Journal of Microbiological Methods 94 (2013) 367–374

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

A simple method for quantifying biomass cell and polymer distribution in biofilms

Wen Zhang [a,*], Eric S. McLamore [b], N.T. Garland [b], J.V. Chavez Leon [b], M. Katherine Banks [c]

[a] Department of Civil Engineering, University of Arkansas, 4190 Bell Engineering Center, Fayetteville, AR 72701, USA
[b] Agricultural and Biological Engineering Department, University of Florida, 105 Frazier Rogers Hall, Gainesville, FL 32611, USA
[c] School of Civil Engineering, Purdue University, 550 Stadium Mall Drive, West Lafayette, IN 47907, USA

ARTICLE INFO

Article history:
Received 10 June 2013
Received in revised form 23 July 2013
Accepted 24 July 2013
Available online 31 July 2013

Keywords:
Biofilm
Detachment
Coulter counter
Exopolymer
Cell count

ABSTRACT

Biofilms are ubiquitous and play an essential role in both environmental processes and hospital infections. Standard methods are not capable of quantifying biomass concentration in dilute suspensions. Furthermore, standard techniques cannot differentiate biomass composition. In this study, a user-friendly technique was developed for measuring biomass cell and polymer content in detached biofilms using a standard coulter counter. The method was demonstrated for an environmentally relevant strain of *Pseudomonas aeruginosa* (Schroeter) Migula grown in a bioreactor and also for a medically relevant strain of *P. aeruginosa* (PAO1) grown on standard growth pegs. Results were compared and validated by standard assays, including EPA method 1684 for measuring biomass, microscopic direct counts, and a crystal violet staining assay. The minimum detection limit for the coulter counter method (0.07 mg-biomass L^{-1}) was significantly lower than the EPA method 1684 (1.9 ± 0.4 mg/L) and the crystal violet assay (1.1 ± 0.2 mg L^{-1}). However, the coulter counter method is limited to dilute biomass samples (below 204 ± 16 mg L^{-1}) due to clogging of the aperture tube. While biomass measurements are useful, the major advantage of the coulter counter method is the ability to directly determine EPS, cell, and aggregate fractions after mild chemical treatment. The rapid technique (4–5 min per sample) was used to measure biomass fractions in dispersed *P. aeruginosa* (Schroeter) and PAO1 biofilms. This technique will be critical for understanding biofilm formation/dispersal.

© 2013 Elsevier B.V. All rights reserved.

1. Introduction

Bacteria exist in planktonic and sessile states surrounded by extracellular polymeric substances (EPS). The quantification of dynamic cell/EPS fragments during biofilm formation/detachment is challenging. One of the major obstacles to accurately characterizing these dynamics is the response time of the technique used to measure EPS and/or cell fractions.

Standard solids analysis of aquatic samples by convection (EPA Method 1684) provides estimates of volatile and fixed biomass (Environmental Protection Agency, 2001). This standard method provides insight into the nature of biomass fractions, but does not provide direct quantitative information about the cell or EPS concentration. Traditional cell counting methods include viable cell counts, optical density measurement (Aydin et al., 2011), and direct counts (Yu et al., 1995). These methods are tedious, time consuming, require extensive user expertise to obtain high-quality data, and are entirely dependent on the dilution ratio. Two-dimensional (2D) cell counts are not viable for estimations of biofilm cell/EPS counts, since biofilms are three-dimensional (3D) structures packed with cells and EPS. Thus, cell clumping (for both planktonic and sessile cells) is a major source of experimental error in direct counts. Viable counts using fluorescent staining techniques and laser scanning confocal microscopy (Cook et al., 2000) offer an improvement over direct counts, although these methods are time-consuming, expensive, and require extensive user training for producing high-quality data.

Molecular immunological techniques and genotyping methods such as real-time quantitative polymerase chain reaction (qPCR) and reverse transcription PCR have been used as an indirect estimate of population density (Bleve et al., 2003; Der Vossen et al., 1996; Zhang et al., 2009). Flow cytometry has also been used as a stand-alone technique or in combination with PCR for direct measurement of cell densities in aquatic samples (Lebaron et al., 2002; Vives Rego et al., 2000). Molecular techniques provide the ability to distinguish between viable, viable but nonculturable, and dead cells, which is a major improvement over traditional PCR; DNA from lysed cells can serve as a template for amplification many days after cell viability is lost (Masters et al., 1994). These molecular techniques are relatively rapid and accurate for quantifying cell counts, but are expensive and provide no information about EPS content.

There are only a few available techniques for estimating EPS content in aquatic samples (Flemming and Wingender, 2010; Sheng et al.,

* Corresponding author at: 4190 Bell Engineering, Fayetteville, AR 72701. Tel.: +1 479 575 6024.
E-mail addresses: wenzhang@uark.edu (W. Zhang), emclamor@ufl.edu (E.S. McLamore), ngarland929@ufl.edu (N.T. Garland), jamesvic@ufl.edu (J.V.C. Leon), k-banks@tamu.edu (M.K. Banks).

0167-7012/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.mimet.2013.07.022

2010). Most techniques require EPS extraction, which causes cell lysis while preserving the structural integrity of the EPS (Frølund et al., 1996). Pretreatment methods require homogenization prior to EPS extraction using physical and/or chemical methods. Extraction techniques such as boiling and strong alkaline treatment have been abandoned due to cell/EPS structural damage. Once EPS are extracted from the sample, there are a number of analytical techniques for characterizing the composition of EPS (Sheng et al., 2010). Colorimetric assays provide information regarding the carbohydrate content. Chromatography/spectrometry is commonly used to provide detailed characterization of saccharide and amino acid content in EPS. DAPI fluorescence, UV absorbance, and the diphenylamine method are commonly used to characterize nucleic acid content of extracted samples. Lectin-tagged fluorophores are used for binding exopolymeric material for microscopic analysis by either laser scanning confocal or epifluorescent microscopy (Flemming and Wingender, 2010; Sheng et al., 2010). The techniques described above are invaluable for understanding the spatial and temporal variation of EPS production, as well as interactions of EPS with the environment (i.e., adsorption, biodegradation, etc.). However, there are currently no rapid techniques for determining EPS and cell content in dispersed biofilm samples without harsh pretreatments that damage the integrity of dispersed cells.

Coulter counters are used to quantify particle size/concentration by measuring changes in electrical conductance at a small aperture as liquid containing particles flows through the orifice of tan aperture tube (Fig. 1). Analog coulter counters have been used to characterize environmental samples such as sediments (Kranck, 1973) and flocculated particles in water treatment plants (Treweek and Morgan, 1977). Ymele-Leki and Ross (2007) used an analog coulter counter (Beckman Multisizer 3) to measure cells and particles during erosion of *Staphylococcus aureus* cells due to fluid shear; all data in this study were validated with direct counts. Ymele-Leki and Ross (2007) noted that only suspended cells were observed in these measurements, and no aggregates or EPS were observed. However, no data was shown to support this finding. State-of-the-art coulter counters digitize and amplify analog signals, providing a major advantage over analog instruments when monitoring complex solutions such as biofilms dispersed in water. Huang et al. (2013) used a digital coulter counter (Beckman Multisizer 4) for monitoring erosion of *Pseudomonas aeruginosa* and *Nitrosomonas europaea* biofilms due to fluid shear. Only total particle counts were reported in the study, and no data were reported on the distribution of biomass fractions.

This paper introduces a coulter counter method for estimating cell, polymer, and aggregate content in detached biofilms. The method is demonstrated for characterizing biofilm detachment in both environmental and medical studies.

2. Materials and methods

2.1. Biofilm Culture

P. aeruginosa (Schroeter) Migula (ATCC 28753) was used for all environmental studies. *P. aeruginosa* (Schroeter) was purchased from the American Type Culture Collection (ATCC, Manassas, VA). Suspended cells were grown in standard glucose media (Ng and Dawes, 1973) at 37 °C overnight. Cells were harvested and allowed to develop biofilms on non-porous polydimethyl siloxane (PDMS) membranes (Dow Corning Co., Midland, Michigan) within a hollow fiber membrane-aerated bioreactor (HfMBR) in an environmental growth chamber (Huang et al., 2013; McLamore et al., 2007). Oxygen was provided through the lumen of the membranes using a Rena air pump (Aquarium Pharmaceuticals, Chalfont, PA). Glucose nutrient solution was pumped to the bioreactors using peristaltic pumps (Cole Parmer, Vernon Hills, Illinois). The bioreactor influent flow rate and hydraulic retention time were 2 mL/min and 1.1 days, respectively. After 30 days of growth, intact, immoblized biofilm samples were removed from bioreactors and transferred to a centrifuge tube with 50 mL phosphate-buffered saline (PBS).

P. aeruginosa PAO1 (ATCC BAA-47) was used to demonstrate the technique for a medically relevant strain. PAO1 was purchased from the American Type Culture Collection. Suspended cells were cultured in Müller–Hinton Broth (MHB), and biofilms were grown using a standard MBEC Assay™ described by (Alkawareek et al., 2012). Briefly, PAO1 optical density (OD550) was adjusted to 1×10^7 CFU mL^{-1}. The bacterial suspension was used to inoculate a Calgary Biofilm Device (Innovotech Inc.) which was then incubated at 37 °C for 48 h in an orbital incubator. The inoculum was replaced with fresh MHB medium every 24 h. After 7 days of incubation, individual pegs were removed with sterile pliers and placed in PBS.

2.2. Particle size analysis

A Multisizer 4 (MS4) Coulter Counter (Beckman, CA) was used to quantify particle size distribution. A 20-μm aperture tube was used to detect particles ranging from 400 nm to 12 μm in diameter. The maximum bin size (400) was used for all experiments, which provided the highest size resolution (±0.029 μm). Suspended cells were diluted with Isoton II solution (Beckman, CA) to a total measured concentration less than 10% reported by the instrument (as recommended by the manufacturer). Isoton II is a balanced electrolytic solution which is approximately 1% total salt concentration (sodium and potassium chloride plus a phosphate buffer). An analytical volume of 75 μL was used for all samples (total diluted sample volume was 20 mL for each sample). Individual samples were analyzed in triplicate and particle size data were averaged for all data reported. A single automatic unblocking mechanism was used in the Beckman software to unclog large aggregates from the aperture tube opening; samples were re-measured immediately after blockage.

For analysis of suspended cultures, three replicate 50 mL samples of *P. aeruginosa* (Schroeter) in glucose media were obtained after overnight growth. Each sample was diluted by adding 10, 50, 100, 200, 400, and 500 μL of cell suspensions to a 20-mL Isoton II solution (Beckman, CA).

For biofilm analysis, biomass was detached by vortexing biofilm cultures in 30 mL of PBS solution on a Vortex Genie 2 (Scientific Industries, Bohemia, NY) for 3 min. After detachment, the membrane (or Calgary peg) was carefully removed with sterile tweezers. The solution with detached particles was diluted to the desired concentration with Isoton II solution (Beckman, CA) and measured immediately. Where applicable, trypsin/EDTA solution (Invitrogen) was added to detached biofilm samples, resulting in a concentration of 0.2% trypsin with 25 mM EDTA. After mixing, samples were maintained at 37 °C and sampled every hour for 24 h. Triplicate experiments were conducted in each method tested here.

2.3. Validation of coulter counter measurements against standard methods

Direct counts were conducted using a Nikon Optiphot fluorescent microscope (Nikon, NY) equipped with the appropriate filter blocks. The suspended cells were diluted and stained with SYTO® 9 green fluorescent nucleic acid stain (Invitrogen, Carlsbad, CA). An aliquot of cells was diluted to a range such that 10 to 50 cells could be counted at a magnification of 1000. An aliquot of sample (1 mL) was stained with 3 μL of SYTO 9 for 20 min, and then filtered through a black Nuclepore polycarbonate membrane filters (Whatman, Sanford, Maine). PBS (4 mL) was then filtered to ensure even distribution of the cells on the filter. For each sample, three aliquots were used and 10 areas were counted for each aliquot.

The optical method described by Shanks et al. (2005) was used to validate measurements of dispersed PAO1 biofilms. Briefly, detached biofilm samples were gently washed in PBS. The remaining biofilms were stained with 1 % crystal violet, and the absorbed crystal violet

Download English Version:

https://daneshyari.com/en/article/10890652

Download Persian Version:

https://daneshyari.com/article/10890652

Daneshyari.com