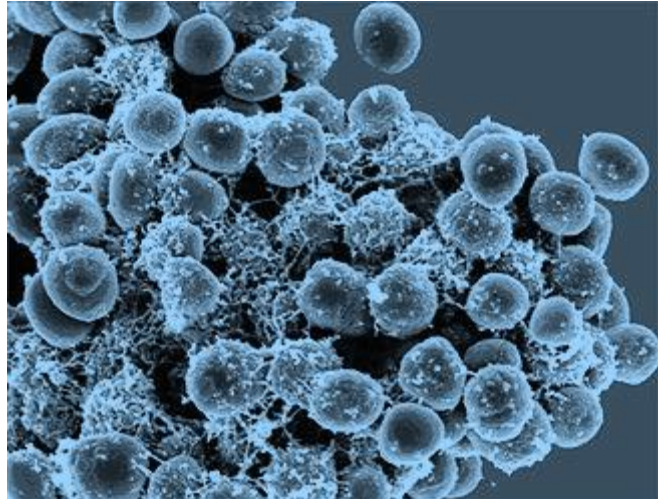


Introduction

According to the Centers for Disease Control and Prevention, biofilms are implicated in more than 80 percent of chronic inflammatory and infectious diseases caused by bacteria, including gingivitis, ear infections, gastrointestinal ulcers, urinary tract infections and pulmonary infections in cystic fibrosis patients.

Scanning and transmission electron microscopy have shown that virtually all in-dwelling central venous catheters are colonized by microorganisms embedded in a biofilm matrix. Microbes may also attach to and develop biofilms on components of mechanical heart valves and urinary catheters.



Bacteria commonly isolated with these devices include gram-positive cocci and diphtheroids, gram-negative bacilli, and the yeast, *Candida albicans*. Among the gram-positive bacteria found in device-associated biofilms are *Enterococcus faecalis*, *Staphylococcus aureus*, and *Staphylococcus epidermidis*. Gram-negative microbes include *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, and *Pseudomonas aeruginosa*.

Microorganisms responsible for the development of biofilms are highly resistant to antimicrobial treatment and tenaciously bind to surfaces. Drug concentrations up to 1000 times higher than those used to treat planktonic bacterial infections may be needed to eradicate microorganisms in biofilms. For this reason, the development of antibiotics and delivery strategies that target biofilm formation and growth has become increasingly important.

To screen new antibiotics for their efficacy against biofilms, we considered two published protocols:

- The Calgary protocol, a microtiter-based method with biofilm formation on pegs (1)
- A microtiter-based protocol described by Kwasny and Opperman with biofilm formation on plastic microtiter plate well surfaces (2)

The Kwasny and Opperman protocol was selected because the protocol involves only a few steps making it ideal for a rapid screen for inhibitors. The organisms chosen for optimization of culture conditions were representative strains of gram-positive cocci. These bacteria were selected because they are commonly found in biofilms on body surfaces and/or implanted medical devices. Two methods for measurement of biofilm formation were evaluated as part of the protocol optimization (2).

Materials and Methods

Strains: The following microbial strains were used for biofilm formation evaluations: *Staphylococcus aureus* ATCC 35556, *Staphylococcus epidermidis* ATCC 35984, and *Enterococcus faecalis* ATCC 29212.

Media for Biofilm Formation: Media formulations used were based on either Trypticase Soy Broth (TSB) or Brain Heart Infusion (BHI) broth with various concentrations of glucose supplementation:

TSB	1X	1X	1X	0.5X	0.5X	0.5X	0.25X	0.25X	0.25X			
BHI										1X	0.25X	0.0625X
+ GLUCOSE	1%	0.5%	0.25%	1%	0.5%	0.25%	1%	0.5%	0.25%	8%	2%	0.5%

Culture Conditions: Cultures of each of the organisms were grown overnight in TSB at 37°C under aerobic conditions. The overnight cultures were diluted in assay media to a concentration of 5×10^5 CFU/mL and 200 μ L was then transferred to the wells of 96-well tissue-culture coated polystyrene flat-bottomed plates. For each strain and each medium, eight wells were inoculated. Plates were incubated at 37°C without agitation (static culture). Each plate included medium-only control wells.

Evaluation of Growth: Total bacterial growth (planktonic + biofilm) was quantified by measuring the optical density (OD) of each well at 600 nm. The average OD₆₀₀ and standard deviation were then calculated for replicate wells.

Evaluation of Biofilm Formation: The culture medium was removed from each well by pipetting and non-adherent cells were removed by washing three times with DPBS. The biofilms were then fixed by incubation of the plates at 60°C for 1 hour. Two methods were used to evaluate biofilm formation:

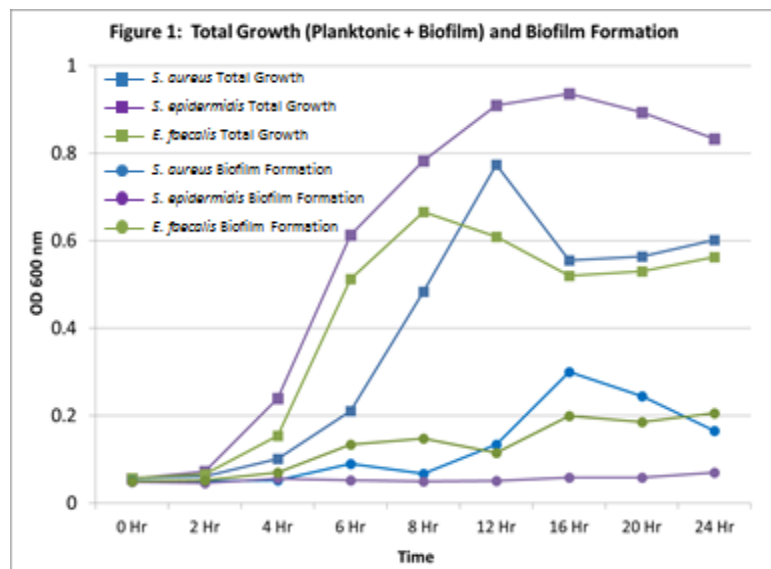
- **Direct Determination:** The formed biofilms in each well were stained with 50 μ L of 0.06% (w/v) solution of crystal violet dissolved in dH₂O for 5 minutes. After staining, excess stain was removed by 3 to 4 washes with dH₂O and the optical density at 600 nm was determined.
- **Indirect Determination:** Biofilm formation was evaluated by adding 200 μ L 30% acetic acid to each well after crystal violet staining and then measuring the OD₆₀₀ of the eluate. Eluates with an optical density > 2.5 were diluted 1:10 in a solution of 30% acetic acid.

Average OD₆₀₀ measurements and standard deviations were calculated for replicate wells.

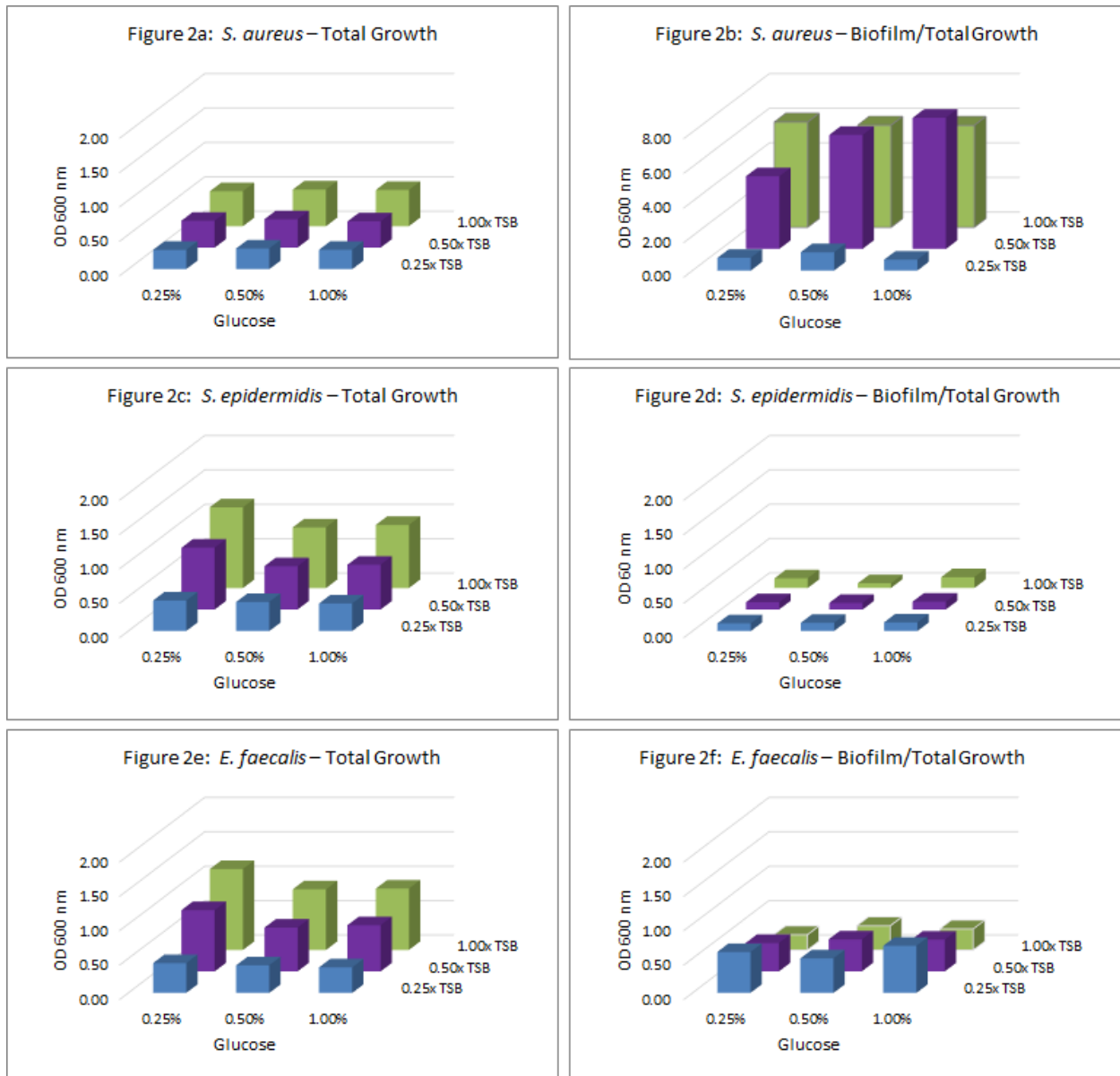
Results

Biofilm Formation Over Time: The results in Figure 1 depict total growth (planktonic + biofilm) and biofilm formation by *S. aureus*, *S. epidermidis* and *E. faecalis* grown in TSB + 1% glucose over a 24-hour period. Exponential growth began approximately two hours after inoculation and continued for 8 to 12 hours. Biofilm formation became detectable at 4 to 6 hours and plateaued after about 16 hours.

The direct method of biofilm measurement was used because it is rapid and correlates with biofilm formation (see below).



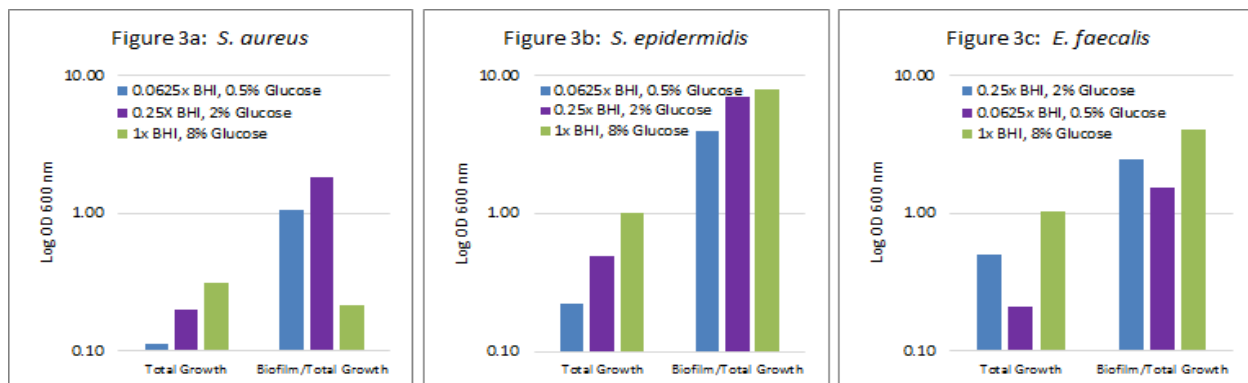
Effect of Culture Medium on Biofilm Formation: The effect of various TSB-based media formulations on biofilm formation (measured using the direct method) was evaluated after incubation of cultures for 16 to 18 hours, the peak time for biofilm formation. Figure 2 shows the amount of growth (planktonic + biofilm) and the ratio of biofilm/total growth.



The ratio of biofilm/total growth was markedly higher for *S. aureus* (Figure 2b; note scale difference) as compared to *S. epidermidis* (Figure 2d) or *E. faecalis* (Figure 2f). Interestingly, total growth was lower for *S. aureus* (Figure 1a) than for *S. epidermidis* (Figure 2c) or *E. faecalis* (Figure 2e). The culture medium which favored the highest ratio of biofilm/total growth varied; higher TSB concentrations resulted in higher ratios for *S. aureus* and lower TSB concentrations produced higher ratios for *E. faecalis*. Media variations had little effect on *S. epidermidis* biofilm formation.

The effect of three BHI-based media formulations was also evaluated (Figure 3). In contrast to the results with TSB-based media, the ratio of biofilm/growth was generally higher for *S. epidermidis* (Figure

3b) and *E. faecalis* (Figure 3c) than for *S. aureus* (Figure 3a). The maximum total growth (OD_{600 nm}) for *S. aureus* was approximately 0.3 whereas the values for *S. epidermidis* and *E. faecalis* were about 1.0.

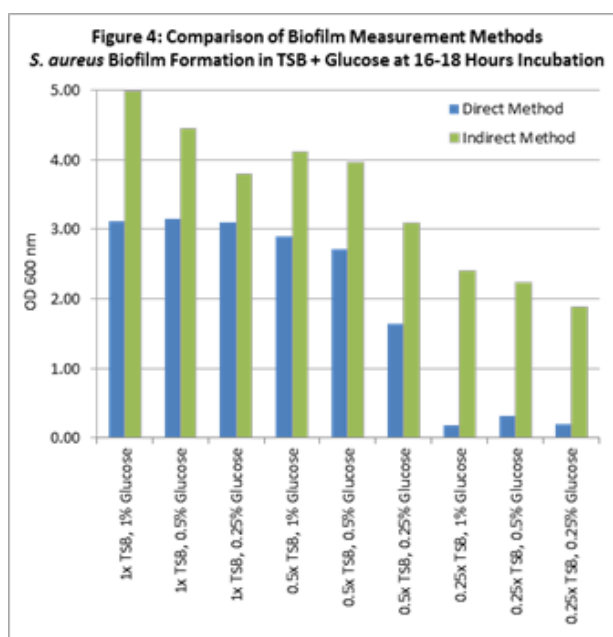


Comparison of Biofilm Measurement Methods:

In general, results using the indirect method of biofilm measurement were higher than with the direct method (Figure 4). However, the two measurements generally correlated with a correlation coefficient of 0.92.

Conclusions

Overall the Kwasny and Opperman protocol as optimized by ImQuest provides a high throughput procedure for studying biofilm formation and the inhibitory effects of antibiotics on this formation. Moreover, the method is less cumbersome than the Calgary method (which we previously evaluated) and requires fewer steps making it a more suitable and cost effective screen to quickly eliminate drug candidates with low activity against biofilms.



Although the indirect biofilm measurement method results in higher measurements, the correlation between the direct and indirect method is sufficient to justify using the direct method to decrease the number of steps in a rapid screen. Staining with the tetrazolium dye, XTT, demonstrated a high percentage of viable cells in biofilms and correlated with biofilm formation (data not shown).

These results demonstrate the need to optimize the media formulation for each organism. TSB-based formulations favored biofilm formation by *S. aureus* as compared to BHI-based formulations whereas the converse was observed for *S. epidermidis* and *E. faecalis*. Although culture media typically used in biofilm protocols support biofilm formation, media which more closely mimics *in vivo* conditions should improve the utility of *in vitro* biofilm protocols.

References

1. Ceri, H., Olson, M. E., Stremick, C., Read, R. R., Morck, D and Buret, A. The Calgary Biofilm Device: New Technology for Rapid Determination of Antibiotic Susceptibilities of Bacterial Biofilms. *J Clin Microbiol.* 1999 Jun; 37(6): 1771–1776. PMID: PMC84946
2. 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.