BioSurface Technologies Corporation

Rotating Disk Biofilm Reactor (RDR) Operator's Manual

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Rev. 12/2022

Contents

1. Purpose

The Rotating Disk Biofilm Reactor Operator's Manual is intended to serve as a guide for researchers interested in growing a laboratory biofilm under continuously stirred flow and medium shear. It is the responsibility of the user to be familiar with basic microbiological concepts and techniques. Although a specific method is presented in the manual, the RDR is suitable for modeling many different environments. Laboratory biofilms are engineered based upon the dynamics in the reactor where they grow. No one biofilm is better than any other, although one reactor may be a better choice for modeling a particular environment. It is the responsibility of the operator to choose which reactor best suits their research needs.

2. Rotating Disk Biofilm Reactor Description

The Rotating Disk Biofilm Reactor consists of a PTFE and Viton disk containing recesses for six 1.25 cm diameter coupons. The removable coupons are manufactured from any machinable material. The bottom of the rotating disk contains a bar magnet to allow disk rotation to create liquid surface shear across surface-flush coupons. The entire disk containing the six coupons is placed in a 1000 ml glass reactor vessel. A liquid growth media/biocide/etc. is circulated through the vessel while the disk is rotated by a magnetic stirrer.

Sampling of the coupons is conducted by aseptically removing the entire disk from the reactor and punching/prying the coupons from the disk (a tool/pick is provided). If proper technique and care is used during sampling, a single coupon can be removed/replaced and the disk returned to the reactor vessel for further biofilm studies. The coupon removed from the reactor vessel is then scraped to collect the biofilm sample for further study or imaging using microscopy.

3. Rotating Disk Biofilm Reactor Standard Operating Procedure

The RDR was designed as a flexible reactor system, meaning that it can be easily adapted to model a variety of conditions in the laboratory. The following standard operating procedure (SOP) is just one suggested use. Operators are encouraged to modify the protocol to model the most appropriate biofilm for their research needs. A series of notes are included in the SOP that alert the operator to situations where special care must be taken.

Caution: All microorganisms should be handled according to safety recommendations for each individual species of microorganism. Decontamination of all media and equipment used during experimentation is required prior to disposal of media or re-use of equipment. It is the responsibility of the operator to inform themselves on these techniques.

A. Summary of Test Method

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.

B. Significance and Use

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.

C. Apparatus

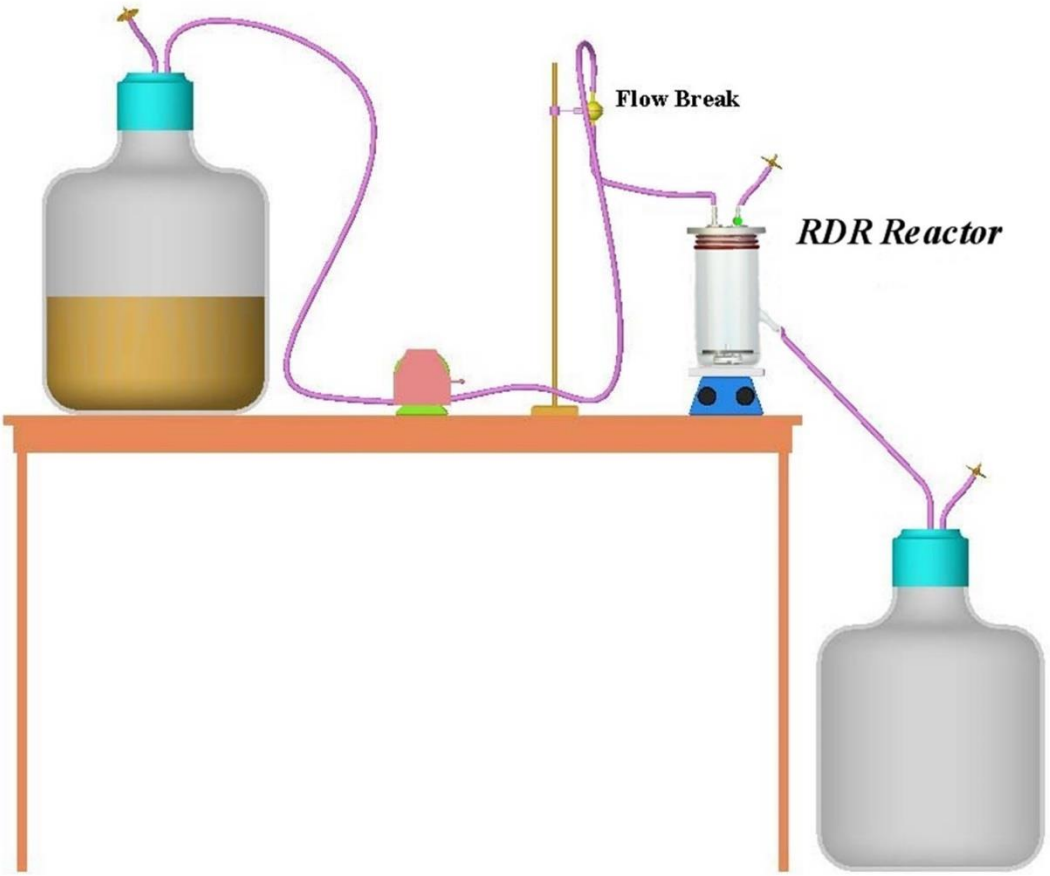

Figure 1: Schematic of the completely assembled reactor system (Norris, 2003).

(a) Wooden Applicator Sticks: Used for scraping biofilm from coupon surface. Sterile.
(b) Inoculating Loop. Used to introduce bacteria into inoculum flask. Sterile.
(c) Petri Dish: 100 x 15mm, plastic, sterile, for transporting rods from reactor to workstation.
(d) Culture Tubes with caps: Any with a volume capability of 10 mL and diameter no less than 6 cm. Recommended size is 16 x 125mm borosilicate glass with threaded opening.
(e) Pipette: Capable of accurately dispensing 1.0 mL.
(f) Vortex: Any vortex mixer that will ensure proper mixing of culture tubes.

(g) Homogenizer Probe: Capable of mixing at 20,500 ± 5000 rpm in a 5-10 mL volume and able to withstand autoclaving or other means of sterilization.
(h) Sonicator: any noncavitating sonicating bath that operates at 50-60 hertz.
(i) Syringe: sterile, 1 ml syringe used during reactor inoculation.
(j) Needle: sterile, 22-gauge needle used with syringe to inoculate reactor.
(k) Bunsen or Alcohol burner: For flame sterilizing inoculating loop and other instruments.
(l) Stainless steel dissecting tools.
(m) Hemostat: Stainless steel hemostat clamp with curved tip to remove and hold coupon in place while scraping. Use after flame sterilizing.
(n) Environmental Shaker: Capable of maintaining temperature of 37˚C.
(o) Top Loading Balance: For weighing reagent ingredients; sensitive to 0.01 gm.
(p) Sterilizers: Any steam sterilizer capable of producing the conditions of sterilization.
(q) Colony Counter: To aid in counting plates.
(r) Peristaltic Pump: For pumping media into reactor during continuous flow phase; pump head capable of holding selected tubing (accommodates tubing ID/OD).
(s) Magnetic Stir plate: Capable of operation at 100-400 rpm. A digital stir plate is recommended. (u) *Silicone tubing:* Two sizes of tubing, one with ID 3.2 mm (port OD=4.75 mm) and the other with ID 7.9 mm (port OD=7.94 mm). Both must withstand sterilization. Use to connect media carboy to reactor inlet; beaker outlet to waste carboy; air vent to reactor top; media carboy air vent.
(t) Glass Flow break: Autoclavable and accommodating tubing of ID 3.2 mm. Insert vertically into media inlet tube to prevent contamination from reactor to media carboy.
(u) Clamp Stand and Clamp: To hold glass flow break and media inlet tube vertically above reactor top. Clamp with 0.5 cm minimum grip size.
(v) Reactor Components:

i. Borosilicate Glass Beaker: 1000mL beaker without conventional pour spout, 9.5 ± 0.5 cm diameter. Barbed outlet spout added so that bottom is at 300 mL ± 15mL mark. Angle the spout 30-45 degrees to ensure drainage. Spout should accommodate silicon tubing with ID of 8-11 mm.

NOTE 1: The rotor will displace approximately 50 ml of liquid. Therefore, an outlet spout at the 300 ml mark will result in approximately a 250 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.

ii. Reactor Top: UHMW polyethylene with 4 holes to accommodate barbed fittings and a Mininert valve as an inoculum port.

iii. Rotor or Disk: 1.6 mm thick PTFE sheet cut into a disk with a diameter of 7.0 cm containing 6 evenly spaced holes with a diameter of 1.27cm. The center of each hole is located 2.55 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 cm containing 6 evenly spaced holes with a diameter of 1.27 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 x 1.4 cm starhead 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 machined to provide a flush mounting surface.

iv) Polycarbonate coupons: Provide a surface for biofilm growth. 6 cylindrical polycarbonate coupons with a diameter of 1.27 cm, thickness of approximately 3.0 mm.

(w) Carboys: 2 15-20L autoclavable carboys, to be used for waste and nutrients.

i. 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

ii. 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.

D. Media and Reagents

(a) Purity of Water: All reference to water as diluent or reagent shall mean distilled water or water of equal purity.

(b) Culture Media:

i. Bacterial Liquid Growth Broth, soybean-casein digest medium, or an equivalent general bacterial growth medium. Tryptic Soy Broth is recommended.

ii. Bacterial Plating Medium. R2A agar is recommended.

NOTE 2: 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.

(c) Buffered Water: 0.0425 g/L KH2 PO4 distilled water, filter sterilized and 0.405 g/L MgCl·6H2O distilled water, filter sterilized.

E. General Instructions

a. Culture Preparation

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 ± 2°C for 20-24 hours. Viable bacterial density should be about 108 CFU/ml, which may be checked by serial dilution and plating.

b. Reactor Preparation

Preparation of polycarbonate coupons:

NOTE 3: 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.

i. Sonicate coupons for 30 seconds in a 1:100 dilution of laboratory soap and tap water. The soapy water must completely cover the coupons.

ii. Rinse coupons with reagent water and sonicate for 30 seconds in reagent water.

iii. Repeat rinsing and sonication with reagent water until, upon visual inspection, no soap is left on the coupon.

iv. Place a coupon into each hole in the rotor, leaving the top of the coupon flush with the viton surface.

v. Place the rotor inside of the beaker.

Preparation of reactor top:

i. 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 fittings on the reactor stopper top.

ii. The glass flow break is spliced into the nutrient tubing line near the reactor stopper top.

Sterilization of reactor system:

i. Assemble the reactor, with the overflow (waste) line clamped and the reactor top securely fastened to the beaker before sterilization.

ii. 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.

iii. Sterilize reactor system using an autoclave for 20 minutes at 121°C.

NOTE 4: Autoclaving longer than 20 minutes could cause unnecessary degradation to the reactor or its components and is not advised. The reactor and its components have not been approved for temperatures above 121°C, and any damage caused by a higher temperature will not be covered under warranty.

c. Procedure

The batch phase:

i. Prepare batch nutrient broth by dissolving bacterial liquid growth medium (300 mg/L) in 250 ml reagent grade water, sterilize.

ii. 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.

iii. Inoculate the reactor with 1 ml of bacteria from the culture prepared previously. Aseptically inject the inoculum into the beaker through the inoculation port using the sterile needle and syringe.

iv. The magnetic stir plate is turned on to allow the rotor to spin freely. The rotation speed should equal approximately 200 revolutions per minute (± 100 rpm). The reactor system is allowed to incubate in batch mode at room temperature (21°C ± 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.

Continuous Flow Operation (CSTR mode):

i. Aseptically connect the nutrient tubing line to the carboy containing the continuous flow nutrient broth.

ii. 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.

iii. A continuous flow of nutrient is pumped into the reactor through a pump set at a flow rate equal to 6.7 ± 0.2 ml/min. Unclamp tubing on the drain spout and place the end into the waste carboy. The drain spout at the 300 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. 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. 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.

iv. The reactor is operated in CSTR mode for 24 hours.

Sampling the biofilm:

i. Prepare sampling materials: vortex, homogenizer, culture tubes, pipettes, empty sterile petri dish, wooden applicator sticks, flame sterilized stainless steel dissecting tools, stainless steel hemostat.

ii. 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.

iii. 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.

iv. 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.

v. 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.

Analyze the biofilm sample:

i. The scraped biofilm sample is homogenized at 20,500 ± 5000 rpm for 30 seconds. If more than one biofilm sample is taken rinse the homogenizing probe by homogenizing 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.

ii. Serially dilute the sample.

NOTE 9: Eq. 1 is accurate only if the culture tube the biofilm was scraped into is referred to as the 100 dilution.

iii. Plate each dilution in duplicate for colony growth using an accepted plating technique such as spread plating or spiral plating.

iv. Incubate the plates for 17-20 hours at 35°C ± 2°C.

Cell Enumeration:

i. Count the appropriate number of colonies according to the plating method used.
ii. Calculate the arithmetic mean of the replicate samples plated.

F. Calculations

a) Calculate the log density for one coupon using **Equation 1**:

$$LOG_{10}\left(\frac{cfu}{cm^2}\right) = LOG_{10}\left[\left(\frac{\left(mean\ cfu \big/ plate\right)}{vol.of\ sample\ plated}\right) \times \left(\frac{volume scraped\ in\ to}{surface\ area\ scraped}\right) \times \left(dilution\right)\right]$$

b) The mean biofilm density is the average of the individual coupon log densities.

G. Repeatability

Randomization is used whenever possible to reduce the potential for systematic bias. 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.

4. Rotating Disk Biofilm Reactor Assembly

NOTE 10: The Rotating Disc Reactor System (RDR) comes with and without a stir plate and support stand. These assembly directions assume a stir plate and support stand are used.

A. Stir Plate and Support Stand

i. Fix the aluminum rod into the back of the stir plate in the support clamp.
ii. Mount the support ring in a clamp and attach to the support rod. Mount the ring low on the stand near the top of the stir plate.
iii. Mount the 3-prong clamp onto the support rod with a clamp. Mount the 3-prong clamp high on the support rod.

B. Reactor Assembly

i. Nutrient Supply Tubing: Attach tubing from your media reservoir to the glass flow break inlets.
ii. Attach effluent tubing. Use minimum 1/4"(6 mm) ID and direct the effluent tubing to the drain or collection reservoir.
iii. Insert the coupons into the disc rotor, flush with the upper surface of the rotor.
iv. Insert the rotor into the glass reactor vessel.
v. Mount the reactor lid onto the glass vessel. Turn the lid slightly while pushing onto the glass vessel to seat the O-ring seal.
vi. Insert the glass vessel bottom through the stand ring support. Adjust the location of the ring support to center the glass vessel in the middle of the stir plate.
vii. Adjust the 3-prong support clamp to hold the influent tubing and glass flow breaks above the reactor vessel.

5. Rotating Disk Biofilm Reactor Ancillary Equipment List

NOTE 11: *BioSurface Technologies does not sell or supply the ancillary equipment described below. This or comparable equipment is required to complete the reactor system set-up. Please check with your local vendors for availability and current pricing. Equipment listed is not recommended equipment, but an aid to help you identify compatible equipment.*

Pump: There are many types of peristaltic pumps available, and you may opt for alternative types and pump head configurations (multi-channel versus single channel, higher/lower rpm range, etc.). Depending on what you need to add to the reactor during operation, you may require more than 1 pump or a multi-head pump (media for growth and biofilm treatment chemical for some duration that may require an additional pump).

- **Masterflex® L/S® Digital Precision Modular Drive with Remote I/O and Benchtop Controller, 1 to 100 rpm; 90 to 260 VAC**
  (VWR Item#: MFLX07557-10)
- **Masterflex® L/S® Easy-Load® II Pump Head for Precision Tubing, PPS Housing, SS Rotor**
  (VWR Item#: MFLX77200-60)

Tubing: The tubing you choose depends on the chemical compatibility, gas permeability, wear resistance in peristaltic pumps, and pricing. You must choose the tubing that best fits your needs. C-Flex tubing (listed below) is similar to silicone tubing but has a low gas-permeability compared to silicone. If gas-permeability is not an issue, standard silicone tubing is acceptable.

- **Masterflex® L/S® Precision Pump Tubing, C-Flex®, L/S 16; 25 ft**
  (VWR Item#: MFLX06424-16)
  - Will fit the 3/16” barbed port fittings on the top of the RDR and connect to the media supply reservoir.
- **Masterflex® L/S® Precision Pump Tubing, C-Flex®, L/S 18; 25 ft**
  (VWR Item#: MFLX06424-18)
  - Used to connect the effluent port (3/8” barbed port) on the reactor to the spent media collection vessel.
- **Masterflex® Transfer Tubing, C-Flex®, Opaque White, 1/4" ID x 7/16" OD; 25 Ft**
  (VWR Item#: MFLX06424-72)
  - A few lengths and adapters to get from the carboy to the smaller diameter tubing, and as a siphon tube inside the carboy.
- **Masterflex® Fitting, Nylon, Straight, Hose Barb Reducer, 1/4" ID x 1/8" ID; 10/PK**
  (VWR Item#MFLX30622-28)
  - Needed to get from the 3/16” or 1/4” ID to the 1/8” tubing.

Carboy: Carboys should be selected based on experiment needs and may be larger or smaller than what is suggested below. Ported lids can be purchased from suppliers, but standard lids are easily converted to ported lids using the following fittings or similar.

- **Azlon® Bottle, Rounded Octagonal, Polypropylene, Dynalon, 10L**
  (VWR Item#: 30620-188)
- **Azlon® Bottle, Rounded Octagonal, Polypropylene, Dynalon, 20L**
  (VWR Item#: 76210-720)
- **Nalgene® Barbed Bulkhead Fittings, Thermo Scientific, 6.4 mm ($^1/_4$")**
  (VWR Item#: 16331-102)

- **Nalgene® Barbed Bulkhead Fittings, Thermo Scientific, 12.7 mm ($^1/_2$")**
  (VWR Item#: 16225-232)
- **Cole-Parmer PTFE Syringe Filters, Non-Sterile; 0.45 µm, 50 mm Diameter**
  (Cole Parmer P/N: EW-02915-30)

Suggested Ancillary Equipment Suppliers:

VWR: 800-932-5000 (www.vwr.com)
Cole Parmer: 800-323-4340 (www.coleparmer.com)
Fisher Scientific: 800-766-7000 (www.fishersci.com)

6. Rotating Disk Biofilm Reactor Reference Material

A. Standard Method

ASTM E2196 (www.astm.org)

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

B. Publications

Cotter, John J., et al. "Characterization of a modified rotating disk reactor for the cultivation of Staphylococcus epidermidis biofilm." Journal of applied microbiology 109.6 (2010): 2105-2117.

Schwartz, Kelly, et al. "The use of drip flow and rotating disk reactors for Staphylococcus aureus biofilm analysis." JoVE (Journal of Visualized Experiments) 46 (2010): e2470.

Gomes, Inês B., et al. "Standardized reactors for the study of medical biofilms: a review of the principles and latest modifications." Critical reviews in biotechnology 38.5 (2018): 657-670.