~~Designation:E2196–07~~ Designation: E2196 – 12

Standard Test Method for ~~Quantification of a~~ *Quantification of Pseudomonas aeruginosa Biofilm Grown with Medium 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

~~1.1This test method is used for growing a repeatable~~

1.1 This test method is used for growing a reproducible **(1)**[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.~~

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

~~1.3Basic 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.*~~ 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 and health 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~~

2.1 *ASTM Standards:*[3]

~~Rotating Disk Reactor—Repeatability and Relevance~~

~~Rotating Disk Reactor—Efficacy Test Method~~

D5465 Practice 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 *biofilm*, *n*—— microorganisms living in a ~~self-organized, cooperative~~ self-organized community attached to surfaces, interfaces, or each other, embedded in a matrix of extracellular polymeric substances of microbial origin, while exhibiting ~~an~~ altered phenotypes 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

---

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

Current edition approved April 1, ~~2007.~~2012. Published ~~May 2007.~~June 2012. Originally approved in 2002. Last previous edition approved in ~~2002~~2007 as E2196 – 0~~2~~7. DOI: ~~10.1520/E2196-07.~~10.1520/E2196-12.

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

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

~~[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.~~

[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.

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*~~coupon, *n*—biofilm sample surface.

4. Summary of Test Method

4.1 This test method is used for growing a ~~repeatable~~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. ~~A steady~~ 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~~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.

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.~~ , 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 *Pipetter*, continuously adjustable pipetter with volume ~~capability~~capacity 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~~that can withstand autoclaving or other means of sterilization.

6.9 *Sonicator*, any ~~noncavitating~~cavitating 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~~

6.11 *Stainless Steel Dissecting Tools*.

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

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

6.13 ~~*Stainless Steel Hemostat Clamp with Curved Tip*.~~

6.14~~*Environmental Shaker*~~, capable of maintaining temperature of ~~35~~36 ± 2°C.

6.15~~6.14~~ *Analytical Balance*, sensitive to 0.01 g.

6.16~~*Sterilizers*~~

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

~~6.17~~

6.16 *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~~

6.17 *Peristaltic Pump*~~, pump head capable of holding tubing with ID 3.1 mm and OD 3.2 mm.~~ , pump head capable of holding tubing with inner diameter of 3.1 mm and outer diameter of 3.2 mm.

~~[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.~~

[4] The sole source of supply of the apparatus (coupon holder) 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, which you may attend. The user may also build the holder.

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

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

~~NOTE1—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.~~ , 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.

6.20 *Norprene Tubing*, inner diameter of 3.1 mm and outer diameter of 3.2 mm.

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

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

6.21.26.23 *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~~

6.24 *Reactor Components*[5]:

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

NOTE 2—The rotor, described in ~~6.22.3~~6.24.3, 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~~ an operating volume of approximately 200 mL. Before use, the user should confirm the actual liquid volume in the reactor, ~~when~~after the rotor is in ~~place, before use.~~ place and the stir plate is turned on. The measured volume is used to calculate an exact pump flow rate.

6.22.26.24.2 *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~~ , size 15 rubber or machined stopper, with three holes bored through top to accommodate 6-cm pieces of stainless steel tubing or other suitable rigid autoclavable tubing with an outside diameter of 4 to 6 mm. One port accommodates tubing for media, the second port is fitted with a short piece of silicone tubing that holds a bacterial air vent, and the third is an inoculum port as shown in Fig. 1~~. Another hole~~

~~[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.~~

[5] 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, which you may attend. The user may also build the reactor.

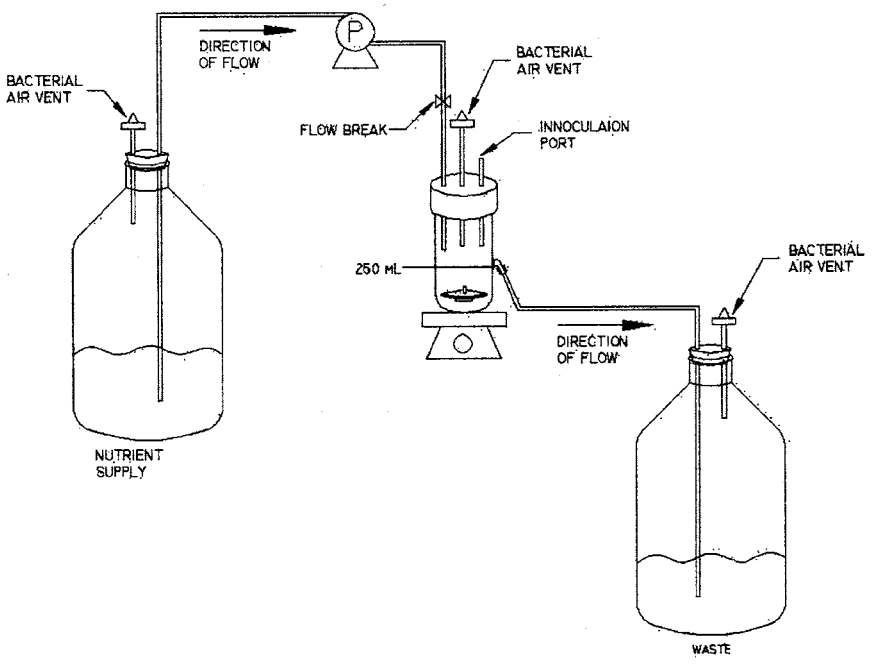

FIG. 1 Rotating Disek Reactor System

~~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~~.

6.24.3 *Rotor or Disk*, nominal ~~(see Note 3) 1.6 mm~~ 1.6-mm thick PTFE sheet cut into a disk with a diameter of 7.0 ± 0.2 cm containing ~~6~~six 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~~rubber sheet, or other suitable autoclavable material, cut into a disk with a diameter of 7.0 ± 0.2 cm containing ~~6~~six evenly spaced holes with a diameter of 1.27 ± 0.15 cm (the holes in the ~~Viton~~rubber 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~~ disk, with holes drilled for assembly ~~with~~using 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*~~

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

~~6.23~~

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

~~6.23.1~~*, two 20-L autoclavable carboys, to be used for waste and nutrients.*

6.25.1 *Carboy Lids*, ~~two carboy lids:~~ two: 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~~vent), one carboy lid with at least ~~2-1 cm~~two 1-cm holes bored in the same fashion (one for effluent waste and one for bacterial air ~~vent (filter)).~~ vent).

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.~~

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

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.

7.2 *Culture Media*:

7.2.1 *Bacterial Liquid Growth Broth*~~, soybean-casein digest medium, or an equivalent general bacterial growth medium. Tryptic Soy Broth is recommended.~~ —Tryptic Soy Broth (TSB) is recommended.

7.2.2 *Bacterial Plating Medium*~~, R2A Agar is recommended.~~ —R2A agar is recommended.

NOTE~~5—Media concentration in this protocol differs from the manufacturer's recommendation. Two~~ 5—Two different concentrations of TSB are used in the protocol, 300 mg/L for the inoculum and batch reactor operation and 30 mg/L for the continuous flow reactor operation.

7.3 *Buffered Water*~~, 0.0425 g/L KH~~—0.0425 g/L KH_2PO_4 distilled water, filter sterilized, and 0.405 g/L $MgCl \cdot 6H_2O$ ~~distilled water, filter sterilized.~~

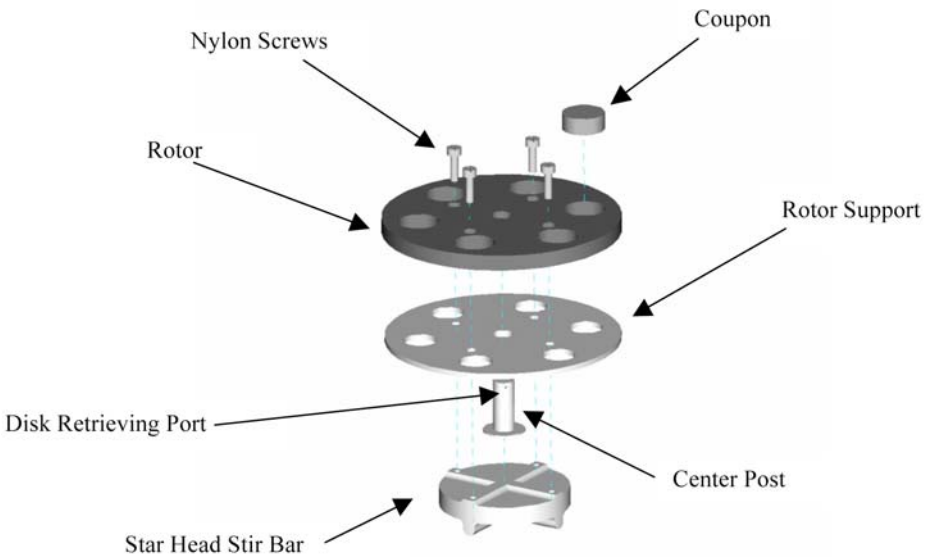

FIG. 2 Rotor Assembly