add the sterile sucrose solution after it has cooled to 50^{o}C.

Decarboxylase Test Media

Basal for use with Lysine, Arginine, Ornithine Moeller method (1954, 1955)

Basal medium

Peptone	5.0 litre
Beef extract	5.0 g
Bromocresol purple (1.6%)	0.625 ml
Cresol red (0.2%)	2.5 ml
Glucose	0.5 g
Pyridoxal	5.0 mg
Distilled water	1.0 liter

The basal medium is divided into four equal portions, one of which is tubed without the addition of any amino acids. These tubes of basal medium are used for control purposes. To one of the remaining portions of basal medium is added 1% of L-lysine dihydrochloride; to the second, 1% of L-arginine monohydrochloride and to the third portion, 1% of L-ornithine dihydrochloride. If DL amino acids are used, they should be incorporated into the medium in 2% concentration, since the microorganisms apparently are active against the L forms only. The pH of the fraction to which ornithine is added should be readjusted after the addition and prior to sterilization. The amino acid medium may be tubed in 3 or 4 ml amounts is small (13x100mm) screw capped tubes and sterilized at 121^{o}C for 10 minutes. A small amount of floccular precipitate may be seen in the ornithine medium. This does not interfere with its use.

Inoculation: Inoculate lightly from a young agar slant culture. After inoculation, add a layer (about 10mm in thickness) of sterile mineral (paraffin) oil to each tube including the control.. A control tube always should be inoculated with each culture under investigation. Incubate at 37^{o}C; examine daily for 4 days. Positive reactions are indicated by alkalization of the medium with a color change from yellow to violet. Weakly positive reactions may be bluish gray.

Dextrose Tryptone Agar

Agar	15.0 g
Pancreatic digest of casein	10.0 g
Glucose	5.0 g
Bromocresol purple	0.04 g

pH: 6.9$\pm$0.2 at 25^{o}C.

Add components to distilled water and bring volume to 1.0 L. Mix thoroughly. Gently heat and bring to boiling. Autoclave at 121°C for 15 min. Pour into sterile tubes or petri dishes.

EC Broth

Trypticase or tryptone	20.0 g
Bile salt No. 3	1.5 g
Lactose	5.0 g
Dipotassium hydrogen phosphate	4.0 g
Potassium dihydrogen phosphate	1.5 g
Sodium chloride	5.0 g
Distilled water	1.0 litre

Adjust pH to 6.9±0.1; dispense 8ml portions into 16 x 150 mm test tubes containing 10 x 75 mm Durham tubes. Sterilize for 15 min at 121°C.

Egg yolk tellurite enrichment

Soak eggs in aqueous mercuric chloride 1:1000 for not less than one minute. Rinse in sterile water and dry with a sterile cloth.

Aseptically crack eggs and separate whites and yolks. Blend yolk and sterile physiological saline solution (3+7 v/v) in high speed sterile blender for 5 seconds. Mix 50.0 ml blended egg yolk to 10.0 ml of filter sterilized 1% potassium tellurite. Mix and store at 2 to 8°C.

Preparation of Plates

Add 5.0 ml pre-warmed (45 to 50°C) enrichment to 95 ml melted basal medium, which has been adjusted to 45 to 50°C. Mix well (avoiding

bubbles), and pour 15.0 to 18.0 ml into sterile 15 x 100 mm Petri dishes. Plates can be stored at 2 to 8°C in plastic bags for 4 weeks. Immediately prior to use spread 0.5 ml per plate of 20% solution of Millipore filter sterilized sodium pyruvate and dry plates at 50°C for 2 hours or 4 hours at 35°C with agar surface uppermost.

If complete medium plates were prepared from commercial or laboratory prepared medium containing sodium pyruvate prior to adding Egg yolk tellurite. These plates must be used within 48 hours while being stored at 2 to 8°C. These plates should also be dried as indicated above prior to inoculating with sample.

L-EMB Agar

Peptone	10.0 g
Lactose	10.0 g
Disodium hydrogen phosphate	2.0 g
Agar	15.0 g
Distilled water	1.0 liter

Make a solution of (a), adjust pH to 7.1 to 7.2. Dispense in 100ml portions. Sterilize for 15 min at 121°C. Before use melt, and to each 100 ml portion add 2.0 ml of aqueous 2% eosin Y solution and 1.3 ml of 0.5% aqueous methylene blue solution.

Gelatin Phosphate Salt Broth

Gelatin	10.0 g
NaCl	10.0 g
$K_2 HPO_4$	5.0 g

PH 7.2+0.2 at 25 °C.

Add components to distilled water (1 L). Antoclave at 121°C for 15 min.

Gram Negative (GN) Broth

Glucose	1.0 g
D. mannitol	2.0 g
Sodium citrate	5.0 g
Sodium deoxycholate	0.5 g
Dipotassium phosphate	4.0 g
Monopotassium phosphate	1.5 g
Sodium chloride	5.0 g
Tryptose	20.0 g
Distilled water	1.0 liter

Dissolve ingredients in distilled water by heat. Dispense in tubes in convenient amounts and sterilize at 116^{o}C for 15 minutes. Final pH is 7.0±0.2. Avoid excessive heating.

Hektoen Enteric Agar

Proteose peptone	12.0 g
Yeast extract	3.0 g
Lactose	12.0 g
Sucrose	12.0 g
Salicin	2.0 g
Bile complex	9.0 g
Sodium chloride	5.0 g
Sodium thiosulfate	5.0 g
Ferric ammonium citrate	1.5 g
Bromthymol blue	0.065 g
Acid fucasin	0.1 g
Agar	14.0 g
Distilled water	1.0 liter

Suspend ingredients in distilled water. Boil with frequent stirring. Do not overheat or autoclave. When completely in solution, cool to 55 to 60^{o}C and distribute into plates. Allow plates to solidify with lids ajar to provide a

dry surface for inoculation. Plates may be refrigerated for future use. Final pH 7.5±0.2.

Hough and Liefson Medium

Peptone……	2g
Sodium Chloride……..	5g
Dipotasium Hydrogen Sulphate……	0.3g
Agar agar……..	3g
Water……	1000ml
pH…….	7.1
Bromothymol blue 0.2% in alcohol……	15 ml

Boil to dissolve and before adding Bromothymol blue and distribute 3 to 4 ml in 14 x100 mm test tubes. Plug and autoclave at 115 ±1 ^{o}C for 20 minutes.

Add aseptically filter sterilized glucose solution to give a final concentration of 1% mix well.

KF Streptococcus Agar

Proteose peptone #3 or polypeptone.....	10.0 g
Yeast extract	10.0 g
Sodium chloride.....	5.0 g
Sodium glycerophosphate.....	10.0 g
Maltose.....	20.0 g
Lactose	1.0 g
Sodium azide	0.4 g
Bromocresol purple	0.015 g
Agar	20.0 g
Distilled water.....	1.0 liter

Dissolve ingredients in distilled water by boiling, and dispense in 100.0 ml portions. Autoclave at 121^{o}C for 10 minutes. When ready to use, cool to 50^{o}C and add 1.0 ml of 1% solution TTC (Triphenyl tetrazolium chloride) per 100.0 ml. Final pH should be 7.2. Do not overheat this medium.

Kligler Iron Agar

Peptone	20 g
Agar	12 g
Lactose	10 g
NaCl	5 g
Beef extract	3 g
Yeast extract	3 g
Glucose	1 g
Ferric citrate	0.3 g
$Na_2S_2O_3$	0.3 g
Phenol red	0.05 g

pH 7.4$\pm$0.2 at 25^{o}C.

Add components to 1 L of distilled water. Distribute into tubes and autoclave at 121^{o}C for 15 min. Make slants with deep butts.

Koser's Citrate Broth

Sodium ammonium hydrogen phosphate	1.5 g
Monopotassium hydrogen phosphate	1.0 g
Magnesium sulphate	0.2 g
Sodium citrate	3.0 g
Distilled water	1.0 liter

Adjust pH to 6.7$\pm$0.1, dispense in 10ml portions in test tubes. Sterilize for 15 min at 121^{o}C.

Lactobacillus MRS Agar

Proteose peptone	10.0 g
Beef extract	10.0 g
Yeast Extract	5.0 g
Dextrose.....	20.0 g
Tween 80	1.0 g
Ammonium citrate.....	2.0 g
Sodium acetate.....	5.0 g
Magnesium sulphate	0.1 g
Manganese sulphate.....	0.05 g
Dipotassium phosphate.....	2.0 g
Agar	12.0 g
Distilled water	1.0 liter

Suspend ingredients in water containing 10 ml glycerol. Boil to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121^{o}C) for 15 minutes. Final pH 6.5 $\pm$ 0.2.

Lactose Broth

Beef extract	3.0 g
Peptone	5.0 g
Lactose	5.0 g
Distilled water	1.0 liter

Adjust pH to 6.8, dispense into fermentation tubes. Sterilize for 15 min at 121^{o}C. Allow temperature in autoclave to drop slowly below 75^{o}C before opening.

Lactose Gelatin Medium

Gelatin	120 g
Tryptone.....	15 g
Lactose.....	10 g
Yeast extract	10 g
Phenol red	10 ml (of 0.5% solution)

pH 7.5+0.2 at 25°C.

Add gelatin to 590 ml distilled water. Gently heat while stirirng and bring to 50 to 60°C. Add phenol red. Add the rest of the components to 400 ml of distilled water and mix with gelatin solution. Dispense 10 ml volumes in test tubes. Autoclave for 10 min at 121°C.

Lauryl Sulphate Tryptose Broth

Tryptose, tryptone or trypticase	20.0 g
Lactose.....	5.0 g
Dipotassium monohydrogen phosphate	2.75 g
Sodium chloride.....	5.0 g
Sodium lauryl sulphate	0.1 g
Disti11ed water.....	1.0 liter
Potassium dihydrogen phosphate	2.75 g

Adjust pH to 6.8±0.1, dispense in 10 ml portions in tubes with inverted Durham tubes. Sterilize for 15 min at 121°C.

Liver Broth

Fresh beef liver	500.0 g
Distilled water.....	1.0 liter
Tryptone.....	10.0 g
Soluble starch.....	1.0 g
Dipotassium phosphate.....	1.0 g

Remove the fat from 1 pound of fresh beef liver, grind, mix with 1000 ml of distilled water, and boil slowly for 1 hour. Adjust the pH to 7.6 and remove the liver particles by straining through cheesecloth. Make the volume of the broth back to 1000 ml with distilled water and add the tryptone, Dipotassium phosphate, and soluble starch, and refilter. Dispense 15ml of the broth into 20 x150 mm tubes and add liver particles previously removed to a depth of one inch in each tube. Autoclave 20 minutes at 121^{o}C.

Lysine Iron Agar (Edwards And Fife)

Peptone.....	5.0 g
Yeast extract	3.0 g
Glucose	1.0
L-lysine	10.0 g
Ferric ammonium citrate	0.5 g
Sodium thiosulfate	0.04 g
Bromocresol purple	0.02 g
Agar	15.0 g
Distilled water.....	1.0 liter

Dissolve ingredients in distilled water and adjust to pH 6.7$\pm$0.2. Dispense in 14 ml amounts in 100 x 13 mm tubes and sterilize at 121^{o}C for 12 minutes. Slant tubes to obtain a deep butt and a short slant.

Lysozyme Broth

Preparation A - Nutrient Broth: Prepare nutrient broth and dispense 99.0.ml amounts in bottles or flasks. Autoclave 15 minutes at 121^{o}C.

Preparation B - Lysozyme solution: Dissolve 0.1g of lysozyme in 65 ml of sterile 0.0IN hydrochloric acid. Heat to boiling for 20 minutes and dilute to 100.0 ml with sterile 0.01N hydrochloric acid. Alternatively

dissolve 0.1 g of lysozyme chloride in 100.0 ml of distilled water and sterilize by filtration. Test solution for sterility before use.

And 1.0 ml of sterile 0.1% lysozyme solution to each 99.0 ml of nutrient broth. Mix thoroughly and aseptically dispense 2.5 ml of complete medium into sterile 13 x 100 tubes.

MacConkey Agar

Peptone	20.0 g
Lactose	10.0g
Bile salts	1.5 g
Sodium chloride	5.0
Agar	15.0 g
Neutral red	0.03 g
Crystal violet	0.001 g
Distilled water	1.0 liter

Adjust pH to 7.1 sterilize for 15 min at 121^{o}C. Pour in petri-dishes.

Malonate Broth

Yeast extract	1.0g
Ammonium sulfate	2.0g
Dipotassiurn phosphate	0.6g
Monopotassiurn phosphate	0.4g
Sodium chloride	2.0g
Sodium malonate	3.0 g
Glucose	0.25g
Bromthymol blue	0.025 g
Distilled water	1.0 liter

Dissolve ingredients in distilled water by heating, if necessary. Dispense into tubes and autoclave for 15 minutes at 121^{o}C. Final pH 6.7$\pm$.0.1.

Malt Agar

Malt Extract	30.00 g
Agar	15.00 g
Distilled water.....	1.0 liter

Dissolve the ingredients in 1.0 liter distilled water with occasional agitation and boil gently for one minute. Dispense into suitable containers and sterilise at 121^{o}C for 15 minutes.

Malt Agar (Acidified)

Malt agar acidified with 10% sterile tartaric acid to pH 3.5$\pm$0.2. Prepare acid solution by weighing 10.0 g of tartaric acid into beaker and bringing up to 100.0 ml with water. Dissolve and sterilize at 121^{o}C for 15 minutes. Acidify the sterile and tempered medium with a predetermined quantity of acid solution immediately before pouring plates. Do not attempt to reheat medium once acid has been added. Determine accuracy of adjusted pH by pouring an aliquot of the medium into a small beaker, cooling to temperature and placing a recently standardized pH directly into the solidified medium.

Malt Agar (With Antibiotic)

Solution A

Prepare malt agar.

Solution B

Add 500.0mg each, of chlorotetracycline HCl and chloramphenicol to 100.0 ml sterile buffered distilled water and mix. (Not all material dissolves.

Therefore, the suspension must be evenly dispersed prior to pipetting into the medium).

To prepare mixture:

Melt medium (solution A above), temperature to 45±1°C and add 2.0ml of antibiotic solution per 100.0 ml medium.

Motility Test Medium (Motility Agar)

(Tittsler and Sandholzer)

Tryptose	10.0 g
Sodium chloride.....	5.0 g
Agar	5.0 g
Distilled H_2O.....	1.0 liter

Suspend ingredients and heat to boiling to dissolve medium completely. Sterilize by autoclaving 15 minutes at 121° C. Final pH 7.2.

MR-VP Broth

Peptone.....	7.0 g
Glucose	5.0 g
Dispotassium hydrogen phosphate	5.0 g
Distilled water.....	1.0 liter

Adjust pH to 6.9±0.2 and dispense in 10 ml portions in tubes. Sterilize for 15 min at 121°C.

Mycological (Mycophil) Agar

Phytone or Soytone	
(papaic digest of soya meal)	10.0 g
Dextrose.....	10.0 g
Agar	18.0 g
Distilled water.....	1.0 litre

Dissolve ingredients in distilled water with heat and autoclave 12 minutes at 118^{o}C (12 Lb steam pressure for 10 minutes).

For yeast and mold counts of carbonated beverages, sugars, and other similar materials, adjust the pH to 4.5 to 4.7 by adding up to 15.0ml of sterile 10 percent lactic acid to each liter of melted medium prior to plating. Do not reheat after acidification.

Mycophil Agar + Antibiotic

Preparation of antibiotic solution: Add 500.0 mg each of chlortetracycline HCl and chloramphenicol to 100.0 ml sterile phosphate buffered distilled water and mix. (Not all material dissolves, therefore the suspension must be evenly dispersed before pipetting into the medium); Two ml of this solution is added per 100.0 mL of tempered agar giving a final concentration in the medium of 100 mg/l of each of the antibiotics. After swirling, the medium is ready for use.

MYP Agar (Mannitol Yolk Polymyxin)

Preparation A-

Meat extract	1.0 g
Peptone	10.0 g
D-mannitol	10.0 g
Sodium chloride	10.0 g
Phenol red	0.025 g
Agar	15.0 g
Distilled water	900.0 ml

Preparation B – Egg Yolk Emulsion: 50%: Wash fresh eggs with stiff brush and drain. Soak 1 hour in 70% alcohol. Aseptically remove yolk and mix (1+1) with sterile 0.85% sodium chloride solution. (Difco Egg Yolk Enrichment 50% is satisfactory).

Preparation C – Polymyxin B sulfate. This selective agent is obtainable in sterile powdered form (500,000 units, i.e., 50 mg per vial) from Pfizer Inc., New York. To use, add aseptically, by syringe, 5.0 ml sterile distilled water. Mix to dissolve powder. Add 1.0 ml by syringe to a liter of final medium.

Mix ingredients of preparation A in distilled water. Adjust to pH 7.1±0.2. Sterilize at 121°C for 20 minutes, cool to 49±1°C and add 100.0 ml of preparation B and 1.0 ml of preparation C. Mix well, pour into petri dishes, allow to solidify and store in a manner to eliminate excess surface moisture. Plates may be stored at 4°C for 7 days.

MY-40 Agar (Malt, Yeast Extract 40% Sucrose)

Malt extract	20.0 g
Yeast extract	5.0 g
Agar	20.0 g
Sucrose	400.0 g
Distilled water	1.0 liter

Dissolve ingredients in distilled water heating gently. Sterilize 20 minutes at 121°C. pH is not adjusted. Do not overheat.

Nitrate Broth

Beef extract	3.0 g
Peptone	5.0 g
Potassium nitrate	15.0 g
Distilled water	1.0 liter

Dissolve ingredients in distilled water. Distribute in tubes and sterilize for 15 minutes at 121°C. The final pH is 7.0.

Nutrient Broth

Beef extract.....	3.0 g
Peptone.....	5.0 g
Distilled water.....	1.0 liter

Suspend ingredients in distilled water and melt agar by gentle boiling. Dispense into suitable flasks or bottles and sterilize 15 minutes at 121^{o}C. Final pH 7.3.

Nutrient Agar

Beef extract.....	3.0 g
Peptone.....	5.0 g
Distilled water.....	1.0 liter

Heat to dissolve, dispense into tubes or flasks, and autoclave 15 minutes at 121^{o}C. Final pH, 6.7$\pm$0.2.

Peptone Water Diluent

Peptone.....	1.0g
Distilled water.....	1.0 liter
pH.....	7.0g

Sterilize for 15 min at 121^{o}C

Plate Count Agar (PCA) (Standard Methods Agar) (TGE Agar)

Dehydrated yeast extract	2.5 g
Pancreatic digest of casein (Tryptone)	5.0 g
Glucose	1.0 g
Agar	15-18 g
Distilled water	1.0 liter

Adjust pH to 7.0±0.1 dispense in 15ml portrions in tubes or flasks. Sterilize for 15 min at 121°C. Before use melt the medium completely in boiling water and keep the tubes or flaks in water bath at 45 to 48°C.

Phenol Red Carbohydrate Broth

Trypticase.....	10.0 g
Sodium chloride.....	5.0 g
Beef extract (optional)	1.0 g
Phenol red (0.25% solution)	7.2 ml
Distilled water.....	800.0 ml

Dissolve ingredients in distilled water. Dispense 2 ml portions into 13 x 100 mm test tubes containing inverted Durham tubes. Autoclave 15 min at 118°C and let cool.

Dissolve 5 g dulcitol, 10g lactose, or 10g sucrose (as specified in title of test) in 200 ml of distilled water and sterilize by passing through bacteria retaining filter. Aseptically add 0.5 ml sterile filtrate to each tube of sterilized broth after cooling to <45°C, shake gently to mix. Final pH 7.4±0.2.

Potassium Cyanide (KCN) Broth

Basal Broth:

Proteose peptone No. 3 or Polypeptone	3.0 g
Disodium phosphate	5.64 g
Monopotassium phosphate	0.225 g
Sodium chloride	5.0 g
Distilled water.....	1.0 liter

Dissolve ingredients in distilled water with stirring. Autoclave 100.0 ml portions 15 minutes at 121°C. Final pH should be 7.6. Prepare 0.5% potassium cyanide by weighing 0.5 g into 100.0 ml sterile distilled water using pipette filter. Transfer 1.5 ml cold potassium cyanide solution to 100 ml basal broth (precooled). DO NO'T PIPETTE BY MOUTH. Mix. Distribute 1.0 ml portions to sterile 13 x 100 mm tubes and stopper immediately with No.2 corks impregnated with paraffin. (Prepare corks by boiling in paraffin for 5 minutes.) Store medium at 5 to 10°C. Storage life is two weeks. Exercise caution because potassium cyanide is lethal.

Potato Dextrose Agar (Acidified)

Infusion from white potatoes.....	200.0 ml
Dextrose.....	20.0 g
Agar	15.0 g
Distilled water.....	1.0 liter

Suspend ingredients in distilled water and heat mixture to boiling to dissolve. Distribute into tubes or flasks, and autoclave 15 minutes at 121°C (15 lb pressure). When used as plating medium for yeasts and molds, melt in flowing steam or boiling water, cool and acidify to pH 3.5 with sterile 10 percent tartartic acid solution. (For use in the cultivation of yeasts and molds, adjust to the desired pH if different from pH 3.5.) Mix thoroughly and pour into plates. To preserve solidifying properties of the agar do not heat medium after the addition of tartartic acid. For preparation of Potato Dextrose Agar with Antibiotic, add antibiotics as described under Mycophil agar with Antibiotic.

Pseudomonas presumptive test broth

	Single strength	concentrated
DL Arginine…….	2g	3.2g
L Proline ……	1g	1.6g
Anhydrous dipotasium hydrogen sulphate…	1g	1.6g
Magnesium sulphate heptahydrate …….. .	0.5g	0.8g
Anhydrous potassium sulphate…….	10g	16.0g
Water…….	1000ml	1000ml
Ethanol……..	25ml	40ml
pH……	7.2±0.2	

Sterilize ethanol by filtration and add required volume after sterilization of medium by autoclaving at 121±1 ^{o}C for 15 minutes. Store at room temperature up to a maximum of three months.

Pseudomonas confirmation medium

Skim milk powder …….	100g
Yeast extract ……...	3g
Peptone…… ..	10g
Sodium chloride…….	5g
Cetrimide……	0.3g
Water …….	1000ml
Agar agar……	15g
pH…….	7.2± 0.2

Dissolve skim milk powder in 250 ml of warm water. Use magnetic stirrer if required.

Sterilize it at 121±1^{o}C for five minutes (to avoid caramalisation). Dissolve rest of ingredients in 750 ml of water. Adjust pH and autoclave at 121±1^{o}C for 15minutes. Cool the medium to 50 ^{o}C and add aseptically the skim milk powder solution.

Selenite Cystine Broth

Tryptone	5.0 g
Lactose	4.0 g
Disodium hydrogen phosphate	10.0 g
Sodium selenite	4.0 g
L.-cystine	0.01 g
Distilled water	1.0 liter

Dissolve by boiling for 5 min. Dispense 10 ml portions into sterile 16 x 150 mm test tubes. Heat 10 min in flowing steam. Do not autoclave. Final pH,7.0±0.2. Medium is not sterile. Use same day as prepared.

Sulfite Agar

(For the Detection of Thermophilic Anaerobes Producing H_2S)

Tryptone or Trypticase	10.0g
Sodium sulfite (anhydrous)	1.0 g
Agar	20.0 g
Distilled water	1.0 liter

Dissolve ingredients in distilled water, dispense into tubes in about 15.0 ml amounts, and into each tube place an iron nail or a small clean strip of iron or base plate. No adjustment of reaction is necessary. Autoclave 20 minutes at 121^{o}C. Tubes should be used within a week after making.

As an alternate for the iron strip or nail, 10.0 ml of a 5% solution of iron citrate may be substituted in the sulfite medium formula. It is necessary to heat the citrate solution to completely dissolve ferric citrate scales or pearls.

Tetrathionate Broth

Basal medium:

Polypeptone or proteose peptone.....	5.0 g
Bile salts.....	1.0 g
Calcium carbonate	10.0 g
Sodium thiosulfate.....	30.0 g
Distilled water.....	1.0 liter
Iodine solution:	
Iodine	6.0 g
Potassium iodide.....	5.0 g
Distilled water.....	20.0 ml

Heat the ingredients of the basal medium in distilled water to boiling temperature, cool to less than 45^{o}C, add 2.0 ml of iodine solution to each 100.0 ml of base. Add 1.0 ml of 1:1000 solution of brilliant green per 100.0 ml of basal medium. The basal medium, with or without added brilliant green, may be tubed, sterilized at 121^{o}C for 15 minutes, and stored. In this case, iodine solution is added (0.2 ml per 10 ml of medium) prior to use.

Sulfathiazole (0.125 mg per ml of medium) may be added to prevent excessive growth of Proteus.

Thiosulfate-Citrate-Bile Salts-Sucrose Agar (TCBS)

Yeast extract	5.0 g
Polypeptone or Proteose Peptone No. 3	10.0 g
Sucrose.....	20.0 g
Sodium thiosulfate ($5H_2O$)	10.0 g
Sodium citrate ($2H_2O$)	10.0 g
Sodium cholate	3.0 g
Oxgall.....	5.0 g
Sodium chloride.....	10.0 g

Ferric citrate	1.0 g
Bromthymol blue	0.04 g
Thymol blue	0.04 g
Agar	15.0 g
Distilled water	1.0 liter

Distilled ingredients in distilled water by bringing to boil. Adjust pH to 8.6+0.2. This medium should not be autoclaved.

T_1N_1 Agar

Trypticase (pancreatic digest of casein)	10.0 g
Sodium chloride	10.0 g
Agar	15.0 g
Distilled water	1.0 liter

Dissolve ingredients in distilled water by bringing to a boil. Dispense in tubes and sterilize at 121^{o}C for 15 min. Allow to solidify in an inclined position (long slant). Final reaction should be pH 7.2±0.2. To prepare T_1N_1 broth, omit the agar.

Thioglycollate Agar

Pancreatic digest of casein USP	15.0g
L-cystine	0.5 g
Dextrose	5.0 g
Yeast extract	5.0 g
Sodium chloride	2.5 g
Resazurin	0.001 g
Agar	20.0 g
Distilled water	1.0 liter

Suspend ingredients in distilled water and heat to boiling to dissolve completely. Distribute approximately 16.0 ml quantities to 20 x 150 mm

screw capped tubes. Add to each tube with head down, an acid cleaned 6d nail. Sterilize at 121°C for 15 minutes. Final reaction should be 7.0 to 7.1. This medium should be used within one week of preparation.

This medium is the same formulation as Fluid Thioglycollate medium except for the addition of 20.0 grams of agar.

Tryptone Glucose Extract Agar

Beef extract	3.0 g
Tryptone	5.0 g
Dextrose	1.0 g
Agar	15.0 g
Distilled water	1.0 liter

Dissolve ingredients in distilled water by boiling gently. Adjust to pH 7.0±0.2. Distribute in tubes or flasks. Sterilize 15 minutes at 121°C.

Thornley's Semi-Solid Arginine Medium

Peptone	0.1 g
Sodium chloride	0.5 g
Dipotassium hydrogen phosphate	0.03 g
Arginine hydrochloride	1.0 g
Phenol red	0.001 g
Distilled water	1.0 liter

Adjust pH to 7.2. Dispense 15 ml in test tubes and sterilize for 15 min at 121°C.

Triple Sugar Iron Agar (TSI)

Meat extract	3.0 g
Yeast extract	3.0 g
Peptone.....	20.0 g
Sodium chloride.....	5.0 g
Lactose	10.0 g
Sucrose.....	10.0 g
Glucose	1.0 g
Ferrous sulfate	0.3 g
Sodium thiosulphate	0:3 g
Phenol red	0.024 g
Agar	12.0 g
Distilled water	1.0 liter

Adjust pH to 7.4±0.2. Dispense in 10 ml portions into tubes. Sterilise for 12 minutes at 121^{o}C. Allow to set in a sloping position to give a butt of 3 cms.

Tryptone (Tryptophane) Broth

Tryptone or trypticase.....	10.0 g
Distilled water.....	1.0 liter

Dissolve with stirring. Autoclave 15 minutes at 121^{o}C Final pH should be 6.9±0.2.

Tryptone (Trypticase) Soy Broth

Tryptone or Trypticase	17.0 g
Phytone or Soytone	3.0 g
Sodium Chloride.....	5.0...g
Dipotassiuin phosphate.....	2.5...g
Dextrose.....	2.5...g
Distilled water.....	1.0...liter

Dissolve ingredients in distilled water; warm slightly if necessary to complete solution. Dispense Into tubes or bottles, and sterilize by autoclaving 15 minutes at 121°C. Final reaction should be pH 7.3±0.2.

Trypticase Soy Polymyxin Broth

Preparation A

Trypticase peptone	17.0g
Phytone peptone.....	3.0 g
Sodium Chloride.....	5.0 g
Dipotassium phosphate.....	2.5 g
Dextrose.....	2.5 g
Distilled water.....	1.0 liter

Preparation B - Polymyxin B sulfate

This selective agent is available in sterile powdered form (500,000 units, i.e., 50 mg per vial) from Pfizer lnc., N.Y. To use, dissolve.500,000 units of sterile powder in 33.3ml of sterile distilled water to give a 0.15% solution. Store solution at 4°C until used.

Suspend ingredients of preparation A in water. Mix thoroughly. Warm slightly if necessary to complete solution. Dispense 15 ml into 20 x 150 mm culture tubes and sterilize by autoclaving for 15 minutes at 121°C. Just before use, add 0.1 ml of sterile 0.15% polymyxin B sulfate solution to each tube and mix thoroughly.

Tryptose-Sulfite Cycloserine (TSC) Agar

Tryptose	15.0 g
Soytone	5.0 g
Yeast extract	5.0 g
Sodium bisulfite (meta)	1.0 g
Ferric ammonium citrate	1.0 g
Agar	20.0 g
Distilled water.....	1.0 liter

Dissolve ingredients in distilled water and adjust the pH to 7.6±0.2 and autoclave for 10 minutes at 121°C. To each liter of autoclaved medium cooled to 50°C, add 10.0 ml of a 4.0% filter sterilized solution of D-cycloserine to give a final concentration of approximately 400 µg per ml and add 40.0 ml of a sterile 50% egg yolk in saline emulsion per 500.0 ml of medium, with the exception of that used to overlay the plates. Egg Yolk enrichment 50% may be obtained from Difco Laboratories, Detroit, Michigan. Dispense the medium in standard Petri dishes for surface plating. Air dry at room temperature for 24 hours or until the surface of the agar is somewhat dry prior to use. Prepare plates fresh each time they are to be used.

Note: SFP agar base available commercially (Difco) is the same as the above basal medium.

Tyrosine Agar

Preparation A - Prepare nutrient agar and dispense 100.0 ml into bottles. Autoclave 15 minutes at 121°C. Cool to 48°C in water bath.

Preparation B - Add 0.5 g of L-tyrosine to a 20 x 150 mm culture tube and suspend in 10.0 ml of distilled water using a Vortex mixer. Sterilize the suspension by autoclaving for 15 minutes at 121°C.

Mix Preparation A (100 ml) with sterile Preparation B (10 ml) and aseptically dispense .3.5 ml of complete medium into sterile 13 x 100 mm tubes. Slant tubes and cool rapidly to prevent separation of the tyrosine.

Urea Broth

Yeast extract	0.1 g
Monobasic potassium phosphate	0.091 g
Dibasic sodium phosphate	0.095 g
Urea.....	20.0g
Phenol red	0.01 g
Distilled water.....	1.0 liter

Mix ingredients in the distilled water. This medium is filter-sterilized and tubed in sterile tubes in 3.0ml amounts. The basal medium (without urea) may be prepared in 900.0 ml of distilled water and sterilized at 121^{o}C for 15 minutes. After cooling, 100.0 ml of 20% sterile urea solution are added and the medium dispensed in sterile tubes in 3.0 ml amounts.

Inoculation

Three loopful (2mm loop) from an agar slant culture are inoculated into a tube of medium and the tube is shaken to suspend the bacteria.

Incubation

Tubes are incubated in a water bath at 37^{o}C, and the results are read after 10 minutes, 60 minutes, and 2 hours.

Violet Red Bile Agar (VRBA)

Yeast extract	3.0 g
Peptone or Gelysate	7.0 g
Sodium chloride.....	5.0 g
Bile salts or Bile salts No. 3.	1.5 g
Lactose.....	10.0 g
Neutral red	0.03 g
Crystal violet.....	0.002 g
Agar	15.0 g
Distilled water.....	1.0 liter

Suspend the ingredients in distilled water and allow to stand for a few minutes. Mix thoroughly and adjust to pH 7.4+0.2. Heat with agitation and boil for 2 minutes. Do not sterilize. Prior to use, cool to 45^{o}C and use as a plating medium. After solidification, add a cover layer above the agar of approximately 3.0 to 4.0 ml to prevent surface growth and spreading of colonies.

Violet Red Bile Agar + Glucose

Prepare 1000 ml VRBA media. Add 10g of glucose. Heat with agitation and boil for 2 min. Do not autoclave.

V-P Broth (Modified For *B. cereus*)

Proteose peptone.....	7.0 g
Glucose	5.0 g
NaCl.....	5.0 g
Distilled water.....	1.0 liter

Dissolve ingredients in distilled water. Dispense 5.0 ml in 20 mm test tubes. Sterilize 15 minutes at 121^{o}C.

Note: The medium is a modified medium and must be formulated in the laboratory.

Xylose Lysine Deoxycholate Agar (XLD)

Yeast extract	3.0 g
L-lysine	5.0 g
Xylose	3.5 g
Lactose	7.5 g
Sucrose	7.5 g
Sodium chloride	5.0 g
Phenol red	0.08 g
Agar	13.5 g
Distilled water	1.0 liter

Heat mixture in distilled water to boiling temperature to dissolve the ingredients. Sterilize at 121^{o}C for 15 minutes, and then cool to 55 to 60^{o}C. Aseptically add 20.0 ml of sterile solution containing.

Sodium thiosulfate	34.0 g
Ferric ammonium citrate	4.0 g
Distilled water	100.0 ml

Mix well to obtain a uniform suspension.

CHAPTER – 3

EQUIPMENT, MATERIALS AND GLASSWARES

1. Autoclave of sufficient size and number. Used for sterilization of media and for discarded plates / used media, etc (with calibrated thermometer and pressure gauge).
2. Anaerobic jars or incubators with equipment and material for obtaining anaerobic conditions.
3. Balance sensitive to 0.1 g with 200 g load.
4. Blenders with steel jar and lid / Stomacher.
5. Bunsen burners.
6. Colony Counter (Quebec or equivalent).
7. Dilution and media storage bottles. 120, 300, 600 and 1500 ml in capacity.
8. Durham's tubes
9. Glass test tubes 16 x150 mm. Rimless
10. Plastic caps for test tubes
11. Serological test tubes
12. Hot air ovens used for sterilization of glass and metal ware. They should have a thermostat range between 150-185^{o}C.
13. Hockey sticks: Glass bent rods (or suitable plastic make) with fine polished edges, 3-4 mm diameter, 15-20 cms long with angled spreading surface 45-55 mm long or disposable plastic material.
14. Howard Mold Counting Chamber
15. Haemocytometer.

16. Incubators. At least 4 incubators are necessary, to be adjusted at 30°C, 37°C, 44.5°C and 55°C of proper size. B.O.D. incubator for temp. Less than ambient temperature.
17. Inoculating loops and wires. (3-5 mm dia of nichrome or platinum or plastic).
18. Magnetic stirrer
19. Membrane Filtration apparatus, for sterilizing fluids which are affected by heat, e.g. Seitz filter operationalzed membrane filters.
20. Microscope binocular with 900 x and higher magnification.
21. Microscopic slides and cover slips.
22. Non-adsorbant cotton.
23. Petri plate (glass or plastic)
24. Petri plate containers. (Stainless steel or aluminium, with covers) for hot air sterilization of glass petri plates.
25. Pipettes. (glass or plastic) Graduated, with 1, 5 and 10 ml total flow type / Automatic pipette with error < ±5 % with sterilisable or Autopipetor with Pre-sterilized plastic tips.
26. Pipette containers (Stainless steel plastic tip containers boxes)
27. pH meter. Electronic pH meter with accuracy of 0.1 pH unit shall be used.
28. Refrigerator and deep freezer.
29. Test tube racks and baskets to hold test tubes.
30. Thermometers.
31. Vortex – mixer.
32. Water bath for holding media at 44-46°C.
33. Serological water bath
34. Laminar flow chamber
35. Biological safety cabinet level II

CHAPTER – 4

BIOCHEMICAL TESTS

1. Carbohydrate fermentation

Inoculate one tube of each of the carbohydrate media (containing the specific sugar). Incubate at 35^{o}C for 24 hours. Acid production is indicated by a change in color and gas production can be detected by observation of gas collection in the inverted Durham tube.

2. Catalase Test

Flood plates of the suspected culture with 3-5% hydrogen peroxide solution. Bubble formation is indicative of a positive reaction.

Or put a colony on glass slide with the help of a wire loop. Bubble formation is indicative of catalase reaction positive.

3. Citrate Test

Inoculate a tube of Koser citrate medium. Incubate at 35^{o}C for 96 hours. Observe for turbidity due to growth.

4. Coagulase Test

4a. Dessicated coagulase plasma (rabbit) with EDTA; Reconstitute according to manufacture's directions.

4b. If plasma containing EDTA is not available, reconstitute desiccated rabbit plasma and add Na_2H_2 EDTA to a final concentration of 0.1% in the reconstituted plasma. Do not store rehydrated plasma longer than 5 days (at 2-8^{o}C).

Transfer suspected *S. aureus* colonies into tubes containing 5 ml of Brain Heart infusion broth. Incubate 18-24 hours at 35-37^{o}C.

Add 0.5 ml of the coagulase plasma with EDTA to 0.2 ml of broth culture. Incubate at 35-37^{o}C and examine periodically during a 6 hours interval for clot formation. A 3+ or 4+ clot formation is considered a positive reaction for *S. aureus*.

5. Decarboxylase Tests

Inoculate the decarboxylase media (containing either lysine, arginine or ornithine) with a young slant culture. Use an oil seal and inoculate a control tube (no amino acid) with each culture under investigation. Incubate at 37^{o}C. Examine daily for four days. The medium first becomes yellow because of acid production. Later if decarboxylation occurs, the medium becomes alkaline (purple). The control tubes remain acid (yellow).

6. **Hydrogen Sulphide Production**

Inoculate a tube of TSI agar by stabbing the butt and streaking the slope. Incubate for 24-48 hours. Observe for blackening due to H_2S production.

7. **Indole Production**

Kovac's Reagent:

p-Dimethylaminobenzaldehyde.....	5.0 g
Amyl alcohol	75.0 ml
Hydrochloric acid (concentrated).....	25.0 ml

Dissolve p-dimethylaminobenzaldehyde in the amyl alcohol, and then slowly add the hydrochloric acid. To test for indole, add 0.2 to 0.3 ml of reagent to 5.0 ml of a 24 hour culture of bacteria in tryptone broth. A dark red color in the surface layer constitutes a positive test for indole.

8. Growth in Potassium Cyanide Broth

Transfer a loopful of young culture to KCN broth. Incubate for up to 48±2 hours. Turbidity indicates growth and a positive test.

9. Methyl Red Reaction

Methyl Red indicator:

Methyl red..... 0.1 g
Alcohol, 95% (ethanol) ..... 300.0 ml

Dissolve methyl red in 300.0 ml of alcohol, and make up to 500.0 ml with distilled water. Incubate test cultures grown in MR-VP broth for 5 days at 30°C. Alternatively incubate at 37°C for 48 hours. Add 5 or 6 drops of reagent to cultures. Do not perform tests on cultures incubated less than 48 hours. If equivocal results are obtained, repeat tests on cultures incubated for 4 or 5 days. Duplicate tests should be incubated at 22 to 25°C.

10. Motility Test

Inoculate tubes of motility medium by stabbing the medium to a depth of about 5 mm. Incubate at the appropriate temperatures. Motile organisms migrate through the medium which becomes turbid; growth of non-motile organisms is confined to the stab.

11. Nitrate Reduction Test

Solution A:

Sulfanilic acid.....	0.5 g
Glacial acetic acid	30.0 ml
Distilled water	120.0 ml

Solution B:

N-(1-naphthyl) ethylenediamine Dihydrochloride

(Marshal's reagent).....	0.2 g
Glacial acetic acid	30.0 ml
Distilled water	120.0 ml

Cleve's acid (5-amino-2 naphthylene sulfonic acid) may be substituted for Marshal's Reagent.

To 3.0 ml of an 18 hour culture in nitrate broth, add two drops of solution A and two drops of solution B. A red violet color which develops within 10 minutes indicates that nitrate has been reduced to nitrite. If the reaction is negative, one must examine for residual nitrate since conceivably the nitrate may have been reduced further. Add a few grains of powdered zinc. If a red violet color does not develop, nitrate has been reduced. Perform tests on uninoculated medium as a control.

12. Oxidase (Cytochrome oxidase) Test (Kovac's method)

Oxidase testing Reagent:

Tetramethylparaphenylenediamine-2-HCl	.....0.25 g
Distilled water.....……	25.0 ml

Store at 4^{o}C. A fresh solution should be made each week.

Soak small pieces of the filter paper in the reagent solution. Some filter paper gives a blue color and these must not be used. Dry or use wet. Scrape some of a fresh young culture with a glass rod and rub on the filter paper. A blue color within 1 minute is a postive oxidase test.

13. Urease test

Inoculate the urea media heavily with the culture being tested and incubate for 24 hours at 35^{o}C. If urease is present, the urea is split to form ammonia, which changes the color of the indicator from yellow to pink.

14. Voges Proskauer (VP) Reaction:

Solution A:

Alpha-naphthol ..... 5.0 g
Absolute ethanol..... 100.0 ml

Solution B:

Potassium hydroxide .....40.0 g
Distilled water to make .....100.0 ml

Perform Voges-Proskauer (V-P) test at room temperature by transferring 1 ml of 48 hour culture to test tube and adding 0.6 ml of alpha-naphthol (Solution A) and 0.2 ml of 40% potassium hydroxide (Solution B); shake after addition of each solution. To intensify and speed reactions, add a few crystals of creatine to test medium. Read results 4 hours after adding reagents. Positive V-P test is the development of an eosin pink color.

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