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Full Length Research Paper

Detection of biofilm on surface using optical methods

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The ability of many bacteria to adhere to surfaces and to form biofilms has major implications in a variety of industries and aquatic ecosystem, where biofilms create a persistent source of contamination. The formation of a biofilm is determined not only by the nature of the attachment surface, but also by the characteristics of the bacterial cell and by environmental factors. This review will focus on the bacterial development on surface, which is the interface of the bacterium with its surroundings, and on the properties of the attachment surface influencing biofilm formation. Optical Coherence Tomomgraphy (OCT) is an interferometric method that can provide two, or three dimensional in-vivo tomographic images of biofilm formation and development. The images revealed the depletion, changes in the density and area of distribution of the biofilm above the surface, and changes of the biofilm aggregate sizes.

Keywords: Optical Coherence Tomography, interferometric method

INTRODUCTION

Biofilm, well organized cooperating community of microorganisms is almost ubiquitous among bacteria and important source for water and waste water treatments. Microbial cells attached to the surfaces and to engage in a multistep process leading to the formation of a biofilm (Donlan, 2002). Therefore, biofilm formation has substantial implications in fields ranging from industrial processes like oil drilling, paper production and food processing, to health-related fields like medicine and dentistry. Biofilm formation depends on an interaction between three main components: the bacterial cells, the attachment surface and the surrounding medium (Caldwell, 1995; Costerton et al., 1994, 1995). A large number of research are currently being carried out on biofilm reactors for the production of bioactive substances, for plant and animal cell cultures, drinking water production and wastewater treatments. Direct observation of a wide variety of natural habitats indicated majority of microbes persist attached to surfaces within a structured biofilm ecosystem and not as free floating organisms (Davey and O'Toole, 2000; Donlan, 2002; Dunne, 2002; Stoodley et al., 2002).

This review will focus on the bacterial development on surface, which is the interface of the bacterium with its surroundings, and on the properties of the attachment surface influencing biofilm formation. It is important to develop methods for fixed biomass activity estimation which is not only simple and rapid, but also sensitive precise and representation. The main purpose of this review is to describe new technology to biofilm investigation. Extraordinary findings have taken place in biofilm research during the past decade, using microscopic and molecular technologies. Otoole et al. (2000) have shown that biofilms are not simple organism and they have complex structure with slime layers on surfaces. In addition, they represent advanced organization where bacteria form structured, coordinated, functional communities. Caldwell et al. (1997) have discussed the complex interactions that form the basis of coexistence in these sessile communities. Biofilm

community perspective is providing us with novel insights into interaction with the environment. Consequently, it seems that the restricted view of bacteria as unicellular life forms is expanding to include their remarkable ability to function as part of a collective.

Biofilms structure

Biofilm community belongs to prokaryotes and the natural habitats of them are remarkably diverse (Pace, 1997; Whitman et al., 1998). They can inhabit any environment that is suitable for higher life forms. Their ability to persist throughout the biosphere is due, in part, to their unequaled metabolic versatility and phenotypic plasticity (Madigan et al., 1997). They have wide range of capacity for their adaptation to the environment themselves in a niche where they can propagate. Numerous adaptation mechanisms have been discovered in bacteria. The most common technique is movement with flagella and other different techniques of surface translocation, including twitching, gliding, darting, and sliding (Henrichsen, 1972). One of the most important lifestyle is aggregation or attachment. Through attachment, the bacteria not only exist themselves on a surface, they can form communities and obtain the additional benefit of the phenotypic versatility of their neighbors.

Other techniques utilizing biofilm communities

They have other mechanisms utilized by bacteria to grow themselves in response to their environment by synthesizing cellulose which can form a fibrous pellicle that places cells near the air-water interface (Ross et al., 1991). Photosynthetic bacteria can grow themselves at different levels in the water column in response to light intensity by producing gas vesicles for buoyancy or synthesizing carbohydrates or forming aggregates in order to sink (Overmann, and Pfennig, 1992). Organisms can exist in an environment independently. Most of the bacterial community can proliferate themselves more effectively by interacting and forming communities (Caldwell et al., 1997).

Metabolic processes

Most of the natural processes require the performance of the bacteria with different metabolic capabilities because bacteria are residing within biofilm communities and carry out many of these complex processes. For example, bacterial communities are important for the degradation of organic matter, pollutants, and the cycling of nitrogen, sulfur, and many metals, in the processing of sewage, in the treatment of groundwater contaminated with petroleum products, and in nitrification (Massol-Deya et al., 1995; de Boer et al., 1991). Biofilms also form in many extreme environments, such as in acid mine drainage (at a pH of 0), where they contribute to the cycling of sulfur (Edwards et al., 2000). For example, *Synechococcus* mat biofilms have been demandingly considered in thermal springs (Ramsing et al., 2000; Ward et al., 1998), and recently, researchers have ongoing investigation on biofilms in the ice covered area in Antarctica (Paerl et al., 1998). Complex structured communities in these extreme environments have been found to conduct a variety of biological processes, such as photosynthesis, nitrogen fixation, and fermentation. Another type of biofilm community that is being investigated is the bacterial assemblages associated with suspended particles of organic and inorganic material in the marine environment. Researchers have shown that these macroscopic particles, often referred to as marine snow, are enriched in microbial biomass, nutrients, and trace metals and are involved in biogeochemical transformation of particulate organic carbon in the pelagic environment (Caron et al., 1986; Paerl and Pinckney, 1996). Microbial communities associated with some of the metabolic process such as methanogenesis (Karl and Tilbrook, 1994), nitrogen fixation (Paerl and Prufert, 1987), and sulfide production (Shanks and Reeder, 1993). Moreover, microbial production of methane or sulfide as well as nitrogen fixation only occurs under anoxic conditions. This condition was examined with oxygen microelectrodes, and steep redox gradients were found in these biofilms, providing additional evidence of anaerobic metabolism (Paerl and Prufert, 1987). Rath et al. (1998) suggested that the phylogenetic diversity of the bacterial community associated with marine snow was evaluated using small-subunit ribosomal DNA (rDNA) fragments from nucleic acids extracted from samples of marine snow. These experiments showed that bacterial colonization of marine snow can result in diverse and complex assemblages.

Optical methods for visualization of biofilm

The application of confocal scanning laser microscopes (CSLM) effectively used to detect biofilm development in flow cells that allows direct observation of biofilm without disrupting the community structure and function (Lawrence et al., 1991). Before the use of CSLM, electron microscopy was the method of choice to examine microbial biofilms under high resolution. Unfortunately, sample preparation for electron microscopy results in dehydrated. Consequently, this approach provided a deceivingly simplistic view of biofilms, since the biofilm distorted when water was removed. On the other hand, CSLM, which allows the visualization of fully hydrated samples, has revealed the complicated three-dimensional structure of biofilms (Costerton et al., 1995; de Beer and Stoodley, 1995; de

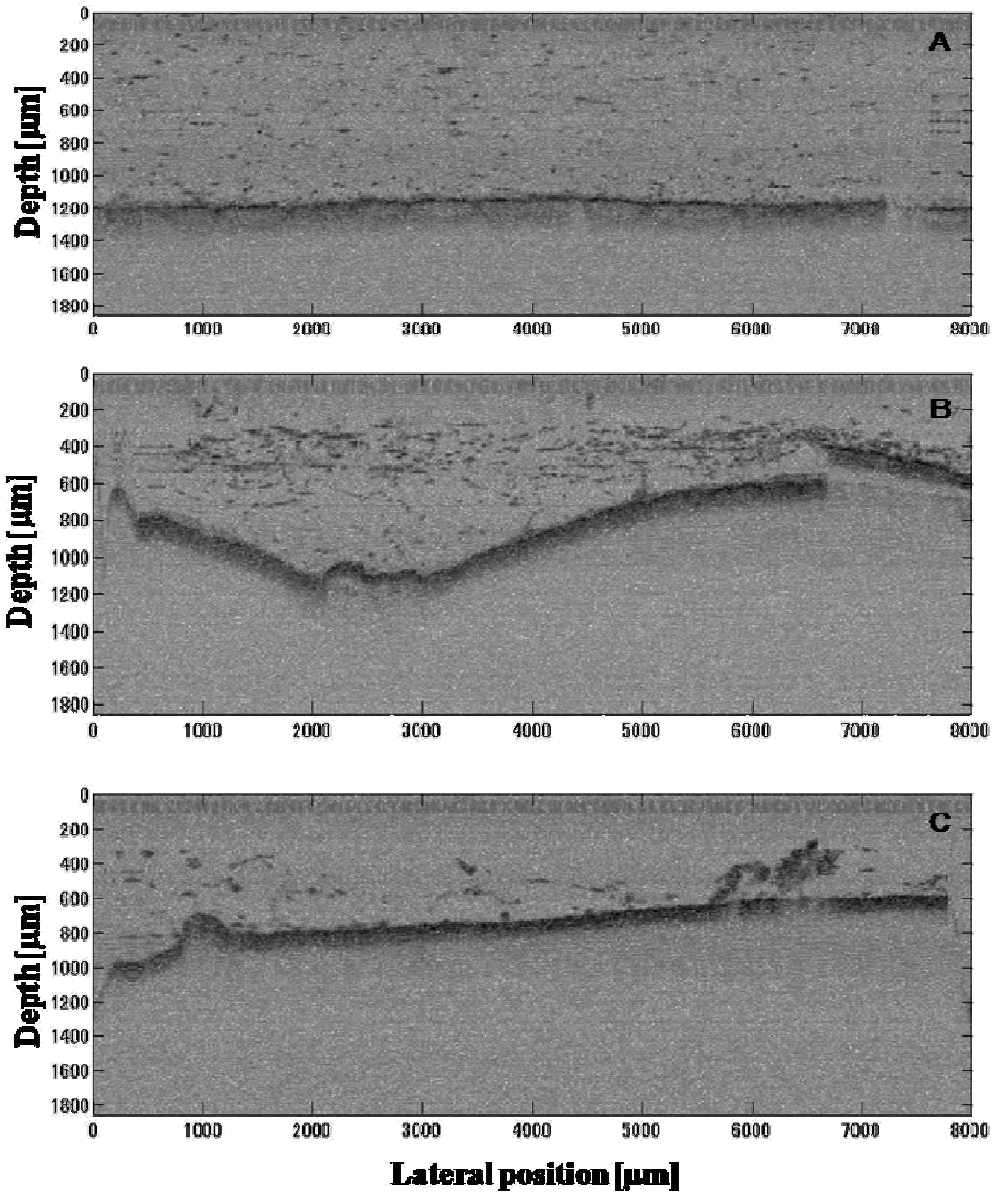

Figure 1. Two dimensional OCT images of samples taken in A- early decomposing state, B- moderately decomposing state, C- later decomposing state.

Beer et al., 1994). CSLM has been used very effectively to monitor biofilm development in flow cells. Flow cells are small continuous flow systems with a viewing port that allows direct observation of the biofilm without disrupting the community. A number of descriptions of flow cell and related techniques have been reported (Doyle, 1999). Interestingly, biofilms formed from single species in vitro and those produced in nature by mixed species consortia exhibit similar overall structural features (Costerton et al., 1995; Danese et al., 2000; Watnick and Kolter, 1999). Most biofilms have been found to exhibit some level of heterogeneity in that patches of cell aggregates, not monolayers, are interspersed throughout an exopolysaccharide matrix that varies in density, creating open areas where water channels are formed. An example of a biofilm on leaf litter is shown in Figure 1. It displays a typical 2D Optical Coherence Tomography (OCT) images of biofilm in early, moderately and later states of leaf litter. The microcolonies that constitute the biofilm can be composed of single-species populations or multimember communities of bacteria, depending on the environmental parameters under which they are formed. Numerous conditions, such as surface and interface properties, nutrient availability, the composition of the microbial community, and hydrodynamics, can affect biofilm

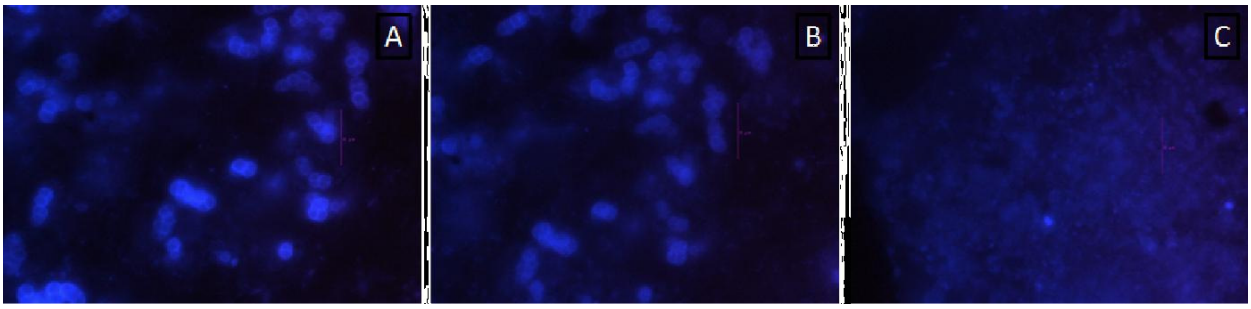

Figure 2. Light microscope observation of biofilm at three different states of decomposing leaf litter (x 1000). A- early decomposing state, B- moderately decomposing state, C- later decomposing state.

structure (Stoodley et al., 1997). For example, under high shear stresses, the biofilm is typically stratified and compacted (Bowden and Li, 1997; Wimpenny and Colasanti, 1997**).** Biofilms have also been examined under various hydrodynamic conditions and it was shown that biofilm structures are altered in response to flow conditions (Stoodley, et bal., 1998). Moreover, by observing biofilm development under continuous flow, this group was able to evaluate the effect of perturbations on established biofilms. They showed that the biofilm was polymorphic and structurally adapted to changes in nutrient availability (MacLeod et al., 1990). This is primarily due to the fact that the degradation of organic materials to methane and carbon dioxide is a community-level process that is driven by the close contact of multiple guilds interacting in a food web (McInerney, 1986; Schink**,** 1997**).** Hence, biofilm structure is affected by both the microbial biology and environmental parameters. Structural organization is clearly a trait of biofilm communities (Stoodley et al., 1994; Costerton, 1995**)**. For instance, in situ measurements of dissolved oxygen using microelectrodes revealed that oxygen is available in the biofilm (Lewansowski et al., 1993**;** Møller et al., 1996**)**. Presumably biofilm structure and function are likely to be mechanisms for the formation as well as the maintenance of these structures. The precise study of the biofilm formation in connection with the leaf structural changes is an important issue. In this paper we demonstrated the capabilities of optical coherence tomography (OCT) as a tool for in-vivo study of biofilm formation and development of leaf litter. The method presented here is suitable for the monitoring of the biofilm structure and leaf litter variation. The specific advantage of this method is that staining is not necessary to monitor biofilm structure on leaf litter and the temporal changes can be visualized *in-vivo*. This study will be devoted especially to quantitative measurements of thickness of leaf litter and biofilm on it. The images are used to determine optical biofilm thickness, on plant litter surface, without disturbing the biofilm. Based on these qualitative observations, we hypothesize that the optical biomass measurement method described here is more sensitive than the methods used to calibrate the measurements.

An argument for this is based on the graphs of the development in biofilm at the three sample positions over time Figure 1 and 2, because the graphs demonstrate a remarkable consistency in the relative magnitudes at the different positions in different decomposing state. If, three states of decomposed litter, the thickness measurement at one sample position are sometimes greater than at another position, it tends to keep the lead throughout the investigation time. If the OCT method had a great internal variability, one should hardly expect such a consistency. The test confirms that there are no systematic differences in the development of biofilm at different sample positions. The early stages of decomposition indicate low internal variability of the biofilm thickness. We therefore conclude that the biofilm measurement method described here is more sensitive to detection of biofilm thickness and that it can be used to detect subtle changes in biofilm accumulation. Fungi and bacteria associated with submerged decaying leaf litter are closely associated spatially even though hyphae can penetrate the substrate, while bacterial cells are thought to be restricted to leaf surfaces with the exception of tunneling bacteria and possible bacterial ‘epiphytes’ on fungal hyphae. Hence, it is plausible to assume that these microorganisms can affect each other through a variety of mechanisms, such as direct resource competition, production of secondary metabolites with antibiotic activity or supply of growth factors. Eutrophication can directly influence microbial activity through elevated nutrient concentrations (especially N and P) and consequently affect plant litter decomposition (Suberkropp and Chauvet, 1995, Sridhar and Bärlocher, 2003, Grattan and Suberkropp, 2001, Gulis and Suberkropp, 2002). To overcome these problems, biofilm thickness measurement is a key area for future investigations.

Nutrient availability and metabolic cooperatives

Biofilm provide an effective means of exchanging

nutrients and metabolites with the bulk aqueous phase, enhancing nutrient availability as well as removal of potentially toxic metabolites (Costerton et al., 1995). The metabolic characteristics of microorganism in a biofilm community are distinct. The sophisticated structural design of biofilm provides the opportunity for metabolic cooperation, and niches are formed within these spatially well-organized systems. Consequently, the bacteria are exposed to an array of distinct environmental signals within a biofilm. For example, cells close to the center of a biofilm structure are adapted to low oxygen tensions. To overcome this problem, microcolonies often consist of a mixture of species (Hirsch 1984; MacLeod et al., 1990). These multispecies microconsortia can result from an association between metabolically cooperative organisms, and their proximity facilitates interspecies substrate exchange and the removal or distribution of metabolic products. For example, the degradation of complex organic matter into methane and carbon dioxide during anaerobic digestion requires the interaction of at least three guilds. Fermentative bacteria initiate the catabolism, producing acids and alcohols that are then readily utilized as substrates by acetogenic bacteria. Finally, the methanogens obtain energy from converting acetate, carbon dioxide, and hydrogen to methane. Hence, very efficient cooperation and mutual dependence can evolve within a biofilm. In fact, biofilms provide an ideal environment for the establishment of syntrophic relationships. Syntrophism is a special case of symbiosis in which two metabolically distinct types of bacteria depend on each other to utilize certain substrates, typically for energy production. Syntrophic associations have been well studied with regard to methanogenic degradation (Schink, 1997; Bryant et al., 1967). Briyant et al., (1967) found that biofilms contain at least two different organisms. They syntrophically interact to convert ethanol to acetate and methane by interspecies hydrogen transfer. The fermenting bacterium is not able to grow on ethanol unless the hydrogen partial pressure is kept sufficiently low because the fermenting organism carries out a reaction that is endergonic under standard conditions. And the methanogen relies on the fermentative bacteria to provide it with an energy source. Therefore, the first reaction can only occur and provide energy for the methanogen if the hydrogen-scavenging methanogen maintains a low hydrogen partial pressure. Therefore, neither partner can grow on ethanol alone, but together they both efficiently derive energy.

CONCLUSION

Single-species or mixed-species biofilms, intercellular interactions and communication are undoubtedly required for biofilm development and persistence. Future challenges in biofilm research particularly need attempt to understand the complexity of the interactions within the biofilm community and to develop accurate and realistic models of natural communities. Communication between species may include extracellular compounds whose sole role is to influence gene expression, metabolic cooperatives and competition (possibly encompassing global changes in gene expression and metabolism), physical contact, and the production of antimicrobial exoproducts. One or all of these interactions may be occurring simultaneously. Collaborative effort will be able to fully discover these complex systems of the microbial world.

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