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USE OF FLOW CELL REACTORS TO QUANTIFY BIOFILM FORMATION KINETICS

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SUMMARY

A parallel plate flow cell reactor is introduced and used to evaluate cell adhesion and biofilm formation kinetics for four different bacterial strains of the species, *E. coli*. The reactor allows biofilm growth under defined, well-controlled fluid dynamics while providing continuous observations and direct sampling of biofilm for biological, chemical and physical analyses as well as immunofluorescent labeling.

INTRODUCTION

Biofilms have been studied extensively due to their detrimental and beneficial effects on natural and engineered system (Bryers, 1987; Characklis and Marshall, 1990). Biofilms create problems in engineered systems (1) by increasing the resistance of mass, momentum, and energy transfer and (2) by way of attached cells reacting chemically or biologically at the substrata. Conversely, biofilms can play a beneficial role in the natural environment and engineered biological systems. Biofilms facilitate the natural removal of dissolved and particulate contaminants from surface and groundwaters, and from domestic and industrial wastewaters by way of fixed film reactors in bioreactor systems. Intentional use of biofilms can facilitate biomass-liquid separation, provide high cell concentrations, eliminate the biomass loss associated with "wash-out", and increase over-all system productivity (Atkinson, 1986; Robinson and Wang, 1986). Recently, concern over the retention and transfer of genetic material within biofilm communities has intensified (Pickup and Saunders, 1990). Regardless of the application (e.g., to eliminate a pollutant, to utilize in biological reactor systems, to control biofouling, or to study gene transfer), biofilm studies require a reactor system that allows control of the growth environment while providing ease of analysis.

There are several kinds of reactor geometries currently used in fundamental biofilm process research: tubular reactor (Bakke, 1986), roto torque reactor or rotating annular reactor (Trulear, 1983; Turakhia, 1986), capillary reactor (Powell and Slater, 1983; Escher, 1986; Caldwell, 1988), a circular or rectangular cross-sectioned Robbins device (McCoy et.

al., 1981), and a parallel-plate flow cell reactor (Sjelloma et. al., 1988), etc. Each configuration has its own advantages and shortcomings. For example, capillary flow cells can be used for non-invasive visual observation of cell adhesion and early biofilm formation, but however they do not allow for invasive sampling and further chemical and biological analyses. Capillary reactors also provide controlled laminar flow hydrodynamics but are too fragile for turbulent flow situations. Robbins devices and parallel-plate flow cells can provide destructive sampling of a biofilm under controlled well-defined fluid conditions (laminar and turbulent). One limitation to a Robbins device and parallel plate reactors is the lack of sufficient sampling surface area; to increase sample area more platens are required which physically increases the length of the reactor. Similarly, operation in turbulent flow requires additional reactor length to compensate for fluid entrance and exit effects. Roto torque or annular rotating reactors do provide high surface area: volume ratios and sufficient sampling area for biofilm analysis. In addition, fluid parameters that may affect biofilm formation (such as fluid velocity and shear stress at the interface) are set by the rotational speed of the moving cylinder and are thus independent of nutrient input flow rates (unlike all other reactor types). The disadvantage of the annular rotating reactor is that the fluid dynamics are not uniform throughout the system leading to non-ideal mixing and non-uniform biofilm formation. Here, we introduce a flow cell reactor design which allows visual surveillance of biofilm formation, is easy to control environmental conditions, and allows periodical sampling, analyses with minimal disturbance to the biofilm samples.

EXPERIMENTAL METHODS

Strains and Cell Growth. *Escherichia coli* HB101, DH5α, MV18 and BK6/pTKW106 were used in the following experimentation. Seed cultures were transferred from slant into 50 mL of M9 medium containing 0.2% glucose, 0.4% casamino acid, and 0.1% thiamine (for MV18 and BK6/pTKW106, 0.0018% tryptophan was added instead of thiamine). Shake flasks were agitated for approximately 12 to 15h at 37°C; inoculum for batch and chemostat cultures were taken from the shake flask culture during early to mid-exponential growth phase. The same medium as above was used in all chemostat cultures. A 1.5 L B. Braun fermenter (1L working volume) was inoculated with 2% (v/v) seed culture and allowed to operate batch wise until the culture was in mid-late exponential growth phase. Then sterile fresh medium was continuously added by peristaltic pump at a flow rate to affect a steady-state, dilution rate of $0.4h^{-1}$. After a minimum of five residence times, the chemostat culture was used as inoculum for the biofilm system.

Flow Cell Reactor. The flow cell reactor, illustrated in Figure 1, is constructed of optically clear polymethylmethacrylate (PMMA) and consists of a cover plate and a bottom piece which was machined with a rectangular indentation to accommodate nine standard microscope glass slides (3x1 in., thickness: 0.97 to 1.07 mm). The slides were washed with 1 M HCl, rinsed in sterilized water and air dried before use. A silicone O-ring was used to prevent the reactor from leaking and to set the gap width at 0.98±0.05 mm. Therefore, the reactor forms a rectangular chamber that can be considered as a parallel plate system to one-dimensional approximation. The assembled flow cell was sterilized by flowing 70% ethanol overnight and rinsing with sterile water three times.

Biofilm System. Bacterial biofilms were cultivated as described previously (Huang and Bryers, 1991). Briefly, the flow cell reactor was inoculated by way of recirculating contents of a chemostat culture through the cell for 2 hours at a flow rate of 45 mL/min. After inoculation, the chemostat was removed from the recycle loop and replaced with a small

mixing vessel (100 mL), the loop rinsed with fresh nutrient solution containing 50 mg/L glucose, 100 mg/l casamino acid, and 25 mg/L thiamine or 0.009 mg/L tryptophan, and recirculation flow resumed. Fresh nutrient solution was delivered to the mixing vessel to affect an overall system dilution rate of $4\ h^{-1}$. The system dilution rate maintained well in excess of the growth rates of the *E. coli* strains which served to minimize cell growth in the fluid phase. The slides with accumulated biofilm were removed from the flow cell reactor every 12 hours for further analysis and replaced with clean slides.

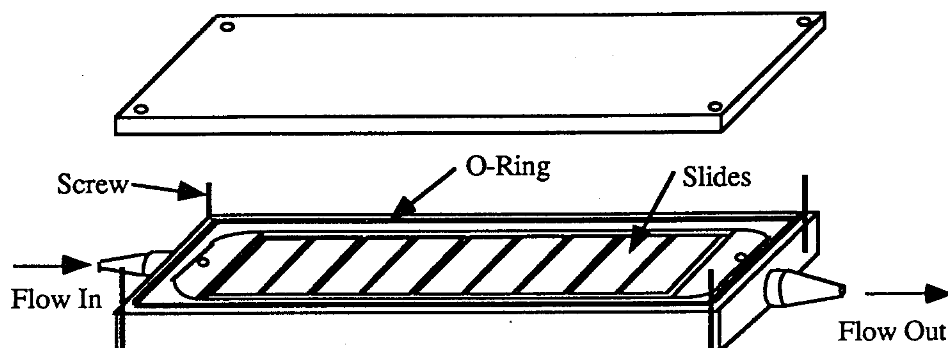

Figure 1. The schematic drawing of the flow cell reactor

Biofilm Analyses. Biofilm net accumulation was observed by measuring biofilm thickness, and total cell number per area and attached biomass dry weight per area. Biofilm thickness was visually measured using 10x objective and micrometer of an UV/VIS microscope (Bakke and Olsson, 1986). Then the biofilm was scraped completely into 50 mL sterile water and vortexed at maximum for 5 min to prevent bacteria aggregation. Total cell number was determined by staining cells in the resultant biofilm suspension with 0.01% acridine orange (Zimmerman and Meyer-Reid, 1974). Biofilm mass (dry weight) per area was determined by filtering the biofilm suspension through a tared 0.2 µm polycarbonate filter and dried at 105°C for 2 hours.

Transmission Electron Microscopy. Biofilm samples were fixed with 0.5% (v/v) formaldehyde in 0.1M cacodylate buffer containing 0.05% (w/v) ruthenium red, treated with 2% (w/v) osmium tetroxide, and dehydrated with a graded series of acetone. Biofilms are then rinsed twice with propylene oxide and embedded in Spurr media. After curing at 60°C overnight, samples are cut by microtome and stained with uranyl acetate and lead citrate.

RESULTS

The parallel plate flow cell was inoculated with one of the four *E. coli* strains under the conditions summarized in Table I. The net accumulation of pure culture biofilms expressed

Table I. The conditions of the four *E. coli* strains for inoculation

Strain	Flow Rate Recycle (mL/min)	Reynolds Number	Shear Stress (Newton/m^2)	Suspended Cell Concentration (cells/mL)
HB101	45	40	8.1×10^5	3.12×10^7
DH5α	45	40	8.1×10^5	1.11×10^7
MV18	45	40	8.1×10^5	9.08×10^7
BK6/pTKW106	45	40	8.1×10^5	2.10×10^7

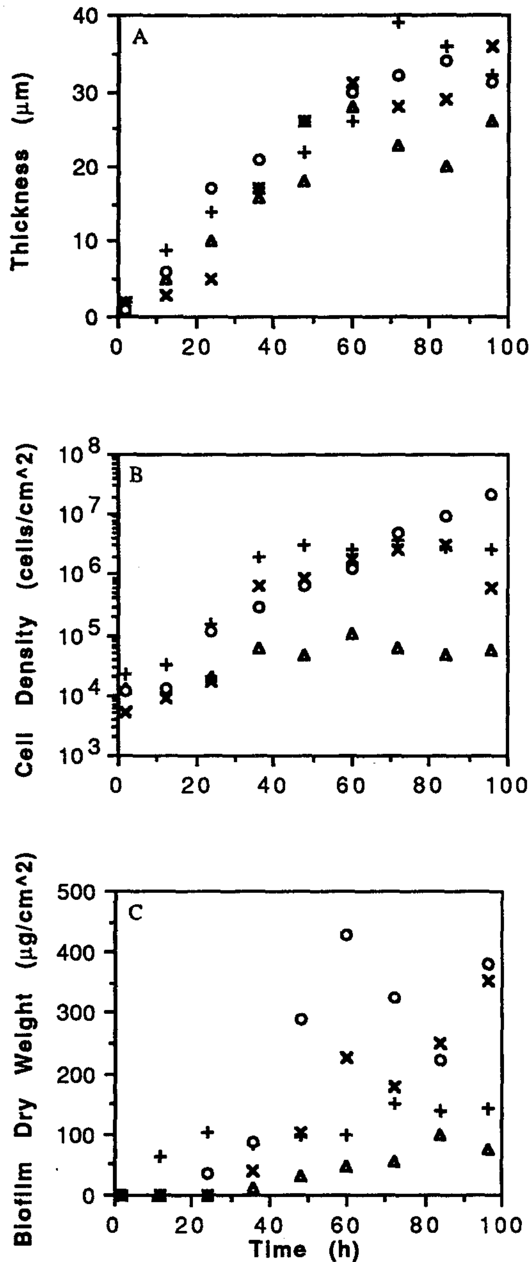

Figure 2. The net accumulation of pure culture biofilms cultivated within a parallel-plate flow cell reactor. (A) Thickness, (B) Total cell numbers/cm^2, and (C) Biofilm biomass dry weight/cm^2.(+) HB101; (o) DH5α; (x) MV18; (Δ) BK6/pTKW106.

by biofilm thickness, cell surface density and biofilm dry weight, of the following *E. coli* strains: HB101, DH5α, MV18 and BK6/pTKW106, are shown in Figure 2A-C. Stoichiometric and kinetic growth parameters obtained from suspended cultures for each stain

are summarized in Table II. *E. coli* BK6/pTKW106, with a lower growth rate and the extra metabolic burden of plasmid retention, accumulates the thinnest biofilm and had the least attached cell density and biofim dry weight.

While there was little difference between biofilm accumulation for each strain in terms of thickness and cell density, *E. coli* DH5α did accumulate most attached biomass dry weight, followed by *E. coli* MV18 and HB101, which suggests *E. coli* DH5α may have produced more extracellular polymers than the other two strains.

Table II. Stoichiometric and kinetic growth parameters for the four *E. coli* strains

Strain	μ_{max} (h^{-1})	Yield (g dry wt/g glucose)
HB101	2.28	0.49
DH5α	1.12	0.53
MV18	0.89	0.43
BK6/pTKW106	0.74	0.38

With the stain ruthenium red, a specific polysaccharide stain, and a higher resolution transmission electron microscopy, one can observe very detailed structure of biofilms formed on the reactor surfaces. Figure 3 shows a sectional image of biofilm formed by *E. coli* DH5α. TEM can also reveal the spatial distribution of bacteria in a biofilm utilizing a modified TEM preparative technique and either cytological fluorescent or immunofluorescent staining.

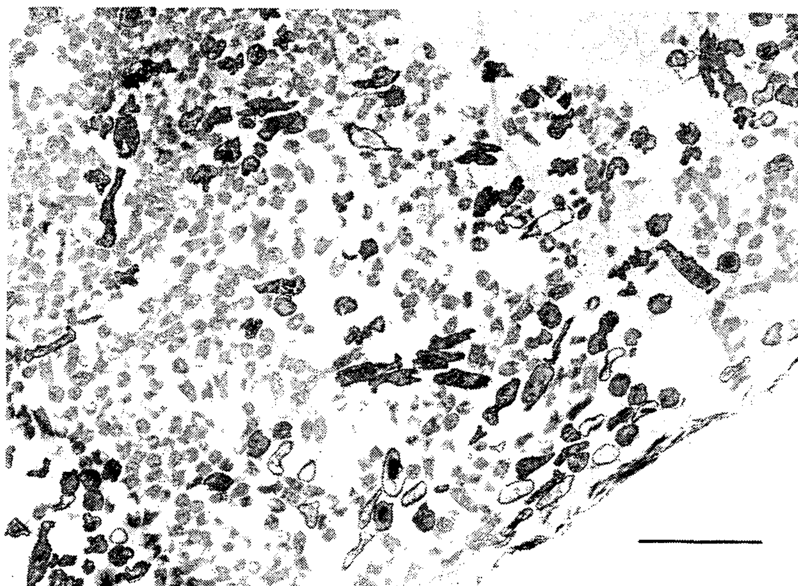

Figure 3. Transmission electron micrograph of ruthenium red-stained biofilm of *E. coli* DH5α after growing 96 hours. Bar represents 5 μm.

DISCUSSION

Compared with other reactor geometries for biofilm studies, the parallel plate flow cell reactor has several advantages: (1) it can accommodate a wide range of flow dynamics, from laminar to turbulent flow, depending on the volumetric flow rate ; (2) the velocity profiles and shear stresses are well defined; (3) biofilm formation can be non-invasively observed or periodically sampled; (4) removable test slides can be fabricated from any desired material; (5) biofilm can be withdrawn for analysis with minimal disturbance; (6) biofilm samples can be destructively removed from slides and used for a variety of biological (biomass, cell count, protein or enzyme activity), chemical (total organic carbon, chemical oxygen demand), physical (biofilm thickness, TEM or SEM photography), and immunological analyses (cell surface antigen marker or cloned-gene protein marker).

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