Designation: D 4783 – 01

Standard Test Methods for Resistance of Adhesive Preparations in Container to Attack by Bacteria, Yeast, and Fungi[1]

This standard is issued under the fixed designation D 4783; 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.

This standard has been approved for use by agencies of the Department of Defense.

1. Scope

1.1 These test methods cover the determination of the resistance of liquid adhesive preparations to microbial attack in the container by challenging adhesive specimens with cultures of bacteria, yeast, or fungi, and checking for their ability to return to sterility. These test methods return qualitative results.

1.2 The values stated in SI units are to be regarded as the standard. The values in parentheses are for information only.

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.* These test methods are designed to be used by persons trained in correct microbiological technique. Specific precautionary statements are given in Section 8.

2. Referenced Documents

2.1 *ASTM Standards:*

D 907 Terminology of Adhesives[2]

D 4299 Test Methods for Effect of Bacterial Contamination on Performance of Adhesive Preparations and Adhesive Films[3]

D 4300 Test Methods for Ability of Adhesive Films to Support or Resist the Growth of Fungi[2]

E 640 Test Method for Preservatives in Water-Containing Cosmetics[4]

NOTE 1—Test Method E 640 is under the jurisdiction of ASTM Committee E35 on Pesticides. The procedure in this method outlines a serial dilution method of determining plate count using a pour plate technique.

2.2 *TAPPI Method:*

T 487 Fungus Resistance of Paper and Paperboard[5]

2.3 *CSMA:*

Cosmetics Preservation, Method 38[6]

3. Terminology

3.1 *Definitions*—Many terms in these test methods are defined in Terminology D 907.

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *adhesive preparation*—the adhesive as packaged for distribution, storage, and use.

3.3 *Abbreviations:Abbreviations:*

3.3.1 *PBS*—phosphate buffered saline.

3.3.2 *PDA*—potato dextrose agar.

3.3.3 *YMPG*—yeast malt peptone glucose (agar).

4. Summary of Test Methods

4.1 The adhesive specimen is challenged by inoculation with a culture of bacteria, yeast, or fungi, which may be a single species or a mixed culture of several species, following the guidelines given in Note 6. The inoculated adhesive specimen is stored at 21 to 27°C (70 to 80°F) for 7 days, during which time cultures (streak plates) are made at preset intervals. See Note 2. At any point in the series of challenges, if the inoculated specimen shows microbial growth on the streak plates made during the week following the challenge (indicating that it has not returned to sterility), the test is discontinued, and the sample is reported as *not resistant to attack in the container* by the species or combination of species used as the inoculum. If the cultures show no growth, the test is repeated with up to four challenges. If the specimen tests out as sterile following the fourth challenge, it is reported to be *resistant to attack in the container* by the species or combination of species of bacteria, fungi, or yeast used as the inoculum. At the discretion of the biological laboratory, the test may be discontinued after the second or third challenge. See Section 16 for further interpretation.

4.2 The time necessary to kill is determined by noting the earliest streak plate to read sterile. If the 4-h plate is positive and the 24-h plate is negative, the kill time could be narrowed down further by repeating the challenge and making streak plates at intervals of 4, 8, 12, and 24 h following the challenge.

4.3 The testing laboratory has the option of changing the

[1] These test methods are under the jurisdiction of ASTM Committee D14 on Adhesives and are the direct responsibility of Subcommittee D14.30 on Wood Adhesives.

Current edition approved Oct. 10, 2001. Published December 2001. Originally published as D 4783 – 88. Last previous edition D 4783 – 98a.

[2] *Annual Book of ASTM Standards*, Vol 15.06.

[3] Discontinued, see *1989 Annual Book of ASTM Standards*, Vol 15.06.

[4] *Annual Book of ASTM Standards*, Vol 11.05.

[5] Available from TAPPI, P.O. Box 105113, Atlanta, GA 30348.

[6] This method is the same as Test Method E 640.

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timing of the challenges, the sterility checks, and the incubation period.

NOTE 2—Two proposed schedules for the challenging and sterility checks are shown in Table 1 and Table 2, Schedule A for bacteria and yeast, and Schedule B for fungi. The exact format to be followed will vary, according to the convenience of the schedule to the testing laboratory and special circumstances relating to the problem being addressed.

NOTE 3—A serial-dilution plate-count method of checking for sterility may be used when numerical information is needed on the population of viable organisms or the reduction in population with increasing levels of biocide. Letheen broth is recommended for the diluent and Letheen agar for the pour plate. See Note 1.

5. Significance and Use

5.1 These test methods are used to demonstrate whether an adhesive preparation is sufficiently protected with biocide to resist attack by bacteria, yeast, and fungi during its storage life. They are patterned after methods used by biological laboratories serving the adhesive industry.

5.2 These test methods may also be used to determine the efficacy of different biocide systems against specific microorganisms.

5.3 These test methods are especially useful when tested against wild-type microorganisms which have been isolated from contaminated adhesives as an aid in determining the amount and type of biocide necessary to kill or inhibit the growth of the contaminants. If an isolated microorganism not generally used as a challenge organism, is chosen as the inoculum, it is important to identify the organism and determine on which medium and under what conditions it will grow, in order to demonstrate the efficacy of the biocide.

5.4 The results obtained when using the procedures given in these methods apply only to the species which are used for the testing. The test species listed in Section 9 are frequently used by laboratories to test for antimicrobial properties, but they are not the only ones which could be used. Selection of the species to use for these test methods requires informed judgment by the testing laboratory or by the party requesting the tests. It is also important that species which commonly attack adhesives be used. See 9.4.

5.5 The presence of an active biocide carried over from the adhesive specimen to the agar could have an inhibiting effect on the growth of microorganisms, resulting in no growth

TABLE 1 Schedule A—Proposed Bacteria and Yeast Testing, Covering 4-h, 24-h, 48-h, 72-h, and 7-Day Tests

Day of Week	Day no.	First Challenge	Second Challenge	Third Challenge	Fourth Challenge
Monday	(−1)	inoculate fresh bacterial or yeast culture	...	...	...
Tuesday	0	prepare suspension	...	...	...
Tuesday	0	inoculate specimens	...	...	...
Tuesday	(0 + 4 h)	streak 4-h plate	...	...	...
Wednesday	1	streak 24-h plate	...	...	...
Thursday	2	streak 48-h plate	...	...	...
Friday	3	streak 72-h plate	...	...	...
Sat./Sun.	4–5	...	...	...	...
Monday	6	...	inoculate fresh bacterial or yeast culture	...	...
Tuesday	7	...	prepare suspension	...	...
Tuesday	7	streak 7-day plate	inoculate specimens	...	...
Tuesday	(7 + 4 h)	read 4-h plate	streak 4-h plate	...	...
Wednesday	8	read 24-h plate	streak 24-h plate	...	...
Thursday	9	read 48-h plate	streak 48-h plate	...	...
Friday	10	read 72-h plate	streak 72-h plate	...	...
Sat./Sun.	11–12	...	...	...	...
Monday	13	...	...	inoculate fresh bacterial or yeast culture	...
Tuesday	14	...	...	prepare suspension	...
Tuesday	14	read 7-day plate	streak 7-day plate	inoculate specimens	...
Tuesday	(14 + 4 h)	...	read 4-h plate	streak 4-h plate	...
Wednesday	15	...	read 24-h plate	streak 24-h plate	...
Thursday	16	...	read 48-h plate	streak 48-h plate	...
Friday	17	...	read 72-h plate	streak 72-h plate	...
Sat./Sun.	18–19	...	...	...	...
Monday	20	...	...	...	inoculate fresh bacterial or yeast culture
Tuesday	21	...	...	...	prepare suspension
Tuesday	21	...	read 7-day plate	streak 7-day plate	inoculate specimens
Tuesday	(21 + 4 h)	...	...	read 4-h plate	streak 4-h plate
Wednesday	22	...	...	read 24-h plate	streak 24-h plate
Thursday	23	...	...	read 48-h plate	streak 48-h plate
Friday	24	...	...	read 72-h plate	streak 72-h plate
Sat./Sun.	25–26	...	...	...	...
Monday	27	...	...	...	...
Tuesday	28	...	...	read 7-day plate	streak 7-day plate
Tuesday	(28 + 4 h)	...	...	...	read 4-h plate
Wednesday	29	...	...	...	read 24-h plate
Thursday	30	...	...	...	read 48-h plate
Friday	31	...	...	...	read 72-h plate
Sat./Sun.	32–33	...	...	...	...
Monday	34	...	...	...	...
Tuesday	35	...	...	...	read 7-day plate

TABLE 2 Schedule B—Proposed Fungi Testing, Covering 4-h, 24-h, 48-h, 72-h, and 7-Day Tests

Day of Week	Day no.	First Challenge	Second Challenge	Third Challenge	Fourth Challenge
Friday	(−10)	inoculate fresh fungal culture	...	...	...
Friday	(−3)	...	inoculate fresh fungal culture	...	...
Monday	0	prepare spore suspension	...	...	...
Monday	0	inoculate specimens	...	...	...
Monday	(0 + 4 h)	streak 4-h plate	...	...	...
Tuesday	1	streak 24-h plate	...	...	...
Wednesday	2	streak 48-h plate	...	...	...
Thursday	3	streak 72-h plate	...	...	...
Friday	4	...	...	inoculate fresh fungal culture	...
Sat./Sun.	5, 6	...	...	...	...
Monday	7	...	prepare spore suspension	...	...
Monday	7	streak 7-day plate	inoculate specimens	...	...
Monday	(7 + 4 h)	read 4-h plate	streak 4-h plate	...	...
Tuesday	8	read 24-h plate	streak 24-h plate	...	...
Wednesday	9	read 48-h plate	streak 48-h plate	...	...
Thursday	10	read 72-h plate	streak 72-h plate	...	inoculate fresh fungal culture
Fri./Sat./Sun.	11, 12, 13	...	...	...	...
Monday	14	...	...	prepare spore suspension	...
Monday	14	read 7-day plate	streak 7-day plate	inoculate specimens	...
Monday	(14 + 4 h)	...	read 4-h plate	streak 4-h plate	...
Tuesday	15	...	read 24-h plate	streak 24-h plate	...
Wednesday	16	...	read 48-h plate	streak 48-h plate	...
Thursday	17	...	read 72-h plate	streak 72-h plate	...
Fri./Sat./Sun.	18, 19, 20	...	...	...	...
Monday	21	...	...	...	prepare spore suspension
Monday	21	...	read 7-day plate	streak 7-day plate	inoculate specimens
Monday	(21 + 4 h)	...	...	read 4-h plate	streak 4-h plate
Tuesday	22	...	...	read 24-h plate	streak 24-h plate
Wednesday	23	...	...	read 48-h plate	streak 48-h plate
Thursday	24	...	...	read 72-h plate	streak 72-h plate
Fri./Sat./Sun.	25, 26, 27	...	...	...	...
Monday	28	...	...	read 7-day plate	streak 7-day plate
Monday	(28 + 4 h)	...	...	...	read 4-h plate
Tuesday	29	...	...	...	read 24-h plate
Wednesday	30	...	...	...	read 48-h plate
Thursday	31	...	...	...	read 72-h plate
Fri./Sat./Sun.	32, 33, 34	...	...	...	...
Monday	35	...	...	...	read 7-day plate

during the span of a normal incubation period, when in fact, viable microorganisms are present, but their growth has been slowed down or held in stasis. The use of Letheen agar and broth is recommended to neutralize the effect of this carry-over.

NOTE 4—Letheen agar may be used for the streak plates, or if another agar is chosen for testing, a Letheen agar plate could be streaked as a control to test against the neutralizing effect. Even more effective would be diluting the challenged adhesive specimen with Letheen broth and running Letheen agar pour plates. See Note 1 and Note 3. Extending the incubation period of negative plates would be another safeguard. To neutralize thiazoline-based preservatives, 10 to 50 ppm of sodium thioglycolate can be added to the medium.

5.6 These test methods are dependent upon the physiological action of living microorganisms under a reported set of conditions. Conclusions about the resistance of the test adhesive to microbiological attack can be drawn by comparing the results to simultaneously run controls of known resistance. See X5.2 for statements regarding test repeatability.

6. Apparatus

6.1 In addition to the standard equipment found in any fully equipped microbiological laboratory, the following items are sometimes needed:

6.1.1 *Autoclave*, capable of producing 103 kPa of steam pressure at 121°C (250°F) and maintaining it for a minimum of 15 min.

6.1.2 *Cell Counting Chamber, Petroff-Hausser*, cell depth—0.02 mm (or equivalent).[7]

6.1.3 *Bottles, Screwcap*, approximately 375 mL, Boston Rounds of flint glass. Mold-A-7232-D, Finish 28-400, and Black Artmold Caps BM-8041, Size 28-400, with rubber ring liners fastened to caps with steamproof adhesive.[8]

6.1.4 *Constant Temperature Chamber*, capable of being

[7] Available from most laboratory supply houses.

[8] Screw cap bottles are available from Owens-Illinois Glass Co., OH Building, Toledo, OH, or equivalent bottles and caps have been found suitable for this purpose.

maintained at 35 ± 0.5°C (95 ± 1°F) for bacteria, or 30 ± 0.5°C (86 ± 1°F) for fungi, or two chambers if needed simultaneously.

6.1.5 *Glass Rods*, 305 mm in length having a diameter of 6.3 mm.

6.1.6 *Hemacytometer, Levy Counting Chamber*, cell depth—0.1 mm, Newbauer rulings.[7]

6.1.7 *Hood*, laminar flow type.[9]

6.1.8 *Jar, Screw Cap*, round, approximately 1 L (1-qt mason type) for samples.

6.1.9 *Pasteur Pipets.*[7]

6.1.10 *Pipet*, 1 mL, disposable, sterile.

6.1.11 *Pipettes*, automatic Oxford or Eppindorf, sterile, with sterile tips.[7]

6.1.12 *Refrigerator*, capable of maintaining temperature of 4 ± 1°C (39 ± 2°F).

6.1.13 *Spectrophotometer*, capable of measuring cell count at a wavelength of 425 nm.[7]

7. Materials

7.1 *Beef Extract*.

7.2 *Deionized or Distilled Water*, sterile.

7.3 *Disinfectant Solution, Amphyll, Alcide*, or equivalent.[7]

7.4 *Glucose*.

7.5 *Letheen Agar*, (Difco or equivalent).[7]

7.6 *Letheen Broth*, (Difco or equivalent).[7]

7.7 *Mycophil Agar*, pH 4.7 (BBL or equivalent).[7]

7.8 *Nutrient Agar*, (BBL or equivalent).[7]

7.9 *Potato Dextrose Agar* (*PDA*)*, Dehydrated*, (Difco or equivalent).[7]

7.10 *Phosphate buffered saline* (*PBS*), sterile.

7.11 *Physiological Saline Solution*, 0.85 % NaCl, sterile.

7.12 *Sorbitan mono-oleate polyoxyethylene.*[10]

7.13 *Tryptone*.

7.14 *Tryptone Glucose Extract Agar*, dehydrated (Difco or equivalent).

7.15 *Yeast Malt Peptone Glucose Agar* (*YMPGA*), dehydrated (Difco or equivalent).

8. Precautions

8.1 These test methods employ live cultures of bacteria, fungi, and yeast, some of which are capable of causing disease, and others allergic reaction in some humans. In addition to other precautions, assign laboratory personnel trained in correct microbiological techniques to run these tests. Use proper microbiological procedures in order to prevent contamination of the cultures or of the work area. Clean and sterilize in an approved manner all spills and all equipment coming into contact with the cultures and the inoculated adhesive specimens. Also sterilize in an approved manner all cultures and contaminated disposable equipment before discarding. See 1.3.

9. Test Organisms[11]

9.1 Cultures of one or more of the following bacterial species are suggested for use:

9.1.1 *Pseudomonas fluorescens* (ATCC 9721).

9.1.2 *Pseudomonas aeruginosa* (ATCC 10145).

9.1.3 *Bacillus subtilis* (ATCC 6984).

9.1.4 *Proteus vulgaris* (ATCC 9920).

9.2 Cultures of one or more of the following yeasts are suggested for use:

9.2.1 *Candida tropicalis* (ATCC 750).

9.2.2 *Kluyveromyces fragilis* (ATCC 8554).

9.2.3 *Candida pseudotropicalis* (ATCC 4135).

9.3 Cultures of one or more of the following species of fungi are suggested for use:

9.3.1 *Aspergillus niger* (ATCC 9642).

9.3.2 *Aspergillus flavus* (ATCC 9643).

9.3.3 *Penicillium pinophilum* (ATCC 9644). See X3.1.6.

9.4 Since a number of other organisms may be of specific interest for certain adhesives or for the environment to which they are to be exposed, such other pure cultures or mixtures of cultures of organisms may be used, if agreed upon between the manufacturer and the purchaser of the adhesive, or upon the recommendation of the testing laboratory.

NOTE 5—See Appendix X1, Appendix X2, Appendix X3, and Appendix X4 for guidelines on selection of species of bacteria, yeast, and fungi, and on problems with use of combined species.

NOTE 6—The use of combined species for the challenge inoculum can result in one species overgrowing the others. When a mixed inoculum is the choice, the recommended groupings are (*a*) fungi and yeast, and (*b*) Gram-positive and Gram-negative bacteria. Group (*a*) organisms prefer a pH of 4.5, while Group (*b*) organisms prefer a pH of 7. Yeast grow faster than fungi, a fact which should be considered in two steps in the test. The mixed inoculum should be used within 2 h of preparation, and for yeast, the streak plates to determine sterility should be read on Schedule A (same as for bacteria), while for fungi, the plates should be read on Schedule B.

10. Sterilization of Equipment and Media

10.1 Follow accepted microbiological practices for sterilizing equipment and media used for the tests. Also, sterilize in an approved manner all cultures and contaminated adhesive specimens before discarding.

NOTE 7—Two references for sterilization methods are TAPPI T487 and Ref (**1**).[12]

11. Preparation of Media

11.1 *Potato Dextrose Agar and Tryptone Glucose Agar*:

11.1.1 Prepare sufficient agar slants and plates for culture propagation and for conducting the tests.

11.1.2 Follow the instructions given for preparation of the commercial product. Dissolve using heat and agitation. Transfer 10 mL of the agar solution to each test tube. Cap tubes loosely with metal, plastic, or foam caps. Autoclave for 15 min at 103 kPa and a temperature of 121°C (250°F). Allow the agar to cool to 48 to 50°C (118 to 122°F) before pouring the plates. Tighten the caps on the tubes and place them in a slanted position to solidify, making a slant of about 51 mm. Store in refrigerator until needed.

11.2 *Other Agar Types—Letheen, Nutrient, and Mycophil—*

[9] The Biogard Hood or similar equipment, The Baker Co., Sanford, ME.

[10] Available commercially as Tween 80.

[11] Cultures may be purchased from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852.

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

Prepare as needed to conduct tests, using commercially available product.

11.3 *Tryptone Glucose Extract Broth and Letheen Broth*:

11.3.1 Prepare sufficient media to conduct the tests.

11.3.2 For the tryptone glucose broth add 1.5 g of beef extract, 2.5 g of tryptone, and 0.5 g of glucose to 500 mL of cold distilled water in a 1000-mL beaker. For the Letheen broth, follow directions for the commercial product.

11.3.3 For both types of broth, heat to boiling to dissolve the medium completely, transfer 10 mL of the dissolved medium to each test tube, and autoclave tubes (with caps loose) for 15 min at 103 kPa and a temperature of 121°C (250°F).

11.3.4 Upon removal from the autoclave, allow the tubes to cool to room temperature, tighten the caps, and refrigerate until needed.

12. Bacterial Cultures

12.1 *Propagation of Bacterial Cultures*:

12.1.1 Prepare on a tryptone glucose agar slant a fresh culture for each species to be used for testing, and label it by species and ATCC Number. Incubate at 30 ± 5°C (86 ± 1°F).

12.1.2 When mature and luxuriant growth is achieved, refrigerate the cultures. Prepare new cultures each month. If contamination occurs, discard the cultures and prepare new ones.

12.2 *Preparation of Bacterial Inoculum*—Use the following procedure for each species to be used in the test procedure:

12.2.1 Twenty four hours before starting the test, inoculate a fresh culture on a tryptone glucose agar slant as described in 12.1.1 and incubate at 30 ± 5°C (86 ± 1°F). On the day the test is started, add 10 mL of sterile PBS to the tube, and using a sterile glass rod as an impinger, mix the bacterial growth into the PBS. See Note 8, Note 9, and Note 10 for details of preparation. Transfer to a sterile container and adjust the bacterial count to 1×10^7 to 1×10^8, preferably using a spectrophotometer. Set the spectrophotometer at 425 nm. Adjust to an optical density of 0.45 using an optical quality test tube measuring 12.7 mm in diameter. This should give approximately 1.0×10^8 bacteria/mL. For yeast, these settings should give approximately 1.0×10^7 yeasts/mL. Use the product insert provided by the manufacturer for the method of determining cell count.

12.2.2 For a mixed-species inoculum, first adjust each suspension of an individual species to the given level, and then combine equal portions. Use the bacterial suspension as the inoculum for the test in 15.3.1. Prepare a fresh suspension for each subsequent challenge described in 15.3.3.

NOTE 8—Prepare the PBS solution by the following formula:

	grams
NaCl	7.2
Na_2HPO_4(anhydrous)	1.48
KH_2PO_4(anhydrous)	0.43

Dilute to 1 L and sterilize. pH should be 7.2.

Instead of PBS, some laboratories use sterile deionized water or sterile physiological saline solution. See 7.2 and 7.11.

NOTE 9—Follow the same general procedure as given in A1.1 to prepare the primary suspension, except for bacteria and yeasts, use PBS as the diluent, do not use a wetting agent, and rinse the pellet only once.

NOTE 10—The alternate method for measuring and adjusting the bacterial and yeast count is use of the Petroff-Hausser counting chamber, which has a depth of 0.02 mm and can handle bacteria and yeasts better than the hemacytometer chamber. Methyl violet may be used to stain the bacteria for better visibility. See the instructions which accompany the counting chamber.

NOTE 11—To meet Schedule A in Table 1, make the inoculation (12.2.1) on Monday, and prepare the suspension (12.2.1 and 12.2.2) on Tuesday, the day the adhesive specimens are inoculated.

13. Yeast Cultures

13.1 Follow the instructions for bacterial cultures in Section 12, except use YMPG agar and determine the cell count using a hemacytometer or a Petroff-Hausser counting chamber. See Note 9.

14. Fungal Cultures

14.1 *Propagation of Fungal Cultures*—Use the following procedure for each species to be used in the tests:

14.1.1 Prepare a fresh culture on potato dextrose agar or other suitable medium for the species, and label by species and ATCC number. Incubate at 25 ± 0.5°C (77 ± 1°F) for 10 days or until full sporulation is achieved.

14.1.2 Refrigerate the cultures. Prepare new cultures each month. If contamination occurs, discard the cultures and prepare new ones.

14.2 *Preparation of Fungal Inoculum*—Use the following procedure for each species to be used in the tests:

14.2.1 Ten days before the test is to be started, follow the procedure in 14.1.1 to prepare fresh cultures.

14.2.2 *Harvesting Fungal Cultures and Dislodging Spores*—On the day of the first challenge, to one tube of each species of fungi, add 15 mL of sterile deionized water, containing 0.05 % sorbitan mono-oleate polyoxyethylene. Harvest fungal cultures and dislodge spores by rubbing and removing the growth gently with a sterile inoculating loop or with a sterile glass rod. Transfer the washings into a sterilized container containing glass beads and shake thoroughly to break up the clumps. Filter through sterile layered cheese cloth or sterile nonabsorbent cotton. Adjust the spore level to 1.0×10^7 spores/mL, ±2000, using a hemacytometer and the procedure in Annex A1. Use this spore suspension of fungi as the inoculum for the test described in 15.4.1 and 15.4.3 when the option is to test with a single species.

14.2.3 For a mixed culture, obtain a spore count on each fungal species and adjust each suspension to the level of 1×10^6 spores/mL. Combine equal portions of the spore suspensions from each of the species in a common sterilized container. Use this spore suspension for the challenge procedures described in 15.4.1 and 15.4.3 when the option is to test with a mixed species.

NOTE 12—To meet Schedule B in Table 2 make the inoculation (14.2.1) on Friday 10 days before first challenge, and prepare the suspension (14.2.2 and 14.2.3) on Monday, the day of the challenge.

15. Test Procedures, Bacterial, Yeast, and Fungal Resistance

15.1 *Adhesive Sample*—Obtain a sufficient sample of adhesive to run the necessary tests, place in a sterilized container, and cap.

15.2 *Adhesive Specimen*—For each test to be run, transfer

300 mL of the adhesive sample to a sterilized 400-mL jar and seal.

NOTE 13—The specimen size may be smaller or larger, but the millilitres of inoculum in 15.3.1 should be adjusted to reflect the millilitres of the adhesive specimen.

15.3 *Test for Resistance to Bacteria or Yeast*:

15.3.1 *Procedure, First Challenge*—Using a sterile pipet, add 5 mL of the aqueous suspension of organisms prepared in 12.2.1 or 12.2.2 to the 300 mL adhesive specimen prepared in 15.2. Mix well using a sterile glass rod or sterile wooden spatula and incubate at 30 ± 5°C (86 ± 1°F).

15.3.2 *Procedure, Sterility Check*:

15.3.2.1 At intervals 4, 24, 48, and 72 h and at 7 days, or at other intervals selected by the testing laboratory, culture the inoculated adhesive specimen onto tryptone glucose agar, making a zig-zag streak plate. For the transfer, use a sterile loop. Incubate each agar plate at 30 ± 5°C (86 ± 1°F) for 7 days.

NOTE 14—Some biological laboratories streak with sterile cotton-tipped swabs. Other types of agar are sometimes selected.

15.3.2.2 Examine all streak plates according to Schedule A on Table 1, or the specially designed schedule being used. Record the presence and degree of colonial growth, or the absence of growth on each of the streak plates. To show the comparative degree of growth on the positive plates, use qualitative gradings: light, medium, and heavy.

15.3.2.3 If colonial growth of the species used for the inoculum appears on the surface of the 7-day streak plate at the end of the 7-day incubation period, the test is complete, and report the adhesive sample to be *not resistant* to the species or group of species of bacteria used for the challenge.

15.3.2.4 If colonial growth of bacteria is not observed at the end of the incubation period for the 7-day streak plate, continue the test as described in 15.3.3.

15.3.3 *Procedure, Subsequent Challenges*—For any specimen testing sterile to the first challenge, repeat the procedure described in 15.3.1-15.3.2.4, reinoculating the same adhesive specimen, following the selected schedule. Discontinue testing at any point in the schedule on a specimen which shows colonial growth on the 7-day streak plate. See Sections 16 and 17 for further guidance on additional challenges, interpretation of results, and reporting.

15.3.4 *Control Streak Plate*—Test the adhesive specimen for presence of microorganisms before challenge by streaking on potato dextrose and tryptone glucose agar plates.

15.3.5 As a control to any test run, challenge an unprotected adhesive specimen.

15.3.6 *Standard Inhibitory Control*—Include in any laddered series or in any test of a single adhesive specimen, a control specimen protected with a level of biocide known to be adequate.

NOTE 15—An inhibitory control is useful as a check on the procedural process, since its performance is predictable. Preferably this should be the same formulation as the test adhesive, but containing a sufficient biocide level to protect. If this is not available, an adequately protected unrelated adhesive formulation which has been pretested may be used.

15.4 *Test for Resistance to Fungi*:

15.4.1 *Procedure, First Challenge*—Follow the procedure given in 15.3.1, except use the aqueous fungal spore suspension prepared in 14.2.2 or 14.2.3 for the inoculum and incubate the challenged specimen at 25± 0.5°C (77 ± 1°F).

15.4.2 *Procedure, Sterility Check*—Follow the procedure given 15.3.2.1 and 15.3.2.2, except use potato dextrose agar plates or other suitable medium chosen for the species. Incubate the streak plates at 25 ± 0.5°C (77 ± 1°F), and follow Schedule B on Table 2.

15.4.3 *Procedure, Subsequent Challenges*—Follow the procedure given in 15.3.3, except use a freshly prepared aqueous suspension of fungal spores.

15.4.4 *Controls*—See 15.3.4, 15.3.5, and 15.3.6.

16. Interpretation of Test Results

16.1 The highest level of biocidal protection is shown by a specimen returning to sterility by the fourth hour following the challenge, holding this sterility for 7 days, and performing in the same manner after four challenges. Consider a specimen that passes this test, however, as protected only against the species used for the challenge. Return to sterility 24 h after the challenge and holding this sterility for seven days, and showing this performance after two to four challenges is considered adequate microbial protection for some adhesives by many biological laboratories.

17. Report

17.1 Report the following information:

17.1.1 The species of bacteria, yeast, or fungi used for each test,

17.1.2 The schedule followed, A or B, or a summary of the testing procedure if it varies from the ones given,

17.1.3 A separate report for each test which used an individual species and each test which used a mixed culture,

17.1.4 The final readings on all streak plates, recording the presence of colonial growth, graded as light, medium or heavy, as positive, and absence of growth as negative,

17.1.5 The time required for the specimen to return to sterility,

17.1.6 The presence or absence of colonial growth on the control and inhibitory streak plates, and

17.1.7 Based on the presence or absence of growth, in accordance with the guidelines given, report the adhesive specimen to be *resistant* or *not resistant* to the organisms used for the challenge, when tested by the procedure as summarized according to 15.3 and 15.4.

18. Precision and Bias

18.1 No information is presented about either the precision or bias of Test Methods in D 4783 for measuring the resistance of adhesive preparations to attack by bacteria, yeast, and fungi since the test results are non quantitative.

19. Keywords

19.1 adhesive; bacteria; biological testing; culture; fungi; growth; yeast

ANNEX

(Mandatory Information)

A1. PROCEDURE FOR DETERMINING FUNGAL SPORE CONCENTRATION IN THE INOCULUM

A1.1 *Preparation of Primary Suspension*—Follow the procedure in 14.3 to harvest the spores. Transfer the washings from an agar slant of each species into a different sterile flask. Using a sterile 50-mL centrifuge tube, wash the spores of each species separately three times in sterile distilled water with the first wash containing a wetting agent, such as Tween 80 or the equivalent, 0.05 % sorbitan mono-oleate polyoxyethylene in deionized water. Centrifuge, decant and re-suspend. Do not use a wetting agent in the final rinse or for the final dilution. After the final centrifugation, leave the pellet in 5 to 6 mL of liquid. Re-suspend with a mechanical shaker, and use this primary suspension to make the spore count, using the hemacytometer.

A1.2 *Loading the Hemacytometer Chamber*—Place the special cover slip for the hemacytometer over the counting areas by centering the slip so that it touches the "V's." Using a sterile Pasteur pipet (6.1.11), or a disposable 1-mL pipet, charge by capillary action both sides of the chamber introducing the spore suspension at the "V's." If the liquid flows into the trough, wash the counting chamber and recharge. Wait 5 min to allow the spores to settle before counting. Make sure the spores are evenly distributed to assure an accurate count.

A1.3 *Dimensions of Hemacytometer Chamber:*

A1.3.1 For the purposes of these test methods, the dimensions given are for the subdivisions of the center primary square. This square is ruled into 25 secondary squares, which are further divided into 16 tertiary squares.

A1.3.2 The Levy hemacytometer chamber is 1⁄10 mm deep and is divided into squares of the following dimensions:

$$\text{Primary square} = 1 \text{ by } 1 \text{ by } 1/10 \text{ mm} = 1/10 \text{ mm}^3$$

$$\text{Secondary square} = 1/5 \text{ by } 1/5 \text{ by } 1/10 \text{ mm} = 1/250 \text{ mm}^3$$

$$\text{Tertiary square} = 1/20 \text{ by } 1/20 \text{ by } 1/10 \text{ mm} = 1/4000 \text{ mm}^3$$

A1.4 *Counting the Spores*—Use a 10× ocular and a 25× objective on the microscope. Focus on the center subdivided primary square, and count the spores in 5 secondary squares, 4 corners and the middle. This will total 80 tertiary squares. It may be necessary to dilute a portion of the primary suspension to make the count. Count all of the spores in the designated areas, but count only the spores that are on the right and lower boundary lines of each square, the center line of triple lines demarking the squares. Typically the primary suspension should have 6 to 10 times the desired 1×10^6 spores per millilitre, making it possible to dilute to the desired concentration.

NOTE A1.1—For a graphic depiction of the grids of the chamber, see the instructions which come with the hemacytometer. These directions give procedures for counting white blood cells and red blood cells, the original use of the chamber. The procedure given here is the one used for the red blood cell count, except the dilution may vary, and a factor of 1000 is used to convert cubic millimetres to millilitres. For further discussion on the spore count, see Refs **(2)** and **(3)**.

A1.5 *Calculation:*

A1.5.1 The volume of 80 tertiary squares equals 80 × 1⁄4000 mm^3.

A1.5.2 When no dilution is used, the dilution factor is one.

A1.5.3 When the spores in 80 tertiary squares are counted, use the two-part formula below:

$$\text{spores per mm}^3 = \frac{(\text{total spores counted}) \times (\text{dilution}) \times 4000}{80}$$

$$(\text{spores per mm}^3) \times 1000 = (\text{spores per mL})$$

Example: When 200 spores are counted, and the dilution factor is one:

$$1.0 \times 10^4 \text{ spores per mm}^3 = \frac{(200) \times (1) \times 4000}{80}$$

$$1.0 \times 10^4 \text{ spores per mm}^3 \times 1000 = 1.0 \times 10^7 \text{ spores per mL}$$

A1.6 After determining the number of spores per millilitre in the primary spore suspension, transfer 1 mL of the suspension to a sterile tube and make the proper dilution to achieve the desired 1.0×10^6 spores per millilitre, ±200 000. Example: To 1 mL of primary suspension at a concentration of 1.0×10^7spores/mL, add 9 mL of the diluent (distilled water or mineral salts solution) in A1.1 to achieve the desired 1.0×10^6 spores/mL concentration.

APPENDIXES

(Nonmandatory Information)

X1. Information on Bacterial Species

X1.1 *Gram Negative Bacteria* are generally used for the challenge. They are more capable of causing biodegradation than Gram positive bacteria.

X1.1.1 *Proteus vulgaris*, ATCC 9920, and *Proteus mirabilis* are found in water, sewage, and decomposing materials.

X1.1.2 *Pseudomonas aeruginosa*, ATCC 10145, is widely distributed in nature, found in water, soil, in the intestinal tract, and contents of man and animal. It is a pathogen.

X1.1.3 *Klebsiella pneumoniae* is an inhabitant of the gut (intestinal tract).

X1.1.4 *Enterobacter aerogenes* is found in soil, air, water, and the gut.

X1.1.5 *Escherichia coli* is found in intestinal tracts.

X1.2 *Gram Positive Bacteria:*

X1.2.1 *Bacillus subtilis* is a commonly occurring aerobic spore former. There are problems associated with using any of the aerobic or anaerobic spore-forming bacteria as test organisms. Bacterial spores are very resistant structures, and very few biocides will kill them. This problem is overcome by using a biocide which kills the cell after it has emerged from the spore. The spores already formed when the culture is used in the challenge inoculum probably will not be killed, but will persist in the adhesive specimen receiving the challenge. When the specimen is tested for sterility, the biocide is usually diluted, sometimes by migrating (leaching) from the adhesive into the agar, so that some living organisms can grow. At this time, the spore, which has been dormant, can germinate. It will appear as though the normal (vegetative) cell has lived through the test, while in reality, only the spores have survived. Of course, if the spores had germinated during the time they were in contact with the biocide while in the adhesive specimen in the container, they would have been killed **(4)**.

X2. Information on Yeast Species

X2.1 *Candida tropicalis*, ATCC 750, is a good general test yeast because it is a vigorous grower and is able to utilize a wide variety of carbon sources.

X2.2 *Kluyveromyces fragilis*, ATCC 8554, and *Candida pseudotropicalis*, ATCC 4135, grow well in the presence of lactose. Yeasts are not generally known for their ability to degrade polymeric materials, but frequently are present when starch is in the adhesive.

X3. Information on Fungal Species

X3.1 The species below include challenge organisms listed in the test methods plus other possible challenge species.

NOTE X3.1—A useful short reference for common industrial fungi and their contaminants is Ref **(5)**.

X3.1.1 *Aspergillus niger*, ATCC 9642, is a commonly occurring black mold.

X3.1.2 *Aspergillus flavus*, ATCC 9643, is a commonly occurring yellow green mold found associated with the decay of organic matter. Some strains of *A. flavus* form mycotoxins, but ATCC 9643 does not. However, all *Aspergilli* should be handled with caution as the inhalation of large quantities of spores can cause severe allergic reactions.

X3.1.3 *Aureobasidium pullulans*, ATCC 9348 and var. *melanigenum* 15233, are very common stain and soft rot fungi on wood products. They deteriorate plastics and paint, are common contaminants on finished surfaces, and might present a problem in adhesive degradation.

X3.1.4 *Chaetomium globosumis*, ATCC 6205, is representative of the genus *Chaetomium* which includes a fairly large number of species commonly encountered in industrial spoilage. They grow readily on paper and other cellulosics and can form mycotoxins.

X3.1.5 *Gliocladium virens*, ATCC 9645, is a common soil mold capable of inducing soft rot in wood. Some laboratories prefer the use of *Gliocladium roseum*, ATCC 48345.

X3.1.6 *Penicillium pinophilum*, ATCC 9644, formerly *Penicillium funiculosum*, is a fungus that deteriorates plastics. It is used in fungal resistance testing of plastics.

X3.1.7 *Phanerochaete chrysosporium*, ATCC 24725, is a common white rot fungus which is found to inhabit wood-chip piles and which will degrade lignin along with other resistant compounds.

X3.1.8 *Trichoderma pseudotkoningii*, ATCC 26801, is a mold commonly found on wood. It degrades fungicides and chlorinated phenols.

X3.1.9 Other commonly occurring fungi on wood are *Ceratocystis spp*., and *Diplodia natalensis*.

X4. Use of Mixed Cultures

X4.1 The use of selected mixed or pure cultures is largely arbitrary. Mixed culture of strains known to be a problem is always a good choice. Such use could lead to overgrowth of one strain or another, but this difficulty is more likely to be encountered if strains which are not normally commensal are employed. Pure cultures would lead to more reproducible less ambiguous results, but it is not clear whether such results would reflect the durability of the adhesive film in the field.

X4.2 Before mixed cultures are used, check for compatibility with each other. Do this by using a cross-hatch streak plate, inoculating the same plate with the selected species and checking for antagonism, that is, incompatibility.

X5. Rationale

X5.1 *Source of Test Methods*—These test methods were adapted from existing microbiological tests currently in use by the adhesive industry, biocide manufacturers, and independent commercial laboratories. A number of microbiologists working actively in this field were consulted. Areas of interest having an input were biocide manufacturers, adhesive manufacturers, and consumers, ASTM Committee G-3 on Durability of Nonmetallic Materials, specifically Subcommittee G03.04 on Biological Deterioration, a U.S.D.A. Microbiological Laboratory at Forest Products Laboratory, and a United States Army biological research laboratory. No precision and bias statements are available from the original test methods.

X5.2 *Commentary on Usefulness and Reproducibility*—In effect, these test methods are pre-set experimental designs which, if carried out properly, will result in data which will, in turn, enable one to arrive at a conclusion about the relative microbial resistance of a product. It is essential that the procedure be thoroughly tested to be certain that given conditions always provide the same results and that appropriate standard unprotected and inhibitory (protected) controls are included. It is also necessary to know the range of materials over which the test is applicable. Although a panel might render an opinion on these matters, nothing can substitute for actual data. As more is known about organisms which cause problems in biodeterioration of adhesives, further refinement should be possible.

REFERENCES

(**1**) Block, S. S., *Disinfection, Sterilization, and Preservation*, Third Edition, Lea and Febiger, Philadelphia, PA, 1983.

(**2**) Henry, John B., ed., *Clinical Diagnosis and Management, by Laboratory Methods*, Seventeenth Edition, Todd-Sanford-Davidson, W. B. Saunders Co., Philadelphia, 1984.

(**3**) Tuite, John, *Plant Pathological Methods, Fungi and Bacteria*, Burgess Publishing Co., Minneapolis, MI 55415, University Microfilms International, Ann Arbor, MI, 1981.

(**4**) Eilender, Albert L., and Opperman, Robert, (for Cosan Chemical Co.), "Biocides: Bactericides and Fungicides," *Handbook of Coating Additives*, Calbo, L. J., ed., Marcel Dekker, Inc., New York and Basel, Chapter 7, 1987, p. 204.

(**5**) Onions, A. H. S., Allsopp, D., and Eggins, H. O. W., eds., *Smith's Introduction to Industrial Mycology*, John Wiley and Sons, Seventh Edition, 1981.

SUMMARY OF CHANGES

Subcommittee D14.30 has identified the location of selected changes to this standard since the last issue, D 4783 - 98a, that may impact the use of this standard.

(1) No significant changes were made during this review.

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