Designation: E2196 – 07

Standard Test Method for Quantification of a *Pseudomonas aeruginosa* Biofilm Grown with Shear and Continuous Flow Using a 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 repeatable[2] *Pseudomonas aeruginosa* biofilm in a continuously stirred flow reactor. In addition, the test method describes how to sample and analyze biofilm for viable cells.

1.2 In this test method, biofilm population density is recorded as log colony forming units per surface area.

1.3 Basic microbiology training is required to perform this test method. *This standard does not claim to address all of the safety problems associated with its use. It is the responsibility of the user of this standard to establish appropriate safety practices and determine the applicability of regulatory limitations prior to use.*

2. Referenced Documents

2.1 *Other Standards:*

Buffered Dilution Water Preparation —Method 9050 C.1a[3]

Rotating Disk Reactor —Repeatability and Relevance[4]

Rotating Disk Reactor —Efficacy Test Method[5]

3. Terminology

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

3.1.1 *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 *coupon*—biofilm sample surface.

4. Summary of Test Method

4.1 This test method is used for growing a repeatable *Pseudomonas aeruginosa* biofilm in a rotating disk reactor. The biofilm is established by operating the reactor in batch mode (no flow) for 24 h. A 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 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, scraping 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

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[2] Ellison, S.L.R., M. Rosslein, A. Williams. (Eds.) 2000. *Quantifying Uncertainty in Anyalytical Measurement*, 2nd Edition. Eurachem.

[3] Eaton, A.D., L.S. Clesceri, Rice, E.W., A.E. Greenberg. (Eds.) *Standard Methods for the Examination of Water and Waste Water* , 21st Edition. American Public Health Association, American Water Works Association, Water Environment Federation. Washington D.C., 2005.

[4] Zelver, N., M. Hamilton, B. Pitts, D. Goeres, D. Walker, P. Sturman, J. Heersink. 1999. Methods for measuring antimicrobial effects on biofilm bacteria: from laboratory to field. In: Doyle, R.J. (Ed.), *Methods in Enzymology-Biofilms* Vol 310, Academic Press, San Diego, CA, pp. 608-628.

[5] Zelver, N., M. Hamilton, D. Goeres, J. Heersink. 2001. Development of a Standardized Antibiofilm Test. In: Doyle, R.J. (Ed.), *Methods in Enzymology-Biofilms* Vol 337, Academic Press, San Diego, CA, pp. 363-376.

parameter. This method will enable a person to grow, sample, and analyze a laboratory biofilm.

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 capability of 10 mL and diameter no less than 6 cm. Recommended size is 16 by 125 mm borosilicate glass with threaded opening.

6.5 *Pipetter*, continuously adjustable pipetter with volume capability of 1 mL.

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

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

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

6.9 *Sonicator*, any noncavitating sonicating bath that operates at 50 to 60 Hz.

6.10 *Syringe*, sterile, 1 mL syringe used during reactor inoculation.

6.10.1 *Needle*, sterile, 22 gauge needle used with syringe to inoculate reactor.

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

6.12 *Stainless Steel Dissecting Tools*.

6.13 *Stainless Steel Hemostat Clamp with Curved Tip*.

6.14 *Environmental Shaker*, capable of maintaining temperature of 35 ± 2°C.

6.15 *Analytical Balance*, sensitive to 0.01 g.

6.16 *Sterilizers*, 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 ID 3.1 mm and OD 3.2 mm.

6.19 *Magnetic Stir Plate*, top plate 10.16 by 10.16 cm, capable of rotating at 200 ± 100 r/min.

NOTE 1—R/min may be measured using a strobe light.

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

6.21 *Glass Flow Break*, any that will connect with tubing of ID 3.1 mm and withstand sterilization.

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

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

6.22 *Reactor Components*[6]:

6.22.1 *Berzelius Pyrex Beaker*, 1000 mL without pour spout, 9.5 ± 0.5 cm diameter. Pyrex barbed outlet spout added at 250 mL ± 15 mL mark at 30 to 45° angle, spout should accommodate silicone tubing with an ID of 8 to 11 mm.

NOTE 2—The rotor, described in 6.22.3, will displace approximately 50 mL of liquid. Therefore, an outlet spout at the 250 mL mark will result in approximately a 200 mL operating volume. The user is encouraged to confirm the actual liquid volume in the reactor, when the rotor is in place, before use. The measured volume is used to calculate an exact pump flow rate.

6.22.2 *Reactor Top*, size 15 rubber or machined stopper, 3 to 4 holes bored through stopper to accommodate 6 cm pieces of fire-polished glass tubing or other suitable rigid autoclavable tubing with OD 4 to 6 mm, as shown in Fig. 1. Another hole can be added to the stopper to contain an inoculum port. The inoculum port consists of a 6 cm piece of fire-polished glass tubing or other suitable rigid autoclavable tubing fitted with a septum.

6.22.3 *Rotor or Disk*, nominal (see Note 3) 1.6 mm thick PTFE sheet cut into a disk with a diameter of 7.0 ± 0.2 cm containing 6 evenly spaced holes with a diameter of 1.27 ± 0.1 cm. The center of each hole is located 2.55 ± 0.03 cm from the center of the disk. 4.5 to 7.0 mm thick Viton sheet, or other suitable autoclavable material, cut into a disk with a diameter of 7.0 ± 0.2 cm containing 6 evenly spaced holes with a diameter of 1.27 ± 0.15 cm (the holes in the Viton are aligned with the holes in the PTFE) and a small hole in the center to house the disk retrieving port. PTFE washer with disk retrieving port. Four nylon screws. PTFE coated 4.0 by 1.4 cm star head magnetic stir bar, set flush against PTFE disk and with holes drilled for assembly with nylon screws. The PTFE ridges on one side of the magnet may be shaved to provide a flush mounting surface. Assemble the pieces conforming to the general details shown in Fig. 2.

NOTE 3—Nominal implies that the manufacturer's tolerance is acceptable.

6.22.4 *Cylindrical Polycarbonate Coupons*, with a diameter of 1.27 ± 0.013 cm and a height of 1.5 to 4.0 mm.

6.23 *Carboys*, two 15 to 20 L autoclavable carboys, to be used for waste and nutrients.

6.23.1 *Carboy Lids*, two carboy lids: one carboy lid with at least 2 barbed fittings to accommodate tubing ID 3.1 mm (one for nutrient line and one for bacterial air vent). One carboy lid with at least 2-1 cm holes bored in the same fashion (one for effluent waste and one for bacterial air vent (filter)).

NOTE 4—Carboy tops can be purchased with fittings.

6.23.2 *Bacterial Air Vent (Filter)*, autoclavable 0.2 µm pore size, to be spliced into tubing on waste carboy, nutrient carboy and reactor top, recommended diameter 37 mm.

7. Reagents and Materials

7.1 *Purity of Water*—All reference to water as diluent or reagent shall mean distilled water or water of equal purity.

[6] Rotating disk reactor is available commercially from BioSurface Technologies, Corp. www.imt.net/~mitbst, or the user may build the reactor.