J Ind Microbiol Biotechnol (2008) 35:1475–1480
DOI 10.1007/s10295-008-0449-z

ORIGINAL PAPER

Colorimetric assay for biofilms in wet processing conditions

Judy W. Arnold

Received: 25 January 2008 / Accepted: 30 July 2008 / Published online: 20 August 2008

Abstract Controlling bacterial biofilms is necessary for food safety and industrial processing in clean room environments. Our goal was to develop a method to quantitatively measure biofilm produced by pathogens under wet poultry production and processing conditions. Stainless steel and glass coupons were incubated in aqueous media containing reduced nutrients and exposed to *Listeria monocytogenes* under static temperature and humidity conditions. Samples were measured separately by biofilm assay and viable cell density, and then confirmed by spectrophotometry and microscopy. The biofilm assay resulted in different *t* groupings from the cell density. The mean from the biofilm assay was 0.50, and the error% was 0.595. The mean of the $\log_{10}$ density (cfu/cm^2) was 5.90, and the standard deviation ranged from 0.127 to 0.438 on 24 coupons. The typical sequence of biofilm development, followed by microscopy of biofilm grown on glass coupons, exhibited a change from dispersed single cells to an all-over pattern of clumps with few dispersed cells. *L. monocytogenes* formed biofilms on all of the substrata tested. Bacterial counts from planktonic cultures at 24, 48, 72, and 144 h confirmed that *L. monocytogenes* remained viable throughout the experiment and reached equilibrium between 6 and 24 h. The cell density $\log_{10}$/ml was 8.01, 8.03, 7.69, and 6.66, respectively; and the standard deviation ranged from 0.156 to 0.394. The data will be used to grow stable biofilms of *Listeria* spp. collected from the food processing environment for further study. This is the first use of the crystal violet assay for measurement of bacterial biofilms on stainless steel under these conditions. The methods tested are applicable to other bacteria and substrata.

J. W. Arnold (✉)
USDA-ARS, Russell Research Center,
950 College Station Road, Athens, GA 30605, USA
e-mail: Judy.Arnold@ars.usda.gov

Keywords Biofilm · Crystal violet · *Listeria monocytogenes* · Stainless steel

Introduction

Bacteria can survive in almost any environment and thrive in a wide variety of temperatures, especially in wet environments, such as food processing and clean room facilities. Control requires multiple intervention strategies and constant monitoring to ensure that the strategies are working. Bacterial adenosine triphosphate (ATP) bioluminescence and conventional microbiological plating methods are most often used to monitor cleaning effectiveness during plant operations [21, 40]. Monitoring methods that are effective in the laboratory can be impractical for the processing environment, including electrical methods such as impedance/conductance [26], chemical methods such as direct epifluorescent filter technique [31], flow cytometry [33], biosensors [18], agglutination or immunological assays [37], and nucleic acid technologies such as polymerase chain reaction [22], ribotyping and microarrays [10]. The difficulty lies in the detection of bacterial cells buried in food and detritus that accumulate in niches near conveyors and other turnaround points in processing areas [2].

Understanding the conditions conducive to bacterial attachment and biofilm formation will provide important information for successful development of sanitation plans that can be monitored. The phenomenon of bacterial attachment to metal, rubber, and plastic surfaces presents a formidable obstacle for surface sanitizing and cleaning treatments [1, 42, 45]. When bacterial cells initially attach to a surface, they produce extracellular fibrils that form a complex matrix conducive to growth and subsequent attachment of more bacteria, other microbes and debris

[5, 16, 24, 38]. The ultimate composite is a biofilm that is resistant to cleaners and sanitizers and is extremely difficult to remove [29, 31, 46]. If microorganisms from food-contact surfaces are not completely removed, they can lead to biofilm formation and also increase the potential for cross contamination [25].

Because of the severity of the illness, *L. monocytogenes* has been a major concern for regulation and research [35]. The food-borne pathogen has a 20–25% mortality rate among its victims and causes many food product recalls [19]. In a study of 41 meat plants, *L. monocytogenes* was found living on 39% of the floors, 29% of the floor drains, 34% of the cleaning equipment, 24% of the wash areas, and 20% of the food-contact surfaces [7]. Each of these sites is a haven for biofilm growth. The growth kinetics of *Listeria* has been studied to determine the optimal medium and temperature for growth [30], surface physical and chemical properties [15], in meat products [27] and in models to predict the biofilm formation process [41].

Polymicrobial biofilms have been grown on coupons made of stainless steel and other materials found in food environments [12, 36, 46]. In our previous studies, imaging techniques from scanning electron microscopy, electron probe microanalysis, and atomic force microscopy, were combined to evaluate bacterial contamination and polymicrobial biofilm formation on stainless steel and other equipment component materials [3, 4].

The development of biofilms has also been followed after inoculation of the bacteria into growth media on microtiter plates that allow rapid and repeatable data collection [14, 34, 43]. Biofilm formation has been indirectly assessed by staining the plastic plates with crystal violet, extracting the dye, and measuring its absorbance by spectrophotometry [13, 32, 39]. However, Djordjevic et al. showed that rates of cell growth in 96-well microtiter plates and final cell densities did not correlate with biofilm formation, indicating that differences in biofilm formation were not due to growth rate differences [17]. The microtiter plates were made of polyvinyl chloride, but most of the surfaces in food processing facilities are made of stainless steel [1]. The biofilm data corresponded more closely to biofilms grown on PVC than to those on stainless steel. Epifluorescence microscopy has been used to directly observe biofilm on stainless steel, although the method may overestimate the area covered by the cells [8]. It is also relatively time-consuming, compared to the PVC microtiter plate assay.

The objective of the present study was to develop a method to measure biofilms under conditions that mimic static niches in processing environments that could accommodate biofilms. This research presents a method that provides quantitative data for analysis of *L. monocytogenes* growth and biofilm production, concomitant measurement of viable cell counts, confirmation by microscopy, and planktonic growth comparison. The methods could be easily used by any laboratory or field facility and applied to other pathogens and surface materials.

Materials and methods

Biofilm preparation

Cultures of the bacterium *Listeria monocytogenes* ATCC #19114 were stored frozen at −70 °C in brain heart infusion broth (BHI), with 10% glycerol. To reconstitute a frozen culture, 200 µl of thawed culture was inoculated into 9 ml of BHI. Reconstituted cultures were incubated at 37 °C for 24 h. One ml of the overnight culture was inoculated into a test tube containing nine ml tryptic soy broth, diluted 1:10 (TSB.1) for each sample coupon to be processed. Sterile stainless steel (304 ANSI, 2B) coupons (0.5 in. d) were aseptically inserted into each of the tubes and incubated at 37 °C for 24, 48, 72, or 144 h. An aerobic plate count was performed to determine the concentration of the initial inoculum, prior to dilution, 8.64 $\log_{10}$ density cfu ml^{-1}.

Biofilm extraction and measurement

The biofilm growth on a portion of the stainless steel coupons was collected at the completion of each time period. Each coupon was rinsed by dipping three times in phosphate buffered saline to remove loose cells, then swabbed three times, and rinsed with a total volume of one ml tryptic soy broth (TSB) into 9 ml at room temperature. The TSB with the biofilm (10 ml) was homogenized with a sterile CAT homogenizer (CAT Scientific, Van Nuys, CA). An aerobic plate count was performed on the homogenate from each coupon to determine the concentration of viable bacteria after each 24-h period. An aliquot of the cultures and any suspect colonies were plated on CHROMagar *Listeria* agar (Becton and Dickinson Co., Sparks, MD) to confirm the continued presence and purity of *Listeria* [23].

An equal portion of the coupons were individually stained with 1% (w:v) crystal violet dye. Controls without bacteria were processed first. Coupons were removed from TSB.1 and washed with water for three times. The coupons were inserted into a tube with 1% (w:v) aqueous crystal violet and allowed to stand 5 min at 23 °C. Coupons were washed with water for three times and placed in a sterile Petri dish. Each coupon was swabbed three times and rinsed with a total volume of 200 µl of 95% ethanol. The ethanol mixtures were loaded into separate wells in a 96-well plate and measured by spectrophotometry (Absorbance = 590 nm) (Bio-Tek Powerwave XS, Bio-Tek Instruments, Winooski,

VT). All samples were duplicated, and the experiments were replicated.

Two glass coupons were processed for each time point. The glass coupons were added in the same batch (processed concomitantly) with the stainless steel coupons as a simple method to visually confirm and monitor biofilm formation during the experiments. When the glass coupons were removed from the incubation tubes, each coupon was rinsed three times, the topmost side of the coupons from the cultures was stained with the Gram stain, and the bottom was cleaned to remove any residual dye or detritus. The stained specimens were observed by brightfield light microscopy (Zeiss Axioskop, Carl Zeiss MicroImaging, Inc. Thornwood, NY). The cells stained purple, and the surrounding matrix stained pink.

L. monocytogenes planktonic growth

Reconstituted cultures were incubated at 37 °C for 24 h. One ml of the overnight culture was inoculated into test tubes containing nine ml TSB.1. Then two ml each of the culture was dispensed as needed into disposable polystyrene cuvettes (10 × 10 × 45 mm), and covered tightly with Parafilm. Cuvettes were placed into the spectrophotometer (Beckman DU640 equipped with Peltier temperature controller and six-position auto cell holder, Beckman Instruments, Fullerton, CA). Optical density (absorbance) was measured at 410 nm for 144 h. At each 24-h interval, two cuvettes were removed from the holder, and 0.1 ml was withdrawn from each, and plated separately for aerobic plate counts.

Statistics

The data obtained was analyzed using analysis of variance (PROC ANOVA, SAS software, Statistical Analysis System Institute, Cary, NC). Significant differences among means were determined by the least significant difference test (LSD) ($P = 0.05$) for analysis of *L. monocytogenes* biofilm formation up to 144 h. The LSD and Duncan's Multiple Range Test control comparisonwise error rate. The means were also compared by the Student–Newman–Keuls Test, Tukey's Studentized Range (HSD) Test, and Scheffe's Test ($P = 0.05$). These tests control Type I experimentwise error rate. All samples were duplicated, and all experiments were replicated.

Results

L. monocytogenes grew well and formed biofilms with the media and substrata tested. Culture material was collected and measured separately for each coupon by aerobic plate counts of viable bacterial cells and for biofilm production by the crystal violet assay. Table 1 shows that, during incubation from 24 to 144 h, the number of viable cells was greater for each succeeding 24 h, but did not increase significantly ($n = 24$, LSD = 0.3544). Comparison of means by Duncan's Multiple Range Test, the Student–Newman–Keuls Test, Tukey's Studentized Range (HSD) Test, and Scheffe's Test also showed no differences among the time points. The standard deviation of the log density (cfu/cm^2) ranged from 0.127 to 0.438 based on 24 coupons for the experiment. Goeres et al. cited a 0.59 standard deviation based on one coupon for an experiment with flow conditions [20]. The overall mean of the density was 5.90, and the error % (SD/mean) for the experiment was 0.048.

The biofilm assay that used crystal violet staining to measure biofilm mass resulted in different *t* groupings than the cell density derived from aerobic plate counts (Table 1). There was not a significant increase in biofilm mass from 24 to 48 h, from 48 to 72 h, or from 72 to 144 h. However, there was a significant difference between 24 and 72 h, and the biofilm mass of the 144-h sample was less than the 72-h sample ($n = 24$, LSD = 0.336). However, comparison of means by Duncan's Multiple Range Test, the Student–Newman–Keuls Test, Tukey's Studentized Range (HSD) Test, and Scheffe's Test showed no differences among the time points for biofilm mass ($P > 0.05$). The variability for the assay was greater than desirable. The overall mean was 0.50, and the error% was 0.595, with the std dev ranging from 0.156 to 0.394.

Figure 1 shows the data generated by measurement of optical density when *L. monocytogenes* was incubated in cuvettes in the spectrophotometer for 144 h. Data were collected from the spectrophotometer every 15 min. Log phase occurred from 0 to 6 h, and equilibrium occurred from 6 to 28 h. The aerobic plate counts taken from cuvettes at 24, 48, 72, and 144 h confirmed that *L. monocytogenes* remained

Table 1 Comparison of the modified crystal violet assay with viable cell density

Hours	Mean (SD)			
	Net absorbance[a]		Cell density[b]	
24	0.359 (0.225)	B	5.820 (0.251)	A
48	0.388 (0.285)	BA	5.878 (0.127)	A
72	0.715 (0.394)	A	5.922 (0.278)	A
144	0.538 (0.156)	BA	5.970 (0.438)	A

[a] Measured by spectrophotometry after staining biomass with crystal violet, absorbance (590 nm) of a control sample with no bacteria was subtracted from the bacterial sample; means with same letter are not different; $n = 24$

[b] Density ($\log_{10}$ cfu/cm^2) of viable sessile cells on stainless steel coupons as determined by aerobic plate counts; means with same letter are not different; $n = 24$

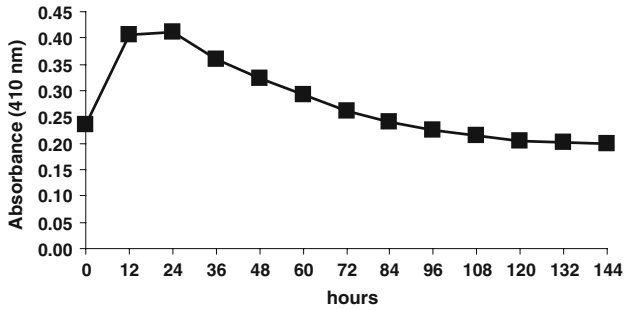

Fig. 1 *L. monocytogenes* growth of planktonic cells in TSB.1 measured by spectrophotometry at 37 °C. Data were collected every 15 min. Log or exponential phase occurred from 0 to 6 h, and stationary phase (equilibrium) occurred from 6 to 28 h, followed by the decline phase

viable throughout the time course of the experiment. The viable cell density $\log_{10}$/ml was 8.01, 8.03, 7.69, and 6.66, respectively.

Examination of the stained biofilm on glass coupons was consistent with the above results. Figure 2 shows biofilm formation at 2, 6, 24, and 48 h. The sequence of biofilm development followed a change from increasing numbers of single cells dispersed over the entire coupon surface before 24 h, to increasing numbers of clumps of cells with fewer surrounding single cells from 24 to 48 h, to an overall pattern of clumps, surrounded by clear areas with few to no dispersed single cells. Consistent with the equilibrium shown by spectrophotometry and the aerobic count data, the numbers of cells did not appear to increase appreciably after 24 h, but greater numbers of cells were found within clumps of biofilm. Spaces remaining following detachment of clumps were observed at 24 h and after.

Discussion

The purpose of this research was to develop a method to monitor and quantify bacterial biofilm formation on stainless steel under static conditions similar to poultry production and processing environments. Methods were needed that mimic wet areas and could be performed by most industrial labs on site. Our model included stainless steel surfaces incubated in aqueous media containing reduced nutrients and exposed to bacteria under temperature and humidity conditions similar to food processing facilities. Stainless steel is the most frequently used material for construction of vessels, piping, valves, and various types of equipment used in food processing and biotechnology industries [3].

TSB was selected as the diluent because it supports the growth of *L. monocytogenes* and most bacterial species previously isolated and grown in polymicrobial biofilms [11]. Therefore, it could be used for future experiments to combine *L. monocytogenes* in biofilms with other bacteria. To enhance biofilm production by reducing the amount of nutrients in the media [1], TSB was diluted 1:10. *L. monocytogenes* grew well and formed biofilms during the entire course of the experiments.

Comparison studies by others [28, 44] have suggested other recovery methods. However, in our hands, the number of bacterial cells recovered by swabbing was not significantly less than scraping or sonication (data not shown). The best recovery method is not always the same, but varies with the applied skills of the technician, the tenacity of the organisms within the biofilm, and the conditions of sample collection and processing.

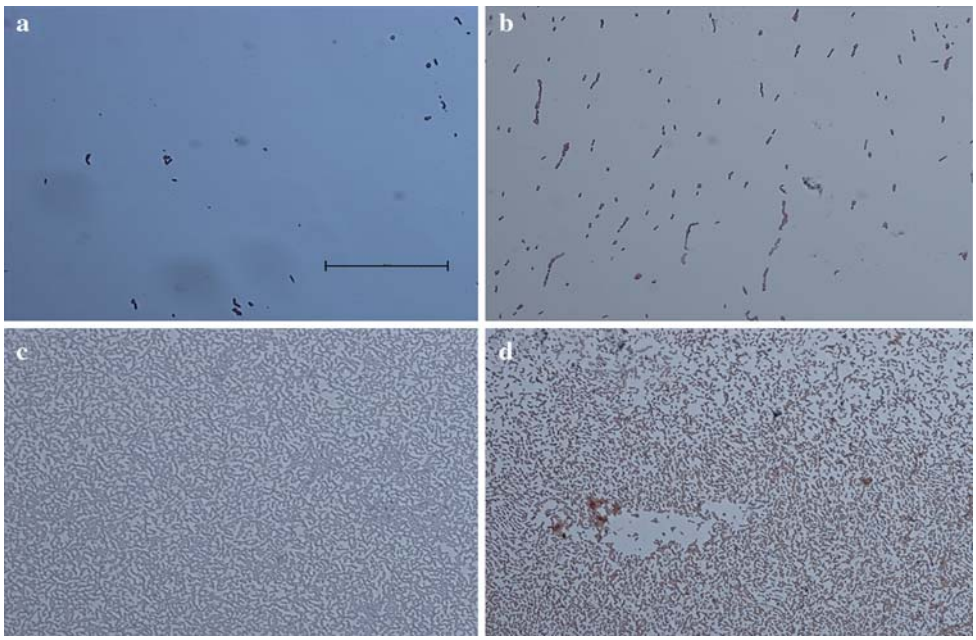

Fig. 2 Biofilm formation by *L. monocytogenes*, grown in TSB.1 at 37 °C on borosilicate glass for 2 h (**a**), 6 h (**b**), 24 h (**c**), and 48 h (**d**). Samples were stained with the Gram stain. Bar = 50 µm. (Zeiss Axioskop)

The crystal-violet based assay was used to quantitatively assess biofilm mass. Criteria for an effective laboratory test include high specificity, low variability, high repeatability, short duration, and low cost. To compare biofilm data and achieve high specificity, the test must target the cells and matrix of a biofilm. Most current tests are based simply on aerobic plate counts, and the methods underestimate the numbers of cells and the amount of biofilm [9]. For low variability, the biofilm data must be statistically comparable from experiment to experiment. Finally, the ultimate criterium is economy.

Use of the assay to measure biofilm production had a limitation. For some tests, the variability of the data was high (Table 1), because of the nonspecific binding of the crystal violet stain [6]. Variability in the coupon surfaces at the microscopic level could have contributed to this. Increasing the number of samples will help reduce the variability in the future. On the positive side, the cost was not prohibitive, and the assay provided a quick and easy way to measure biofilm mass.

An interesting comparison can be made among the results for the spectrophotometric assay for planktonic cell growth, viable cell density by aerobic plate counts, biofilm assay, and microscopic observations. Trials for each method were run for 144 h, gave positive results, and each indicated that equilibrium was reached by 24 h. The planktonic cells reached equilibrium from approximately 6–28 h, followed by decline (Fig. 1). The cell density on the coupons remained level after 24 h, and the biofilm mass was not reduced after 24 h, as shown in Table 1. Visual observation by microscopy showed that clumps of biofilm were forming by 24 h with increasingly fewer dispersed cells thereafter. The growth and detachment of clumps remained about the same thereafter.

The growth curve represents planktonic cells that are suspended in the medium, in contrast to sessile biofilm cells that were measured from coupons in Table 1. The cell density (plate counts) on the coupons remained level after 24 h, and the biofilm mass reached a peak at 72 h, but was not reduced after 24 h (Table 1). Visual observation by microscopy showed that clumps of biofilm were forming by 24 h with increasingly fewer dispersed cells thereafter (Fig. 2). These results indicate that sanitation programs must be constant in their vigilance to reduce contamination and prevent biofilm formation.

The purpose of this study was to model wet areas in a poultry processing environment, and the crystal violet assay was modified and improved to do so. This is the first use of the crystal violet-based assay to measure bacterial biofilms on stainless steel under these conditions. Spectrophotometry and microscopy were used separately to confirm the results. The data provides information about *L. monocytogenes* that will be used to grow stable biofilms of *Listeria* spp. collected from the poultry production and processing environments. The methods could be used with other bacteria and surface materials in other laboratories, but improvements are needed that increase label specificity and thereby reduce variability.

Acknowledgments The technical assistance of Manju Amin and Tommy Tucker is gratefully acknowledged.

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