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STRATEGIES TO COMBAT BIOFILM FORMATION

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ABSTRACT

Biofilm formation in healthcare is an issue of considerable concern, as it results in increased morbidity and mortality, imposing a significant financial burden on the healthcare system. Biofilms are highly resistant to conventional antimicrobial therapies and lead to persistent infections. Hence, there is a high demand for novel strategies other than conventional antibiotic therapies.

Keywords: Biofilm formation, Combat strategies

INTRODUCTION

Biofilm are surface attached groups of microbial cells that are embedded in a selfproduced extracellular matrix and are highly resistant to antimicrobial agents (Subhadra *et al*., 2018). Biofilm may be formed on biotic and abiotic surfaces (Donlan, 2002). As many as 80 percent of pathogens that form biofilm are associated with persistent infections. Approximately 90 percent of the biofilm mass is composed of extracellularpolysaccharides (EPS), proteins and extracellular DNA (eDNA). Extra cellular polysaccharides provides stability to the cells, mediates surface adhesion and serves as a scaffold for cells, enzymes and antibiotics to attach (Stewart and Costerton, 2001). *Pseudomonas aeruginosa*, associated with cystic fibrosis and *Staphylococcus aureus* which is responsible for most wound infections are typical examples of persistent pathogens that form biofilm.

The biofilm formation allows the bacteria to withstand hostile environmental conditions like starvation, desiccation and makes them capable, to cause a broad range of chronic diseases. Hence, it is considered as a major cause of persistent nosocomial infections in immunocompromised patients (Singh *et al*., 2000). Biofilm are associated with many medical conditions including indwelling medical devices, dental plaque, upper respiratory tract infections, peritonitis and urogenital infections. Both Gram positive and Gram negative bacteria

have the capability to form biofilm. Bacteria commonly involved include *Enterococcus faecalis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus viridans*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis* and *Pseudomonas aeruginosa* (Donlan, 2001). Biofilm protect the invading bacteria against the immune system of host via impaired activation of phagocytes and complement system and also increase their resistance against the conventional antibiotics by around 1000 fold (Brandl *et al*., 2008). Virulence and pathogenicity of microorganisms is often enhanced when growing as a biofilm and strategies are therefore required to control biofilm formation.

The study of biofilm and the strategies to eliminate them is one of the most important area of research. The manipulation of individual environmental factors to prevent biofilm formation has been met with limited success. Control over surface chemistry has been used to reduce cell attachment, including the development of dynamic surface that degrade or reorganize in response to temperature and other environmental conditions and shed adsorbed bacteria into bulk fluid (Renner and Weibel, 2011).

Biofilm Formation

The biofilm formation mainly includes four stages: bacterial attachment, microcolony formation, bacterial biofilm maturation and dispersion.

Bacterial attachment

Bacterial attachment consists of reversible and irreversible attachment. During the process of attachment, the organism must be come in close proximity of the surface, propelled either randomly or in a directed fashion via chemotaxis and mobility (Prakash *et al*., 2003). In the case of reversible attachment, bacteria casually sticks to the carrier surface by some extracellular organelles, such as flagellum, pili and a small amount of EPSs (Klausen *et al*., 2003). The main components of the EPSs include polysaccharide intercellular adhesin (PIA), eDNA, protein, lipids etc. When these environmental factors change, it promotes the attachment to the surface, driving attachment toward irreversible attachment. In the stage of reversible attachment, the level of EPSs secreted by bacteria reaches a certain degree, which generates a strong interaction between the bacteria and the surface and then the biofilm enters the stage of irreversible attachment. From the stage of reversible attachment to the irreversible attachment, the time is

as short as several minutes (Palmer *et al*., 2007).

Microcolony formation

After irreversible attachment, with the accumulation of a certain number of bacteria and their secretion of extracellular polymers, the binding between bacteria and the surface becomes close under appropriate growth conditions . In the meantime, the process of microcolonies being formed increases significantly and gradually small colonies are formed. For *Staphylococcus epidermidis*, in the irreversible phase, the PIA encoded by the *ica*ADBC locus is the main component mediating intercellular adhesion (Qin *et al*., 2007). Quorum sensing occurs mainly in the microcolony formation stage. Quorum sensing is a bacterial intercommunication system which is controlled by population density of bacteria. The result of quorum sensing is the secretion of signal molecules regulating the expression of the corresponding gene and secreting EPSs (Hammer and Bassler, 2003).

Table 01: Methods of Biofilm Detection

S. No	Methods	Principle
1.	Crystal violet (CV) assay	The CV assay quantifies the dye bound to biofilm. It actually quantifies all biomass (Djordjevic *et al*., 2002).
2.	Tissue culture plate method	It is a standard method for biofilm detection. It simply involves the staining of cells with crystal violet dye (Christensen *et al*., 1985).
3.	Tube method	Crystal violet staining A visible lining appears on the bottom and wall of tube confirms biofilm formation (Christensen *et al*., 1982) .
4.	Congo red agar method	Congo red staining black colonies in crystalline form appears confirms biofilm production (Freeman *et al*., 1989).
5.	Scanning Electron Microscopy	This is used to study the morphology of bacteria attached on the surface and for enumeration of adhered bacteria (Eighmy *et al*., 1983) .
6.	Fluorescent *in situ* Hybridization	This is used to visualize the patterns of microbial colonization and the composition of microbial communities (Stahl and Amann, 1991).
7.	Confocal scanning laser microscopy	This gives the 3D view of the microbial community. It can show the focused part as well as the part out of focus (Gomes *et al*., 2011).

Table 02: Strategies to Combat Biofilm Formation

Strategy	Method/Agents	Examples
Inhibition of initial biofilm attachment	I) Altering chemical Properties of biomaterials	I) Antibiotics, biocides, iron coatings
	II) Changing physical properties of biomaterials	II) Use of hydrophilic polymers, superhydrophobic coatings, hydrogel coatings, heparin coatings
Removal of Biofilm	I) Matrix degrading Enzymes	I) Polysaccharide degrading enzymes (Dispersin B, Endolysins); Nucleases (Deoxyribonuclease I) and Proteases (Proteinase K, trypsin)
	II) Surfactants	II) Sodium dodecyl sulfate (SDS), cetyl tri methyl ammonium bromide (CTAB), Tween 20 and Triton X-100, surfactin and rhamnolipids
	III) Free fatty acids, amino acids and nitric oxide donors	III) Cis-2-decenoic acid, D-amino acids, nitric oxide generators such as sodium nitroprusside (SNP), S-nitroso-L-glutathione (GSNO) and S-nitroso-N-acetylpenicillamine (SNAP)
Biofilm inhibition by quorum quenching	I) Degradation of QS signals	I) Lactonases, acylases and oxidoreductases
	II) Inhibition of signal synthesis	II) Use of analogues of AHL precursor S-adenosyl-methionine (SAM), S-adenosyl-homocysteine (SAH), sinefugin, 5-methylthioadenosine (MTA), butyryl-SAM; SAMbiosynthesis inhibitor cycloleucine, AHL synthesis inhibitors such as nickel and cadmium
	III) Antagonizing signal molecules	III) AHL analogues (bergamottin, dihydroxybergamottin, cyclic sulfur compounds, phenolic compounds including baicalin hydrate and epigallocatechin); AI-2 analogues (ursolic acid, isobutyl-4,5-dihydroxy-2,3-pentanedione (isobutyl-DPD) and phenyl-DPD); AIP analogues (cyclic peptides such as cyclo (L-Phe-L-Pro) and cyclo(L-Tyr-L-Pro), RNAIII inhibiting peptide (RIP) and its homologues.
	IV) Inhibition of signal transduction	IV) Use of halogenated furanone or fimbrolide, cinnamaldehyde, virstatin gues
	V) Inhibition of signal transport	V) Use of copper or silver nanoparticles, Phe-Arg- β naphthylamide (PAN)

Biofilm maturation

The attachment of small colonies grows into the mature biofilm with the characteristic three dimensional (3D) biofilmstructure. The attachment between cells and carriers and cells to cells mainly

Table 03: Some of the most relevant studies about the *in vitro* efficacy of phages against biofilms

S. No	Bacteria	Phage	Experimental approach	Result	Reference
1.	*K. pneumoniae*	KPO1K2	12h old *K. pneumoniae* biofilms were subjected to the combined treatment of phage (MOI of 1) and ciprofloxacin (1 mg/L).	No significant differences in biofilm removal efficacies between phage treatment alone or combined with ciprofloxacin were observed.	(Verma *et al*., 2009)
2.	*S. aureus*	SAP- 26	Phage (10-PFU) was applied together with azithromycin (80 mg/L), vancomycin (10mg/L) and rifampicin (0.6mg/L) against 24h old *S. aureus* biofilms.	Phage alone was able to kill approximately 28% of the biofilm bacteria after 24h. Azithromycin and vancomycin could kill 25% and 17%, respectively and when biofilms were treated with phage and rifampicin 35% of the live cells remained after this treatment. Phage/ azithromycin and phage/ vancomycin treatments showed 40% and 60% cells alive after 24h, respectively.	(Rahman *et al*., 2011)
3.	*E. coli*	T4	The antimicrobial synergy between T4 phage and Cefotaxime in the eradication of *E. coli* biofilms was evaluated.	The use of phages (titres of 10- and 10- PFU/mL) reduced the MBEC of cefotaxime against *E. coli* biofilms by 2 and 8 folds, respectively.	(Ryan *et al*., 2012)
4.	*P. aeruginosa*	Cocktail of RNA phages	A mixture of phages and chlorine with different concentrations was tested to control and remove *P. aeruginosa* biofilms.	The phage cocktail (3×10- PFU/mL) and chlorine (2×10mg/L) reduced biofilm growth by~94% and removed ~88% of existing Biofilms.	(Zhang and Hu, 2013)
5.	*E. coli* and *P. aeruginosa*	T4 and PB1	*E. coli* and *P. aeruginosa* 48 h biofilms were exposed to a combination of tobramycin (2 mg/mL) and T4 phage (MOI of 0.01) or tobramycin (0.5 mg/ mL) and PB-1 phage (MOI of 0.01) for 24h, respectively.	The combination of phage and antibioticled to ~ 99.99% decrease on the survival of *E. coli* biofilms compared to the use of tobramycin alone, while the combination of tobramycin and PB-1 on *P. aeruginosa* biofilms was just as effective as tobramycin alone in decreasing biofilm cells. However, phage infection in combination with tobramycin reduced the emergence of antibiotic and phage resistant cells.	(Coulter *et al*., 2014)
6.	*P. mirabilis*	Cocktail of two RNA phages	Catheters were pretreated with the phage cocktail before bacterial inoculation.	A significant reduction in the number of *P. mirabilis* biofilm cells was observed after 96h and 168h of biofilm formation in phage coated catheters.	(Melo *et al*., 2016)

Table 04: Quorum Sensing Inhibitory Activities of Endophytes

S. No	Endophytic Strain	Host Plant	Type of Organism	Test	Reference
1.	*Bacillus amyloliquefaciens* PEBA20	*Populus tomentosa* Carr	*Botryosphaeria dothidea*	Cut shoots assay for antagonistic activity	(Yin *et al*., 2011)
2.	*Fusarium graminearum* and *Lasidiplodia* spp	*Ventilago madraspatana* Gaertn	*C. violaceum* wild-type and mutant CV026	Violacein inhibition assay	(Rajesh and Ravishankar Rai, 2013)
3.	*Bacillus* spp strain B3, *Bacillus megaterium* strain B4, *Brevibacillus borstelensis* strain B8 and *Bacillus* spp Strain B11	*Cannabis sativa*	*Chromobacterium violaceum*	Violacein inhibition assay	(Kusari *et al*., 2014)
4.	*Fusarium* spp, *Epicoccum* spp, *Trichoderma* spp, *Aspergillus* spp and *Fusarium* spp	*Diploria strigosa*	*C. violaceum* mutant CV026	Violacein inhibition assay	(Martin-Rodriguez *et al*., 2014)
5.	*Alternaria* spp, *Aspergillus* spp and *Fusarium* spp	*Siderastrea siderea*	*C. violaceum* mutant CV026	Violacein inhibition assay	(Martin-Rodriguez *et al*., 2014)

relies on the EPSs so that the colony can withstand a certain degree of mechanical pressure to prevent shedding from the carrier surface. Cell lysis and released eDNA are critical for the initial biofilm attachment and released eDNA remains an important matrix component in biofilm maturation. The regulation of quorum sensing and surfactants has extensive importance for biofilm maturation processes. The mature biofilm is composed of three layers, where the inner layer is a regulating film, the middle layer is a compact microbial basement membrane; the outermost surface film is where the plankton lives. The mature biofilm, with pathogenicity, which increases resistance to antibacterial agents, is more difficult to contact and remove bacteria and the detachment of the biofilm often leads to human infection because of *S. epidermidis* and other foodborne pathogens (Otto, 2013).

Dispersion

As the biofilm gets older, cells detach, disperse and colonize a new niche. It has been shown that biofilm bacteria can be detached by disruptive factors, such as catabolite repression, nutrient limitation and secretory proteins. The reasons for biofilm separation include external environmental effects, such as increased shear stress, a lack of nutrient supply and internal biochemical changes in bacteria, such as endogenous enzyme degradation, EPS or surface binding

protein releasing (Sauer *et al*., 2004). *P. aeruginosa* is regulated by two quorum sensing systems, LasI/LasR and RhlI/RhlR and quorum sensing promotes biofilm dispersion at least by reducing the synthesis of rhamnolipids. The detachment from the biofilm is thought to be a key reason for the spread of pathogens, so it is important to study the mechanism of biofilm detachment and its inhibition for preventing foodborne infection.

Methods of Biofilm Detection–Biofilm production can be assessed by several methods as summarized in Table no. 01.

Strategies to Combat Biofilm Formation

There have been three major strategies considered so far to control biofilm formation or to target different stages of biofilm development. The first approach is inhibiting the initial attachment of bacteria to biofilm forming surfaces, thereby reducing the chances of biofilm development. The second approach targets the disruption of biofilm during the maturation process (Kalia and Purohit, 2011). The third strategy is the signal interference approach, in which the bacterial communication system or the quorum sensing (QS) system is interfered with as QS coordinates biofilm formation/ maturation in pathogenic bacteria (Wright *et al*., 2004).

I. Inhibition of Initial Attachment

The initial attachment of cells to the biofilm forming surfaces happens within an average of the first 02 days of biofilm formation. Inhibition of initial attachment of cells to the surfaces is a potential strategy to prevent biofilm formation rather than targeting the dispersal of established biofilm. The attachment of bacteria to surfaces is mediated by several factors, including adhesion surface proteins, pili or fimbriaeand exopolysaccharides. The surfaces that are rough, coated with surface conditioning films and more hydrophobic are prone to ease biofilm formation. Thus, the initial attachment of cells can be prevented by altering the chemical or physical properties of indwelling medical devices.

(a) By Altering the Chemical Properties of Biomaterials

The commonly used chemical methods to modify the surface of biomedical devices in order to prevent biofilm formation include antibiotics, biocides and ion coatings. Catheters coated with antibiotics such as minocycline and rifampin have been shown to decrease the incidence of biofilm associated bloodstream infection by *S. aureus* in healthcare. In addition, catheters impregnated with different antibiotics, including nitrofurazone, gentamicin, norfloxacin etc.,are suggested to have a role

in preventing biofilm associated urinary tract infections.

In *Streptococcus pyogenes*and *S. aureus* a series of small molecules inhibited the expression of many key virulence factors that are involved in biofilm formation and infection. The early stages of biofilm formation in *S. aureus*, *S. epidermidis* and *Enterococcus faecalis* were inhibited by several aryl rhodamines. In *Vibrio cholera* small molecules inhibited the induction of cyclic di GMP, which is a second messenger controlling the switch between planktonic and sessile lifestyle of bacteria (Jenal and Dorman, 2009).

Several antimicrobial peptides are also known to interfere with biofilm formation in different bacterial pathogens. For example peptide 1018 is considered to be a biofilm inhibitor in *P. aeruginosa*, *E. coli*, *A. baumannii*, *K. pneumoniae*, *S. aureus*, *Salmonella typhimurium*, *Burkholderia cenocepacia* (De la Fuente Nunez *et al*., 2014).

In addition, antibiotics (nisin, subtilin, epidermin and gallidermin), a class of peptide antibiotics, are reported to inhibit biofilm formation in *S. aureus*, *Lactococcus lactis* and *S. epidermidis*. Chelators that interfere with the function of metal ions in biofilm formation are also considered to be biofilm inhibitors. Metallic silver, silver salts and silver nanoparticles have been widely used as antimicrobial agents in medical implants against bacteria such as *E. coli*, *S. aureus*, *Klebsiella* spp, *P. aeruginosa*, *S.* Typhimurium and *Candida albicans*. The silver treatment inhibits the replication of DNA, expression of ribosomal and cellular proteins and respiration process, leading to celldeath. It has been reported that silver ion coated implants inhibited *S. aureus* biofilm formation without causing silver accumulation in host tissues (Secinti *et al*., 2011). In addition, in the presence of nanoparticles,antibiotics such as penicillin G, amoxicillin, erythromycin, clindamycin and vancomycin displayed increased antibacterial activity against *S. aureus*.

(b) By Changing the Physical Properties of Biomaterials

Biofilm formation begins with a weak reversible adhesion of bacterial cells to the surface of medical devices. Hydrophobicity and surface charge of implant materials play an important role in determining the ability of bacteria to attach to surfaces. Thus, modification of the surface charge and hydrophobicity of polymeric materials using several backbone compounds and antimicrobial agents has proven to be effective for biofilm prevention. Hydrophilic polymers such as hyaluronic acid and poly N-vinylpyrrolidone on polyurethane catheters and silicone shuts, respectively have been known to reduce the adhesion of *S. epidermidis*. Superhydrophobic surfaces are reported to reduce bacterial adhesion and biofilm formation due to their extremely low wettability (Falde *et al*.,

2016). Surface roughness can also influence biofilm formation, as rough, high energy surfaces are more conducive to biofilm formation compared to smooth surfaces. It is noted that the surface roughness can alter the hydrophobicity thus in turn affecting bacterial adherence (Meiron and Saguy, 2007). Formation of biofilms by *S. aureus* is a major concern for the dairy industry and is frequently associated with a lack of monitoring of operational standards established for processing milk (Zadoks *et al.,* 2002). Lee *et al.*(2014) stated that 45% of *S. aureus* isolated from different sources on dairy farms produce biofilms on microplates, stainless steel or rubber, indicating possible persistence of this pathogen in the milking environment. However, none of the isolate produced biofilms on silicone.

II. Biofilm Removal

Mature biofilm are highly tolerant to antimicrobials due to the altered growth rate of cellsin the biofilm and the emergence of resistant subpopulations. Also, biofilm favour the horizontal transfer of antibiotic resistance genes among cells. Hence, the agents that interfere with the initial biofilm development or biofilm structure have great potential in controlling biofilm related infections.

(a) Matrix degrading enzymes

The biofilm matrix is usually composed of EPS, eDNAs and proteins. The EPS and eDNAs contribute to antibiotic resistance by preventing the diffusion of antimicrobials or by inducing antibiotic resistance. Dissociation of the biofilm matrix is an effective antibiofilm approach.Biofilm matrix degrading enzymes fall into three categories: polysaccharide degradingenzymes, nucleases and proteases (Li and Lee, 2017). Dispersin B is a bacterial glycoside hydrolase produced by *Actinobacillus actinomycetemcomitans* which hydrolyzes poly-N-acetylglucosamine (PNAG), a major matrix exopolysaccharide of *Staphylococcus* spp and *E. coli*. Deoxyribonuclease I which is capable of digesting eDNA is known to disperse biofilm in several bacteria including *Staphylococcus* strains, *A. baumannii*, *E. coli*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Psuedomonas aeruginosa* etc. The matrix proteins can be effectively cleaved by Proteinase K contributing to biofilm prevention and biofilm dispersal. It was demonstrated that the treatment with dispersin B followed by Proteinase K or trypsin successfully eradicated *Staphylococcus* biofilm (Chaignon *et al*., 2007).

(b) Surfactants

Surfactants are reported to have antimicrobial and antibiofilm activities. The surfactants sodium dodecyl sulfate

(SDS), cetyltrimethylammonium bromide (CTAB), Tween 20 and Triton X-100 are known to promote either biofilm dispersal or detachment (Boles *et al*., 2005). A biosurfactant, surfactin which is a cyclic lipopeptide produced by *B. subtilis*, is reported to inhibit biofilm formation and induce biofilm dispersal in *S.* Typhimurium, *E. coli* and *P. mirabilis*.

(c) Free fatty acids, amino acids and nitric oxide donors

Free fatty acids are shown to have antibiofilm activity against several pathogenic bacteria. It was reported that *P. aeruginosa* produces an organic compound cis-2-decenoic acid which is capable ofdispersing the already established biofilm by *E. coli*, *K. pneumoniae*, *P. mirabilis*, *S. pyogenes*, *B. subtilis*, *S. aureus* and *C. albicans*.In *S. aureus*, *B. subtilis* and *P. aeruginosa*a mixture of D-amino acids triggered the disassembly of biofilm by releasing amyloid fibers, which are the proteinaceous component of the extracellular matrix. While many L-amino acids promote biofilm formation in *P. aeruginosa* in the case of tryptophan, both D and L isoforms inhibited biofilm formation and caused biofilm dispersal (Brandenburg *et al*., 2013). Nitric oxide (NO) generators such as sodium nitroprusside (SNP), S-nitroso L-glutathione (GSNO) and S-nitroso N-acetylpenicillamine (SNAP) are reported to induce biofilm dispersal in *P. aeruginosa*.

III. Biofilm Inhibition by Quorum Quenching

Quorum sensing (QS) is an important cellular communication system in many Gram negative and Gram positive bacteria. QS mediates the regulation of various genes according to the density of signaling molecules in the surrounding environment. The signaling molecules of the QS system are denoted as autoinducers. Based on signaling molecules, the QS system is categorized into three; N-acyl homoserine lactones (AHLs) based (Gram negative bacteria), autoinducing peptide (AIP) based (Gram positive bacteria) and autoinducer-2 (AI-2) based (both Gram negative and Gram positive bacteria). QS plays a crucial role in biofilm formation, it has been suggested that QS inhibition (quorum quenching; QQ) is an interesting strategy to prevent biofilm formation (Brackman and Coenye, 2015). The major advantage of controlling biofilm by QQ is that this strategy reduces the risk of multidrug resistance.

(a) Degradation of QS signals

N-acyl homoserine lactones (AHLs) can be degraded by specific enzymes such as lactonases that hydrolyze the lactone ring in the homoserine moiety and acylases that cleave off the acyl side chain and the activity can be altered by reductases and oxidase. Quorum quenching enzymes disrupt the biofilm architecture, which

increases the antibiotic susceptibility of the cells. Significant reduction of biofilm formation and increased sensitivity to antibiotics was noticed in *P. aeruginosa* after treatment with lactonase (Kiran *et al*., 2011). The oxidoreductases reduced the signaling moleculesAHL and AI-2 to QS inactive hydroxyl derivatives in *K. pneumoniae*.

(b) Inhibition of signal synthesis

Several reports have shown that mutations affecting AHL synthesis have an adverse effect on biofilm formation. The mutants of *Vibrio* spp, *Streptococcus* spp and *Staphylococcus* spp that are deficient in AI-2 synthesis were not able to produce biofilm properly. Analogues of AHL precursor molecule S-adenosyl methionine (SAM), such as S-adenosyl homocysteine (SAH), sinefugin, 5-methylthioadenosine (MTA) and butyryl SAM are known to inhibit biofilm formation in *P. aeruginosa*. Also, the SAM biosynthesis inhibitor cycloleucine is reported to inhibit AHL production. The antibiotic azithromycin interferes with signal synthesis in *P. aeruginosa* and thus significantly clears biofilm in mouse model of cystic fibrosis. In addition, several inhibitors for the key enzymes (50-methylthioadenosine/S-adenosylhomo cysteine nucleosidase (MTAN) and S-ribosylhomocysteinase (LuxS) involved in AI-2 synthesis are shown to reduce biofilm formation. In *Burkholderiamultivorans*,nickel(Ni2+)and cadmium(Cd2+) inhibited the expression of genes responsible for AHL production thereby inhibiting cell to cell signaling and subsequently biofilm formation (Vega *et al*., 2014). The inhibitory effect of Cd2+ in quorum sensing was also reported in *Chromobacterium violaceum* (Thornehill *et al*., 2017).

(c) Antagonizing the signal molecules

AHL analogues in which the lactone ring was replaced by a cyclopentyl or a cyclohexanone ring adversely affected biofilm formation in *Serratia marcescens* and *P. aeruginosa*. In addition, some phenolic compounds including baicalin hydrate and epigallocatechin blocked AHL QS and affected biofilm formation of *B. cenocepacia*, *B. multivorans* and *P. aeruginosa*. It was noted that the antibiotic susceptibility of *B. cenocepacia* and *P. aeruginosa* increased after treatment with baicalin hydrate in different*in vitro* biofilm models (*et al*., 2011). The concept of combining QS inhibitor (QSI) and antibiotics was a better strategy to control biofilm formation by pathogenic bacteria. In addition, it has been noticed that biofilm formation can be effectively controlled by combining QSIs and QQ enzymes.

The AI-2 analogues ursolic acid, isobutyl-4,5-dihydroxy 2,3 pentanedione (isobutyl-DPD) and phenyl-DPD inhibited biofilm formation and removed preformed

biofilm in *E. coli* and *P. aeruginosa*. Although other compounds, including pyrogallol and its derivatives, some nucleoside analogues, boronic acids and sulfones have been identified to antagonize AI-2 signaling. The most investigated QS inhibiting peptide is the RNAIII inhibiting peptide (RIP), which is produced by coagulase negative *Staphylococci*. RIP interferes with the QS response by inhibiting the production of RNAIII, a key component of QS response in *S. aureus* (Gov *et al*., 2001). RIP and several RIP homologues have been reported to have anti QS and antibiofilm activity against *Staphylococcus* spp.A RIP analogue, FS3 prevented *S. aureus* biofilm formation in a rat vascular graft model. In addition, a nonpeptide RIP analogue, hamamelitannin blocked QS in *Staphylococcus* spa nd potentially inhibited biofilm formation in *in vitro* and *in vivo* rat model of graft infection.

(d) Inhibition of signal transduction by interfering with response regulator activity

The QS system can also be hindered at the level of signal transduction cascade. The natural compounds, halogenated furanone or fimbrolide and cinnamaldehyde which are isolated from red algae *Delisea pulchra* and Cinnamon bark respectively, interfere with signal transduction and affect biofilm formation, thereby increasing antibiotic susceptibility in several pathogenic bacteria. Both compounds block AI-2 and AHL-type QS systems and thereby affect biofilm formation in *Vibrio harveyi*. The halogenated furanone and cinnamaldehyde inhibits AI-2 QS and AHL QS by decreasing the DNA binding ability of the response regulator LuxR, which is important for the signal transduction cascade or by displacing AHL from its receptor, respectively (Niu *et al*., 2006). In addition, the natural furanone inactivates LuxS and accelerates LuxR turnover, thereby blocking AI-2 and AHL QS signaling system, respectively. Cinnamaldehyde is widely used as a flavoring agent in food and beverages, while the application of furanones is limited because of their toxicity.

(e) Inhibition of signal transport

The signaling molecules need to be exported and released into the extracellular space to be sensed by other bacteria for effective cell to cell communication. The role of multidrug resistant (MDR) efflux pumps in signal traffic was first reported in *P. aeruginosa*, in which AHLs with long side chains are actively transported across the cell membrane through the MexAB-OprM efflux pump. In *P. aeruginosa*, the expression of the autoinducer producing gene and the genes encoding the virulence factors is limited by the intracellular concentration of the autoinducer. The involvement of the MDR efflux pump in the QS system has also been reported in *E. coli*, in which the overexpression of the QS regulator SdiA led to the increased

expression of the AcrAB efflux pump. The inhibition of the efflux pump would be a promising strategy to alter QS signaling cascade, thereby preventing biofilm formation and virulence.

Several studies have provided evidence to show the link between the physiological function of efflux pump and biofilm formation. In *E. coli* and *Klebsiella* strains, the inhibition of the efflux pump activityusing efflux pump inhibitors (EPIs) reduced biofilm formation. The genetic inactivation or the chemical inhibition of efflux pump activity resulted in impaired biofilm formation in *S. enteric* serovar Typhimurium. The effect of efflux pump inhibitors to prevent biofilm formation was also demonstrated in *P. aeruginosa* and *S. aureus* (Baugh *et al*., 2013) inwhich copper nanoparticles work well as EPI and antibiofilm agents (Christena *et al*., 2015).

AlternateStrategies to Combat Biofilm Formation

I. Biofilm Inhibition by Bacteriophages

Phages are natural predators of bacteria causing cell lysis with the release of several virion progeny. The role of bacteriophages in controlling bacterial population dynamics within biofilms has been extensively studied. Phages as lytic agents of biofilms form the most direct means of controlling bacterial populations. In order to circumvent phage limitations and improve their performance for an efficient biofilm control, different approaches, such as synergistic combinations with other phages or antimicrobials, mechanical debridement of biofilms and genetic engineering of phage genomes have been addressed.

II. Biofilm Inhibition by Endophytes

Many natural compounds are also reported to antagonize AHL-based QS signaling and those include bergamottin and dihydroxybergamottin from grapefruit juice, cyclic sulfur compounds from garlic, patulin and penicillic acid from a variety of fungi etc. (Galloway *et al*., 2011). Endophytes are the group of microbes that reside intercellularly or/and intracellularly within any tissues (root, stem, leaf, and others) of host plant without showing any infection. Endophytes are a prospective novel source of QS inhibitors. Thus, many researchers have started bioprospecting endophyticmicrobes based on their QS inhibitor biosynthesiscapacity.

CONCLUSION

The various approaches for modulating biofilm formation on medical devices are discussed in detail, with special emphasis on quorum-quenching strategies. Several studies have shown that multidrug efflux pumps play a potential role in controlling biofilm formation. Strategies that do not induce antibiotic

resistance, can be of great potential in the future for the treatment of biofilm based infections in human health and in toto in veterinary practice. Bacteriophage therapy and endophytes can serve as an alternate to control biofilm formation.

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