Designation: E2196 – 23

Standard Test Method for Quantification of *Pseudomonas aeruginosa* Biofilm Grown with Medium Shear and Continuous Flow Using Rotating Disk Reactor[1]

This standard is issued under the fixed designation E2196; 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 is used for growing a reproducible (1)[2] *Pseudomonas aeruginosa* biofilm in a continuously stirred tank reactor (CSTR) under medium shear conditions. In addition, the test method describes how to sample and analyze biofilm for viable cells.

1.2 Although this test method was created to mimic conditions within a toilet bowl, it can be adapted for the growth and characterization of varying species of biofilm (rotating disk reactor—repeatability and relevance (2)).

1.3 This test method describes how to sample and analyze biofilm for viable cells. Biofilm population density is recorded as $\log_{10}$ colony forming units per surface area (rotating disk reactor—efficacy test method (3)).

1.4 Basic microbiology training is required to perform this test method.

1.5 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.6 *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, health, and environmental practices and determine the applicability of regulatory limitations prior to use.*

1.7 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.*

2. Referenced Documents

2.1 *ASTM Standards:*[3]

E2756 Terminology Relating to Antimicrobial and Antiviral Agents

D5465 Practices for Determining Microbial Colony Counts from Waters Analyzed by Plating Methods

2.2 *Other Standards:*

Method 9050 C.1.a Buffered Dilution Water Preparation (4)

3. Terminology

3.1 For definitions of terms used in this test method refer to Terminology E2756.

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *biofilm, n*—microorganisms living in a self-organized community attached to surfaces, interfaces, or each other, embedded in a matrix of extracellular polymeric substances of microbial origin, while exhibiting altered phenotypes with respect to growth rate and gene transcription.

3.2.1.1 *Discussion*—Biofilms may be comprised of bacteria, fungi, algae, protozoa, viruses, or infinite combinations of these microorganisms. The qualitative characteristics of a biofilm, including, but not limited to, population density, taxonomic diversity, thickness, chemical gradients, chemical composition, consistency, and other materials in the matrix that are not produced by the biofilm microorganisms, are controlled by the physiochemical environment in which it exists.

3.2.2 *coupon, n*—biofilm sample surface.

4. Summary of Test Method

4.1 This test method is used for growing a reproducible *Pseudomonas aeruginosa* biofilm in a rotating disk reactor. The biofilm is established by operating the reactor in batch mode (no flow) for 24 h. Steady state growth (attachment is equal to detachment) is reached while the reactor operates for an additional 24 h with continuous flow of the nutrients. The

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

[3] 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.

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

residence time of the nutrients in the reactor is set to select for biofilm growth, and is species and reactor parameter specific. During the entire 48 h, the biofilm is exposed to continuous fluid shear from the rotation of the disk. At the end of the 48 h, biofilm accumulation is quantified by removing coupons from the disk, harvesting the biofilm from the coupon surface, disaggregating the clumps, then diluting and plating for viable cell enumeration.

5. Significance and Use

5.1 Bacteria that exist in a biofilm are phenotypically different from suspended cells of the same genotype. The study of biofilm in the laboratory requires protocols that account for this difference. Laboratory biofilms are engineered in growth reactors designed to produce a specific biofilm type. Altering system parameters will correspondingly result in a change in the biofilm. The purpose of this method is to direct a user in the laboratory study of biofilms by clearly defining each system parameter. This method will enable a person to grow, sample, and analyze a laboratory biofilm. The method was originally developed to study toilet bowl biofilms, but may also be utilized for research that requires a biofilm grown under moderate fluid shear.

6. Apparatus

6.1 *Wooden Applicator Sticks,* sterile.

6.2 *Inoculating Loop.*

6.3 *Petri Dish,* 100 by 15 mm, plastic, sterile and empty to hold rotor while sampling.

6.4 *Culture Tubes and Culture Tube Closures,* any with a volume capacity of 10 mL and minimum diameter of 16 mm. Recommended size is 16 by 125 mm borosilicate glass with threaded opening.

6.5 *Pipette(s),* continuously adjustable pipette(s) with volume capacity of 1 mL.

6.6 *Micropipette(s),* continuously adjustable pipette(s) with a volume capacity of 10 – 250 µL.

6.7 *Vortex,* any vortex that will ensure proper agitation and mixing of culture tubes.

6.8 *Homogenizer,* any capable of mixing at 20 500 ± 5000 r/min in a 5 to 10 mL volume.

6.9 *Homogenizer Probe,* any capable of mixing at 20 500 ± 5000 r/min in a 5 to 10 mL volume that can withstand autoclaving or other means of sterilization.

6.10 *Sonicating Bath,* any cavitating sonicating bath that operates at 45 to 50 kHz for cleaning the coupons.

6.11 *Bunsen Burner,* used to flame inoculating loop and other instruments.

6.12 *Stainless Steel Dissecting Tools,* for removing the coupons.

NOTE 1—Alternatively, a coupon manipulation tool[4] may be used.

6.13 *Stainless Steel Hemostat Clamp,* with curved tip.

6.14 *Environmental Shaker,* capable of maintaining temperature of 36 ± 2 °C.

6.15 *Analytical Balance,* sensitive to 0.01 g.

6.16 *Sterilizer,* any steam sterilizer capable of producing the conditions of sterilization is acceptable.

6.17 *Colony Counter,* any one of several types may be used, such as the Quebec, Buck, and Wolfhuegel. A hand tally for the recording of the bacterial count is recommended if manual counting is done.

6.18 *Peristaltic Pump,* pump head capable of holding tubing with inner diameter of 3.1 mm and outer diameter of 3.2 mm.

6.19 *Digital Magnetic Stir Plate,* top plate 10.16 by 10.16 cm, capable of rotating at 200 ± 5 r/min.

6.20 *Silicone Tubing,* two sizes of tubing: one with inner diameter of 3.1 mm and outer diameter of 3.2 mm, and the other with inner diameter of 7.9 mm and outer diameter of 9.5 mm. Both sizes must withstand sterilization.

6.21 *Norprene*[5] Tubing, inner diameter of 3.1 mm and outer diameter of 3.2 mm.

6.22 *Glass Flow Break,* any that will connect with tubing of inner diameter 3.1 mm and withstands sterilization.

6.23 *Clamp,* used to hold flow break, extension clamp with 0.5 cm minimum grip size.

6.24 *Clamp Stand,* height no less than 76.2 cm, used with clamp to suspend glass flow break vertically and stabilize tubing above reactor.

6.25 *Reactor Components*[6]:

6.25.1 *Berzelius Borosilicate Glass Beaker,* 1000 mL without pour spout, 9.5 ± 0.5 cm diameter. Borosilicate barbed outlet spout added at 300 ± 15 mL mark at 30 to 45° angle, spout should accommodate silicone tubing with an inner diameter of 8 to 11 mm.

NOTE 2—The rotor, described in 6.25.3, will displace approximately 25 mL of liquid. Therefore, an outlet spout at the 300 mL mark will result in an operating volume of approximately 275 mL. Before use, the user should confirm the actual liquid volume in the reactor, after the rotor is in place and the stir plate is turned on. The measured operating volume is used to calculate an exact pump flow rate.

6.25.2 *Reactor Top,* size 15 rubber or machined stopper, with three holes bored through top to accommodate 6 cm

[4] The sole source of supply of the apparatus (coupon manipulation tool) known to the committee at this time is BioSurface Technologies, Corp., www.biofilms.biz. If you are aware of alternative suppliers, please provide this information to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee,[1] which you may attend. The user may also build the holder,

[5] Trademarked by the Saint-Gobain Performance Plastics Corporation.

[6] The sole source of supply of the apparatus (rotating disk reactor) known to the committee at this time is BioSurface Technologies, Corp., www.biofilms.biz. If you are aware of alternative suppliers, please provide this information to ASTM International Headquarter. Your comments will receive careful consideration at a meeting of the responsible technical committee,[1] which you may attend. The user may also build the reactor.