Revisiting an agar-based plate method: What the static biofilm method can offer for biofilm research

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Abstract

The development of biofilms in static plates was monitored. Glass coupons were placed on agar covered with filter paper, which was inoculated with suspended bacteria. The viable cell density, biofilms matrix and biomass were quantified. The method is excellent for adhesion and material studies, due to its simplicity and flexibility.

The unique properties of bacterial biofilms call for the development of reliable and specific research methods, different to the ones optimized for planktonic bacteria (Cos et al., 2010). Accordingly, various biofilm culturing methods have been developed, in which model biofilms grow under various fluid shear conditions in specific reactors. The choice of the biofilm reactor is based upon the research question under investigation (Buckingham-Meyer et al., 2007; Coenye and Nelis, 2010).

Biofilm formation in liquid cultures has been studied in detail, and it is thought to involve several distinctive phases. At the initial phase, planktonic bacteria are irreversibly attached to the surface. The attachment is followed by aggregation and proliferation of the cells. The production and accumulation of extracellular polymeric substances then result in the formation of a mature biofilm, where the cells are organized into a three-dimensional community surrounded by the biofilm matrix. At the final phase, cells return into a planktonic stage and detach from the biofilm (Dunne, 2002; Kiedrowski and Horswill, 2011; Otto, 2008).

Although bacterial biofilms may also form on surfaces that are not immersed in liquid, the solid-state growth methods are not as common as the well-studied liquid cultures. Among the reported solid-state methods are the colony biofilm model (Anderl et al., 2000) and the static biofilm method (Charaf et al., 1999). In the colony biofilm model, polycarbonate membrane filters are inoculated and regularly transferred onto fresh agar medium, while in the static biofilm method the biofilm is left to develop on a single filter-covered agar plate for the whole incubation period (Fig. 1). The static biofilm method has been shown to be useful in antimicrobial efficacy testing against Pseudomonas aeruginosa PAO1, ATCC 15442 and Staphylococcus aureus ATCC 6538 biofilms (Buckingham-Meyer et al., 2007; Charaf et al., 1999). However, the formation of the biofilms on static method plates has not been thoroughly characterized, nor its potential as a research choice fully explored.

Consequently, in this contribution we set off to gain a deeper understanding of the development of static method biofilms. For this purpose, the kinetics of biofilm development was studied for the Gram-positive S. aureus ATCC 25923, a model biofilm-forming organism. Furthermore, the results were compared with S. aureus ATCC 6538, Staphylococcus epidermidis ATCC 35984, and the Gram-negative Escherichia coli XL-1 Blue and P. aeruginosa ATCC 15442. In all cases, biofilms were grown exactly according to the previously described method (Buckingham-Meyer et al., 2007).

Briefly, sterile borosilicate glass coupons (diameter 1.27 cm, height 0.4 cm, BioSurface Technologies Corporation) were placed on an agar plate covered with an inoculated filter paper (Whatman Qualitative Grade 2, 70-mm diameter, GE Healthcare). The purpose of the filter paper is to provide a barrier between the coupons and the agar surface that allows for diffusion of nutrients while

Abbreviations: CFU, colony forming unit; CV, crystal violet; TSB, tryptone soy broth; WGA, wheat germ agglutinin.
The work with three of the strains consisted of 1.5 mL of a 1:10 dilution of an 18–24 h old preculture in either tryptone soy broth (TSB, Sigma-Aldrich) or in a 100-fold diluted TSB (only for P. aeruginosa ATCC 15442). After 24 h of incubation in a humidified incubator at 37 °C, the filters were remoistened with 1.5 mL of 10-fold or 1000-fold (P. aeruginosa ATCC 15442) diluted TSB (Fig. 1, Buckingham-Meyer et al., 2007).

In this contribution, longer incubation periods for growing the biofilm were adopted (1, 2, 4, 24, 32 or 48 h), and the coupons were analyzed by either viable cell counts or crystal violet (CV) staining of the total biomass. In the case of S. aureus ATCC 25923 the coupons were additionally analyzed by staining with a wheat germ agglutinin (WGA) Alexa Fluor 488 conjugate (Molecular Probes, Thermo Fisher Scientific). At the end of the selected incubation period, each coupon was rinsed by dipping it in TSB, and subsequently analyzed according to a specific protocol. Differences in the protocols were only owed to practical reasons, as the work with three of the strains (S. aureus ATCC 25923, S. epidermidis ATCC 35984 and E. coli XLI-1 Blue) was performed in Finland while the other strains (S. aureus ATCC 6538 and P. aeruginosa ATCC 15442) were studied in the United States.

For viable cell counting, the biofilms (except those formed by S. aureus ATCC 6538 and P. aeruginosa ATCC 15442) were removed and dispersed by a sonication-based method, simpler than the one originally reported (Buckingham-Meyer et al., 2007; Charaf et al., 1999), which was based on scraping biofilm off the surface. Coupons were fully immersed in 1 mL of 0.5% (wt/vol) Tween 20 (Sigma-Aldrich) in TSB, followed by quick vigorous mixing and 5 min of sonication in a water bath sonicator (Sonorex Digitec, Bandelin) at 25 °C, 35 kHz. In the case of S. epidermidis ATCC 35984 the coupons were ad-
voronic acid.

For WGA staining of the S. aureus ATCC 25923 biofilms, a previously described protocol (Skogman et al., 2012) was followed with minor modifications: the coupons were washed three times in a 12-well plate with 3 mL of PBS, and the dye was dissolved in 1 mL of 95% (vol/vol) ethanol (10 min). For WGA staining of the S. aureus ATCC 25923 biofilms, the staining was performed for 15 min in 5 mL of 0.5% (wt/vol) CV (Acros) in Milli-Q water, followed by washing three times in 30 mL of sterile buffered water. The dye was dissolved in 5 mL of 95% (vol/vol) ethanol (10 min).

Table 1

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Log (CFU/cm²)</th>
<th>CV staining (A595)</th>
<th>WGA staining (fluorescence)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Humidified</td>
<td>No humidification</td>
<td>Humidified</td>
</tr>
<tr>
<td>1</td>
<td>5.55 (±0.24)</td>
<td>5.84 (±0.06)</td>
<td>0.14 (±0.00)</td>
</tr>
<tr>
<td>2</td>
<td>5.77 (±0.03)</td>
<td>5.94 (±0.02)</td>
<td>0.14 (±0.01)</td>
</tr>
<tr>
<td>4</td>
<td>7.36 (±0.03)</td>
<td>7.38 (±0.22)</td>
<td>0.20 (±0.04)</td>
</tr>
<tr>
<td>24</td>
<td>8.23 (±0.62)</td>
<td>7.61 (±0.18)</td>
<td>0.58 (±0.30)</td>
</tr>
<tr>
<td>32</td>
<td>8.08 (±0.33)</td>
<td>7.75 (±0.09)</td>
<td>0.85 (±0.09)</td>
</tr>
<tr>
<td>48</td>
<td>8.01 (±0.25)</td>
<td>7.49 (±0.30)</td>
<td>1.31 (±0.54)</td>
</tr>
</tbody>
</table>

N.a. = not analyzed.

The results of the viable counts, CV staining and WGA staining of S. aureus ATCC 25923 coupons incubated in a humidified vs. non-humidified incubator. In the latter case, the volume of the inoculum was increased from 1.5 mL to 2.25 mL. The WGA stain was quantified at λabsorption = 495 nm and λemission = 520 nm. The average value of two biological replicates is presented with the standard deviation in parentheses. Samples obtained in each time point were compared in humidified vs. non-humidified conditions with an unpaired comparison t-test with Welch’s correction (p < 0.05) (GraphPad Prism program, San Diego, USA).
level. The accumulation of the extracellular substances was quantified by staining with the WGA conjugate that binds to the (poly-)N-acetylglucosamine residues (PNAG, Sharon, 2007). The PNAG content was determined by staining with the WGA conjugate that binds to the (poly-)

Fig. 2. The kinetics of biofilm formation for various bacterial species in a humidified incubator. The results from the viable counts are presented as average log values of the CFUs per cm² (red circles and red lines). The accumulation of the total biomass on the coupons is shown as an average absorbance value of the dissolved CV stain measured at 595 nm (blue crosses and blue lines). The dotted lines were only used to connect the actual experimental data points. Due to the differences in the staining protocols, the exact values for P. aeruginosa ATCC 15442 and S. aureus 6538 are not directly comparable with the values obtained for the other strains. The average value and the standard deviation for two biological replicates are presented.

Despite the robustness and the flexibility of the static biofilm method, there are obvious limitations related to its applicability. Since the biofilms are formed under no fluid shear, the method can only provide useful models of biofilms that are involved in, for instance, the infections of the ear and the skin. However, there are also numerous benefits related to the use of the method. It is simple and economical to use, and suitable for any laboratory equipped for basic microbiology work, which makes it a very convenient investigational tool for a wider audience. The method can be directly applied for studies on bacterial adhesion and biofilm formation on various materials. It is very flexible in terms of composition, size and shape of the test coupons, and it is currently used for material testing in our laboratory.

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References


