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BIOFILM-A REVIEW

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ABSTRACT

Biofilm is formed when microorganisms universally attach to any natural and man-made surface by producing extracellular polysaccharides. Everywhere we can see the presence of biofilms. Biofilms creates a serious problem for public health because of the increased resistance to antimicrobial agents. Biofilm formation is a life threatening one to humans.

INTRODUCTION

The Roman philosopher Lucretius (about 98-55 B.C.) and the physician (1478-1553) suggested that disease was caused by invisible living creatures.[1] When life arose on Earth about 4 billion years ago, the first types of cells to evolve were prokaryotic cells. For approximately 2 billion years, procaryotic-type cells were the only form of life on Earth.[2] Bacteria are a large domain of single-cell, prokaryote microorganisms.[3]

Among the earliest forms of life on earth, bacteria have evolved to thrive in a variety of environments. Some can withstand searing heat or frigid cold, and others can survive radiation levels that would be lethal to a human being. Many bacteria, however, prefer the mild environment of a healthy body.[4] In 1665, the first drawing of a microorganism was published in Robert Hooke's micrographia.[1] Bacteria were first observed by Antonie van Leeuwenhoek in 1676, using a single-lens microscope of his own design.[5]

Bacteria are some of the most diverse life forms on Earth and are capable of living in a huge variety of conditions. In order to accomplish this feat, bacteria have developed unique survival techniques and growth patterns. One of these is the ability to form biofilms.[6,7]

Biofilm-associated organisms are able to adapt to environmental changes by altering their gene expression and general performance, of which increased resistance to antibiotics is an example.[8] Biofilms can lead to an increase in friction and heat transfer resistance. They can cause material deterioration through microbial corrosion, and they can contaminate artificial organs and catheters.[9]

Biofilms have important roles in many diseases. Prosthetic device colonization, dental plaque formation, infection of the cystic fibrosis, food spoilage and unusual resistance to antibiotics are important conditions originating from biofilms.[10] Microorganisms can attach to and colonize any biomaterial surface, putting patients at risk for local and systemic infections.[11]

The National Institutes of Health (NIH) has estimated that up to 80% of human infectious diseases are biofilm related.[12] More than 99% of bacteria found in nature exist in these stable, persistent biofilms and there are reasons to believe this bacterial theme also holds true in the wound environment.[13-16]

WHAT IS BIOFILM?

Biofilms are densely packed communities of microbial cells that grow on living or inert surfaces and surround themselves with secreted polymers.[17]

WHY AND HOW DO THESE BIOFILMS FORM?

Bacteria become attracted to surfaces for a number of reasons. One may be gravity organisms may just settle out and end up resting on a surface. Or bacteria (who often have a negative charge associated with their outer envelop) may be attracted to the positive charges on some inorganic surfaces. But there is evidence that biofilm formation is much more than random physical forces. Many surfaces attract and concentrate nutrients, and many bacteria have the capacity to detect and move toward high concentrations of nutrients (an ability called chemotaxis).[18]

HOW DO BACTERIA DEVELOP INTO A TEEMING, ACTIVE COMMUNITY?

Some cells are able to produce copius amounts of polysaccharides, which act as mucus layers and hold the cells to the surface. These are called the primary colonizers. This external slime captures other bacteria (secondary colonizers), who live and grow off the waste products produced by the primary colonizers. Before you know it, there's an extensive and complex microbial community, all tangled up inside the polysaccharide slime. This is the biofilm.[18]

Different species of bacteria, protozoans, algae, yeasts and fungi can form biofilms. With most biofilms ranging from a few microns to hundreds of microns (one micron being one-millionth of a meter) in thickness.[19]

EXTRACELLULAR POLYMERIC SUBSTANCES (EPS)

The biofilm is held together and protected by a matrix of excreted polymeric compounds called EPS[20] and amyloid fibers composed of the protein TasA.[21] EPS is an abbreviation for either extracellular polymeric substance or exopolysaccharide.[20] The exopolysaccharide is produced by the epsA-O operon, and the TasA protein is encoded by the yqxM-sipW-tasA operon.[21] The EPS is a highly hydrated, gel-like biopolymer that immobilizes bacteria creating the three-dimensional structures characteristic of biofilms and microbial aggregates. The EPS composition is important not only for adhesion and biofilm matrix stabilization, but also for creating heterogeneity and increasing nutrient availability within the biofilm.[22]

They are formed by adhesion of cells to surfaces through an exopolymeric matrix. This matrix is important both in the formation and structure of the biofilm and also on the protection of the cells since it may prevent the access of antimicrobials and xenobiotics to the cells inside the biofilm and confer protection against environmental stresses such as UV radiation, pH shifts, osmotic shock and desiccation.[23] Biofilms, more commonly called slime, can corrupt any solid surface.[24] This slime is composed of polysaccharides, proteins and nucleic acids and often makes up 80% of the biofilm. The remaining 20% are microbial cells that reside within a microbial community encased within the EPS matrix.[8,25] It well known that bacteria grow and develop into “tight-knit” communities on biotic and abiotic surfaces. The innate features of a biofilm can protect bacteria from antibiotic damage. Biofilms are so efficient in preventing antibiotic damage that concentrations of antibiotics must be increased by 100-1000 fold to be effective.[26]

COMPONENT OF BIOFILMS

The major component of the biofilm matrix is water and is believed to constitute approximately 95–99% of the biofilm. The microbial content is only approximately 2–5%, surrounded by EPS that may reach up to 2% of the total matrix. Other substances often found in the biofilm matrix include DNA, RNA, proteins and enzymes reaching levels of approximately 2% in total.[22,27] The components of biofilm are given in the table 1.[28]

Table 1: Component of biofilms

Component	% of Matrix
Water	Up to 97%
Microbial Cells	2-5% (many species)
Polysaccharide (homo and heteropolysaccharides)	1-2% (neutral & polyanionic)
Proteins (extracellular and resulting from lysis)	<1-2% (many, including enzymes)
DNA & RNA	<1-2% (from lysed cells)
Ions	? (bound and free)
Host	Fibrin, RBCs, WBCs

WHERE DO BIOFILMS GROW?

Biofilms grow virtually everywhere, in almost any environment where there is a combination of moisture, nutrients and a surface.[29]

Natural environments

Biofilms grow in rain forests and in deserts, as "desert varnish." They have been found at the bottom of the ocean as early colonizers of new deep-sea vents and living on glaciers in the Antarctic. Bacteria that live in these very hot or very cold environments are called *extremophiles*.[29]

Environmental biofilms

Biofilm grow in marine and freshwater hull fouling, wastewater treatment, hot springs, deep sea vents, pollution remediation, ocean energy capture-thermal,-mechanical, deep earth rock, underground caves, Arctic and Antarctic.

Biomedical biofilms

Biofilm grow in indwelling medical implants (MRSA), surgical and hospital environmental surfaces, bathroom surfaces in home and hospital, air-handling and water-handling systems (Legionella), biological fluid-handling machines (dialysis equipment), chronic wounds (diabetic ulcers), infections (ear infections) and diseases (cystic fibrosis). Studies now show that the majority of chronic infections arising from hospitals is biofilm related.

Industrial biofilms

Biofilm grow in cooling towers, nuclear reactor cooling water, water systems, (including ultrapure, RO, DI), pulp and paper mills, Petrochemicals (jet fuel, oil pipelines, metalworking fluids), food and beverage processing, pharmaceutical manufacturing, cosmetics manufacturing, bulk and fine chemicals.[30]

WHY DO BACTERIA TALK TO EACH OTHER?

Quorum Sensing enables bacteria to co-ordinate their behaviour. As environmental conditions often change rapidly, bacteria need to respond quickly in order to survive. These responses include adaptation to availability of nutrients, defence against other microorganisms, which may compete for the same nutrients and the avoidance of toxic compounds potentially dangerous for the bacteria. It is very important for pathogenic bacteria during infection of a host (e.g. humans, other animals or plants) to co-ordinate their virulence in order to escape the immune response of the host in order to be able to establish a successful infection.[31]

CELL-CELL COMMUNICATION OR CELL-CELL SIGNALING

Bacteria can produce chemical signals ("talk") and other bacteria can respond to them ("listen") in a process commonly known as cell-cell communication or cell-cell signaling.[32] Bacteria produce and release chemical signals-autoinducers-in search of similar cells in their close surroundings. This is also called "cell-cell communication." Other bacteria release the same autoinducers in response.[31] Cell-to-cell signalling activity takes place through a quorum-sensing pathway.[33]

QUORUM-SENSING

This phenomenon has been known to scientists since the 1960s, only now they are able to study it in detail. This new branch of microbiology, quorum sensing, discovered by Bonny Bassler, professor of microbiology from Princeton, is dedicated to studying this phenomenon. Professor Bassler discovered that bacteria could send signals not only to their own kind, but to other bacteria as well. She describes this phenomenon as "bacterial ESPERANTO".[31]

When certain bacteria are in wet environments, they attach and send out signals to attract other bacteria called “quorum sensing” molecules. When the other bacteria start congregating, they start differentiating into bacteria that attach, that transport nutrients, that digest, that form protective films or crusts, adjust resistance and become far more formidable than any bacterium alone.[34] Quorum-sensing molecules are continuously secreted from each individual bacterium and act on the same bacterial species, interspecies and even on the cells of their mammalian host.[33] The quorum-sensing pathway can express over 800 new proteins not seen with planktonic phenotype bacteria.[35]

The population density is detected by the concentration of signal molecules (autoinducers) which are secreted by the cells consitutively. The autoinducers diffuse into the cells and by reaching a threshold concentration an induction of quorum sensing regulated genes takes place. This phenomenon has been called autoinduction and now it is known as quorum sensing.[36] Bacteria that use quorum sensing produce and secrete certain signalling compound, (called as autoinducers or pheromones).[31] The two most common auto-inducers seen in bacteria are known as AHL (N-Acyl Homoserine Lactones) and Auto-inducer family (AI-2, AI-3).

AHL (N-Acyl Homoserine Lactones)

AHL are signaling molecules found in many gram negative bacteria. Common gram negative bacteria include *H. pylori* (ulcer causing bacteria), *Salmonella* (food poisoning), *Neisseria gonorrhoeae* (gonorrhea) *Spirochetes* (Lyme disease), *Proteus* and *E. Coli* (urinary tract infections). They are inactivated by an enzyme called lactonase.[37]

Auto-inducers AI-2, AI-3

The second series of signaling molecules used in quorum sensing are the 'auto-inducer' family, typically number 1 through 3. Unlike the AHL family the auto-inducers work on both gram negative and gram positive bacteria. Common gram positive bacteria include *Staphylococcus*, *Streptococcus* and *Clostridium* (botulism). The auto-inducers are one of the very few biologically active family of molecules that contain the element boron. Some evidence indicates that foods containing furocoumarins inhibit their signaling. Common furocoumarins in the diet include grapefruit juice and bergamot.[38] Some species of seaweed are also being investigated for their ability to 'jam' bacterial signaling.[39]

Biofilm Formation

Formation of a biofilm begins with the attachment of free-floating microorganisms to a surface. These first colonists adhere to the surface initially through weak, reversible adhesion via van der Waals forces. If the colonists are not immediately separated from the surface, they can anchor themselves more permanently using cell adhesion structures such as pili. Some species are not able to attach to a surface on their own but are often able to anchor themselves to the matrix or directly to earlier colonists. It is during this colonization that the cells are able to communicate via quorum sensing using such products as Acyl-Homoserine Lactone. Once colonization has begun, the biofilm grows through a combination of cell division and recruitment. The final stage of biofilm formation is known as development, and

is the stage in which the biofilm is established and may only change in shape and size. The development of a biofilm may allow for an aggregate cell colony (or colonies) to be increasingly antibiotic resistant.[20]

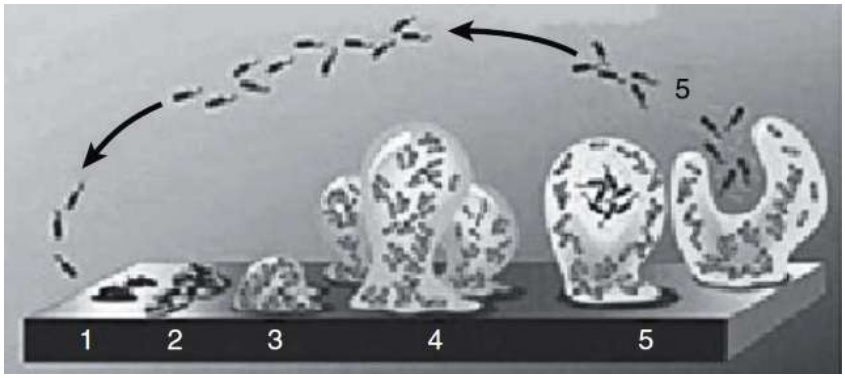

Fig. 1: The biofilm life cycle: attachment, adhesion, aggregation, growth and maturation and detachment.

BIOFILM DEVELOPMENT

The biofilm developmental cycle is believed to include the processes:[40]

The five stages of biofilm development. Stage 1: Planktonic (free floating) bacteria adhere to the biomaterial surface. Stage 2: Cells aggregate, form micro colonies and excrete extracellular polymeric substances (EPS), i.e. slime. The attachment becomes irreversible. Stage 3: A biofilm is formed. It matures and cells form multi-layered clusters. Stage 4: Three-dimensional growth and further maturation of the biofilm, providing protection against host defense mechanisms and antibiotics. Stage 5: The biofilm reaches a critical mass and disperses planktonic bacteria, ready to colonize other surfaces.[40]

i) Transport of microbes to a surface

Free floating or planktonic bacteria encounter the conditioned surface and form a reversible, sometimes transient attachment often within minutes.[41]

ii) Initial attachment

The bacterium approaches the surface so closely that its motility is slowed and its forms a transient association with the surface and/or other microbes previously attached to the surface. The solid–liquid interface between a surface and an aqueous medium (e.g. water, blood) provides an ideal environment for the attachment and growth of microorganisms to a surface. In general, attachment will occur most readily on surfaces that are rougher, more

hydrophobic and coated by surface 'conditioning' films. An increase in flow velocity, water temperature or nutrient concentration may also equate to increased attachment, if these factors do not exceed critical levels. Properties of the cell surface, specifically the presence of fimbriae, flagella and surface-associated polysaccharides or proteins, are also important and may possibly provide a competitive advantage for one organism where a mixed community is involved.[42]

Reversible surface attachment

Bacterial cells attached reversibly to surfaces produce EPS due to stimulation of membrane - bound sensory proteins of the bacterial cell, which allows for the development of cell – cell bridges that, in turn, cement the cells to the surface.[43] Microorganisms are commonly perceived to be free-floating and solitary (ie planktonic). However, under natural conditions most microorganisms tend to attach to surfaces and eventually form biofilms. The initial attachment is reversible.[44]

Irreversible Adherence

If the association between the bacterium and its substrate persists long enough, other types of chemical and physical structures may form which transform the reversible adsorption to a permanent and essentially irreversible attachment.[41]

iii) Formation of microcolonies

After the bacteria adhere to the inert surface/living tissue, the association becomes stable for microcolony formation. The bacteria begin to multiply while emitting chemical signals that 'intercommunicate' among the bacterial cells. Once the signal intensity exceeds a certain threshold level, the genetic mechanisms underlying exopolysaccharide production are activated. In this way, the bacteria multiply within the embedded exopolysaccharide matrix, thus giving rise to the formation of a microcolony.[42]

iv) Biofilm maturation

It includes cell growth (and potential reproduction) within a given microenvironment, as determined by exopolysaccharide substances, neighboring cells and proximity to a water channel. The open water channels represent a primitive circulatory system for the preservation of homeostasis within the biofilm. In the mature biofilm, more volume is being occupied by the EPS matrix (70 – 95%) than by bacterial cells (5 – 25%). At this stage,

secondary colonizers (other bacteria or fungi) can become associated with the biofilm surface.[43]

A mature biofilm can contain as many 100 billion bacterial cells per milliliter. Complex diffusion channels deliver nutrients, oxygen and other elements that cells need to grow and carry away metabolic waste products, debris, and cells. The thriving and well protected colony provides a continuous supply of cells that easily slough away and contaminate other surface.[45]

v) Biofilm dispersal

Finally, bacteria can be detached from the biofilm either by external forces or as a part of a wavelike migrating physical movement or even as a self - induced process to disseminate to the environment.[43] Biofilm cells may be dispersed either by shedding of daughter cells from actively growing cells, detachment as a result of nutrient levels or quorum sensing or shearing of biofilm aggregates (continuous removal of small portions of the biofilm) because of flow effects. The mechanisms underlying the process of shedding by actively growing cells in a biofilm are not well understood.[46]

HOW TO PREVENT BIOFILM FORMATION

The challenge for clinicians is how to manage biofilms. There appears to be five key factors,

1) Interfere with matrix formation
2) Digest EPS
3) Prevent cell-to cell-communication
4) Remove already formed biofilm
5) Stop bacteria attaching to host cells

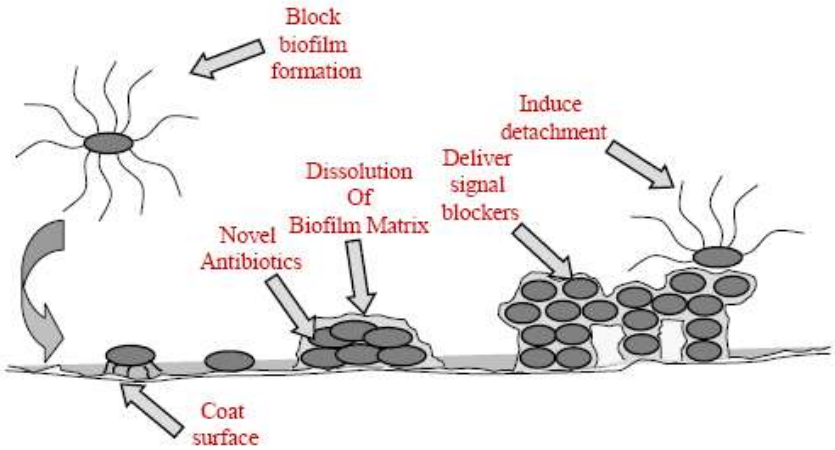

Fig. 2-Prevention of biofilm

Surface coating

Many approaches have been proposed to inhibit bacterial biofilm formation, including several major directions. The most widely studied way is material surface coating. An example is a photocatalytic titanium dioxide (TiO_2) surface, which has potential as a self-cleaning technology to reduce the adhesion of Deinococcus geothermalis cells in warm water- using industries.

Novel antibiotic

Antibiotic coating systems are sometimes effective in preventing biofilm formation, such as the application of ciprofloxacin-releasing bioabsorbable polymer in fighting Staphylococcus epidermidis biofilms. Application of antimicrobial compounds has also been examined. For example, hydrogen peroxide-based disinfectants were proven effective for inhibiting biofilm formation in dental unit waterlines.

Signal blocker

Physical methods showed that ultrasound improved the transport of gentamicin through colony biofilms of *P. aeruginosa* and *Escherichia coli*. Recently a research report showed that an efficient way to prevent *P. aeruginosa* biofilm formation is by releasing ciprofloxacin from self- assembled coatings by ultrasonic control. Other novel approaches are also being examined, such as utilizing bacteriophage to degrade exopolysaccharide (EPS), and the application of cell- to-cell signaling inhibitors.[47]

REASON FOR BIOFILM DIFFICULT TO TREAT

Many characteristics of biofilms contribute to the difficulty in treating these types of infections with normal antibiotic doses. First, the exopolysaccharide matrix produces a physical barrier, which reduces the amount of antibiotic that can enter into this microbial community. Second, even if antibiotic treatments infiltrate the exopolysaccharide matrix, the antibiotic may still have difficultly accessing the internal cells of the mushroom-like structures. Third, during the formation of a biofilm, specialized virulence gene expression can occur. These virulence factors include the expression of antibiotic denaturing enzymes, efflux pump, and increased plasmid exchange. The development and expression of many of these virulence features is typically under quorum sensing control.[48]

REFERENCES

1. Prescott, Harley and Klein's. Microbiology. Joanne M. Willey, Linda M. Sherwood and Christopher J. Woolverton. 7th edition. Mc Graw Hill international edition; 2008, 3.
2. Todar's Online Textbook of Bacteriology.
3. Fredrickson JK, Zachara JM, Balkwill DL et al. "Geomicrobiology of high-level nuclear waste-contaminated vadose sediments at the Hanford site, Washington state". Applied and Environmental Microbiology, 2004; 70(7): 4230–41.
4. http://altered-states.net/barry/newsletter406/bacteria.htm
5. Porter JR. "Antony van Leeuwenhoek: tercentenary of his discovery of bacteria". Bacteriological Reviews, 1976; 40(2): 260–9.
6. http://www.bionewsonline.com/index.html
7. http://www.biofilm.montana.edu/node/2390
8. Costerton, J.W., Stewart, P.S., Greenberg, E.P. Bacterial biofilms: a common cause of persistent infections. Science, 1999; 284: 1318-1322.
9. Zhou Ji. Quantitative Analysis of Biofilm Images Using Fractal Dimensions. Thesis. August 2000. 1.
10. H. Hosain Zadegan and B. Delfan. Evaluation of Antibiofilm Activity of Dentol. Acta Medica Iranica, 2009; 47(1): 35-40.
11. Euan Burton, Purushottam V. Gawande, Nandadeva Yakandawala, Karen LoVetri, George G. Zhanel, Tony Romeo, Albert D. Friesen, and Srinivasa Madhyastha. Antibiofilm Activity of GlmU Enzyme Inhibitors against Catheter-Associated Uropathogens. Antimicrobial Agents and Chemotherapy, 2006; 50(5): 1835–1840.
12. Minutes of the National Advisory Dental and Craniofacial Research Council, 153rd Meeting. National Institutes of Health 9 September 1997. http://www.nidcr.nih.gov/ AboutNIDCR/CouncilAndCommittees/ NADCRC/Minutes/ Minutes153.htm
13. Saye, DE. Recurring and antimicrobial-resistant infections: considering the potential role of biofilms in clinical practice. Ostomy Wound Manage, 2007; 53(4): 46-48.
14. Davis. SC, Ricotti. C, Cazzaniga A. et al. Microscopic and physiologic evidence for biofilm-associated wound colonization in vivo. Wound Repair Regen., 2008; 16(1): 23-29.
15. James, G.A., Swooger, E., Wolcott, R. et al. Biofilms in chronic wounds. Wound Repair Regen 2008; 16(1): 37-44.
16. Ngo, Q, Vickery, K, Deva, AK. Role of bacterial biofilms in chronic wounds. Abstract: PR21. ANZ J Surg 2007; 77: Suppl 1: A66.

17. Nadell CD, Xavier JB and Foster KR. The sociobiology of biofilms. FEMS Microbiol Rev. 2009; 33: 206-24.
18. https://courses.cit.cornell.edu/biomi290/z.OldWebSite/Horror/Biof.tutorial.HTML
19. http://science.howstuffworks.com/life/cellular-microscopic/biofilm1.htm
20. https://en.wikipedia.org/wiki/Biofilm
21. Ilana Kolodkin-Gal, Diego Romero, Shugeng Cao, Jon Clardy, Roberto Kolter, Richard Losick. D-Amino Acids Trigger Biofilm Disassembly. Science, 2010; 328: 627-629.
22. Jana Jass, Susanne Surman and James Walker. Medical Biofilms. Detection, Prevention and Control. John Wiley & Sons Ltd, USA, 2003, Page no. 5.
23. Carla C. C. R. de Carvalho. Biofilms: Recent Developments on an Old Battle. Recent Patents on Biotechnology 2007; 1: 49-57.
24. http://ehp.niehs.nih.gov/docs/1998/106-12/innovations.html
25. Costerton, J.W., Stewart, P.S. Battling biofilms. Sci Am 2001; 285(1): 74-81.
26. Costerton, J. W., Z. Lewandowski, D. De Beer, D. Caldwell, D. Korber and G. James. Biofilms, the customized microniche (minireview). J. Bacteriol. 1994; 176: 2137-2142.
27. https://microbewiki.kenyon.edu/index.php/Stream_biofilm
28. Thesis. Biofilms 101 Biofilms Made Easy: "A Picture Tutorial" Understanding the Impact of Microbiology, "Focusing" on Biofilms. John G. Thomas, PhD, Sara B. Posey, MPH, and Staff. WVU School of Medicine Department of Pathology Biofilm Research Laboratory for Translational Studies Morgantown, WV 26506-9203. P. No 20.
29. http://www.biofilm.montana.edu/node/2408/
30. Mark Fornalik. Industrial Biofouling Science. Introduction to Biofilms and Biofouling in Pharmaceutical Manufacturing. www.biofoulingscience.com
31. Madhumathi Seshadri and Lakshmi Sivasubramaniam. Quorum Sensing- Communication Between Bacteria. 2005; 3(6): Pharmainfo.net
32. http://www.biofilm.montana.edu/node/2410
33. Mikkelsen, H., Duck, Z., Lilley, KS., Welch, M. Interrelationships between colonies, biofilms and planktonic cells of Pseudomonas aeruginosa. J Bacteriol., 2007; 189(6): 2411-2416.
34. Karen Vaughan, L. Biofilms and Disease: Why should I care? 2009. http://www.acupuncturebrooklyn.com/uncategorized/biofilms-and-disease-why-should-i-care
35. Sauer, K., Camper, AK., Ehrlich, GD. et al. Pseudomonas aeruginosa displays multiple phenotypes during development as a biofilm. J Bacteriol., 2002; 184(4): 1140-1154.

36. Fuqua, WC., Winans, SC and Greenberg, E.P. Quorum sensing in Bacteria: the LuxR-LuxI family of cell density-response transcriptional regulators. J Bacteriol., 1994b; 176: 269-275.
37. Tavori H, Vaya J and Aviram M. Paraoxonase 1 attenuates human plaque atherogenicity: relevance to the enzyme lactonase activity. Adv. Exp. Med. Biol., 2010; 660: 99-111.
38. Girennavar B, Cepeda ML, Soni KA, Vikram A, Jesudhasan P, Jayaprakasha GK, Pillai SD, Patil BS. Grapefruit juice and its furocoumarins inhibits autoinducer signaling and biofilm formation in bacteria. Int J Food Microbiol., 2008; 125(2): 204-8.
39. University Of New South Wales. New Weapon in Germ Warfare: 'Jamming' Bacteria Signals Stops Cholera. Science Daily. from http://www.sciencedaily.com /releases/2004/12/041208083233.htm
40. Erik Unosson. Antibacterial Strategies for Titanium Biomaterials. Research Gate. Thesis. 2015.
41. http://www.personal.psu.edu/faculty/j/e/jel5/biofilms/primer.html
42. B. Prakash, B. M. Veeregowda and G. Krishnappa. Biofilms: A survival strategy of bacteria. Review articles. Current science, 2003;85 (9): 1299-1307.
43. Tamilvanan Shunmugaperumal. Biofilm eradication and prevention. A pharmaceutical approach to medical device infections. A John Wiley & Sons, Inc., Publication. Hoboken, New Jersey. 2010.
44. http://www.woundsinternational.com/article.php?issueid=303&contentid=123&articleid=8851&page=1
45. http://www.hycator.com/domain/functional-sectors/biofouling/steps-in-biofilm-formation.html
46. Rodney M. Donlan. Biofilms: Microbial Life on Surfaces. Emerging Infectious Diseases, 2002; 8(9): 881-890.
47. Chen Niu. The role of autoinducer-2 in *Escherichia coli* biofilm formation and the discovery of a plant-derived quorum sensing inhibitor. Dissertation. 2006; 3-4.
48. Maria M. Nagy. Quorum sensing inhibitory activities of various folk-medicinal plants and the thyme-tetracycline effect. Biology Dissertations. 2010.