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Bacterial Biofilm Quantification: Using a Crystal Violet Assay to Assess Student-Selected Remedies

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Biofilms are communities of surface-associated bacteria existing in an extracellular matrix. Within such protective communities, bacterial species are more resistant to antimicrobial agents than their planktonic counterparts, often resulting in higher levels of virulence and chronic infection. *Staphylococcus aureus* is a hearty biofilm former that has been linked to many infections. In this inquiry-based exercise, students examine the anti-biofilm properties of natural oil-based soaps by performing a hands-on wet lab adaptation of the crystal violet quantitative biofilm assay (O'Toole, 1998), using *S. aureus*. This method is an important and low-budget tool in assessing natural remedies, a common interest of biology research students. This is a highly adaptable protocol which can be tailored to a variety of species and test agents.

Keywords: Biofilms, natural antibiotics, inquiry-based learning

Introduction

With increased antibiotic resistance observed among many pathogenic bacterial species, a common research interest of biology students is natural alternative treatments of bacterial infections. Investigation of bacterial growth has revealed two main patterns including free-living (planktonic) and their community based-counterparts known as biofilms. Biofilms are complex aggregates of surface associated bacterial cells existing in a secreted extracellular matrix, which may consist of proteins, sugars and/or DNA. Formation of biofilms involves a complex mechanism which is mainly regulated by cell density and nutrient availability (Donlan, 2002). Bacteria growing within a biofilm are often protected from their external environment, which may result in increased resistance to antimicrobial agents and the immune response of the host (Brady, 2008). Biofilms may consist of one species of bacteria, or several species as in the case of dental plaque and surface wounds on the skin. In human infections, bacterial biofilms may form on living tissue or on non-living tissue including catheters and prosthetic limbs. Further, it is estimated that 60 - 80 % of all bacterial infections are caused by biofilms, necessitating the need to examine natural anti-biofilm remedies (Lewis, 2001). In this inquiry-based wet laboratory experiment, students examine the effect of selected natural remedies on bacterial biofilm development *in vitro* using an adapted form of the previously described crystal violet biofilm assay (O'Toole, 1998, O'Toole, 2011, Ge, 2008). Using polystyrene wells as a biofilm attachment matrix students grow bacteria under biofilm inducing conditions in the presence of test agents. After sufficient biofilm growth of approximately 20 hours, non-adherent cells are decanted, biofilms stained with crystal violet, solubilized, and absorbance readings quantified using a spectrophotometer. Mean absorbance values are proportional to the amount of biofilm in each well and are compared to positive and negative controls.

We have had much success performing this procedure with the biofilm-forming species *Staphylococcus aureus*. *S. aureus* is a robust biofilm former, implicated in many nosocomial infections and skin wounds ranging from mild to severe. This experiment is ideal for a microbiology or student research laboratory course and is highly adaptable for a variety of student-selected anti-biofilm test agents and species. This laboratory exercise is introduced in an investigative

manner following classroom discussions of antibiotic resistance, biofilm formation and their roles on human infections. Students will then review primary literature examining natural treatments. Many plant-based treatment methods have been examined with the crystal violet biofilm assay including coconut oil. Coconut oil is rich in lauric acid, a medium chained fatty acid, effective in destroying the lipid membrane of many bacterial species (DebMandal, 2011). We have observed that allowing our students flexibility in selecting such test agents has been an invaluable engagement tool in student research and interest. Often students will investigate remedies from their family or cultural background. During the initial study of each test agent students perform a "dose response assay" examining serial dilutions of the treatment agent to determine the minimal inhibitory concentration on biofilm formation. This adapted procedure is an inexpensive, hands-on approach in which students use a spectrophotometer to individually measure optical density readings of solubilized stained biofilms. They then perform statistical analysis of their absorbance readings to examine the test agent's effect on *S. aureus* biofilm development. In the following interdepartmental collaborative laboratory module, students enrolled in Saint Peter's University's BI497 Biology Research course used the crystal violet assay to assess the anti-biofilm activity of coconut oil-based soaps on *S. aureus* biofilms. The soaps were produced by their student colleagues enrolled in Organic Chemistry laboratory CH252L.

Student Outline
Assessment of the Antibacterial Properties of Natural Oil-Based Soaps In *Staphylococcus Aureus* Using Crystal Violet Biofilm Assay

In this three day assay, we will examine the inhibitory effect of natural oil-based soaps prepared by your peers in Organic Chemistry labs on *S. aureus* biofilm formation. To determine a minimal inhibitory concentration, 10-fold serial dilutions will be prepared. Treatment agents will be assessed relative to an untreated control, sterile water and an antibiotic, tetracycline.

Day 1. Bacterial Inoculation

1. Using aseptic technique inoculate 5 mL Tryptic Soy Broth (TSB) with a discrete colony of *S. aureus* and incubate at 37°C overnight aerobically.

Day 2. Serial Dilutions

2. Measure 1 g of selected soap agent and dissolve in a final volume of 10 mL sterile water. Vortex tube until soap is dissolved. Using a 0.22 µm filter attached to a 15-mL syringe, filter-sterilize your soap mixture into a sterile 15 mL falcon tube. Again vortex to ensure sample is thoroughly mixed. This will be Dilution A (10%). Follow the steps seen in the flow chart below. Using a new sterile 1- mL pipette, transfer 1 mL from dilution tube A to dilution tube B which contains 9 mL sterile water. Cap and vortex tube. Using a new sterile 1-mL tip pipette, transfer 1 mL from dilution tube B to dilution tube C which contains 9 mL sterile water. Cap and vortex tube. Using a new sterile 1-mL pipette tip, transfer 1mL from dilution tube C to dilution tube D which contains 9 mL sterile water. Cap and vortex tube.

To determine the minimal inhibitory concentrations (MIC) effect of the soap you will be testing 10-fold serial dilutions ranging from 1% to 0.001% final w/v concentration

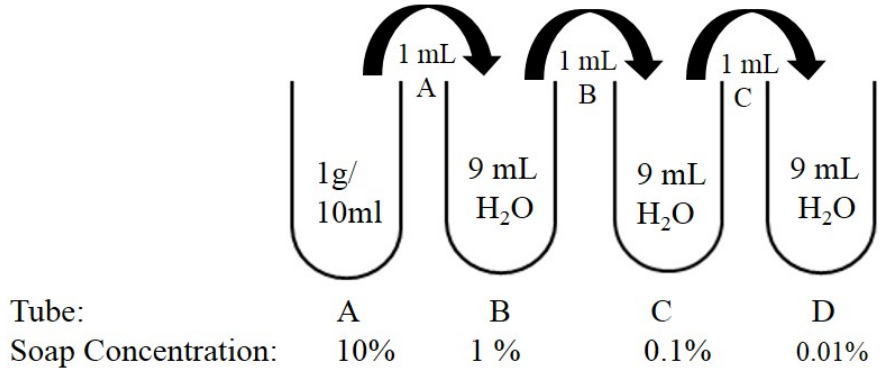

Figure 1. Serial dilution of soap test agents.

3. Using a sterile 24-well polystyrene plate and a sterile tip, pipette 900 µl of freshly prepared Tryptic Soy Broth with 0.25% glucose (TSBG) to all 24 wells. As indicated in Table 1, add 100 µL of each soap dilution test agent in quadruplicate to each well in respective columns A-D. This additional 10-fold dilution will result in the following (w/v) soap concentrations 1%, 0.1%, 0.01% and 0.001%. Be sure to use a fresh pipette for each solution. Add 100 µL of sterile water plus a tetracycline disk to the wells in Column E. This will serve as a positive control. Add 100 µL of sterile water to the wells in Column F. This will serve as a negative control. Inoculate by adding 10µL of overnight culture to each well in the top 3 rows. Leave bottom row un-inoculated. This row will test for contamination and will be resuspended in 30% acetic acid for the blank.

Table 1. 24-well plate setup guidelines.

A	B	C	D	E	F
1% soap *S. aureus*	0.1% soap *S. aureus*	0.01% soap *S. aureus*	0.001% soap *S. aureus*	Tetracycline *S. aureus*	Untreated *S. aureus*
1% soap *S. aureus*	0.1% soap *S. aureus*	0.01% soap *S. aureus*	0.001% soap *S. aureus*	Tetracycline *S. aureus*	Untreated *S. aureus*
1%1 soap *S. aureus*	0.1% soap *S. aureus*	0.01% soap *S. aureus*	0.001% soap *S. aureus*	Tetracycline *S. aureus*	Untreated *S. aureus*
1% soap *Uninoculated*	0.1% soap *Uninoculated*	0.01% soap *Uninoculated*	0.001% soap *Uninoculated*	Tetracycline *Uninoculated*	Untreated *Uninoculated*

4. Cover the 24-well plate and incubate aerobically at 37°C for ~ 20 hours.

Day 3. Biofilm Viewing and Staining

5. The following day, carefully remove 24 -well plates from the incubator. Slowly and carefully pour off the planktonic growth into a biohazard waste container using a paper towel to guide in and prevent splashing. Softly blot plate with paper towel. Then carefully rinse each well twice by pipetting 1 mL sterile distilled water. Add the water to the side of the wells, to avoid dislodging the biofilm. Gently shake off excess water by blotting dry into paper towel. Dry the plate with lid off for approximately 15 min in 37º C incubator. Take picture of biofilm plate.

6. Pipette 300 µL of a 1% crystal violet (CV) (prepared in 100% ethanol) to each well of the plate, and incubate at room temperature for 15 minutes.

7. Carefully pour off the CV into a waste tray using a paper towel to guide in. Rinse the plate 3 times by adding 1 mL sterile distilled water to each well, and as before, carefully add to the side of the well. Use caution not to apply too much force or you may dislodge the biofilm. Lightly blot excess water with paper towel between rinses. Place uncovered and upright in the 37º C incubator. Dry in the incubator for approximately 20 minutes. After drying take a picture of the CV-stained plate.

8. Dissolve the CV in each well by adding 1.5 mL 30% glacial acetic acid (vol/vol). Set up a duplicate plate and label. Transfer 1 mL from each well of the solubilized CV to the same location in the duplicate plate. To create the blank, use 1 mL acetic acid solubilized CV from each uninoculated condition against its respected inoculated wells. Pipet 1 mL from each treated well into a cuvette to determine the OD_{600} and record in Table 2. ***Wear goggles when working with acetic acid!!***

Table 2. Raw data for solubilized biofilm absorbance readings.

1%	0.1%	0.01%	0.001%	Tetracycline	Water
Treatment A	Treatment B	Treatment C	Treatment D	+ Control	Untreated

Analysis and Assessment

9. Using the data obtained for each condition, use Excel to calculate the mean and standard deviation of triplicate readings. Create a bar graph plotting the independent variable (soap concentration) on the X-axis and the dependent variable (OD_{600}) on the Y-axis. Under your graph, align plate image with respective wells under labeled treatment condition. Mean values are proportional to amount of biofilm in each well. Using an unpaired two tailed T-test determine which soap dilution significantly reduced biofilm formation relative to an untreated control. Using your graph, determine the range of the MIC of soap solution.

Sample Results

Crystal Violet Assay of Staphylococcus aureus Biofilms Treated with Coconut Oil-Based Soap

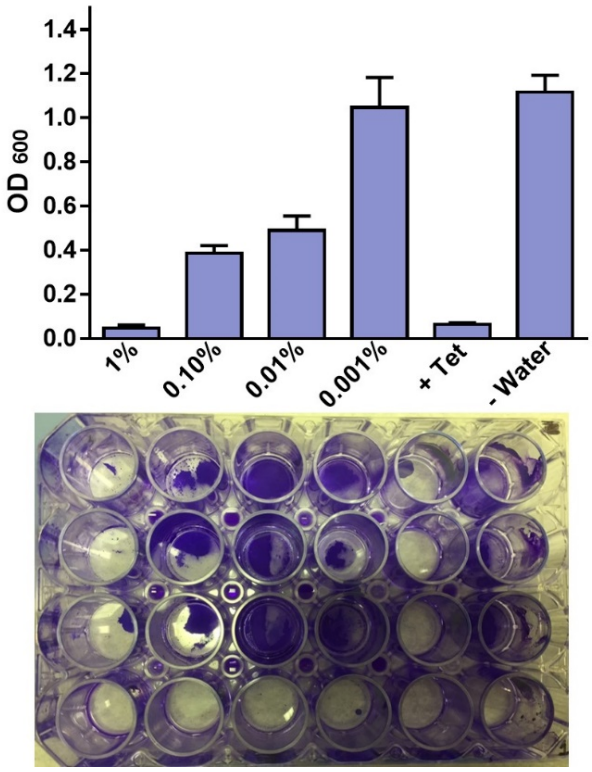

Figure 2. Typical results for a *Staphylococcus aureus* crystal violet biofilm assay incubated 20 hours with indicated coconut soap dilutions. Values indicated are the mean of triplicate readings + SD and are aligned with their respect stained columns on a dried plate prior to solubilization. Results indicated a dose dependent inhibition with MIC between 0.01% and 0.001%.

Materials

Needed Materials:

- Sterile solid growth media- Tryptic Soy Agar
- Sterile liquid growth media- Tryptic Soy Broth
- *Staphylococcus aureus* culture
- Grainger plastic syringe 20 mL
- 0.22µm PVDF 33mm syringe filters
- 40% glucose (filter sterilized)
- Sterile capped 15-mL culture tubes
- 24-well assay plates with lids (Thermo Scientific™ BioLite Microwell Plate polystyrene, sterile, tissue culture treated)
- Test solutions diluted and filter sterilized
- Rinsing solution sterile distilled water
- 1% crystal violet (w/v)
- 30% acetic acid
- Antibiotic Disks (Tetracycline)
- Micropipetter and sterile tips (P10, P100, P1000)
- 10% bleach
- Waste disposal dish
- Spectrophotometer and cuvettes
- Camera

Notes for the Instructors

This adapted technique has been very useful as an inquiry-based laboratory where students select agents to test. It is appropriate for a microbiology or research methods lab. It is important to have students research selected agents first with a literature review to determine their modes of action. The student outline provided is set up for a three-day consecutive procedure, but is very adaptable. If students will be performing this experiment as part of a weekly class meeting, day one can be prepared by the instructor the day before the class and each group provided with an overnight culture. Once the biofilm has formed for 20 hours it can be stored at 4º C for several days to slow growth. When instructing this procedure, emphasize the importance of aseptic technique. Contamination in the biofilms may be an issue, so remind students not to keep plates open while adding media and just lift up lid when pipetting. If OD_{600} readings are too high (>1.5) to determine differences among test conditions, solubilized dilutions can be prepared by adding 1mL of solubilized biofilm to a duplicate plate containing 1 to 2 mL of 30% acetic acid. You can then measure the absorbance of 1 mL of this dilution. In addition, the crystal violet stain can be added at a concentration of 0.1%, which may lower the range of absorbance readings. During the rinsing steps, you may carefully use a distilled water squirt bottle to gently rinse the biofilms, instead of pipetting. If 10-fold serial dilutions show too drastic an inhibition, two to five-fold dilutions may be preferred and exhibit a more gradual decrease.

During the ABLE mini-workshop, participants inquired about performing this experiment with less pathogenic bacterial species. *Staphylococcus aureus* is a BSL-2 pathogen and students should be instructed to wear gloves and use precautions particularly during the decanting step. For disposal and disinfection of the non-adherent cells, a 10% bleach solution placed in a wide disposal reservoir (we use a Pyrex™ dish) works well. This method can also be performed using *Staphylococcus epidermidis* a BSL-1 species. Other species may have different conditions that induce biofilm formation. An initial comparison of media and sugar concentrations (generally ranging from 0.25% to 1%) can assess conditions which induce biofilms. Oral bacteria such as *Streptococcus mutans* and *Streptococcus sanguinis* produce hearty biofilms when incubated under reduced oxygen conditions in a minimal media supplemented with 1% sucrose (Ge, 2008). Motile species are more likely to adhere to sides of wells while non-motile species are more likely to adhere to bottom of wells (O'Toole, 2011). This protocol is an inexpensive manual introduction to the crystal violet staining method that could also be performed in an automated 96-well plate reader. For the solubilization step, 100% ethanol can replace acetic acid for safety but the biofilms don't dissolve as well. To prepare the TSBG, Tryptic Soy Broth media is first autoclaved separately. Glucose stock solutions are prepared in sterile water, filtered, and diluted in cooled TSB. I generally begin with a 40% stock.

Methods of Soap Preparation Used by Chemistry Lab

Weigh a 150-mL beaker and record the mass (g). Measure out approximately 5.0 g of coconut oil and add it to the beaker. Add 15-mL of ethanol and 15-mL of 20% NaOH to the beaker and add a magnetic stir bar. Heat mixture to 70°C-80° C on a hot plate for 30 minutes. To keep a constant volume, add equal amounts of water and ethanol. Pour the solution containing the 15mL of ethanol and 15 mL of 20% NaOH and coconut oil into a 400-mL

beaker. Add 50 mL of saturated NaCl into the beaker and stir the solution. Place the beaker on top of a 1000-mL beaker filled with ice. The 400-mL beaker will stay on top of the ice for a few hours. Then it will be taken out of the ice and it will sit for 24 hours or more. While the soap solution is on top of the beaker filled with ice, the soap will start becoming solid. Collect the soap curds using vacuum filtration and an air aspirator for an hour. After the soap has dried sufficiently, a spatula is used to put the soap curds into a watch glass or Ziploc bag. (Adapted from Lehman, 2002)

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