B－38 Development of an Experimental Methodology for Biofilm Structure Investigation under Different Hydrodynamic Conditions
異なる流体力学的条件における生物膜構造調査の実験的方法論の構築

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1.0 INTRODUCTION

Biofilms are colonies of microbial cells encased in slime and attached to a surface called the substratum. Growth of biofilm is a very complex process in which interactions of hydrodynamic and biochemical factors occur. Turbulence and substrate concentration in the vicinity of biofilm are external factors affecting the substrate profiles and hence the structure of the biofilm. Until recently, very little information was available on the microstructure of a biofilm mainly due to the difficulty in in-situ observation, limitations of the experimental methods followed and the lack of appropriate tools for experimentation. With the advent of modern equipment, intensive research has been stimulated especially on microbial biofilm microstructure and bacterial population dynamics. Biofilm structures on planar substrata are widely researched as they are convenient for microscopic examination in the fully hydrated state (De Beer et al., 1993). Cryo-sectioning of biofilms parallel to the planar substratum into thin sections of 10 - 20 μm using a cryomicrotome have enabled the study of microbial stratification in a nitrifying biofilm (Okabe et al., 1996). However, the examination of biofilm architecture requires the non-destructive visualization of the total biofilm. New experimental methodologies are required to overcome, the artifacts that are introduced by various preparatory techniques prior to microscopy, which distort the structure considerably thereby nullifying the advantages gained by Confocal Scanning Laser Microscopy (CSLM).

Investigations into the effect of hydrodynamics on biofilm structure require that the entire biofilm surface be under the same hydraulic condition. Hydraulic regime inside a tube or a pipe is uniform and well defined and therefore a tubular reactor could be an ideal substratum for hydrodynamics-structure relationship investigation. We successfully combined the techniques of vertical (perpendicular to the substratum) micro slicing of a tubular biofilm with CSLM and image analysis to visualize the structural features of the biofilm. This micro-sliced section was hybridized with fluorescently labeled group specific oligonucleotide probes and visualized by epifluorescent photomicrography to evaluate the stratification of the microbial species. Here we describe the methodology adopted and present some preliminary results of biofilm structures obtained under different hydraulic shear.

2.0 MATERIALS AND METHODS

2.1 Substrate Composition. The concentrated synthetic substrate consisted of the following (in g / l): $NaHCO_3$, 1.46; $(NH_4)_2SO_4$, 1.23; K_2HPO_4, 0.53; $MgSO_4.7H_2O$, 0.26; NaCl, 0.10; and $CaCl_2.2H_2O$, 0.03. The substrate was diluted in de-chlorinated tap water and $NaHCO_3$ was used for pH adjustment. The NH_4^+-N loading rate was 2.0 g-NH_4^+-N/m^2/d and the hydraulic retention time was 1.5 hrs.

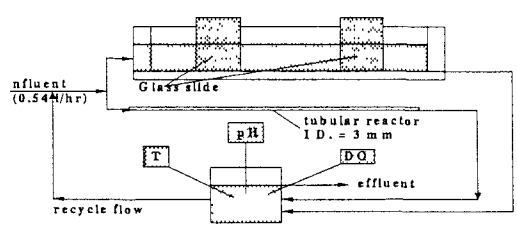

Fig. 1 Schematic Diagram of Biofilm Reactor

2.2 Experimental Reactor. The reactor set-up, as shown in Fig. 1, consisted of a horizontal flow biofilm reactor with glass slides (coverslips) providing the surface for biofilm growth, in parallel with a tubular biofilm reactor (Tygon tubing, MasterFlex, Cole-Palmer, Illinois) of 3 mm internal diameter. Two identical reactor configurations were operated with different flow rates through the reactors. The fluid was re-circulated to give high (Reynolds Number = 2000) and low (Reynolds Number = 100) hydraulic shear and to maintain uniformly mixed conditions throughout. In the mixing vessel, the temperature, DO and pH was controlled for optimum nitrification.

2.3 Preparation of biofilm samples. Approximately 1 cm long piece of tube was cut away from the down flow end of the tubular reactor and the cells were fixed immediately by immersing in freshly prepared 4% paraformaldehyde solution for 3 hours at 4° C. The fixed biomass was washed twice with PBS (130 mM NaCl, 10 mM Na_2HPO_4 [pH 7.2]), and forthwith

embedded in OCT (Tissue-Tek II; Miles, Elkhart, Ind.) freezing compound and was frozen to -40° C. The frozen sample was mounted on the cutting stage of microtome and cut, perpendicular to the direction of flow in the tube, into thin sections of 10 - 20 μm size. The slices were carefully picked up, placed on the wells of a gelatin coated microscopic glass slide and were immobilized by air-drying. Further fixing and immobilizing was carried out by washing successively with 50%, 80% and 98% ethanol for 3 minutes each. The biofilms grown on the glass slides were similarly fixed and treated but without subjecting to freezing or micro slicing.

2.4 Staining and Hybridization. DNA staining fluorochromes such as 4', 6-diamidino-2-phenylindole (DAPI) and YOYO-1-iodide (molecular probes) were used for epifluorescent observation of the biofilm. The hybridizations were done as described by Manz et al. The universal probe EUB 338 (complementary to a conserved region of all bacteria) and group specific probes complementary to alpha, beta, gamma subclass of the class *proteobacteria*, gram positive high mol % G + C group, and Cytophaga-Flavobacteruim group were used to phylogenetically characterize the biomass. The probes were fluorescently labeled with either fluorescein isothiocyanate (FITC) or tetramethyl rhodamine isothiocyanate (TRITC).

2.5 Microscopy. The vertical structure of the tubular biofilm and the 'XY' structure of the biofilm on glass slide were visualized by Episcopic Differential Interference Contrast (EDIC) microscopy using a Leica PL APO 63X oil immersion lens and 40X dry lens attached to a CSLM (Leica, TCS NT). Sections stained with fluorochromes or hybridized with fluorescently labeled oligonucleotide probes were visualized by epifluorescent microscopy using appropriate filter sets. Digital pictures were acquired with the program TCS NT (Leica). The pictures were subsequently exported as TIFF files, and were edited by the Paint Shop Pro (version 4.14, Eden Prairie, MN 55344) soft wear.

3.0 RESULTS AND DISCUSSION

3.1 Biofilm Structure on Glass Slide. After inoculation, the glass surface became colonized in a few days and a biofilm was formed after switching to continuous operation. Photomicrographs taken with DAPI staining and fluorescent oligonucleotide probe hybridization showed that biomass exist as cell clusters as opposed to the traditional concept of uniformly covered layer of attached microorganisms. Such isolated clusters were observed even in 60-day-old biofilms. There observed the existence of large "voids" in between cell clusters. In fully-grown mature biofilms, the clusters were found to be interconnected with each other by thin sections (Fig. 2). By confocal observations, it was noted that the dehydrated biofilm was almost 60 μm in thickness. No "conduits" (voids in the horizontal direction) were observed by confocal scanning. Due to technical considerations, the biofilm structure along the vertical direction could not be properly preserved even after freezing and vertical micro slicing.

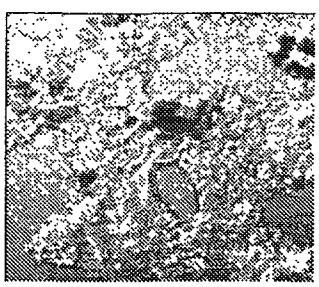

Fig. 2 Horizontal structure of a 60-day-old biofilm on glass slide, with interconnected cell clusters and large voids, visualized by EDIC microscopy

Close observation of DAPI stained photomicrographs and EDIC images showed that cell clusters are microbial aggregates cemented with EPS, where as the "voids" are open structures not clogged with EPS. Such a structure has a profound effect on the supply and transfer of substrates and oxygen to the microorganisms located in the interior of the cell cluster.

3.2 Limitations of the Technique. A major limitation of this technique, as far as the observation of vertical biofilm structure is concerned, is the distortion of the vertical structure by dry fixation and dehydration. Such distortions are very significant in the vertical "Z" direction, yet less severe in the "X" and "Y" direction, which could, if at all, affect the size and shape of the pores and clusters. Air-drying leads to the collapse of the hydrated biofilm structure in the vertical direction there by eliminating all the conduits and the vertical stratification of the microorganisms. Ethanol dehydration causes severe shrinkage and alters the architecture of the biofilm. Further, the hydrodynamic conditions along and across the slide can be non-uniform and complex there by affecting the structure in an unknown manner.

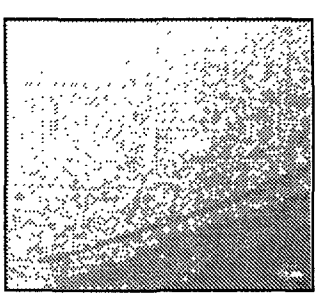

Fig. 3 Vertical microstructure showing the pores and protrusions of a 70 μm thick tubular biofilm produced under low shear conditions, taken by microslicing normal to the substratum and visualized by EDIC microscopy

3.3 Structure Preservation. It is important that the biofilm structural features like pores, protruding arms, or simply biofilm thickness is preserved from any distortion due to the preparation techniques (Fig. 3).

The greatest impact on structure is caused by ethanol dehydration (Schramm et al., 1996) leading to severe shrinkage and consequently collapse of pores. This problem can be minimized (for X-Y, not Z) if biofilm slices are attached to gelatin coated slides and thereby stabilized. By freezing followed by micro slicing the biofilm vertically and immobilizing in the 'X-Y' plane, the collapsing of the vertical structure in the above manner was avoided. Slicing the biofilm to thinner sections as possible mitigated the distortions arising from vertical contractions. A change of the spatial distribution of microorganisms due to fixation or dehydration is not very likely but can not completely be excluded.

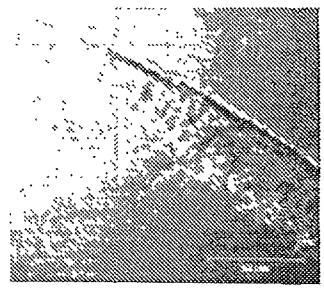

Fig. 4 High cell density 50 μm thick biofilm base produced under high shear conditions and visualized by EDIC microscopy

3.4 Effect of Substratum. Solid substrata possess characteristic surface properties that are important in microbial adhesion and colonization, particularly surface free energy, surface charge and surface roughness. We have examined the colonization of microorganisms in various tube materials, with identical tube diameter, such as silicone and derivatives of polyvinyl tubes and have found that biofilm formation is least encouraged in silicone tubes. This could be due to the difference in the chemical properties of the material. The adhesion strength was found more dependent on the applied hydraulic shear force than on substrata surface properties. Higher adhesion strengths were found in biofilms grown under higher hydraulic shear.

3.5 Tubular Biofilm Structure and Microbial Stratification. The well-preserved vertical slice of biofilm with substratum displayed the intricate details of biofilm structural heterogeneity (Fig. 3). Photomicrographs of biofilms taken for the two different hydraulic conditions showed distinct structural features. Biofilms produced under low shear had a lower cell density with a porous structure whereas under high hydraulic shear, the film had a dense structure (Fig. 4). Growth was manifested, as protrusions extending from the thick base, which understandably were less dense. The hydrodynamics and kinetics in biofilm systems are intimately related by the fact that most biofilm reactions are diffusion limited. Consequently, the shapes of the concentration gradients are determined by molecular diffusion and convection. The fluid velocity has three major influences: it affects the boundary layer thickness, the biofilm density and sloughing of the biofilm. Theoretically, the increase in biofilm density at higher fluid velocity could be explained by mass transfer correlation. Under increasingly higher turbulence, higher rate of mass transfer results.

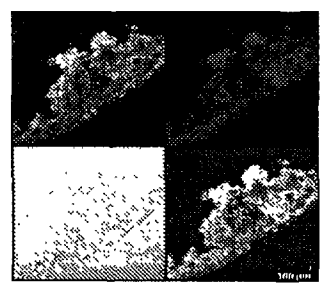

Fig. 5 Multichannel SCLM image of the exact spatial register of a biofilm, showing the morphology by staining with YOYO-1 (top left), abundance of beta subclass of *proteobacteria* by hybridizing with beta subclass probe, XRITC, (top right), EDIC image (bot. left) & merged view of top 2 images (bot.right).

Fluorescent in-situ hybridization with group specific probes showed that beta subclass of *proteobacteria* was the more abundant group although no clear pattern of stratification could be observed with any of the probes used (Fig. 5). The use of genus and species specific probes would probably lead to more detailed information on the stratification of microbial species in a multi-species biofilm.

4.0 CONCLUSIONS

The developed methodology circumvents the lumping of biomass in the vertical direction thus enabling the preservation and visualization of the biofilm structure in a more desirable way. Initial results show that the tubular biofilm is ideally suited for the investigation of biofilm structure-hydrodynamic relationship.

5.0 REFERENCES

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