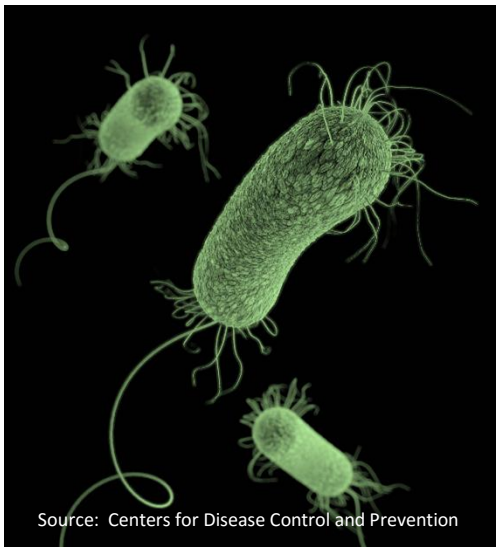
ImQuest BioSciences

Biofilm Protocol Optimization For *Pseudomonas aeruginosa*

Culture Media, Incubation Time, and Biofilm Measurement

Introduction

In addition to the conventional arsenal of antibiotic resistance mechanisms such as efflux pumps, β-lactamases, and aminoglycoside modifying enzymes, *Pseudomonas aeruginosa* forms biofilms that have a minimum inhibitory concentration (MIC) for antibiotics up to 1,000-fold higher than that of planktonic bacteria.


Source: Centers for Disease Control and Prevention

Multiple biofilm-specific mechanisms, poor antibiotic penetration, nutrient limitation, slow growth, adaptive stress responses, and formation of persister cells contribute to the high levels of antibiotic resistance. Therefore, *P. aeruginosa* biofilm-associated infections, such as those common in cystic fibrosis and burn patients, present huge clinical challenges which have spurred interest in the development of novel drugs and drug delivery strategies to treat these infections.

To screen new drugs for efficacy against biofilms formed by *P. aeruginosa*, we optimized a microtiter-based protocol described by Kwasny and Opperman. This methodology was selected because few steps are required, making it ideal as a primary screen for drug candidates with activity against biofilms. In addition, two commonly used methods of biofilm measurement were evaluated to determine which of these should be included as part of an optimized process to identify biofilm inhibitors.

Materials and Methods

Strains: The following strains of *P. aeruginosa* were chosen for optimization of culture conditions.

- PA01 – a widely studied strain, the genome of which has been sequenced
- PA6077 – a toxin producing strain
- ATCC 27853 – a strain commonly used for antimicrobial susceptibility testing

Media for Biofilm Formation: Media formulations used were based on Tryptic Soy Broth (TSB) supplemented with 0.2% glucose, Brain Heart Infusion (BHI) Broth, and Luria-Bertani (LB) Broth supplemented with 2% glucose.

Culture Conditions: Cultures were grown overnight at 37°C under aerobic conditions. Overnight cultures were diluted in assay media, and 100 µL was transferred to the wells of a 96-well tissue-culture coated polystyrene flat-bottomed plate. For each strain and medium, eight wells were inoculated. Plates were incubated at 37°C without agitation (static culture). Each plate included medium-only control wells.

Evaluation of Biofilm Formation: The culture medium was removed from each well by pipetting; non-adherent cells were removed by washing three times with Dulbecco's Phosphate-Buffered Saline. Biofilms were fixed by incubating the plates at 60°C for 1 hour. Two methods were used to measure biofilm formation:

- Crystal Violet 1 (CV1): Biofilm formation was evaluated by adding 200 µL of 30% acetic acid to each well after staining with 50 µL of a 0.1% (w/v) crystal violet solution and then measuring the OD_{600} of the eluate. Eluates with an optical density > 2.5 were diluted 1:10 in a solution of 30% acetic acid.
- Crystal Violet 2 (CV2): Biofilm formation was evaluated by adding 200 µL of 70% ethanol to each well after staining with 50 µL of a 0.4% (w/v) crystal violet solution and then measuring the OD_{600} of the eluate. Eluates with an optical density > 2.5 were diluted 1:10 in a solution of 70% ethanol.

Evaluation of Cell Viability in Biofilms: Ninety microliters of XTT (2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide) solution at 1 mg/mL and 10 µL of phenazine methosulfate at 0.34 mg/mL were added to each well. Cultures were incubated for 2 hours and the absorbance at 450 nm was determined. Background absorbance was measured at 650 nm and then subtracted from absorbance at 450 nm to obtain normalized absorbance values.

Figure 1a. Effect of Media on Biofilm Formation
P. aeruginosa PA01

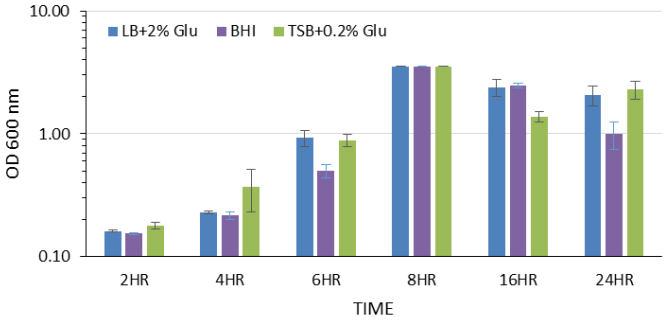


Figure 1b. Effect of Media on Biofilm Formation
P. aeruginsa 6077

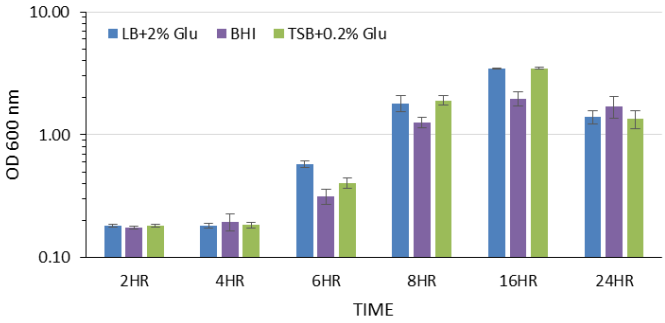
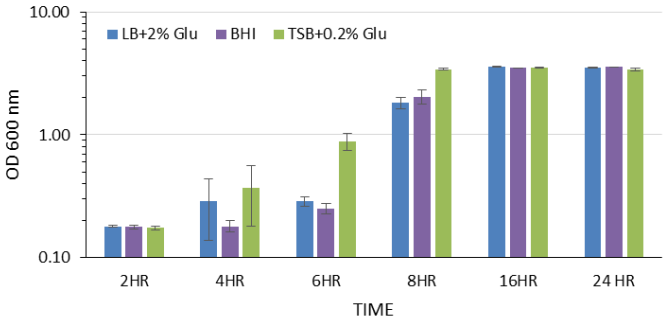


Figure 1c. Effect of Media on Biofilm Formation
P. aeruginosa 27853



Results

Effect of Culture Media

Figure 1 depicts the effects of three different culture media on biofilm formation by three strains of *P. aeruginosa*. Cultures were initiated with an inoculum of 1 x 10^6 CFU/mL.

The results were similar for all media evaluated, although biofilm formation was slightly lower with cells grown in BHI broth than cells grown in LB + 2% glucose or TSB + 0.2% glucose. Biofilm formation by each of the three strains increased and reached a plateau between 8 and 16 hours of incubation. A decrease in biofilm mass was observed for two of the three strains after 16 hours.

Results in Figure 1 were obtained with the CV2 method but the pattern was similar to that obtained using the CV1 method. For a comparison of CV1 vs CV2 see Figure 4.

TSB + 0.2% glucose was selected for protocol optimization because it is a commonly used medium for the cultivation of aerobic microbes like pseudomonads, is used for inoculum preparation prior to antibiotic sensitivity testing, and has been reported to support biofilm formation by *P. aeruginosa*.

Effect of Inoculum Concentration

The effect of inoculum concentration on biofilm formation in TSB + 0.2% glucose (Figure 2) as well as BHI and LB + 2% glucose (data not shown) was determined for three strains of *P. aeruginosa*. Biofilm formation was measured using the CV2 method.

Biofilm formation over time was a function of inoculum – at higher concentrations of bacteria a biomass plateau was reached more quickly.

With an inoculum of 1 x 10^8 CFU/mL, biofilm formation reached maximum levels after 4 to 6 hours of incubation. At 1 x 10^6 and 1 x 10^7 CFU/mL, biofilm formation reached maximum levels at 6 to 8 hours with the exception of *P. aeruginosa* strain 6077 inoculated at 1 x 10^6 CFU/mL which reached a maximum at 16 hours. Biofilm formation with an inoculum of 1 x 10^5 CFU/mL generally increased during the early hours and reached maximum levels between 8 and 16 hours.

An inoculum of 1 x 10^6 CFU/mL was selected as the starting inoculum concentration for protocol optimization because biofilm formation steadily increases and reaches maximal or near maximal biomass levels within 8 hours.

Figure 2a. Biofilm Formation at Various Inoculation Levels
P. aeruginosa PA01

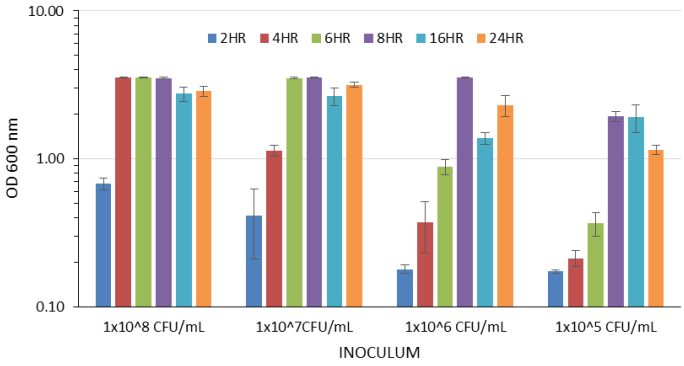


Figure 2b. Biofilm Formation at Various Inoculation Levels
P. aeruginosa 6077

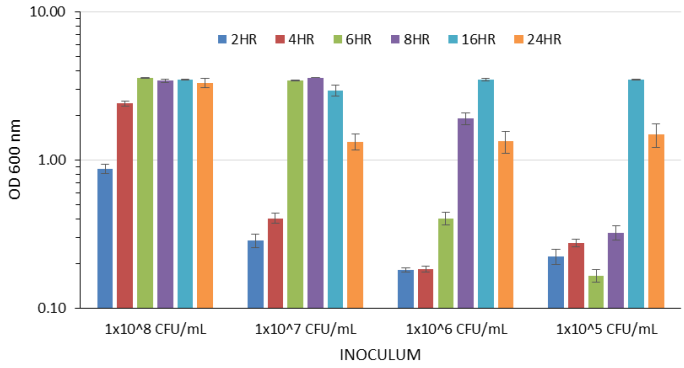
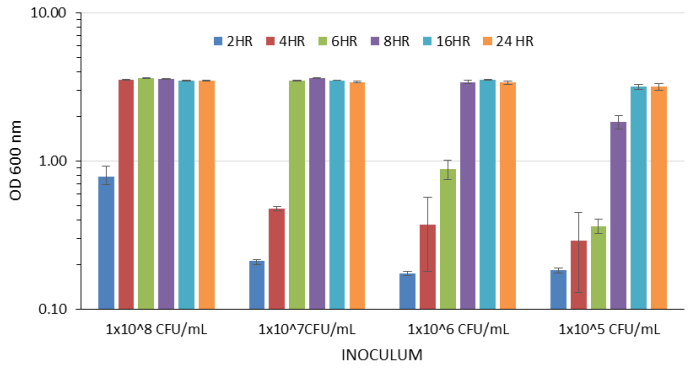
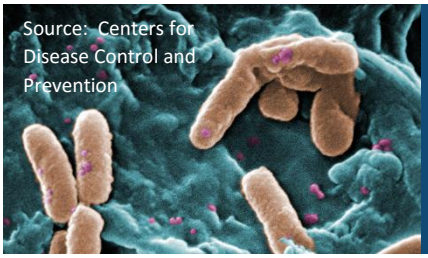


Figure 2c. Biofilm Formation at Various Inoculation Levels
P. aeruginosa 27853




Source: Centers for Disease Control and Prevention

Pseudomonas aeruginosa and Cystic Fibrosis

Cystic fibrosis patients are predisposed to *P. aeruginosa* infection of the lungs. Among those CF patients age 18 and older, about 80% are infected with *P. aeruginosa,* and about 18% of these patients are infected with a multidrug-resistant strain of *P. aeruginosa*.

Biofilm Formation and Viability

Figure 3 shows biofilm formation and cell viability for three strains of *Pseudomonas* during a 24-hour incubation in TSB + 0.2% glucose initiated with an inoculum of 1 x 10^6 CFU/mL.

Staining with the tetrazolium dye, XTT, demonstrated a high percentage of viable cells in biofilms and correlated with biomass formation.

Viability increased during the first 8 to 16 hours of incubation. During this time, biomass formation also increased. Some decrease in viability was observed between 16 and 24 hours of culture. Similar results were obtained with BHI and LB + 2% glucose inoculated at 1 x 10^6 CFU/mL (data not shown).

Figure 3a. Biofilm Formation and Viability
P. aeruginosa PA01

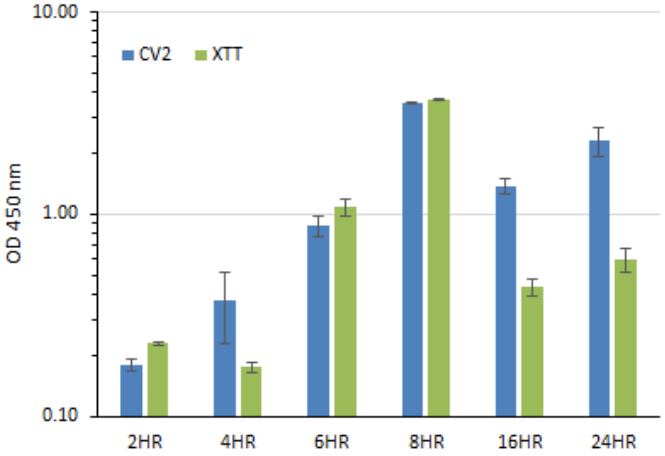


Figure 3b. Biofilm Formation and Viability
P. aeruginosa 6077

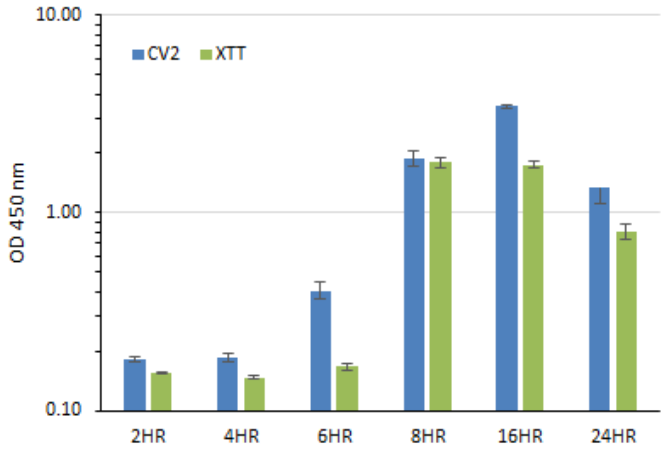
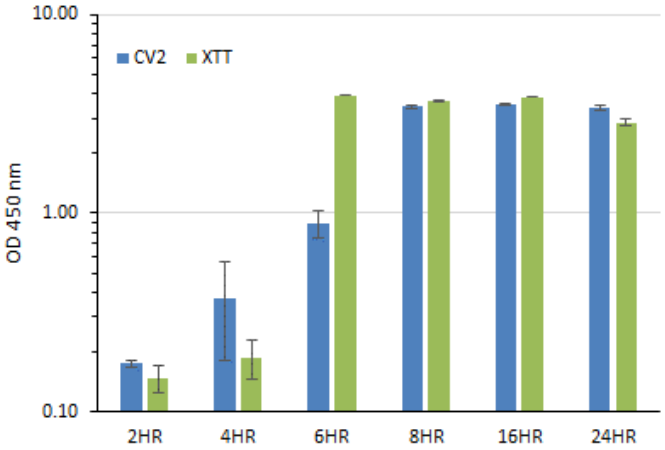


Figure 3c. Biofilm Formation and Viability
P. aeruginosa 27853



Pseudomonas aeruginosa Nosocomial Infections

According to the CDC, an estimated 51,000 healthcare-associated *P. aeruginosa* infections occur in the United States each year. More than 6,000 (13%) of these are multi-drug-resistant, with roughly 400 deaths per year attributed to these infections.

Patients in hospitals, especially those on ventilators, those with devices such as catheters, and patients with wounds from surgery or from burns are potentially at risk for serious, life-threatening infections caused by *P. aeruginosa*.

Source: Centers for Disease Control and Prevention

Biofilm Measurement Method

Two methods of biofilm biomass measurement, CV1 and CV2, were used to determine the level of biofilm formation by *P. aeruginosa* grown in TSB + 0.2% glucose (Figure 4) at an inoculum of 1 x 10^6 CFU/mL.

Whether the CV1 or the CV2 method was used to measure biofilm formation, biofilm biomass increased over 8 to 16 hours. However, the biofilm level was generally higher with the CV2 method. Similar results were obtained when the strains were grown in BHI broth or LB + 2% glucose (data not shown).

The CV2 method was selected for use in the optimized protocol because it may have resulted in a more complete extraction of crystal violet than CV1.

Figure 4a. Comparison of Biofilm Measurement Method
P. aeruginosa PA01

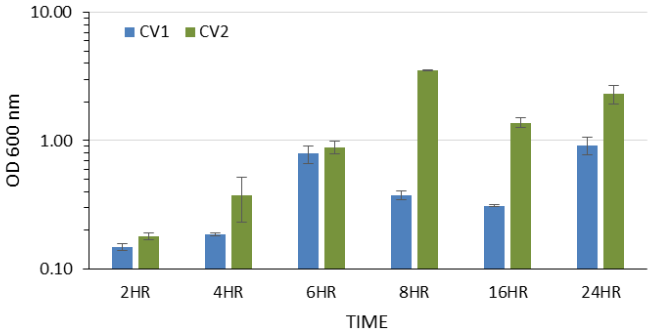


Figure 4b. Comparison of Biofilm Measurement Method
P. aeruginosa 6077

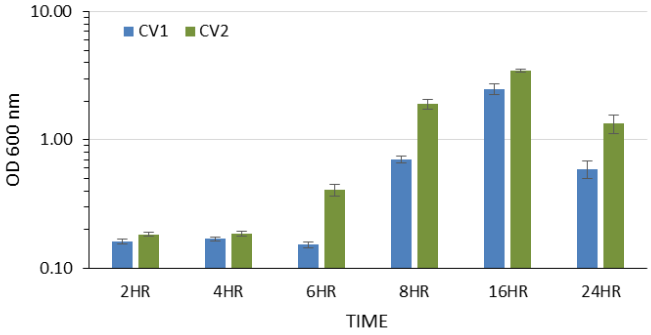
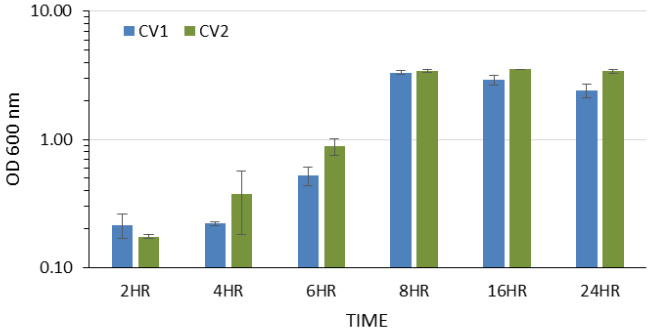


Figure 4c. Comparison of Biofilm Measurement Method
P. aeruginosa 27853



Conclusions

We report herein the optimization of the Kwasny and Opperman protocol for biofilm formation by *P. aeruginosa*. The results demonstrate that all three media used support robust biofilm formation and that cells in the biofilm were viable. With an inoculum level of 1 x 10^6 CFU/mL, biofilm formation generally reaches maximum levels within 8 hours, thereby providing a one-day time frame for a rapid screening biofilm inhibition assay. The results also demonstrate that cell viability generally correlates with biofilm biomass formation and that the CV2 method of biofilm measurement is probably more suitable than the CV1 method.

In summary, the protocol optimized by ImQuest BioSciences provides a high throughput procedure to screen drug candidates for activity against *P. aeruginosa* biofilms and is especially suitable to rapidly identify highly active inhibitors of biofilm formation.

References

1. Kwasny, SM and Opperman, TJ. Static biofilm cultures of gram-positive pathogens grown in a microtiter format used for anti-biofilm drug discovery. *Curr Protoc Pharmacol.* 2010; Chapter 13: Unit 13A.8.
2. Sabaeifard, P. *et al*., Optimization of tetrazolium salt assay for *Pseudomonas aeruginosa* biofilm using microtiter plate method. Journal of Microbiological Methods. 2014 (105), 134-140.

Antimicrobial Development Services

ImQuest BioSciences' MicroSENS antimicrobial product development services uniquely combine *in vitro* and animal model evaluations.

Our Micro*SENS* services facilitate the rapid screening of novel antimicrobial agents using established *in vitro* assays and the evaluation of active compounds in well-defined animal models.

In Vitro Antimicrobial Evaluations

- CLSI broth-based microdilution for MIC and MBC determination
- Kill-curve analysis
- Analysis of bactericidal versus bacteriostatic activity
- Post-antibiotic effect evaluation
- Inhibition of biofilm development and disruption
- Persister cell assays
- Minimal biofilm inhibitory concentration (MBIC)
- Combination antimicrobial evaluations
- Resistance selection and characterization
- Mechanism of action studies
- β-lactamase assays

In Vivo Models

- Neutropenic thigh model
- Peritonitis-sepsis model
- Systemic sepsis model
- Custom models (upon request)
- CLSI-based serum bactericidal effect (for some models)

Microbe Panels

The foundation of ImQuest's Micro*SENS* platform consists of a select panel of clinically relevant wild-type and antibiotic resistant microorganisms for use in both *in vitro* assays and *in vivo* models.

Bacterial/fungal pathogens and clinical specimens

- Indication-specific microbes
- Clinically-relevant gram-negative and gram-positive organisms
- Anaerobic organisms
- Sexually transmitted organisms

ESKAPE and other drug-resistant pathogens

- Carbapenam resistant
- Extended spectrum beta-lactam resistant (ESBL)
- Methicillin resistant
- Penicillin resistant
- Vancomycin resistant
- Linezolid resistant
- NDM-1 resistant
- Multi-drug resistant