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Standard Test Methods for Ability of Adhesive Films to Support or Resist the Growth of Fungi[1]

This standard is issued under the fixed designation D 4300; 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 test the ability of adhesive films to inhibit or support the growth of selected fungal species growing on agar plates by providing means of testing the films on two agar substrates, one which promotes microbial growth, and one which does not.

1.2 These test methods are not appropriate for all adhesives. The activity of certain biocides may not be demonstrated by these test methods as a result of irreversible reaction with some of the medium constituents.

NOTE 1—As an example, quaternary ammonium compounds are inactivated by agar.

1.3 A test method is included for use with low-viscosity adhesives along with an alternative method for use with mastic-type adhesives. Also, a method approved by the government is given.

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

1.5 *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 techniques. Specific precautionary statements are given in Section 7 and in Note 9.

2. Referenced Documents

2.1 *ASTM Standards:*

D 907 Terminology of Adhesives[2]

D 1286 Test Method for Effect of Mold Contamination on Permanence of Adhesive Preparations and Adhesive Bonds[3]

G 21 Practice for Determining Resistance of Synthetic Polymeric Materials to Fungi[4]

2.2 *TAPPI Method:*

T487 Fungus Resistance for Paper and Paperboard[5]

3. Terminology

3.1 *Definitions:*

3.1.1 *resist, vt, as related to fungi*—to fend off or withstand the growth.

3.1.2 *support, vt, as related to fungi*—to submit to the growth.

3.2 *Definitions of Terms Specific to This Standard:*

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

3.2.2 *adhesive film, n*—the small portion of the adhesive preparation, as prepared for use by the consumer, either with additives or as received, which is cast on a substrate, cured 24 h, and represents the glue line.

3.2.2.1 *Discussion*—For purposes of these test methods the *adhesive film* is the thin layer of adhesive spread on either the 21-mm fiberglass disk as described in 14.2, or the adhesive layer 3 mm thick which is cast on the tile squares as described in 15.1.

3.2.3 *zone of inhibition, n*—the area on an inoculated agar plate surrounding the adhesive-coated disk or tile, showing a reduced fungal growth or an absence thereof.

3.3 *Abbreviations:*

3.3.1 *PDA*—potato dextrose agar.

3.3.2 *MSA*—mineral salts agar.

3.3.3 *ZI*—zone of inhibition.

4. Significance and Use

4.1 These test methods are designed to be used to determine the susceptibility of the adhesive film to biodegradation and whether the adhesive will carry into the glue line sufficient anti-fungal properties to prevent growth of fungi frequently present on the gluing equipment, on adherends, or in the adhesive as applied.

4.2 Potato dextrose agar (PDA) provides a complete medium for the growth of fungi, while mineral salts agar (MSA) lacks a carbohydrate source and provides a less favorable

[1] These test methods are under the jurisdiction of ASTM Committee D-14 on Adhesives and are the direct responsibility of Subcommittee D14.30 on Wood Adhesives.

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

[3] Discontinued; see *1983 Annual Book of Standards*, Vol 15.06.

[4] *Annual Book of ASTM Standards*, Vol 14.02.

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

medium. Use of PDA tests the adhesive film for its ability to resist the growth of fungi on its surface as well as its ability to repel a copious growth of fungi on the adjacent agar surface. Use of MSA tests the adhesive film primarily for its ability to resist the growth of fungi on its surface. When it is used, there is a reduced possibility that the growth from the agar will be mis-read as coming from the adhesive film, since fungal growth on the adjacent agar will be scant.

NOTE 2—The method given here using the MSA is based on Practice G 21, adapted to be used with adhesives. Requirements to meet the approval of government specifications are the use of the MSA described in 10.2, and a mixed species of fungi described in 8.2 for the inoculum.

4.3 The results obtained when using the procedures given in this method apply only to the species used for the testing. The test species listed in Section 8 are frequently used by laboratories to test for antifungal properties, but they are not the only ones which could be used. Selection of the fungal species to test against requires informed judgment by the testing laboratory or by the party requesting the tests. These methods are especially useful when species that have been isolated from contaminated adhesives are used as the test species (see Section 8) to aid in the selection of more effective fungicides.

4.4 The efficacy of some biocides may change in storage due to the chemical and thermal environment to which they are subjected as components of certain adhesives. These test methods are not appropriate for determining the effect of fungal contamination on adhesives under water-soaking conditions, because they are not designed to cover the possibility of water-soluble biocides leaching out of the glue line.

4.5 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 fungal attack can be drawn by comparing the results to simultaneously run controls of known resistance. See X5.2 for statements regarding test reapeatability.

5. Apparatus

5.1 In addition to the standard equipment found in any fully equipped microbiological laboratory, items from the following list are needed for various tests. Not all items are needed for each test.

5.1.1 *Chromist Laboratory Spray Unit.*[6]

5.1.2 *Constant Temperature Chamber*, capable of being maintained at 35 ± 0.5°C (95 ± 1°F) or 25 ± 0.5°C (77 ± 1°F), or two chambers if needed simultaneously.

5.1.3 *Filter Disk, Glass Microfibre*, 934-AM, diameter-21 mm.[7]

5.1.4 *Filter Disk, Sterile Whatman No. 1.*[7]

5.1.5 *Filter Paper Assay Disk*, 1.5 cm diameter, sterile. Schleicher and Schnell, Inc., or the equivalent, has been found satisfactory for this purpose.[7]

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

5.1.7 *Glove Bag*, 68 cm in length and width, 38 cm in height.[8]

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

5.1.9 *Hood, Laminar-Flow Type*, Class II Type I.[9]

5.1.10 *Jar, Screw Cap*, round, approximately 1 L (1 qt, mason type).

5.1.11 *Pipet, Pasteur.*[7]

5.1.12 *Petri Dishes*, sterile, disposable, top-diameter of 150-mm, bottom-height of 15-mm.

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

5.1.14 *Teflon Paper or Grid*, pressure sensitive overlay, coated with TFE-fluorocarbon (PTFE), vinyl sheet backing, to be used at up to 93°C (200°F).[10]

6. Materials

6.1 *Potato Dextrose Agar*, Difco or equivalent.

6.2 *Sterile Deionized or Distilled Water*.

6.3 *Disinfectant Solution*—Amphyll, Alcide, or comparable product.

6.4 *Materials for Mineral Salts Agar*. (See list in 10.2.1.)

6.5 *Sorbitan mono-oleate polyoxyethylene*. [11]

7. Precautions

7.1 Assign laboratory personnel trained in correct microbiological techniques to run these tests. These test methods employ live cultures of fungi, some of which are capable of causing disease or allergic reaction in some humans. Use proper microbiological procedures in order to prevent contamination of the cultures or of the work area. Disinfect and sterilize in an approved manner all spills and all equipment coming into contact with the cultures. Also sterilize in an approved manner all cultures and contaminated disposable equipment before discarding. See 1.5 and Note 9.

7.2 In addition to other precautions, the use of a Class II, Type I containment hood is highly recommended for all procedures that would cause formation of fungal aerosols. This type of laminar flow hood prevents the spread of fungal spores throughout the laboratory and inhalation of spores by the operator. The hoods should be monitored by a biological safety officer or a health physicist if they are to be used with hazardous agents. Refer to the operating manual supplied by the manufacturer for detailed information. This warning applies specifically to the use of the Chromist laboratory spray unit listed in 5.1.1, the instructions in 14.3.2, and Note 9.

8. Test Species of Fungi[12]

8.1 Cultures of one or more of the following species are suggested for use when PDA is the medium:

[6] Available from Gelman Sciences, Ann Arbor, MI.

[7] Available from laboratory supply houses.

[8] Available from Instruments for Research and Industry, 108 Franklin Ave., Cheltenham, PA, or most laboratory supply houses.

[9] The Biogard Hood or similar equipment is available from laboratory supply houses.

[10] Gelman Sciences, or most laboratory supply houses.

[11] Available commercially as Tween 80.

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

		ATCC No.
8.1.1	*Aspergillus niger*	9642
8.1.2	*Aspergillus flavus*	9643
8.1.3	*Penicillium pinophilum*	9644 (See X1.6)
8.1.4	*Phanerochaete chrysosporium*	24725
8.1.5	*Aureobasidium pullulans* Var.—*melanigenum*	15233

NOTE 3—The choice of test organisms is often made from the fungal species listed above. Information on these and other species is given in Appendix X1.

8.2 Cultures of the following species are used for the government requirements described in Section 16, using MSA:

		ATCC No.
8.2.1	*Aspergillus niger*	9642
8.2.2	*Aureobasidium pullulans* Var.—*melanigenum*	15233
8.2.3	*Chaetomium globosum*	6205
8.2.4	*Gliocladium virens*	9645
8.2.5	*Penicillium pinophilum*	9644 (See X1.6)

NOTE 4—The species listed in 8.2 are used in Practice G 21. The following optional species are also sometimes used: *Aspergillus flavus*, (ATCC No. 9643) and *Aspergillus versicolor* (ATCC No. 11730). See 13.2 and Appendix X1.

8.3 Other pure cultures or mixed cultures of fungal species may be used, if agreed upon between the interested parties and upon the recommendation of the testing laboratories.

9. Sterilization of Equipment and Media

9.1 Follow accepted microbiological practices for sterilizing equipment and media.

NOTE 5—Two references for sterilization methods are TAPPI T 487 (see 2.2) and Ref **(1)**.[13]

10. Preparation of Media

10.1 *Potato Dextrose Agar*:

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

10.1.2 Follow the instructions given for preparation of the commercial product. Dissolve using heat and agitation. Transfer an appropriate amount of the agar solution to each flask used for pouring plates, and 10 mL per test tube. Plug flask with appropriate closures. 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, filling to an approximate depth of 3 mm. Allow plates to solidify. Tighten the caps on the tubes and place them in a slanted position to solidify, making a slant of about 51 mm. Store slants and plates in refrigerator until needed.

10.2 *Mineral Salts Agar*—Prepare sufficient medium for tests as described below:

10.2.1 Dissolve in 1 L of water the designated amounts of the following reagents:

	Grams
Potassium phosphate (KH_2PO_4)	0.7
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	0.7
Ammonium nitrate (NH_4NO_3)	1.0
Sodium chloride (NaCl)	0.005
Ferrous sulfate ($FeSO_4 \cdot 7H_2O$)	0.002
Zinc sulfate ($ZnSO_4 \cdot 7H_2O$)	0.002
Manganous sulfate ($MnSO_4 \cdot 4H_2O$)	0.001
Agar	15.0

10.2.2 Adjust the pH of the medium by the addition of 0.01N NaOH solution so that after sterilization the pH is between 6.0 and 6.5, and sterilize by autoclaving at 103 kPa, and 121°C (250°F) for 15 min.

10.2.3 Prepare plates as described in 10.1.2, and store in the refrigerator until needed.

11. Fungal Cultures

11.1 *Propagation of Fungal Cultures*:

11.1.1 Prepare a fresh culture for each species on PDA and label by species and ATCC Number. Incubate at 25 ± 0.5°C (77± 1°F) for a minimum of 10 days or until full sporulation is achieved.

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

11.2 *Preparation of Fungal Inoculum*:

11.2.1 Follow the procedure in 11.1.1 to prepare fresh cultures on PDA slants for each species to be used to conduct the tests.

11.2.2 *Harvesting Fungal Cultures and Dislodging Spores*—To one tube of each species of fungi, add 15 mL of sterile distilled or deionized water, containing 0.05 % sorbitan mono-oleate polyoxyethylene. Harvest fungal cultures and dislodge spores by rubbing the growth gently with a sterile inoculating loop or by removing it 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 1.0×10^6 per mL, using a hemacytometer and the procedure in Annex. Use this spore suspension of a single species of fungi as the inoculum for the tests described in Sections 14 and 15 when using the option given in 13.1.1.1.

11.2.3 For a mixed culture, obtain a spore count on each fungal species, and adjust each suspension to the level of 1×0^6 per mL. Combine equal portions of the spore suspensions from each of the species in a common sterilized container. Use this mixed spore suspension for the tests described in Sections 14, 15, and 16 when using the option given in 13.1.1.2.

12. Adhesive Sample

12.1 For ready-to-use liquid adhesives, obtain an approximate 250-mL sample. For adhesives to be mixed at the time of use, obtain a sufficient sample of each component, mix in accordance with the manufacturer's instructions, and run the tests on the prepared adhesive mix. For mastics, use the adhesive as packaged for the consumer, directly from the applicator tube.

NOTE 6—The sample size given is for convenience in handling. The test may be run on only a few millilitres of material. When several components are to be mixed to yield the sample to be tested, the amount of each component should be sufficient for remixing should a retest be necessary.

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

13. Selection of Conditions of Testing

13.1 Select one option from each of the testing conditions given below:

13.1.1 *Fungal Species for Inoculum*—Select the fungal species to use for the tests based on the informed decision of the testing laboratory or on the requirements of specifications which are to be met, using one of the options below:

13.1.1.1 Testing with spore suspensions of pure cultures of single species, or

13.1.1.2 Testing with a mixed spore suspension of two or more species.

NOTE 7—See Section 8 and Appendix X1 for options and help in selecting the species to use. See Appendix X2 for guidelines on use of mixed cultures.

13.1.2 *Agar Medium*—Select the medium based on the informed decision of the testing laboratory or on specifications to be met:

13.1.2.1 Potato dextrose agar,

13.1.2.2 Mineral salts agar, or

13.1.2.3 Other medium of choice.

13.1.3 *Procedure, Based on Viscosity or Consistency of the Adhesive*:

13.1.3.1 Low-viscosity adhesives, or

13.1.3.2 Mastic-type adhesives.

13.2 *Testing to Meet Government Specifications*—To comply with government specifications, select the following options: the mixed fungal species designated in 13.1.1.2, using the species listed in 8.2, and the MSA designated in 13.1.2.2. Follow the procedure given in Section 16.

14. Film Test for Low Viscosity Adhesives

14.1 *Number of Specimens and Plates per Test*—For each species or mixed species of fungi to be tested against, run two agar plates using three adhesive-coated test specimens described in 14.2, per 150-mm diameter plate and one plate with three uncoated Whatman No. 1 filter disks as a control.

14.2 *Preparation of Adhesive Specimens*—On the day before the tests are to be initiated, prepare the adhesive-coated fiber-glass disks (see 5.1.3). Coat both sides of each disk with the adhesive. Allow the disks to dry until no longer tacky by resting on a sheet of TFE-fluorocarbon-coated paper or a TFE-fluorocarbon-coated grid. Then dry for 24 h at 40 ± 2°C (104 ± 3.6°F) to allow the volatiles to dissipate. Store in sterile petri dishes until used.

NOTE 8—The drying time may need to be reduced for some adhesives to avoid curling of the disks. See Appendix X3 for a discussion on handling adhesives.

14.3 *Inoculation and Placement of Specimens*—Seed the duplicate test plates and the control plate, using one of the following procedures:

14.3.1 Inoculate a 150-mm agar plate by placing 0.1 mL of the spore suspension prepared in 11.2.2 on the surface of the plate, dropping from a pipet. Using a sterile L-shaped rod, seed the total surface. Prepare two plates, placing the three adhesive-coated fiber-glass disks equidistant and flat on the surface. Prepare the control plate by placing three uncoated Whatman No. 1 filter paper disks on the inoculated surface. Using a sterile capillary dropping pipet, place three drops of the spore suspension on the surface of each disk on the test plates and the control plate.

14.3.2 As an alternate method, prepare duplicate plates by placing three adhesive coated fiberglass disks equidistant and flat on the surface of each of two 150-mm agar plates. For the control, place three uncoated Whatman No. 1 filter paper disks on the agar surface of a third plate. Then inoculate the test plates and the control plate by spraying the fungal spore suspension over the entire surface of the agar and the disks. Use the apparatus in 5.1.1 for the spraying, and conduct this operation in the confines of a Class II Type I Biological Safety Cabinet or laminar-flow hood which has been properly monitored by a biological safety officer. Follow the precautions given in 7.2 and Note 9.

NOTE 9—**Precaution:** In addition to other precautions, use special care with the spray inoculation described in 14.3.2, which has a potential for gross contamination of the work area. Before inoculation place the open plates inside the described biological safety cabinet. Inside the hood, use a secondary containment, such as an empty 10-gal aquarium, partially covered, or a glove bag. Use disposable surgical gloves and a respirator when inoculating by the spray technique and when handling the inoculated plates. Invert the plates (if this will not disturb the disks) and enclose them in a sealed plastic bag to avoid contaminating the incubator. Use of a respirator provides an extra measure of safety when inoculating by the spray technique.

14.4 *Incubation and Examination*—Seal the plates with parafilm to prevent drying out during incubation. Incubate both the test and control culture plates at 25 ± 0.5°C (77 ± 1°F). When PDA is the culture medium, examine at 3, 7, and 14 days for zone of inhibition (ZI) or overgrowth on the adhesive coated disks, and record results using the grading system given in 14.5. For slow growing species, extend the incubation period to 21 days. Examine the control plate and the outer edges of the test plates for presence of a normal confluent growth pattern of the fungal species being tested and an absence of contaminants. When MSA is the culture medium, examine at 7, 14, and 21 days.

NOTE 10—To avoid transfer of developing spores during the incubation period, handle the plates with care for the preliminary readings.

14.5 *Grading System*:

14.5.1 When PDA is the culture medium use the following grading system:

OC—No growth on disk, clear zone of inhibition

OM—No growth on disk, inhibited growth zone around disk with mycelia, but no spores

NG—No growth on disk, no ZI

SG—Sparse growth on disk

LG—Light growth on disk

MG—Moderate growth on disk

HG—Heavy growth on disk

14.5.2 The passing ratings are OC, OM, and NG. The failing ratings are SG, LG, MG, and HG. Determine whether any peripheral growth around the disk is from the agar or from the adhesive-coated surface of the disk. Only growth on the surface of the disk constitutes failure.

14.5.3 Measure the OC zone from the edge of the disk to the edge of the OM zone. Measure the OM zone from the edge of the OC zone, if there is one; otherwise measure from the disk. Enter the average or the range of the measurements in mm, and

record readings for all three disks.

14.5.4 When MSA is used (Section 16), use the NG, SG, LG, MG, and HG ratings.

NOTE 11—When used with inhibitory (protected) and unprotected controls, the grading system in the hands of an experienced microbiologist makes possible a judgment as to the degree of protection offered against the growth on the adhesive film of the species of fungi used for the test.

NOTE 12—The fungal growth on MSA will be sparse.

14.6 *Interpretation of Results*:

14.6.1 The primary judgment of the test results is on the basis of the presence or absence of any fungal growth of test species on the adhesive specimen. Also, any ZI around a given specimen indicates antifungal activity. The formation of these zones, however, indicates that the fungicide in the adhesive film is capable of migrating when subjected to wet conditions. Frequently it is considered important that the fungicide demonstrate only minimal migration, and remain in the adhesive film to provide continuous protection.

14.6.2 When running a laddered series of a specific biocide to test a range of levels of the biocide in an adhesive, as the levels increase, there should be an increased ZI if the fungicide is capable of migrating. When adhesives are protected by different biocides, a greater ZI for one adhesive over others in the series of competitive adhesives does not necessarily indicate that the superior protection is present in the adhesive with the greater ZI. See Appendix X5.

14.6.3 If the adhesive supports growth, regardless of whether or not it is a contaminant, then that adhesive is susceptible. If one species overgrows another when a mixed culture is the inoculum, or if a contaminant grows, report these circumstances. The test may be repeated using individual species if more information is needed.

NOTE 13—See Appendix X2 for further comments on use of mixed species.

15. Test Using Mastics or High-Viscosity Adhesives

15.1 *Preparation of Specimen and Agar Plate*—For each species of fungus, sterilize three 25-mm square pieces of tile at 103 kPa for 15 min. Cover the unglazed side of two of the sterile tiles with a 3-mm layer of adhesive. Dry for 24 h at 40 ± 2°C (104 ± 3.6°F) to allow the volatiles to dissipate. Place the coated tiles, adhesive side up, each in a sterile petri dish (100-mm diameter). Place the remaining tile in the center of a third petri dish for use as a control. Pour sterile agar around the tile, PDA or MSA, according to the selected option. Fill to a level even with the top edge of the tile, or the adhesive-coated tile. Allow the agar to solidify before inoculation.

15.2 *Inoculation*—Inoculate the two test plates and one control by the means described in 14.3.1, or by the alternate spray method described in 14.3.2. Seed the entire surface of the agar plate and the surface of the coated tile and control tile. Follow the precautions in 7.2 and Note 9 if the spray method is used.

NOTE 14—See X3.2 for an alternative to the use of the tiles in testing mastic adhesives.

15.3 *Interpretation of Results*—See 14.5, 14.6, and 16.4.

16. Procedure for Meeting Government Specifications (2)

16.1 *Adhesive Specimens*:

16.1.1 Cut adhesive tapes and sheets into approximately 5-cm^2 pieces. For adhesive formulations and base polymers used in the preparation of adhesives, use glass microfibre disks (see 5.1.3), dipped or coated with the material to be tested.

16.1.2 Run a test on a known protected adhesive as a control.

16.1.3 Use sterile filter-paper assay disks, 1.5 cm in diameter, as the unprotected control (see 5.1.5).

16.1.4 Place the control disk at the 12:00 position on the MSA plate (150-mm diameter), and the test specimens (prepared as described in 16.1.1 and conditioned as described in 14.2.1) at the 3, 6, and 9 o'clock positions. Prepare in triplicate.

16.2 *Inoculation*—Inoculate the plates as described in 14.3.2, using the spray technique. Follow the precautions given in 7.2 and Note 9.

16.3 *Incubation Period*—Hold at 30 ± 0.5°C (86 ± 1°F) for 21 days, examining the specimens and controls for presence or absence of fungal growth on their surfaces at weekly intervals. See 14.4.

16.4 *Interpretation of Test Results and Grading*—See 14.5.4, 14.6, Note 12, Note 13, and Appendix X2.

17. Report

17.1 For each test and the controls, report the following:

17.1.1 Type of agar used,

17.1.2 Fungal species used, or all species included in the inoculum, if following the use of a mixed-spore suspension,

17.1.3 Inoculation procedure used,

17.1.3.1 Pipet and L-shaped rod,

17.1.3.2 Spray method,

17.1.4 Procedure followed,

17.1.4.1 Low viscosity adhesives, Section 14,

17.1.4.2 Mastic type adhesive, Section 15,

17.1.4.3 Government procedure, Section 16,

17.1.5 Zone of inhibition (ZI) in millimetres for each disk, reported by plate and disk number,

17.1.6 Grading for each disk as described in 14.5,

17.1.7 Nature of the growth of the fungi on the control plate and on the periphery of the test plates, as normal, scant, or profuse,

17.1.8 The presence or absence of contaminants, and

17.1.9 Summary of test results and statement as to whether the adhesive shows resistance to the species used for the tests.

18. Precision and Bias

18.1 No information is presented about either the precision or bias of Test Method D 4300 for measuring the growth of fungi on adhesives since the result is nonquantitative.

19. Keywords

19.1 adhesive film; biodegradation; biological testing; fungi

ANNEX

(Mandatory Information)

A1. Procedure for Determining Spore Concentration in the Inoculum

A1.1 *Preparation of Primary Suspension*—Follow the procedure in 11.2.2 to harvest the spores. Transfer the washings from an agar slant of each species into a different sterile flask (see Note A1.1). 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 monooleate polyoxyethylene. Centrifuge, decant, and re-suspend. To prepare a suspension which may be used for a longer period of time, use a mineral salt solution as the final rinse. (Follow the directions in 10.2.1, deleting the agar.) This will slow down the germination of the spores while in the suspension. 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.

NOTE A1.1—*Chaetomium globusum* will not yield sufficient spores from a single agar slant.

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 (5.1.11), 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:

Primary square = $1 \times 1 \times 1/10$ mm = 1⁄10 mm^3

Secondary square = $1/5 \times 1/5 \times 1/10$ mm = 1⁄250 mm^3

Tertiary square = $1/20 \times 1/20 \times 1/10$ mm = 1⁄4000 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 five secondary squares, four 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 cubic millilitre, making it possible to dilute to the desired concentration.

NOTE A1.2—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 **(3)** and **(4)**.

A1.5 *Calculation*:

A1.5.1 The volume of 80 tertiary squares equals 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 given below:

$$\text{spores per mm}^3 = \frac{(\text{total spores counted}) \times (\text{dilution}) \times 4000}{80} \quad \text{(A1.1)}$$

$$(\text{spores per mm}^3) \times 1000 = (\text{spores per mL}) \quad \text{(A1.2)}$$

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

$$1.0 \times 10^4 \text{ spores per mm}^3 = \frac{(200) \times (1) \times 4000}{80} \quad \text{(A1.3)}$$

$$1.0 \times 10^4 \text{ spores per mm}^3 \times 1000 = 1.0 \times 10^7 \text{ spores per mL} \quad \text{(A1.4)}$$

A1.6 After determining the number of spores per mL 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 mL, ±200 000.

Example: To 1 mL of primary suspension at a concentration of 1.0×10^7, add 9 mL of the diluent (distilled water or mineral salts solution) in A1.1 to achieve the desired 1.0×10^6 concentration.

APPENDIXES

(Nonmandatory Information)

X1. Information on Fungal Species (5)

X1.1 The species below include challenge organisms listed in the test methods plus other possible challenge species. See Ref **(5)** for a useful short reference for common industrial fungi and their contaminants.

X1.1.1 *Aspergillus niger*, ATCC 9642, is a commonly occurring black mold.

X1.1.2 *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. ATCC 9643 does not. However, all *Aspergilli* should be handled with caution as the inhalation of large quantities of spores can cause severe allergic reaction.

X1.1.3 *Aureobasidium pullulans*, ATCC 9348, and var. *melanigenum*, ATCC 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.

X1.1.4 *Chaetomium globosum*, 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.

X1.1.5 *Gliocladium virens*, ATCC 9645, or *Gliocladium reoseum*, ATCC 48395 (preferable), are both common soil molds capable of inducing soft rot in wood.

X1.1.6 *Penicillium pinophilum*, ATCC 9644, formerly *Penicillium funiculosum*, is a fungus that deteriorates plastics. It is used in fungal resistance testing of plastics.

X1.1.7 *Phanerochaete chrysosporium*, ATCC 24725, is a common white rot fungus which has been found to inhabit wood-chip piles and which will degrade lignin along with other resistant compounds.

X1.1.8 *Trichoderma pseudotkoningii*, ATCC 26801, is a mold commonly found on wood. It degrades fungicides and chlorinated phenols.

X1.1.9 Other commonly occurring fungi on wood are *Ceratocystis spp*., and *Diplodia natalensis.*

X2. Use of Mixed Cultures

X2.1 The use of selected mixed or pure cultures is largely arbitrary. Mixed cultures 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.

X2.2 Before mixed cultures are used they should be checked for compatibility with each other. This could be done by using a cross hatch streak plate, inoculating the same plate with the selected fungi and checking for antagonism, that is, incompatibility.

X3. Handling Adhesives

X3.1 Adhesives vary greatly in handling properties, and casting a film to perform these tests sometimes requires ingenuity. In the absence of recommendations from the manufacturer, the following means of applying the adhesives to the disks may be considered: dipping, painting on, and dropping on and allowing to spread unassisted. A convenient means of transferring the adhesive to both sides of the disk is to anchor the fiberglass disk on the tip of one index finger with a drop of adhesive. (Hands are fitted with a clean pair of surgical gloves.) Spread the adhesive on one side and then the other with a glass rod or with the other index finger. Some laboratories prefer to lay down a film of adhesive on a TFE-fluorocarbon coated surface, dry it, and cut a square of film to serve as the specimen. On drying, some adhesives will curl a cellulosic disk. Use of the prescribed fiberglass disk will alleviate this problem to some extent. Some laboratories spread the film on a sterile glass microscope slide, allow it to dry, and place the slide in a sterile petri dish. Then they fill the dish with agar to the surface of the glass slide, in the manner of the described test for mastics in Section 15. Inoculation is handled by dropping several millilitres of a fungal suspension over the entire area of the slide and the agar surface, or by spraying a suspension of fungal spores over the total surface.

X3.1.1 To achieve repeatable and accurate results, a laboratory should use the same disk coating procedure for all tests and should judge all testing by the same criteria.

X3.2 Some laboratories prefer to apply a mastic type adhesive to the agar plate by dropping directly onto the agar plate, by-passing the cumbersome use of the tiles. The disadvantage of this technique lies in the lack of control over the size of the drop. Also, the adhesive film does not have the opportunity to dry out, so the adhesive specimen can contain

volatiles of a nature that would interfere with the test results.

X4. Advantage of Fiber Glass Disk

X4.1 These methods employ the use of a fiber glass disk as a surface for the adhesive film. This substrate was chosen over the previously used cellulosic disk because cellulose can support the growth of certain fungi and could contribute to a misinterpretation of results.

X5. Rationale

X5.1 *Source of Test Methods*—These test methods were adapted from Test Method D 4300 and existing microbiological tests currently in use by the adhesive industry, biocide manufacturers, and independent commercial laboratories. A number of microbiologists working actively in their fields were consulted. Areas of interest having an input were biocide manufacturers, adhesive manufacturers and consumers, ASTM Committee G-3 on Durability of Nonmetallic Materials, Subcommittee G03.04 on Biological Deterioration, a U.S.D.A. Microbiological Laboratory at Forest Products Laboratory, and a U.S. Army biological research laboratory. No precision and bias statements are available from the original methods.

X5.2 *Interpretation of Test Results*—The interpretation given in 14.6.2 was taken in part from Ref **(6)**, a document prepared as part of a study and resulting in the recommendation in 1978 of the agar plate method of testing for biocides in adhesives.

X5.3 *Commentary on Usefulness and Reproducibility*—In effect, these standard test procedures 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 absolutely essential that the procedure be thoroughly tested to be certain that given conditions always return the same results and that appropriate standard unprotected and protected (inhibitory) 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 fungal species which cause problems in biodeterioration of adhesives, further refinement should be possible.

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