3.3.2 MICROBIOLOGICAL EXAMINATION OF NON-STERILE PRODUCTS: TESTS FOR SPECIFIED MICROORGANISMS

Final text for addition to The International Pharmacopoeia

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3.3.2 MICROBIOLOGICAL EXAMINATION OF NON-STERILE PRODUCTS: TESTS FOR SPECIFIED MICROORGANISMS

The tests described hereafter will allow determination of the absence of, or limited occurrence of specified microorganisms which may be detected under the conditions described.

The tests are designed primarily to determine whether a substance or preparation complies with an established specification for microbiological quality. When used for such purposes follow the instructions given below, including the number of samples to be taken and interpret the results as stated below.

Alternative microbiological procedures, including automated methods may be used, provided that their equivalence to the pharmacopoeial method has been demonstrated.

GENERAL PROCEDURES

The preparation of samples is carried out as described in 3.3.1 Microbial enumeration tests.

If the product to be examined has antimicrobial activity, this is insofar as possible removed or neutralized as described in 3.3.1 Microbial enumeration tests.

If surface-active substances are used for sample preparation, their absence of toxicity for microorganisms and their compatibility with inactivators used must be demonstrated as described in 3.3.1 Microbial enumeration tests.
GROWTH PROMOTING AND INHIBITORY PROPERTIES OF THE MEDIA, SUITABILITY OF THE TEST AND NEGATIVE CONTROLS

The ability of the test to detect microorganisms in the presence of the product to be tested must be established. Suitability must be confirmed if a change in testing performance, or the product, which may affect the outcome of the test is introduced.

Preparation of test strains

Use standardized stable suspensions of test strains or prepare as stated below. Seed-lot culture maintenance techniques (seed-lot systems) are used so that the viable microorganisms used for inoculation are not more than 5 passages removed from the original master seed-lot.

**Aerobic microorganisms**

Grow each of the bacterial test strains separately in containers containing casein soya bean digest broth or on casein soya bean digest agar at 30–35 °C for 18–24 h. Grow the test strain for *Candida albicans* separately on Sabouraud-dextrose agar or in Sabouraud-dextrose broth at 20–25 °C for 2–3 days.

*Staphylococcus aureus* such as ATCC 6538, NCIMB 9518, CIP 4.83 or NBRC 13276, *Pseudomonas aeruginosa* such as ATCC 9027, NCIMB 8626, CIP 82.118 or NBRC 13275, *Escherichia coli* such as ATCC 8739, NCIMB 8545, CIP 53.126 or NBRC 3972, *Salmonella enterica* subsp. *enterica* serovar Typhimurium such as ATCC 14028 or, as an alternative, *Salmonella enterica* subsp. *enterica* serovar Abony such as NBRC 100797, NCTC 6017 or CIP 80.39, *Candida albicans* such as ATCC 10231, NCPF 3179, IP 48.72 or NBRC 1594.

Use buffered sodium chloride-peptone solution, sterile, pH 7.0, TS or phosphate buffer sterile, pH 7.2, TS to make test suspensions. Use the suspensions within 2 h or within 24 h if stored at 2–8 °C.

**Clostridia**

Use *Clostridium sporogenes* such as ATCC 11437 (NBRC 14293, NCIMB 12343, CIP 100651) or ATCC 19404 (NCTC 532 or CIP 79.3). Grow the clostridial test strain under anaerobic conditions in reinforced medium for Clostridia at 30–35 °C for 24–48 h. As an alternative to preparing and then diluting down a fresh suspension of vegetative cells of *Cl. sporogenes*, a stable spore suspension is used for test inoculation. The stable spore suspension may be maintained at 2–8 °C for a validated period.

**Negative control**

To verify testing conditions a negative control is performed using the chosen diluent in place of the test preparation. There must be no growth of microorganisms. A negative control is also performed when testing the products as described under *Testing of products*. A failed negative control requires an investigation.
Growth promotion and inhibitory properties of the media

Test each batch of ready-prepared medium and each batch of medium prepared either from dehydrated medium or from ingredients.

Verify suitable properties of relevant media as described in Table 1.

Table 1. Growth-promoting, inhibitory and indicative properties of media

<table>
<thead>
<tr>
<th>Medium</th>
<th>Property</th>
<th>Test strains</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Test for bile-tolerant gram-negative bacteria</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Enterobacteria enrichment broth-Mossel</em></td>
<td>Growth-promoting</td>
<td><em>E. coli</em></td>
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<tr>
<td></td>
<td></td>
<td><em>P. aeruginosa</em></td>
</tr>
<tr>
<td></td>
<td>Inhibitory</td>
<td><em>S. aureus</em></td>
</tr>
<tr>
<td><em>Violet red bile glucose agar</em></td>
<td>Growth-promoting</td>
<td><em>E. coli</em></td>
</tr>
<tr>
<td></td>
<td>+ Indicative</td>
<td><em>P. aeruginosa</em></td>
</tr>
<tr>
<td><strong>Test for <em>Escherichia coli</em></strong></td>
<td></td>
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</tr>
<tr>
<td><em>MacConkey broth</em></td>
<td>Growth-promoting</td>
<td><em>E. coli</em></td>
</tr>
<tr>
<td></td>
<td>Inhibitory</td>
<td><em>S. aureus</em></td>
</tr>
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<td><em>MacConkey agar</em></td>
<td>Growth-promoting</td>
<td><em>E. coli</em></td>
</tr>
<tr>
<td></td>
<td>+ Indicative</td>
<td></td>
</tr>
<tr>
<td><strong>Test for <em>Salmonella</em></strong></td>
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<td></td>
</tr>
<tr>
<td><em>Rappaport Vassiliadis Salmonella enrichment broth</em></td>
<td>Growth-promoting</td>
<td><em>Salmonella enterica subsp.</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>enterica serovar Typhimurium</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>or <em>Salmonella enterica subsp.</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>enterica serovar Abony</em></td>
</tr>
<tr>
<td></td>
<td>Inhibitory</td>
<td><em>S. aureus</em></td>
</tr>
<tr>
<td><em>Xylose, lysine, deoxycholate agar</em></td>
<td>Growth-promoting</td>
<td><em>Salmonella enterica subsp.</em></td>
</tr>
<tr>
<td></td>
<td>+ Indicative</td>
<td><em>enterica serovar Typhimurium</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>or <em>Salmonella enterica subsp.</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>enterica serovar Abony</em></td>
</tr>
<tr>
<td><strong>Test for <em>Pseudomonas aeruginosa</em></strong></td>
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<tr>
<td><em>Cetrimide agar</em></td>
<td>Growth-promoting</td>
<td><em>P. aeruginosa</em></td>
</tr>
<tr>
<td></td>
<td>Inhibitory</td>
<td><em>E. coli</em></td>
</tr>
</tbody>
</table>
Test for Staphylococcus aureus

<table>
<thead>
<tr>
<th>Mannitol salt agar</th>
<th>Growth-promoting + Indicative</th>
<th>S. aureus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inhibitory</td>
<td>E. coli</td>
</tr>
</tbody>
</table>

Test for Clostridia

<table>
<thead>
<tr>
<th>Reinforced medium for Clostridia</th>
<th>Growth-promoting</th>
<th>Cl. sporogenes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Columbia agar</td>
<td>Growth-promoting</td>
<td>Cl. sporogenes</td>
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</tbody>
</table>

Test for Candida albicans

<table>
<thead>
<tr>
<th>Sabouraud dextrose broth</th>
<th>Growth-promoting</th>
<th>C. albicans</th>
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</thead>
<tbody>
<tr>
<td>Sabouraud dextrose agar</td>
<td>Growth-promoting + Indicative</td>
<td>C. albicans</td>
</tr>
</tbody>
</table>

Test for growth promoting properties, liquid media: inoculate a portion of the appropriate medium with a small number (not more than 100 CFU) of the appropriate microorganism. Incubate at the specified temperature for not more than the shortest period of time specified in the test. Clearly visible growth of the microorganism comparable to that previously obtained with a previously tested and approved batch of medium occurs.

Test for growth promoting properties, solid media: perform surface-spread method, inoculating each plate with a small number (not more than 100 CFU) of the appropriate microorganism. Incubate at the specified temperature for not more than the shortest period of time specified in the test. Growth of the microorganism comparable to that previously obtained with a previously tested and approved batch of medium occurs.

Test for inhibitory properties, liquid or solid media: inoculate the appropriate medium with at least 100 CFU of the appropriate microorganism. Incubate at the specified temperature for not less than the longest period of time specified in the test. No growth of the test microorganism occurs.

Test for indicative properties: perform surface-spread method, inoculating each plate with a small number (not more than 100 CFU) of the appropriate microorganism. Incubate at the specified temperature for a period of time within the range specified in the test. Colonies are comparable in appearance and indication reactions to those previously obtained with a previously tested and approved batch of medium.

Suitability of the test method

For each product to be tested perform sample preparation as described in the relevant paragraph in the section below on Testing of products. Add each test strain at the time of mixing, in the prescribed growth medium. Inoculate the test strains individually. Use a number of microorganisms equivalent to not more than 100 CFU in the inoculated test preparation.

Perform the test as described in the relevant paragraph in Testing of products section using the shortest incubation period prescribed.

The specified microorganisms must be detected with the indication reactions as described below under Testing of products.

Any antimicrobial activity of the product necessitates a modification of the test procedure (see
Neutralization/removal of antimicrobial activity of 3.3.1 *Microbial Enumeration Tests*).

If for a given product the antimicrobial activity with respect to a microorganism for which testing is prescribed cannot be neutralized, then it is to be assumed that the inhibited microorganism will not be present in the product.

**TESTING OF PRODUCTS**

**Bile-tolerant gram-negative bacteria**

*Sample preparation and pre-incubation*
Prepare a sample using a 1 in 10 dilution of not less than 1 g of the product to be examined as described in 3.3.1 *Microbial enumeration tests*, but using casein soya bean digest broth as the chosen diluent, mix and incubate at 20–25 °C for a time sufficient to resuscitate the bacteria but not sufficient to encourage multiplication of the organisms (usually 2 h but not more than 5 h).

*Test for absence*
Unless otherwise prescribed use the volume corresponding to 1g of the product, as prepared in *Sample preparation and pre-incubation* to inoculate *enterobacteria enrichment broth-Mossel*. Incubate at 30–35 °C for 24–48 h. Subculture on plates of *violet red bile glucose agar*. Incubate at 30–35 °C for 18–24 h.

The product complies with the test if there is no growth of colonies.

*Quantitative test*

*Selection and subculture.* Inoculate suitable quantities of *enterobacteria enrichment broth-Mossel* with the preparation as described under *Sample preparation and pre-incubation* and/or dilutions of it containing, respectively 0.1 g, 0.01 g and 0.001 g (or 0.1 ml, 0.01 ml and 0.001 ml) of the product to be examined. Incubate at 30–35 °C for 24–48 h. Subculture each of the cultures on a plate of *violet red bile glucose agar*. Incubate at 30–35 °C for 18–24 h.

*Interpretation.* Growth of colonies constitutes a positive result. Note the smallest quantity of the product that gives a positive result and the largest quantity that gives a negative result. Determine from Table 2 the probable number of bacteria.

Table 2. Interpretation of results

<table>
<thead>
<tr>
<th>Results for each quantity of product</th>
<th>Probable number of bacteria per gram or ml of product</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 g or 0.1 ml</td>
<td>0.01 g or 0.01 ml</td>
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<tr>
<td>+</td>
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<tr>
<td>+</td>
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<td>+</td>
<td>-</td>
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</tbody>
</table>
**Escherichia coli**

*Sample preparation and pre-incubation*

Prepare a sample using a 1 in 10 dilution of not less than 1 g of the product to be examined as described in 3.3.1 *Microbial enumeration tests* and use 10 ml or the quantity corresponding to 1 g or 1 mL to inoculate a suitable amount (determined as described under *Suitability of the test method*) of *casein soya bean digest broth*, mix and incubate at 30–35 °C for 18–24 h.

*Selection and subculture*

Shake the container, transfer 1 mL of *casein soya bean digest broth* to 100 mL of *MacConkey broth* and incubate at 42–44 °C for 24–48 h. Subculture on a plate of *MacConkey agar* at 30–35 °C for 18–72 h.

*Interpretation*

Growth of colonies indicates the possible presence of *E. coli*. This is confirmed by identification tests.

The product complies with the test if no colonies are present or if the identification tests are negative.

**Salmonella**

*Sample preparation and pre-incubation*

Prepare the product to be examined as described in 3.3.1 *Microbial enumeration tests* and use the quantity corresponding to not less than 10 g or 10 mL to inoculate a suitable amount (determined as described under *Suitability of the test method*) of *casein soya bean digest broth*, mix and incubate at 30–35 °C for 18–24 h.

*Selection and subculture*

Transfer 0.1 mL of *casein soya bean digest broth* to 10 mL of *Rappaport Vassiliadis Salmonella enrichment broth* and incubate at 30–35 °C for 18–24 h. Subculture on plates of *xylose, lysine, deoxycholate agar*. Incubate at 30–35 °C for 18–48 h.

*Interpretation*

The possible presence of *Salmonella* is indicated by the growth of well-developed, red colonies, with or without black centres. This is confirmed by identification tests.

The product complies with the test if colonies of the types described are not present or if the confirmatory identification tests are negative.

**Pseudomonas aeruginosa**

*Sample preparation and pre-incubation*

Prepare a sample using a 1 in 10 dilution of not less than 1 g of the product to be examined as described in 3.3.1 *Microbial enumeration tests* and use 10 ml or the quantity corresponding to 1 g or 1 mL to inoculate a suitable amount (determined as described under *Suitability of the test method*) of *casein soya bean digest broth* and mix. When testing transdermal patches, filter the volume of sample corresponding to 1 patch of the preparation described under *Preparation of the sample in 3.3.1 Microbial enumeration tests* through a sterile filter membrane and place in 100 mL of *casein soya bean digest broth*. Incubate at 30–35 °C for 18–24 h.
Selection and subculture
Subculture on a plate of cetrimide agar and incubate at 30–35 °C for 18–72 h.

Interpretation
Growth of colonies indicates the possible presence of *P. aeruginosa*. This is confirmed by identification tests.

The product complies with the test if colonies are not present or if the confirmatory identification tests are negative.

Staphylococcus aureus

Sample preparation and pre-incubation
Prepare a sample using a 1 in 10 dilution of not less than 1 g of the product to be examined as described in 3.3.1 Microbial enumeration tests and use 10 ml or the quantity corresponding to 1 g or 1 ml to inoculate a suitable amount (determined as described under Suitability of the test method) of casein soya bean digest broth and homogenize. When testing transdermal patches, filter the volume of sample corresponding to 1 patch of the preparation described under Preparation of the sample in 3.3.1 Microbial enumeration tests through a sterile filter membrane and place in 100 ml of casein soya bean digest broth. Incubate at 30–35 °C for 18–24 h.

Selection and subculture
Subculture on a plate of mannitol salt agar and incubate at 30–35 °C for 18–72 h.

Interpretation
The possible presence of *S. aureus* is indicated by the growth of yellow/white colonies surrounded by a yellow zone. This is confirmed by identification tests.

The product complies with the test if colonies of the types described are not present or if the confirmatory identification tests are negative.

Clostridia

Sample preparation and heat treatment
Prepare a sample using a 1 in 10 dilution (with a minimum total volume of 20 ml) of not less than 2 g or 2 ml of the product to be examined as described in 3.3.1 Microbial enumeration tests. Divide the sample into two portions of at least 10 ml. Heat 1 portion at 80 °C for 10 minutes and cool rapidly. Do not heat the other portion.

Selection and subculture
Use 10 ml or the quantity corresponding to 1 g or 1 ml of the product to be examined of both portions to inoculate suitable amounts (determined as described under Suitability of the test method) of Reinforced clostridium medium. Incubate under anaerobic conditions at 30–35 °C for 48 h. After incubation, make subcultures from each container on Columbia agar and incubate under anaerobic conditions at 30–35 °C for 48–72 h.

Interpretation
The occurrence of anaerobic growth of rods (with or without endospores) giving a negative catalase reaction indicates the presence of *Clostridia*. This is confirmed by identification tests.
The product complies with the test if colonies of the types described are not present or if the confirmatory identification tests are negative.

**Candida albicans**

*Sample preparation and pre-incubation*

Prepare the product to be examined as described in 3.3.1 *Microbial enumeration tests* and use 10 ml or the quantity corresponding to not less than 1 g or 1 ml to inoculate 100 ml of *Sabouraud-dextrose broth* and mix. Incubate at 30–35 °C for 3–5 days.

*Selection and subculture*

Subculture on a plate of *Sabouraud-dextrose agar* and incubate at 30–35 °C for 24–48h.

*Interpretation*

Growth of white colonies may indicate the presence of *C. albicans*. This is confirmed by identification tests.

The product complies with the test if such colonies are not present or if the confirmatory identification tests are negative.

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**RECOMMENDED TEST SOLUTIONS AND CULTURE MEDIA**

The following test solutions and culture media have been found satisfactory for the purposes for which they are prescribed in the test for microbial contamination in the pharmacopoeia. Other media may be used provided that their suitability can be demonstrated.

*Stock buffer solution.* Transfer 34 g of potassium dihydrogen phosphate to a 1000 ml volumetric flask, dissolve in 500 ml of purified water, adjust to pH 7.2 ± 0.2 with sodium hydroxide, add purified water to volume and mix. Dispense in containers and sterilize. Store at a temperature of 2–8 °C.

*Phosphate buffer, sterile, pH 7.2, TS.* Prepare a mixture of purified water and stock buffer solution (800:1 V/V) and sterilize.

- **Buffered sodium chloride-peptone solution pH 7.0**
  - Potassium dihydrogen phosphate 3.6 g
  - Disodium hydrogen phosphate dihydrate 7.2 g equivalent to 0.067 M phosphate
  - Sodium chloride 4.3 g
  - Peptone (meat or casein) 1.0 g
  - Purified water 1000 ml

  Sterilize in an autoclave using a validated cycle.
- **Casein soya bean digest broth**
  Pancreatic digest of casein 17.0 g
  Papaic digest of soya bean 3.0 g
  Sodium chloride 5.0 g
  Dipotassium hydrogen phosphate 2.5 g
  Glucose monohydrate 2.5 g
  Purified water 1000 ml

  Adjust the pH so that after sterilization it is 7.3 ± 0.2 at 25 °C. Sterilize in an autoclave using a validated cycle.

- **Casein soya bean digest agar**
  Pancreatic digest of casein 15.0 g
  Papaic digest of soya bean 5.0 g
  Sodium chloride 5.0 g
  Agar 15.0 g
  Purified water 1000 ml

  Adjust the pH so that after sterilization it is 7.3 ± 0.2 at 25 °C. Sterilize in an autoclave using a validated cycle.

- **Sabouraud-dextrose agar**
  Dextrose 40.0 g
  Mixture of peptic digest of animal tissue and pancreatic digest of casein (1:1) 10.0 g
  Agar 15.0 g
  Purified water 1000 ml

  Adjust the pH so that after sterilization it is 5.6 ± 0.2 at 25 °C. Sterilize in an autoclave using a validated cycle.

- **Potato dextrose agar**
  Infusion from potatoes 200 g
  Dextrose 20.0 g
  Agar 15.0 g
  Purified water 1000 ml

  Adjust the pH so that after sterilization it is 5.6 ± 0.2 at 25 °C. Sterilize in an autoclave using a validated cycle.

- **Sabouraud-dextrose broth**
  Dextrose 20.0 g
  Mixture of peptic digest of animal tissue and pancreatic digest of casein (1:1) 10.0 g
  Purified water 1000 ml
Adjust the pH so that after sterilization it is 5.6 ± 0.2 at 25 °C. Sterilize in an autoclave using a validated cycle.

- **Enterobacteria enrichment broth-Mossel**
  Pancreatic digest of gelatin 10.0 g
  Glucose monohydrate 5.0 g
  Dehydrated ox bile 20.0 g
  Potassium dihydrogen phosphate 2.0 g
  Disodium hydrogen phosphate dihydrate 8.0 g
  Brilliant green 15 mg
  Purified water 1000 ml

  Adjust the pH so that after heating it is 7.2 ± 0.2 at 25 °C. Heat at 100 °C for 30 minutes and cool immediately.

- **Violet red bile glucose agar**
  Yeast extract 3.0 g
  Pancreatic digest of gelatin 7.0 g
  Bile salts 1.5 g
  Sodium chloride 5.0 g
  Glucose monohydrate 10.0 gl
  Agar 15.0 g
  Neutral red 30 mg
  Crystal violet 2 mg
  Purified water 1000 ml

  Adjust the pH so that after heating it is 7.4 ± 0.2 at 25 °C. Heat to boiling; do not heat in an autoclave.

- **MacConkey broth**
  Pancreatic digest of gelatin 20.0 g
  Lactose monohydrate 10.0 g
  Dehydrated ox bile 5.0 g
  Bromocresol purple 10 mg
  Purified water 1000 ml

  Adjust the pH so that after sterilization it is 7.3 ± 0.2 at 25 °C. Sterilize in an autoclave using a validated cycle.

- **MacConkey agar**
  Pancreatic digest of gelatin 17.0 g
  Peptones (meat and casein) 3.0 g
  Lactose monohydrate 10.0 g
  Sodium chloride 5.0 g
Bile salts 1.5 g
Agar 13.5 g
Neutral red 30.0 mg
Crystal violet 1 mg
Purified water 1000 ml

Adjust the pH so that after sterilization it is 7.1 ± 0.2 at 25 °C. Boil for 1 minute with constant shaking then sterilize in an autoclave using a validated cycle.

- **Rappaport Vassiliadis Salmonella Enrichment Broth**
  Soya peptone 4.5 g
  Magnesium chloride hexahydrate 29.0 g
  Sodium chloride 8.0 g
  Dipotassium phosphate 0.4 g
  Potassium dihydrogen phosphate 0.6 g
  Malachite green 0.036 g
  Purified water 1000 ml

  Dissolve, warming slightly. Sterilize in an autoclave using a validated cycle, at a temperature not exceeding 115 °C. The pH is to be 5.2 ± 0.2 at 25 °C after heating and autoclaving.

- **Xylose, lysine, deoxycholate agar**
  Xylose 3.5 g
  L-Lysine 5.0 g
  Lactose monohydrate 7.5 g
  Sucrose 7.5 g
  Sodium chloride 5.0 g
  Yeast extract 3.0 g
  Phenol red 80 mg
  Agar 13.5 g
  Sodium deoxycholate 2.5 g
  Sodium thiosulfite 6.8 g
  Ferric ammonium citrate 0.8 g
  Purified water 1000 ml

  Adjust the pH so that after heating it is 7.4 ± 0.2 at 25 °C. Heat to boiling, cool to 50 °C and pour into Petri dishes. Do not heat in an autoclave.

- **Cetrimide agar**
  Pancreatic digest of gelatin 20.0 g
  Magnesium chloride 1.4 g
  Dipotassium sulfate 10.0 g
  Cetrimide 0.3 g
Agar       13.6 g
Purified water       1000 ml
Glycerol       10.0 ml

Heat to boiling for 1 minute with shaking. Adjust the pH so that after sterilization it is 7.2 ± 0.2 at 25 °C. Sterilize in an autoclave using a validated cycle.

- **Mannitol salt agar**
  - Pancreatic digest of casein      5.0 g
  - Peptic digest of animal tissue      5.0 g
  - Beef extract      1.0 g
  - D-Mannitol      10.0 g
  - Sodium chloride      75.0 g
  - Agar      15.0 g
  - Phenol red      0.025 g
  - Purified water      1000 mL

Heat to boiling for 1 minute with shaking. Adjust the pH so that after sterilization it is 7.4 ± 0.2 at 25 °C. Sterilize in an autoclave using a validated cycle.

- **Reinforced medium for Clostridia**
  - Beef extract      10.0 g
  - Peptone      10.0 g
  - Yeast extract      3.0 g
  - Soluble starch      1.0 g
  - Glucose monohydrate      5.0 g
  - Cysteine hydrochloride      0.5 g
  - Sodium chloride      5.0 g
  - Sodium acetate      3.0 g
  - Agar      0.5 g
  - Purified water      1000 ml

Hydrate the agar, dissolve by heating to boiling with continuous stirring. If necessary, adjust the pH so that after sterilization it is about 6.8 ± 0.2 at 25 °C. Sterilize in an autoclave using a validated cycle.
- **Columbia agar**
  - Pancreatic digest of casein: 10.0 g
  - Meat peptic digest: 5.0 g
  - Heart pancreatic digest: 3.0 g
  - Yeast extract: 5.0 g
  - Maize starch: 1.0 g
  - Sodium chloride: 5.0 g
  - Agar, according to gelling power: 10.0 g to 15.0 g
  - Purified water: 1000 ml

  Hydrate the agar, dissolve by heating to boiling with continuous stirring. If necessary, adjust the pH so that after sterilization it is 7.3 ± 0.2 at 25 °C. Sterilize in an autoclave using a validated cycle. Allow to cool to 45–50 °C; add, where necessary, gentamicin sulfate corresponding to 20 mg of gentamicin base and pour into Petri dishes.

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