Microbial Biofilms: Microbes in Social Mode

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Abstract

Biofilm is a community of microorganisms embedded in extracellular polymeric substances. Organisms in their biofilm form get many advantages over their planktonic counterparts, making them prefer to exist as biofilm. Biofilm formation involves adhesion of microbes on surfaces, followed by maturation stage which is controlled by quorum sensing (QS). Organisms present in biofilm can be 10-1000 times more resistant to antimicrobials compared to their planktonic stage. This may be due to incomplete penetration of antibiotics into the biofilm, slow growth rate of organisms in biofilm, or certain phenotypic changes. Biofilm forming ability of microorganisms has been a source of problems for human health, industry, and agriculture. Natural products including plant extracts, and quorum sensing inhibitors seem to be good alternatives of conventional antibiotics against biofilms. Biofilm forming microbes can be exploited for beneficial applications viz. waste water treatment, N₂ fixation, oil degradation, and heavy metal sequestration. This review describes salient features of microbial biofilms, quorum sensing and drug-resistance in biofilms, problems caused by biofilms, their potential applications, and methods available for their study.

Key words: Planktonic; Sessile; Quorum sensing inhibitors; Antibiotic resistance

Introduction

Microorganisms for long were studied in their planktonic stage. Initially not much attention was paid to their capacity to exist as a community. However, in recent times their ability to form biofilms in various environments, and its impact on ecology, medicine, and industry has attracted considerable attention. A microbial biofilm is a sessile community composed of cells that attach to a substratum or interface or to each other, with the help of gelatinous extracellular polymeric substances (Elvers and Lappin-scott, 2000; Jain et al., 2011). As noted by Andre Levchenko and Johns Hopkins, “There is a perception that single-celled organisms are asocial, but that is misguided.” When bacteria are under stress- which is the story of their lives- they team up and form this community called biofilm. If you look at naturally occurring biofilms, they have very complicated architecture.“They are like cities with channels for nutrients to go in, and waste to go out” (Proal, 2008, p. 1; http://mpkb.org/home/pathogenesis/microbiota/biofilm). Biofilm normally forms on both biotic (plant surfaces, human body parts) and abiotic (in aquatic environment, and on inanimate objects, e.g. ship hull, industrial pipelines, etc.) surfaces. Biofilm build up in a wide variety of environments, ranging from the sebum that builds up in toilet bowls, and walls of swimming pools, to the constant deposition of plaque on teeth. It is this prmeval tendency of making biofilms, occurring from billions of years, through which microbes have been able to colonize most habitats on earth (Talaro, 2008). Biofilm formation by normal human flora has also been recorded. In nature,
biofilms generally exist as a mixed bacterial consortium, but they may also consist of a single bacterial species. In multispecies biofilm many type of positive (coaggregation, conjugation, and protection to eradication by antimicrobial agents) and negative (bacteriotoxin production, lowering of pH) interactions take place (Burmølle et al., 2006; Perumal et al., 2007).

A wide majority of plant and human pathogens have been reported for their ability to form biofilm, e.g. *Streptococcus mutans*, *Staphylococcus epidermidis*, *Candida albicans*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Agrobacterium tumefaciens*, *Xanthomonas campestris*, *Pseudomonas syringae*, *Erwinia caratovorum*, *Aeromonas hydrophila*, etc. (Burmølle et al., 2006; Canals et al., 2006; Høiby et al., 2011; Li Chen, 2011; Perumal et al., 2007; Ramey et al., 2004; Rukayadi and Hwang, 2006; Saito et al., 2012).

Several descriptions of microbial biofilms (Table 1) have appeared since 17th century, when van Leeuwenhoek first observed microorganisms from his own teeth surface, but general theory of biofilm did not emerge until 1978 (Donlan and Costerton, 2002).

Microorganisms within biofilms display features distinct from their planktonic counterparts (Costerton et al., 1999; Donlan, 2002; De Beer and Stoodley, 2006; Elvers and Lappin-Scott 2002; Vu et al., 2009; Wilson, 2005), such as:

- Increase adherence to surfaces
- High population densities (around $10^{10}$ cells per ml of hydrated biofilm)
- Enhanced production of extracellular polymeric slime matrix (glycocalyx)
- Wide range of physical, metabolic and chemical heterogeneities
- Elevated tolerance to antimicrobial agents
- Higher level of nutritional interactions between microorganisms
- Higher order of communication through quorum sensing
- Less susceptibility to host defence mechanism
- Organisms in biofilm may display some novel phenotype(s)

### Table 1

<table>
<thead>
<tr>
<th>Year</th>
<th>Investigator</th>
<th>Contribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>17th Century</td>
<td>van Leeuwenhoek</td>
<td>First examined microorganisms from his own teeth surfaces</td>
</tr>
<tr>
<td>1930</td>
<td>Claude ZoBell</td>
<td>Research on bacterial adhesion to surfaces</td>
</tr>
<tr>
<td>1976</td>
<td>Marshall</td>
<td>Observed the role of “very fine extracellular polymer fibrils” that attaches bacteria to surfaces</td>
</tr>
<tr>
<td>1978</td>
<td>Costerton et al.</td>
<td>Examined that communities of attached bacteria in aquatic systems were enclosed in a “glycocalyx” matrix which was polysaccharide in nature, and this matrix material mediates adhesion</td>
</tr>
<tr>
<td>1987</td>
<td>Costerton et al.</td>
<td>Pointed that biofilm comprises of single cells and microcolonies which are embedded in a highly hydrated, predominantly anionic extracellular polymeric matrix</td>
</tr>
<tr>
<td>1990</td>
<td>Characklis and Marshall</td>
<td>Described characteristics of spatial and temporal heterogeneity and role of inorganic or abiotic substances held together in the biofilm matrix.</td>
</tr>
<tr>
<td>1995</td>
<td>Lappin-Scott</td>
<td>Investigated adhesion triggered expression of genes that control production of bacterial components which are necessary for adhesion and biofilm formation.</td>
</tr>
</tbody>
</table>
Why microorganisms prefer to exist in biofilm?

Microorganisms residing in biofilms get many advantages as compared to freely swimming planktonic stage, and that’s the reason for them to prefer biofilm mode of living (Annous et al. 2009; Costerton et al. 1999; Donlan, 2002; Vu et al., 2009). Some of these potential advantages are:

- Microorganisms in biofilms exhibit elevated antimicrobial tolerance and also get protected from environmental stresses such as extreme pH, oxygen, osmotic shock, heat, freezing, UV radiation, predators, and so on.
- Extracellular polymeric matrix formed from the secreted exopolysaccharides (EPS) increases the binding of water resulting in decreased chance of dehydration (desiccation) of the bacterial cells, which is a common stress condition experienced by planktonic cells.
- The adherent nature of microbial cells in biofilms allows rapid exchange of nutrients, metabolites, and genetic material.

Biofilm Formation

Biofilm formation is a multistage process (Figure 1). The initial step in biofilm formation involves reversible attachment of planktonic (freely moving individual cell) bacteria to a surface (colonization) by using adhesins (Donlan and Costerton, 2002; De Beer and Stoodley, 2006; Høiby et al. 2011). For example, polysaccharide adhesin (PS/A) of S. epidermidis initiates adhesion on naked or coated polymer surface (expression is controlled by the inter-cellular adhesion operon (Ica) (Li Chen, 2011; Tojo et al., 1988; Zhang et al., 2003). In Streptococcus pyogenes various cell surface molecules such as proteins and lipoteichoic acid are important for adherence on cultured human cells (Nobbs, 2009). The adhesin SpaP (PAc) in Streptococcus mutans is important for adhesion on teeth surfaces, and its expression is enhanced by sucrose or pre existing biofilm (Li Chen, 2011). In Vibrios, lateral flagella provide mechanism for attachment on surfaces (Atlas and Bartha, 1998). In P. aeruginosa, one of the virulence determinants, alginate plays important role in the adherence of the organism on trachael epithelium (Anwer et al., 1992; Marcus et al., 1989). In S. aureus, SasC protein factor plays important role in colonization during infection (Schroeder et al., 2009). The adhesion process is also affected by physiological state of the organism, in some organisms attachment is high in log phase, while in others attachment is high in stationary phase (Fletcher, 1999). The bacteria are still susceptible to antibiotics at this stage.

The next step in biofilm formation is turning the initial reversible binding of organisms to a surface into irreversible binding, followed by multiplication of the bacteria resulting in microcolony formation, after which production of a polymer matrix around the microcolony converts it into a mature biofilm (Annous et al. 2009; De Beer and Stoodley, 2006; Høiby, 2011).

Figure 1: Events during biofilm formation
(Anwer, 1992; Annous, 2009; Høiby, 2011; Proal, 2008; Scheie and Petersen, 2004)
Mature biofilm architecture varies from flat homogeneous layer of cells, to organized mushroom-like or tower-like structures (Folkesson et al., 2008; Høiby et al., 2011). This maturation stage is controlled by quorum sensing (QS) systems, such as N-acyl-homoserine lactone (AHL) and 4-quinolone systems (in gram-negatives), AgrD peptide systems (in gram-positives), AI2/LuxS system (in both gram-negatives), and farnesol systems (in fungi) (Høiby et al., 2011; Vu et al., 2009). The subsequent biofilm development involves focal dissolution, liberating bacterial cells (erosion), that can then spread to other locations where new biofilms can be formed from these liberated bacteria. This liberation process may be triggered by bacteriophage activity within the biofilm. The mature biofilm matrix may contain water-filled channel like structures and thereby resemble primitive, multicellular organisms (Annous et al., 2009).

Gene regulation and expression in biofilm of a microbial species may be notably different from that in the planktonic members of the same species. Davies and Geesey (1995) showed that in P. aeruginosa gene algC controlling phosphomannomutase, involved in alginate (exopolysaccharide) synthesis, is up regulated within minutes of adhesion to a solid surface. Recent studies have shown that algD, algU, rpoS, and the genes controlling polyphosphokinase synthesis are all up regulated in biofilm formation, and that as many as 45 genes differ in expression between sessile cells and their planktonic counterparts (Donlan and Costerton, 2002). Ram-age et al. (2002) reported that in C. albicans expression of CDR1, CDR2, and MDR1 genes was increased during biofilm formation.

**Quorum Sensing in Biofilm**

It is interesting to investigate how microbes establish communication network among themselves in a biofilm. They employ sufficiently complex communication mechanism termed as ‘quorum sensing’ (QS) (Joshi et al., 2010). QS plays key role in cell attachment and detachment from biofilms (Donlan, 2002). For example, in P. aeruginosa two different cell-to-cell signaling systems lasR-lasI and rhlR-rhlI are involved in biofilm formation (Davies et al., 1998, Donlan, 2002). At sufficiently high population densities, these signals reach concentrations enough for activation of genes involved in biofilm differentiation (Annous et al., 2009; Smith et al., 2004; Vu et al., 2009). Several QS signals are implicated in biofilm formation, e.g. (a) acylatehomoserine lactones (AHLs) among proteobacteria, (b) gamma butyrolactones in Streptomyces species, (c) cis-11-methyl-2-dodecanolic acid (also called DSF) in species of Xanthomonas, Xylella and other related spp., and (d) oligopeptides among gram-positive microbes (Joshi et al., 2010; Smith et al., 2004). Quorum sensing plays role in antibiotic production, toxin release (responsible for virulence), and horizontal gene transfer (HGT) (Smith et al., 2004). The frequencies of gene transfer are 10–600 times higher in biofilms than among planktonic cells (Donlan, 2002). Microbial biofilms provide a fertile ground for HGT, which can be a strong driving force for acquisition, development and spread of drug-resistance among microbial populations.

QS signals are not only responsible for communication among microorganisms in biofilm, but they also control production of virulence factors in biofilms. In P. aeruginosa AHLS control the production of cellular lysins (e.g., rhamnolipid-important for pathogenesis) and certain extracellular enzymes (Høiby et al. 2011; Jensen, 2007). QS inhibitors can reduce virulence of a biofilm. Antibiotics like ceftazidime, ciprofloxacin, macrolides, azithromycin, and clarithromycin inhibit QS in P. aeruginosa at sub-MIC (minimum inhibitory concentration) concentrations, which ultimately leads to loss of virulence in these bacteria (Høiby et al. 2011; Skindersoe et al. 2008). Brominated farnones interfere with cell-cell communication and are able to inhibit biofilm formation (Bridier et al., 2011). These quorum sensing inhibitors breakdown cell-cell communication and bacterial cells remain in planktonic stage, retaining their susceptibility to antimicrobials. Some plant products like ginseng and garlic (Estrela and Abraham, 2010) extracts are able to inhibit quorum sensing in bacterial community (Høiby et al. 2011). Curcumin (a well-known plant metabolite) at 1 µg /l, caused a 25% reduction in 3-oxo-dodecanoyl-AHL 6, and a 2%
reduction in butanoyl-AHL 2, resulting in reduction of P. aeruginosa pathogenicity (Estrela and Abraham, 2010). QS Inhibitors can prove effective at arresting biofilm formation, or for eradication of already existing biofilms.

**Antibiotic Resistance among Biofilms**

When bacteria exist in biofilm, the well-known mechanisms of antibiotic resistance, such as efflux pumps, modifying enzymes, and target mutations, do not always seem to be responsible for the protection of bacteria (Li Chen, 2011; Stewart and Costerton, 2001). Even sensitive bacteria which do not have a known genetic basis for resistance can have profoundly reduced susceptibility when they are present in a biofilm. When cells exist in a biofilm, they can become 10–1000 times more resistant to the effects of antimicrobial agents (Costerton et al., 1999; Davey and O'Toole, 2000; Hiorth et al., 2007; Lewis, 2001; Mah and O'Toole, 2001; Stewart and William, 2001; Scheie and Petersen, 2004). Many mechanisms (Figure 2) have been proposed for antibiotic resistance in biofilms.

**Figure 2:** Mechanisms of antibiotic resistance in biofilms
(Hosmin et al., 1992; Mah and O'Toole, 2001; Stewart and Costerton, 2001)

One of the proposed resistance mechanisms is based on the possibility of slow or incomplete penetration of the antibiotics into the biofilm (Donlan and Costerton, 2002), due to EPS matrix in which microorganisms are embedded in biofilm (Mah and O'Toole, 2001; Stewart and Costerton, 2001). A well known disinfectant chlorine was able to reach only upto 20% of that in bulk media within a mixed species biofilm of P. aeruginosa and Klebsiella pneumoniae (De Beer et al., 1994). Al-Fattani and Dauglas (2004) investigated penetration of antifungal drugs through Candida biofilms, and concluded that poor antifungal penetration is not a major drug resistance mechanism for Candida biofilms. They found that mixed species biofilm of bacteria (S. epidermidis) and yeast (C. albicans) allowed slower penetration of drugs than single species biofilm of C. albicans. Many researchers reported that penetration of aminoglycosides is retarded in P. aeruginosa biofilm, it is due to binding of aminoglycoside with alginate (polysaccharide) (Donlan and Costerton, 2002; Stewart and Costerton, 2001).

Another resistance mechanism focuses on altered chemical microenvironment within the biofilm. Microscale gradient formation in nutrient concentrations is a well-known feature of biofilms. Oxygen can be completely consumed in the surface layers of a biofilm, which ultimately leads to anaerobic environment in the deeper layers. Local accumulation of acidic waste products might lead to pH differences greater than 1 between the bulk fluid and the biofilm interior. Physiological heterogeneity and gradient formation is very well recorded within the biofilms. All the cells present in the biofilm are not in the same physiological or metabolic state (Joshi et al., 2010). Combined with this heterogeneity in microenvironment, slower growth rate of the microbes in biofilm than its planktonic stage, antibiotic action may be antagonized (Donlan and Costerton, 2002; Stewart and Costerton, 2001). Most antibiotics are best effective against actively growing cells. There can be significant differences in the metabolic and/or growth rates of biofilm bacteria compared to their planktonic counterparts. Welch et al. (2012) derived the specific growth rate of S. mutans bacterial biofilm. They found the specific growth rate of S. mutans in biofilm mode of growth was 0.70 h⁻¹, compared to 1.09 h⁻¹ in planktonic growth. Growth related effect of antibiotics in mixed species biofilms of P. aeruginosa, Escherichia coli and S. epidermidis were reported by Mah and O'Toole (2001). They observed increase in sensitivity to tobramycin or ciprofloxacin with increasing growth rate in both- planktonic and biofilm mode. This indicates that slow growth rate
of organisms in biofilm may offer them protection from antimicrobial agents (Folsom, 2010; Mah and O’Toole, 2001).

Microorganisms in a biofilm may form a unique and highly protected phenotypic state resembling cell differentiation during spore formation (Stewart and Costerton, 2001). This type of resistant phenotype can arise due to nutrient limitation, certain types of stress, and high cell density (Perumal et al., 2007; Hosmin et al., 1992). Perumal et al., (2007) reported that high cell density among C. albicans biofilm is responsible for antifungal drug resistance. Phenotypic change like alteration in membrane composition in response to antimicrobial agents, may ultimately lead to decrease in permeability to various antimicrobial agents. Mutation in ompB (regulator of ompF and ompC genes encoding the outer membrane porin proteins) and ompF increases resistance against various β-lactam antibiotics in E. coli (Mah and O'Toole, 2001).

**Problems Associated with Biofilms**

Biofilms have been associated with a wide range of problems in industry, medicine (dental plaque formation, clinical infections), and agriculture (plant infections). A brief description of the same follows:

**Biofilms in Public Health**

What’s alarming about biofilm is the fact that the organisms living in biofilm are more difficult to eradicate than their planktonic form. When cells exist in a biofilm, they can become much more resistant to the effects of antimicrobial agents than the planktonic cells (Costerton et al., 1999; Chen, 2011; Davey and O’toole, 2000; Hirsch et al., 2007; Lewis, 2001; Mah and O’Toole, 2001; Stewart and William, 2001). Biofilm infections are marked by recurrence of symptoms after cycles of antibiotic therapy. Most antibiotics are able to eliminate only planktonic cells and remaining sessile cells continue to disseminate when the treatment is terminated (Aparna and Yadav, 2008; Mah and O’Toole, 2001). It has been estimated that biofilms are associated with more than 60-65% of nosocomial infections (Talaro, 2008) and that treatment of these biofilm-associated infections costs >$1 billion annually (Mah and O’Toole, 2001). Biofilms from various indwelling medical devices and other clinical sources have been studied extensively over last 4 decades (Donlan and Costerton, 2002). Many pathogenic organisms have been noted to form biofilm on indwelling medical devices within the human body (Table 2).

### Table 2

Common organisms forming biofilms on medical implants (Aparna and Yadav, 2008; Chen, 2011; Donlan, 2001; Donlan, 2002; Donlan and Costerton, 2002; Kokare et al., 2009; McCann et al., 2008)

<table>
<thead>
<tr>
<th>Implant</th>
<th>Organism(s) forming biofilms on these implants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prosthetic valves</td>
<td>S. epidermidis, Streptococcus sanguis, S. aureus</td>
</tr>
<tr>
<td>Contact lenses</td>
<td>P. aeruginosa, S. epidermidis and other gram-positive cocci</td>
</tr>
<tr>
<td>Central venous catheters</td>
<td>S. epidermidis, S. aureus, E. faecalis, K. pneumoniae, P. aeruginosa, C. albicans</td>
</tr>
<tr>
<td>Artificial heart valves</td>
<td>P. aeruginosa, S. aureus, S. epidermidis, Enterococci</td>
</tr>
<tr>
<td>Urinary catheters</td>
<td>E. coli, E. faecalis, P. mirabilis, P. aeruginosa, K. pneumoniae</td>
</tr>
<tr>
<td>Orthopedic devices</td>
<td>Hemolytic Streptococci, Enterococci, P. mirabilis, Bacteroides sp., P. aeruginosa, E. coli</td>
</tr>
<tr>
<td>Endotracheal tube</td>
<td>S. aureus, S. epidermidis, E. coli, P. aeruginosa</td>
</tr>
<tr>
<td>Artificial voice prosthesis</td>
<td>Streptococci, Staphylococci, C. albicans</td>
</tr>
<tr>
<td>Intrauterine devices (IUDs)</td>
<td>S. epidermidis, S. aureus, Corynebacterium sp., Micrococcus sp., Enterococcus sp., C. albicans, Group B Streptococci</td>
</tr>
</tbody>
</table>

Biofilm formation can lead to malfunction of the device and destruction of adjacent tissue. Biofilms on indwelling medical implants may be composed of gram-positive or gram-negative bacteria or yeasts. Bacteria commonly isolated from these devices include the gram-positive Enterococcus faecalis, Staphylococcus aureus, S. epidermidis, Streptococcus viridans; and the gram-negative Escherichia coli, K. pneumoniae, Proteus
mirabilis, P. aeruginosa, etc. These microorganisms may be originated from the skin of patients, or health-care workers, or sometimes from various other sources in the environment. Depending on the device and its duration of use in the patient, biofilms may be composed of a single species or multiple species. Biofilms found on urinary catheter may initially be composed of single species, but later develop into multispecies type (Donlan, 2001).

Biofilms are also associated with non implant diseases (Table 3) ranging from a common earache to specific bacterial infections, e.g. cystic fibrosis, native valve endocarditis, otitis media, periodontitis, and chronic prostatitis. Biofilms in these cases may be composed of single or mixed species of bacteria or fungi (Donlan, 2002).

### Table 3

Human infections involving microbial biofilms (Aparna and Yadav, 2008; Donlan and Costerton, 2002; Kokare et al., 2009)

<table>
<thead>
<tr>
<th>Infection or disease</th>
<th>Commonly implicated species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cystic fibrosis pneumonia</td>
<td><em>P. aeruginosa</em>, <em>Burkholderia cepacia</em></td>
</tr>
<tr>
<td>Periodontitis</td>
<td><em>Porphyromonas gingivalis</em> and gram-negative oral bacteria</td>
</tr>
<tr>
<td>Otitis media</td>
<td><em>Streptococcus pneumoniae</em>, <em>Haemophilus influenzae</em>, <em>S. aureus</em>, <em>S. epidermidis</em>, <em>P. aeruginosa</em> and other organisms</td>
</tr>
<tr>
<td>Native valve endocarditis</td>
<td>Viridans <em>Streptococci</em>, <em>Enterococci</em>, <em>Pneumococci</em>, <em>Escherichia coli</em> and other organisms</td>
</tr>
<tr>
<td>Chronic Bacterial prostatitis</td>
<td><em>E. coli</em> and other gram-negative bacteria</td>
</tr>
<tr>
<td>Musculoskeletal infections</td>
<td>gram-positive cocci (e.g. <em>Staphylococci</em>)</td>
</tr>
<tr>
<td>Dental caries</td>
<td>Acidogenic gram-positive cocci (e.g. <em>Streptococcus</em>)</td>
</tr>
<tr>
<td>Osteomyelitis</td>
<td>Various bacterial and fungal species – often mixed</td>
</tr>
<tr>
<td>Biliary tract infection</td>
<td>Enteric bacteria (e.g. <em>E. coli</em>)</td>
</tr>
</tbody>
</table>

### Biofilms in Industry

Practically, every industry faces problem of biofilming. Biofilms can form in pipelines carrying various fluids. The level of biofilm formation in a given system is difficult to monitor. Levels higher than the permitted limit of coliforms, *Pseudomonas*, and *Flavobacterium* spp. are reported in water due to detachment from biofilm. Excessive biofilm formation on porous media, on heat exchanger surfaces, and in storage tanks is responsible for reduction in efficiency of heat transfer, and reduction of flow rate. Biofilms on ship hulls consist of diatoms, single celled algae and other gram-positive and gram-negative organisms. They are associated with reduced vessel speed in water, and increased fuel consumption (Elvers and Lappin-Scott, 2000; Fletcher, 1999). Biofilms have been associated with corrosion of metals such as iron, steel, manganese, and copper. Bacteria in biofilm are responsible for degradation of marble rocks, and mineral oxidation. *Thiobacillus ferroxidans* is responsible for oxidation of arsenopyrite (Fletcher, 1999). Biofilms have been associated with food spoilage and food poisoning (Elvers and Lappin-Scott, 2000). In dairy industries, *Listeria monocytogenes* is responsible for post-pasteurization contamination of food, as their biofilm forming ability provides for heat resistance and spore survival (Chmielewski and Frank, 2006; Elvers and Lappin-Scott, 2000). It forms biofilm on stainless steel, plastic and many other food contact surface materials. *Pseudomonas* spp. are also found in food processing environments such as drains, floors, meat surfaces, vegetables and in low acid dairy products. *Bacillus* spp. and *Salmonella* spp. have also been implicated in food spoilage in food processing industry (Chmielewski and Frank, 2006). Genes for biofilm formation were reported to be present in majority of *Aeromonas hydrophila* strains isolated from patients with gastroenteritis and diarrhoea in Brazil (Ljungh et al. 1977). Biofilm formation helps *A. hydrophila* in polar flagellar assembly and bacterial adhesion to host tissues. This organism is recognized as one of the most challenging, ubiquitous and opportunistic food borne pathogens, and is capable of growth even at low temperatures used for food storage and preservation (Canals et al., 2006; Kothari et al. 2010a; Patel et al., 2010).
### Table 4
Various plant diseases involving biofilms (Dow et al., 2003; Danhorn and Fuqua, 2007; Eberl et al., 2007; Ramey et al., 2004)

<table>
<thead>
<tr>
<th>Plant pathogen</th>
<th>Host plant</th>
<th>Colonization site</th>
<th>Disease caused</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. tumefaciens</td>
<td>Walnuts, tomatoes, and roses</td>
<td>Roots and crown tissue</td>
<td>Crown-gall disease</td>
</tr>
<tr>
<td>P. syringae</td>
<td>Arabidopsis thaliana, Nicotiana benthamiana, and tomato</td>
<td>Leaves</td>
<td>Brown spot disease</td>
</tr>
<tr>
<td>Erwinia chrysanthemi</td>
<td>Potato</td>
<td>Fruit, leaves and flowers</td>
<td>Soft rot and black leg</td>
</tr>
<tr>
<td>X. Campestris pv. Campestris</td>
<td>Cruciferous plants</td>
<td>Xylem vessels</td>
<td>Black rot</td>
</tr>
<tr>
<td>Ralstonia solanacearum</td>
<td>Tobacco, tomato, pepper, Irish potato</td>
<td>Roots to xylem</td>
<td>Lethal wilt</td>
</tr>
<tr>
<td>Clavibacter michiganensis subsp. sepedonicus</td>
<td>Potato</td>
<td>Xylem vessels</td>
<td>Ring rot</td>
</tr>
<tr>
<td>Pantoea stewartii subsp. Stewartii</td>
<td>Maize</td>
<td>Xylem vessels</td>
<td>Stewart’s wilt disease</td>
</tr>
</tbody>
</table>

### Biofilms in Agriculture
Formation of biofilm is important for effective disease transmission to the plant by phytopathogenic microbes (Eberl et al., 2007). Various plant pathogens like A. tumefaciens, X. campestris, P. syringae, and Erwinia spp. are able to form biofilm on plant surfaces (Danhorn and Fuqua, 2007; Ramey et al., 2004). Each year a large proportion of crop is lost due to these plant pathogens and in order to maintain the productivity, more and more chemicals are being added in the natural environment. These chemical pesticides enter the food chain resulting in serious harmful effects on human health. According to a survey made by the WHO more than 50,000 people in developing countries are annually poisoned and 5000 die as result of the effect of toxic agents used in agriculture (Bhardwaj and Laura, 2009). Biofilms may form on various parts of plants such as leaves, roots, seeds, and internal vasculature (Table 4) (Danhorn and Fuqua, 2007; Eberl et al., 2007; Ramey et al., 2004).

In X. Campestris pv. campestris, responsible for black rot on cruciferous plants, the polysaccharide xanthan gum (EPS forms biofilm matrix) and degradative exoenzymes are primary virulence factors that govern vascular blockage and migration of the organism through the vasculature. For many other plant pathogens too, production of exopolysaccharide has been shown to be essential for establishment of disease (Eberl et al., 2007).

### Applications of Biofilm
Besides their adverse impact on human health and industrial productivity, it is important to note biofilms as an integral part of nature. Quite a few beneficial applications of microbial biofilms have been proposed viz. their use in drinking water/waste water treatment, detoxification of toxic and hazardous wastes, etc. Bacterial biofilms can also be engineered in vitro for specific biotechnological applications.

**Water and wastewater treatment:** Biofilms have been used successfully in water and wastewater treatment for over a century. Engineers have exploited natural biofilm forming ability of microbes in developing water-treatment systems like trickling filters for removal of biological pollutants. (De Beer and Stoodley, 2006; Fletcher, 1999) Biological filters are employed for reducing the concentration of biodegradable organic carbon entering the water distribution systems (http://biofilmbook.hypertextbookshop.com/v03/r002/conte...
Phototrophic cyanobacterial biofilms can be used for additional nutrient removal from secondary effluents of wastewater treatment plants. An increase in pH affected by photosynthetic activity of biofilm, causes precipitation of dissolved phosphate (Roeselers et al., 2008). Removal of phosphorus this way helps in reducing the possibility of eutrophication. Immobilized biofilm may be used as biobarriers for trapping ground water contaminants (De Beer and Stoodley, 2006).

**Fungal-rhizobial biofilm:** Fungal-rhizobial biofilm may be used as inoculum to improve nodulation and nitrogen fixation in Rhizobium-legume symbiosis. Higher nodulation and enhanced N₂-fixation was observed in *Pezicillium-Rhizobium* biofilm treated plants (Ariyaratne et al., 2011; Seneviratne et al., 2008). In *Pleurotus ostreatus*-Bradyrhizobial biofilms nitrogenase activity was detected in the biofilm, but not in the fungus or *Bradyrhizobium* alone (Rinaudi and Giordano, 2010). The biofilm inocula can be used in biosolubilisation of rock phosphate. Mixed species biofilm composed of *Penicillium* spp., *Xanthoparmelia mexicana* and *P. ostreatus* - a lichen fungus- increased the phosphate solubilisation up to ca. 230% compared to the monocultures. The biofilm inocula can also be used for successful establishment of introduced beneficial microorganisms in plants for biocontrol of diseases. *P. ostreatus-Pseudomonas fluorescens* biofilm increased endophytic colonisation of tomato by *P. fluorescens*, a biocontrolling agent, by over 1,000% compared to inoculation with *P. fluorescens* alone (Seneviratne et al., 2008).

**Oil degradation/recovery:** Biofilms are used for microbially enhanced oil recovery (MEOR) (De Beer and Stoodley, 2006; Hiorth et al., 2007). Biofilm of *Clostridium acetobutylicum* was employed to enhance oil recovery from fields in Arkansas, USA (Jack, 1985). Biofilms of cyanobacteria such as, *Oscillatoria* spp., sulfate reducing bacteria and aerobic heterotrophs are also used for oil degradation in oil contaminated beaches. Cyanobacterial N₂ fixation can provide sufficient nitrogen source for heterotrophic oil degradation, reducing the need for exogenous supply of nutrients. Free radicals formed during oxygenic photosynthesis carried out by cyanobacteria can indirectly increase photochemical oil degradation (Roeselers et al., 2008).

**Removal of heavy metals:** Phototrophic biofilms have important role in the detoxification of waste water polluted with heavy metals. Mucilage sheaths of cyanobacteria, *Microcystis aeruginosa* and *Aphanthece halophyta* are known to have high affinity to heavy metal ions including copper, lead, and zinc (Roeselers et al., 2008). These types of applications are based on biosorption or bioaccumulation of metal ions by microbial biomass. The elevated pH inside the photosynthetically active biofilms helps removal of metals by precipitation, as most metals remain in solution only at acidic pH.

**Methods for Study of Biofilms**

A number of methods are available to assess biofilm forming ability of microorganisms and/or to test their susceptibility to antimicrobial agents, but no method is universally applicable because of inherent analytical limitations associated with measurements of bacterial adhesions. Some of these methods are described below:

**(a) Tissue culture plate (TCP) method** (Hirshfield et al., 2009; Mathur et al., 2006; Rukayadi and Hwang, 2006): TCP assay described by Christensen et al. (1985) is the most widely used assay and is considered as standard test for detection of biofilm formation. In this method biofilm formation is initiated by addition of nutrient media and inoculum in surface treated polystyrene tissue culture plate, followed by detection of biofilm by crystal violet staining (after incubation for definite period). Biofilm-coated wells of microtiter plate are vigorously shaken in order to remove all nonadherent bacteria. The remaining attached bacteria are washed twice with phosphate buffer saline (PBS) and then air-dried. Then, each of the washed wells is stained with aqueous crystal violet solution (0.1- 0.4 %). Afterwards, each well is washed twice with sterile distilled water and immediately de-stained with 95% ethanol. De-staining solution is then transferred to a new well and the amount of the crystal violet extracted in the de-staining solution is estimated with a microplate reader at 500 - 600 nm. TCP method allows a quantitative measure of the mass of biofilm cells. This method has found
use in antimicrobial susceptibility tests. TCP method was used for determination of effect of xanthorrhizol (XTZ) (purified from the rhizome of Curcuma xanthorrhiza Roxb.) on the S. mutans biofilms (Rukayadi and Hwang, 2006). This method was used for evaluating eradication of S. mutans biofilms, when challenged with certain plant extracts prepared by microwave assisted extraction (data yet unpublished). However, this method can only measure eradication of biofilm from the plate surface, and does not give indication about viability of the biofilm, as crystal violet stains peptidogycan of both live and dead cells within biofilm. Thus TCP method, though quantitative, fails to determine viability of bacterial cells in biofilm, but is suitable for assessing eradication potential of test antimicrobials.

(b) Tube method (TM) (Christensen et al, 1982): In this method nutrient media is inoculated in a test tube with a loop full of activated culture, and incubated for 24 hour at optimum growth temperature of respective organism. The tubes are then decanted and washed with PBS (pH 7.3) and dried. Dried tubes are stained with crystal violet (0.1%). Excess stain is removed and tubes are washed with deionized water. Dried Tubes are observed for biofilm formation. Biofilm formation is considered positive when a visible film lines the wall and bottom of the tube. Ring formation at the liquid interface is not considered indicative of biofilm formation. Tubes are examined and the degree of biofilm formation is scored qualitatively on a scale of 0-3 (0-absent, 1-weak, 2-moderate, 3-strong). This method does not allow for quantification of biofilm formation. It was used for studying biofilms of clinical isolates of the genus Staphylococcus (Mathur et al., 2006).

(c) Congo red agar method (CRA) (Freeman et al., 1989): A specially prepared solid medium brain heart infusion (BHI) agar supplemented with 5% sucrose and congo red is used in this qualitative method. Plates are inoculated and incubated aerobically for 24 to 48 hour at optimum temperature of respective isolate. Efficient biofilm formation is positively indicated by black colonies with a dry crystalline consistency. Weak slime producers usually remain pink, though occasional darkening at the centers of colonies is observed. A darkening of the colonies with the absence of a dry crystalline colonial morphology indicates an indeterminate result. Utility of this method for screening of biofilm formation by Staphylococci was evaluated by Mathur et al. (2006).

(d) Flow cell Method (Lewis, 2001): In this method, cells are allowed to adhere to 24 detachable discs and grow into a biofilm. Once a biofilm is formed, the feeding liquid can be replaced with a culture medium that contains test compound. After incubation, the device is taken apart and the cells are dislodged by sonication and plated. Reproducible biofilm formation and the observation of biofilm dynamics are advantages of this method. The discs can also be used for microscopic observations of biofilm structure. But this method is less suited for large scale susceptibility studies in which hundreds (often thousands) of samples are to be examined. This method was used for investigating efficacy of ciprofloxacin against P. aeruginosa and S. aureus biofilms (Gupta et al. 2011).

(e) Calgary biofilm Device (Lewis, 2001): This disposable apparatus combines the shearing force that makes a robust biofilm with a microtiter plate capability. A 96-prong replicator with plastic pins is inserted into a grooved tray filled with growth medium inoculated with cells. Following inoculation the apparatus is placed on a tilting shaker platform and the growing cell suspension washes the pins, on which growth of biofilms occurs. Once a biofilm is formed, the lid with pins can be placed into a microtiter plate for susceptibility testing. After incubation with antibiotics, by mild sonication the cells can be dislodged and plated for determination of colony counts. This method was used to examine effect of the MUC7 peptides on S. mutans biofilm (Wei et al., 2006).

(f) Confocal laser scanning microscopy (CLSM) (Xavier et al., 2003): This method allows continuous monitoring of biofilm development in flow cell reactors, and optical sectioning of the structure of biofilm. The in vivo reconstruction of the three-dimensional structure of microbial biofilms in their naturally hydrated and undisturbed form is possible by CLSM. It can be used for monitoring of morphological parameters of biofilm development. Using CLSM S. epidermidis biofilm on contact lens was studied (Leshem et al., 2011). This method is usually used in biocorrosion studies.

(g) Adenosine triphosphate (ATP)-bioluminescence assay (Jin et al., 2004): In this assay after
formation of biofilm, each well is washed four times with PBS, followed by addition of 200 µl of PBS into each well. Then biofilm mass is scraped off from the well wall by using a sterile scalpel. This suspension containing detached biofilm mass is transferred to a sterile tube, vortexed for 3 minutes and subjected to ATP assay. A commercially available ATP analyser can be used for quantification of ATP in viable cells within biofilms. An aliquot of 100 µl, each of the sample (cell suspension) and the extractant is added into a new container which is inserted into the ATP analyser for 30 seconds for extraction of intracellular ATP. It is followed by addition of 100 µl of luminescent reagent to this mixture, and result is recorded in form of ATP concentration (mol/l) as measured by a luminometer. This method was used for antibiotic susceptibility assay of S. aureus biofilms (Amorena et al., 1999).

Natural Products as Potent Anti-Biofilm Agents
Continuous appearance of drug-resistant strains of pathogenic microorganisms makes it necessary to search for novel natural or synthetic antimicrobial compounds. Reduced susceptibility of biofilms to conventional antibiotics makes this challenge more daunting. Natural products have been a significant source of commercial medicines and drug leads. Plants are naturally gifted at the synthesis of bioactive compounds. Screening of crude plant extracts is the first step in the long process of discovery of novel bioactive compounds. Isolation of active principle(s) from these crude extracts followed by successful structure elucidation can provide novel lead compounds (Kothari and Seshadri, 2010). Plants synthesize several bioactive compounds such as polyphenols, terpenoids, essential oils, alkaloids, saponins, peptides and proteins, with antibacterial, antifungal, antioxidant and other properties (Kothari et al., 2009b). Several plant leaf extracts e.g. betel, banana, tea, curry, aloe vera, piper mint, etc. have been used to degrade the bacterial biofilms (Saito et al., 2012). Aqueous leaf extract of Cassia alata could inhibit biofilm formation of S. epidermidis (at 0.5 mg/mL) and P. aeruginosa (at 0.025 mg/ml) (Agrawal, 2011). Cranberry extract (500 µg/ml) could reduce the formation of S. epidermidis biofilm on soft contact lenses (Leshem et al., 2011). 50 µmol l -1 of xanthorrhizol (XTZ) (purified from the rhizome of Curcuma xanthorrhiza Roxb.) removed 76% of biofilm at plateau accumulated phase (at 24 h) when applied to S. mutans biofilm for 60 minutes (Rukayadi and Hwang, 2006). In our lab (data yet to be published) extracts of Tamarindus indica and Syzygium cumini seeds were found to be capable of killing S. mutans in biofilm. Acetone and methanol extract of T. indica seeds were able to kill more than 95% cells of S. mutans in biofilm. Hydroalcoholic extract of S. cumini seeds were able to cause a loss of 97.72% in viability of S. mutans biofilm. Ampicillin up to a conc of 30 µg/ml failed to cause any effect on S. mutans biofilm; at 40 µg/ml it was able to kill 97.81% of the cells without eradicating the biofilm. Eradication caused by the seed extracts was in all cases lesser than the loss of viability. This indicates that these extracts were able to penetrate the biofilm matrix without fully eradicating it.

Final Comments
Microorganisms, in their planktonic form, have excited generations of microbiologists and still continue to do so. Biofilms of these tiny creatures are generating even more excitement. Their inherent heterogeneity makes them more challenging to study, and their inherent reduced susceptibility to antimicrobial agents makes it difficult to control them. Many potential applications of microbial biofilms in waste treatment, bioremediation, and agriculture await the attention of current and future generations of microbiologists. It will be important for the investigators in this field to learn to cope with the heterogeneous nature of biofilms, in order to generate more reproducible results during biofilm experiments. Development of novel methods for study of processes occurring inside a biofilm, and for accurate determination of their viability status will make faster growth of this field of biology possible. Laying down widely accepted guidelines for antimicrobial susceptibility testing against biofilms will be of crucial importance for medical microbiologists.
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