# ⟨2021⟩ MICROBIAL ENUMERATION TESTS—NUTRITIONAL AND DIETARY SUPPLEMENTS

## INTRODUCTION

This chapter provides tests for the estimation of the number of viable aerobic microorganisms present in nutritional supplements of all kinds, from raw materials to the finished forms. Alternative methods may be substituted for the tests, provided that they have been properly validated as giving equivalent or better results. In preparing for and in applying the tests, observe aseptic precautions in handling the specimens. The term "growth" is used in a special sense herein, i.e., to designate the presence and presumed proliferation of viable microorganisms.

## PREPARATORY TESTING

The validity of the results of the tests set forth in this chapter rests largely upon the adequacy of a demonstration that the test specimens to which they are applied do not, of themselves, inhibit the multiplication, under the test conditions, of microorganisms that may be present. Therefore, preparatory to conducting the tests on a regular basis and as circumstances require subsequently, inoculate diluted specimens of the material to be tested with separate viable cultures of the challenge microorganisms.

For the *Soybean–Casein Digest Agar Medium* used for *Total Aerobic Microbial Count*, inoculate duplicate plates with 25–250 cfu of *Staphylococcus aureus* (ATCC[1] No. 6538), *Escherichia coli* (ATCC No. 8739), and *Bacillus subtilis* (ATCC No. 6633) to demonstrate a greater than 70% bioburden recovery in comparison to a control medium. For the *Sabouraud Dextrose Agar Medium* used for *Total Combined Molds and Yeasts Count*, inoculate duplicate plates with 25–250 cfu of *Candida albicans* (ATCC No. 10231) and *Aspergillus brasiliensis* (ATCC No. 16404) to demonstrate a greater than 70% bioburden recovery in comparison to a control medium. For *Enterobacterial Count (Bile-Tolerant Gram-Negative Bacteria)*, appropriate dilutions of *Escherichia coli* (ATCC No. 8739) and *Salmonella typhimurium* (ATCC No. 13311) are used. Failure of the organism(s) to grow in the relevant medium invalidates that portion of the examination and necessitates a modification of the procedure by (1) an increase in the volume of diluent, the quantity of test material remaining the same, or by (2) the incorporation of a sufficient quantity of suitable inactivating agent(s) in the diluents, or by (3) an appropriate combination of modifications to (1) and (2) so as to permit growth of the inoculum.

The following are examples of ingredients and their concentrations that may be added to the culture medium to neutralize inhibitory substances present in the sample: soy lecithin, 0.5%; and polysorbate 20, 4.0%. Alternatively, repeat the test as described in the preceding paragraph, using *Fluid Casein Digest–Soy Lecithin–Polysorbate 20 Medium* to demonstrate neutralization of preservatives or other antimicrobial agents in the test material. Where inhibitory substances are contained in the product and the latter is soluble, a suitable, validated adaptation of a procedure set forth under *Procedure* using the *Membrane Filtration Method* may be used.

If, in spite of the incorporation of suitable inactivating agents and a substantial increase in the volume of diluent, it is still not possible to recover the viable cultures described above, and where the article is not suitable for the employment of membrane filtration, it can be assumed that the failure to isolate the inoculated organism is attributable to the bactericidal or bacteriostatic activity of such magnitude that treatments are not able to remove the activity. This information serves to indicate that the article is not likely to allow proliferation or contamination with the given species of microorganism. Monitoring should be continued in order to determine the inhibitory range and bactericidal activity of the article.

## BUFFER SOLUTION AND MEDIA

Culture media may be prepared as follows, or dehydrated culture media may be used provided that, when reconstituted as directed by the manufacturer or distributor, they have similar ingredients and/or yield media comparable to those obtained from the formulas given herein.

In preparing media by the formulas set forth herein, dissolve the soluble solids in the water, using heat if necessary to effect complete solution, and add solutions of hydrochloric acid or sodium hydroxide in quantities sufficient to yield the desired pH in the medium when it is ready for use. Determine the pH at 25 ± 2°.

Where agar is called for in a formula, use agar that has a moisture content of NMT 15%. Where water is called for in a formula, use *Purified Water*.

### pH 7.2 Phosphate Buffer

Prepare a stock solution by dissolving 34 g of monobasic potassium phosphate in about 500 mL of water contained in a 1000-mL volumetric flask. Adjust to a pH of 7.2 ± 0.1 by the addition of sodium hydroxide TS (about 175 mL), add water to volume, and mix. Dispense and sterilize. Store under refrigeration. For use, dilute the stock solution with water in the ratio of 1–800, dispense as desired, and sterilize.

[1] Available from ATCC, 10801 University Boulevard, Manassas, VA 20110-2209. Equivalent microorganisms, provided that they are from a national collection repository, can be used in lieu of ATCC strains. However, the viable microorganisms used in the test must not be more than five passages removed from the original ATCC or national collection culture.

## Media

Prepare media for the tests as described below. Alternatively, dehydrated formulations may be used provided that, when reconstituted as directed by the manufacturer or distributor, they meet the requirements of *Growth Promotion Testing*. Unless otherwise indicated elsewhere in this chapter, media are sterilized in autoclaves using a validated process. The exposure time within the autoclave at 121° will depend on the volume of media to be sterilized. Thus, for example, a 500-mL volume would need to be autoclaved using a temperature and time relationship that will ensure that the medium has attained at least an $F_0$ of 12–15 in the sterilization process. However, the appropriate time and temperature duration for sterilizing prepared media at any given volume should be confirmed by a thermal penetration study using a thermocouple or thermoprobe placed within the liquid medium.

### FLUID CASEIN DIGEST–SOY LECITHIN–POLYSORBATE 20 MEDIUM

| | |
|---|---|
| Pancreatic Digest of Casein | 20 g |
| Soy Lecithin | 5 g |
| Polysorbate 20 | 40 mL |
| Water | 960 mL |

Dissolve *Pancreatic Digest of Casein* and *Soy Lecithin* in 960 mL of water, heating in a water bath at 48°–50° for about 30 min to effect solution. Add 40 mL of *Polysorbate 20*. Mix, dispense as desired, and sterilize.

### SOYBEAN–CASEIN DIGEST–AGAR MEDIUM

| | |
|---|---|
| Pancreatic Digest of Casein | 15.0 g |
| Papaic Digest of Soybean Meal | 5.0 g |
| Sodium Chloride | 5.0 g |
| Agar | 15.0 g |
| Water | 1000 mL |

pH after sterilization: 7.3 ± 0.2.

### FLUID SOYBEAN–CASEIN DIGEST MEDIUM

| | |
|---|---|
| Pancreatic Digest of Casein | 17.0 g |
| Papaic Digest of Soybean Meal | 3.0 g |
| Sodium Chloride | 5.0 g |
| Dibasic Potassium Phosphate | 2.5 g |
| Dextrose | 2.5 g |
| Purified Water | 1000 mL |

Dissolve the solids in the water, heating slightly to effect a solution. Cool the solution to room temperature, and adjust the pH with 1 N sodium hydroxide so that after sterilization it will have a pH of 7.3 ± 0.2. Filter, if necessary, and dispense into suitable containers. Sterilize at a temperature and time relationship that will ensure that the medium has attained at least an $F_0$ of 12–15 in the sterilization process, or by a validated filtration process.

### SABOURAUD DEXTROSE–AGAR MEDIUM

| | |
|---|---|
| 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 |
| Water | 1000 mL |

Mix, and boil to effect solution.
pH after sterilization: 5.6 ± 0.2.

### VIOLET-RED BILE AGAR WITH GLUCOSE AND LACTOSE

| | |
|---|---|
| Yeast Extract | 3.0 g |

| | |
|---|---|
| Pancreatic Digest of Gelatin | 7.0 g |
| Bile Salts | 1.5 g |
| Lactose | 10.0 g |
| Sodium Chloride | 5.0 g |
| D-Glucose Monohydrate | 10.0 g |
| Agar | 15.0 g |
| Neutral Red | 30 mg |
| Crystal Violet | 2 mg |
| Water | 1000 mL |

Adjust the pH so that it is 7.4 ± 0.2 after heating. Heat to boiling, but do not heat in an autoclave. Pour onto plates.

MOSSEL–ENTEROBACTERIACEAE ENRICHMENT BROTH

| | |
|---|---|
| Pancreatic Digest of Gelatin | 10.0 g |
| D-Glucose Monohydrate | 5.0 g |
| Dehydrated Ox Bile | 20.0 g |
| Monobasic Potassium Phosphate | 2.0 g |
| Dibasic Potassium Phosphate | 8.0 g |
| Brilliant Green | 15 mg |
| Water | 1000 mL |

Suspend the solids in water, and heat to boiling for 1–2 min. Transfer 120-mL portions to 250-mL volumetric flasks or 9-mL portions to test tubes, all being capped with cotton plugs or loose-fitting caps. Heat on a steam bath for 30 min. Adjust the pH so that it is 7.2 ± 0.2 after heating.

GROWTH PROMOTION TESTING

Each lot of dehydrated medium bearing the manufacturer's identifying number or each lot of medium prepared from basic ingredients must be tested for its growth-promoting qualities. Cultures of *Staphylococcus aureus* (ATCC No. 6538), *Escherichia coli* (ATCC No. 8739), *Bacillus subtilis* (ATCC No. 6633), *Candida albicans* (ATCC No. 10231), and *Aspergillus brasiliensis* (ATCC No. 16404) are used. A $10^{-3}$ dilution of a 24-hour broth culture of the microorganism to the first dilution (in *pH 7.2 Phosphate Buffer* or *Fluid Soybean–Casein Digest Medium*) may be used as the inocula. Serially streak plates of the media with the appropriate inocula to obtain isolated colonies to demonstrate the growth-promotion qualities of the *Soybean–Casein Digest Agar* and *Sabouraud Dextrose Agar* media. Inoculate the *Fluid Soybean–Casein Digest Medium* and *Mossel–Enterobacteriaceae Enrichment Broth* with 10–100 cfu of the appropriate challenge organisms to demonstrate their growth-promotion qualities.

## SAMPLING

Provide 10-mL or 10-g specimens for the tests called for in the individual monograph.

## PROCEDURE

Prepare the specimen to be tested by a treatment that is appropriate to its physical characteristics and that does not alter the number and kind of microorganisms originally present, in order to obtain a solution or suspension of all or part of it in a form suitable for the test procedure(s) to be carried out.

For a solid that dissolves to an appreciable extent but not completely, reduce the substance to a moderately fine powder, suspend it in the vehicle specified, and proceed as directed under *Total Aerobic Microbial Count*.

For a fluid specimen that consists of a true solution, or a suspension in water or a hydroalcoholic vehicle containing less than 30% of alcohol, and for a solid that dissolves readily and practically completely in 90 mL of *pH 7.2 Phosphate Buffer* or the media specified, proceed as directed under *Total Aerobic Microbial Count*.

For water-immiscible products, prepare a suspension with the aid of a minimal quantity of a suitable, sterile emulsifying agent (such as one of the polysorbates), using a mechanical blender and warming to a temperature not exceeding 45°, if necessary, and proceed with the suspension as directed under *Total Aerobic Microbial Count*.

## Total Aerobic Microbial Count

For specimens that are freely soluble, use the *Membrane Filtration Method* or *Plate Method*. For specimens that are sufficiently soluble or translucent to permit use of the *Plate Method*, use that method; otherwise, use the *Multiple-Tube Method*. With either method, first dissolve or suspend 10.0 g of the specimen if it is a solid, or 10 mL, accurately measured, if the specimen is a liquid, in *pH 7.2 Phosphate Buffer, Fluid Soybean–Casein Digest Medium*, or *Fluid Casein Digest–Soy Lecithin–Polysorbate 20 Medium* to make 100 mL. For viscous specimens that cannot be pipeted at this initial 1:10 dilution, dilute the specimen until a suspension is obtained, i.e., 1:50 or 1:100, etc., that can be pipeted. Perform the test for absence of inhibitory (antimicrobial) properties as described under *Preparatory Testing* before the determination of *Total Aerobic Microbial Count*. Add the specimen to the medium NMT 1 h after preparing the appropriate dilutions for inoculation.

### MEMBRANE FILTRATION METHOD

Dilute the fluid further, if necessary, so that 1 mL will be expected to yield 30–300 colonies. Pipet 1 mL of the final dilution into 5–10 mL of *pH 7.2 Phosphate Buffer, Fluid Soybean–Casein Digest Medium*, or *Fluid Casein Digest–Soy Lecithin–Polysorbate 20 Medium*. Wash each membrane with an appropriate amount of one of the above diluents. Transfer each membrane to a Petri dish containing *Soybean–Casein Digest–Agar Medium*, previously solidified at room temperature. Incubate the plates at a temperature 30°–35° for 48–72 h. Following incubation, examine the plates for growth, count the number of colonies, and express the average for the two plates in terms of the number of microorganisms per g or per mL of specimen. If no microbial colonies are recovered from the dishes representing the initial 1:10 dilution of the specimen, express the results as "less than 10 microorganisms per g or per mL of specimen".

### PLATE METHOD

Dilute the fluid further, if necessary, so that 1 mL will be expected to yield 30–300 colonies. Pipet 1 mL of the final dilution onto each of two sterile Petri dishes. Promptly add to each dish 15–20 mL of *Soybean–Casein Digest–Agar Medium*, previously melted and cooled to about 45°. Cover the Petri dishes, mix the sample with agar by gently tilting or rotating the dishes, and allow the contents to solidify at room temperature. Invert the Petri dishes and incubate for 48–72 h. Following incubation, examine the plates for growth, count the number of colonies, and express the average for the two plates in terms of the number of microorganisms per g or per mL of specimen. If no microbial colonies are recovered from the dishes representing the initial 1:10 dilution of the specimen, express the results as "less than 10 microorganisms per g or per mL of specimen".

### MULTIPLE-TUBE METHOD

Into each of 14 test tubes of similar size, place 9.0 mL of sterile *Fluid Soybean–Casein Digest Medium*. Arrange 12 of the tubes in four sets of three tubes each. Put aside one set of three tubes to serve as the controls. Into each of three tubes of one set ("100") and into a fourth tube (A) pipet 1 mL of the solution or suspension of the specimen, and mix. Pipet 1 mL from tube A into the one remaining tube (B), not included in a set, and mix. These two tubes contain 100 mg or 100 µL and 10 mg or 10 µL of the specimen, respectively. Into each of the second set ("10") of three tubes pipet 1 mL from tube A, and into each tube of the third set ("1") pipet 1 mL from tube B. Discard the unused contents of tubes A and B. Close well, and incubate all of the tubes. Following incubation, examine the tubes for growth: the three control tubes remain clear, and the observations in the tubes containing the specimen, when interpreted by reference to *Table 1*, indicate the most probable number of microorganisms per g or per mL.

**Table 1. Most Probable Count by Multiple-Tube Method**

| Observed Combinations of Numbers of Tubes Showing Growth in Each Set | | | Most Probable Number of Microorganisms per g or per mL |
|---|---|---|---|
| Number of mg or µL of specimen per tube | | | |
| 100 | 10 | 1 | |
| 3 | 3 | 3 | More than 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 |

**Table 1. Most Probable Count by Multiple-Tube Method** *(continued)*

| Observed Combinations of Numbers of Tubes Showing Growth in Each Set | | | Most Probable Number of Microorganisms per g or per mL |
|---|---|---|---|
| Number of mg or µL of specimen per tube | | | |
| 100 | 10 | 1 | |
| 3 | 0 | 2 | 60 |
| 3 | 0 | 1 | 40 |
| 3 | 0 | 0 | 23 |
| 2 | 2 | 0 | 21 |
| 2 | 1 | 1 | 20 |
| 2 | 1 | 0 | 15 |
| 2 | 0 | 1 | 14 |
| 2 | 0 | 0 | 9 |
| 1 | 2 | 0 | 11 |
| 1 | 1 | 0 | 7 |
| 1 | 0 | 0 | 4 |
| 0 | 1 | 0 | 3 |
| 0 | 0 | 0 | <3 |

## Total Combined Molds and Yeasts Count

PROCEDURE

Proceed as directed for *Membrane Filtration Method* or *Plate Method* under *Total Aerobic Microbial Count*, except to use the same amount of *Sabouraud Dextrose–Agar Medium* instead of *Soybean–Casein Digest–Agar Medium* and to incubate the plates for 5–7 days at 20°–25°.

RETEST

For the purpose of confirming a doubtful result by any of the procedures outlined in the foregoing tests following their application to a 10-g specimen, a retest on an additional 10-g specimen from the original sample and a 10-g specimen from the new sample of the nutritional supplement may be conducted. Proceed as directed under *Procedure*.

## Enterobacterial Count (Bile-Tolerant Gram-Negative Bacteria)

Dissolve or suspend the sample in a sufficient volume of *pH 7.2 Phosphate Buffer* or *Fluid Soybean–Casein Digest Medium* and dilute with *Fluid Soybean–Casein Digest Medium* to 100 mL. Pre-incubate for 2–5 h at 20°–25° in soybean–casein digest broth diluent; inoculate suitable quantities of *Mossel–Enterobacteriaceae Enrichment Broth* to contain 0.1, 0.01, or 0.001 g or mL of the product. Incubate at 30°–35° for 24–48 h. Subculture onto a plate of *Violet-Red Bile Agar with Glucose and Lactose*, and incubate at 30°–35° for 18–24 h. Growth of well developed, generally red or reddish, colonies of Gram-negative bacteria reveal the presence of enterobacteria. Determine the most probable number of microorganisms per g or per mL by reference to *Table 2*.

**Table 2. Most Probable Enterobacterial Count**

| Observed Presence of Enterobacteria | | | Most Probable Number of Enterobacteria per g or per mL |
|---|---|---|---|
| Number of g or mL of specimen per tube | | | |
| 0.1 | 0.01 | 0.001 | |
| + | + | + | More than $10^3$ |
| + | + | – | Fewer than $10^3$ but more than $10^2$ |
| + | – | – | Fewer than $10^2$ but more than $10^1$ |
| – | – | – | Fewer than $10^1$ |