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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, N_2 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 primeval 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,

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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

History of biofilm research (Costerton *et al*., 1978; Donlan, 2002; Donlan and Costerton, 2002; Elvers, and Lappin-Scott 2000)

Year	Investigator	Contribution
17th Century	van Leeuwenhoek	First examined microorganisms from his own teeth surfaces
1930	Claude ZoBell	Research on bacterial adhesion to surfaces
1976	Marshall	Observed the role of "very fine extracellular polymer fibrils" that attaches bacteria to surfaces
1978	Costerton *et al*.	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
1987	Costerton *et al*.	Pointed that biofilm comprises of single cells and microcolonies which are embedded in a highly hydrated, predominantly anionic extracellular polymeric matrix
1990	Characklis and Marshall	Described characteristics of spatial and temporal heterogeneity and role of inorganic or abiotic substances held together in the biofilm matrix.
1995	Lappin-Scott	Investigated adhesion triggered expression of genes that control production of bacterial components which are necessary for adhesion and biofilm formation.

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).

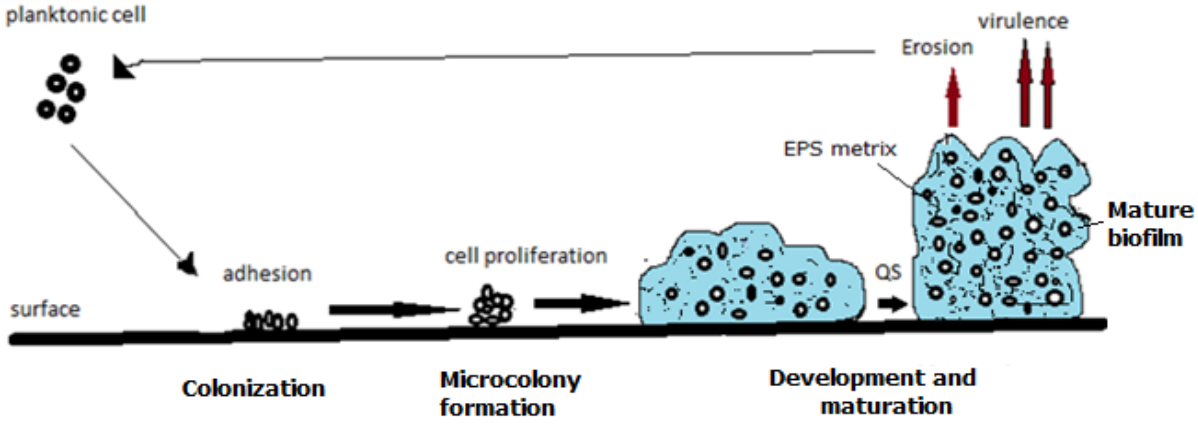

Figure 1: Events during biofilm formation
(Anwer, 1992; Annous, 2009; Høiby, 2011; Proal, 2008; Scheie and Petersen, 2004)

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 gram-positives), and farnesol systems (in fungi) (Høiby *et al*., 2011; Li Chen, 2011). 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 phospho-mannomutase, involved in alginate (exopoly-saccharide) 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-dodecanoic 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 faranones 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.

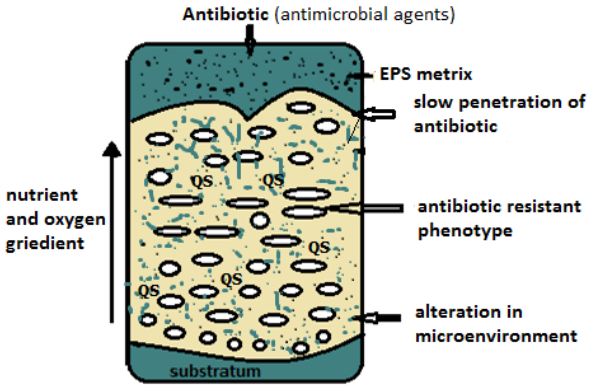

Figure 2: Mechanisms of antibiotic resistance in biofilms
(Hosmin *et al*., 1992; Mah and O'Toole, 2001; Stewart and Costerton, 2001)

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 to1.09 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; Hiorth *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)

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

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 microrganisms 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)

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

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)

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

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/v003/r002/conte

nts/chapters/chapter001/section003/blue/page001.html). 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-rhozobial biofilm: Fungal-rhozobial biofilm may be used as inoculum to improve nodulation and nitrogen fixation in Rhizobium-legume symbiosis. Higher nodulation and enhanced N_2-fixation was observed in *Penicillium-Rhozobium* 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 biofilmed 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 biofilmed 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 N_2 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 *Aphanothece halophytica* 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 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 aliquote 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*., 2010b). Several plant leaf extracts e.g. betel, banana, tea, curry, aloevera, 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 upto 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.

References

Agrawal I. (2011), "Susceptibility of bacterial biofilm against some leaf extracts", *Plant Sciences Feed*, Vol. 1 No. 5, pp. 69-73

Al-Fattani and Douglas, L. J. (2004), "Penetration of *Candida* biofilms by antifungal agents", *Antimicrobial Agents and Chemotherapy*", Vol. 48 No. 9, pp. 3291- 3297

Amorena, B., Gracia, E., Monzón, M., Leiva, J., Oteiza, C., Pérez, M. and Hernández-Yago, J. (1999), "Antibiotic susceptibility assay for *Staphylococcus aureus* in biofilms developed in vitro", *Journal of Antimicrobial Chemotherapy*, Vol. 44 No. 1, pp. 43-55

Annous, B., Fratamico, P. and Smith, J. L. (2009), "Quorum sensing in biofilms: why bacteria behave the way they do", *Journal of Food Science*, Vol. 74 No. 1, pp. 24–37

Anwer, H., Strap, J. L. and Costerton, J. W. (1992), "Establishment of aging biofilms: possible mechanism of bacterial resistance to antimicrobial therapy", *Antimicrobial Agents and Chemotherapy*, Vol. 36 No. 7, pp. 1347-1351

Aparna, M. S. and Yadav, S. (2008), "Biofilms: microbes and disease", *Brazilian Journal of Infectious Diseases*, Vol. 12 No. 6, pp. 526-530

Ariyaratne, M. D., Seneviratne, G. and Kulasooriya, S. A. (2011), "Comparison of rhizobial inoculants and fungal-rhizobial biofilms on nodulation, N-yield, growth and development of green gram (*Vigna radiate*)", *Proceedings of the Peradeniya University Research Sessions*, Sri Lanka, pp. 186

Atlas, Ronald and Bartha, Richard (1998), *Microbial Ecology: Fundamentals and Applications*, Pearson, South Asia

Bhardwaj, S. K. and Laura J. S. (2009), "Anti-bacterial activity of some plant extracts against plant pathogenic bacteria *Xanthomonas campestris* pv. *campestris*", *Indian Journal of Agricultural Research*, Vol. 43 No. 1, pp. 26-31

Bridier, A., Briandet, R., Thomas, V. and Dubois-Brissonnet, F. (2011), "Resistance of bacterial biofilms to disinfectants: a review", *Biofouling*, Vol. 27 No. 9, pp. 1017-1032

Burmølle, M., Webb, J. S., Rao, D., Hansen, L. H., Sørensen, S. J. and Kjelleberg, S. (2006), "Enhanced biofilm formation and increased resistance to antimicrobial agents and bacterial invasion are caused by synergistic interactions in multispecies biofilms", *Applied and Environmental Microbiology*, Vol 72 No. 6, pp. 3916–3923

Canals, R., Altarriba, M., Vilches, S., Horsburgh, G., Shaw, J. W., Tom J. M. and Merino S. (2006), "Analysis of the lateral flagellar gene system of *Aeromonas hydrophila* AH-3", *Journal of Bacteriology*, Vol. 188 No. 3, pp. 852–862

Chmielewski, R. A. N., and Frank, J. F. (2006), "Biofilm formation and control in food processing facilities", *Comprehensive Reviews in Food Science and Food Safety*, Vol. 2 No. 1, pp. 22-32

Christensen, G. D., Simpson, W. A., Bisno, A. L. and Beachey, E. H. (1982), "Adherence of slime-producing strains of *Staphylococcus epidermidis* to smooth surfaces", *Infection and Immunity*, Vol. 37 No.1, pp. 318-326

Christensen, G. D., Simpson, W. A., Younger, J. J., Baddour, L. M., Barrett, F. F., Melton, D. M. and Beachey, E. H. (1985), "Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices", *Journal of Clinical Microbiology*, Vol. 22 No. 6, pp. 996-1006

Costerton, J. W., Geesey, G. G. and Cheng, G. K. (1978), "How bacteria stick", *Scientific American*, Vol. 238 No. 48, pp. 86–95

Costerton, J. W., Stewart, P. S. and Greenberg, E. P. (1999), "Bacterial biofilms: a common cause of persistent infections", *Science*, Vol. 284 No. 5418, pp. 1318-1322

Danhorn, T. and Fuqua, C. (2007), "Biofilm formation by plant-associated bacteria", *Annual Review of Microbiology*, Vol. 61, pp. 401-422, DOI 10.1146/annurev.micro.61.080706.093316

Davey, M. E. and O'toole, G. A. (2000), "Microbial biofilms: from ecology to molecular

genetics", *Microbiology and Molecular Biology Reviews*, Vol. 64 No. 4, pp. 847-867

Davies, D. G., Parsek, M. R., Pearson, J. P., Iglewski, B. H., Costerton, J. W. and Greenberg, E. P. (1998), "The involvement of cell-to-cell signals in the development of a bacterial biofilm", *Science*, Vol. 280 No. 5361, pp. 295–298

De Beer, D., Srinivasan, R. and Stewart, P. S. (1994), "Direct measurement of chlorine penetration into biofilms during disinfection" *Applied Environmental Microbiology*, Vol. 60 No. 12, pp. 4339- 4344

De Beer, D. and Stoodley, P. (2006), "Microbial biofilms", in Dworkin, M., (Ed.) *The prokaryotes*, Springer, New York. pp. 904-937

Donlan, R. M. (2001), "Biofilms and device-associated infections", *Emerging Infectious Diseases*, Vol. 7 No. 2, pp. 277-328

Donlan, R. M. (2002), "Biofilms: microbial life on surfaces", *Emerging Infectious Diseases,* Vol. 8 No. 9, pp. 881-890

Donlan, R. M. and Costerton, J. W. (2002), "Biofilms: survival mechanisms of clinically relevant microorganisms", *Clinical Microbiology Reviews*, Vol. 15 No. 2, pp. 167-193

Dow, J. M., Crossman, L., Findlay, K., He, Y. Q., Feng, J. X. and Tang, J. L. (2003), "Biofilm dispersal in *Xanthomonas campestris* is controlled by cell–cell signaling and is required for full virulence to plants", *Proceedings of the National Academy of Sciences*, Vol. 100 No. 19, pp. 10995-11000

Eberl, L., Von Bodman, S. B. and Fuqua, C. (2007), "Biofilms on plant surfaces", in Kjelleverg, S. and Givskov, M. (Ed.) *The Biofilm Mode of Life: Mechanisms and Adaptations*, Horizon Bioscience, U.K., pp. 215-233

Elvers, K. T. and Lappin-Scott, H. M. (2000), "Biofilms and biofouling", in Lederberg, J. (Ed.) *Encyclopedia of Microbiology*, Academic press, USA, pp. 478 -485

Estrela, A. B. and Abraham, W. (2010), "Combining biofilm-controlling compounds and antibiotics as a promising new way to control biofilm infections", *Pharmaceuticals,* Vol. 3 No. 5, pp. 1374-1393

Fletcher, M. (1999), "Biofilm and biocorrosion", in Demain, A. L. and Davies, J. E. (Ed.) *Industrial Microbiology and Biotechnology*, American Society for microbiology, Washington, pp.704-714

Folkesson, A., Haagensen, J. A. J., Zampaloni, C., Sternberg, C. and Molin, S. (2008). "Biofilm induced tolerance towards antimicrobial peptides", *Public Library of Science*, Vol. 3 No. 4, pp. 1-11

Folsom, J. P., Richards, L., Pitts, B, Roe, F., Ehrlich, G. D., Parker, A., Mazurie, A. and Stewart, P. S. (2010), "Physiology of *Pseudomonas aeruginosa* in biofilms as revealed by transcriptome analysis", *BMC Microbiology*, Vol. 10 No. 294, pp.1-17

Freeman, D. J., Falkiner, F. R. and Keane, C. T. (1989), "New method for detecting slime production by coagulase negative *staphylococci*", *Journal of Clinical Pathology*, Vol. 42 No. 8, pp. 872-874

Gupta, S., Agarwal, S, Sahoo, D. R. and Muralidharan, S. (2011), "In vitro production of biofilm in a flow cell system in a strain of *Pseudomonas aeruginosa* and *Staphylococcus aureus* and determination of efficiency of ciprofloxacin against them", *Indian Journal of Pathology and Microbiology*, Vol. 54 No. 3, pp. 569-571

Hiorth, A., Kaster, K., Lohne, A., Siqveland, O. K., Berland, H., Giske, N. H. and Stavland, A. (2007), "Microbial enhanced oil recovery-mechanism", International symposium of the society of core analysts, Calgary, Canada, 10-13 September.

Hirshfield, I. N., Barua, S. and Basu, P. (2009), "Overview of biofilms and some key methods for their study", in Goldman, E. and Green, L. H. (Ed.) *Practical Handbook of Microbiology*, CRC press, Boca Raton, pp. 675-688

Høiby, N., Ciofu, O., Johansen, H. K., Song, Z. J., Moser, C., Jensen, P. and Bjarnsholt, T. (2011), "The clinical impact of bacterial biofilms", *International Journal of Oral Sciences*, Vol. 3 No. 2, pp. 55-65

Jack, T. R. (1985), "Microbially Enhanced Oil Recovery", in Moo-Young, M. (Ed.) *Comprehensive Biotechnology*, pp. 295-303

Jain, A., Marsili, E. and Bhosle, N. B. (2011), "The Biofilm Returns: Microbial Life at the Interface", *Microbes and Microbial Technology*, Springer, New York, pp. 59-85

Jensen, P. Ø., Bjarnsholt, T., Phipps, R., Rasmussen, T. B., Calum , H., Christoffersen, L., Moser, C., Williams, P., Pressler, T., Michael Givskov, M. and Høiby, N. (2007), "Rapid necrotic killing of polymorphonuclear leukocytes is caused by quorum- sensing-controlled production of rhamnolipid by *Pseudomonas aeruginosa*", *Microbiology*, Vol. 153 No. 5, pp. 1329–1338

Jin, Y., Samaranayake, L. P., Samaranayake, Y. and Yip, H. K. (2004), "Biofilm formation of *Candida albicans* is variably affected by saliva and dietary sugars", *Archives of Oral Biology*, Vol. 49 No. 10, pp. 789-798

Joshi, P., Wadhwani, T., Bahaley, P. and Kothari, V. (2010), "Microbial Chit-Chat: Quorum Sensing", *The IUP Journal of Life Sciences*. Vol. 4 No. 1, pp. 59-72

Kokare, C. R., Chakraborty, S., Khopade, A. N. and Mahadik, K. R. (2009), "Biofilm: importance and applications", *Indian Journal of Biotechnology*, Vol. 8 No. 2, pp. 159-168

Kothari, V. and Seshadri, S. (2010), "In vitro antibacterial activity in seed extracts of *Manilkara zapota, Anona squamosa*, and *Tamarindus indica*", *Biological Research*, Vol. 43 No. 2, pp. 165-168

Kothari, V., Naraniwal, N. and Gupta, A. (2010a), "Effect of certain phytochemicals on *Aeromonas hydrophila*", *Research in Biotechnology*, Vol. 2 No. 4, pp. 20-25

Kothari, V., Shah, A., Gupta, S., Punjabi, A. and Ranka, A. (2010b), "Revealing the antimicrobial potential of plants", *International Journal of Biosciences and Technology*, Vol. 3 No.1, pp. 1-20

Leshem, R., Maharshak, I., Jacob, E. B., Ofek, I. and Kremer, I. (2011), "The effect of nondialyzable material (NDM) cranberry extract on formation of contact lens biofilm by *Staphylococcus epidermidis*", *Investigative Ophthalmology and Visual Science*, Vol. 52 No. 7, pp. 4929-4934

Lewis, K. (2001), "Riddle of biofilm resistance", *Antimicrobial Agents and Chemotherapy*, Vol. 45 No. 4, pp. 999-1007

Li Chen, Y. H. (2011), "The role of bacterial biofilm in persistent infections and control strategies", *International Journal of Oral Science*, Vol. 3 No. 2, pp. 66-73

Ljungh A, Popoff M, and Wadstrŏm T. (1977), "*Aeromonas hydrophila* in acute diarrheal disease: Detection of enterotoxin and biotyping of strain", *Journal of Clinical Microbiology*, Vol. 6, pp. 96-100

Mah, T. F. C. and O'Toole, G. A. (2001), "Mechanisms of biofilm resistance to antimicrobial agents", *Trends in Microbiology*, Vol. 9 No. 1, pp. 34-39

Marcus, H., Austria, A. L. and Baker, N. R. (1989), "Adherence of *Psudomonas aeruginosa* to trachel epithelium", *Infection and Immunity*, Vol. 57 No. 4, pp. 1050-1053

Mathur, T., Singhal, S., Khan, S., Upadhyay, D. J., Fatma, T. and Rattan, A. (2006), "Detection of biofilm formation among the clinical isolates of staphylococci: an evaluation of three different screening methods", *Indian Journal of Medical Microbiology*, Vol. 24 No. 1, pp. 25-29

McCann, M. T., Gilmore, B. F. and Gorman, S. P. (2008), "Staphylococcus epidermidis device related infections: pathogenesis and clinical management*" Journal of Pharmacy and Pharmacology*, Vol. 60 No. 12, pp. 1551-1571

Nobbs, A. H., Lamount, R. J. and Jenkinson, H. (2009), "Streptococcus adherence and colonization", *Microbiology and Molecular Biology Reviews*, Vol. 73 No. 3, pp. 407-505

Patel, D., Desai K., Lawani D. and Kothari, V. (2010), "*Aeromonas hydrophila*: a challenging food borne pathogen", *International Journal of Life Sciences and Technology,* Vol. 3 No 4, pp. 39-43

Perumal, P., Mekala, S. and Chaffin, W. L. (2007), "Role for cell density in antifungal drug resistance in *Candida albicans* biofilms",

Antimicrobial Agents and Chemotherapy, Vol. 51 No. 7, pp. 2454-2463

Proal, A. (2008), "Understanding biofilms", Available at: http://bacteriality.com/2008/05 /26 /biofilm. (Accessed; 26 march 2013)

Ramage, G., Bachmann S., Patterson T. F., Wickes B. L. and Lopez- Ribot J. L. (2002), "Investigation of multidrug efflux pumps in relation to fluconazole resistance in *Candida albicans* biofilms", *Journal of Antimicrobial Chemotherapy,* Vol. 49 No. 6, pp. 973–980

Ramey, B. E., Koutsoudis, M., Von Bodman, S. B. and Fuqua, C. (2004), "Biofilm formation in plant-microbe associations", *Current Opinion in Microbiology*, Vol 7, pp. 602–609, DOI 10.1016/j.mib.2004.10.014

Rinaudi, L. V. and Giordano, W. (2010), "An integrated view of biofilm formation in rhizobia", *FEMS Microbiology Letters*, Vol. 304 No. 1, pp. 1-11

Roeselers, G., Loosdrecht, M. C. V. and Muyzer, G. (2008), "Phototrophic biofilms and their potential applications", *Journal of Applied Phycology*, Vol. 20 No. 3, pp. 227-235

Rukayadi, Y. and Hwang, J. K. (2006), "In vitro activity of xanthorrhizol against *Streptococcus mutans* biofilms", *Letters in Applied Microbiology*, Vol. 42 No. 4, pp. 400-404

Saito, S. T., Trentin, D. D. S., Macedo, A. J., Pungartnik, C., Gosmann, G., Silveira, J. D. D. and Brendel, M. (2012), "Bioguided fractionation shows *cassia alata* extract to inhibit *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* growth and biofilm formation", *Evidence-Based Complementary and Alternative Medicine*, Vol. 2012, pp. 1-13, DOI 10.1155/2012/867103.

Scheie, A. A. and Petersen F. C. (2004), "The biofilm concept: consequence for future prophylaxis of oral diseases", *Critical Review in Oral Biology and Medicine*, Vol. 15 No. 1, pp. 4-12

Schroeder, K., Jularic, M., Horsburgh S. M., Hirschhausen, N., Neumann, C., Bertling, A., Schulte, A., Foster, S., Kehrel, B. E., Peters, G. and Heilmann, C. (2009), "Molecular characterization of a novel *Staphylococcus aureus* surface protein (SasC) involved in cell aggregation and biofilm accumulation", *Public Library of Science,* Vol. 4 No. 10, pp. 1-14

Seneviratne, G., Kecskés, M. L. and Kennedy, I. R. (2008), "Biofilmed biofertilisers: Novel inoculants for efficient nutrient use in plants", Available at: aciar.gov.au/files/node/9817/ PR130%20Part%205.pdf (Accessed: 4 April 2013).

Skindersoe, M. E., Alhede, M. and Phipps, R. (2008), "Effects of antibiotics on quorum sensing in *Pseudomonas aeruginosa*", *Anti-microbial Agents and Chemotherapy*, Vol. 52 No. 10, pp. 3548–3663

Smith, J. L., Fratamico, P. M. and Novak, J. S. (2004), "Quorum sensing: a primer for food microbiologists", *Journal of Food Protection*, Vol. 67 No. 5, pp. 1053-1070

Stewart, P. S. and Costerton, J. W. (2001), "Antibiotic resistance of bacteria in biofilms", *The Lancet*, Vol. 358 No. 9276, pp. 135-138

Talaro, Kathleen, Park (2008), *Foundation in Microbiology: Basic Principles*, McGraw-Hill, New York

Tojo, M., Yamashita, N., Goldmann, D. A. and Pier, G.B. (1988), "Isolation and characteri-zation of a capsular polysaccharide adhesin from *Staphylococcus epidermidis*", *Journal of Infectious Diseases*, Vol. 157 No. 4, pp. 713–722

Vu, B., Chen, M., Crawford, R. J. and Ivanova, E. P. (2009), "Bacterial Extracellular Polysac-charides Involved in Biofilm Formation", *Molecules*, Vol. 14 No. 7, pp. 2535-2554

Wei, G. X., Campagna, A. N. and Bobek, L. A. (2006), "Effect of MUC7 peptides on the growth of bacteria and on *Streptococcus mutans* biofilm", *Journal of Antimicrobial Chemo-therapy*, Vol. 57 No. 6, pp. 1100-1109

Welch, K., Cai, Y. and Strømme, M. (2012), "A method for quantitative determination of biofilm viability", *Journal of Functional Biomaterials,* Vol. 3, pp. 418-431; DOI.10. 3390/jfb3020418

Wilson, Michael (2005), *Microbial Inhabitants of Humans: Their Ecology and Role in Health and*

Disease, Cambridge University Press, New York

Xavier, J. B., White, D. C. and Almeida, J. S. (2003), "Automated biofilm morphology quantification from confocal laser scanning microscopy imaging", *Water Science and Technology*, Vol. 47 No. 5, pp. 31-37

Zhang, Y. Q, Ren, S. X., Li, H. L., Wang, Y. X., Fu, G., Yang, J. Qin, Z. Q., Miao, Y. G., Chen, R. S., Shen, Y., Chen, Z. H., Zhao, G. P., Qu, D., Danchin, A. and Wen Y. M. (2003), "Genome-based analysis of virulence genes in a non-biofilm-forming *Staphylococcus epidermidis* strain (ATCC 12228)", *Molecular Microbiology*, Vol. 49 No. 6, pp. 1577–1593