Current Research in Microbiology

Chapter 1

Microbial Biofilms

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1. Introduction and Historical Perspective

Biofilm exhibit two types of growth mode i.e. planktonic cell and sessile aggregate. In biofilm (association of micro-organisms), cells stick to each other on a surface encased within matrix of extracellular polymeric substance produced by bacteria themselves [1]. A Dutch researcher, Antoni van Leeuwenhoek, for the first time observed 'animalcule' on surfaces of tooth by using a simple microscope and this was considered as the microbial biofilm discovery [2]. For marine microorganism i.e. bacterial growth and activity were substantially enhanced by the incorporation of a surface to which these microorganisms could attach is known as "bottle effect" observed by Heukelekian and Heller [3]. Zobell observed that the number of bacteria on surfaces was higher than in the surrounding medium [4]. Zo Bell introduced first about multicellular prokaryotic communities on submerged surfaces who stated the presence of adherent microbial associations in all natural environments [5,6].

The extensive physical and chemical analysis of bacterial biofilms did not begin until the late 1960s and early 1970s, when some of the investigators identified the extensiveness of bacterial biofilms. Scanning and transmission electron microscopy was used by Jones *et al.* to examine biofilms on trickling filters in a wastewater treatment plant and showed them to be composed of a variety of organisms (based on cell morphology). By using a specific polysaccharide-stain such as ruthenium red when coupled with osmium tetroxide fixative to show that the matrix material surrounding and enclosing cells in these biofilms was polysaccharide. In 1973 Characklis studied microbial slimes in industrial water systems and reported that they were not only adhering very closely but also highly resistant to disinfectants such as chlorine. Costerton *et al.* in 1978 gives a theory of biofilms based on observations of dental plaque and sessile communities in mountain streams that explain the mechanisms whereby microorganisms adhere to living and nonliving materials and the benefits arises by

this ecologic niche [7-9].

Costerton and Geesey specified that glycocalyx acted as an ionic exchange matrix, trapping nutrients that were transported into cells by highly efficient permeases [10]. In 1981 glycocalyx was characterised as a hydrated polyanionic polysaccharide matrix which is produced by polymerases that is attached to the lipopolysaccharide component of the bacterial cell wall. Biofilm production of glycocalyx in aqueous environment is prevalent with organic and inorganic nutrients being concentrated at the solid/liquid interface. The glycocalyx provides a physical/chemical barrier, offers partial protection against antibacterial agents [11]. The structures of different biofilms have distinct features because it forms under diverse conditions and composed of single or multiple species. The study related to biophysical, structural and chemical properties of biofilm have led to a useful basic concept of "biofilm model" [12].

The important advances of the development and behavior of biofilms were made in 1998, when molecular genetics approaches combined with confocal laser scanning microscopy (CLSM). Traditionally, microbiologists have performed physiological experiments with microorganisms grown in liquid monocultures where the cells are "free swimming" or planktonic [13]. It is now widely accepted that 99% of all micro-organisms attach to a surface and grow as a bioflim. An important survival strategy for micro-organisms in the healthcare environment is the growth of biofilm mode. According to the Centers for Disease Control and Prevention, the association of biofilms is approximately 65% of all healthcare-associated infections. Thus, their presence in medical devices, chronic wounds and surgical site infections is of growing concern [14].

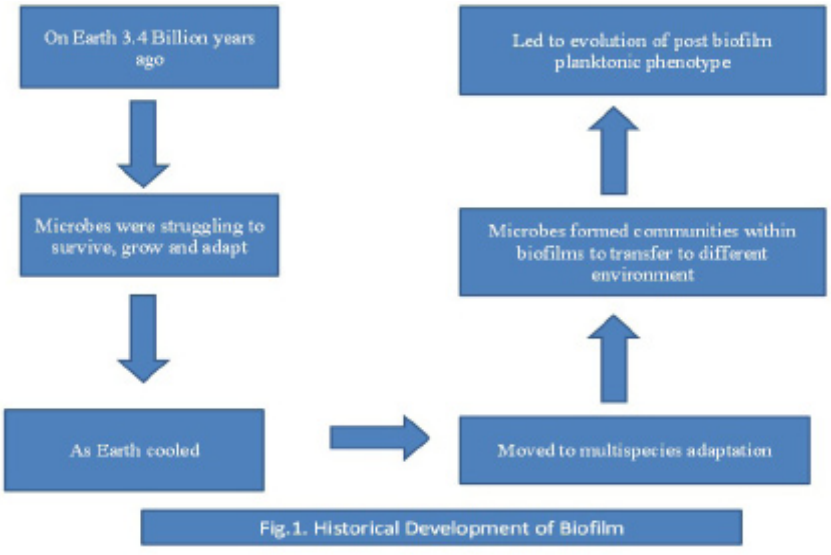

Figure 1: Historical Development of Biofilm

2. Definition

Many novel, organic compounds have been developed in last few years that are released into the environment. These compounds include heavy metals, poly-aromatic hydrocarbons, polychlorinated biphenyls, pesticides, chemical fertilizers, detergents, paints, disinfectants,

lubricants, antibiotics and nanoparticles. Many of them are toxic to humans and other organisms. Managing the harmful effects of these pollutants is a challenge to sustainable development globally. Using biofilms in bioremediation can allow new technologies to remain environmentally sustainable if integrated methods are correctly developed and applied [15]. Biofilms vary greatly in structure and composition from one environmental condition to another so that they are not easily defined. Microbial biofilms are extremely complex microbial ecosystems consisting of microorganisms attached to a surface and embedded in an organic polymer matrix of microbial origin. Non-cellular materials such as mineral crystals, corrosion particles, and clay or silt particles, blood components may also be found in the biofilm matrix. Therefore biofilm may be defined as "microbial cells immobilized in a matrix of extracellular polymers acting as an independent functioning ecosystem, homeostatically regulated" [16]. Biofilm is a community of bacteria that attach to a surface by excreting a sticky, sugary substance that encompasses the bacteria in a matrix. Bacteria, fungi and protists are the microorganisms that form biofilms. Biofilms are complex systems that are sometimes compared to multicellular organisms. Biofilms have been found growing on minerals and metals. They have been found underwater, underground and above the ground. They can also grow on plant tissues and animal tissues, implanted medical devices such as catheters and pacemakers etc [17,18]. Bacterial biofilms can be considered to be an emergent form of bacterial life, in which communal life is completely different from bacteria that live as free-living cells [19]. Biofilms may form on living or non-living surfaces and can be prevalent in natural, industrial and hospital settings [1,20]. The microbial cells grows on biofilm are physiologically distinct from planktonic cells of the same organism, which, by contrast, are single-cells that may float or swim in a liquid medium [21]. The morphological structures of biofilm are shown in **Figure 2** [22].

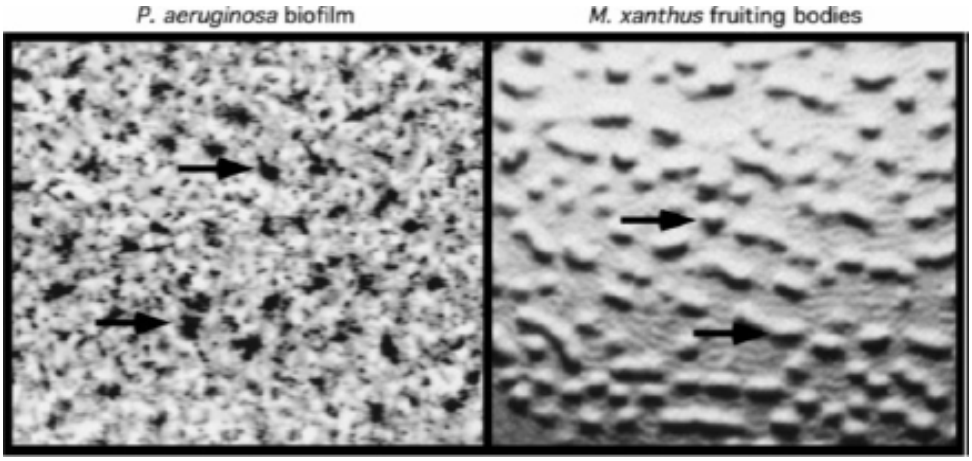

Figure 2 The morphological similarity in the structure of a *P. aeruginosa* biofilm and a *Myxococcus* fruiting body is evident in these top-down photographs. Both organisms form distinct aggregates of cells that are well separated from their neighbors. *Left*: 8-h-old biofilm of *P. aeruginosa* grown on PVC plastic at 400_ magnification. *Right*: Fruiting bodies of *Myxococcus xanthus* after 6 h on starvation agar plates at 5_ magnification. Microcolonies and fruiting bodies are indicated by *arrows.*

3. Classification of Biofilms [23].

3.1 On basis of its location:

a. Supragingival - Present coronal to the gingival margin

b. Subgingival - Present apical to the gingival margin

3.2. On basis of pathogenicity

a. Cariogenic - Generally acidogenic and gram-positive

b. Periopathogenic - Mostly basophilic and gram-negative

4. Composition of Biofilm

A biofilm comprises any syntrophic consortium of microorganisms in which cells stick to each other and also to a surface. These adherent cells become embedded within a slimy extracellular matrix that is composed of extracellular polymeric substances (EPS). The cells within the biofilm produce the EPS components, which are typically a polymeric conglomeration of extracellular polysaccharides, proteins, lipids and DNA [1, 24, 25]. They have been described (metaphorically) as "cities for microbes" because they have three-dimensional structure and represent a community lifestyle for microorganisms [26, 27]. The EPS has a complex biochemical composition, comprising predominantly carbohydrates and proteins, although lipids and extracellular DNA (eDNA) have also been identified [28], along with exogenous inorganic or organic substances which may become entrapped within the EPS, for example, iron or manganese [29]. EPS primarily composed of polysaccharides and may vary in chemical and physical properties. For the EPS gram-negative bacteria, some of these polysaccharides are neutral or polyanionic. The presence of uronic acids (such as D-glucuronic, D-galacturonic, and mannuronic acids) or ketal-linked pryruvates confers the anionic property of EPS [30]. This property allows association of divalent cations such as calcium and magnesium, which have been shown to cross-link with the polymer strands and provide greater binding force in a developed biofilm [31]. A biofilm is an immobile microbial community composed of cells immersed in a matrix of EPS attached to a substratum or interface. Essentially the matrix is of microbial origin and the cells encased in this matrix present a modified phenotype, especially with regard to growth rate and gene transcription [32]. The term of *slime* was used to define the glycocalix produced by the strongly adherent strains of *Staphylococcus epidermidis* isolated from the infected surface of medical implants [33, 34].

Biofilms are group or micro-organisms in which microbes produced extracellular polymeric substances (EPS) such as proteins including enzymes, DNA, polysaccharides and RNA and in addition to these components water (up to 97%) is the major part of biofilm which

is responsible for the flow of nutrients inside the matrix of biofilm. The complex structure of biofilm consists of two main components i.e. water channel (for transport of nutrients) and densely packed cells (a region having no prominent pores in it) [35]. The components of biofilms (Table 1) have the capacity to make it resistant against various environmental factors and signify the biofilm integrity [36,37]. The chemical composition of biofilm is shown in **Table 1**.

Table 1: Chemical Composition of Biofilms

S. No.	Components	Percentage
1	Microbial cells	2-5%
2	Polysaccharides	1-2%
3	DNA and RNA	<1-2%
4	Proteins including enzymes	<1-2%
5	Water	Up to 97%

5. Role and Importance of Biofilm in Different Field

5.1 In Medical Field

Microorganisms are able to adhere to various surfaces and to form a three-dimensional structure known as biofilm. Bacteria embedded in the biofilm can escape and form well known planktonic cells (free flowing bacteria in suspension), that are only a part of the bacterial life cycle. Bacteria also adhere to medical devices such as catheters, either urinary or intravenous, artificial heart valves, orthopedic implants that causes device-related infections like cystitis, catheter-related sepsis, endocarditis etc. Once a biofilm has been established on a surface, the bacteria hold inside are less exposed to the host's immune response and less susceptible to antibiotics. As an important cause of nosocomial infections the biofilm must remain in the centre of the microbiologist's attention [38].

The fourth leading cause of death in the United States is nosocomial infections (infections acquired at a hospital). About 65% of these infections are due to biofilms on implanted medical devices [39]. Biofilms differ from an infection of planktonic bacteria is due to the EPS matrix of the biofilm, which is important in cell adhesion and aggregation. This EPS matrix also hinders the normal functions of antibodies and the phagocytic cells of the host's immune system [40]. Another key factor that makes biofilms particularly difficult in medical situations is their heightened resistance to antibiotics. There are three proposed methods [41]:

a**.** The antibiotic is deactivated faster than it can diffuse and also not able to penetrate the surface layers of the biofilm.

b.The different chemical environments of biofilm can affect the action of the antibiotic. The cause of non-growing state of bacteria is low level of nutrients in the lower layers of biofilm.

c. About 1% of the population may exhibit a phenotypic state (which persists under continued exposure to an antibiotic), even when the biofilm is too thin to inhibit diffusion of the antibiotic or of nutrients.

Because of these properties, cells (exist in biofilms) can be 1000 times more resistant to antimicrobial agents than the same cells in planktonic form. Cells at the surface of the biofilm can infect the host when detach from the biofilm matrix. Therefore, biofilms can act as a reservoir of protected bacteria (on inserted medical devices) often persists until the removal of the infected devices [42, 43]. To get rid completely from the Infections associated with the biofilm growth are challenging task due to the fact mature biofilms display tolerance towards antibiotics and the immune response. The rapidly growing industry for biomedical devices and tissue engineering related products is already at $180 billion per year worldwide. These industries continue to suffer from microbial colonization [44, 45]. Various microorganisms developed on medical devices are shown in **Table 2**.

Table 2: Microorganisms associated with biofilm developed on medical devices

S. No.	Microorganism	Medical Devices
1.	*Psudomonas aeuginosa*	Artificial hip prosthesis, Central venous catheter, Urinary catheter
2.	*Candida albicans*	Artificial hip prosthesis, Central venous catheter, Prosthetic heart valves, Intra-uterine devices
3.	*Staphylococcus aureus*	Artificial hip prosthesis, Central venous catheter, Prosthetic heart valves, Intra-uterine devices
4.	*Enterococcus Spp.*	Artificial hip prosthesis, Urinary catheter, Prosthetic heart valves
5.	*Klebsiella pneumoniae*	Central venous catheter, Urinary catheter
6.	*Coagulase-negative staphylococci*	Central venous catheter, Urinary catheter, Intra-uterine devices, Prosthetic heart valves

5.2. In Industry

Biofilm formed when bacteria are able to attach to and colonize environmental surfaces which allow the organisms to persist in the environment and resist desiccation, UV light and treatment with antimicrobials and sanitizing agents. Biofilms are formed when microbes attach to a solid support and to each other by extracellular polymeric substances (EPS) on a wide variety of surfaces including metal, plastic, rock and living or dead tissue. Bacteria can be several orders of magnitude in biofilm which is more resistant to antimicrobials than their planktonic forms [46]. In marine and other aquatic environments algae, diatoms and bacteria that are able to attach and form biofilms on ships' hulls and become resistant to the different antifouling paints (developed to prevent the initial colonization) results in increased fluid frictional resistance and fuel consumption. In the food industry, contamination of food processing and/or food contact equipment often leads to post-process contamination and reduce the shelf life of products [47].

Biofilms can also be utilized for useful purposes. Sewage treatment plants include a secondary treatment stage in which waste water passes over biofilms grown on filters which extract and digest organic compounds. In such condition of biofilms, bacteria are mainly responsible for removal of organic matter, while protozoa and rotifers are mainly responsible for removal of suspended solids, including pathogens and other microorganisms. Slow sand filters depends on biofilm development to filter surface water from lake, spring or river sources for drinking purposes. To eliminate petroleum oil from contaminated oceans or marine systems, biofilms can be helpful by the hydrocarbon degrading activities of microbial communities [48]. Biofilms are used to generate electricity from a variety of starting materials, including complex organic waste and renewable biomass in the form of in microbial fuel cells (MFCs). Biofilms are also used to enhance the metal dissolution in bioleaching industry [49-52].

5.3. In Food industry

Biofilm formation is a dynamic process in which various mechanisms are involved in their attachment and growth. Biofilms have been a matter of interest in the context of food hygiene. If the microorganisms from food-contact surfaces are not completely removed, they may lead to form biofilm which increases the biotransfer potential [53]. Biofilms are complex microbial ecosystems formed by one or more species immersed in an extracellular matrix of different compositions which depends on the food manufacturing conditions and the colonizing species [54]. The formation of Biofilms in food industry environments is very fast. The first two steps are; a) the conditioning of the materials surfaces b) the reversible binding of the cells to that surface. The binding becomes irreversible that causes development of microbial colonies. Finally, the tridimensional structure of biofilm is formed, and this complex ecosystem is ready for dispersion [55-57]. The extracellular matrix is mainly composed of polysaccharides, such as cellulose, proteins or exogenous DNA and it can be fixed to hard surfaces such as food industry equipment, transport, dispensing and storage surfaces, soil, etc. or to biological structures viz. vegetables, meat, bones, fruits. The extracellular matrix is responsible for the strong persistence of these biofilms in the food industry. This generates complex gradients with respect to nutrients and oxygen diffusion, contains extracellular enzymes used for nutritional purposes. These complex gradients allow for the transfer of cell communication molecules, and protect the embedded cells against toxic compounds [58].

The biofilm layer is found on the mesocarp inherently formed by various yeast and lactic acid bacteria. These bacteria and yeast strains play a role in the fermentation of olive and also they become a dominant flora on the fruit which prevents the olive from microbial spoilage originated by Gram negative bacteria. In this regard, the quality and safety of the table olive and also the taste and flavour of the last product has been determined by biofilm forming microorganisms found on the mesocarp of the fruit. Biofilm forming ability is a desired property of fermented fruit products [59]. Beneficial effect of the biofilm formation is about the

yeast strains used commonly in the food industry. Some yeast species having biotechnological relevance such as *Saccharomyces cerevisiae* might regulate the QS type. In the QS mechanism of the yeast strains, aromatic alcohols are the most observed signal molecules which can result in modification and improvement of industrial processes [60]. The microbial interactions have an importance for food industry. Fermentation, brewing and cheese ripening are some areas where microbial interactions have been observed. Mixture of fungi, yeast and bacterial species play a led role in the production of wine, from ripening of grapes in vineyards to wine bottling. The growth of some bacterial species, such as *Leucobacter* sp. or *Brevibacterium aurantiacum*, significantly relies on the presence of the yeast [61].

5.4 In aquaculture

Aquaculture is defined as the production of aquatic plants and animals and this is a fastest growing food industry (FAO-Fisheries and Aquaculture Department 2012). Aquaculture has expanded 12-fold with an annual growth rate of 8.8% and this data was observed during the last 30 years. In 2010, it has reached a total volume of 60 million tonnes per year. A major share of global aquaculture production is covered by freshwater fish (56.4%), most notably by carp culture in China (16 million tonnes). Approximately 38% of the total aquaculture production is from marine aquaculture [62]. In fish culture, presently the most common form is floating net cages, which contains large amounts of fish at minimal costs. Tank and pond cultures are more expensive; however they are easier to access and are thus the best choice for labor-intensive cultures such as larvae, juveniles and brood stock. A re-circulating aquaculture system are a further development of pond or tank cultures and is a relatively new culture technique that presupposed the availability of durable technical equipment as well as biological and technical knowledge originating from wastewater treatment research [63]. Microbial community of biofilm occurs in blocks of 20-60u in water and sediment, harvestable by many planktonic fish like silver carp, rohu, catla, mullets and milkfish. The microbial community flourishes using organic and mineral fractions of organic manure as source of energy and nutrients. Fishes are able to harvest these organisms directly in significant quantities. The microbial film coating that is relatively indigestible substrate of the detritus and it is digested while the substrate itself passes through the fish gut which then get re-colonized by microbes and re-harvested by fish [64]. Numerous studies have shown that biofilm can be a reservoir for potentially pathogenic bacteria in freshwater aquaculture [65, 66].

6. Functions of Biofilm in Microbial Communities

6.1 Environmental protection

Extracellular Polymeric Substances (EPS) plays different roles in structure and function of biofilm communities. EPS act as an anion exchanger to prevent the access of certain antimicrobial agents into the biofilm. It restricts the diffusion of compounds from surroundings

into the biofilm. Antibiotics that are hydrophilic and positively charged such as amino-glycosides show more pronounced attraction towards this effect. EPS has also been reported to sequester metal ions, cations and toxins that provide protection from variety of environmental stresses such as pH-shift, UV radiation, osmotic shock and desiccation [34, 67-69].

6.2 Availability of nutrients

The effective means of exchanging nutrient and metabolites is water channel. Aqueous phase enhances the availability of nutrient and also removes the potentially toxic metabolites. Fermentive bacteria produce acids and alcohols initiated by the process of catabolism, which are then utilized as substrate by acetogenic bacteria. Biofilms provides an ideal environment for the establishment of syntrophic relationship. Syntrophism is a symbiosis in which two metabolically distinct bacteria depends on each other to utilize certain substrates typically for energy requirements [70, 71].

6.3. Acquisition of new genetic trait

Acquisition of new genetic trait gives chances to the microbial communities to transcribe the necessary games to become the active member of biofilm communities. The production of alginate which involves the transcription of algC gene is increased approximately fourfold in biofilm associated cells as compared to planktonic cells [12, 72].

6.4. Penetration of antimicrobial agent

Diffusion is the rate limiting step to inactivate the biofilm forming microbial community by antimicrobial agents. EPS acts as diffusion barrier for these molecules that influences the rate of transport of the reaction of antimicrobial agents with the matrix material. Advantages of biofilm growth towards the microbial community are:

a. As the growth is restricted all the energy is used up by the bacteria in making the EPS that will give protection to the microbial community [73].

b. As the growth is restricted, bacteria will remain in dormant stages that will give protection to the microbial community against antibiotics (most of the antibiotics are active against the growth phase of the bacteria) [74].

7. **Formation of Biofilm**

Biofilm formation begins with planktonic (free-swimming) bacteria which can attach to a variety of surfaces, from woods, metals, and plastics to living tissues and stagnant water. The cells are excreted a sugary molecule called extracellular polymeric substance or EPS has a strand-like structure that holds the cells together and attaches them to the surface and creating a matrix. This matrix of cells and strands can be quite complex: the cells may share

genetic material and have organized structure. A biofilm can be as thin as a single cell or as thick as several inches depend on the conditions of the environment. Biofilms become mature and thickens as they grows and develop. In the presence of sufficient water and nutrients, the biofilm will develop until small portions detach and float to another surface and colonize [75]. Complex process of biofilm formation involves several distinct phases start with adsorption on to the tooth surface of a conditioning film derived from bacterial and host molecules forms tooth eruption or tooth cleaning. This adsorption process is followed by passive transport of bacteria mediated by weak long-range forces of attraction. Covalent and hydrogen bonds create strong, short-range forces that result in irreversible attachment. The primary colonizers form a biofilm by auto-aggregation (attraction between same species) and co-aggregation (attraction between different species). Co-aggregation results in a functional organization of plaque bacteria and formation of different morphological structures such as Corncobs and Rosettes. The microenvironment now changes from aerobic/capnophilic to facultative anaerobic. The attached bacteria multiply and secrete an extracellular matrix (EPS), which results in a mixed-population of mature biofilm. Organization takes place within biofilm after one day. Formation of a climax community takes place during transmission that occurs from other sites, leading to incorporation of new members into the biofilm.The thickness of the plaque increases slowly with time, increasing to 20 to 30 μm after three days [76].

The formation of a biofilm begins with the attachment of free-floating microorganisms (Planktonic) to a surface [77]. The first colonist bacteria of a biofilm may adhere to the surface initially by the weak van der Waals forces and hydrophobic effects. If they are not immediately separated from the surface, they can anchor themselves more permanently using cell adhesion structures such as pili. Hydrophobicity affects the ability of bacteria to form biofilms. With increased hydrophobicity bacteria have reduced repulsion between the substratum and the bacterium. Bacteria with increased hydrophobicity have reduced repulsion between the substratum and the bacterium. Motile bacteria can recognize surfaces and aggregate together easily than non-motile bacteria. Bacteria cells are able to communicate using quorum sensing (QS) products such as N-acyl homoserine lactone (AHL) during surface colonization process. Bacterial biofilms encloses polysaccharide matrices that also contain material from the surrounding environment [78]. Biofilms are the product of a microbial developmental process. The diagram of biofilm formation is shown in Figure 3 [79].

The process is summarized by five major stages of biofilm development [80]:

1. Initial/ reversible attachment (binding of 1st colonist)

2. Irreversible attachment (they anchor themselves using pili)

3. Maturation I (inter communication through quorum sensing)

4. Maturation II/Development (final stage of modification)

5. Dispersion (essential stage for biofilm formation and life cycle)

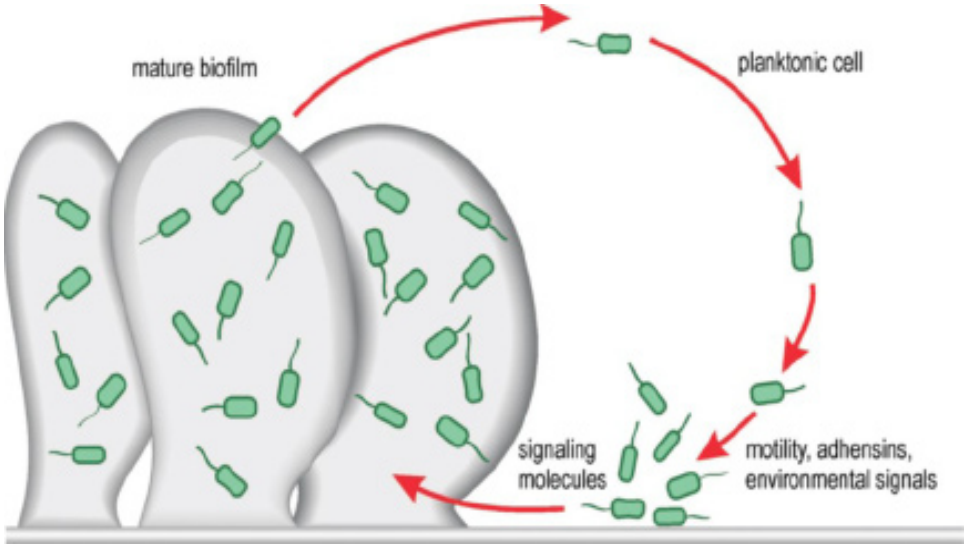

Figure 3: Biofilm Formation

8. Chracterization/Evaluation of Biofilm

Most commonly used methods of biofilm characterization are quantitative characterization and qualitative characterization.

8.1 Quantitative Characterization Methods

Biofilm dynamics and complex architecture creates challenges for basic measurements regarding the number of viable cells, mass accumulation, biofilm morphology, and other critical properties. These challenges are not in the measurements themselves but in the lack of standardized protocols for characterization and uniform training availability for individuals. One of the most basic and most commonly acquired types of bacterial measurements, whether in planktonic or biofilm cultures is the determination of how much is present. A variety of direct and indirect methods have been used to quantify cells in biofilms [81].

8.1.1. Direct Quantification Methods

Direct counting methods permit enumeration of cells that can be cultured, including plate counts, microscopic cell counts, Coulter cell counting, flow cytometry, and fluorescence microscopy. Direct methods for biofilm quantification are those that rely on direct observation for quantification of the desired parameter (number of cells, total biofilm volume, etc.). Imaging and automated cell counting are the most common methods of biofilm quantification. Furthermore, the use of stains or fluorescent markers, in order to more accurately identify cells of interest and distinguish from culture debris, allow for easier and increased accuracy of cell counting and data interpretation. Imaging methods, including light and confocal microscopy provide manual platforms to count cells and determine total biofilm volume. Instruments

incorporating flow, such as automated cell counters and flow cytometers, provide mechanized methods. Different direct methods for the characterization of biofilm are [82]:

8.1.1.1. Plate Count Method (colony forming units/ml or CFUs)

This method is used for the determination of viable cell numbers by aka CFU/ml assay or aerobic plate count [83-86]. This assay is used to separate the individual cells on an agar plate and grow colonies from cells, therefore differentiates and quantifies living from dead cells without use of dyes or instrumentation. The first step of this procedure starts with a liquid planktonic culture or a mature biofilm which is suspended and homogenized in liquid medium via scraping, vortexing or sonicating. The plating method involves the aseptic removal of aliquots of the suspended biofilm, followed by serial dilution and plating onto nutrient agar. After 24-72 hours (when incubation is complete) colonies are counted on the plates and the number of cells per milliliter (cfu/mL) are calculated using the mean colony counts. During the process it is important to note the incubation time and keep it uniform to expand each culture by the same amount. It is advisable to have an experiment control with no treatment [85]. Optical density (OD) can be measured prior to plating to obtain a calibration curve used to correlate cell number and absorbance in pure culture by enumeration method. Thereby absorbance of a sample of unknown cell number can then be measured to determine the cell concentration [87, 88]. The CFU technique can be performed by trained individual in laboratory scale and does not require highly specialized advanced equipment. However, this technique is time and labor intensive, sometimes require days to perform enough replicates to obtain reproducible results. This technique is also vulnerable to counting error especially when the given number of colonies is high and/or the count is done manually [89].

8.1.1.2. Flow-based Cell Counting

In this method cells in liquid culture flow through narrow apertures and are measured as they pass. Coulter counting and flow cytometry both require homogenized and suspended biofilm in liquid cultures. The Coulter method involves passing of charged particles in an electrolyte solution through an aperture (part of an electrical circuit). Flow cytometry gives more information about cells during measurement while Coulter counters are less expensive [90, 91]. The voltage pulses are then counted over a period of time and correlated with cell number. This technique is very simple but cannot differentiate live and dead cells [92]. In flow cytometer technique, cells flow through a narrow opening (to pass through single file). A laser is requred to detect the cells as they pass via scattering, absorbance or intrinsic and extrinsic fluorescence measurements. The major advantages of this method are the speed, simplicity and accuracy associated with measurements. Additional information about the cells also gathered by using this method including the cell dimensions, surface properties metabolic activity and the differentiation state of the cells with endogenous fluorescent tags (such as GFP). The

main disadvantage of this method is the cost of the instrument approximate between $50,000-100,000 [93].

8.1.1.3. Light and Fluorescence Microscopy

Biofilm 3D characterization and cell counting can be done by using several microscopy methods ranging from simple light microscopy to confocal laser scanning microscopy (CLSM) [81].

Compound light and fluorescence microscopes

Small structures of bacterial cells can be visualized by a compound light microscope. Resolution of bacterial cells (2-8μm in length) requires total magnification of 200x or greater. Use of Contrast enhancement methods such as phase contrast or differential interference contrast (DIC) can improve total quality of the images. Fluorescence microscopy enlarges the optical capabilities of light microscopy to intrinsic or added fluorescent light emission [94].

Confocal laser scanning microscopy

Confocal laser scanning microscopy (CLSM) produces high-resolution, sharp images of biofilms in three dimensions [97-100]. The area of focus is scanned across the sample to produce high-resolution 2-D “slices” at various heights that are assembled to produce a final 3D image. Confocal microscopy can utilize single or multiple excitation lasers to view multiple fluorescent markers simultaneously. These instruments also require experienced and highly trained users for accurate measurement and analysis [95].

Fluorescent dyes and proteins

Intrinsic biomolecules, such as NADH and NAD(P)H or chlorophyll which have fluorescent properties can be used in fluorescence microscopy. Fluorescent dyes and proteins are used to introduce fluorescence into a sample. Fluorescent dyes are fluorescent molecules (known as fluorophores) absorbs and emits light while incorporated in the biological structure. The emitted light is detected to analyze biofilm features, such as spatial cellular viability, shape and function [96]. Some examples of fluorescent dyes are DAPI (4’,6-Diamidino-2-phenylindole dilactate), lipophilic dyes such as FM 4-64, SYTO 9 and Propidium Iodide (PI) [97]. Green fluorescent protein (GFP), enhanced green fluorescent protein (EGFP) [98], Cyan Fluorescent Protein (CFP) and Yellow Fluorescent Protein (YFP) are the examples of fluorescent protein [99].

8.1.2. Indirect Quantification Methods

The growth of biofilm (quantity of biofilm) can be determined indirectly using a proxy marker such as dry mass, total protein content, DNA, RNA, polysaccharides or metabolites.

All the indirect quantification methods involve basic assumption that the substance or property to be quantified correlates to the number of cells or amount of protein/DNA/mass [100].

8.1.2.1. Dry Mass Measurement

Dry mass (mass per unit area) or biofilm density is a widely used marker for quick growth quantification. The biofilm together with growth substrate is placed in an oven at a constant temperature (depends on substrate heat tolerance capacity) until the water is removed and a constant weight is achieved to find the dry mass. If the substrate is heat sensitive, the biofilm can be scraped from the surface then suspended in physiological saline after that precipitated with cold ethanol and precipitates are collected for analysis. After complete drying the sample is weighed, the biomass is scraped from the substrate and then substrate is weighed. Dry biomass is calculated as the difference in weight between biomass on the substrate and the substrate with no biomass [101, 102].

8.1.2.2. Total Organic Carbon (TOC) Quantification

Total organic carbon (TOC) is an indirect measurement of the amount of carbon in a sample associated with organic compounds or carbon compounds derived from living things such as proteins, lipids, urea etc. This is opposed to elemental carbon (EC) such as graphite or coal, and inorganic carbon (IC) consisting of simple compounds including simple carbon oxides (CO and CO2), carbonates, carbides, and cyanides [103]. TOC measurement is generally used to determine the quality of environmental water and for testing of instrument cleanliness used in the pharmaceutical industry. This method is also used in the quantification of biofilm accumulation [104, 105]. The TOC quantification of biofilms follows a two-step process in which total carbon (TC) and IC are measured and TOC is calculated by the difference between these two values (TOC = TC – IC). The exact method is determined using instruments such as the Oceanic International Carbon Analyzer, Analytik Jena Multi N/C 2100S, or a UIC incorporated Model CM5012 CO2 coulometer [106, 107].

8.1.2.3. Crystal violet assay

The primary component and commonly used dye for gram staining (identification and visualization of bacteria) is crystal violet, a basic tri-aniline dye which is cell membrane permeable [108]. For both gram positive and negative cells, the crystal violet is used and the dye will freely pass from the cell during the de-decolorization step allowing for the quantification of crystal violet via spectroscopy. This quantification has proven extremely useful as a cell estimate for biofilm growth [109, 110].

8.1.2.4. Tetrazolium salt

Tetrazolium salts are most widely used in biology for monitoring metabolism *in vitro*

[111]. A variety of salts successfully utilized for biofilm evaluation which allow for quantification and visualization of cellular viability and metabolism with the help of UV-Vis and fluorescence spectroscopy. The tetrazolium salt is diluted into a physiologically relevant solution, such as media or saline, and the biofilm is allowed to incubate for 1-3 hours at culture temperature or room temperature and cellular viability is detected by visual or fluorescent spectrometers or microscopes [112, 113]. The reduction can result in water soluble or water insoluble formazan, water soluble formazans solubilize in the treatment buffer used for real-time evaluation of cellular viability and metabolism [114, 115]. Water-insoluble formazan crystallizes and trapped within the cell membrane, crystals can be evaluated via flow cytometry and microscopy [116]. Some examples of commonly used tetrazolium salts are given in **Table 3**.

Table 3. Commonly used tetrazolium salts used for *in vitro* study of biofilms

S. No.	Name
1.	2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide (MTT)
2.	5-Cyano-2,3-di-(p-tolyl)tetrazolium chloride (CTC)
3.	2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (INT)
4.	2,3,5-TriphenylTetrazolium Chloride (TTC)
5.	(2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide) (XTT)

8.1.2.5. ATP bioluminescence test

ATP bioluminescence is a well-established microbial test used to detect the presence of microbial contamination on surfaces in food and biomedical communities. Adenosine triphosphate (ATP) is a nucleoside triphosphate which acts as the primary energy source in all organisms, so it is used as a prime marker for viability. In the process of bioluminescence organisms convert chemical energy to light and the amount of light can be used infer biofilm viability and biomass. This assay is very reliable, can be performed quickly, and only requires a luminometer for analysis. The assay is highly accurate at low ATP levels [117-119].

8.1.2.6. Total protein determination

Protein content has been found to correlate with the number of cells in biofilms. Total protein content determination is widely accepted method to detect the growth of biofilm [107]. In this process the biofilms are removed from their substrate and homogenized in a liquid suspension and the cells are lysed. Some protocols require incubation (at 55°C) in the presence of a strong base or detergent solution and protein precipitates with trichloroacetic acid (TCA). This lysis made protease free in the presence of proteases enzyme that break down proteins. After lysis, the protein content can be measured by color change (eg. Coomassie Brilliant Blue G-250 dye), and colour change result from the dye-protein interaction. The change in absorbance of the colored species at a particular wavelength is proportional to the concentration of protein by the Beers-Lambert law. Bradford, Lowry, and bicinchoninic acid (BCA) are some

established methods used for total protein determination [120].

8.1.2.7. Quartz crystal microbalance

Quartz crystal microbalance (QCMs) is used for the nondestructive measurement of biofilm accumulation. The instrument consists of a small disc of Astatine (AT)-cut single crystal quartz that is driven at the resonant frequency by an applied oscillating potential difference. The disc may be coated by Gold (Au) or Silicon Oxide (SiO2) and serves as the growth substrate. In this study, a direct correlation between wet mass of the film and QCM frequency shift is shown, giving a quantitative measure of mass from the QCM device. The major advantage of this technique is the monitoring of mass accumulation to ng/cm2 accuracy in real-time without sacrificing the sample and allows for the investigation with multiple analytic techniques [121-123].

8.2. Qualitative Characterization Methods

The characteristics which are helpful in the qualitative determination of biofilm are imaging the physiological biofilm surface, structure evaluation of surface roughness, morphology, spatial organization, and interaction of the biofilm with the environment. Surface structure analysis is done by light and fluorescent microscopy, Scanning Electron Microscopy (SEM) methods through high resolution imaging.

8.2.1. Scanning Electron Microscopy (SEM)

SEM is used for high resolution magnified image of surface topography. The magnification range of SEM is about 10-500,000 times which makes this technique invaluable in the analysis of microscopic structures and biofilm morphology. SEM utilizes a concentrated beam of electrons to observe a sample through a number of electromagnetic lenses [124, 125]. An advantage of electron microscopy is the easy availability of tandem spectroscopic techniques for quantitative elemental analysis and the high resolution of the surface images can reveal details about biofilm structure and topography. SEM analysis cannot be performed on living samples and testing is done under high vacuum, extensive preparation is required prior to the analysis of biological samples [126].

8.2.2. Alternative Qualitative Characterization Methods

Alternative methods used for qualitative characterization of biofilm growth are scanning electrochemical microscopy (SECM) [127], Infrared (IR) and Raman spectroscopic characterization[128], Surface Enhanced Raman Spectroscopy (SERS) [129], Small angle x-ray scattering (SAXS) [130], Surface Plasmon Resonance imaging (SPRi) and Electrochemical Surface Plasmon Resonance (EC-SPR) [131].

9. Management of Biofilms

The importance from a public health perspective is the role of biofilm in antimicrobial drug resistance, poses a serious threat to the Pharmaceutical industries. Therefore prevention of biofilm formation is recommended rather than treatment [132]. Biofilm formation can be prevented by signaling molecules that block the attachment of bacterial cells to substrate surface [133] and by chemical reactions that prevent synthesis of polymers in extracellular matrix [134]. Substances that block communication between bacteria can prevent biofilm formation or stimulate its dispersion [135, 136]. Biofilm dispersion can be induced by the use of enzymes that break down polymers in extracellular matrix [137].

Treatment of periodontal biofilms- In these treatment individual considerations must be taken care of. Biofilm control is fundamental to the maintenance of oral health and to the prevention of dental caries gingivitis and periodontitis [138].

9.1. Possible strategies to control oral biofilms [138]

- Inhibition of bacterial colonization
- Inhibition of bacterial growth and metabolism
- Disruption of established plaque
- Modification of plaque biochemistry
- Alteration of plaque ecology

9.2. Clinical approaches

a. Mechanical plaque control [139]

- Tooth brushes
- Manual
- Electrical
- Interdental cleaning aids/brushes
- Wooden and rubber tips
- Dental floss
- Oral irrigation devices

b. **Chemical plaque control** [140]

- Enzymes (Mucinase, Dehydrated pancrease, Lactoperoxidase hypothiocyanate)
- Antibiotics (Penicillin, Vancomycin, Erythromycin)
- Phenols (Thymol, Delmopenol)
- Quaternary Ammonium Compounds (Benzalkonium chloride, Cetylpyridinium chloride)
- Bisbiguanides (Chlorhexidine, Alexidine)
- Bispyridines (Octenidine)
- Metallic Salts (Zinc, Tin, Copper)
- Amino alchohols (Octapenol, Decapenol)
- Herbal extracts (Sanguinarine)
- Surfactant (Sodium lauryl sulfate)

10. Application of Biofilm

Specific applications of bound bioactive molecules to surfaces (biofilm) in different sectors or scientific disciplines are described below;

10.1. Food industry application

In food processing industry antimicrobial polymers (active packaging) can be used to improve the safety of food [141]. Immobilized lysozyme, glucose oxidase and chitosan have been used as packaging films. These packaging technologies play an important role in extending shelf-life of foods and reduce the risk of growth of pathogenic microorganisms [142]. Material/compounds proposed and tested for antimicrobial activity in food packaging includes organic acids, antibacterial peptides and fungicides [143-145]. Triclosan containing food contact surfaces such as include cutting boards and dishcloths effectively reduces the bacterial contamination. Enzyme immobilization reduced the overall bioactivity after denaturation [146]. When surface modification strategies are applied to obtain antibacterial food processing surfaces, they can help reduce biofouling and cross-contamination [147]. The effectiveness of coating SS with anticorrosion undercoat paint was reported in various studies [148]. Biofilm formation in food may be avoided by equipment design, temperature control and by reduction of water and nutrients. Effective cleaning (alkali compounds) is the main focus to control the growth of biofilm. The sanitizers used in food industry are halogens, acids, peroxygens and

quaternary ammonium compounds (cationic surfactant sanitizers) [132].

10.2. Biomedical application

Modified materials are not recommended for the medical purpose because if the substances will leach out it may cause cytotoxicity [149]. A metallic material which is implanted into human body release metal ions may cause various health problems due to metal accumulation in organs, allergy and carcinoma [150-152]. Biocompatibility is the most important property that must involve in a modified abiotic surface. Biocompatibility can be divided into two kinds, one is the bulk property of the biomaterial and other is its surface property. The rigidity of modified implants must match with that of the adjacent tissue otherwise hyperplasia or absorption of the tissue will occur resulting in failure of implantation [153].

11. A Future Prospectus for Research

The biofilm is viscoelastic in nature which is universal but when exposed to different environment, hydrodynamic conditions will change the structure, composition and physical properties of their matrix. Biofilm science is highly exciting research area because it is a mixture of biology, microbiology, biotechnology, biophysic, chemistry and much more [154]. Research on microbial biofilms opens many fronts with special attention on elucidation of the genes expressed by biofilm-associated organisms, evaluation of control strategies to control or prevent biofilm colonization of medical devices and development of new methods for assessing or evaluating the efficacy of these treatments. The focused research area should be on the role of biofilms in antimicrobial resistance, biofilms as a reservoir for pathogenic organisms and the participation of biofilms in chronic diseases. As the pharmaceutical and health-care industries embrace this approach, novel strategies for biofilm prevention and control will definitely emerge in future. The key to success may depend upon a complete understanding of what makes the biofilm phenotype so different from the planktonic phenotype [155].

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