Dissolve all the solid components in the water, and add the glycerin. Heat, with frequent agitation, boil for 1 minute, and sterilize by heating in an autoclave at 121°C for 15 to 20 minutes. pH after sterilization: 7.0 – 7.4.

(xx) Vogel-Johnson Agar Medium

Casein peptone	10.0 g
Yeast extract	5.0 g
D-Mannitol	10.0 g
Dipotassium hydrogenphosphate	5.0 g
Lithium chloride	5.0 g
Glycine	10.0 g
Phenol red	0.025 g
Agar	16.0 g
Water	1000 mL

Mix all the components, and boil for 1 minute to effect solution. Sterilize by heating in an autoclave at 121°C for 15 to 20 minutes, and cool to between 45°C and 50°C. pH after sterilization: 7.0 – 7.4. To this solution add 20 mL of sterile potassium tellurite solution (1 in 100), and mix.

(xxi) Baird-Parker Agar Medium

Casein peptone	10.0 g
Meat extract	5.0 g
Yeast extract	1.0 g
Lithium chloride	5.0 g
Glycine	12.0 g
Sodium pyruvate	10.0 g
Agar	20.0 g
Water	950 mL

Mix all the components. Heat the mixture with frequent agitation, and boil for 1 minute. Sterilize by heating in an autoclave at 121°C for 15 to 20 minutes, and cool to between 45°C and 50°C. pH after sterilization: 6.6 – 7.0. To this solution add 10 mL of sterile potassium tellurite solution (1 in 100) and 50 mL of egg-yolk emulsion. Mix gently, and pour into petri dishes. Prepare the egg-yolk emulsion by mixing egg-yolk and sterile saline with the ratio of about 30% to 70%.

(xxii) Mannitol-Salt Agar Medium

Casein peptone	5.0 g
Animal tissue peptone	5.0 g
Meat extract	1.0 g
D-Mannitol	10.0 g
Sodium chloride	75.0 g
Phenol red	0.025 g
Agar	15.0 g
Water	1000 mL

Mix all the components. Heat with frequent agitation, and boil for 1 minute. Sterilize by heating in an autoclave at 121°C for 15 to 20 minutes. pH after sterilization: 7.2 – 7.6.

36. Microbial Limit Test for Crude Drugs

This chapter provides tests for the qualitative and quantitative estimation of viable microorganisms present in crude drugs. It includes tests for total viable count (aerobic bacteria and fungi) and specified microbial species (Enterobacteria and other gram-negative bacteria, *Escherichia coli*, *Salmonella*, and *Staphylococcus aureus*). Microbial limit test must be carried out under conditions designed to avoid accidental microbial contamination of the preparation during the test. When test specimens have antimicrobial activity, or contain antimicrobial substances, any such antimicrobial properties must be eliminated by means of procedures such as dilution, filtration, neutralization or inactivation. For the test, use a mixture of several portions selected at random from the bulk or from the contents of a sufficient number of containers. If test specimens are diluted with fluid medium, the test should be performed quickly. In performing the test, precautions must be taken to prevent biohazard.

1. Total viable aerobic count

This test determines mesophilic aerobic bacteria and fungi (molds and yeasts) which grow under aerobic conditions. Psychrophilic, thermophilic, basophilic, and anaerobic bacteria, and microorganisms which require specific ingredients for growth, may give a negative result, even if a significant number exists in the test speciments. The test may be carried out using one of the following 4 methods, i.e., pour plate method, spread plate method, serial dilution method (most probable number method) or membrane filtration method. Use an appropriate method depending on the purpose. An automated method may be used for the test presented here, provided it has been properly validated as giving equivalent or better results. Different culture media and temperature are required for the growth of bacteria and fungi (molds and yeasts). The serial dilution method is applicable only to the enumeration of bacteria.

Sampling and Preparation of the test specimens

Unless otherwise specified, samples should be taken by the following methods.

(1) When crude drugs to be sampled are small-sized, cut or powdered, 50 to 250 g of sample should be taken after mixing thoroughly.

(2) When crude drugs to be sampled are large-sized, 250 to 500 g of sample should be taken after mixing thoroughly and cutting.

(3) When the mass of each single piece of the crude drug is not less than 100 g, not less than 5 pieces should be taken for a sample, or not less than 500 g of the sample should be taken after cutting to a suitable size and mixing thoroughly. If necessary, cut more for use.

(4) When crude drugs to be sampled are in the form of a solution or a preparation, the sample should be taken after mixing thoroughly.

(5) An insoluble solid should be taken after reducing the substance to a moderately fine powder.

Preparation of the test fluid

Phosphate Buffer, pH 7.2, Buffered Sodium Chloride-Peptone Solution, pH 7.0 or fluid medium used for the test is used to suspend or dilute the test specimen. Unless otherwise specified, usually take 10 g or 10 mL of the test specimen, and suspend or dissolve it in 90 mL of the buffer or fluid medium specified. A test specimen as a suspension must be shaken for 10 minutes. If necessary, for crude drugs to which microorganisms might adhere, repeat the same method and use this as the test fluid. A different quantity or volume may be used if the nature of the test specimen requires it. The pH of the test fluid is adjusted to between 6 and 8. The test fluid must be used within an hour after preparation.

Fluid specimen: Take 10 mL of the test specimen, and suspend or dissolve it in 90 mL of the buffer or fluid medium specified. A different quantity or volume may be used if the nature of the test specimen requires it.

Insoluble solids: Reduce the substance to a moderately fine powder, take 10 g of the test specimen, and suspend it in 90 mL of the buffer or fluid medium specified. A different quantity or a larger volume of buffer and fluid medium than indicated may be used for the suspension, if the nature of the test specimen requires it. The suspension may be dispersed well using, if necessary, a mechanical blender. A suitable surface active agent (such as 0.1 w/v% Polysorbate 80) may be added to aid dissolution.

Test procedures

(1) Pour Plate Method

Use petri dishes 9 to 10 cm in diameter. Use at least two petri dishes for each dilution. Pipet 1 mL of the test fluid or its diluted solution onto each petri dish aseptically. Promptly add to each dish 15 to 20 mL of sterilized agar medium that has previously been melted and kept below 45°C, and mix. Primarily for the detection of aerobic microbes, use Soybean-Casein Digest Agar Medium. For specimens that consist of fragments of crude drugs, or to control the growth of fungi, 2,3,5-triphenyl-2*H*-tetrazolium chloride (TTC) TS for aerobic bacterial strains and amphotericin B TS as an antimycotic may be added to the agar. Just prior to use, add 2.5–5 mL of TTC TS or 2 mL of amphotericin B TS per liter of sterile medium and mix. Primarily for the detection of fungi, use one of Sabouraud Glucose Agar with antibiotics, Potato Dextrose Agar with antibiotics, and GP Agar Medium with antibiotics. For an agar medium that is suffused with fungi, Rose Bengal TS may be added to the agar. Add 5 mL of Rose Bengal TS per liter of agar medium, mix and sterilize by heating in an autoclave at 121°C for 15 to 20 minutes. After the agar solidifies, incubate the plates for at least 5 days at between 30°C and 35°C for aerobic bacteria, and between 20°C and 25°C for fungi. If too many colonies are observed, dilute the fluid as described above so that a colony count of not more than 300 per plate may be expected in the case of aerobic bacteria, and not more than 100 per plate in the case of fungi. If a reliable count is obtained in a shorter incubation time than 5 days, this may be adopted.

(2) Spread Plate Method

On the solidified and dried surface of the agar medium, pipet 0.05 to 0.2 mL of the test fluid and spread it on the surface with a spreader. The diameter of petri dishes, the kind and volume of the medium to be used, TS to be added, temperature and time of incubation, and the method for calculation of total viable count are the same as described in the Pour Plate Method section.

(3) Serial Dilution Method (Most Probable Number Method)

Prepare tubes each containing 9 to 10 mL of Fluid Soybean-Casein Digest Medium. To each of the first three tubes add 1 mL of the test fluid (containing 0.1 g or 0.1 mL of specimen), resulting in 1 in 10 dilution. To the next three tubes add 1 mL of a 1 in 10 dilution of the fluid, resulting in 1 in 100 dilution. To the next three tubes add 1 mL of a 1 in 100 dilution of the fluid, resulting in 1 in 1000 dilution. If necessary, dilute further. To the last three tubes add 1 mL of the diluent as a control. Incubate the tubes between 30°C and 35°C for not less than 5 days. The control tubes should show no microbial growth. If the reading of the results is difficult or uncertain, transfer about 0.1 mL to a liquid or solid medium and read the results after a further period of incubation between 30°C and 35°C for 24 to 72 hours. Determine the most probable number of microorganisms per g or per mL of the specimen from Table 1.

Table 1. Most probable number of microorganisms

Number of tubes in which microbial growth is observed for each quantity of the specimen			Most probable number of microorganisms per g or per mL
0.1 g or 0.1 mL per tube	0.01 g or 0.01 mL per tube	1 mg or 1 μL per tube	
3	3	3	>1100
3	3	2	1100
3	3	1	500
3	3	0	200
3	2	3	290
3	2	2	210
3	2	1	150
3	2	0	90
3	1	3	160
3	1	2	120
3	1	1	70
3	1	0	40
3	0	3	95
3	0	2	60
3	0	1	40
3	0	0	23

If, for the first column (0.1 g or 0.1 mL of specimen), the number of tubes showing microbial growth is two or less, the most probable number of microorganisms per g or per mL is likely to be less than 100.

(4) Membrane Filtration Method

This method employs membrane filters of appropriate materials, having a normal pore size not greater than 0.45 μm. Filter discs about 50 mm in diameter are recommended, but filters of a different diameter may also be used. Filters, the filtration apparatus, media, etc., should be well sterilized. Usually, take 20 mL of the test fluid (containing 2 g of test specimen), transfer 10 mL of the solution to each of two membrane filters, and filter. If necessary, dilute the pretreated preparation so that a colony count of 10 to 100 may be expected. After the filtration of the test fluid, wash each membrane by filtering through it three or more times with a suitable liquid such as Buffered Sodium Chloride-Peptone Solution, pH 7.0, Phosphate Buffer, pH 7.2, or the fluid medium to be used. The volume of the washing to be used is approximately 100 mL each time, but if the filter disc is not about 50 mm in diameter, the volume may be adjusted according to the size of the filter. For fatty substances, the washings may contain a suitable surface-active agent such as Polysorbate 80. Put one of the membrane filters, intended primarily for the enumeration of aerobic bacteria, on the surface of a plate of Soybean-Casein Digest Agar and the other, intended primarily for the enumeration of fungi, on the surface of a plate of one of Sabouraud Glucose Agar with antibiotics, Potato Dextrose Agar with antibiotics, and GP Agar Medium with antibiotics. After incubation of the

plates for at least 5 days, at between 30°C and 35°C in the test for the detection of aerobic bacteria and between 20°C and 25°C in the test for fungi, count the number of colonies that are formed. If a reliable count is obtained in a shorter incubation time than 5 days, this may be adopted.

Effectiveness of culture media and confirmation of antimicrobial substances

Use microorganisms of the following strains or their equivalent. Grow them in Fluid Soybean-Casein Digest Medium between 30°C and 35°C for aerobic bacteria and between 20°C and 25°C for *Candida albicans*.

Escherichia coli,	IFO 3972, ATCC 8739, NCIB 8545, etc.
Bacillus subtilis,	IFO 3134, ATCC 6633, NCIB 8054, etc.
Staphylococcus aureus,	IFO 13276, ATCC 6538, NCIB 8625, etc.
Candida albicans,	IFO 1393, IFO 1594, ATCC 2091, ATCC 10231, etc.

Dilute portions of each of the cultures using Buffered Sodium Chloride-Peptone Solution, pH 7.0, or Phosphate Buffer, pH 7.2 to prepare test suspensions containing 50 to 200 cfu per mL. Growth-promoting qualities are tested by inoculating 1 mL of each microorganism into each medium. The test media are satisfactory if clear evidence of growth appears in all inoculated media after incubation at the indicated temperature for 5 days. When a count of test organisms with a test specimen is less than 1/5 of that without the test specimen, any such effect must be eliminated by dilution, filtration, neutralization or inactivation. To confirm the sterility of the medium and of the diluent and the aseptic performance of the test, carry out the total viable count method using sterile Buffered Sodium Chloride-Peptone Solution, pH 7.0 or Phosphate Buffer, pH 7.2 as the control.

2. Test for the detection of specified microorganisms

Enterobacteria and certain other gram-negative bacteria, *Escherichia coli, Salmonella* and *Staphylococcus aureus*, are included as target strains of the test.

Sampling and Preparation of the test specimens

Refer to the paragraph on Sampling and Preparation of the Test Solution in Total viable aerobic count.

Preparation of the test fluid

If necessary, refer to the paragraph on Preparation of the Test Fluid in Total viable aerobic count. When test specimens are prepared, use the medium designated in each test, unless otherwise specified. If necessary, eliminate antimicrobial substances so as to permit growth of the inocula, and adjust the quantity of test specimen or increase the volume of medium to suitable values.

Test Procedure

(1) Enterobacteria and certain other gram-negative bacteria

(i) Detection of bacteria

To 10 g or 10 mL of the test specimen add 90 mL of Fluid Lactose Medium to form a suspension or solution. Transfer 10 mL to 90 mL of Enterobacteria enrichment broth Mossel and incubate at between 35°C and 37°C for 18 to 24 hours. Mix by gently shaking the container, take a portion by means of an inoculating loop, and streak it on the surface of Crystal violet, Neutral red, Bile Agar with glucose. Incubate at between 35°C and 37°C for 18 to 24 hours. If red or reddish colonies are found, the specimen may contain Enterobacteria and certain other gram-negative bacteria.

(ii) Quantitative evaluation

If Enterobacteria and certain other gram-negative bacteria are found, to 10 g or 10 mL of the test specimen add 90 mL of Fluid Lactose Medium to form a suspension or solution. Transfer 1 mL of the test fluid (containing 0.1 g or 0.1 mL of specimen) to a tube containing 9 mL of the fluid, and mix. Next, transfer 1 mL of the test fluid (containing 0.01 g or 0.01 mL of specimen) to a tube containing 9 mL of the fluid, and mix. Furthermore, transfer 1 mL of the test fluid (containing 1 mg or 1 μL of specimen) to a tube containing 9 mL of the fluid, and mix. If necessary, repeat the same method with 0.1 mg or 0.1 μL of specimen. Incubate the tubes at between 35°C and 37°C for 18 to 24 hours, take a portion by means of an inoculating loop, and streak it on the surface of Crystal violet, Neutral red, Bile Agar with glucose. Incubate at between 35°C and 37°C for 18 to 24 hours. If red or reddish colonies are found, this constitutes a positive result. Note the smallest quantity of the product which gives a positive result and the largest quantity that gives a negative result. Determine from Table 2 the probable number of microorganisms.

Table 2. Most probable number of microorganisms

Results for each quantity of product				Probable number of microorganisms (cfu) per g or per mL
0.1 g or 0.1 mL	0.01 g or 0.01 mL	1 mg or 1 μL	0.1 mg or 0.1 μL	
+	+	+	−	more than 10^3
+	+	−	−	less than 10^3 and more than 10^2
+	−	−	−	less than 10^2 and more than 10^1
−	−	−	−	less than 10^1

(2) *Escherichia coli*

(i) Detection of bacteria

To 10 g or 10 mL of the test specimen add 90 mL of Fluid Lactose Medium to make a suspension or solution. Transfer 1 mL to a fermentation tube containing 9 to 10 mL of EC broth and incubate the tube at 44.5±0.2°C for 24±2 hours in a water bath. If gas bubbles are not found, the specimen meets the requirements of the test for absence of *Escherichia coli*. If gas bubbles are found, take a portion by means of an inoculating loop, and streak it on the surface of EMB Agar Medium. Incubate at between 30°C and 35°C for 18 to 24 hours. Upon examination, if none of the colonies exhibits both a characteristic metallic sheen and a blue-black appearance under transmitted light, the specimen meets the requirements of the test for absence of *Escherichia coli*. Confirm any suspect colonies on the plate by means of the IMViC test. (Indole production test, Methyl red reaction test, Voges-Proskauer test, and Citrate utilization test); colonies which exhibit the pattern of either [+ + − −] or [− + − −] are judged as *Escherichia coli*. Rapid detection kits for *Escherichia coli* may also be used.

(ii) Quantitative evaluation

If *Escherichia coli* is found, prepare tubes each containing 9 to 10 mL of EC broth. Use three tubes for each dilution. To 10 g or 10 mL of the test specimen add 90 mL of Fluid Lactose Medium and suspend or dissolve. To each of

the first three fermentation tubes add 1 mL of the test fluid (containing 0.1 g or 0.1 mL of specimen), resulting in 1 in 10 dilution. To the next three fermentation tubes add 1 mL of a 1 in 10 dilution of the fluid, resulting in 1 in 100 dilution. To the next three fermentation tubes add 1 mL of a 1 in 100 dilution of the fluid, resulting in 1 in 1000 dilution. To the last three fermentation tubes add 1 mL of the diluent as a control. Incubate the tubes at 44.5±0.2°C for 24±2 hours in a water bath. If gas bubbles are found, take a portion by means of an inoculating loop, and streak it on the surface of EMB Agar Medium. Incubate at between 30°C to 35°C for 18 to 24 hours. Upon examination, colonies of Gram-negative organisms show both a characteristic metallic sheen and a blue-black appearance under transmitted light. Determine the most probable number of microorganisms per g or per mL of the specimen from Table 1.

(3) *Salmonella*

As in the case of the detection of *Escherichia coli*, to 10 g or 10 mL of the test specimen add 90 mL of Fluid Lactose Medium to form a suspension or solution. Incubate at between 30°C to 35°C for 24 to 72 hours. Examine the medium for growth, and if growth is apparent, mix by gentle shaking, then pipet 1 mL portions, respectively, into 10 mL of Fluid Selenite-Cystine Medium and Fluid Tetrathionate Medium, and incubate for 12 to 24 hours. 10 mL of Fluid Selenite-Cystine Medium may be replaced by the same volume of Fluid Rappaport Medium. After the incubation, streak portions from both the fluid media on the surface of at least two of Brilliant Green Agar Medium, XLD Agar Medium, and Bismuth Sulfite Agar Medium, and incubate at between 30°C and 35°C for 24 to 48 hours. Upon examination, if none of the colonies conforms to the description given in Table 3, the specimen meets the requirements of the test for the absence of the genus *Salmonella*. If colonies of Gram-negative rods matching the description in Table 3 are found, transfer suspect colonies individually, by means of an inoculating wire, to a slant of TSI Agar Medium using both surface and deep inoculation. Incubate at between 35°C and 37°C for 18 to 24 hours. The presence of genus *Salmonella* is confirmed if, in the deep culture but not in the surface culture, there is a change of color from red to yellow and usually formation of gas with or without production of hydrogen sulfide. Precise identification and typing of genus *Salmonella* may be carried out by using appropriate biochemical and serological tests additionally, including an identification kit.

Table 3. Morphologic characteristics of *Salmonella* species on selective agar media

Medium	Description of colony
Brilliant Green Agar Medium	Small, transparent and colorless, or opaque, pink or white (often surrounded by a pink to red zone)
XLD Agar Medium	Red, with or without a black center
Bismuth Sulfite Agar Medium	Black or green

(4) *Staphylococcus aureus*

To 10 g or 10 mL of the test specimen add 90 mL of Fluid Soybean-Casein Digest Medium, or another suitable fluid medium without antimicrobial activity, to form a suspension or solution. Incubate the fluid containing the specimen at between 30°C and 35°C for 24 to 48 hours, and pipet 1 mL into 9 mL of Fluid Soybean-Casein Digest Medium with 7.5% sodium chloride. If, upon examination, growth is apparent, use an inoculating loop to streak a portion of the medium on the surface of one of Vogel-Johnson Agar Medium, Baird-Parker Agar Medium, or Mannitol-Salt Agar Medium, and incubate at between 30°C and 35°C for 24 to 48 hours. Upon examination, if no colonies of Gram-positive rods having the characteristics listed in Table 4 are found, the specimen meets the requirements of the test for the absence of *Staphylococcus aureus*. Confirm any suspect colonies as *Staphylococcus aureus* by means of the coagulase test. With the aid of an inoculating loop, transfer suspect colonies to individual tubes, each containing 0.5 mL of mammalian, preferably rabbit or horse, plasma with or without suitable additives. Incubate in a thermostat at 37±1°C. Examine the coagulation after 3 hours and subsequently at suitable intervals up to 24 hours. Test positive and negative controls simultaneously. If no coagulation is observed, the specimen meets the requirements of the test for the absence of *Staphylococcus aureus*.

Table 4. Morphologic characteristics of *Staphylococcus aureus* on selective agar media

Medium	Colonial characteristics
Vogel-Johnson Agar Medium	Black surrounded by a yellow zone
Baird-Parker Agar Medium	Black, shiny, surrounded by a clear zone
Mannitol-Salt Agar Medium	Yellow colonies surrounded by a yellow zone

Effectiveness of culture media and confirmation of antimicrobial substances

Grow the test strains listed in Table 5 in the media indicated at between 30°C and 35°C for 18 to 24 hours. Dilute portions of each of the cultures using Buffered Sodium Chloride-Peptone Solution, pH 7.0, Phosphate Buffer, pH 7.2, or medium indicated for each bacterial strain to make test suspensions containing about 1000 cfu per mL. As occasion demands, using a mixture of 0.1 mL of each suspension of *Escherichia coli*, *Salmonella* and *Staphylococcus aureus* containing about 1000 cfu, test the validity of the medium and the presence of antimicrobial substances in the presence or absence of the specimen.

Table 5. Bacteria strains and media used for confirmation of the effectiveness of culture medium and validity of the test for specified microorganisms

Microorganism	Strain number	Media
Escherichia coli	IFO 3972, ATCC 8739, NCIB 8545 or equivalent strains	Fluid Lactose Medium
Salmonella	No strain number is recommended*	Fluid Lactose Medium
Staphylococcus aureus	IFO 13276, ATCC 6538, NCIB 8625 or equivalent strains	Fluid Soybean-Casein Digest Medium

**Salmonella* strains of weak or no pathogenicity may be used. *Salmonella* Typhi may not be used.

Retest

For the purpose of confirming a doubtful result, a retest is conducted using a test specimen 2.5 times larger than the first test specimen. Proceed as directed under Test procedure, but make allowance for the larger specimen size, for example by adjusting the volume of the medium.

3. Buffer solution, media and test solution (TS)

Buffer solution, media and TS used for the microbial limit test are described below. Other media may be used if they have similar nutritive ingredients, and selective and growth-promoting properties for the microorganisms to be tested.

(1) Buffer solution

(i) Phosphate Buffer, pH 7.2

Stock Solution: Dissolve 34 g of monobasic potassium phosphate in about 500 mL of water. Adjust to pH 7.1 to 7.3 by the addition of 175 mL of sodium hydroxide TS, add water to make 1000 mL, and use this solution as the stock solution. After sterilization by heating in an autoclave, store under refrigeration. For use, dilute the Stock Solution with water in the ratio of 1 to 800, and sterilize at 121°C for 15 to 20 minutes.

(ii) Buffered Sodium Chloride-Peptone Solution, pH 7.0

Monobasic potassium phosphate	3.56 g
Disodium hydrogenphosphate 12-water	18.23 g
Sodium chloride	4.30 g
Peptone	1.0 g
Water	1000 mL

Mix all the components, and sterilize by heating in an autoclave at 121°C for 15 to 20 minutes. pH after sterilization: 6.9 - 7.1. Polysorbate 20 or 80 (0.1 to 1.0 w/v%) may be added.

(2) Media

(i) Soybean-Casein Digest Agar Medium

Casein peptone	15.0 g
Soybean peptone	5.0 g
Sodium chloride	5.0 g
Agar	15.0 g
Water	1000 mL

Mix all the components, and sterilize by heating in an autoclave at 121°C for 15 to 20 minutes. pH after sterilization: 7.1 - 7.3.

(ii) Fluid Soybean-Casein Digest Medium

Casein peptone	17.0 g
Soybean peptone	3.0 g
Sodium chloride	5.0 g
Dipotassium hydrogenphosphate	2.5 g
Glucose	2.5 g
Water	1000 mL

Mix all the components, and sterilize by heating in an autoclave at 121°C for 15 to 20 minutes. pH after sterilization: 7.1 - 7.5.

(iii) Sabouraud Glucose Agar Medium with Antibiotics

Peptone (animal tissue and casein)	10.0 g
Glucose	40.0 g
Agar	15.0 g
Water	1000 mL

Mix all the components, and sterilize by heating in an autoclave at 121°C for 15 to 20 minutes. pH after sterilization: 5.4 - 5.8. Just prior to use, add 0.10 g of benzylpenicillin potassium and 0.10 g of tetracycline per liter of medium as sterile solutions or, alternatively, add 50 mg of chloramphenicol per liter of medium.

(iv) Potato Dextrose Agar Medium with Antibiotics

Potato extract	4.0 g
Glucose	20.0 g
Agar	15.0 g
Water	1000 mL

Mix all the components, and sterilize by heating in an autoclave at 121°C for 15 to 20 minutes. pH after sterilization: 5.4 - 5.8. Just prior to use, add 0.10 g of benzylpenicillin potassium and 0.10 g of tetracycline per liter of medium as sterile solutions or, alternatively, add 50 mg of chloramphenicol per liter of medium.

(v) GP (Glucose-peptone) Agar Medium with Antibiotics

Glucose	20.0 g
Yeast extract	2.0 g
Magnesium sulfate	0.5 g
Peptone	5.0 g
Monobasic potassium phosphate	1.0 g
Agar	15.0 g
Water	1000 mL

Mix all the components, and sterilize by heating in an autoclave at 121°C for 15 to 20 minutes. pH after sterilization: 5.6 - 5.8. Just prior to use, add 0.10 g of benzylpenicillin potassium and 0.10 g of tetracycline per liter of medium as sterile solutions or, alternatively, add 50 mg of chloramphenicol per liter of medium.

(vi) Fluid Lactose Medium

Meat extract	3.0 g
Gelatin peptone	5.0 g
Lactose monohydrate	5.0 g
Water	1000 mL

Mix all the components, and sterilize by heating in an autoclave at 121°C for 15 to 20 minutes. pH after sterilization: 6.7 - 7.1. After sterilization, cool immediately.

(vii) Fluid EC Medium

Peptone	20.0 g
Lactose monohydrate	5.0 g
Bile salts	1.5 g
Dipotassium hydrogenphosphate	4.0 g
Monobasic potassium phosphate	1.5 g
Sodium chloride	5.0 g
Water	1000 mL

Mix all the components, and sterilize by heating in an autoclave at 121°C for 15 to 20 minutes. pH after sterilization: 6.8 - 7.0. After sterilization cool immediately. If after cool, do not use the tube that gas remains in durham tube.

(viii) EMB (Eosin-Methylene Blue) Agar Medium

Gelatin peptone	10.0 g
Dipotassium hydrogenphosphate	2.0 g
Lactose monohydrate	10.0 g
Agar	15.0 g
Eosin	0.40 g
Methylene blue	0.065 g
Water	1000 mL

Mix all the components, and sterilize by heating in an autoclave at 121°C for 15 to 20 minutes. pH after sterilization: 6.9 - 7.3.

(ix) Fluid Enterobacteria Enrichment Broth Mossel Medium

Gelatin peptone	10.0 g
Glucose	5.0 g

Bile salts	20.0 g
Monobasic potassium phosphate	2.0 g
Disodium hydrogenphosphate 12-water	8.0 g
Brilliant green	0.015 g
Water	1000 mL

Mix all the components, boil at 100°C for 30 minutes, and cool immediately. pH after boiling: 7.0 - 7.4.

(x) VRB (Violet/Red/Bile) Agar with glucose

Yeast extract	3.0 g
Gelatin peptone	7.0 g
Bile salts	1.5 g
Lactose monohydrate	10.0 g
Sodium chloride	5.0 g
Glucose	10.0 g
Agar	15.0 g
Neutral red	0.030 g
Crystal violet	0.002 g
Water	1000 mL

Mix all the components, and boil to effect solution. pH after boiling: 7.2 - 7.6. Do not sterilize in an autoclave.

(xi) Fluid Selenite-Cystine Medium

Gelatin peptone	5.0 g
Lactose monohydrate	4.0 g
Trisodium phosphate 12-water	10.0 g
Sodium acid selenite	4.0 g
L-Cystine	0.010 g
Water	1000 mL

Mix all the components, and heat to effect solution. Final pH: 6.8 - 7.2. Do not sterilize.

(xii) Fluid Tetrathionate Medium

Casein peptone	2.5 g
Animal tissue peptone	2.5 g
Sodium desoxycholate	1.0 g
Calcium carbonate	10.0 g
Sodium thiosulfate pentahydrate	30.0 g
Water	1000 mL

Heat the solution of solids to boiling. On the day of use, add a solution prepared by dissolving 5 g of potassium iodide and 6 g of iodine in 20 mL of water. Then add 10 mL of a solution of brilliant green (1 in 1000), and mix. Do not heat the medium after adding the brilliant green solution.

(xiii) Fluid Rappaport Medium

Soybean peptone	5.0 g
Sodium chloride	8.0 g
Monobasic potassium chloride	1.6 g
Malachite green	0.12 g
Magnesium chloride hexahydrate	40.0 g
Water	1000 mL

Dissolve malachite green and magnesium chloride hexahydrate, and the remaining solids separately in the water, and sterilize by heating in an autoclave at 121°C for 15 to 20 minutes. For the use, mix the both solutions after sterilization. Final pH: 5.4 - 5.8.

(xiv) Brilliant Green Agar Medium

Peptones (animal tissue and casein)	10.0 g
Yeast extract	3.0 g
Sodium chloride	5.0 g
Lactose monohydrate	10.0 g
Sucrose	10.0 g
Phenol red	0.080 g
Brilliant green	0.0125 g
Agar	20.0 g
Water	1000 mL

Mix all the components, and boil for 1 minute. Sterilize just prior to use by heating in an autoclave at 121°C for 15 to 20 minutes. pH after sterilization: 6.7 - 7.1. Cool to about 50°C and pour to petri dishes.

(xv) XLD (Xylose-Lysine-Desoxycholate) Agar Medium

D-Xylose	3.5 g
L-Lysine monohydrochloride	5.0 g
Lactose monohydrate	7.5 g
Sucrose	7.5 g
Sodium chloride	5.0 g
Yeast extract	3.0 g
Phenol red	0.080 g
Sodium desoxycholate	2.5 g
Sodium thiosulfate pentahydrate	6.8 g
Ammonium iron (III) citrate	0.80 g
Agar	13.5 g
Water	1000 mL

Mix all the components, and boil to effect solution. pH after boiling: 7.2 - 7.6. Do not sterilize in an autoclave or overheat. Cool to about 50°C and pour to petri dishes.

(xvi) Bismuth Sulfite Agar Medium

Meat extract	5.0 g
Casein peptone	5.0 g
Animal tissue peptone	5.0 g
Glucose	5.0 g
Trisodium phosphate 12-water	4.0 g
Iron (II) sulfate heptahydrate	0.30 g
Bismuth sulfite indicator	8.0 g
Brilliant green	0.025 g
Agar	20.0 g
Water	1000 mL

Mix all the components, and boil to effect solution. pH after boiling: 7.4 - 7.8. Do not sterilize in an autoclave or overheat. Cool to about 50°C and pour to petri dishes.

(xvii) TSI (Triple Sugar Iron) Agar Medium

Casein peptone	10.0 g
Animal tissue peptone	10.0 g
Lactose monohydrate	10.0 g
Sucrose	10.0 g
Glucose	1.0 g
Ammonium iron (II) sulfate hexahydrate	0.20 g
Sodium chloride	5.0 g
Sodium thiosulfate pentahydrate	0.20 g
Phenol red	0.025 g
Agar	13.5 g
Water	1000 mL

Mix all the components, and boil to effect solution. Distribute in small tubes and sterilize by heating in an autoclave at 121°C for 15 to 20 minutes. pH after sterilization: 7.1 - 7.5. Use as a slant agar medium. The medium containing 3 g of meat extract or yeast extract additionally, or the medium containing ammonium iron (III) citrate instead of ammonium iron (II) sulfate hexahydrate may be used.

(xviii) Fluid Soybean-Casein Digest Medium with 7.5% Sodium Chloride

Casein peptone	17.0 g
Soybean peptone	3.0 g
Sodium chloride	75.0 g
Dibasic potassium phosphate	2.5 g
Glucose	2.5 g
Water	1000 mL

Add the sodium chloride 70.0 g for (ii) Fluid Soybean-Casein Digest Medium (containing 5 g of sodium chloride), mix all the components, and sterilize by heating in an autoclave at 121°C for 15 to 20 minutes. pH after sterilization: 7.1 - 7.5.

(xix) Vogel-Johnson Agar Medium

Casein peptone	10.0 g
Yeast extract	5.0 g
D-Mannitol	10.0 g
Dipotassium hydrogenphosphate	5.0 g
Lithium chloride	5.0 g
Glycine	10.0 g
Phenol red	0.025 g
Agar	16.0 g
Water	1000 mL

Mix all the components, and boil for 1 minute to effect solution. Sterilize by heating in an autoclave at 121°C for 15 to 20 minutes, and cool to between 45°C and 50°C. pH after sterilization: 7.0 - 7.4. To this solution add 20 mL of sterile potassium tellurite solution (1 in 100), and mix.

(xx) Baird-Parker Agar Medium

Casein peptone	10.0 g
Meat extract	5.0 g
Yeast extract	1.0 g
Lithium chloride	5.0 g
Glycine	12.0 g
Sodium pyruvate	10.0 g
Agar	20.0 g
Water	950 mL

Mix all the components. Heat the mixture with frequent agitation, and boil for 1 minute. Sterilize by heating in an autoclave at 121°C for 15 to 20 minutes, and cool to between 45°C and 50°C. pH after sterilization: 6.6 - 7.0. To this solution add 10 mL of sterile potassium tellurite solution (1 in 100) and 50 mL of egg-yolk emulsion. Mix gently, and pour into petri dishes. Prepare the egg-yolk emulsion by mixing egg-yolk and sterile saline with the ratio of about 30% to 70%.

(xxi) Mannitol-Salt Agar Medium

Casein peptone	5.0 g
Animal tissue peptone	5.0 g
Meat extract	1.0 g
D-Mannitol	10.0 g
Sodium chloride	75.0 g
Phenol red	0.025 g
Agar	15.0 g
Water	1000 mL

Mix all the components. Heat with frequent agitation, and boil for 1 minute. Sterilize by heating in an autoclave at 121°C for 15 to 20 minutes. pH after sterilization: 7.2 - 7.6.

(3) Reagent·Test solution

Amphotericin B powder Amphotericin B added sodium deoxycholic acid, sterilized by γ-ray.

Amphotericin B TS Dissolve 22.5 mg of amphotericin B powder in 9 mL of sterile purified water.

Bile salts Yellow-brown powder made from dried bile of animal, consist of sodium taurocholic acid and sodium glycocholic acid, and containing not less than 45% of cholic acid. pH of 5% solution: 5.5 - 7.5.

Rose bengal $C_{20}H_2Cl_4I_4Na_2O_5$ [Special class] Red-brown powder, purple-red solution in water.

Rose bengal TS Dissolve 1 g of rose bengal in water to make 100 mL.

2,3,5-Triphenyl-2*H*-tetrazolium chloride TS (TTC TS) Prepare a 0.8% TTC solution (dissolve 0.8 g of TTC in water to make 100 mL), distribute in small tubes and sterilize by heating in an autoclave at 121°C for 15 to 20 minutes. Store in light-resistant containers.

(4) Preparation

Preparation of agar medium with TTC

Just prior to use, add 2.5 to 5 mL of TTC TS per liter of sterile medium and mix.

Preparation of agar medium with amphotericin B

Dissolve 22.5 mg of amphotericin B sterilized by γ-ray in 9 mL of sterile purified water. Just prior to use, add 2 mL of this solution in a liter of sterile medium and mix.

Preparation of agar medium with rose bengal

Add 5 mL of rose bengal TS in a liter of medium and mix. Sterilize in an autoclave at 121°C for 15 to 20 minutes.

37. Mineral Oil Test

The Mineral Oil Test is a method to test mineral oil in nonaqueous solvents for injections and for eye drops.

Procedure

Pour 10 mL of the sample into a 100-mL flask, and add 15 mL of sodium hydroxide solution (1 in 6) and 30 mL of ethanol (95). Put a short-stemmed, small funnel on the neck of the flask, and heat on a water bath to make clear, with frequent shaking. Then transfer the solution to a shallow porcelain dish, evaporate the ethanol on a water bath, add 100 mL of water to the residue, and heat on a water bath: no turbidity is produced in the solution.

38. Nitrogen Determination (Semimicro-Kjeldahl Method)

The Nitrogen Determination is a method to determine ammonia in ammonium sulfate obtained by decomposition of organic substances containing nitrogen with sulfuric acid.

Apparatus

Use the apparatus illustrated in the figure. It is thoroughly constructed of hard glass, and ground glass surfaces may be used for joints. All rubber parts used in the apparatus should be boiled for 10 to 30 minutes in sodium hydroxide TS and for 30 to 60 minutes in water, and finally washed thoroughly with water before use.

Procedure

Unless otherwise specified, proceed by the following method. Weigh accurately or pipet a quantity of the sample corresponding to 2 to 3 mg of nitrogen (N:14.01), and place in the Kjeldahl flask A. Add 1 g of a powdered mixture of 10 g of potassium sulfate and 1 g of cupper (II) sulfate pentahydrate. Wash down any adhering sample from the neck of the flask with a small quantity of water. Add 7 mL of sulfuric acid, allowing it to flow down the inside wall of the flask.