

# Comparison of the reflectance and Crystal Violet assays for measurement of biofilm formation by *Enterococcus*

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## ABSTRACT

In previous work, we described a protocol for measuring biofilm formation on opaque and non-opaque surfaces using reflected light. In the present work, we used the reflectance assay to assess biofilm formation for 14 strains of *Enterococcus* and compared our results with those obtained using a Crystal Violet (CV) assay. In general, the results for the two assays were in agreement: both identified the same *Enterococcus* strain as the highest biofilm-forming strain and the same two strains as the lowest biofilm-forming strains. However, two *Enterococcus* strains identified as high biofilm formers by the CV assay were not identified as such by the reflectance assay. A single numerical value was obtained for each of the reflectance assay results that corresponded well with the confluence of a biofilm (and presumably its depth) using phase contrast microscopy.

## INTRODUCTION

Bacteria exist in the environment as planktonic cells or as sedentary communities within a biofilm matrix. Bacteria within biofilms are better able to survive stressful environmental conditions (O'Toole *et al.*, 2000). Furthermore, many natural and synthetic surfaces are capable of harboring biofilms of at least one bacterial species (Chmielewski & Frank, 2003, and references therein). Although the mechanisms for biofilm production are phylogenetically conserved, specific mechanisms may exist for habitation on selective surfaces (Costerton *et al.*, 1995; Davey & O'Toole, 2000; O'Toole *et al.*, 2000; Shirtliff *et al.*, 2002; Schachter, 2003). Consequently, it is desirable to have a common screening method capable of accommodating multiple substrata. Such a method would allow evaluation of intra- and interspecies variation relative to substratum type and growth conditions.

The benchmark assay for measuring biofilm formation involves quantification of dye (Crystal Violet) bound to cells within a biofilm. The Crystal Violet (CV) assay described by Djordjevic *et al.* (2002) for quantification of *Listeria monocytogenes* biofilm formation can be used to detect biofilm formation on many types of substrata, but quantification on surfaces other than polystyrene requires transferring liberated dye (following cell lysis) to polystyrene plates. A previous paper details the protocol

for a new assay that uses reflected light to measure biofilm formation (Broschat *et al.*, 2005). The reflectance assay was developed as an alternative assay capable of rapid screening for biofilm growth on both opaque and non-opaque surfaces. It is an inexpensive and non-destructive assay and measurements are made directly from the substratum of interest. In addition, the reflectance assay requires the use of less rinsing than does the CV assay and, because variation between measurements is smaller than for the CV assay, fewer replicates are needed. Here, we present a comparison of measurements made of biofilm formation using the reflectance assay and a CV assay for 14 different *Enterococcus* isolates.

## MATERIALS AND METHODS

In a previous study, 44 different strains of *Enterococcus* were obtained from the Washington State University Field Disease Investigation Unit (Pullman, WA, USA) and screened for biofilm development using a CV assay based on the method described by Djordjevic *et al.* (2002). Of these strains, the seven identified as the highest biofilm formers (largest optical density values) and the seven identified as the lowest biofilm formers (smallest optical density values) were chosen for the current study. To eliminate the transfer step required for polyvinylchloride

(PVC) plates and thereby to minimize the differences between the CV and reflectance assays, polystyrene plates were used for both assays. The CV assay used for the present study was based on that of Tendolkar *et al.* (2004) for *Enterococcus* species. For each of the 14 strains, glycerol stocks were streaked for isolation on tryptic soy agar (TSA) plates (Difco, Becton Dickinson, Sparks, MD, USA) and a single colony was subsequently transferred to 3 ml of sterile tryptic soy broth (TSB) + 0.125% glucose in a glass culture tube and incubated at 37 °C with agitation for 18–24 h. Each culture was mixed by light vortexing, and a 1:40 dilution in fresh TSB + 0.125% glucose was prepared. This procedure was used to prepare samples for both assays.

For the CV assay, aliquots (200 µl) of a single cell dilution were dispensed into each of eight wells of a sterile 96-well polystyrene plate (Costar, Corning, NY, USA). Each plate also included a set of eight wells filled only with 200 µl of TSB + 0.125% glucose to serve as a control. The plates were incubated at 37 °C for 24 h without agitation. After 24 h the medium was removed and the wells washed three times in 1 × phosphate-buffered saline (PBS). The plates were then inverted and blotted on paper towels and allowed to air dry for 15 min. Aliquots (200 µl) of a 0.1% CV solution (1:10 dilution of 1% CV in 1 × PBS) were added to each well and allowed to incubate at room temperature for 15 min (0.2% was used by Tendolkar *et al.*, but 0.1% was found to be sufficient, giving clean results (Borucki *et al.*, 2003)). After incubation, the CV solution was removed and the wells washed three times with 1 × PBS to remove unbound dye. Ethanol (95%; 200 µl) was added to each well and gently swirled to release the dye from the cells. To minimize evaporation, the plates were quickly measured colorimetrically with a Spectramax Plus 384 (Molecular Devices, Sunnyvale, CA, USA). Optical density wavelength measurements of 595 nm were obtained and stored using Softmax Pro 4.0 (Molecular Devices). The data were transferred to the Microsoft-Excel program (Microsoft Corporation, Redmond, WA, USA), and the values of the eight control wells were averaged. The control-well average was used to normalize the average of each set of replicate wells for each strain of *Enterococcus*, and the resulting value was subtracted from unity. All values were thus in a range from 0 to 1, with 0 indicating the negative control (that is, the absence of a biofilm) and larger values indicating greater biofilm formation. The assay was repeated on three separate days and the replicate values were averaged to obtain a final value and standard deviation for each strain.

For the reflectance assay, aliquots (80 µl) of cell dilution were dispensed into each of four wells of a sterile 96-well polystyrene lid (Costar, Corning, NY, USA). In addition, a set of four wells with 80 µl TSB + 0.125% glucose was included to serve as a control. The control set was also used for calibration during the reflectance measurements. The lid was incubated at 37 °C without agitation for 24 h. This was followed by successive 1 min washes using submersion into 50% and 95% ethanol to dislodge unattached cells and to fix the attached cells. Fixation is not necessary to obtain reflectance measurements, but it was used to dehydrate the cells for imaging and long-term storage. Reflectance

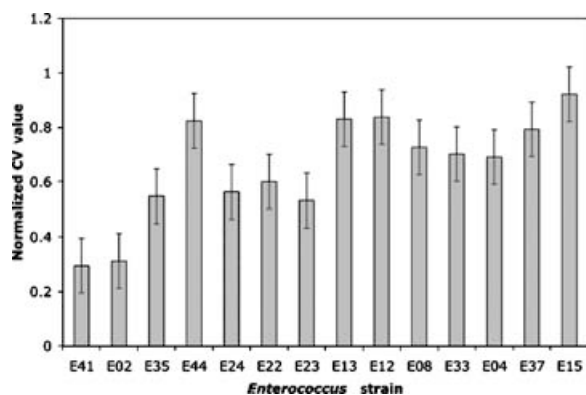
measurements were then performed. This process was repeated two more times on separate days to yield a total of three sets of replicates. For one isolate, E44, the third replicate was discarded due to culture contamination.

The protocol for the reflectance assay is detailed by Broschat *et al.* (2005). Briefly, the reflectance data were obtained using an Ocean Optics USB 2000 spectrometer with a polychromatic light source (Ocean Optics, Dunedin, FL, USA). A fiber optic cable was used both for illumination and collection, and Ocean Optics OOIBase32 Platinum software was used for preliminary processing of the data. Reflectance measurements were taken for each set of replicates. The results for one replicate, i.e. for four wells of a bacterial strain or control, were averaged to yield one reflectance value for each of 601 wavelengths between 200 and 800 nm. The control values were used for normalization of the data, including the control values themselves. This produced a constant reflectance value of 1 for the control data and wavelength-dependent reflectance values of less than 1 for each isolate. Finally, the three replicate sets of normalized data were averaged to obtain one data file of the average reflectance value as a function of wavelength for each isolate.

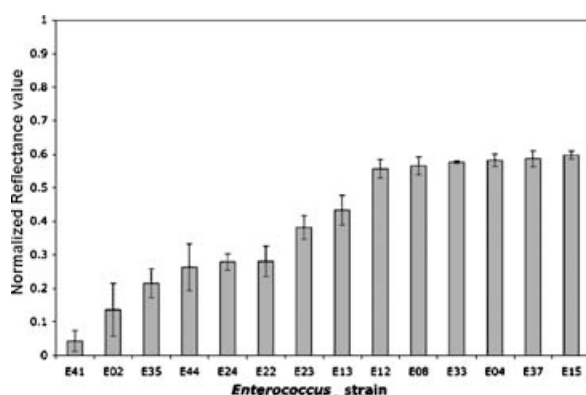
Reflectance can be plotted as a function of wavelength as described by Broschat *et al.* (2005) and the resulting profiles analyzed to determine biofilm formation. For comparison with the CV assay results, however, it is more convenient to obtain a single value that indicates whether a biofilm has formed. This can be accomplished by averaging reflectance values over a range of wavelengths. For the present study with a polystyrene substratum and *Enterococcus* isolates, a single value was obtained by averaging all the normalized reflectance values for wavelengths between 250 and 800 nm inclusive, with the exception of the value at 655 nm. This value was excluded because of a characteristic spike that occurs at 655 nm – an artifact of the tungsten light source in the instrument. Between 200 and 250 nm, time series reflectance measurements provide information on attachment but not on the volume of a biofilm (Broschat *et al.*, 2005). Values below 250 nm were therefore excluded from the average. After being averaged over wavelengths, the resulting reflectance value for each strain was subtracted from 1 such that the larger the value, the greater the formation of biofilm in terms of thickness and coverage.

## RESULTS

Figures 1 and 2 show the normalized results of the CV and reflectance assays, respectively, for the 14 different *Enterococcus* isolates under study. The reflectance results are given in order of lowest to highest biofilm-forming isolates and, to facilitate comparison, the CV results are given in the same order as the reflectance results. It should be noted that the normalized results for the two assays cannot be compared directly because the values for each are relative values for the particular assay. With the reflectance assay, four wells were used for each strain,



**Fig. 1:** Normalized results for the CV assay for 14 different strains of *Enterococcus*. Each bar represents an average of three replicated experiments, and larger values correspond to greater biofilm formation. Error bars give the standard error of the mean for three replicate experiments.



**Fig. 2:** Normalized results for the reflectance assay for 14 different strains of *Enterococcus*. Each bar represents an average of three replicated experiments, and larger values correspond to greater biofilm formation. Error bars give the standard error of the mean for three replicates.

whereas eight wells were used with the CV assay. From previous work it was known that the reflectance assay requires a smaller number of wells for each replicate because there is less variability in the measurements. This is demonstrated by the percentage coefficients of variation shown in Table 1. The average coefficient for the reflectance assay (%CV = 9.7) was significantly lower than

the average for the CV assay (%CV = 26.4) ( $P = 0.007$ ; Mann–Whitney U-test).

Both the CV and reflectance assays identified the two lowest biofilm-forming isolates as E41 and E02, and both are in agreement that the highest biofilm-forming isolate is E15. However, the CV assay indicates that isolates E44 and E13 are high biofilm formers, while the reflectance assay does not. The reason for this difference is not clear. The reflectance assay also indicates more extensive biofilm formation than does the CV assay for several of the strains. Two possible reasons for this are, first, that the difference is attributable to the different rinsing methods and, secondly, that the CV assay measures the volume of bacterial cells in a biofilm but not the volume of exopolysaccharide whereas the reflectance assay measures both (Broschat *et al.*, 2005). It is possible that some isolates created more exopolysaccharide than others and this exopolysaccharide was measured by the reflectance assay, but, because the CV assay quantifies only the amount of dye bound to the bacteria, the extra exopolysaccharide was not detected.

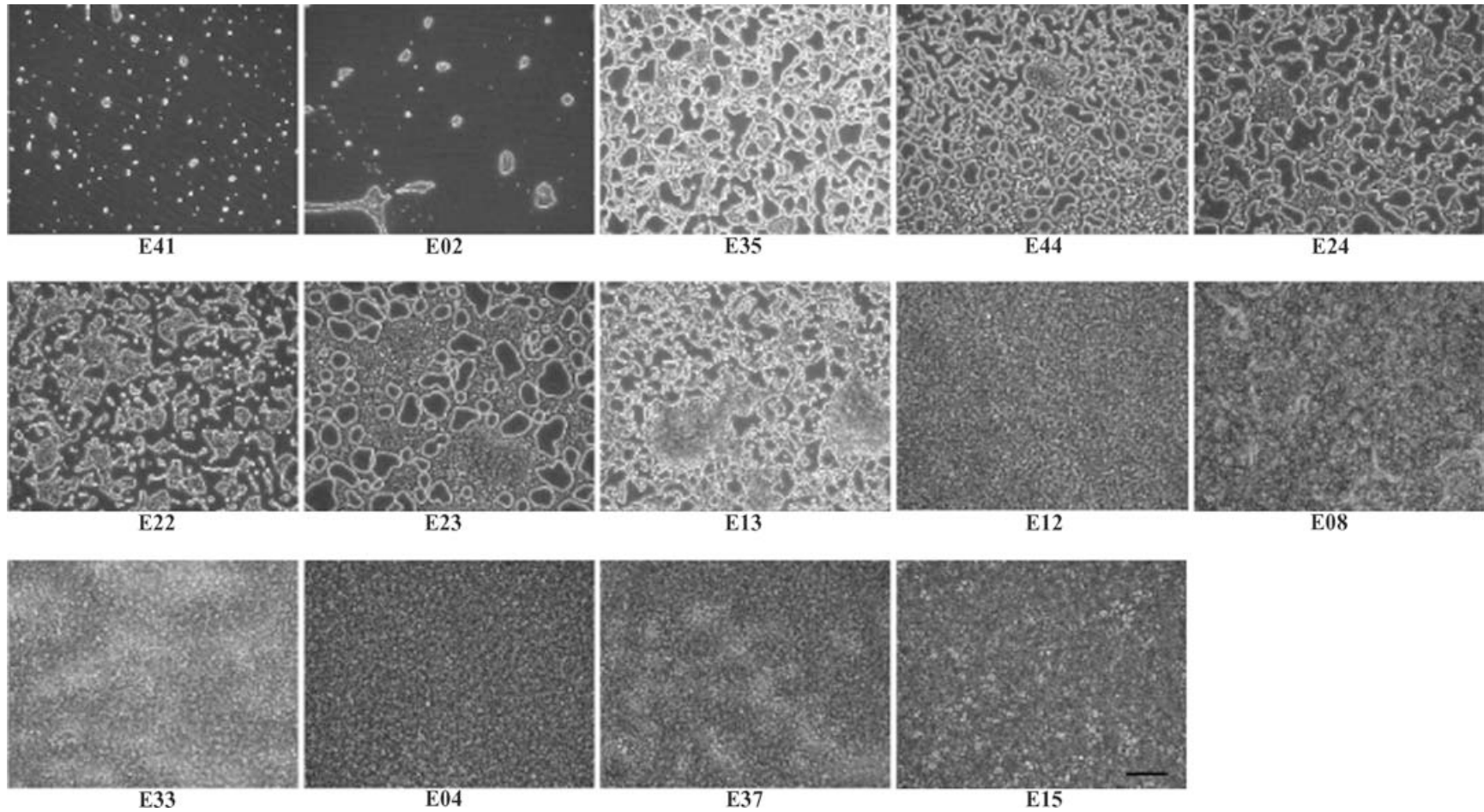
All 164 wells that had been spotted with the 14 different isolates were imaged with a phase contrast microscope. A description of biofilm growth in each well was noted and, where possible, an image was captured as a typical example for each isolate. These images are shown in Fig. 3 and are arranged in the same order as the reflectance results shown in the bar graph of Fig. 2. The images correspond well to the reflectance values in terms of the apparent depth and confluence of biofilm coverage as assessed qualitatively. Images of isolates E41 and E02 show sparse cells and small amounts of biofilm consistent with their identification as low biofilm formers. Images of isolates E12, E08, E33, E04, E37 and E15 show solid matrices of biofilm growth that completely covered each well, consistent with their identification as strong biofilm formers. Images of isolates E35, E44, E24, E22, E23 and E13 show intermediate biofilm formation. The dark patches visible in the images are the polystyrene substratum.

On the basis of the phase contrast images, we assigned the following thresholds for interpreting average normalized reflectance values. For the 14 strains considered, final values greater than 0.5 indicate a mature biofilm that completely covers each well. Values less than 0.15 indicate virtually no significant biofilm formation, while values greater than 0.15 and less than 0.5 indicate

**Table 1:** Percentage coefficients of variation for individual replicates. The CV results are for eight wells per strain per replicate while the reflectance (RR) results are for four wells per strain per replicate. In the third reflectance assay replicate, strain E44 was discarded because of culture contamination

| Assay | Enterococcus strain |      |      |      |      |      |      |      |      |      |      |      |      |      |
|-------|---------------------|------|------|------|------|------|------|------|------|------|------|------|------|------|
|       | E02                 | E04  | E08  | E12  | E13  | E15  | E22  | E23  | E24  | E33  | E35  | E37  | E41  | E44  |
| CV 1  | 14.6                | 23.4 | 17.3 | 49.4 | 34.6 | 52.9 | 10.0 | 1.5  | 24.5 | 6.3  | 14.7 | 7.0  | 15.1 | 39.3 |
| CV 2  | 20.0                | 63.1 | 47.7 | 34.6 | 44.1 | 30.1 | 54.0 | 13.1 | 14.9 | 42.5 | 14.2 | 18.2 | 14.6 | 24.6 |
| CV 3  | 1.2                 | 21.5 | 32.6 | 28.4 | 29.8 | 22.3 | 35.8 | 48.1 | 37.4 | 6.1  | 13.4 | 16.6 | 14.0 | 57.2 |
| RR 1  | 28.2                | 18.8 | 5.8  | 14.9 | 8.0  | 20.3 | 8.9  | 23.3 | 19.5 | 7.4  | 4.6  | 11.2 | 9.0  | 14.7 |
| RR 2  | 3.0                 | 11.5 | 7.8  | 16.6 | 5.3  | 4.3  | 6.7  | 23.8 | 15.7 | 2.8  | 10.2 | 5.5  | 2.7  | 5.6  |
| RR 3  | 3.0                 | 6.8  | 18.3 | 1.1  | 11.1 | 4.4  | 7.8  | 11.0 | 4.3  | 2.9  | 2.7  | 6.1  | 1.0  | n.d. |

n.d., not determined.



**Fig. 3:** Phase contrast images of the 14 *Enterococcus* strains used in this study shown in the same order (left to right, top to bottom) as the reflectance results in Fig. 1 (left to right). The scale bar on the E15 image represents 50  $\mu\text{m}$ , and all other images have the same scale.

either biofilm growth of increasing degree or inconsistent biofilm growth among the wells. While these threshold values have been demonstrated only for *Enterococcus* on a polystyrene substratum, it is believed they will be the same for other bacterial species and other substrata. However, this remains to be shown, although as discussed in the next section, partial data support this hypothesis.

## DISCUSSION

A new reflectance technique has been developed for the detection of biofilm formation as described in detail by Broschat *et al.* (2005). The method was developed to provide a rapid means of screening for biofilm on multiple types of substrata, both opaque and non-opaque. An additional feature of the reflectance assay is that specimens can be fixed and stored for later examination. Phase contrast images have been captured 6 months after initial fixation, with no evident degradation.

In this study, 14 *Enterococcus* isolates were examined for biofilm formation using both the standard CV assay and the reflectance assay. Both assays identified the same two biofilm negative isolates, and both identified the same isolate as the strongest biofilm positive strain. There were some differences between the two assays in identification of biofilm positive strains. Representative phase contrast microscopy images of the 14 isolates were observed that corresponded well with the reflectance assay results. A single value was obtained using the reflectance assay that distinguished between low biofilm-forming and high biofilm-forming strains.

This study was limited to *Enterococcus* on a polystyrene substratum. The paper by Broschat *et al.* (2005) used both polystyrene and glass substrata. The reflectance method has also been used successfully to measure biofilm formation for *Enterococcus*, *Listeria* and *Salmonella* on several different substrata, including stainless steel, porcelain and PVC, as well as glass and polystyrene. Complete reflectance data are not available for comparison with the CV assay, but partial data, i.e. two or four wells for one replicate rather than four wells each for three replicates, indicate that single values are applicable for these as well. For example, *L. monocytogenes* strain 39503A has been identified as a high biofilm-forming strain using the CV assay (Borucki *et al.*, 2003). The value obtained using the reflectance method for *L. monocytogenes* 39503A on stainless steel using four wells and one replicate for 40 h of incubation is 0.684. For this same strain on glass using two wells and one replicate for 48 h of incubation, the reflectance value is 0.543. Both these values are greater than the threshold value of 0.5 obtained from the *Enterococcus* data. In addition, for *Enterococcus faecalis* strain E15 on porcelain using four wells and one replicate for 24 h of incubation the reflectance value is 0.747, again greater than the threshold value.

There are several advantages to using the reflectance assay for detecting biofilm formation. The reflectance assay has significantly lower variance, making it possible

to generate a more precise estimate of biofilm formation using fewer intra-assay replicates. It is also amenable to multiple substrata. The reflectance assay requires some preliminary measurements of a particular substratum to understand its inherent reflectance properties, but this needs to be done only once for a given substratum. Afterwards any experiments using this substratum can be performed without additional calibration of the substratum itself. Finally, the reflectance assay does not require multiple washings, destruction of a specimen, or the use of chemical dyes. Experiments have been restricted to static growth, but there is no obvious reason why the reflectance assay cannot be used to measure biofilm grown under other conditions.

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