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An American National Standard

Standard Test Methods for Mildew (Fungus) Resistance of Paper and Paperboard[1]

This standard is issued under the fixed designation D 2020; 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 These test methods cover the qualitative determination of mildew (fungus) resistance of paper and paperboard, particularly those types which have been given a fungus resistant treatment.

1.2 The two test methods appear in the following order:

	Sections
Method A—Direct Inoculation, Pure Culture, Nonsterile Specimen	6 to 12
Method B—Soil Burial	13 to 17

1.3 The direct inoculation, pure culture, nonsterile specimen method is applicable to paper products that are expected to be used or stored in a damp, warm atmosphere, but out of contact with damp soil and is the preferred method. The soil burial method may be useful, and is recommended, for papers, with or without fungus resistant treatment, which may be in contact with damp soil for long periods of time.

1.4 *This standard does not purport to address all of the safety problems, 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.*

2. Referenced Documents

2.1 *ASTM Standards:* [2]

D 585 Practice for Sampling and Accepting a Single Lot of Paper, Paperboard, Fiberboard, or Related Product

D 828 Test Method for Tensile Breaking Strength of Paper and Paperboard

D 1193 Specification for Reagent Water

D 1968 Terminology Relating to Paper

3. Terminology

3.1 *Definitions*—Definitions shall be in accordance with Terminology D 1968 and the *Dictionary of Paper*.[3]

4. Significance and Use

4.1 Paper products used or stored in damp warm atmospheres or in contact with damp soil are subject to attack by fungus and other microorganisms. These test methods cover procedures for evaluating the degree of resistance to attack and for evaluating the degree and permanency of protection to attack by paper treatments.

5. Sampling

5.1 Obtain a sample of the paper to be evaluated in accordance with Practice D 585.

METHOD A—DIRECT INOCULATION, PURE CULTURE, NONSTERILE SPECIMEN

6. Apparatus

6.1 *Autoclave*, capable of being operated at a pressure of 15 psi (103 kPa) steam pressure and an exhaust temperature of 121°C (250°F), for sterilization of media.

6.2 *Bottles for Inoculum*—Several 250-mL, narrow-mouthed, square-sectional glass bottles fitted with screw caps are used for water blanks. Standard milk-dilution bottles scribed at the 99-mL level are recommended. The blanks are prepared by adding to each bottle approximately 13 mm of glass beads, 100 mL of water, and three or four drops of suitable wetting agent. The water blanks are then ready for sterilizations.

6.3 *Plugs*, nonabsorbent cotton or other suitable closures.

6.4 *Flaming Equipment*—Depending upon the circumstances, an alcohol lamp or a Bunsen burner may be used to flame the inoculating needle and the mouths of sterile containers.

6.5 *Containers*—Erlenmayer flasks, 250 mL, or bottles are convenient containers for sterile media.

6.6 *Oven*, capable of maintaining a temperature of 165°C.

6.7 *Incubator*, capable of maintaining a temperature of 28 ± 1°C to provide proper incubation of the inoculum and for the inoculated specimens.

6.8 *Inoculating Needle*—A standard inoculating needle fitted with either 22- or 24-gage Nichrome wire.

6.9 *Test Tubes*—18 by 150-mm rimless bacteriological test tubes are used for growing the test organisms.

6.10 *Petri Dishes*, disposable, 100 by 15 mm are recommended.

[1] These test methods are under the jurisdiction of ASTM Committee D06 on Paper and Paper Products and are the direct responsibility of Subcommittee D06.92 on Test Methods.

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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] Formerly published by American Paper & Pulp Assoc. (API), New York, NY (4th ed., 1980).

6.11 *Pipets*—Graduated 5- or 10 mL Mohr pipets, with the tips cut off and fire-polished to give an opening about 3 mm in diameter at the delivery end, are best for pipetting the spore-mycelial inoculum and for measuring the volume of culture medium added to each Petri dish. The Kolmer is also a satisfactory type of pipet. Presterilized disposable pipets may be used.

6.12 *Scissors*—A satisfactory type has approximately 100 mm cutting edges.

6.13 *Colony Counter*—Either of the standard bacterial colony counters or a large magnifying lens is helpful but not required for the examination of fungus growth on the test specimen.

7. Reagents and Materials

7.1 *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, where such specifications are available.[4] 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.

7.2 *Purity of Water*—Unless otherwise indicated, references to water shall be understood to mean freshly boiled and cooled reagent water, Type I or II, as described in Specification D 1193.

7.3 *Test Organisms*—The fungi recommended as standard organisms for this test are: *Chaetomium globosum*[3] (A.T.C.C.-6205), *Aspergillus terreus*[3] (QM-82J), and *Aspergillus niger*[3] (BL-89). Other species of fungi may be included in the test which have been isolated from paper products that have failed under certain use conditions. These additional test organisms may require the addition of dextrose to the nutrient salt agar before they will grow on the test specimens.

7.4 *Nutrient Media*—Two kinds are needed, as follows:

7.4.1 *Potato-Dextrose Agar*—This medium is used to maintain the test organisms in stock and to grow the test organism for the inoculum. This standard medium can be obtained from many biological supply houses.

7.4.2 *Mineral-Salt Agar*—This medium is used in the test to provide a portion of the nutrient requirements of the test organisms. The composition is as follows:

Ammonium nitrate (NH_4NO_3)	3.0 g
Dipotassium phosphate (K_2HPO_4)	1.4 g
Potassium chloride (KCl)	0.25 g
Magnesium sulfate ($MgSO_4$ $7H_2O$)	0.25 g
Agar	10.0 g
Tap water	1000.0 mL

7.4.3 After the ingredients are dissolved, the hot mixture is dispensed into appropriate containers. For the medium described in 7.4.1, approximately 10 mL of the mixture are dispensed into each test tube, and then the tube is plugged with cotton. For the medium described in 7.4.2, the mixture may be dispensed into either Erlenmeyer flasks or bottles and plugged with cotton, or the bottles may be capped with suitable screw caps. The culture media are then ready to be sterilized.

7.4.3.1 The mineral-salt agar may be modified by adding 0.5 % of dextrose, provided this sugar is necessary to fulfill the nutritive requirements of the test organisms. The use of this modified mineral-salt agar must be stated in the report.

7.5 *Preparation of Inoculum*:

7.5.1 *Spore-Mycelial Method*:

7.5.1.1 Inoculate two potato-dextrose agar slants with each organism to be used in the test and incubate for 14 days at 28°C. At the end of this incubation period, check to see that each agar slant is covered with mycelium and fruiting bodies of the test organism.

7.5.1.2 To each agar slant, add 5 mL of sterile water and suspend the surface growth in the liquid by gently scraping the surface of the slant with a sterile inoculating needle. Pool the suspension from each set of two slants in the water blank from which the water was removed. Shake the spore-mycelial suspension thoroughly to break the spore and mycelial clumps. Repeat this procedure for each organism used in the test.

7.5.2 *Spore-Cloud Method*:

7.5.2.1 If desired, this method of preparing the inoculum may be substituted for the spore-mycelial method. Thoroughly mix 30 g of wheat brand with an equal weight of 0.1*N* HCl and sterilize in a loosely capped 947 mL (1-qt) Mason jar for 30 min at 103 kPa (15 psi). Inoculate the cooled sterilized bran by intimate mixing with 10 mL of a spore-mycelial suspension of the test organism which has been grown on a potato-dextrose agar slant. Prepare the suspension by washing the surface of the slant with 10 mL of sterile water. If necessary, loosen the spores and mycelium with a sterile inoculating needle.

7.5.2.2 To secure optimum surface, spread out the inoculated bran in the Mason jar, which is set on its side, and incubate in this position at 28°C. After about 48 h of incubation, the fungus will have grown rapidly through the bran. Then, using aseptic techniques, break the mat which has formed into small clumps with a sterile knife or spatula and incubate until the fungus has sporulated throughout the bran. After sporulation, spread out the bran in the closed container and allow to dry. Then stopper the dried bran tightly and store at room temperature. It is recommended that the inoculated bran be prepared every 6 months.

8. Viability Control

8.1 When possible, use untreated material similar in all other respects to the treated material, for testing in the same manner as the test specimen to verify the viability of test organisms. When such material is not available, use material known to support the growth of the particular organism, such as filter paper. If this untreated material fails to show any abundant growth of the test organisms, consider the test inconclusive and repeat it.

9. Test Specimens

9.1 Select at random from the sample test specimens by cutting under aseptic conditions (preferably under a laminar-flow hood), 50 mm squares at random from the sample, the

[4] "Reagent Chemicals, American Chemical Society Specifications," Am. Chemical Soc., Washington, D.C. For suggestions on the testing of reagents not listed by the American Chemical Society, see "Reagent Chemicals and Standards," by Joseph Rosin D. Van Nostrand Co., Inc., New York, N.Y., and the "United States Pharmacopeia."

number of squares cut being governed by the number of test organisms used in the test.

9.2 Test each sample in triplicate with at least three different test organisms and determine the fungistatic properties of both sides of the test specimen.

9.3 For lightweight papers [weight per unit area 19.5 g/m^2 or less (12 lb or less, $24 \times 36 - 500$)], treat two plies of the paper as a single test specimen. However, the use requirements should dictate the number of plies used in the test. Always include suitable untreated controls in each series of tests.

9.4 The control specimen should be identical to the test specimens, except for the presence of the preservative. If this is not possible, untreated paper similar to the treated specimens should be used as the control.

10. Procedure

10.1 *Sterilization of Equipment and Media*—Depending upon the nature of the equipment to be sterilized, use one of the three following methods:

10.1.1 *Steam Sterilization (Autoclave)*—Sterilize culture media or water blanks by autoclaving for 20 min at a minimum of 121°C, corresponding to 103 kPa (15 psi). After placing items in autoclave, sufficient time (5 min) should be allowed for steam to completely replace the air in autoclave; then sterilize for an additional 15 min. Include a biological or chemical indicator strip to evaluate autoclave performance.

10.1.2 *Dry-Heat Sterilization (Electric Oven)*—Sterilize the pipets by heating in a closed metal container for at least 2 h at 165°C.

10.1.3 *Flaming*—Sterilize the inoculating needle by heating the Nichrome wire in an open flame until it becomes red hot. The hot needle is cooled by jabbing it into the agar slant from which the inoculum is to be collected.

10.2 *Procedure*:

10.2.1 Pour cooled or remelted sterilized mineral-salt agar into the required number of Petri dishes (one dish for each test specimen). The amount of agar dispensed into each Petri dish is dependent upon the thickness of the paper under test. For testing paper and paperboard up to 0.43 mm thick, measure 15 mL of the mineral-salt agar into each Petri dish; for paperboard 0.46–1.65 mm thick, use 25 mL. After the mineral-salt agar has solidified, inoculate the test specimens with the test organisms in accordance with either one of two methods, as follows:

10.2.1.1 *Spore-Mycelial Method*—After the nutrient salt agar has solidified in the Petri dish, place one of the 50 mm square test specimens on the surface of the agar. After the replicates for each treatment have been placed on the hardened mineral-salt agar, inoculate each square with one of the test organisms by distributing 0.5 mL of the prepared spore-mycelial suspension over the surface of each test specimen. The distribution of the inoculum can be facilitated with the use of a sterile glass rod bent into the shape of an "L." Also, inoculate suitable untreated specimens with test organisms.

10.2.1.2 *Spore-Cloud Method*—This method is best carried out in an enclosed chamber constructed of transparent plastic. Two round openings should be provided to allow the technician to work with hands and forearms in the chamber. Place the jar containing the wheat-bran inoculum in the chamber, shake, and inoculate the test specimens by suspending them momentarily in the mouth of the jar. After inoculation, place the test specimens on the surface of the solidified nutrient salt agar.

10.3 *Incubation*—Incubate the Petri dishes containing the inoculated test specimens for a period of two weeks at 28 ± 1°C, preferably in a humid atmosphere.

11. Interpretation of Results

11.1 The resistance to fungus growth is determined by visual examination. Examine the test specimens several times during the first week of incubation for the growth of the test organisms or other fungi.

11.2 If the test specimens show growth of either of the test organisms after seven days' incubation, discontinue the test and report the sample as not fungus-resistant. If no growth is observed on the test specimens after the first week of incubation, incubate them for an additional seven days. If the sample then supports the growth of any of the test fungi, report it as unsatisfactory or not fungus-resistant. If the specimens support no growth during the first week of incubation and only sparse growth after two weeks, report the sample as moderately fungus-resistant. If there is no growth of the test organisms or other species of fungi on the specimens after two weeks, or both, report the sample as fungus resistant. However, the growth of fungi other than the test organisms shall always be reported. In the case of a coated paper sample, no growth may be observed on the surface of the specimen but may be quite prolific along the cut edges. Such tests will be considered as satisfactory for coating but not for the complete specimen.

12. Report

12.1 The report shall include the following:

12.1.1 The names of the test organisms,

12.1.2 The method of inoculation, and

12.1.3 The number of days the test specimens were incubated.

METHOD B—SOIL BURIAL

13. Apparatus

13.1 *Soil Beds*—Soil composed of equal parts of good top soil or leaf mold, well rotted and shredded manure, and coarse sand, maintained at a moisture content of 25 ± 2 % (dry mass), a pH of 5.5 to 7, and a temperature of 30 ± 2°C. The soil should be sufficiently porous in texture to permit ready penetration of air and moisture, should be highly favorable to microbial activity, and rich in cellulose destroying organisms.

NOTE 1—To verify the microbiological activity of the soil, untreated controls should be deteriorated completely after 14 days.

13.2 *Soil Container*—A container of wood, glass, porcelain, or plastic, in a size convenient to handle, having a depth of at least 5 in. (127 mm), and provided with glass plate covers to reduce evaporation from the soil burial beds.

13.3 *Incubator*—A temperature-controlled room or cabinet from which direct natural light is excluded.

14. Test Specimens

14.1 Select at random from the sample sufficient paper (five sheets) to prepare ten specimens 1 by 10 in. (25.4 by 254 mm)

long. Unless otherwise specified, cut two adjacent specimens from a sheet, one specimen for the test and one to be used as a not-buried, treated control.

15. Procedure

15.1 Unless otherwise specified in the material specification, determine resistance to mildew by the change in tensile breaking strength after the specimens have been exposed in the soil beds for two weeks. Place the specimens for soil burial in a shallow trough approximately 4 in. (102 mm) wide by 2 in. (51 mm) deep and as long as necessary to keep the individual specimens about 2 in. apart. Lay each specimen across the trough, depressing the center of the specimen gently until it reaches the bottom of the trough and assumes a "U" shape.

15.1.1 Sprinkle soil over the specimen until the trough is filled. Leave about 2 in. at each end of each specimen exposed. Of greatest importance is the disposition of the specimens, so that they will have uniform and firm contact with the soil.

NOTE 2—Bury nonflexible papers and paperboard in a vertical or inclined position, with one end exposed.

15.2 At the end of the exposure period of 14 days, remove the specimens from the soil bed, and if not completely degraded, gently wash to remove the soil, dry in a vented oven at 60°C for 4 to 6 h and then condition for 24 h at 23 ± 2°C and 50 ± 2 % relative humidity. Wash, dry, and condition the controls in like manner and at the same time.

15.3 At the end of the conditioning period, determine the tensile breaking strength in accordance with Test Method D 828.

16. Calculation

16.1 Compute the average tensile breaking strength for the controls and the average tensile breaking strength for the test specimens that were buried, and from these averages compute the average percent loss in strength.

17. Report

17.1 The report shall include the following:

17.1.1 Number of specimens tested and

17.1.2 Average percent loss in tensile breaking strength.

18. Precision and Bias

18.1 No statement is made about either precision or bias as the test method is only intended for qualitative use.

19. Keywords

19.1 fungus resistance; mildew resistance; paperboard