Designation: D 2574 – 00

Standard Test Method for Resistance of Emulsion Paints in the Container to Attack by Microorganisms[1]

This standard is issued under the fixed designation D 2574; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope *

1.1 This test method covers the determination of the relative resistance of emulsion paints to attack in the container by microorganisms.

1.2 The values stated in SI units are to be regarded as the standard. The values given 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.*

2. Summary of Test Method

2.1 This test method is designed to challenge samples of one or more paints containing various levels of one or more biocides with a known amount of bacteria and rate the ability of the test paint(s) to control the "contamination."

3. Significance and Use

3.1 Spoilage of paint in the container can result in putrefaction, lowered pH, gas formation, and decrease in viscosity. This test method provides a standard procedure for the evaluation of the resistance of emulsion paints to microbial deterioration. The results should enable: (*1*) the paint manufacturer to select an effective preservative and (*2*) the supplier of preservatives to evaluate the performance in emulsion paints of competitive and developmental preservatives.

3.2 This test method should be used preferably by persons who have had basic microbiological training.

NOTE 1—The reliability of the results obtained from this test method is extremely dependent on the techniques employed. Improper techniques can result in a sterile sample appearing to be contaminated, and even worse, a contaminated sample appearing to be sterile (see also Note 2). It is recommended that you consult with your biocide supplier, raw material supplier, or an independent testing laboratory to confirm questionable results. Formulation and raw materials' quality may also vary and thereby affect the test results.

4. Apparatus and Materials

4.1 *Balance*, capable of weighing to 0.10 g.

4.2 *Incubator*, or other device capable of maintaining a constant temperature between 28 and 32°C.

4.3 *Refrigerator*, maintained at 10 to 13°C.

4.4 *Screwcap Borosilicate Test Tubes*, 125 by 15-mm.

4.5 *Borosilicate Flasks*, 1-L.

4.6 *Screwcap Bottles*, 150-mL.

4.7 *Autoclave*, capable of producing 103 kPa (15 psi) of steam pressure at 121°C and maintaining it for a minimum of 15 min. An autoclave is not necessary if prepared agar slants are used.

4.8 *Pipettes or an Automatic Pipettor*, sterile, 1-mL, with sterile disposable pipette tips for 1 mL.

4.9 *Petri Dishes*, sterile.

4.10 *Dehydrated Tryptic Soy Agar (TSA)*, medium, or pre-prepared slants, plates, and broth tubes.[2]

4.11 *Swabs*, sterile cotton.

4.12 *Laminar Flow Hood, Sterile Room, or at Least a Laboratory Testing Area*, relatively clean, free of blowing dust and dirt, etc., which can be used for streaking plates.

4.13 *Antiseptic Solution*, to help maintain sterility of testing area surfaces (4.12) (for example, 70% ethanol solution).

4.14 A minimum of 235 mL (½ pt) of each paint sample under test (pre-loaded with biocide).

4.15 A minimum of 475 mL (1 pt) of paint identical to 4.14, but containing no biocide.

4.16 *Twenty-four Hour Cultures of a Pseudomonas sp.* (for example, *Pseudomonas aeruginosa* ATCC #10145) and an *Enterobacter sp.* (for example, *Enterobacter aerogenes, ATCC #13048)*—These should be grown separately in tryptic soy broth. If a spoiled paint of a similar type as that under test is available, organisms cultured from this material can be used.

NOTE 2—See Appendix X1 for a method to spoil paint for use as an inoculum. Also *Bacillus sp.* for example, *Bacillus subtilis*, ATCC #27328 or other organisms as agreed upon between the parties involved may be

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[2] Available from microbiological supply companies. Media with TTC indicator dye may be used. In general, the TTC helps visualize contamination, but it has been reported on occasion to inhibit the growth of some bacteria. Interferences from pigments in materials being tested may make the color change difficult to see. If self-prepared plates are used with the TTC indicator, 0.01 % TTC indicator should be used and it must be added *after* autoclaving.

***A Summary of Changes section appears at the end of this standard.**

employed. When using spore-forming bacteria, care must be taken to ensure only vegetative cells are used in the inoculation (early log phase of growth).

5. Preparation of Materials

NOTE 3—Observe conventional microbiological techniques in making these tests. Handle all materials so as to avoid contamination from the air, fingers, or work surfaces.

5.1 *Preparation of Tryptic Soy Agar Plates and Slants*:

5.1.1 Follow the instructions on the container for preparation, or purchase prepared plates and slants.

5.1.2 Distribute 10 mL of the dissolved medium into each of 50 test tubes and 100-mL medium in 250-mL conical flasks.

5.1.3 Autoclave tubes (with caps loose) and the flask for 15 min at 103 kPa (15 psi) and a temperature of 121°C.

5.1.4 Upon removal from the autoclave, tighten caps and place the tubes at an approximate 30° angle position to prepare the slants with a slope of about 50 mm (2 in.) long.

5.1.5 For preparing TSA plates, pour 30 mL of the agar medium from the flask into sterile petri dishes and allow to set.

5.1.6 Store the prepared TSA slants and plates in a refrigerator at 10 to 13°C until needed.

5.2 *Preparation of Tryptic Soy Broth Tubes (TSB)*:

5.2.1 Follow the instructions on the container for preparation, or purchase prepared tubes.

5.2.2 Distribute 10 mL of the dissolved medium into each of 50 test tubes.

5.2.3 Autoclave tubes (with caps loose) for 15 min at 103 kPa (15 psi) and a temperature of 121°C.

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

5.3 *Inoculation of Tryptic Soy Broth Tubes with the Pseudomonas sp. and the Enterobacter sp*:

5.3.1 Above organisms are stored on tryptic soy agar slants in a refrigerator. To prepare a 24-h culture of each of the above organisms, the surface of a slant of each organism is scraped off with a sterile inoculating loop. This material is inoculated into a tube of TSB each and incubated in a 30 ± 2°C incubator overnight.

5.3.2 The overnight cultures are used to reinoculate fresh TSB tubes using a sterile inoculating loop.

5.3.3 Incubate the cultures to their log phase of growth as previously determined by standard microbiological technique and growth curves using a plate count usually 16 to 24 h.

5.3.4 Soak a sterile cotton swab or a loop in the inoculated broth culture following the incubation period described in 5.3.3.

5.3.5 Remove the swab or loop and prepare a second broth culture by repeating 5.3.2 and 5.3.3.

5.3.6 Following the incubation period, use the broth culture prepared in 5.3.5 to proceed as in Section 6 to inoculate the paint.

NOTE 4—Maintenance of cultures for future use: The purity of the bacterial inoculum prepared in 5.3.2 is verified by streaking a loopful from the growth onto a prepared TSA plate. A single isolated colony from the plate is then transferred to a previously prepared TSA slant using an inoculating loop. Incubate the slant for 24 h at 30 ± 2°C or until a luxuriant growth occurs on the slant surface. The slant is then stored in the refrigerator as a working stock culture until further use.

NOTE 5—The inoculum preparation for *Bacillus substilis* differs from the other cultures. *Bacillus subtilis*, ATCC 27328 has been shown to produce extracellular cellulase enzymes in the TSB medium.[3] Hence, it is advised that for *Bacillus* inoculum, the broth culture from 5.3.5 should be centrifuged at 4000 r/m for 10 min, the supernatant containing the cellulase enzymes is discarded and the bacterial pellet is re-suspended in equal volume of sterile water and then used as the inoculum in Section 6.

5.4 *Preparation of Paints for Test*:

5.4.1 Paints may be previously loaded with biocide as provided, or ladders of levels of biocide may be added as agreed upon by the parties involved. In all testing, a negative control (sample containing no biocide) should be included and appropriately identified.

5.4.2 Weigh 100 g of each paint sample to be tested into a suitable container (screwcap glass jars have been found suitable).

6. Procedure

6.1 *Inoculation of Paint Samples*:

6.1.1 Remove 0.1 mL from each of the individual bacterial inocula at $\sim 10^9$ colony forming units/mL CFU/mL and inoculate into 100 g of the test paint (provides $\sim 10^6$ CFU/g of the paint).

6.1.2 Incubate the paint at 30 ± 2°C for one week, and check for bacterial recovery or paint sterility after 1, 2, or 3, 5, and 7 days as described in 6.3.

6.1.3 For those samples which were sterile after the seventh day of the first week, repeat the inoculation using 1 mL of a $\sim 10^9$ inoculum and repeat incubation in accordance with 6.1.2.

6.2 *Preliminary Examination of Paint Under Test*:

6.2.1 Examine the container for evidence of swelling. If the container/lid is swollen, exercise caution in removing the lid.

6.2.2 Remove the lid and carefully smell the contents of the container. Deterioration of paint by microorganisms is often characterized by distinct odors. Such odors may be either putrefactive or fermentative.

6.2.3 Observe the contents of the container for the presence of stringy structures characteristic of the presence of certain microorganisms.

6.2.4 Observe the contents for noticeable losses in viscosity. This physical change frequently occurs as the result of microbiological deterioration.

6.3 *Determination of Recovery Microorganisms from the Paint Under Test*:

6.3.1 Soak a sterile cotton swab in the paint under test. Remove excess paint by pressing gently against inside of container (approximately, 200-mg quantity of paint is retained on the cotton swab).

6.3.2 Evenly spread the paint from the cotton tip onto the surface of a TSA plate (out of 200-mg quantity of paint retained on the swab, only about 50 mg of paint gets spread on the plates).

[3] Sadasivan, L. and Hinkle, J., "Extracellular Production of Cellulase by *Bacillus* Isolates from Spoiled Paints," *Proceedings of the 9th International Biodeterioration and Biodegradation Symposium*, held 5–10 September 1993, The University of Leeds, U. K. (in press)