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Biofilm: A Sequential Analysis

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***Abstract*: Biofilm is the stepping stone towards the establishment of a hard fouling community. Often swept off as a slimy mass, they are a community in totality, posing grave threat to man and materials. The intricate process of biofilm development, including conditioning film formation, initial attachment and aggregation, maturation and subsequent dispersal has been discussed in detail in this review.**

***Keywords:* Biofilm; microfouling; conditioning film; microcolonies, exopolysaccharide.**

I. INTRODUCTION

Microfouling is a major component of biofouling, as much as macrofouling. While the hard macrofoulers cause visible damage, the microbes are equally harmful. The establishment of a '**biofilm**' is a pivotal step in the microfouling process. Biofilms are the oldest and most successful form of life on Earth with fossils dating back to 3.5 billion years and represent the first signs of life on Earth (Schopf et al., 1983). Biofilm is an assemblage of microbial cells that is irreversibly associated with a surface and enclosed in a matrix of primarily polysaccharide material (Donlan, 2002). "Biofilm" includes a wide variety of manifestations of microbial aggregates (Flemming, 2011). Clusters of different microbial populations are found in almost all moist environments where nutrient flow is available and surface attachment is possible.

In aqueous environments the majority of microbes grow as biofilms. These biofilms can be pathogenic, releasing harmful products and toxins, which become encased within the biofilm matrix. In marine environment biofilm is a surface accumulation which is not necessarily uniform in time or space and comprises of cells immobilised at a substratum, frequently embedded in an organic polymer matrix of microbial origin (Characklis et al., 1990). Of all the life forms, microbial biofilms are ubiquitous and most successful with highest survival potential.

In natural conditions, monospecies biofilms are relatively rare; thus most biofilms are composed of mixtures of micro-organisms. The exact structure of any biofilm is probably a unique feature of the environment in which it develops. Biofilm formation protects and enables single-cell organisms to assume a multicellular lifestyle, in which "group behaviour" facilitates survival in adverse environments (Maria, 2014). Since biofilms form under diverse conditions, and may be composed of single or multiple species, the structures of various biofilms will necessarily have distinct features. Nevertheless, biophysical, structural and chemical studies have led to a useful basic concept of a "biofilm model" (Costerton et al., 1995). In this model, microorganisms form microcolonies surrounded by copious amounts of exopolysaccharide. Between the microcolonies are water-filled channels, and it has been proposed that these promote the influx of nutrients and the efflux of waste products. The major component in the biofilm matrix is water up to 97% (Zhang et al., 1998).

In the ecological perspective, biofilms are involved in the biogeochemical cycles of virtually all elements and are carriers of the environmental "self-purification" processes. The process is always the same: microorganisms on surfaces convert dissolved or particulate nutrients from the water phase and/or from their support into metabolites and new biomass.

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II. A BRIEF HISTORY OF BIOFILM

The concept of biofilm starts from the microorganism. Hence it is considered that discovery of microorganism or bacteria must be the steppingstone for the development of biofilm. The major milestones in biofilm research are listed in Table I.

TABLE I. Major milestones in biofilm research

Year	Milestone
1684	Antonie van Leeuwenhoek was the first to display the animalcule (bacteria) found in the plaque of teeth.
1922	Angst reported that slime on the bottom of ships was caused to a large extent by bacteria, when biofouling occurred on surfaces.
1933	Henrici used direct microscopy to study biofouling in fresh water and he observed that 'it is quite evident that for the most part water bacteria are not free floating organisms, but grow attached upon submerged surfaces
1935	ZoBell and Allen studied the adherence and growth of bacteria on submerged glass slides in sea water
1940	Heukelekian and Heller observed the "bottle effect" for marine microorganisms and wrote about the development of bacterial slime and colonial growth attached to surfaces
1969	Jones et. al. Used scanning and transmission electron microscopy to examine biofilms on trickling filters in a wastewater treatment plant and showed them to be composed of a variety of organisms
1973	Characklis investigated microbial slimes in industrial water and revealed that biofilms were both tenacious and highly resistant to the antimicrobial effects of chlorine.
1978	Costerton et.al. published a theory of biofilms that explained the mechanisms whereby microorganisms adhere to living and non-living materials and the benefits accrued by this ecological niche.

After 1978 the study of biofilm became renowned to the scientific, medical, industrial and marine researchers. Hence many researchers focused to understand the mechanism of biofilm formation followed by the antifouling strategies being developed.

III. BIOFILM FORMATION

Biofilm formation is a cycle of events; it varies from place to place based on the availability of nutrients and suitability of the environment. Biofilm formation occurs in a sequential manner, involving formation of conditioning layer or film, initial attachment of microorganisms and biofilm maturation followed by dispersal of biofilm.

A. CONDITIONING FILM:

An important phenomenon in the initial adhesion of bacteria to non-living substrate is surface conditioning (Carpentier & Cerf 1993; Korber et al., 1995). Generally water contains a large number of organic, macro and micro molecules such as polysaccharides, proteins, lipids, humic acids, nucleic acids and amino acids resulting from the breakdown of dead organisms and animals (Fig. I). When a clean solid surface is immersed in water, it is quickly coated by a thin film of organic molecules. This is known as the conditioning film (Siboni et al., 2007). Loeb and Neihof (1975) were the first to report the formation of these conditioning films on surfaces exposed in seawater. These researchers found that films were organic in nature, formed within minutes of exposure, and continued to grow for several hours. The conditioning film is formed rapidly, as significant organic deposits have been detected after even 15 minutes of submergence. Film thicknesses ranging from 30 to 80 nm have been measured (Korber et al., 1995, Siboni et al., 2007).

The composition of the conditioning film differs depending on the environment. In order to understand the fouling process it is necessary to study the kinetics and composition of conditioning film, based on the composition of organic molecules the bacterial consortia may vary. Also based on the property of substrate, adsorption of organic molecules may differ. For example proteins were the first compounds followed by carbohydrates to adsorb onto the stainless steel panels immersed in seawater (Compere et al., 2001, Poleunis et al., 2002). Sometimes these organic molecules may react with the substratum and the chemical composition may be modified (Garg et al., 2009). A study by Jain and Bhosle (2009) showed that without the conditioning film, the number of bacterial cells that attach to a surface is significantly reduced. They also found that depending on the composition of the conditioning film, different species were more successful at attachment. For example the concentration of carbohydrates in a marine conditioning film was positively correlated to *Pseudomonas* species attachment, but negatively correlated to *Bacillus* species attachment. This may have been a result of the differences in cell hydrophobicity between the different species (Jain and Bhosle, 2009) or due to chemical attraction or

repulsion (Chet et al., 1975). Helke et al., 1993 found that proteins forming part of the conditioning film can competitively inhibit attachment of bacterial cells by occupying suitable binding sites on the attachment surface. However many of the molecules (such as some proteins) present in a conditioning film are made up of long chains of monomers which themselves may provide additional binding sites for microorganisms (Lappin-Scott and Costerton, 1995). As well as providing attachment sites for microorganisms, the conditioning film may increase attachment by altering the physical properties of the attachment surface like surface tension, surface free energy and surface roughness (Schneider, 1996, Callow and Callow, 2006).

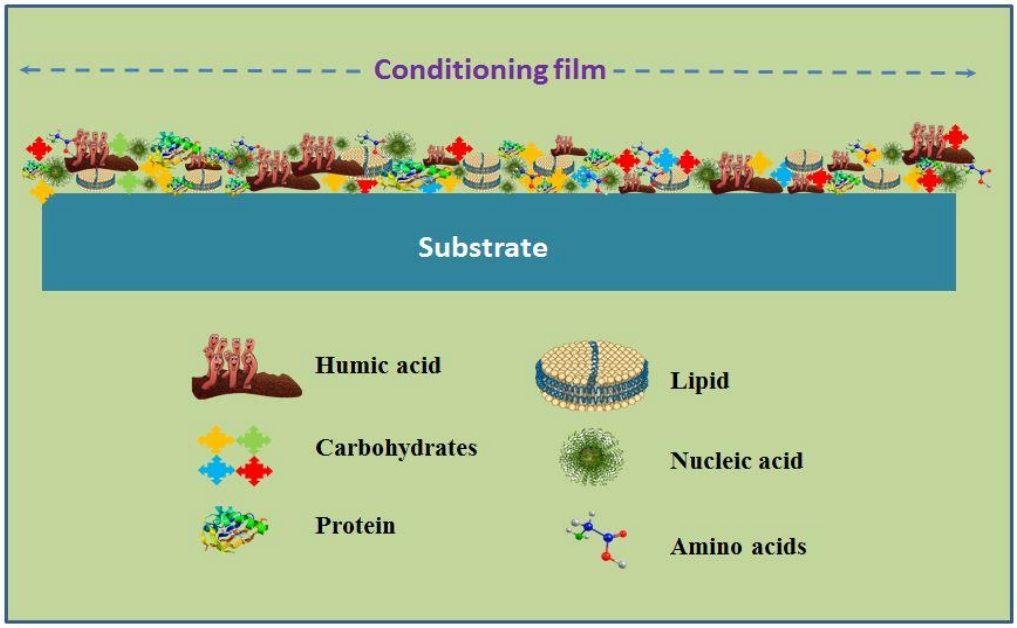

Figure I: Schematic diagram of conditioning film

B. INITIAL ATTACHMENT:

Biofilm formation is thought to begin when bacteria sense environmental conditions like physico-chemical properties and nutrients that trigger the transition to life on a surface (Fletcher and Pringle, 1986, Wimpenny and Colasanti, 1997). Microbial adhesion to surfaces is initially reversible, but even in the absence of metabolic processes becomes essentially irreversible shortly after first contact. Initial attachment may be single microbial species or multiple microbial species and can form on a range of biotic and abiotic surfaces. Initial adhesion between bacteria and non-living surfaces is usually mediated by non-specific (e.g. hydrophobic) interactions, whereas adhesion to living surfaces is usually accomplished through specific molecular docking mechanisms (Dunne, 2002). Korber et al., 1995 explained the interaction between the cell and the conditioned surface by two different theories (Wetting theory and DLVO theory). The "wetting" theory is based on surface thermodynamics, relies on determining critical surface tension of the bacteria and substratum. DLVO theory (named after Derjaguin and Landau, Verwey and Overbeek) equates repulsive and attractive forces acting on an adhering particle.

Microbial aggregation is an important initial phenomenon, which further leads to mature biofilm formation (Basson et al., 2008). Higher incidence of microcolonies, formed by autoaggregation as well as coaggregation, leads to enhanced biofilm formation (Windt et al., 2006). Autoaggregation is the term used to describe the adhesion of genetically identical cells (Elliot et al., 2006), while coaggregation is the highly specific recognition and adhesion of different bacterial species to one another (Min and Rickard, 2009). Macroscopically, the phenomenon can usually be detected as clumping when the different cell types are mixed. Microscopically, the clumps of cells formed consist of a network of interacting cell types (Kolenbrander et al., 1993). Coaggreagtion facilitates structural and metabolic codependences, subsequently resulting in the development of complex biofilm communities (Elliott et al., 2006).

The environmental signals vary among organisms. For example, *P. aeruginosa* and *P. Fluorescens* will form biofilms under almost any conditions that allow growth (O'Toole and Kolter, 1998). On the other hand, *Escherichia coli* K-12 (Pratt and Kolter, 1998) will not form biofilms in minimal medium unless supplemented with amino acids. Further Freter

and O'Brien, 1981 proved that *V. cholerae* has been shown to be chemotactic toward all amino acids, suggesting that proteins, peptides, or amino acids may be important nutrient sources in the aquatic environment. In contrast, *E. coli* O517:H7 is reported that needs low-nutrient media to make a biofilm (Dewanti and Wong, 1995).

Many studies have shown that sometimes the seasonal changes affecting the chemical composition of the conditioning film will impact the initial adhering of microbial community in the marine environment (Bakker et al., 2003; Bhosle et al., 2005). Initial interaction being established, stable connection between bacteria and substrate surface is maintained by specific cell membrane proteins and adhesins. If adhesion activity is inhibited, there will be no biofilm formation, which was proved by studies carried out by Pratt and Kolter, 1998 on *E. coli* and Watnick and Kolter, 1999 on *Vibrio cholera*.

C. BIOFILM DEVELOPMENT OR MATURATION:

Continued growth of bacterial cells on a surface leads to the development of mature biofilm colonies containing millions of tightly packed cells gathered into pillar and mushroom shaped masses that project outward into the surrounding medium for hundreds of microns (Hall-Stoodley et al., 2004)**.** The maturation of a biofilm, resulting in the complex architecture with water channels, is influenced by a number of biological factors and by hydrodynamic features (Stoodley et al. 2002). The biological factors include cell-to-cell signaling between the biofilm bacteria, growth rates of the bacteria, extent of Extracellular Polymeric Substances (EPS) production, motility of the biofilm bacteria as well as possible competition or cooperation between the bacteria.Once in initial contact with a surface, microbes develop different types of attachment behaviours such as motile attachment, reversible attachment and irreversible attachment. Bacterial mobility is enabled by two types of protein growths on the cell surface, flagella and fimbriae. Bacterial mobility enabled by flagella is necessary for establishing the connection between the bacteria and the surface, while the mobility enabled by fimbriae is necessary for the formation of microcolonies. Flagella are long, spiral growths that enable bacteria to float in liquid medium, and fimbriae are short, straight growths that enable limited, twitching movements of bacteria on substrate surface. Many studies have been conducted to look at the role of flagella on the development of bacterial biofilms (Hossain and Tsuyumu, 2006, Kim et al., 2008, Lemon et al., 2007). For example *Pseudomonas fluorescence* uses the motile attachment of flagellated cells to move along surfaces in a semi-attached condition within the hydrodynamic boundary layer, independent of the flow direction (Korber et al. 1995). The reversible adhesion of *E. coli* cells with residence times of over 2 min on a surface has been described as **"near-surface swimming" (**Vigeant and Ford, 1997). "Irreversible attachment" is exhibited when microbes can no longer move perpendicularly away from the surface (Busscher et al., 1998). Microbes can attach irreversibly, while retaining active motility by mechanisms known as gliding, swarming, twitching, swimming, darting and sliding. *Vibrio cholera* and *E. coli* first utilize the flagella to spread across the surface, and then anchor onto the surface with pili and possibly outer membrane proteins. *P. aeruginosa* requires type IV pili for twitching motion on a surface and for the subsequent build-up of stagnant microcolonies (O'Toole et al., 1998).

Once cells have adhered to the surface by any one of the kind of motility mentioned above, bacteria undergo further adaptation to life in a biofilm. They often proliferate by binary division to form clusters of microcolonies which spread over the surface (Lappin-Scott and Costerton, 1995). As the initial colonisers grow and divide, two properties are often associated with surface-attached bacteria, increased synthesis of EPS and the development of antibiotic resistance. Generally polysaccharides mediate cell-to-surface and cell-to-cell interactions that are critical for biofilm formation and stabilisation. The EPS matrix of a biofilm community can also contain microzones with different charge and hydrophobicity (Wolfaardt et al., 1998).

A mature biofilm with its complex architecture provides niches with distinct physicochemical conditions, differing e.g. in oxygen availability, in concentration of diffusible substrates and metabolic byproducts, in pH and in the cell density. Consequently, cells in different regions of a biofilm can exhibit different patterns of gene expression (Costerton et al., 1999). The development of a biofilm is a highly regulated process, for which communication between cells is essential. Many bacteria possess ability to communicate with one another and to organize into communal groups with characteristics not exhibited by individual cells (Greenberg, 1999). Bacteria produce diffusible extracellular signaling molecules, e.g., Acylated homoserine lactones (AHLs; gram-negative bacteria), cyclic oligopeptides (gram-positive bacteria) to monitor their own population density and to coordinate expression of specific sets of genes in response to the cell density (Waters and Bassler, 2005). This type of cell-density-dependent gene regulation is termed quorum sensing. Watnick and Kolter (2000) summarised that a mixed species biofilm is a dynamic community harboring bacteria that stay and leave with purpose, compete and cooperate, share their genetic material, and fill distinct niches within the biofilm.

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They stated: "The natural biofilm is a complex, highly differentiated, multicultural community much like our own city" (Watnick and Kolter, 2000).

IV. BIOFILM DISPERSAL

Dispersal of biofilm denotes the disassociation of bacteria from the matured biofilm to colonise a new surface. The bacterial biofilm dispersal can be divided in to three distinct phases

(i) Detachment of cells from the biofilm colony

(ii) Translocation of the cells to a new location; and

(iii) Attachment of the cells to a substrate in the new location

Various factors are responsible for the dispersal of bacteria in biofilm, including physical, environmental and extracellular secretion. Additionally cell death often becomes the reason for dispersal of biofilm. In general, mechanisms of biofilm dispersal can be divided into two broad categories: active and passive. Active dispersal refers to mechanisms that are initiated by the bacteria themselves, whereas passive dispersal refers to biofilm cell detachment that is mediated by external forces (Lawrence et al., 2002; Choi and Morgenroth, 2003). The two type of biofilm dispersal mechanism is successfully carried out by following the three distinct mode of dispersion such as erosion, sloughing and seeding. Erosion refers to the continuous release of single cells or small clusters of cells from a biofilm at low levels over the course of biofilm formation. Sloughing refers to the sudden detachment of large portions of the biofilm, usually during the later stages of biofilm formation.The process of dispersal from the interior of microcolonies has been termed "seeding dispersal" which is the passive removal of cells from the biofilm by fluid shear (Kaplan, 2010). Seeding dispersal has been well extensively studied in oral bacterium *Aggregatibacter actinomycetemcomitan*s (Kaplan et al., 2003) and in *P. aeruginosa* biofilms (Sauer et al., 2002; Hunt et al., 2004; Schooling et al., 2004)

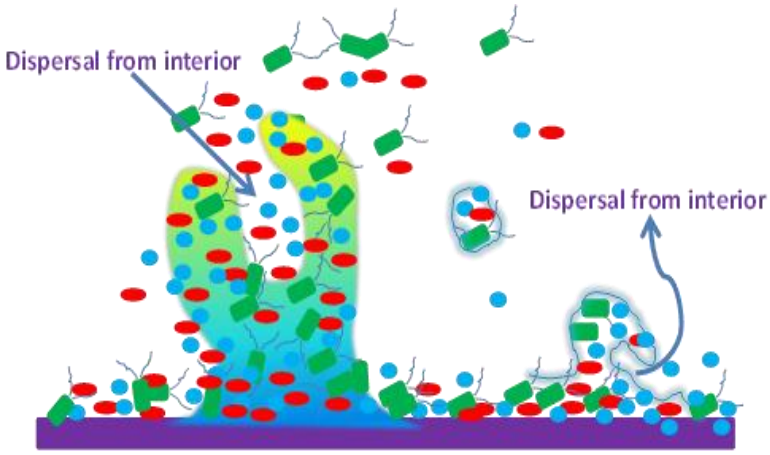

Figure II: Schematic diagram of seeding dispersal

Erosion and sloughing can be either active or passive processes, whereas seeding dispersal is always an active process.The physical processes such as shearing result in sloughing or erosion of clusters of cells. Environmental cues are also sometimes responsible for the dispersal of biofilm; for example nutrient starvation can induce dispersal in *P. aeruginosa* biofilms (Gjermansen et al., 2005, Hunt et al., 2004). On the contrary, Sauer and colleagues (2004) showed that rapidly increasing nutrient availability can also induce dispersal in *P. aeruginosa.* Some of the dispersal events are regulated by the several inter- or intra-cellular signalling mechanisms including quorum sensing systems. Increased levels of the intracellular messenger cyclic-di-GMP determine the transition from planktonic to biofilm growth, while a reduction causes biofilm dispersal (Chua et al., 2014). Extracellular secretion of polysaccharides, enzymes, antimicrobial peptides, anti-adhesion agents, and chelating agents play a vital role in the culmination of biofilm processes. A pattern of cell lysis within biofilms has now been observed as a normal feature of development across a broad range of biofilm forming bacteria. In biofilms, cell death commonly occurs with spatial organization inside mature microcolony structures, and kills only a proportion of cells within the biofilm which facilitates conversion of surviving cells to the motile dispersal phenotype. It was believed that cells dispersed from biofilms, immediately go into the planktonic growth phase. Recently,

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Chua et al (2014) proved that when expression of the small regulatory RNAs RsmY and RsmZ is down regulated, secretion genes are induced in dispersed cells. In connection with this they found that the physiology of dispersed cells from *Pseudomonas aeruginosa* biofilms is highly different from those of planktonic and biofilm cells using single nucleotide resolution transcriptomic analysis (Fig. III)

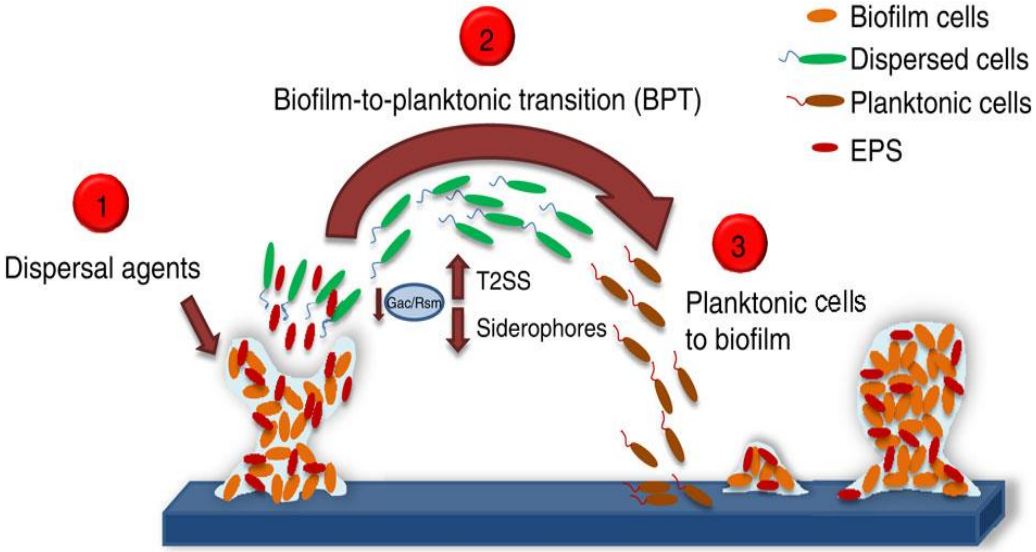

Figure III: The process of biofilm-dispersal, stage by stage (Chua et al., 2014)

V. BIOFILM CONTROL MEASURES

Extracellular polysaccharides secreted by the bacteria exhibit anti-biofilm properties by a) altering the physical characteristics of bacterial cells or abiotic surfaces, b) acting as signalling molecules that impact the gene expression patterns of susceptible bacteria or c) competitively inhibit multivalent carbohydrate–protein interactions, thereby interfering with adhesion (Abdel-Aziz and Aeron, 2014). Enzymes that degrade biofilm extracellular matrix may play a role in biofilm dispersal and may also be useful as anti-biofilm agents. For example, Dispersin-B is a glycoside hydrolase that cleaves β 1–6 N-acetyl glucosamine polymer in the bacterial peptidoglycan layer. Dispersin-B treatment has been shown to be effective against *S. aureus* and *S. epidermidis* biofilms and bacteria (Kaplan, 2010). Chelating agents of Sodium citrate, tetrasodium-EDTA and lytic peptides of antimicrobial peptides have been shown to destabilize biofilm architecture besides interfering with bacterial membrane stability.

VI. CONCLUSION

The study of biofilms has grown markedly in recent years due to increased awareness of the pervasiveness and impact of biofilms on natural and industrial systems, as well as human health. Understanding the mechanism of biofilm formation will be a significant approach to solve the problem of biofilm formation.Containment of biofilm formation will eventually slow down macrofouling assemblage, since biofilms provide a conducive environment for the settlement of larval forms. On the whole, the sequence of biofilm formation is an extended process which significantly varies with changes in environmental factors, and therefore requires monitoring at each stage to device successful control mechanisms.

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