Designation: D 4249 – 83 (Reapproved 2005)

An American National Standard

Standard Test Method for Enumeration of *Candida albicans* in Water[1]

This standard is issued under the fixed designation D 4249; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This test method covers the detection and enumeration of the yeast *Candida albicans* in raw sewage, waste waters, and natural waters.

1.2 It is the responsibility of the analyst to determine if this test method yields satisfactory results in waters of other matrices.

1.3 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.* For a specific hazard statement, see Section 9.

2. Referenced Documents

2.1 *ASTM Standards:* [2]

D 1129 Terminology Relating to Water

D 1193 Specification for Reagent Water

D 3870 Practice for Establishing Performance Characteristics for Colony Counting Methods in Microbiology[3]

E 200 Practice for Preparation, Standardization, and Storage of Standard and Reagent Solutions for Chemical Analysis

3. Terminology

3.1 *Definitions*— For definitions of terms used in this test method, refer to Terminology D 1129.

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *germ tubes*—elongated extensions, 3 to 4 µm wide and up to 20 µm in length, which originate from the yeast cell when incubated for 1 to 3 h in serum. There is no constriction of the germ tube at its point of origin; this is a critical diagnostic feature (**1**). [4] Similar structures (elongate buds, pseudohyphae) may be produced by *C. albicans* and other yeasts but all have discrete constrictions at the base where the structure is formed at the cell surface.

4. Summary of Test Method

4.1 This test method consists of filtering appropriate volumes of raw sewage, waste water, or natural water through 1.2-µm retentive porosity gridded membrane filters and placing the membranes on the surface of a selective medium, herein referred to as mCA (**2**).

4.2 Cultures are incubated for 2 to 4 days at 37°C and typical colonies are observed and counted using a dissecting microscope.

4.3 At least initially, suspect colonies may be confirmed as *C. albicans* by picking to bovine (calf) serum, incubating for 2 to 3 h, and observing cells using microscopy (preferably phase) for the presence of germ tubes that are diagnostic for *C. albicans* (**1**,**3**).

5. Significance and Use

5.1 *C. albicans* is a yeast that is found as a commensal in the gastrointestinal, genitourinary, and alimentary tracts of healthy individuals, both human and lower animals (**3**, **4**, **5**). As such, it is a serious opportunistic pathogen of humans and may cause superficial or deep mycotic infections. Consequently, the yeast is found in raw sewage and in natural waters receiving human and animal wastes. *C. albicans* can survive *in situ* in seawater for at least six days (**6**). *In vitro* survival of the yeast in distilled (**7**) and lake water (**8**) has been demonstrated also. While there is at present no epidemiological evidence connecting human disease caused by *C. albicans* and use of water, the organism may be a useful indicator of recreational water quality (**9**). The test method may be applied to the monitoring of various treatment processes for efficiency in removing particular pathogens in waste water prior to discharge in receiving waters which in turn may be used again for a variety of purposes. Both public health and sanitary engineering interests should be aware of the presence of this yeast in wastewater and the potential for disease in contiguous waters.

5.2 Future studies between the incidence of *C. albicans* and traditional water quality indicators (for example, total and fecal coliforms, fecal streptococci) may reveal a correlation of value

[1] This test method is under the jurisdiction of ASTM Committee D19 on Water and is the direct responsibility of Subcommittee D19.24 on Water Microbiology.

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[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

[3] Withdrawn.

[4] The boldface numbers in parentheses refer to references at the end of the standard.

in the assessment of potential health risks of swimming or other recreational waters.

6. Interferences

6.1 In some waters, "false positive" colonies resembling *C. albicans* may develop on mCA medium. Generally, however, these can be differentiated by colony shape, color, or texture, or a combination thereof, using a high-power dissecting microscope. Also, they may be detected by the germ tube procedure described below.

6.2 Germ tubes have been reported to occur in *C. stellatoidea*, a yeast closely resembling *C. albicans* in all respects. However, *C. stellatoidea* is human-associated and apparently rare in natural waters; its occurrence probably assumes the same significance as that of *C. albicans.*

7. Apparatus

7.1 *pH Meter* (expanded scale preferable).

7.2 *Magnetic Stirrer*.

7.3 *Water Bath*, 45 to 50°C.

7.4 *Membrane Filtration Apparatus* (holder, tubing, trap, flasks, vacuum pump).

7.5 *Incubator*, 37±1°C.

7.6 *Binocular Dissecting Microscope (Stereozoom Preferable) and External Light Source (Nicholas or Spot Type).*

7.7 *Research Microscope (Phase Contrast Preferable).*

7.8 *Culture Tubes*, disposable, 10 by 75 mm.

7.9 *Hollow Plastic Straws*, approximately 13.5 by 3 mm (cocktail sippers).

7.10 *Sterile Petri Dishes*.

7.11 *Microscope Slides and Cover Slips*.

8. Reagents and Materials

8.1 *Purity of Reagents*—Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, it is intended that all reagents shall conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society. [5] Other grades may be used provided it is first ascertained that the reagent is of sufficiently high purity to permit its use without lessening the accuracy of the determination.

8.2 *Purity of Water*—Unless otherwise indicated, references to water shall be understood to mean reagent water conforming to Specification D 1193, Type II.

8.3 *Hydrochloric Acid (1 + 9)*—Refer to Practice E 200.

8.4 *Ammonium Hydroxide (1 + 9).*

8.5 *Bovine (calf) Serum.*

8.6 *Sterile Membrane Filters*, white, gridded, 47-mm diameter, 1.2-µm retentive porosity.

9. Precautions

9.1 *Candida albicans* is a human pathogen; thus, handle all culture material (plates, slides, serum tubes, and straws) using accepted microbiological technique including the sterilization of all discards.

10. Procedure

10.1 *Preparation of mCA Medium*:

10.1.1 Combine the following ingredients:

Glycine	1.0 g
Maltose	3.0 g
Sodium sulfite; Na_2SO_3	0.3 g
Bismuth ammonium citrate; $Bi[(NH_4)\ C_6H_5O_7]_3$ [6]	0.5 g
Chloramphenicol	50 mg
Cycloheximide	150 mg
Reagent water	90 mL

10.1.2 With stirring, warm to about 50°C (slight turbidity).

10.1.3 While stirring, adjust the pH to 7.1 with 1.0 *N* HCl or NH_4OH.

10.1.4 Add 1.5 g of agar, and bring slowly to the boiling point by swirling constantly over a flame. Continue to boil gently for 2 min and cool to 45 to 50°C in a water bath. Do not autoclave.

10.1.5 Add 10 mL of membrane-filtered (0.45-µm) commercially available yeast nitrogen base prepared at a 10× concentration.

10.1.6 While stirring, adjust the pH to 6.5 with 1.0 *N* HCl. This is a critical step since colony color development is dependent on proper pH adjustment.

10.1.7 With frequent swirling of the medium, pour into petri dishes to a depth of 4 to 5 mm. Allow to solidify.

10.1.8 Store plates in the dark (foil-wrapped) at about 4°C. The medium is stable for about 2 weeks under these conditions. White crystal formation indicates that the medium should be discarded.

10.2 *Collection of Samples*:

10.2.1 Use clean, sterile containers.

10.2.2 Obtain sample so as to preclude contamination.

10.2.3 Large volumes of some waters will be required (for example, several litres if replicate plates of recreational waters are to be prepared).

10.2.4 If chlorinated waters are sampled, add sodium thiosulfate to the collection bottle before sterilization, at a concentration of 0.1 mL of 10 % solution for each 125-mL of sample volume.

10.2.5 Filter sample as below as soon as possible after collection.

10.3 *Filtration of Sample*:

10.3.1 Sample volumes will vary depending on the water sampled; 10 to 40 mL may be appropriate for raw sewage while up to 1 L or more of relatively clean and clear recreational water should be examined.

10.3.2 Shake sample thoroughly.

10.3.3 Filter sample through 1.2-µm retentive porosity membrane filter. Rinse holder with 20 to 30 mL of sterile reagent grade water.

10.3.4 Aseptically remove membrane and place grid side up on plates of mCA medium.

10.3.5 Incubate at 38°C for 2 to 4 days.

10.4 *Counting Procedure*:

[5] "Reagent Chemicals, American Chemical Society Specifications," Am. Chemical Soc., Washington, DC. For suggestions on the testing of reagents not listed by the American Chemical Society, see "Analar Standards for Laboratory Chemicals," BDH Ltd., Poole, Dorset, and the "United States Pharmacopeia."

[6] Bismuth ammonium citrate is the most critical and least readily available ingredient in mCA medium. Best results are obtained when the crystalline or powder form rather than the liquid is used.

10.4.1 Examine and count colonies using a dissecting microscope with an external light source placed to the side of the stage with the light beam directed at an oblique angle to the membrane surface such that a shadowing effect is created.

10.4.2 *C. albicans* colonies on mCA medium are dark (chocolate) brown, approximately 1 mm in diameter, distinctly raised, domelike in appearance, and have a matte, rather than a perfectly smooth and glossy, surface texture when viewed under high magnification.

10.5 *Confirmation of C. albicans Colonies*:

10.5.1 It is recommended that, at least initially, a laboratory confirm developing colonies on mCA medium to ensure accuracy and confidence. With experience, this becomes unnecessary.

10.5.2 Commercially available bovine (calf) serum is dispensed in 0.3 mL amounts in nonsterile disposable glass culture tubes (10 by 75 mm). These may be frozen and subsequently thawed as needed.

10.5.3 Using a dissecting microscope, suspect colonies are touched with a nonsterile, plastic cocktail straw and a small amount of growth is transferred to a tube of serum. Twirl the straw to dispense cells.

10.5.4 The straw is left in the serum tube and incubated at 37°C for 2 to 3 h.

10.5.5 The straw is twirled again and the amount of serum contained in the straw (a drop or two) is placed on a glass microscope slide, covered with a glass cover slip, and examined for germ tubes by phase microscopy, if possible, at a magnification of about 400×. If 18-mm ^{2} cover slips are used, three preparations can be placed on one slide.

11. Precision and Bias [7]

11.1 The following precision and bias statements are based upon statistical calculations on data from a collaborative study involving two operators in each of four laboratories performing triplicate analyses on nine samples; three raw sewages of choice, three recreational waters of choice and three standardized seawater samples. Each participant was provided with a pure culture and instructions for spiking standard amounts into the seawater samples. The raw sewages and the recreational waters were not spiked. It should be recognized that these data may not apply to waters of other matrices.

11.2 Since each participant analyzed different samples, it is impossible to estimate the overall precision (S_T) of this test method. However, it is possible to estimate the single-operator standard deviation (S_o) from each set of replicate analyses and pool different S_o estimates for similar waters and count levels. As a result, the single-operator precision of this test method, within the range studied, varies with the average sample count according to Fig. 1.

11.3 There are no known count levels for the samples used in this collaborative study, so a bias statement is impossible.

12. Keywords

12.1 *Candida albicans*; detection; enumeration; sewage; water; yeast

[7] Supporting data have been filed at ASTM International Headquarters and may be obtained by requesting Research Report RR: D19–1097.

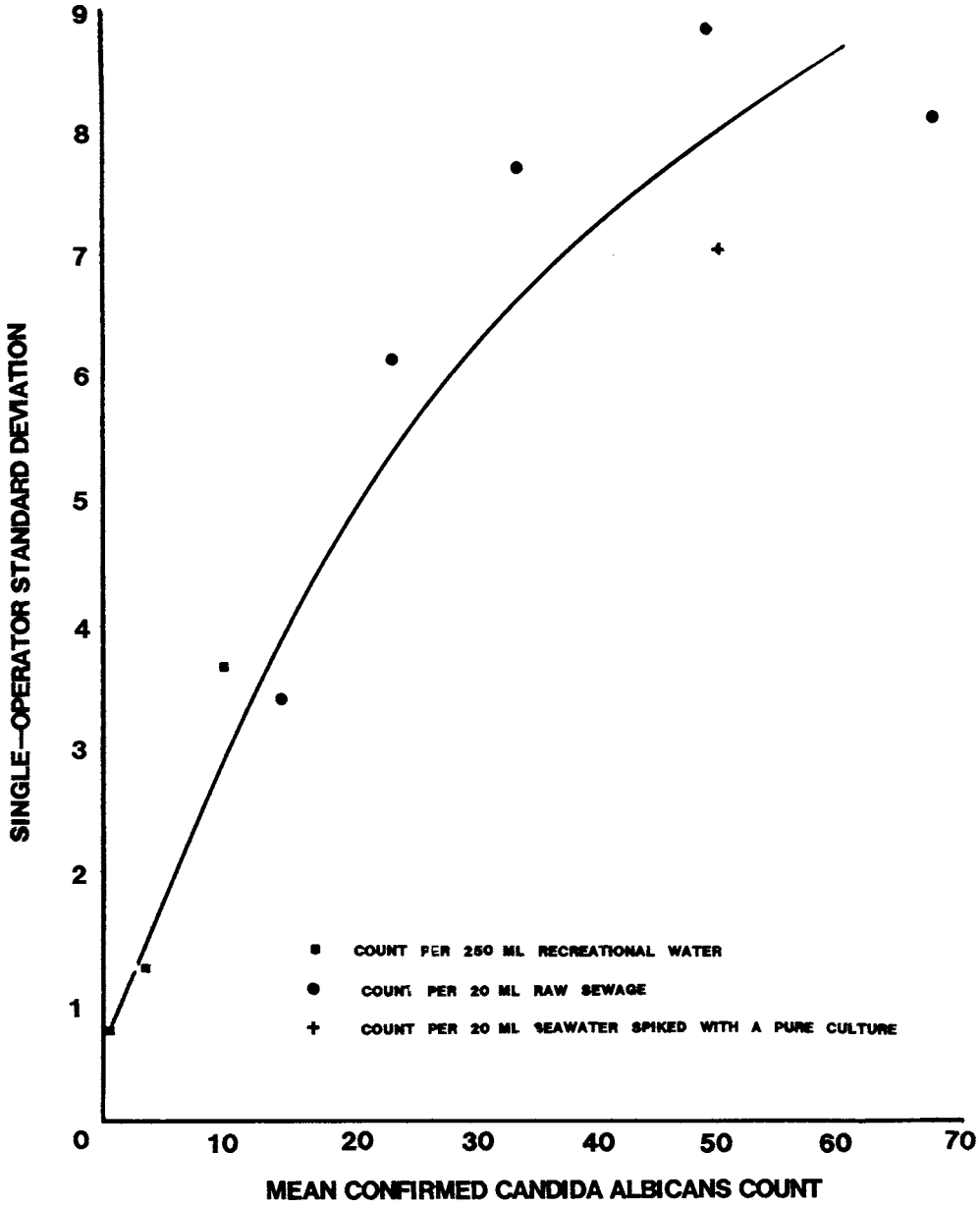

FIG. 1 Single Operator Precision for Enumeration of Candida Albicans in Water

APPENDIX

(Nonmandatory Information)

X1. PERFORMANCE CHARACTERISTICS

X1.1 The performance characteristics given below are in accordance with Practice D 3870.

X1.1.1 *Precision*—Using duplicate plates in recovery experiments (a total of 34 sets of data), square roots of mean counts = 15.25 and 15.46.

X1.1.2 *Bias*—A total of 54 trials were made using nine strains of *C. albicans* (field and laboratory). Washed cell suspensions were held in seawater at 4°C overnight. Membrane filtration platings were made on mCA medium; controls were spread plates on Sabauroud-dextrose agar. Percent recoveries were calculated.

Average percent recoveries = 81.7 % (stressed)

Overall range of recovery = 38.5 − 300 %

TABLE X1.1 Summary of Performance Characteristics of mCA

% Accuracy:	
Unstressed	—
Stressed	81.7 %
Specificity and Selectivity:	
False positive error	10 %
Undetected target error	3 %
Selectivity index (%)	49 %
Counting range	100 colonies
Upper limit	
Comparability:	
Target recovery	NIL
Background	—

Range in percent recovery between strains = 54.3 − 148.5 %

X1.1.3 *Specificity*— A total of 64 samples of raw sewage and water from rivers, estuaries, and marine bathing beaches were used. "Typical" and "atypical" colonies were examined by the germ tube test, specific for *C. albicans.*

Presumptive target colonies examined	644
Presumptive nontarget colonies examined	670
False positive colonies	65
Undetected target colonies	20

Indices of Specificity:

$$\textit{False positive error} = \frac{65}{644} = 0.10$$

$$\textit{Undetected target error} = \frac{20}{644 - 65 + 20} = \frac{20}{599} = 0.03$$

X1.1.4 *Selectivity*—

Presumptive target colonies	644
Total countable colonies	644 + 670 = 1314
Index of selectivity	

$$\frac{644}{1314} = 0.490$$

X1.1.5 *Upper Counting Limit*—This is related to volume of sample filtered. Maximum volume of raw sewage filterable through 1.2-µm membrane is 30 to 40 mL. These volumes often yield several hundred colonies per plate resulting in crowding and difficulty in observing characteristic colony shape and texture. In these cases, an upper limit of about 100 per plate is a workable guideline instead of a well-defined upper counting limit. With clean (clear) natural waters, up to 1 L can be filtered and less than 100 colonies per plate develop. Usually, the problem is filtering enough water to detect the organism.

X1.1.6 *Comparability*— Commercially available media for detection of *Candida* species are not designed for use in natural waters and will overgrow rapidly with bacteria or filamentous fungi, or both; the occurrence of false positives are high. (Also see Table X1.1).

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