Video Article

Protocol for Biofilm Streamer Formation in a Microfluidic Device with Micro-pillars

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Abstract

Several bacterial species possess the ability to attach to surfaces and colonize them in the form of thin films called biofilms. Biofilms that grow in porous media are relevant to several industrial and environmental processes such as wastewater treatment and CO_2 sequestration. We used *Pseudomonas fluorescens*, a Gram-negative aerobic bacterium, to investigate biofilm formation in a microfluidic device that mimics porous media. The microfluidic device consists of an array of micro-posts, which were fabricated using soft-lithography. Subsequently, biofilm formation in these devices with flow was investigated and we demonstrate the formation of filamentous biofilms known as streamers in our device. The detailed protocols for fabrication and assembly of microfluidic device are provided here along with the bacterial culture protocols. Detailed procedures for experimentation with the microfluidic device are also presented along with representative results.

Video Link

The video component of this article can be found at https://www.jove.com/video/51732/

Introduction

Recently, we demonstrated bacterial biofilm formation dynamics in a microfluidic device that mimics porous media[1]. Bacterial biofilms are essentially colonies of surface aggregated bacteria that are encased by extracellular polymeric substances (EPS)[2-4]. These thin films of bacteria can form in almost every conceivable niche ranging from smooth surfaces to the much more complex habitat of porous media. Valiei *et al*.[1] used a microfluidic device with an array of micro-pillars to simulate a porous media structure and studied biofilm formation in this device as a function of fluid flow rate. They found that in a certain flow regime, filamentous biofilms known as streamers began to emerge between different pillars. Streamers can be tethered at one or both ends to solid surfaces, but the rest of the structure is suspended in liquid. Streamer formation typically starts after an initial layer of biofilm has formed and its formation can dictate the long-term evolution of biofilm in such complex habitats. Recently, several researchers have investigated the dynamics of streamer formation. Yazdi *et al*.[5] showed that the streamers can form in vortical flows originating from an oscillating bubble. In another experiment, Rusconi *et al*.[6] investigated the effect of channel curvature and channel geometry on the formation of streamers. They found that the streamers can form in curved sections of microchannels, and streamer morphology is related to motility. Recent research has demonstrated that streamers can have wide repercussions in various natural and artificial scenarios as they can act as precursors to the formation of mature structures in porous interfaces, lead to rapid and catastrophic biofilm proliferation in a biomedical systems, and also cause substantial flow-structure interactions, *etc*[1,7-9].

Biofilm streamers often form in complex habitats such as porous media. Understanding biofilm growth in porous media environment is relevant to several environmental and industrial processes such as biological wastewater treatment[10], maintaining well-bore integrity in situations such as CO_2 capture[11] and plugging of pores in soil[12]. Observing biofilm formation in such complex habitats can often be challenging due to the opacity of porous media. In such situations, microfluidics based porous media platforms can prove extremely advantageous as they allow real-time and *in situ* monitoring. Another advantage of microfluidics is the ability to build multiple bioreactors on a single bio-microfluidic platform and simultaneously allow for online monitoring and/or incorporation of sensors. The flexibility to implement multiple laboratory experiments in one device and the ability to collect significant pertinent data for accurate statistical analysis is an important advantage of microfluidic systems[13,14].

In the context of the above discussion, understanding streamer formation dynamics in a porous media environment would be beneficial to several applications. In this study, we develop the protocol for investigating streamer formation in a device that mimics porous media. Fabrication of the microfluidic platform, necessary steps for cell culture and experimentation are described. In our experiments, the wild type bacterial strain of *Pseudomonas fluorescens* was employed. *P. fluorescens,* found naturally in soil, plays a key role in maintaining soil ecology[15]. The bacterial strain employed had been genetically engineered to express green fluorescent protein (GFP) constitutively.

Protocol

Perform the experimental protocols here in the order described below. Microfabrication protocols for creating the microfluidic platform are discussed in Step 1. Step 2 describes the bacterial culture protocol (**Figure 2**), and Step 3 pertains to assembly of the experimental setup (**Figure 3**). Finally, the actual experimental step is described in Step 4.

1. Chip Fabrication Procedure

NOTE: Proper safety procedures must be followed for the processes described below. Consult the institutional safety officer for details.

1. Design the mask with an appropriate software (*e.g.*, L-Edit). The channel design consists of a main micro-channel of width 625 µm. The central region of the channel contains an array of micro-posts 50 µm in diameter, spaced 25 µm apart (See Supplemental Files).
2. Print this design on glass (5" x 5" soda lime glass), which has a thickness of 0.09" and is coated by approximately 70 nm thick layer of Chromium (Cr), using masking in order to prepare a photo mask. Use AZ400K developer for 1 min. Then, etch the Cr layer using Cr etchant for about 1 min. Use acetone to strip the resist and clean it with cold piranha solution (H_2SO_4 and H_2O_2 in a ratio of 3:1).
3. Photolithography
    1. Clean a standard 4" silicon wafer chemically with piranha solution for 20 min.
    2. Rinse the wafer with DI water and dry it.
    3. Heat the wafer on a hot plate (200 °C for 15 min).
    4. Coat the silicon wafer with photoresist. Here, the positive photoresist AZ4620 was spin-coated on a silicon wafer at 2,000 rpm for 25 sec to obtain a 12.5 µm thick layer.
    5. Remove all the solvent by soft baking of the wafer on a hot plate by floating the wafer for 90 sec on nitrogen flow at 100 °C. Then, keep it in vacuum at the same temperature for 60 sec.
    6. Place the wafer in a dark box for 24 hr for dehydration.
    7. Expose the wafer to UV light in order to transfer the designed pattern to the photoresist.
    8. Immerse the wafer in photoresist developer solution (AZ400K) for 240 sec. Then, rinse the wafer with isopropyl alcohol and dry it by placing in a stream of nitrogen gas.
4. ICP-DRIE (Inductively Coupled Plasma - Deep Reactive Ion Etching) Process
    1. Apply DRIE etching. Choose the appropriate etch depth according to final depth required for device (50 µm in this investigation). Photoresist acts as a masking layer during this process.
    2. Remove the remaining photoresist with acetone and clean the wafer.
5. PDMS (polydimethylsiloxane) Casting
    1. Use trichloromethylsilane (TCMS) for silanizing the silicon master mold. Pour 2 or 3 drops of trichloromethylsilane in a vial and place it in a desiccator beside the silicon master mold. Allow 2-3 hr for the silanizing process to complete.
    2. In a separate container, mix the Sylgard 184 silicone base with curing agent by weight ratio of 10:1 to prepare PDMS. Degas the PDMS by subjecting it to vacuum conditions (about 2 hr).
    3. Put the silicon master mold in a holder. Then, pour the PDMS on the silicon master mold to form the PDMS stamp. Ensure that bubbles do not form in the PDMS during this process.
    4. Cure the PDMS for 2 hr at 80 °C.
    5. Peel off the PDMS stamp from the master mold. Then, cut the PDMS stamp into separate microchips. Finally, use a cutting core to drill holes for the inlet(s) and outlet(s).
6. Bonding of PDMS to Glass
    1. Expose the cover slip and PDMS stamp to oxygen plasma for 30 sec. Bond PDMS stamp to the cover slip.
    2. To achieve proper sealing between the PDMS stamp and the cover slip, anneal the device by putting it in oven at 70 °C for 10 min.

2. Bacterial Culture

NOTE: Proper biosafety protocols must be followed for Steps 2-4. Consult the institutional safety officer for details.

1. Prepare LB Agar Plates
    1. Add 20 g Luria-Bertani (LB) agar (Miller) powders and 500 ml of ultrapure water to a 1 L flask. Stir to dissolve the powder.
    2. Sterilize by autoclaving at 15 psi, 121 °C for 15 min.
    3. Allow the flask to cool down to 50-55 °C on a bench or in a water bath in the biosafety hood.
    4. Add the antibiotic Tetracycline to achieve a final concentration of 50 µg/ml. Mix well by swirling.
    5. Pour the mixture into plates. Fill each plate till 1/2 - 2/3 full.
    6. Flame air bubbles briefly to pop them if they form. Solidified air bubbles are difficult to spread bacterial culture over.
    7. Allow the plates to cool at room temperature overnight.
    8. When they are cool, put plates back into their sleeve, seal the bag, label (antibiotic and date), and store at 4 °C.
    NOTE: Cover the stock of plates with tin foil, as light deactivates many antibiotics.
2. LB Broth Preparation
    1. Add 20 g Luria-Bertani (LB) broth (Miller) powders and 1 L of ultrapure water to a flask. Stir to dissolve the powder.
    2. Sterilize by autoclaving at 15 psi, 121 °C for 15 min.
    3. Allow flask to cool down to 50-55 °C on a bench or in a water bath in the biosafety hood.

4. Add the antibiotic tetracycline to achieve a final concentration of 50 µg/ml. Mix well by swirling.
5. When cool, place the labeled bottle at 4 °C.
   NOTE: Cover the bottle with tin foil, as light deactivates many antibiotics.

3. Culture Bacteria on an LB Agar Plate (This protocol uses *Pseudomonas fluorescens*)
   1. Take the bacterial stock from the freezer (-80 °C) and place it on ice.
   2. Place the -80 °C bacterial stock and an LB agar plate inside a biosafety hood.
   3. Streak the bacterial strain onto an LB agar plate in a zigzag pattern. Cover the agar plate and incubate it at 30 ºC overnight. Finally, store the plate in the refrigerator at 4 °C.

4. Prepare the Bacterial Solution (S1)
   1. Pour 50 ml LB broth media to an autoclaved flask. Perform this operation inside a biosafety hood.
   2. Transfer a single bacterial colony from the LB agar plate to the flask. This operation should also be performed inside a biosafety hood.
   3. Put the flask in a shaker incubator at 30 °C and 150 rpm for adequate time (4 hr).

5. Prepare the Dilute Bacterial Solution (S2)
   1. Pour 5 ml LB broth media into a sterilized plastic tube.
   2. Dilute S1 by mixing with LB broth media. Then, vortex the solution. Dilute the solution achieve the desired optical density (OD measured at 600 nm = 0.1).
      NOTE: Biofilm experiments typically employ OD values in this vicinity.

3. Prepare the Experimental Setup

1. Using tweezers connect flexible plastic tubes (0.20" ID) into the inlet(s) and outlet(s) of the microchip. The inlets and outlets were previously drilled into the PDMS portion of the microchip (step 1.5.5). In this investigation, the microchip consists of two inlets and one outlet.
2. Fill syringe(s) with bacterial solution (S2 solution) and remove all the bubbles in the syringe(s).
3. Connect syringe tip(s) (30 G 0.5" blunt needle) into inlet tube(s). Then, connect outlet tube(s) to waste container.

4. Run the Experiment

1. Connect the syringe(s) to the syringe tip(s).
2. Place and fix syringe(s) onto the syringe pump. Then place the microchip under an optical microscope with objective lenses of desired magnification (*e.g.*, 40X). Cover the microchip with a live cell chamber device to maintain a constant temperature environment for bacterial growth (30 °C for *P. fluorescens*).
3. Set the pump to the desired flow rate level (say 10 µl/hr) and initiate fluid pumping.
4. Once bacteria are introduced into the chamber, biofilm formation is also initiated. Biofilm formation and maturation typically occur over a period of several hours or even days. Observe and take images of biofilm growth through the microscope.

Representative Results

Using the above mentioned microfabrication protocol, a PDMS based microfluidic device was constructed. **Figure 1** shows the scanning electron microscope (SEM) images of the PDMS device. **Figure 1a** shows the entrance section of the device. A fork-like entrance is created to equalize pressure head across the device. Further SEM imaging also showed that the pillar walls are almost vertical (**Figure 1b**). The cultured bacterial solution (**Figure 2**) was diluted and its optical density was adjusted to a value of 0.1. We examined biofilm formation in the microfluidic device as a function of input flow rate. When *P. fluorescens* was injected into the device at a low flow-rate of 0.8 µl/hr, bacterial attachment and biofilm formation occurred at the walls of the device. Even after a prolonged period of time (>20 hr), no other bacterial structures other than surface-hugging biofilms were observed. Next, the same experiment was repeated at a flow rate of 8 µl/hr. In this case, biofilm formation again started after a few minutes of infusion of the diluted bacterial culture. However, after a few hours, appearance of filamentous structures extending between micro-pillars was observed near the mid-section of the device (**Figure 4**). These filamentous structures could be visualized through the presence of immobile bacteria. These structures are known as streamers and they are filamentous biofilms that are only tethered at one or both ends to surfaces. The rest of the structure is often suspended in the liquid medium (as in this case). **Figure 4** shows the time-evolution of biofilm streamer structure. Streamers usually form due to the effect of fluid shear on the visco-elastic biofilm. **Figure 5** shows the streamlines and velocity contours for flow past a series of pillars. The simulation shows that the streamers that form in our microfluidic system are essentially aligned along the fluid flow streamlines. The correlation between the flow structures and formation of biofilm streamers is not yet well understood. However, Das and Kumar[16] have recently proposed that these streamers form as highly viscous liquid state of the intrinsically viscoelastic biofilms. They based their conjecture on the observation that the time-scale of biofilm streamer formation typically far exceeds the viscoelastic relaxation time scales of biofilms. Biofilms are known to behave as viscoelastic liquids and hence at time-scales much larger than the viscoelastic relaxation time scale, they essentially behave as highly viscous liquids[17]. According to this formulation, streamers can be expected to originate at locations of high shear stresses. **Figure 5** shows the locations of high velocity in the channel, and these locations coincide with locations of high shear stresses. In the initial phase of growth, streamers are observed to originate near these locations (**Figure 4**).

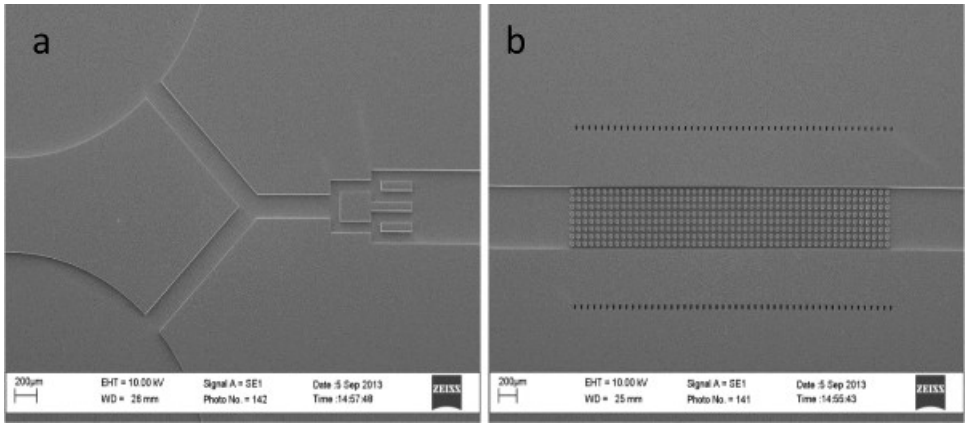

Figure 1. Scanning electron microscope (SEM) images of the microfluidic channel (top-view). a) Inlet section, **b)** Region containing micro-pillars. Please click here to view a larger version of this figure.

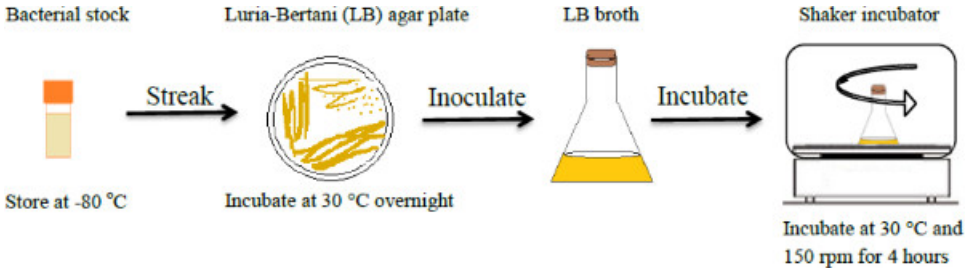

Figure 2. Sequential steps involved in bacterial culture. Please click here to view a larger version of this figure.

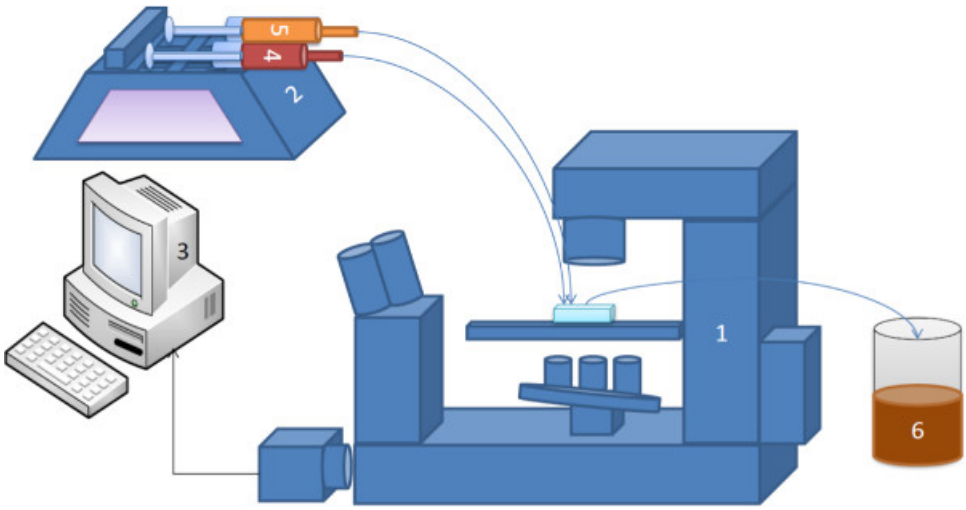

Figure 3. Set up for microfluidic experiments. 1—Optical microscope (inverted), 2—Syringe pump, 3—Image and data acquisition, 4—Syringe containing dye (optional), 5—Syringe containing bacteria, 6—Waste reservoir. Please click here to view a larger version of this figure.

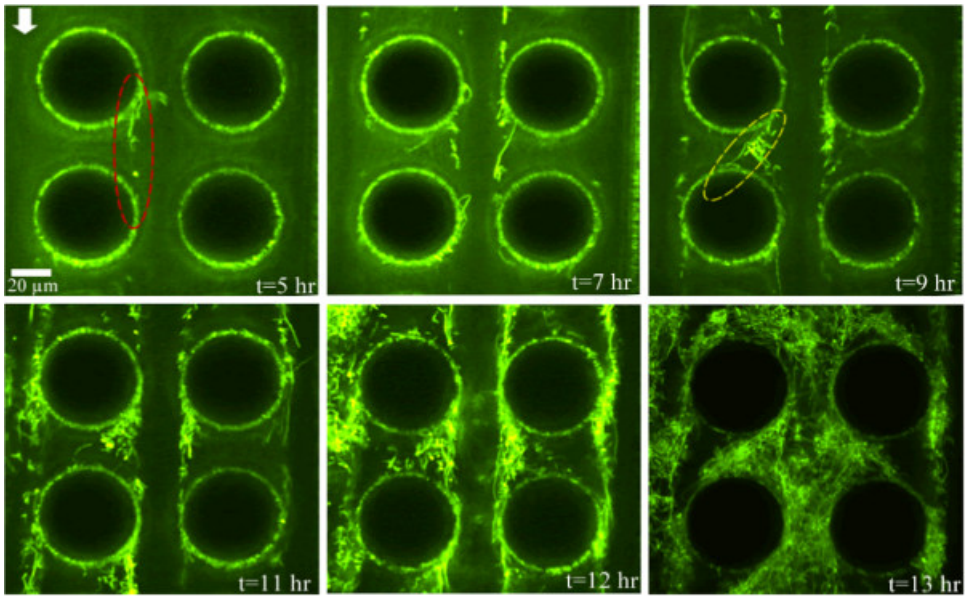

Figure 4. Time-lapse confocal imaging of evolution of streamers. Image plane corresponds to z = 25 µm *i.e.* middle of device. Dashed ellipses demonstrate biofilm streamers. Please click here to view a larger version of this figure.

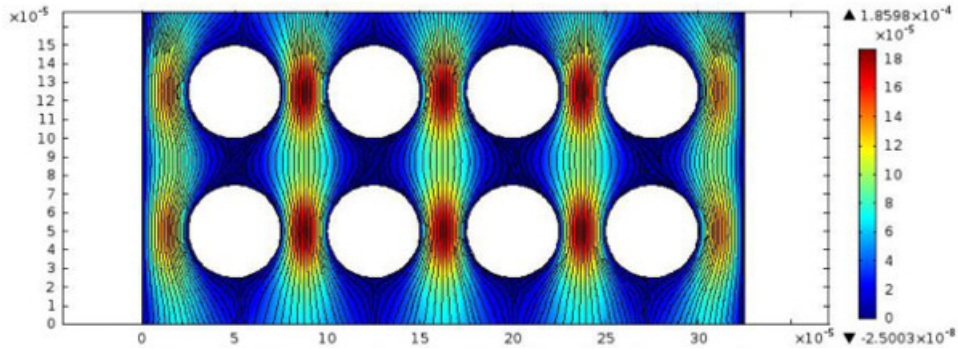

Figure 5. Computational fluid mechanical simulations showing streamlines and velocity contours of flow past micro-pillars. Fluid flow is from top to bottom and velocity scale is in m/sec. Please click here to view a larger version of this figure.

Discussion

We demonstrated a simple microfluidic device that mimics porous media for studying biofilm development in complex habitats. There are several critical steps that dictate the outcome of the experiments. They include device geometry. While the post geometry can vary, adequate pore-space for streamers to form is necessary. Moreover, Valiei *et al*.[1] have demonstrated that streamer formation occurs only in a certain flow rate range. At flow rates lower than a threshold value, deformation of biofilms into streamers may not be observed. Yet above a certain another threshold flow rate value, biofilm fracture can dominate and not allow formation of streamers. Another issue that can plague these experiments is gas bubbles that can become trapped in the micro-pillar array. Usually these bubbles have to be removed by increasing the flow rate initially and then gradually decreasing it to the desired value.

Microfluidic platforms such as these offer several advantages and few limitations. The platform enables us to work with small culture volumes, and has the flexibility of incorporating user-defined features. For example, different porous structures can be simulated by altering the geometrical parameters of the micro-pillar array. Even structures that mimic the random structure of real porous media can be fabricated on microfluidic platforms[18]. Moreover, several such channels can be implemented on a single device allowing for collection of significant pertinent data for accurate statistical analysis. However, microfluidic systems typically mimic two-dimensional structure of porous media. Devices that can mimic the three-dimensional nature of porous media are usually quite challenging to fabricate.

Formation and evolution of streamers are not well understood yet, and further research is required in this direction. Understanding of how streamers form and lead to the formation of mature biofilm structures will be relevant to a wide variety of scenarios including clogging of biomedical devices such as heart stents, biofilms in soil, and filtration systems. Our microfluidic platform is a step in that direction.

Disclosures

The authors have nothing to disclose.

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References

1. Valiei, A., Kumar, A., Mukherjee, P.P., Liu, Y., & Thundat, T., A web of streamers: biofilm formation in a porous microfluidic device. *Lab Chip.* **12**, 5133-5137, (2012).
2. Costerton, J.W., Bacterial Biofilms: A Common Cause of Persistent Infections. *Science.* **284**, 1318-1322, (1999).
3. Flemming, H.C., & Wingender, J., The biofilm matrix. *Nat Rev Microbiol.* **8**, 623-633, (2010).
4. Wong, G.C.L., & O'Toole, G.A., All together now: Integrating biofilm research across disciplines. *MRS Bulletin.* **36**, 339-342, (2011).
5. Yazdi, S., & Ardekani, A.M., Bacterial aggregation and biofilm formation in a vortical flow. *Biomicrofluidics.* **6**, 044114, (2012).
6. Rusconi, R., Lecuyer, S., Guglielmini, L., & Stone, H.A., Laminar flow around corners triggers the formation of biofilm streamers. *J R Soc Interface.* **7**, 1293-1299, (2010).
7. Drescher, K., Shen, Y., Bassler, B.L., & Stone, H.A., Biofilm streamers cause catastrophic disruption of flow with consequences for environmental and medical systems. *P Natl Acad Sci USA.* **110**, 4345-4350, (2013).
8. Marty, A., Roques, C., Causserand, C., & Bacchin, P., Formation of bacterial streamers during filtration in microfluidic systems. *Biofouling.* **28**, 551-562, (2012).
9. Taherzadeh, D. *et al.* Computational Study of the Drag and Oscillatory Movement of Biofilm Streamers in Fast Flows. *Biotechnol Bioeng.* **105**, 600-610 (2010).
10. Vrouwenvelder, J.S. *et al.* Impact of flow regime on pressure drop increase and biomass accumulation and morphology in membrane systems. *Water Res.* **44**, 689-702, (2010).
11. Mitchell, A.C. *et al.* Biofilm enhanced geologic sequestration of supercritical CO2. *International Journal of Greenhouse Gas Control.* **3**, 90-99, (2009).
12. Soleimani, S., Van Geel, P.J., Isgor, O.B., & Mostafa, M.B. Modeling of biological clogging in unsaturated porous media. *J Contam Hydrol.* **106**, 39-50, (2009).
13. Kumar, A., *et al.* Microscale confinement features can affect biofilm formation. *Microfluid Nanofluid.* **14**, 895-902, (2013).
14. Neethirajan, S., *et al.* in *Encylopedia of Nanotechnology* (ed B. Bhushan) Springer (2012).
15. Barathi, S., & Vasudevan, N., Utilization of petroleum hydrocarbons by *Pseudomonas fluorescens* isolated from a petroleum-contaminated soil. *Environ Int.* **26**, 413-416, (2001).
16. Das, S., & Kumar, A., Formation and post-formation dynamics of bacterial biofilm streamers as highly viscous liquid jets. *arXiv preprint arXiv:1312.6056.* (2013).
17. Shaw, T., Winston, M., Rupp, C.J., Klapper, I., & Stoodley, P., Commonality of elastic relaxation times in biofilms. *Phys Rev Lett.* **93**, (2004).
18. Berejnov, V., Djilali, N., & Sinton, D., Lab-on-chip methodologies for the study of transport in porous media: energy applications. *Lab Chip.* **8**, 689-693, (2008).

