**Abstract**

Through experience and surveying undergraduate microbiology curriculum, we found that most schools discuss biofilms in lecture classes but not laboratory courses. Biofilms are a concern for both industry and the medical field and should be studied in student laboratories. To study this at an institution, there would need to be an affordable method to assay them. As a sample specimen, Pseudomonas aeruginosa was used as it proliferates into biofilms when it is starved for nutrients, which can be easily simulated in a laboratory environment. Known assays for studying biofilms are expensive, and most departments do not have the materials. In Trypticase Soy Broth (TSB), P. aeruginosa can also be induced to form a biofilm, but the technique is not seen very often because it has not been sufficiently standardized for undergraduate microbiology education laboratories. To account for the absence of specialized reagents, we incubated bacteria for longer periods of time as a means to diminish nutrients or starve the specimen. Using a two-tailed t-test, we were able to show that glass tubes inoculated with P. aeruginosa in TSB for 48 hours were forming distinct biofilms on the glass surface, which is appropriate for undergraduate microbiology laboratory studies.

**Key Words:** Pseudomonas aeruginosa; biofilm; spectrophotometry; extracellular polymeric substance (EPS); Trypticase Soy Broth (TSB); crystal violet (CV); microbiology technique.

**Introduction**

The undergraduate biological science educational curriculum does not tailor to biofilm assay methods in the microbiology laboratory environment. Biofilms proliferating from pathogenic and opportunistic species are responsible for infection primarily in the food manufacturing and health care industries. The former results when planktonic “streamers” are shed into the production line, ultimately contaminating food products either from produced toxins (intoxication) or from viable pathogens (infection) that may enter and replicate inside a consumer's body. Moreover, biofilms are extremely difficult to completely eradicate as they sequester debris and integrate them within their EPS (extracellular polymeric substance), exponentially increasing their resistance to disinfecting strategies (Cramer, 2012). Health-care-acquired infections are a concern, especially for immunocompromised patients, and result when a biofilm has established itself onto some medical device that penetrates through a patient’s portals of entry or skin. Examples of this include needles, catheters, and ventilators (Bauman, 2016; Lima et al., 2017). There is a lack of affordable and standardized instructional material to accomplish such a feat, yet it is integral that students are exposed to the robustness of these biofilms and rapid methods to analyze them (Konrat et al., 2016). Textbooks for general microbiology undergraduate courses include a wealth of information about biofilms across a wide variety of topics, meaning they are important enough to study extensively in the conceptual lecture environment (Bauman, 2016).

Since biofilms pose alarming threats to public health (in addition to being exemplary models for demonstrating resistance factors and adaptability), it stands to reason that they should be incorporated into the undergraduate microbiology laboratory experience in some capacity. Many microbiology students are going to college to enter health-care fields. Understanding how a biofilm can adversely affect their patients can be key to understanding diagnoses for diseases such as urinary tract infections, pneumonia, or secondary infections in immunocompromised patients (Donlan, 2002; Lima et al., 2017; Moskowitz et al., 2004). This would offer students a hands-on experience with something commonly found in a number of prominent disciplines, such as health care and food manufacturing. This encourages the students to form critical hypotheses for biofilm removal strategies. For the purpose of this experiment, we concentrate on a fast polysaccharide biofilm-producing specimen, *Pseudomonas aeruginosa*, which can produce a viable biofilm in under 10 hours with the right conditions (Harrison-Balestra et al., 2003). This allows it to be used easily in a classroom setting.

**Materials List**

1. Reagents
   a. Glacial acetic acid

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b. Concentrated hydrochloric acid
c. Concentrated nitric acid
d. Deionized water
e. 0.41% crystal violet (CV) staining solution suspended in 30% acetic acid (0.41 mg of CV/100 mL of water)
f. 4 mL Trypticase Soy Broth (TSB) in glass test tubes
g. Sterile Trypticase Soy Broth

II. Tools
a. P1000 micropipette and sterile pipet tips
b. Laboratory-grade safety gloves
c. Safety goggles
d. Metal forceps

III. Equipment
a. Ambient air incubation system at 37˚C
b. 4 mL borosilicate glass test tubes
c. Test tube racks
d. Shaker table
e. UV-VIS spectrophotometer (GENESYS 30 is what we used.)
f. Clear plastic tape
g. Drying oven
h. Aluminum foil
i. Vortex

IV. Biologicals
a. Pseudomonas aeruginosa on tryptic soy agar (TSA)
   [Alternatively, you can use Pseudomonas fluorescens if BSL 2 organisms are not allowed in your lab.]
b. Proteus vulgaris on tryptic soy agar

○ Experimental Design

Prelab Instructor Setup

1. Glass Cleaning Procedure
   a. Obtain two glass beakers, a large glass graduated cylinder, a glass funnel, and 4 mL borosilicate glass test tubes that have all been sterilized in an autoclave.
   b. Arrange the glass test tubes at the bottom of a 1000 mL glass beaker with the openings facing upwards until test tubes are not able to move out of position when shaken.
   c. Obtain a second glass beaker, graduated cylinder, and funnel.
   d. Under a fume hood, pour 300 mL concentrated hydrochloric acid (HCl) into the glass beaker.
   e. Pour 100 mL concentrated nitric acid into the glass graduated cylinder using the glass funnel and slowly mix this into the glass beaker with HCl.
   f. Leave the solution to sit under a fume hood for about 15 minutes.
   g. Add acid solution to glass test tubes until all are covered.
   h. Remove acid, rinse the beaker three or four times using DI H₂O. Leave tubes in the beaker, cover with aluminum foil, and dry overnight in a drying oven.
   i. Alternatively, new unused borosilicate glass test tubes can be employed instead of following the above cleaning procedure.

2. Inoculate Stock Tubes
   a. Inoculate tryptic soy agar plate with stock Pseudomonas aeruginosa and Proteus vulgaris.
   b. Take a loop full of P. aeruginosa from a new stock tryptic soy agar (TSA) plate and aseptically transfer it to a 4 mL tryptic soy broth (TSB) tube.
   c. Incubate the TSB inoculum for a period of 24 hours in an ambient air incubation system at 37˚C.
   d. Repeat steps b and c for P. vulgaris if you require a positive control as we did.

Student Procedure Day 1

1. Biofilm Inoculation
   a. Place clean 4 mL test tubes onto racks aseptically using sterile forceps. Tubes need to be covered with tape or caps.
   b. Grab the stock TSB tube of P. aeruginosa and resuspend cells using the vortex.
   c. Dilute 1:100 with sterile TSB in a separate sterile container using a micropipette for a total of 100 mL.
   d. Vortex the diluted inoculum for 5 seconds.
   e. Dispense 1 mL of the inoculum into each of the 4 mL test tubes using a P1000 micropipette with sterile tips. Make sure that you have enough tubes for the amount of replicate you are preforming. Twenty tubes per student are preferred.
   f. Apply clear plastic tape to the openings of each row of inoculated 4 mL test tubes or cap each tube individually. Tape is preferred to limit loss of fluid within the tubes.
   g. Place test tube racks on a shaker table that resides within a 37˚C ambient air incubation system. To increase humidity in the incubator, place a tray of water inside of it. Alternatively, tubes can be placed into a sterile plastic box with wet paper towels during incubation.
   h. Set the shaker table to medium speed. The solution should be able to move but not slosh up the sides of the tubes.
   i. Incubate test tubes for a period of 48 hours, supplying water to the incubation system initially and halfway through the incubation period. Tubes may be left in a refrigerator for 7 days if needed after incubation.
   j. Repeat this process for negative uninoculated control tubes and P. vulgaris for a positive control.

Student Procedure Day 2

2. Biofilm Assay
   a. Remove test tubes from incubation or refrigeration.
   b. Remove liquid media from each of the test tubes using a sterile plastic transfer pipette and discard into a waste beaker. This step must be done cautiously so you do not disrupt the biofilm during transfer.
c. Prepare a 1:600 crystal violet staining solution, diluting with 30% glacial acetic acid in DI H₂O.

d. Dispense 1 mL of the diluted CV into each of the test tubes and leave test tubes to incubate at room temperature for 15 minutes.

e. During this waiting period, zero the GENESYS 30 by dispensing 1 mL of 30% acetic acid in DI H₂O into a clean cuvette and measuring its absorbance at OD₅₉₀. This cuvette should be put aside and used to blank the spectrophotometer in between reads.

f. Using a transfer pipette, remove the CV solution from each of the test tubes and dispense into a waste beaker.

g. Leave test tubes to dry for at least 10 minutes.

h. Transfer 1 mL of 30% acetic acid into each of the test tubes after the drying period. Let the solution sit for 5 minutes.

i. Pour the contents of each tube into a cuvette and measure for absorbance in the GENESYS 30.

j. Record absorbance measurements.

k. Convert absorbance values to concentrations using a previously generated crystal violet standard curve.

l. Repeat this process for the negative uninoculated control tubes and P. vulgaris for a positive control.

○ Standardization Methods

To construct a crystal violet (CV) standard curve, we first needed to ascertain what concentrations of the solution were detectable by our spectrophotometer, a GENESYS 30, at OD₅₉₀. The CV stock solution was serial-diluted in 1:2 increments until the upper threshold of detectability was ascertained, which was determined to be 1:640. A total dilution of 1:40960 would yield a value close to the lower threshold. The curve produced depicts the relationship between absorbance and concentration (mg/mL of crystal violet) for CV between the limits of detection. (The regression equation is y = 16.336x + 0.0027, where y = Absorbance and x = Concentration.)

○ Results

The biofilm experimental tubes were compared to the control tubes to determine if the concentrations arising from the assay were statistically different between the two treatments. Using a two-tailed t-test and assuming unequal variances, the treatment was determined to be significantly different from both controls; therefore, the assay is valid. A p < 0.05 was used as the metric to ascertain validity and reliability for this assay. The statistical analyses are included in Table 1. This shows a viable experiment using simple materials found in an education lab that can use positive and negative control if desired. A comparison was made only between controls and the experimental condition because a comparison between controls does not answer the question.

It is important to note that the polysaccharide films are easily disrupted if proper care is not taken with the sample. As such, students should be made aware of the possible experimental error. Other concerns with the procedure include improper dilution of cells and remnants of cleaning chemicals on any glassware that can interact with crystal violet, such as sodium hypochlorite. Tubes that are not new or were cleaned with acid will not work. These tube surfaces do not hold the Pseudomonas biofilm, which will come off when extracting the broth.

○ Discussion

The success of this assay shows that its incorporation into the undergraduate educational biology environment is something to be considered and easy to accomplish without costly equipment. Many biofilm assays use expensive and harder-to-find dye or a microplate reader (Konrat et al., 2016). These assays are not often available to educational labs due to the lack of equipment or will not fit within a budget. Our assay allows for the study of biofilms at an affordable cost. In our lab, the consumables cost is under $4 per student. This is less than many of the dyes used in other procedures (Wilson et al., 2017). The procedure is something that could easily be accomplished in two subsequent two-and-a-half-hour laboratory periods; one period of inoculation and one period of reading results in a spectrophotometer with time to spare. The intention of this method is to utilize reagents and equipment available in most undergraduate institutions to teach students about the robustness of biofilms and their rapid proliferation in the appropriate environment. To confirm this, we surveyed many microbiologists at colleges within the United States. All surveyed institutions showed interest in the assay for their classroom. Over 70% had all materials presented here.

This type of learning objective can be interesting to many areas of study within the college setting and not just a general microbiology classroom. It can help provide an understanding of biofilms and how they concern the health care and food manufacturing

| Table 1. Statistical analysis of results using two separate variances for control tubes and inoculated tubes in a two-tailed t-test. |
|-------------------------------------------------|-----------------|-----------------|-----------------|
| Mean Concentration mg/mL | Variance | N = |
| Negative Control | 4.519E-03 | 4.559E-06 | 90 |
| Positive Control | 4.271E-03 | 3.798E-06 | 40 |
| Pseudomonas Biofilms | 7.745E-03 | 1.010E-05 | 168 |
| Negative vs. Pseudomonas Biofilms | 9.693 | -6.61549 |
| Positive vs. Pseudomonas Biofilms | <.001 | <.00001 |
industries, which makes this type of experiment valuable in a health-care-based microbiology or biotechnology laboratory class.

References

Bauman, R.W. (2016). *Microbiology with diseases by taxonomy*, 5th ed. United States: Pearson.

Cramer, M. (2012). Biofilms: Impact on the Food Industry. *Food Safety Magazine*, June/July. https://www.foodsafetymagazine.com/magazine-archive1/junejuly-2012/biofilms-impact-on-the-food-industry/.

Donlan, R.M. (2002). Biofilms: Microbial life on surfaces. *Emerging Infectious Diseases*, 8(9), 881–890.

Harrison-Balestra, C., Cazzaniga, A.L., Davis, S.C. & Mertz, P.M. (2003). A Wound-Isolated *Pseudomonas aeruginosa* Grows a Biofilm In Vitro Within 10 Hours and Is Visualized by Light Microscopy. *Dermatologic Surgery*, 29(6), 631–635.

Konrat, K., Schwebeke, I., Laue, M., Dittmann, C., Levin, K., Andrich, R., et al. (2016). The Bead Assay for Biofilms: A Quick, Easy and Robust Method for Testing Disinfectants. *Plos One*, 11(6).

Lima, J., Alves, L.R., Paz, J., Rabelo, M.A., Maciel, M. & Morais, M. (2017). Analysis of biofilm production by clinical isolates of *Pseudomonas aeruginosa* from patients with ventilator-associated pneumonia (Análise da produção de biofilme por isolados clinicos de *Pseudomonas aeruginosa* de pacientes com pneumonia associada à ventilação mecânica). *Revista Brasileira de terapia intensiva*, 29(3), 310–316.

Moskowitz, S.M., Foster, J.M., Emerson, J. & Burns, J.L. (2004). Clinically Feasible Biofilm Susceptibility Assay for Isolates of *Pseudomonas aeruginosa* from Patients with Cystic Fibrosis. *Journal of Clinical Microbiology*, 42(5), 1915–1922.

Wilson, C., Lukowicz, R., Merchant, S., Valquier-Flynn, H., Caballero, J., Sandoval, J., et al. (2017). Quantitative and Qualitative Assessment Methods for Biofilm Growth: A Mini-review. *Research & Reviews: Journal of Engineering and Technology*, 6(4).

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