Designation: D 5247 – 92

Standard Test Method for Determining the Aerobic Biodegradability of Degradable Plastics by Specific Microorganisms[1]

This standard is issued under the fixed designation D 5247; 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 describes the procedures required to carry out a pure-culture study for evaluating the biodegradation of degradable plastics in submerged culture under aerobic conditions. Degradation will be evaluated by weight loss, tensile strength loss, percent-elongation loss and changes in molecular-weight distribution.

1.2 *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:*

D 638 Test Method for Tensile Properties of Plastics[2]

D 882 Test Methods for Tensile Properties of Thin Plastic Sheeting[2]

D 3536 Test Method for Molecular Weight Averages and Molecular Weight Distribution of Polystyrene by Liquid Exclusion Chromatography (Gel Permeation Chromatography—GPC)[3]

G 22 Practice for Determining Resistance of Plastics to Bacteria[4]

3. Terminology

3.1 *Definitions of Terms Specific to This Standard:*

3.1.1 *degradable plastic*—a plastic designed to undergo a significant change in its chemical structure under specific environmental conditions resulting in a loss of some properties that may vary as measured by standard test methods appropriate to the plastic and the application in a period of time that determines its classification.

3.1.2 *residual plastic*—the remaining plastic material in the culture medium after incubation with shaking.

3.1.3 *uninoculated control*—a plastic that is chemically disinfected and incubated with shaking in the same manner as test samples but without exposure to microorganisms.

3.1.4 *zero control*—a plastic without disinfection or cultural incubation; the starting material.

4. Summary of Test Method

4.1 The test method described herein follows the method developed by Lee et al.[5] and consists of the following steps:

4.1.1 Cut degradable plastic samples into appropriate lengths suitable for tensile strength determinations as specified in Test Methods D 882 and a suitable size for a 250-mL Erlenmeyer shake-flask.

4.1.2 Plastic strips are chemically disinfected, aseptically dried, and initial weights determined.

4.1.3 Disinfected plastic strips are aseptically added to the appropriate growth medium, incubated with shaking for 24 h (125 r/min; orbital shaker) at the appropriate temperature for the specific microorganism being used. If not contaminated (contaminated medium becomes cloudy) the medium is then inoculated with a pure culture.

4.1.4 Inoculated and uninoculated-control flasks are incubated with shaking for specified period of time. Each film is evaluated with a minimum of four replicates with and without each microorganism.

4.1.5 Residual plastic strips are washed in 70 % ethanol for 30 min and dried.

4.1.6 Biodegradation is determined by weight loss, tensile-strength loss, percent-elongation loss, and average-molecular-weight distribution changes.

4.1.7 Significant differences between the inoculated flask and its corresponding uninoculated-control film are determined by performing an analysis of variance ($p < 0.05$).

5. Significance and Use

5.1 Several states in the United States and many European countries have required the use of degradable plastics in disposable products. As a result, several regulatory agencies

[1] This test method is under the jurisdiction of ASTM Committee D-20 on Plastics and is the direct responsibility of Subcommittee D20.96 on Environmentally Degradable Plastics.

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[2] *Annual Book of ASTM Standards*, Vol 08.01.

[3] *Annual Book of ASTM Standards*, Vol 08.02.

[4] *Annual Book of ASTM Standards*, Vol 08.03.

[5] Lee, B., Pometto III, A. L., Fratzke, A., and Bailey Jr., T. B.," Biodegradation of Degradable Plastic Polyethylene by *Phanerochaete* and *Streptomyces*," *Applied Environmental Microbiology* 57, 1991, pp. 678–685.

have the need to evaluate degradable products for compliance with state and local regulations. In today's market there are three main actions by which degradable plastics are designed to degrade: (*1*) biological, (*2*) chemical, or (*3*) photo degradation, or combination thereof. The final degradation of all degradable plastic materials, whether they be initially degraded by photo- or chemical-degradation, will be by microorganisms. This test method provides an indirect way to evaluate the biodegradability of degradable plastics before and after exposure to the environment or artificially accelerated tests. Furthermore, if the degradable plastic consists of more than one component (for example, starch and polyethylene) the biodegradation of each component needs to be evaluated.

5.2 This test method describes a procedure that can be used to indicate the aerobic biodegradability of degradable plastics. The microorganisms described in the following test method are lignin degraders that are common to forest litter and composting sites (Lee et al., 1991).[5] However, the test is not limited to these described microbes.

5.3 This test method is also appropriate for the evaluation of degradable plastics that have undergone specific chemical, thermal, or photo degradation, or combination thereof, as specified by ASTM procedures. Research has demonstrated that the initiation of biodegradation of degradable plastic (starch/polyethylene with pro-oxidant blends) requires chemical-degradation pretreatment of 4 to 8 days 70°C heat treatment or photodegradation pretreatment of 2 to 4 week exposure to ultraviolet light (365 nm) (Lee et al., 1991).[5] Therefore, a pretreatment of the degradable plastic by heat, light or other appropriate treatment could be a component for evaluating the biodegradability of the different materials.

5.4 A pure-culture method provides a test that is reproducible in almost any laboratory. The use of an uninoculated control allows for the separation of degradable plastic changes due to incubation condition from those that are the results of biological activity.

5.5 Plastics not significantly degraded in this test may degrade at acceptable rates in a natural environment under mixed microbial conditions. Degradation of these unique polymers may require some biological capabilities not possessed by the pure cultures used in this test but which are commonly available in a natural environment. The test cultures recommended by this test method are organisms widely found in the environment. However, observation of significant degradation rates when using pure cultures that are not ubiquitously present in the natural environment would not necessarily indicate that the plastic will biodegrade at an acceptable rate in a natural environment.

6. Apparatus

6.1 *Glassware*—Glass 250-mL Erlenmeyer flask and culture tubes with sterilizable biological closures.

6.2 *Plastic Ware*—Sterilized petri dishes, autoclavable 2-L beakers, and specimen jars with lids.

6.3 *Incubator*—Incubator orbital shaker which operates at a temperature range of room temperature to 65°C.

6.4 *Universal Tester*—Tensile-strength and percent-elongation measurements with a 2-kg load cell.

6.5 *High-Temperature Gel-Permeation Chromatograph*—Determining changes in average-molecular-weight distribution.

6.6 *Analytical Balance*, capable of measurement to 0.1 mg.

6.7 *Autoclave*—Sterilization of disinfection solutions and culture medium.

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 reagents shall conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society, where such specifications are available.[6] 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 deionized water.

7.3 *Streptomyces Medium*.

7.3.1 This medium is a carbon- and nitrogen-free solution to be used whenever the medium for *Streptomyces* calls for a buffered mineral salts solution (pH 7.1). Prepare this medium by dissolving in 1 L of water the designated amounts of the following reagents:

Sodium phosphate, dibasic (Na_2HPO_4)	5.03 g
Potassium phosphate, monobasic (KH_2PO_4)	1.98 g
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	0.20 g
Sodium chloride (NaCl)	0.20 g
Calcium chloride ($CaCl_2 \cdot 2H_2O$)	0.05 g

7.3.2 Prepare bacterial trace-elements solution separately, and store refrigerated. One mL of trace-element solution is added to 1 L of mineral-salts solution.

Cupric sulfate ($CuSO_4 \cdot 7H_2O$)	6.1 g
Iron sulfate ($FeSO_4 \cdot 7H_2O$)	1.1 g
Manganese chloride ($MnCl_2 \cdot 4H_2O$)	7.9 g
Zinc sulfate ($ZnSO_4 \cdot 7H_2O$)	1.5 g

7.3.3 To buffered-mineral salts solution add 0.6 % (w/v) yeast extract, hereafter referred to as 0.6 % yeast-extract medium.

7.4 *Fungal Medium*—Prepare a solution of 3 % (w/v) malt extract in water and adjust the pH to 4.5, hereafter referred to as 0.3 % malt-extract medium.

7.5 Add 100 mL of medium to a 250-mL flask, fitted with a cotton plug. Wrap the flask cotton plug and neck with aluminum foil to reduce evaporation and then sterilize by autoclaving.

7.5.1 *Medium Sterilization*—Sterilization will be at 121°C (250°F) for 20 min.

8. Microbial Inoculum

8.1 *Bacterial-Cell Suspension*:

8.1.1 Use the following test organisms, or a suitable bacterium as agreed upon among parties concerned:

[6] "Reagent Chemicals, American Chemical Society Specifications," Am. Chemical Soc., Washington, DC. 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, NY, and the "United States Pharmacopeia."

8.1.1.1 *Streptomyces badius* 252 (ATCC 39117).

8.1.1.2 *Streptomyces setonii* 75Vi2 (ATCC 39115).

8.1.1.3 *Streptomyces viridosporus* T7A (ATCC 39115).

8.1.2 Maintain stock cultures of *S. viridosporus* on agar slants of 0.4 % (w/v) yeast extract 1.0 % (w/v) malt extract, 1.0 % (w/v) dextrose, 0.5 % (w/v) peptone pH adjusted to 7.2 and 2.0 % (w/v) agar (yeast-extract, malt-extract, glucose agar). *S. badius* and *S. setonii* are maintained on agar slants of 0.6 % (w/v) yeast extract medium in mineral-salts solution with 2.0 % Bacto agar. Incubate cultures at 37°C for about one week until characteristic bacterial sporulation is observed. Stock culture can then be stored at 4°C for 2 to 8 weeks.

8.2 *Fungal-Cell Suspension*:

8.2.1 Use the following test organism, or a suitable fungus as agreed upon among parties concerned:

8.2.1.1 *Phanerochaete chrysosporium* (ATCC 34541).

8.2.2 Stock cultures of *P. chrysosporium* are maintained on agar slants of 3.0 % (w/v) malt-extract agar or potato-dextrose agar. Stock culture can then be stored at 4°C for 2 to 8 weeks.

8.3 Culture medium inoculation is accomplished by adding a loop full of spores/cells or by adding 0.5 mL of a spore/cell suspension. Prepare the latter by adding 5 mL of sterile water to a stock slant, aseptically suspending the spores in the water by using a sterile inoculating loop.

8.4 Any substitution of microorganisms or medium must be stated in report.

9. Test Procedure

9.1 Cut degradable plastic specimens into strips, which meets the requirement for tensile-strength determination as described in Test Method D 882. Recommended minimum size is 4 by 1 in. strips, and maximum size is 6 by 1 in. strips.

9.1.1 Suggested degradable and nondegradable control films are cellophane and polyethylene, respectively, to ensure culture viability and for interlaboratory comparisons.

9.2 *Plastic-Specimen Disinfection*:

9.2.1 Add 1 L of distilled water to two 2-L beakers containing a stir bar and cover with aluminum foil. Autoclave the container at 15 psi and 121°C for 20 min. After sterilization and cooling, the volume will reduce to about 980 mL. To one of the sterile-water beakers, aseptically add 14.0 mL of filter sterilized Tween 80 detergent (polyoxyethylene [20] sorbitan monooleate) and 20 mL of chlorine bleach, which makes a fresh solution of universal disinfectant.[7]

9.2.2 Place plastic strips into universal disinfectant and stir at room temperature for 30 to 60 min. To ensure that both sides of the film are disinfected, mix the solution occasionally with a sterile pipet. (Place no more than twenty-one 4-in. or ten 6-in. plastic strips into one universal disinfectant solution at one time.)

9.2.3 Using sterile forceps, remove each film and place it into the beaker of fresh sterile water at room temperature for at least 60 min, mixing occasionally.

9.2.4 Aseptically place films into a fresh solution of 70 % ethanol in a covered specimen jar for 30 min standing at room temperature.

9.2.5 Aseptically place each film into a sterile pre-weighed petri dish. To ensure each dish is dry, store at least overnight at a temperature sufficiently below the samples T_m to avoid morphological alterations, equilibrate to room temperature, then weigh to ±0.1 mg accuracy to determine the initial weight of the empty sterile dish. Wear gloves while handling dishes.

9.2.6 Store dishes with films at room temperature until dry (24 to 72 h). Upon removal from the incubator, and after equilibration at room temperature for at least 1 h, reweigh the dishes (±0.1 mg). Determine the weight of the film by subtracting the initial weight of the empty dish from the dish now containing the film.

9.3 *Pure-Culture Incubation*:

9.3.1 *Culture Medium*—Autoclave 100 mL of bacterial and fungal culture broths in 250-mL flasks fitted with cotton plugs and wrapped with aluminum foil covering the entire neck of the flask.

9.3.2 Aseptically add the film to the desired sterile culture broth incubated with shaking in an orbital shaker at 125 r/min and 37°C for the bacteria and 30°C for the fungus for 24 h prior to inoculation. If the film has been properly disinfected, no bacterial growth will be observed after that 24-h incubation. Discard contaminated flasks (cloudy or turbid). Inoculate sterile culture broths with film with their respective microorganism.

9.3.3 *Uninoculated-Control Flask*—Prepare sterile uninoculated-control culture flasks for both the bacterial and fungal mediums as described previously by adding a disinfected preweighed plastic strip without microorganisms. Discard contaminated flasks.

9.3.4 *Incubation*—Incubate each culture and control flask with shaking (125 r/min) at 37°C for bacteria or 30°C for fungus for specified period of time (minimum of 14 days). Perform each culture incubation (inoculated and uninoculated-control) and degradable plastic sample in replicates of at least four. Always incubate the sterile uninoculated-control at the same time.

9.3.5 *Zero-Control*—Evaluate films prior to disinfection and incubation for initial tensile strength, percent elongation, and average molecular weight distribution.

9.4 *Observation*:

9.4.1 *Weight-Loss Determination*—After incubation, remove residual films with forceps and wash with fresh 70 % ethanol for 30 min standing at room temperature. Place each plastic residue in a sterile petri dish and dry at room temperature (24 to 72 h). Determine its final weight, taking care to use forceps and gloves. Determine the average weight-loss for each specific culture, and compare it to the weight-loss of the uninoculated-control.

9.4.2 *Tensile Strength and Percent Elongation Loss*—Determine tensile strength and percent elongation changes in machine direction for each of samples using Test Method G 882. Compare the average tensile strength and percent elongation loss for each culture to their corresponding uninoculated-control flask and zero-control film.

[7] *Manual of Methods for General Bacteriology*, American Society for Microbiology, 1981, p. 499.

9.4.3 *High-Temperature Gel-Permeation Liquid Chromatography*—Changes in weight-average molecular-weight distribution for each residue are determined by Test Method D 3536 or Lee et al. (1991).[5] Compare the molecular-weight distribution for each culture to their corresponding uninoculated-control flask and zero-control film.

9.4.4 Any change or modification in the test method and additional test methods must be described in the report.

10. Report

10.1 Report the following information:

10.1.1 Description of the degradable plastic and the positive and negative control films being evaluated,

10.1.2 The organisms used for the evaluation,

10.1.3 The time, temperature, and agitation speed used for the incubation,

10.1.4 The number of replicates performed,

10.1.5 Average percent weight loss for the inoculated and uninoculated control,

10.1.6 The average changes in tensile strength and percent elongation for the inoculated, uninoculated-controls, and the initial materials, and

10.1.7 Changes in weight-average molecular weight ($\bar{M}_w$), number-average molecular weight ($\bar{M}_n$) and polydispersity ($\bar{M}_w / \bar{M}_n$) for the inoculated materials to the uninoculated and initial materials.

10.1.8 Compare all observations for each culture to their corresponding uninoculated-control and zero-control. Significant differences between the inoculated flask and its corresponding uninoculated-control film are determined by performing an analysis of variance ($p < 0.05$).

11. Precision and Bias

11.1 The precision of the procedure in this test method has not been determined. Precision will be determined by round-robin study.

12. Keywords

12.1 aerobic biodegradation; bacterial biodegradation; degradable plastic; fungal biodegradation; molecular-weight distribution; percent-elongation; pure culture; tensile strength