THIRD DRAFT (November 2001)

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

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 (Buffered dilution water preparation – Method 9050 C.1*a*)[3]
2.2 (Rotating Disk Reactor – repeatability and relevance)[4]
2.3 (Rotating Disk Reactor – efficacy test method)[5]

3. Terminology

3.1 Biofilm – an accumulation of bacterial cells immobilized on a substratum and embedded in
an organic polymer matrix of microbial origin.

Note 1: Biofilm is a dynamic, self-organized accumulation of microorganisms and microbial and environmental by-products that is determined by the environment in which it lives. Definition 3.1 does not capture every biofilm type that is known to exist.

3.2 Coupon – biofilm sample surface.

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[1] This test method is under the jurisdiction of ASTM Committee E-35 on Pesticides and is the direct responsibility of Subcommittee E-35.15 on Antimicrobial Agents
[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, A.E. Greenberg. (Eds.) 1995. Standard Methods for the Examination of Water and Waste Water, 19th Edition. American Public Health Association, American Water Works Association, Water Environment Federation. Washington D.C.
[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.

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 hours. A steady state growth (attachment is equal to detachment) is reached while the reactor operates for an additional 24 hours 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 hours, the biofilm experiences continuous fluid shear from the rotation of the disk. At the end of the 48 hours, 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 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 $\pm$ 5000 rpm in a 5-10 ml volume.
6.8 Homogenizer probe – any capable of mixing at 20,500 $\pm$ 5000 rpm in a 5-10 ml volume and can withstand autoclaving or other means of sterilization.
6.9 Sonicator – any noncavitating sonicating bath that operates at 50-60 hertz.
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˚C $\pm$ 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 10.16x10.16cm, capable of rotating at 200 ± 100 rpm.[6]
6.20 Silicon 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 withstands 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.[7]
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 – 45 degree angle, spout should accommodate silicon tubing with an ID of 8 – 11 mm.

Note 2: The rotor, described in 6.21.3, 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. 3-4 holes bored through stopper to accommodate 6 cm pieces of fire-polished glass tubing or other suitable rigid autoclavable tubing with OD 4-6 mm. 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, as shown in Figure 1.
6.22.3 Rotor or Disk – Nominal[8] 1.6 mm thick Teflon 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 – 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 Teflon) and a small hole in the center to house the disk retrieving port. Teflon washer with disk retrieving port. Four nylon screws. Teflon coated 4.0 x 1.4 cm starhead magnetic stir bar, set flush against Teflon disk and with holes drilled for assembly with nylon screws. The Teflon 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 Figure 2.

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[6] rpm may be measured using a strobe light.
[7] Rotating disk reactor is available commercially from BioSurface Technologies, Corp. www.imt.net/~mitbst, or the user may build the reactor.
[8] Nominal implies that the manufacturer's tolerance is acceptable.

6.22.4 Cylindrical polycarbonate coupons with a diameter of 1.27 $\pm$ 0.013 cm and a height of 1.5 – 4.0 mm.
6.23 Carboys – 2 15-20L autoclavable carboys, to be used for waste and nutrients.
6.23.1 Carboy Lids – 2 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).[9]
6.23.2 Bacterial Air Vent – autoclavable 0.2 micrometer 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.
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.
7.2.2 Bacterial Plating Medium. R2A agar is recommended.

Note 3: Media concentration in this protocol differs from the manufacturer's recommendation. Two different concentrations 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_2PO_4 distilled water, filter sterilized and 0.405 g/L $MgCl{\cdot}6H_2O$ distilled water, filter sterilized.[10]

8. Culture Preparation

8.1 *Pseudomonas aeruginosa* (ATCC 700888) is the organism used in this test. An isolated colony is aseptically removed from an R2A plate and placed into 100 ml of sterile bacterial liquid growth broth (300 mg/L) and incubated in an environmental shaker at 35˚C $\pm$ 2˚C for 20-24 hours. Viable bacterial density should be about 10^8 CFU/ml, which may be checked by serial dilution and plating.

9. Reactor Preparation

9.1 Preparation of polycarbonate coupons.

Note 4: Coupons can either be used once and discarded or used repeatedly with proper cleaning and sterilization in-between each use. Check each coupon for scratching, chipping, other damage or accumulated debris before each use by screening under a dissecting microscope at a magnification of at least 20X. Discard those with visible damage to surface topography.

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[9] Carboy tops can be purchased with fittings.
[10] Prepared according to the document referenced in 2.1.

9.1.1 Sonicate coupons for 30 seconds in a 1:100 dilution of laboratory soap and tap water. The soapy water must completely cover the coupons.
9.1.2 Rinse coupons with reagent water and sonicate for 30 seconds in reagent water.
9.1.3 Repeat rinsing and sonication with reagent water until, upon visual inspection, no soap is left on the coupon.
9.1.4 Place a coupon into each hole in the rotor, leaving the top of the coupon flush with the viton surface.
9.1.5 Place the rotor inside of the beaker.
9.2 Preparation of reactor stopper top.
9.2.1 Connect the bacterial air vent by fitting the vent to a small section of appropriately sized tubing, which is then attached to one of the pieces of glass tubing on the reactor stopper top.
9.2.2 The glass flow break is spliced into the nutrient tubing line near the reactor stopper top.[11]
9.3 Sterilization of reactor system.
9.3.1 Assemble the reactor, with the overflow (waste) line clamped and the reactor top securely fastened to the beaker before sterilization.
9.3.2 Cover the end of the nutrient tubing that connects to the nutrient carboy and the end of the overflow (waste) tubing with aluminum foil. Cover any extra openings on the reactor top with aluminum foil.
9.3.3 Sterilize the reactor system.

10. Procedure

10.1 The batch phase.
10.1.1 Prepare batch nutrient broth by dissolving bacterial liquid growth medium (300 mg/L) in 250 ml reagent grade water, sterilize.
10.1.2 The top of the sterile reactor is removed and 200 ml of the batch nutrients are aseptically poured into the beaker. The reactor top is secured and the reactor is placed onto a stir plate.
10.1.3 Inoculate the reactor with 1 ml of bacteria from the culture prepared previously (see 8.1). Aseptically inject the inoculum into the beaker through the inoculation port using the sterile needle and syringe.
10.1.4 The magnetic stir plate is turned on to allow the rotor to spin freely. The rotation speed should equal approximately 200 revolutions per minute ($\pm$ 100 rpm). The reactor system is allowed to incubate in batch mode at room temperature (21˚C $\pm$ 2˚C) for 24 hours.

Note 5: The rotational speed of the disk dictates the fluid shear in the reactor. Fluid shear affects biofilm formation.

10.2 Continuous Flow Operation (CSTR mode).
10.2.1 Aseptically connect the nutrient tubing line to the carboy containing the continuous flow nutrient broth.

[11] The other ports on the reactor top may be adapted for anaerobic use, dilution water, or treatment solutions as necessary.

10.2.2 Prepare continuous flow nutrient broth by dissolving bacterial liquid growth medium (30 mg/L) in 15 L sterile reagent grade water. Dissolve and sterilize the broth in a smaller volume to prevent caramelization. Aseptically pour the concentrated broth into the carboy of sterile water to make a total of 15 L.

Note 6: The concentration of nutrients in the bacterial liquid growth medium will affect the biofilm density that accumulates on the coupons.

10.2.3 A continuous flow of nutrient is pumped into the reactor through a pump set at a flow rate equal to 6.7 $\pm$ 0.2 ml/min. Unclamp tubing on the drain spout and place the end into the waste carboy. The drain spout at the 250 ml mark on the beaker allows overflow to occur, maintaining a constant bacterial liquid growth broth concentration of 30 mg/L in the reactor during CSTR (continuously stirred tank reactor) mode.

Note 7: Flow rate is calculated by dividing the reactor volume by the residence time. The residence time is 30 minutes. The reactor volume is approximately 200 ml (see Note 2). The operator should set an exact flow rate based upon the measured fluid volume in the reactor when the rotor is in place to achieve an exact 30 minute residence time. The reactor residence time is a critical parameter that is specific to the bacterial species used during the experiment. In order to select for biofilm growth in the reactor, the residence time must be less than the doubling time for the suspended cells. This will result in the suspended cells washing out of the reactor, leaving only biofilm.

10.2.4 The reactor is operated in CSTR mode for 24 hours.
10.3 Sampling the biofilm.
10.3.1 Prepare sampling materials: vortex, homogenizer, culture tubes, pipettes, empty sterile petri dish, wooden applicator sticks, flame sterilized stainless steel dissecting tools, stainless steel hemostat.
10.3.2 The reactor is opened and the rotor is retrieved using a stainless steel dissecting tool via the retrieving port. The rotor is placed into an empty, sterile petri dish.
10.3.3 Holding the rotor by the Viton using a flame-sterilized hemostat, a randomly chosen coupon is removed from the rotor using a stainless steel dissecting tool. Care should be given to not disturb the top coupon surface in any way until it is scraped. If other coupons are to be sampled, replace rotor in reactor to prevent the biofilm from drying.
10.3.4 The coupon is secured by holding it with the flame-sterilized hemostat. The top coupon surface is scraped for approximately 15 seconds using the flat end of a wooden applicator stick held perpendicular to the coupon surface. The stick is rinsed by stirring it in the dilution buffer in the culture tube. Repeat the scraping and rinsing process 3-4 times, ensuring full coverage of the coupon surface.
10.3.5 The coupon is rinsed by holding the coupon at a 60° angle over the culture tube and pipetting 1 ml of sterile dilution water over the surface of the coupon. The final volume in the culture tube is 10 ml.[12]
10.4 Analyze the biofilm sample.
10.4.1 The scraped biofilm sample is homogenized at 20,500 $\pm$ 5000 rpm for 30 seconds. If more than one biofilm sample is taken rinse the homogenizing probe by homogenizing

[12] Each culture tube originally contains 9 ml of buffered water.

a dilution blank for 30 seconds at the same rpm, homogenize a tube containing 70% ethanol for 15 seconds, then remove the probe and let the probe set in the ethanol tube for 1 minute. Shake any remaining ethanol off the probe, reattach probe and homogenize a dilution blank for 30 seconds. Homogenize a second dilution blank and then homogenize the next sample tube. Always repeat this cleaning process between samples.

Note 8: Homogenizing the sample disaggregates the biofilm clumps to form a homogeneous cell suspension. Improper disaggregation will result in an underestimation of the viable cells present in the sample.

10.4.2 Serially dilute the sample.

Note 9: The equation in section 10.6 is accurate only if the culture tube the biofilm was scraped into is referred to as the 10^0 dilution.

10.4.3 Plate each dilution in duplicate for colony growth using an accepted plating technique such as spread plating or spiral plating.
10.4.4 Incubate the plates for 17-20 hours at 35˚C $\pm$ 2˚C.
10.5 Cell Enumeration.
10.5.1 Count the appropriate number of colonies according to the plating method used.
10.5.2 Calculate the arithmetic mean of the replicate samples plated.
10.6 The log density for one coupon is calculated as follows:

LOG_{10} (CFU/cm^2) = LOG_{10} [(mean CFU/volume plated per sample)(dilution)(volume scraped into/surface area scraped)]

10.7 Calculate the overall biofilm accumulation by taking the mean of the log densities calculated in section 10.6.

11. Precision and Bias

11.1 Randomization is used whenever possible to reduce the potential for systematic bias.
11.2 During development, the protocol was repeated 27 times. The internal reproducibility standard deviation for this protocol, when the biofilm density is based on a single coupon, was calculated to equal 0.5. The sources of variability were:

- 40% attributable to technician-to-technician variability,
- 40% attributable to experiment-to-experiment within technician variability,
- 20% attributable to coupon-to-coupon, within experiment variability.