Designation: E 1839 – 96 (Reapproved 2002)

Standard Test Method for Efficacy of Slimicides for the Paper Industry—Bacterial and Fungal Slime[1]

This standard is issued under the fixed designation E 1839; 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 presents a procedure to evaluate the efficacy of slimicides for the control of bacterial and fungal slimes in paper mill systems and their counterparts.

1.2 It is the responsibility of the investigator to determine whether Good Laboratory Practices (GLP) are required and to follow them where appropriate (40 CFR 160).

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. Referenced Documents

2.1 *ASTM Standards:*

D 1193 Specification for Reagent Water[2]

E 1054 Practices for Evaluating Inactivators of Antimicrobial Agents Used in Disinfection, Sanitizer, Antiseptic, or Preserved Products[3]

2.2 *TAPPI Standard:*

T 205 Forming Handsheets for Physical Tests of Pulp[4]

2.3 *CFR Standard:*

Title 40, Code of Federal Regulations (CFR), Part 160, Good Laboratory Practice Standards[5]

3. Terminology

3.1 *Definitions:*

3.1.1 *furnish*, *n*—pulp slurry fed to a paper machine. The type of pulp (sulfite, Kraft, mechanical), the source of fiber (virgin, recycled including pre- or post-consumer waste paper), and the pH are used to designate a specific type of furnish.

3.1.2 *pulp*, *n*—wood separated by chemical or mechanical means into their fibrous components. The pulp is used to make paper, paper board, or pulp sheets after specific treatments. Hardwood pulp is made from trees, such as maples or oaks, and softwood pulp is produced from trees, such as pines.

3.1.3 *pulp slurry*, *n*—an aqueous combination of cellulosic fibers, fillers, and other additives used for specific grades of paper.

3.1.4 *slimicides*, *n*—chemicals added during pulp and paper processing to control the growth of slime-forming microorganisms.

4. Summary of Test Method

4.1 Bacterial cells or fungal spores are added to acid or alkaline pulp slurries, or both, treated with slimicides to achieve final concentrations of 2×10^6 to 1×10^7 bacteria/mL or 10^5 to 10^6 fungal spores/mL, and incubated at appropriate temperature for determined time periods. Aliquots of the test suspension are then neutralized, plated onto bacterial or fungal medium, and observed for growth. Results with biocide are compared to results without biocide (control).

4.2 As a performance standard, an effective slimicide is one that shows a continued reduction in bacterial and fungal counts relative to the control over the duration of the test.

5. Significance and Use

5.1 This test method is to be used to determine if a slime control agent has application in the paper industry for control of bacterial or fungal slime.

5.2 This test method is run in acid, alkaline, or acid and alkaline conditions to determine the efficacy of the slime control agent.

5.3 The test conditions may be modified to reflect intended use patterns in typical paper mill systems, including use of actual paper mill furnish.

6. Apparatus

6.1 *Balance*:

6.1.1 *Plant Balance*, sensitive to 0.1 g and used to weight furnish.

[1] This test method is under the jurisdiction of ASTM Committee E35 on Pesticides and Alternative Control Agents and is the direct responsibility of Subcommittee E35.15 on Antibacterial Agents.

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[2] *Annual Book of ASTM Standards*, Vol 11.01.

[3] Discontinued. See 1999 *Annual Book of ASTM Standards*, Vol 11.05.

[4] Forming Handsheets for Physical Tests of Pulp, TAPPI Test Method T 205 on-88, 1994–1995, TAPPI, Atlanta, GA, 30348.

[5] Available from U.S. Government Printing Office, Superintendent of Documents, Mail Stop: SSOL, Washington, DC 20402-9328.

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6.1.2 *Analytical Balance*, sensitive to 0.1 mg and employed to weigh the candidate slime control agent to be used in the preparation of the stock solutions.

6.2 *Sample Containers (Sterile)*, 120-mL plastic specimen containers with screw-cap lids are ideal for holding test materials. Other suitable containers include 150/160-mL milk dilution bottles or WHIRL-PAKS.

6.3 *Culture Containers*, Petri plates, tissue culture bottles or glass tubes (15 × 125 mm or 18 × 150 mm without lip, preferably of borosilicate glass).

6.4 *Closures*, for tubes and containers.

6.5 *Disintegrators*[6]

6.6 *Flaming Equipment*—Depending upon circumstances, either an alcohol lamp, a bunsen burner, or electric incinerator may be used to flame inoculating needles and other equipment.

6.7 *Reliable incubators* that control at the temperature required, ± 2°C. Temperatures used should be consistent with the temperatures of the systems.

6.8 *pH Meter*—Any reliable pH meter is suitable to standardize the pH of the culture.

6.9 *Pipets*—1.1-mL milk dilution type, 1.0 mL graduated in 0.01 mL, and 10 mL graduated in 0.1 mL. Pipetters may be used, but not for highly viscous materials.

6.9.1 *Pipetting Aid*—Rubber bulb or other device to accomplish the transfer of liquid.

6.10 *Sterilizers*, steam sterilizer (121°C) or hot-air oven (180 ± 2°C for 2 h), or both.

6.11 *Filter Apparatus for Filter Sterilizing*, Disposable filter units, appropriate volume, 0.22-μm pore size.

6.12 *Sterile Funnel*, with sterile glass wool or sterile cotton gauze for filtration of spores.

6.13 *Colony counter*, manual, such as the Quebec, Buck, Wolffhuegel, or equivalent; or a colony image analyzer (electronic/scanner type) are suitable for counting plates after incubation. A hand tally for recording of bacteria count is recommended.

6.14 *Swabs*, sterile, for aiding in removal of fungal spores from agar surface.

6.15 *Hemacytometer*, for counting spore suspension.

6.16 *Microscope*, that provides a magnification of 400 to 1000× and is complete with a suitable light source. Phase contrast or dark field capability is desirable.

6.17 *Constant Temperature Shaker*—A reliable constant-temperature shaker (water bath or incubator type), shall be used to provide mixing and aeration and to maintain a selected temperature (± 2°C) during the contact period.

6.18 *Mechanical Stirrer*—Magnetic or propeller-type stirrers or any other suitable device.

7. Reagents and Materials

7.1 *Purity of Reagents*—Reagent 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.[7] 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 distilled water or water of equal purity (see Specification D 1193, Type III).

7.3 *Buffer for Suspending Spores and for Dilutions*, sample containers having 100-mL phosphate buffer dilution water, sterile, for spore suspension have solid, sterile glass beads in container.

7.3.1 *0.25 M Phosphate Buffer Stock Solution*—Dissolve 34 g of reagent grace KH_2PO_4 in 500 mL of distilled water and mix. Adjust to pH 7.2 with 1 *N* NaOH and dilute to 1 l.

7.3.2 Phosphate buffer dilution water. Add 1.25 mL of 0.25 *M* phosphate buffer stock solution to 1 L of distilled water and mix. Dispense to sample container and sterilize.

7.4 *Aluminum Sulfate (Alum)* $[Al_2(SO_4)_3\ 18H_2O]$—Prepare a 0.4 % solution of the hydrated aluminum in distilled water and sterilize in an autoclave. Any loss of water during sterilization is made up by adding sterile distilled water. Alternately, the solution may be filter sterilized.

7.5 *Acid and Base for pH Adjustment to Make Acid and Alkaline Furnish*:

7.5.1 Prepare a 2 *N* solution of sulfuric acid in water. Sterilize by filtration.

7.5.2 Prepare a 2.0 *N* solution of sodium hydroxide in water. Sterilize by filtration.

7.6 *Pulp*—A two-third hardwood and one-third softwood pulp, typical of current production techniques, and that has been produced without slimicide is needed.[8] Disintegrate the sheet in distilled water until free of fiber clots and undispersed fiber bundles.[6] Avoid methods which involve extensive cutting of fibers. The concentration of the pulp in water should be 1 %.

7.7 *Bacterial and Fungal Culture Medium*:

7.7.1 *Bacteria*—Standard dehydrated tryptone glucose extract agar or equivalent is recommended. Adjust pH of culture medium to pH of the test system.

7.7.2 *Fungi*—Sabouraud Dextrose Agar or Potato Dextrose Agar are recommended for enumeration. Adjust pH of culture medium to pH of the test system.

7.8 *Stock Slimicide Solution*—Weigh appropriate amount of slimicide and add to distilled water so that when 1 mL is added to the furnish it gives the desired final concentration.

[6] Forming Handsheets for Physical Tests of Pulp. Appendix A: Specifications and Care of Apparatus (Disintegrator), TAPPI Test Method T 205 on-88. 1994–1995. TAPPI, Atlanta, GA 30348.

[7] *Reagent Chemicals, American Chemical Society Specifications*, American Chemical Society, Washington, DC. For suggestions on the testing of reagents not listed by the American Chemical Society, see *Analar Standards for Laboratory Chemicals*, BDH Ltd., Poole, Dorset, U.K., and the *United States Pharmacopeia and National Formulary*, U.S. Pharmacopeial Convention, Inc. (USPC), Rockville, MD.

[8] The sole source of supply of the apparatus known to the committee at this time is Zellerbach, 808 Rhodes Ave., Columbus, OH 43205. If you are aware of alternative suppliers, please provide this information to ASTM Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee,[1] which you may attend.

8. Test Organisms

8.1 *Bacteria: Enterobacter aerogenes* (ATCC 13048) and *Pseudomonas aeruginosa* (ATCC 15442)—Maintain these organisms on tryptone glucose extract agar at 32 ± 2°C.

8.2 *Fungi: Aspergillus niger* (ATCC 6275)—Maintain and grow for sporulation on Sabouraud Dextrose Agar, grow at 25 ± 2°C. *Chaetomium globosum* (ATCC 6205)—Maintain and grow for sporulation on Mineral Slats Agar[9] with sterile filter paper at 25 ± 2°C.

9. Preparation of Inoculum

9.1 *Bacteria*:

9.1.1 Grow the bacterial cultures on tryptone glucose extract agar at 32 ± 2°C for 24 h. Add 1 mL of sterile water to the surface of the slant. Gently scrape and suspend the organisms in the liquid.

9.1.2 Transfer this volume to a tryptone glucose extract agar milk dilution bottle flat and incubate at 32 ± 2°C for 24 h. The organisms harvested from this bottle flat will serve as inoculum for the pulp cultures.

9.1.3 To obtain this inoculum, add 10 mL of sterile water to the bottle flat from a 99 ± 2-mL sterile water blank. Disperse the growth on the flat throughout the water by gently moving it over the surface of the agar medium. Transfer 5 mL of this suspension to the remaining 89 mL of sterile water in the water blank and shake the contents of the water blank vigorously. Add 1 mL of this inoculum (2×10^8 to 1×10^9 cells/mL) to each bottle containing the pulp substrate in the test. This inoculum can also be cultured on tryptone glucose agar petri plates and removed in a similar fashion.

9.2 *Fungal Spores*:

9.2.1 Fungi are grown on appropriate agar slants at 25± 2°C for 7 days. The fungal spores are harvested by adding 2 mL of sterile, distilled water to the surface of the slant. Gently scrape and suspend the spores in the liquid.

9.2.2 Inoculate these spores onto appropriate agar and incubate at 25 ± 2°C for 7 days. Spores are harvested and serve as the inoculum for the pulp cultures.

9.2.3 To obtain this inoculum, add 5 to 10 mL of sterile, phosphate buffer dilution water to the surface of the agar. Disperse the fungal spores and mycelia in the bottle by gently scraping the surface of the medium. Alternately, remove the growth from the surface of the medium, add it to a screw-cap container with buffer and glass beads, and shake vigorously. Set aside for 5 to 10 min to let the hyphae settle.

9.2.4 Carefully remove the supernate with a pipet and filter through cotton gauze or glass wool plugged sterile funnel.

9.2.5 Count the conidial suspension with a hemacytometer and standardize to 10^7 to 10^8/mL with phosphate dilution buffer.

10. Pulp Cultures

10.1 Weigh 50 g of pulp slurry into a sample container. To ensure a uniform pulp suspension in each container, constantly agitate the stock solution with a mechanical stirrer. Use only pulp suspension prepared the same day. Stopper or cap and sterilize. After sterilization, allow the bottles to cool to approximately 25 ± 2°C or the temperature used for the test.

10.2 Make the following additions aseptically to each bottle in the order named and shake vigorously after each addition, using 20 complete cycles in a vertical motion.

10.3 *Furnish*:

10.3.1 For acid furnish, add a sufficient amount of 2.0 *N* sulfuric acid to adjust the pH to between pH 5.0 and 5.5. Add 0.1 mL of 0.4 % solution of Alum.

10.3.2 For alkaline furnish, add a sufficient amount of 2.0 *N* sodium hydroxide to adjust the pH to between pH 8.0 and 8.5.

10.3.3 Other furnishes, appropriate for the specific paper mill applications.

10.4 The time interval between the preparation of the suspension of the test organism for inoculating the bottles and the addition of the suspension to each bottle may have some effect on the resistance of the organism. The proper aliquots of biocides shall be promptly added to each bottle of substrate.

10.5 Add 1 mL of slimicide stock solution of sufficient concentration to each furnish to achieve the desired final concentration in parts per million. To the negative controls, add 1 mL of sterile water. Include in each test, as a positive control, a reference biocide that is known to have activity in this test. Include a minimum of five concentrations of the biocide under test and the reference biocide in each experiment. Run each test and reference biocide in duplicate.

10.6 Add the amount of sterile distilled water that is necessary to bring the total weight of the contents of the bottle to 99 ± 1 g after all other additions have been made.

10.7 Add 1 mL of an aqueous suspension of the test organism to each pulp slurry. Prepare this suspension just before it is to be used. Final bacterial concentration should be between 2×10^6 to 1×10^7 bacterial/mL of pulp slurry. Final fungal spore concentration should be between 10^5 to 10^6 spores/mL of pulp slurry.

10.8 After the final addition of the test organism, place the inoculated bottles on an incubator shaker and maintain at a temperature of 32 ± 2°C for bacteria and 25 ± 2°C for fungi. An alternate test temperature may be used reflecting appropriate use conditions.

10.9 *Bacterial Incubation and Enumeration*:

10.9.1 Incubate for at least 3 h. If desired, additional contact times may be included in the test. However, the 3-h contact period shall always be included in the test. A control flask with bacteria but without biocide must be run, and bacterial numbers determined at $T = 0$ and at least 3 h after bacterial addition.

10.9.2 After incubation, vigorously shake the pulp cultures and immediately plate it in tryptone glucose extract agar in accordance with standard dilution plating techniques. Make standard dilutions in phosphate buffer. Neutralization of biocide at the end of the contact period should be demonstrated (see Practice E 1054). It is important that the pulp cultures be plated in the same order as they were inoculated, thus ensuring a uniform contact time. After the medium has solidified in the petri dishes, incubate them at 32 ± 2°C for 48 ± 2 h.

[9] American Type Culture Collection, ATCC Medium 329.

10.9.3 At the completion of the incubation period, count the number of bacterial colonies, following standard microbiological protocols.

10.10 *Fungal Incubation and Enumeration*—Incubate for up to 24 h. Remove aliquots for fungal enumeration after $T = 0$ and at least 2 additional contact times. (See Note 1). Serially dilute on the appropriate agar. Initial serial dilutions should be done in neutralizer, followed by dilutions in phosphate buffer dilution water. Neutralization process should be shown to be effective against the slimicide being tested (see Practice E 1054). Incubate at 25 ± 2°C or appropriate temperature of test for up to 14 days. After completion of the incubation period, count the number of fungal colonies following standard microbiological protocols.

NOTE 1—Suggested contact times are any two of the following 3, 6, and 24 h.

11. Calculation

11.1 *Bacteria*:

11.1.1 Report the results as the percent kill using the following equation:

$$\text{Kill, \%} = \frac{(A - B)}{(A)} \times 100 \quad (1)$$

where:

A = count of initial control, and

B = count after 3-h incubation or other selected contact time interval.

11.1.2 Plot the data and determine the percent kill for the desired slimicide concentration. If more than one contact time is used, a reasonable progression in percent kill should be found. The control should either remain unchanged or increase over time. If it does not, then the test must be repeated.

NOTE 2—It has been observed that an effective slimicide gives a reduction in bacterial counts (kills) of 99 % relative to the control. Data generated in the laboratory, however, is only indicative of biocide performance in the filed. Effective bacterial control may require adjustments in biocide dosages due to specific environmental conditions and application procedures in the paper systems.

11.2 *Fungi*:

11.2.1 An effective slimicide is one that shows a continued reduction in fungal counts relative to the control over the duration of the test.

11.2.2 If there is any question regarding the growth of the test organism in the test series, repeat the test.

NOTE 3—An effective agent should give a reduction in fungal counts of 90 % relative to the control. Data generated in the laboratory, however, is only indicative of biocide performance in the field. Biocide dosages and feed points need to be determined in the actual paper system.

12. Precision and Bias

12.1 A precision and bias statement cannot be made for this test method at this time.

13. Keywords

13.1 acid furnish; alkaline furnish; *Aspergillus niger* (ATCC 6275); bacteria; *chaetomium globosum* (ATCC 6205); *enterobacter aerogenes* (ATCC 13048); fungi; paper pulp; *pseudomonas aeruginosa* (ATCC 15442); slimicide

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