Growing reproducible biofilms with respect to structure and viable cell counts

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Abstract

We have developed a new method of growing 4-day-old biofilms that are reproducible, with respect to viable cell number and biofilm structure. To demonstrate the utility of the method, we grew biofilms composed of Pseudomonas aeruginosa (ATCC#700829), P. fluorescens (ATCC#700830) and Klebsiella pneumoniae (ATCC#700831), 18 times in flat-plate reactors under well-defined conditions of: flow rate, nutrient concentration, temperature, inoculum and growth rate. The resulting 4-day-old biofilms were approximately 200–300 μm thick and exhibited a high degree of reproducibility. The number of viable cells that accumulated per unit surface area and the biofilm areal porosity were reproduced within 10% error. We have also quantified other parameters characterizing biofilm structure using biofilm-imaging techniques: fractal dimension, textural entropy and diffusion distance as auxiliary parameters characterizing the reproducibility of biofilm accumulation. As a result of analysis, we have introduced a new parameter to better quantify and characterize the number of viable cells in biofilms, “specific number of viable cells” (SNVC). This parameter is the viable cell number normalized with respect to the surface area covered by the biofilm and with respect to the biomass of the biofilm. This new descriptor represents the dynamics of biofilm accumulation better than the traditionally used colony-forming unit (CFU) per surface area covered by the biofilm because it accounts not only for the surface coverage but also for the biofilm thickness. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

The lack of standard procedures in biofilm research negatively affects the quality of information generated by biofilm researchers and makes the comparison of results produced in different laboratories difficult or impossible (Heydorn et al., 2000). As an example illustrating this problem, we cite the results of several studies showing biocide efficacy of different substances against Pseudomonas aeruginosa biofilms. Qian et al. (1997) reported that their biofilms had \(1 \times 10^6\) CFU/cm²; Stickler and Hewett (1991) had \(1 \times 10^9\) CFU/cm²; Ketyi (1995) had CFU < \(10^5\); Benson et al. (1996) did not present any number of cells, instead, they used log reduction of cell numbers when the antimicrobials were used;
Fujiwara et al. (1998) did not show the cell number per unit surface area, but instead, the number of cells in “a well” \(10^3\) CFU/well. Although all of these papers refer to testing antimicrobials against biofilms of the same microorganism, \(P.\ aeruginosa\), it is impossible to compare the data because the studies were done using biofilms grown and characterized by different methods. Identical biofilms are not only composed of the same microorganisms, but also have the same number of viable cells, the same amount of extracellular biopolymers, and the same structure. Results obtained using such biofilms can be directly compared, but growing such biofilms is not trivial.

We often test the activity of antimicrobial agents against biofilms. The first problem to be solved in such tests is to make sure the biofilms, before treatment, are “the same”. This statement is related to the problem of biofilm reproducibility. It is difficult to define biofilm reproducibility in general terms because researchers may be interested in reproducing different features of the biofilms. For our purposes, we defined reproducible biofilms as those that, when grown under similar conditions, exhibited similar dynamics of accumulation expressed in terms of viable cell number per unit surface area, viable cell number per unit surface area to biomass ratio, and temporal development of structural features, such as areal porosity, fractal dimension, textural entropy, and average diffusion distance Yang et al., 2000. These parameters are described further in Section 2.

The goal of this work was to demonstrate that growing reproducible biofilms is possible. We used two parameters to quantify biofilm accumulation. First, the number of viable cells per unit surface area of biofilm (CFU/cm²), and second, the number of viable cells per unit surface area of biofilm was divided by the dry weight of biomass (mg) to determine the CFU/mg biomass/cm², which we termed the “specific number of viable cells” (SNVC). We grew mixed population biofilms consisting of \(P.\ aeruginosa\) (ATCC#700829), \(P.\ fluorescens\) (ATCC#700830) and \(Klebsiella pneumonie\) (ATCC#700831) in flat-plate, open channel reactor, which were designed and fabricated in our laboratory. The reactors were designed to maximize the growth potential of the biofilms on the microscope slides, which were located at the bottom of the reactor. The reproducibility of biofilm processes, to a large extent, depends on the reproducibility of growth conditions (unpublished results). Therefore, we precisely controlled the nutrient delivery rate and hydrodynamics in the reactor. Using the system described here, we could grow in a short, 4-day period of time, a biofilm that was reproducible with respect to our criteria. It appears that for longer growth times, more than 4 days, detachment and attachment become more relevant than they are during the initial phases of biofilm growth. We presume that other researchers must have noticed this as well, since many studies in the literature used young biofilms, e.g. Biedlingmaier et al. (1998), 5 days; Fujiwara et al. (1998), 1 day; Gagnon et al. (1992), 3 days; Richards et al. (1990), overnight.

2. Materials and methods

2.1. Experimental set-up and biofilm reactor

The experimental set-up is presented in Fig. 1(a). To grow biofilms, we used a flat-plate, open channel reactor. The reactor was a polycarbonate channel, 2.5 cm wide, 4.0 cm deep and 34.5 cm long. Fig. 1(b) shows a detailed schematic drawing of the reactor. The reactor had a working volume of 150-ml, including the volume in all of the tubings. The tubing for the growth media and the sterile deionized (DI) water were Masterflex® 6402-14 (Cole Parmer, Chicago, IL), while the recycle, air and waste line were all Masterflex® 6402-16 (Cole Parmer). Peristaltic pumps (Cole Parmer) were used to maintain nutrient flow and recycle rates. The air line had an in-line Bacterial Air Vent filter (Pall–Gelman Laboratory, Ann Arbor, MI, USA) with a pore size of 1 \(\mu\)m.

2.2. Reactor preparation

To simplify biofilm analysis, we grew biofilms on glass microscope slides placed on the bottom of the reactor. These slides were then removed one by one and treated separately, according to the specific protocol of the experiment. To facilitate this option, the width of the reactor is exactly equal to the width of the microscope slides (2.5 cm). For each run, three
Fig. 1. (a) Schematic view of the experimental set-up. (1) Growth medium; (2) recycle loop; (3) peristaltic pump; (4) inverted microscope integrated with a digital camera; (5) flat-plate reactor; (6) computer; (7) air filter; (8) feed line; (9) air in; (10) outflow; (11) flow breaker. We placed three glass microscope slides 2.5 cm on the bottom of the reactor and used the biofilms on these slides for further analysis. (b) The reactor was constructed of polycarbonate (1/4 in. thick). All fittings had 3/8 in. opening width with 1/8 in. plastic pipe thread (ACE® Hardware). All fittings were centered and placed close to the edge except the output line, which was positioned above the recycle line. All dimensions are in centimeters and the figure is sketched without scale.
microscope slides (2.5 × 7.5 × 0.1 cm), etched with 20% hydrogen fluoride (HF) for 5 s and rinsed with excess deionized water, were positioned at the bottom of the reactor. Etching roughens the surface of the glass and helps microbial attachment. The slides were placed on the bottom of the reactor and held in place using stainless steel brackets to prevent the slides from rising off the bottom of the reactor if gas bubbles formed underneath them. To prevent microbial contamination, we sealed the reactor lid with 100% silicone rubber (ACE® Hardware, Oak Brook, IL) and then bleached the reactor for 2 h using 20% Clorox®. After bleaching, the reactor was flushed with 10 l of sterile deionized water (autoclaved at 121°C, 1 atm absolute pressure), allowing the rinse water to entirely fill the volume of the reactor, and repeating the cycle several times. The sterile water was allowed to flush the reactor overnight.

2.3. Growth medium

The growth media consisted of (reagents from Fisher®): Na₂HPO₄ (1.825 g/l), KH₂PO₄ (0.35 g/l), MgSO₄·7H₂O (0.01 g/l), (NH₄)₂SO₄ (1.0 g/l), glucose (5 g/l) and yeast extract (0.1 g/l). The glucose and yeast extract were autoclaved separately and added to the media after autoclaving. A 1-ml/l mixture of micronutrients: MnCl₂·4H₂O (527 mg/l), CuCl₂·2H₂O (228 mg/l), CoCl₂·6H₂O (317 mg/l), (NH₄)₆Mo₇O₂₄·4H₂O (231 mg/l), Na₂B₄O₇·10H₂O (127 mg/l), ZnCl₂ (363 mg/l), CaCl₂ (3.7 g/l), and FeCl₃ (2.16 g/l), dissolved in 1 l of 0.1 M HCl, was added to the media after sterilization. To prevent microbial contamination, the micronutrient solution was aseptically filtered using a 0.2 μm syringe filter (Corning, New York) into the media, immediately after the media had been autoclaved. The pH of the growth medium was 7.2.

2.4. Microorganisms

Following the protocol established in our lab; Beyenal and Lewandowski, 2000; Yang et al., 2000; Lewandowski et al., 1999; Beyenal et al., 1998; Xia et al., 1998; Yang and Lewandowski, (1995), we used a three-species biofilm as a model biofilm that consisted of: P. aeruginosa (ATCC#700829), P. fluorescens (ATCC#700830) and K. pneumoniae (ATCC#700831). The microorganisms were environmental isolates cultured by our research group and registered with the ATCC.

2.5. Reactor inoculation

A frozen stock sample of each species was inoculated into separate flasks and the cultures were grown for 24–30 h in 100 ml of growth medium on a shaker set to 150 rpm at room temperature (approximately 25°C). Typical cell concentrations (CFU/ml of solution) of the individual cultures, when plated on R2A agar and grown for 24 h in the incubator at 30°C, were 9.4 × 10⁸ for P. aeruginosa, 1.4 × 10⁸ for K. pneumoniae and 1.3 × 10⁸ for P. fluorescens. The reactor was inoculated with 20 ml of each culture, aseptically, via needle and syringe, through the line in which the growth media entered the reactor. Just before inoculation, the sterile water in the reactor was replaced with freshly prepared sterile growth medium. During the inoculation, the flow of growth media, the recycle loop, and the airflow were stopped and the waste line was clamped. Approximately 15 min after inoculation, the recycling was resumed and continued for 6–12 h before the feed pump was turned on. Also, at that point, the airflow was restored to the reactor, the effluent clamping was resumed and continued for 6–12 h before the feed pump was turned on.

2.6. Operating the reactor

The fresh feed flow rate was 0.4 ± 0.1 ml/min. We used a 6-h retention time. To maintain a reasonable flow velocity and shear stress, the recycle ratio was set to 300 for the duration of the experiments. The high recycle ratio also provided uniform substrate concentration along the reactor (Smith, 1970). Twice a day, the reactor was flushed with sterile, deionized water to remove suspended microorganisms. The 6-h retention time alone was not enough to prevent the accumulation of microorganisms in suspension. The reactor was operated at room temperature (≈ 25°C). Glucose concentration in the reactor was measured using procedure 510 by Sigma® Diagnostics. The reactor was continuously aerated from the surface using filtered air (3 l/h). The dissolved
oxygen concentration was checked in some experiments using a dissolved oxygen electrode and it was around saturation value.

2.7. Biofilm imaging

To capture the images of biofilm structure, the reactor was placed on an Olympus CK2 inverted microscope. UV light (Cambridge Instruments, Buffalo, NY) was used to illuminate the reactor. The best quality images we could obtain were just after the reactor was flushed with sterile water. The images were taken through the bottom of the reactor using the 40× magnification and were captured by a COHU® camera using Flashpoint, a frame grabber integrated with a computer. The images were in 8-bit gray-scale TIFF format, consisting of 640×480 pixels, and were viewed with a customized software package, ImagePro Plus® (Media Cybernetics, Version 3). Each day, 30 images were taken at random locations within the biofilm. Previous statistical analyses, conducted in a similar project in our laboratory, have shown that 30 images are adequate to represent the average biofilm structure (Yang et al., 2000; Lewandowski et al., 1999).

2.8. Quantifying biofilm structure

To quantify biofilm structure, we measured the collected biofilm images. These images were used to calculate areal porosity, fractal dimension, textural entropy and average diffusion distance using our custom-written image analysis program (Yang et al., 2000). Definitions of the parameters and the exact procedures of their calculation were given in the paper by Yang et al. (2000). Below, we briefly define the parameters that we have used in this paper.

Parameters that quantify biofilm structure are either textural or areal. Textural parameters quantify the distribution of optical density in biofilms; we measured textural entropy in this work. Areal parameters quantify the morphology of biofilm microcolonies; in this work, we also measured areal porosity, fractal dimension, and diffusion distance. In our previous works (Lewandowski et al., 1999), we have demonstrated that the selected parameters were good descriptors of biofilm structure.

2.8.1. Areal porosity

Areal porosity is the ratio of interstitial void area to the total area of the image. As a biofilm matures, the areal porosity of a biofilm decreases, sometimes approaching a steady state (Lewandowski et al., 1999). It gives information about the relative coverage of microorganisms on the microscope slide surface. Lower porosity indicates more coverage of microorganisms on the slide surface.

2.8.2. Fractal dimension

Fractal dimension is an indication of the “raggedness” of the biofilm microcolonies’ perimeter boundary. The higher the value, the more irregular the boundary of the microcolony. The irregularity of the boundary increases the surface area of the microcolonies, thus allowing for greater interaction with the surrounding bulk fluid. Fractal dimension can be used as a tool for the morphological analysis of biofilm structure. However, the fractal dimension is not a unique characteristic and two objects that vary vastly may have the same fractal dimension (Hermanowicz et al., 1995).

2.8.3. Textural entropy

Texture is broadly defined as the rate and direction of change of the chromatic properties of the image, and could be subjectively described as fine, coarse, smooth, random, rippled, irregular, etc. (Yang et al., 2000). Textural entropy is a parameter that measures the variation of gray levels in biofilm images. The gray level varies because of local thickness or cell density variations. Higher textural entropy indicates more variation in local thickness or density. This parameter can be used as a universal measure of biofilm heterogeneity. The higher the textural entropy, the greater the heterogeneity of the biofilm (Lewandowski et al., 1999).

2.8.4. Average diffusion distance

This is an average distance a nutrient must travel from the cell cluster edge to reach the microorganisms at different positions in the microcolonies. Average diffusion distance takes into consideration the size of the cluster and its general shape. The larger the diffusion distance, the greater the distance a substance must diffuse to reach the inner parts of a cluster. It can be considered as the average diameter
of the cell clusters. Hence, the greater the average diffusion distance, the bigger the cell cluster.

2.9. Specific number of viable cells

The specific number of cells were calculated according to the spread plate method described by Brock et al. (1986). After 4 days, when the biofilms reached a thickness of approximately 200–300 μm, we opened the reactors by removing the silicone rubber with a razor knife. During this procedure, the air and nutrient flows were stopped. Once the reactor was opened, the stainless steel brackets holding the slides were removed. The slides were carefully removed from the reactor and put into sterile centrifuge tubes containing a phosphate buffer at pH 7.2. The biofilm from the microscope slide was aseptically scraped in the buffer using a sterile Teflon scraper. The sample biofilm (in liquid) in the test tubes was dispersed using a probe homogenizer (Ika Labortechnik, Wilmington, NC) with a probe diameter of 1.5 cm. The sample was homogenized for 30 s at 20,500 rpm, then centrifuged for 20 min at 6000 rpm to pellet the microorganisms. The supernatant was poured off, the microorganisms (pellet) resuspended in 10 ml of phosphate buffer, and the sample serially diluted, generally out to 10⁻⁷. A 100 μl aliquot of each serial dilution was spread plated, in triplicate, on R2A agar (Fisher Scientific). The plates were inoculated at 30°C and the colonies counted after 24 h. After plating, the remaining sample was vacuum-filtered through Whatman Glass Microfibre Filters, 2.5 cm in diameter, with a 0.7 μm pore size. Prior to filtering, the clean filters were dried to a constant weight in a drying oven at 110 ± 5°C for 24 h and weighed on a Mettler AE2000 balance. The trapped cells and the filter paper were re-dried at the same temperature for 24 h and the weight difference recorded. The weight difference represented the dry biomass that accumulated on a given microscope slide. To determine the final number of viable cells in terms of colony forming units (CFU) per unit surface area of biofilm, the number of colonies counted on the spread plate, after accounting for dilutions, was divided by the total surface area. The resulting CFU per unit area was divided by the dry biomass to yield the CFU/mg biomass/surface area, which we have termed the “specific number of viable cells” (SNVC) in biofilm, with a dimension of CFU/mg biomass/cm².

3. Results and discussions

The variability in the biofilm structure based on 18 runs is presented in Figs. 2–5. In general, the areal porosity (Fig. 2) decreases with time. After 3 days of growth, the areal porosity reached a pseudosteady state and its value remained constant, around 0.5, showing that 50% of the microscope slide surface area was covered with biofilm. The standard deviation from the average was 0.1, which testified that the areal porosity was reproducible within 20%.

The fractal dimension was increasing steadily with time (Fig. 3). However, the standard deviation from the average (error bars) showed a tendency to decrease with time (20% the first day and 4% the fourth day), which indicated that the fractal dimension was reaching a steady state. Since the fractal dimension reflects the extent of the raggedness of the microcolonies’ perimeters, the increasing fractal dimension indicated that the perimeter of the biofilm microcolonies was becoming more ragged, and was effectively increasing the cluster perimeter. As a consequence, during biofilm growth, the surface roughness of the microcolonies increased. An increase in the surface roughness increases the surface area exposed to the nutrients, and possibly, increases...
the local mass transfer coefficient to the cell clusters. A rougher surface has been associated with higher mass transfer coefficients (Dawson and Trass, 1972).

Textural entropy increased during the first 2 days of biofilm growth, reached a maximum, and then decreased (Fig. 4). The standard deviation from the average was high at the beginning of biofilm development and lower by the end of each run.

Textural entropy is influenced by the variation in gray level of the images. The gray level variations may be caused by (1) biofilm thickness variations, (2) local biofilm density variations. During the first two days of biofilm growth, only a small number of cells detached from the surface, indicating that the process was dominated by growth and attachment. The local areas of biofilm deposition were influencing the distribution of the gray level in the images, which resulted in high textural entropy. After day 3, the biofilm started to reach maturity, and the variations in local biofilm thickness and density began to lessen. This correlated well with fairly constant surface coverage by the biofilm (see Fig. 3). As a result, textural entropy decreased.

As mentioned previously, textural entropy can also be considered as a universal parameter characterizing biofilm heterogeneity. At the beginning of biofilm growth, there was a small number of cell clusters attached to the microscope slide surface and they were unevenly distributed. The areal porosity was high and the cell clusters were well separated from each other; the biofilm was highly heterogeneous. However, as time progressed, the cell clusters grew and the areal porosity decreased. Also, the variations in local density and thickness decreased. As a result, biofilm heterogeneity decreased, which is reflected by the decrease in textural entropy. Finally, when the surface was uniformly covered with biofilm, the textural entropy decreased to low values because there was no spatial distribution between the cell clusters.

Both the average diffusion distance and the standard deviation from the average increased with time (Fig. 5). The average diffusion distance reflected the
average size of the cell clusters. The standard deviation from the average reflected the variability in sizes of the cell clusters. As time progressed, the attached cell clusters grew and increased their size, which was reflected by the increasing diffusion distance. Also, as time progressed, the sizes of the cell clusters differentiated because old clusters got bigger and new clusters started to grow. This is reflected in the increasing standard deviation from the average.

Although Figs. 2, 3, 4, and 5 show error bars, these error bars reflect the extent of biofilm heterogeneity, not experimental errors. Because we do not know the true values of the measured parameters: fractal dimension, areal porosity, and textural entropy, we cannot estimate the experimental errors. The parameters plotted in Figs. 2, 3, 4, and 5 were evaluated from biofilm images and their values are reported to show the level of consistency with which we could reproduce the biofilm structure, and not necessarily to report the absolute value of the measured parameters.

Fig. 6 shows the number of viable cells per surface area vs. the number of repeated runs. The two continuous horizontal lines in the figure mark the 95% confidence limits calculated according to Rasmussen (1992). These data were generated using the standard procedure for enumerating viable cells in a biofilm, and one can see that approximately 45% of the cell counts fell outside of the 95% confidence limit. This suggests that a new method must be used to decrease the deviations associated with enumerating biofilms.

To eliminate this kind of experimental error, we introduced a new parameter called “specific number of viable cells”, for which the viable cell number was normalized with respect to the surface area and with respect to the biomass accumulation (Fig. 7). The number of viable cells per unit surface area of biofilm was divided by the dry biomass (mg), which we termed the “specific number of viable cells” in biofilm, (SNVC).

The new parameter “specific number of viable cells” takes into account the dry weight of biomass present on a microscope slide (test coupon). Intuitively, it is obvious that the number of viable cells present on a certain surface area should depend on the biomass accumulation, not only on the area covered by the biofilm. Therefore, we believe that the number of viable cells should be normalized with respect to two factors: surface area covered by the biofilm and the biomass weight.

Although the viable cells per surface area presented in Fig. 6 showed respectable reproducibility of our biofilms, the new parameter, SNVC (Fig. 7), showed better reproducibility. Almost all of the data were within the 95% confidence limit (indicated by the two horizontal lines), in fact, only 11% fell outside of this confidence limit. Noticeably more data points were within the 95% confidence interval in Fig. 7 than in Fig. 6, thus indicating that the procedure we are promoting, to normalize the cell count with respect to biofilm volume and the surface area covered by the biofilm, is superior to the estab-
Table 1
The coefficient of variation for viable cells per surface area and SNVC

<table>
<thead>
<tr>
<th>Method</th>
<th>Coefficient of variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNVC</td>
<td>0.336</td>
</tr>
<tr>
<td>Viable cells per surface area</td>
<td>0.589</td>
</tr>
</tbody>
</table>

To compare the two data sets, those in Figs. 6 and 7, we calculated the coefficient of variation as:

\[
\text{Coefficient of variation} = \frac{\text{Standard deviation}}{\text{Average value}}.
\]

The coefficients of variation for both methods are presented in Table 1. SNVC showed almost two times less deviation than the standard method used to quantify viable cells per surface area in biofilms.

Our goal was (1) to grow reproducible biofilms and (2) to use cell counts to quantify reproducibility. Although choosing cell counts to quantify biofilm reproducibility is subjective, for many purposes, e.g. testing the effects of antimicrobial agents against biofilms, having reproducible cell counts is quite adequate. However, in some biofilm studies, e.g. the effect of biofilm structure on microbial activity, cell count alone may not be an adequate descriptor of biofilm reproducibility, and other factors should be included to quantify biofilm reproducibility. In this paper, we have quantified a few parameters reflecting the evolution of biofilm structure. The temporal changes of these parameters indicate that the structures of our biofilms were developed according to similar patterns. However, there are fundamental problems about quantifying these parameters that have to be resolved, for example, a reproducible method of image thresholding (Yang et al., 2000, 2001) before we may recommend them as reliable tools in quantifying reproducibility.

In our past studies, we have determined that young biofilms (a few days old) can be reproduced quite accurately. For the purpose of this demonstration, we chose 4-day-old biofilms. It is sometimes possible to extend this period by a day or two and still achieve a respectable level of cell count reproducibility, but finally, each biofilm starts to develop its own characteristics and the level of reproducibility decreases.

4. Conclusions

(1) We have demonstrated that, following the procedures described in this paper, it is possible to grow biofilms that are reproducible with respect to viable cell number per surface area.

(2) We have demonstrated that biofilms grown using the described procedures show consistent and predictable patterns of temporal changes in areal porosity, fractal dimension, average diffusion distance and textural entropy. However, these parameters reflect fundamental features of biofilm processes that are, at present, not entirely understood, and therefore, we recommend them only as auxiliary parameters, to check if the pattern of their changes is qualitatively consistent and reflects the expectations.

(3) To improve quantifying biofilm reproducibility, we suggest using a new parameter to quantify the number of viable cells accumulated in biofilms, “specific number of viable cells”, which is calculated by dividing the viable cell number per unit surface area and by the biomass. In our study, this new parameter, SNVC, reduced the variability in the viable cell numbers that may have been caused by the fact that biofilms developed on seemingly identical surfaces may have different amounts of biomass, e.g. because of different biofilm thicknesses.

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