

## Microbial biofilms and their control by various antimicrobial strategies

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In nature, bacteria as opposed to the planktonic form predominantly exist as biofilms. Biofilms are consortium of microorganisms and can be considered as complex communities of microorganisms which are embedded in a self-produced matrix of extracellular polymeric substances attached to a substratum which can be inert or living surfaces. In the recent years, there is an increasing interest in studying biofilms. This is mainly due to the reason that biofilms are known to cause persistent and chronic infections. They are also a major problem in industries and food processing units. Their resistance to biocides, disinfectants and host immune responses makes their study important. In this review, the development and dynamics in microbial biofilms and the strategies employed to effectively control biofilms in industrial and medical settings as well as food processing units will be discussed in detail. The various environmental cues, genetics and molecular mechanisms involved in biofilm formation will be assessed in the review. The role of cell to cell communication or quorum sensing in biofilm formation and the use of anti-quorum sensing strategies in biofilm control will also be focused. The mechanism of action of various antibiofilm strategies including the use of phytochemicals, nanoparticles, quorum sensing inhibitors, enzymes and other novel biological approaches will be reviewed. The techniques used in studying biofilms will be briefly discussed.

**Keywords** biofilm; antimicrobials; quorum sensing; phytochemicals; nanoparticles

### 1. Introduction

A biofilm is a sessile microbial community that are irreversibly attached to a substratum and embedded in an extracellular polymeric matrix [1]. There is a generalized model for biofilm formation. Initially, the microorganisms colonize and attach to an abiotic or biotic surface. The microorganisms then transform from planktonic form to immobilized status. Finally, the microcolony is formed and continuous proliferation of microorganisms will result in a three dimensional architecture of biofilm, which is considered as mature biofilm [2].

The transition from planktonic growth to biofilm occurs in response to environmental changes. Multiple regulatory networks are involved which translate signals to concerted gene expression changes thereby mediating the spatial and temporal reorganization of the bacterial cells into biofilms [3]. The cellular reprogramming alters the expression of surface molecules, nutrient utilization ability and virulence factor production to prepare the bacteria for survival in unfavorable conditions [4]. Within the biofilm, bacteria are embedded in a self-produced extracellular matrix, which accounts for 90% of the biomass. The matrix is composed of extracellular polymeric substances (EPS) and carbohydrate-binding proteins, pili, flagella, other adhesive fibers, and extracellular DNA (eDNA). The matrix acts as a stabilizing scaffold for the three dimensional biofilm structure [5]. Nutrients get trapped in the matrix for metabolic utilizations by the resident bacteria and with the aid of hydrophilic polysaccharides, water is efficiently retained through H-bond interactions. Enzymes secreted by the bacteria modify EPS composition in response to changes in nutrient availability, resulting in changes in biofilm architecture to the specific environment. The structural components of the matrix are responsible for a highly hydrated, robust structure with high tensile strength that keeps bacteria in close proximity, enabling intimate cell-to-cell interactions and DNA exchange, and also protects the biomass from desiccation, predation, oxidizing molecules, radiation, and antimicrobials [6]. The resistant nature of biofilms can also be attributed to the presence of environmental gradients within the biomass, which give rise to community with subpopulations of bacteria showing differential gene expression in response to local nutrient and oxygen availability [7].

From a medical perspective, both commensal and pathogenic microorganisms form biofilm-like conglomerates. These are associated with the epithelial or endothelial lining, embedded in the lung, intestinal or vaginal mucus layer, attached to the teeth or medical implant surfaces, or formed intracellularly. Biofilm formation and persistence has profound implications for the patient, as microorganisms in biofilms are significantly less susceptible to antimicrobials and host immune responses than the planktonic forms of the same microorganisms. Many biofilm infections are persistent and difficult to eradicate and they further manifest as chronic or recurrent infections. Biofilm infections constitute a number of clinical challenges, including diseases involving uncultivable species, chronic inflammation, impaired wound healing, rapidly acquired antibiotic resistance, and the spread of infectious emboli [8].

It is well known that many pathogenic microorganisms form biofilms on food and food-contact surfaces under appropriate environmental conditions. There are a number of studies reporting the ability of foodborne pathogens to attach to food and food-contact surfaces, such as *Salmonella*, *Listeria monocytogenes*, *Yersinia enterocolitica*, *Campylobacter jejuni* and *Escherichia coli* O157:H7. Their attachment to food-contact surfaces causes hygienic problems as these biofilms provide a reservoir of contamination. There is also an increase in the risk for microbial

contamination in food plants. The biofilms of foodborne pathogens is often established by various microorganisms on the equipment surfaces of the production line. The biofilms containing pathogens like *L. monocytogenes* can become one of the major causes of contamination of food products or transmission of diseases [9]. Therefore, it is very important to develop cleaning and disinfection methods and control systems in food-processing plants and environments.

Biofouling or biofilms in industrial and environmental setting has been defined as the undesirable accumulation of microorganisms, plants and animals on artificial surfaces immersed in a common matrix. The problem could manifest itself as adverse effects from the biological settlement process. Biofouling can cause damage to equipments, resulting in their failure leading to economic losses to industries. Biofouling causes damages to optical based systems, i.e. sensors having measurements and accuracy can be depreciated by surface adherence of bacteria [10]. Biofilms represent the most prevalent type of microbial growth in nature and are crucial to the development of clinical infections. Intense interest in biofilms studies is due to their resistance to the immune system and tolerance to antimicrobials and stresses.

## 2. Tools and techniques used to study biofilms

Biofilm model systems are essential to gain a better understanding of the mechanisms involved in biofilm formation and resistance so as to develop effective antibiofilm strategies.

### 2.1. *In vitro* biofilm model systems

Microtiter plate (MTP)-based systems are among the most frequently used biofilm model systems. They are closed systems and with time the nutrients become depleted and signalling molecules accumulate unless the growth media is replaced. MTP-based assays are inexpensive as only small volumes of reagents are required and a large number of tests can be simultaneously performed and this system is ideal for distinguishing biofilm-deficient mutants from biofilm forming wild type strains and to screen for the antimicrobial and anti-biofilm compounds. The effects of modification, coating or impregnation of materials on various stages of biofilm development can also be carried out in MTP systems. By varying multiple parameters including the composition of growth media, incubation temperatures, humidity, presence or absence of shear stress and O<sub>2</sub> and CO<sub>2</sub> concentrations biofilm formation can be studied [11]. A variant of the MTP system is the “Calgary Biofilm Device”. In this system, pegs are attached to the top lid of a microtiter plate and by closing the microtiter plate, these pegs are immersed in the media present in the wells of the 96-well MTP. Following biofilm growth, the lid is transferred to a plate containing antibiotics to be tested. After the treatment the pegs are incubated for regrowth or the biofilm biomass or the number of sessile cells present in the biofilm is quantified. It is a rapid and miniaturized biofilm assay used to evaluate the effects of antimicrobials on biofilm eradication [12]. Biofilm Ring Test (BioFilm Control SAS) is a commercially available MTP-based method. In this method, biofilm formation is studied using the immobilised inert paramagnetic beads included in the culture medium. The non-immobilised beads are collected into a single spot using a magnet which is then quantified through specialised image algorithms. It is used to study the kinetics of biofilm formation, of *Listeria monocytogenes*, *E. coli*, *Staphylococcus carnosus* and *Staphylococcus xylosoxylosus*, influence of matrix components on *Leuconostoc mesenteroides* biofilm formation, evaluate the effect of co-administration of antibiotics on *Pseudomonas aeruginosa* biofilms, compare the biofilm formation between *Campylobacter coli* and *Campylobacter jejuni* and to study early phases of *P. aeruginosa* biofilm formation [13-14].

The flow displacement biofilm model systems are “open” systems in which there is continuous replacement of growth medium. In the “continuous flow stirred tank reactor” (CFSTR) systems there is perfect mixing and the rate at which growth medium is added to the reactor is identical to the effluent removal rate. As the dilution rate is higher than the doubling time of the microorganism(s) present in the reactor, planktonic cells are washed out of the reactor and only the sessile cells attached to a surface will remain and will be able to multiply. In the “plug flow reactor” (PFR) approach the influent moves as a single “plug” in the direction of the flow (axial direction), with mixing (through diffusion) only occurring in the radial direction. Here, the environmental conditions change progressively through the reactor [15].

Cell-culture-based model systems include the human cell lines that mimic the *in vivo* situation. Well-studied examples of mucosal biofilms include those formed by *Candida albicans* on oral and vaginal tissues. The other *in vitro* cell-culture-based models that have been used for the study of the interaction of human cells with bacterial biofilms are airway epithelial cells for *P. aeruginosa* biofilms, EA. hy926 endothelial cells and primary human umbilical vein endothelial cells for *Streptococcus gallolyticus* biofilms, HeLa cells for enterohemorrhagic *E. coli* and cystic fibrosis derived IB3-1 bronchial cells to grow *Stenotrophomonas maltophilia* biofilms. The damage inflicted by the biofilms and the internalisation of microbial cells to human cells can be determined by microscopy studies [11].

## 2.2. *In vivo* biofilm model systems

*Caenorhabditis elegans* model which is used to assess virulence of pathogens can also be used to study biofilm formation. The biofilms of *Yersinia pestis* were studied in *C. elegans* [16]. Biofilms of staphylococci (*S. epidermidis* and *S. aureus*) and *Xenorhabdus nematophila* have been tested in the *C. elegans* model and the study revealed the importance of the extracellular matrix for biofilm formation and its immunoprotective role during infection [17].

The vertebrate animal models include the central venous catheter models, subcutaneous foreign body infection models, intraperitoneal foreign body infection models, urinary tract infection models and the respiratory tract infection models. As microbial biofilm formation on central venous catheters (CVC) causes considerable morbidity and mortality, animal models have been developed to study biofilm formation on CVC *in vivo*. These models are useful for studying fundamental aspects of microbial biofilm formation in *in vivo* conditions, the dissemination of the microorganism to various organs and the efficacy of various antimicrobials [18]. The subcutaneous foreign body infection models makes use of guinea pigs, hamsters, mice, ponies, rabbits and rats to insert a foreign body in subcutaneous pockets and to allow biofilm formation on the implanted material. These models have been used to study the biofilms of *S. aureus*, *S. epidermidis*, *E. coli*, *Actinomyces radidentis*, *P. aeruginosa* and *C. albicans* [19]. Intraperitoneal foreign body infection models using rabbits or mice are studied for the biofilms that are formed on biomaterials inserted in the the peritoneal cavity. The materials are either precolonised with microbes or are injected intraperitoneally following implantation. These model systems are used to study chronic infections, the effect of antibiotics against bacterial biofilms of *S. aureus*, *S. epidermidis*, *P. aeruginosa* and evaluate the importance of quorum sensing in *P. aeruginosa* biofilm persistence [20]. The surgical and non-surgical urinary tract infection models have been developed in rats, rabbits and mice. Zinc disc implants or catheters are inserted in the bladder of rats, followed by transvesical inoculation with pathogens and further studied for biofilm formation and matrix production in the development of urinary tract infections [21]. Several animal models for chronic respiratory tract infections have been developed such as the bacteria embedded in agar or agarose beads to establish pulmonary infections in laboratory animals. Using this model the chronic infection with histological damage similar to cystic fibrosis or chronic obstructive pulmonary diseases can be studied. A plastic tube precoated with *P. aeruginosa* and subsequently inserted in the trachea of a murine model can be used to study chronic respiratory tract infection caused by the organism, and the efficacy of various (combinations of) antibiotics (including clarithromycin, levofloxacin and erythromycin) against *in vivo P. aeruginosa* biofilms [22-23].

## 2.3. Visualization of biofilms

Advancements in imaging techniques for studying the formation of single and mixed species biofilms have contributed significantly to the current status of the biofilm research community. The techniques including laser scanning microscopy, scanning transmission X ray microscopy, and magnetic resonance imaging have made it possible to study the formation and dynamics of biofilms *in situ*. The application of fluorescent reporters in imaging techniques is useful in studying activation of specific genes in biofilms, biofilm formation on surfaces that are not penetrable by light, distribution of organisms in mixed species biofilms and the dynamics in biofilm matrix structures.

The laser scanning microscopy (LSM) technique is widely used in biofilm imaging studies for three reasons: visualization of multiple features in different channels and spectrally resolved analysis of structure, composition, microhabitats, activity and processes using a variety of specific probes and volumetric and structural quantification of multichannel signals in four dimensions. The LSM technique can be performed in a variety of ways. It can be equipped with lasers using one-photon excitation which is achieved using continuous UV and visible lasers. Recently white lasers also known as super-continuum light sources have been used to obtain confocal laser scanning microscopy. The system can be set up with a laser using two-photon excitation (pulsed infrared laser) to get two-photon or multiphoton laser scanning microscopy [24].

The main application of LSM comprises the structural examination of biofilms and bioaggregates. Most importantly, LSM imaging has revealed the overall three-dimensional and internal structures of biofilms including voids and channels, which resulted in a new concept of biofilm architecture. Imaging of extracellular polymeric substances (EPS) has also increased the understanding of the understudied component of biofilms [25].

The most frequently used fluorochromes to study composition and functionality of biofilms are nucleic acid-specific stains. Apart from the traditional fluorochromes, for example acridine orange and 6-diamidin-2-phenylindole, cell-permeable and cell-impermeable stains with different excitations and emissions such as the Syto™ series can also be used. Due to the complexity of EPS composition, an *in situ* approach is used to analyse the glycoconjugate fraction of the EPS by means of fluorescence lectin-binding analysis. The identification of community members in biofilm can be studied by imaging of FISH using rRNA-targeted oligonucleotide probes [26]. Reporter gene techniques using fluorescent reporter genes also present a valuable tool to study microbial communities as it does not require staining of the sample. The green fluorescent protein (GFP) as a cell marker for ecological and environmental studies has been useful. The applicability of various GFP types having different excitation and emission characteristics for specific labelling of different bacterial strains has been discussed. The combination of GFP labelling of bacteria and LSM

examination of the communities has led to the major progress in the structure–function of microbial biofilm systems [27].

The two-photon laser scanning microscopy overcomes the limitations of confocal LSM. It allows for deeper penetration into light scattering samples and an improved resolution in deeper sample locations. It also prevents out of focus area from bleaching. However, confocal LSM with one-photon excitation is still the major choice for visualizing and imaging studies [28].

### 3. Resistance to antimicrobials

The highly recalcitrant nature of bacterial biofilms to the antibiotic treatments makes their study important. The conventional drug resistance mechanisms which include upregulated efflux pumps, mutations in drug target sites and receptors,  $\beta$  lactamase production are also expressed in biofilms and do contribute to antibiotic resistance and survival of biofilms. Apart from these there are other factors which also impart resistance to antimicrobials in drugs and needs to be identified to develop antibiofilm strategies [29].

The genetic mechanisms of the biofilm antibiotic resistance have been recognized as innate resistance factors and induced resistance factors [30]. The innate mechanisms get activated during the development of biofilm, the factors being integral parts of biofilm structure and physiology. The decreased diffusion of antibiotics through the biofilm matrix, decreased oxygen and nutrient availability accompanied by altered metabolic activity, formation of persisters, increased production of oxidative stress and other specific molecules are identified as some of the intrinsic factors imparting resistance to the antimicrobials in biofilms. The induced resistance factors are due to the induction by the antimicrobial agent. For example, exposure to antibiotics causes lysis of bacteria in the upper strata of biofilm and release of defensive enzymes such as  $\beta$  lactamase. Drugs such as imipenem and piperacillin are also able to induce overproduction of  $\beta$ -lactamase in *P. aeruginosa* biofilms, which hydrolyse the  $\beta$ -lactam antibiotics before reaching the bacterial cells [31]. However, the resistance to antibiotic in biofilms is due to both the innate and induced mechanisms.

The mechanism of antibiotic resistance in biofilms has been attributed to the slow penetration or failure to penetrate beyond the surface layer of the cells, development of resistance phenotypes in biofilms and altered microenvironment [32]. Though there is no generic barrier for the diffusion of antibiotics through the biofilm matrix, yet, its penetration decreases if the antibiotic is deactivated. It has been shown that ampicillin penetrates a biofilm formed by a  $\beta$ -lactamase-negative strain of *Klebsiella pneumoniae* but is unable to diffuse the biofilm formed by the  $\beta$ -lactamase-positive wildtype strain of the same micro-organism as it gets deactivated in the surface layers more rapidly than it diffuses [33]. Another possibility is that the antibiotics can adsorb onto the biofilm matrix accounting for its slow penetration. Aminoglycoside antibiotics are positively charged and bind to the negatively charged polymers in the biofilm matrix [34].

Altered microenvironment can also lead to the reduced susceptibility of biofilms to the antibiotics. For example, in biofilms the oxygen concentration is high at the surface but low in the centre of the biofilm. The resulting anaerobic condition causes decreased growth, protein synthesis and metabolic activity or no growth of bacteria in the centre and lower layers of biofilms. Usually antibiotics are effective active against metabolically active cells and antibiotics such as  $\beta$ -lactams active against dividing *P. aeruginosa* cells fail to completely eradicate the biofilms [35].

The oxidative stress in biofilms have also been implied to cause hypermutation in bacteria, transmission of drug resistance genes and tolerance to antibiotics [36]. The increased production of endogenous reactive oxygen species (ROS) and lack of antioxidant causes oxidative stress in biofilms. The oxidative stress results in enhanced mutability and an increased horizontal gene transmission in biofilms. It has been observed that due to the occurrence of endogenous oxidative stress in the microcolony structures of biofilms, there is an enhanced genetic adaptation and evolutionary changes in these specific sites. Thus, the endogenous oxidative stress in biofilms produces diversity and adaptability in biofilm community and promotes antibiotic resistance. This has also been confirmed by the addition of antioxidants which reduces such diversity [37].

Bacterial populations can produce persister cells which do not grow or die in the presence of microbicidal antibiotics. The ability to avoid killing is the key feature of persisters. It has been hypothesized that tolerance to antimicrobials in persister is not by preventing antibiotic binding, but by interfering with the lethal action of cidal compounds. For example, streptomycin, a bactericidal aminoglycoside, causes translational misreading, which apparently produces truncated toxic peptides, leading to cell death. Therefore, shutting down the ribosome in a persister cell would produce tolerance to aminoglycosides [38]. Thus, persisters are also responsible for high levels of biofilm tolerance to antimicrobials such as fluoroquinolones that can kill slow-growing cells [39].

Quorum sensing or cell to cell communication in bacteria also contributes to biofilms tolerance to antimicrobials. It has been reported that *P. aeruginosa* tolerance to tobramycin, hydrogen peroxide and polymorphonuclear leukocytes is quorum-sensing dependent. The biofilm of lasR rhlR quorum sensing mutant of *P. aeruginosa* was 100-fold more efficiently cleared in a pulmonary infection mouse model compared to the wildtype *P. aeruginosa* strain [40]. The quorum sensing inhibitors furanone C-30 and C-56 on biofilm increased the sensitivity to tobramycin treatment. A synergistic effects of C-30 and the antibiotic tobramycin is seen as biofilms grown in the presence of C-30 were less tolerant to tobramycin treatment compared to their untreated counterparts [41]. Thus, the role of a functional quorum

sensing system in the development of the characteristic biofilm tolerance to a variety of antimicrobial treatments is explained.

#### 4. Antimicrobial strategies to control biofilms

Biofilm formation can be controlled by antimicrobial strategies using chemical and physical methods (Table 1). However, bacteria living in biofilms are more resistant or tolerant to antibiotics and disinfectants and more difficult to remove mechanically when compared to planktonic forms [42].

**Table 1** Various antimicrobial strategies used to control microbial biofilms.

Antimicrobials	Nature of Compounds	Mechanism of Action	References
Quorum sensing inhibitors	Furanones C-30 and C-56	Inhibition of signal production, reception or dissemination and disruption of quorum sensing process and biofilm formation.	[43-46]
	RNAIII-inhibiting peptide (RIP)	Phosphorylation of TRAP (target of RNAIII activating protein involved in quorum sensing and biofilm formation).	
Bacteriophages (T4 and T7 phages)	Dispersin B polymerase	Production of depolymerases that hydrolyze biofilm extracellular polymers.	[47-51]
	Lysins and endolysins	Antibacterial activity against Gram-positive bacteria.	
Enzymes	Glycosidases, proteases, and deoxyribonucleases.	Degradation of biofilm matrix.	[52-59]
	Alginate lyase	Degradation of alginate and dispersal of <i>P. aeruginosa</i> cells.	
	Thermonuclease	Cell detachment in <i>S. aureus</i> biofilms	
Surfactants and biosurfactants	Surfactin	Modification of hydrophobicity of surfaces and prevention of microbial adhesion.	[60-64]
	Rhamnolipids		
Nanoparticles	Magnesium fluoride, NO- releasing nanoparticles, superparamagnetic iron oxide NPs (SPIONs).	Generation of reactive oxygen species. Hyperthermia. Bactericidal.	[65-71]
Phytochemicals and functional foods	Catechin, Polyphenolics, Aqueous extracts, Milk proteins (casein and lactoferrin)	Inhibition of enzymes such as glucosyl transferases (GTFs) involved in EPS production.	[72-77]
		Antiadhesive action (inhibition of bacterial interaction with surfaces by masking, coating etc).	

##### 4.1. Quorum sensing inhibitors

It is evident that resistance of biofilm to antimicrobial agents is acquired as a multicellular strategy that relies on exchange of chemical signals between cells in a process known as “quorum sensing” [43]. Interfering with this mechanism of bacterial cell-cell communication can provide a novel approach to prevent biofilm formation. The application of quorum sensing inhibitors (QSIs) is one of the promising techniques for disrupting quorum sensing and biofilm formation. The prime example of such QSIs is the bromated furanones from the red alga *Delisea pulchra*, which were first discovered to inhibit bacterial colonisation on the algae. Further research enabled scientists to synthesise a series of halogenated-furanone compounds whose structures were similar to those found in *D. pulchra*. Among them, furanones C-30 and C-56 have been widely studied since they can inhibit QS-controlled behaviours, in particular the production of extracellular virulence factors and the development of biofilms in microorganisms such as oral streptococci [44]. Synthetic furanone C-30 can inhibit biofilm formation by *S. mutans* and its luxS mutant strain, although it does not affect the bacterial growth rate itself. The quantities of biofilm formed by both strains significantly decreased ( $P < 0.05$ ) and the biofilms became thinner and looser as revealed by CLSM with increasing concentrations of furanone C-30. The addition of furanone C-30 down regulated expression of certain genes involved in biofilm formation [45].

*Staphylococcus aureus* and *S. epidermidis* virulence is often associated with its ability to form a biofilm, which is regulated through a quorum sensing mechanism. It has been shown that adhesion and biofilm formation can be prevented both *in vitro* and *in vivo* by the quorum-sensing inhibitor RNAIII-inhibiting peptide (RIP). This heptapeptide prevents *S. aureus* infections by inhibiting the phosphorylation of TRAP (target of RNAIII activating protein), a 21-kDa protein that is unique to staphylococci and is highly conserved among staphylococcal strains and species [46].

#### 4.2. Bacteriophages

Lytic bacteriophages can be an alternative or adjunct to antibiotics for bacterial infections, particularly for biofilm reduction or disruption. Phage therapy is based on the use of lytic phages to combat bacterial infections. It has many advantages compared to antibiotics. Phages are very specific and efficient for their target bacteria, they are not pathogenic for man and they persist only as long as the targeted bacteria are present [47]. Phages have been examined as potential agents for biofilm control. For example, phage T4 can infect and replicate within *E. coli* biofilms and disrupt biofilm topography by killing bacterial cells [48]. It is suggested that although lytic phages showed considerable inhibitory effects on growth and biofilm formation in *P. aeruginosa*, their effect on mature biofilms was very limited [49]. Many phages produce depolymerases that hydrolyze biofilm extracellular polymers. A phage-induced depolymerase was specific for the EPS and capsular polysaccharides of *Klebsiella aerogenes* strains [50]. Phage-associated depolymerases positively influence the adsorption of phage to the bacterial cell surface in several different bacteria including *P. aeruginosa*, *Lactococcus lactis*, *Alcaligenes faecalis*, *Azotobacter agilis* and *Bacillus subtilis*. These enzymes are associated with the phage particles as free enzyme that is produced during the lytic cycle of the phage or as both free and bound enzyme. There is evidence that phage-induced depolymerases could affect biofilms [51]. Bacteriophages can be engineered to express a biofilm-degrading enzyme during infection. The gene for dispersin B (*dspB*) was cloned into T7, an *E. coli*-specific phage, to express *dspB* and produced an engineered enzymatic phage which was more efficacious than wild-type phage at attacking biofilms [52]. Alternatively, phage lysins, or endolysins, are possible antimicrobial agents against Gram-positive bacteria and have been applied to a variety of pathogens [53]. These studies indicate that some of the most important obstacles to biofilm control by antimicrobial agents, such as antimicrobial tolerance, presence of the biofilm extracellular matrix and the effect of biofilm age might be overcome by phage. However, drawbacks of phage to consider include narrow host range, bacterial resistance to phage and phage-encoded virulence genes that can incorporate into the host bacterial genome. The immune system might inactivate phage, and impure phage preparations could contain endotoxin. Phage mixtures or engineered phages could provide effective strategies to overcome these obstacles [51].

#### 4.3. Enzymes

As the biofilm matrix encases the bacterial cells within the biofilm colony, degradation of the matrix results in the detachment of cells from the colony and their release into the environment. Matrix-degrading enzymes implicated in active biofilm dispersal include glycosidases, proteases, and deoxyribonucleases [54]. Dispersin B is a well-studied biofilm-matrix-degrading enzyme. It is a glycoside hydrolase produced by the periodontopathogen *Actinobacillus actinomycetemcomitans*. Dispersin B degrades poly-*N*-acetylglucosamine (PNAG), a biofilm matrix polysaccharide that mediates attachment of *A. actinomycetemcomitans* cells to abiotic surfaces, intercellular adhesion (autoaggregation), and resistance to killing by detergents and human phagocytic cells [55]. *P. aeruginosa*, produces both alginate, a biofilm matrix polysaccharide composed of mannuronic and guluronic acids, and alginate lyase, an enzyme that degrades alginate. Increased expression of alginate lyase promotes the detachment of cells from *P. aeruginosa* biofilms and exogenously added alginate lyase increases the effectiveness of some antibiotics against *P. aeruginosa* biofilms [56]. The deoxyribonuclease known as thermonuclease or micrococcal nuclease has been implicated in cell detachment in *S. aureus* biofilms [57]. *S. aureus* biofilms are readily detached from microplate wells by exogenously added deoxyribonucleases, including thermonuclease, indicating that extracellular DNA is a major biofilm matrix adhesin in this species [58].

In food industry, enzymes and detergents have been used synergistically to improve disinfectant efficacy. The combination of proteolytic enzymes with surfactants increased the wettability of biofilms formed by a thermophilic *Bacillus* species and, therefore, enhanced the cleaning efficiency. Formulations containing several different enzymes seem to be fundamental for a successful biofilm control strategy. Basically, proteases and polysaccharide hydrolysing enzymes may be useful [59]. However, the specificity in the enzymes mode of action makes it a complex technique, increasing the difficulty of identifying enzymes that are effective against all the different types of biofilms. Moreover, the use of enzymes in biofilm control is still limited due to the low prices of the chemicals used today compared with the costs of the enzymes. In fact, the technology and production of these enzymes and the enzyme-based detergents are mostly patent-protected. Moreover, the low commercial accessibility of different enzyme activities limits their current usage [42].

#### 4.4. Surfactants

The pre-conditioning of surfaces using biosurfactants can be done to avoid the adhesion of bacteria and the formation of biofilms. Biosurfactants are microbial compounds that can modify the physicochemical properties of surfaces changing bacterial interactions and consequently adhesion. Surfactants are the chemical products usually utilized for cleaning food contact surfaces. The biosurfactants have low toxicity and high biodegradability when compared to synthetic surfactants [60]. Surfactin, a lipopeptide produced by *Bacillus subtilis* and rhamnolipid, a glycolipid from *Pseudomonas aeruginosa* have strong surface activity, emulsion forming ability and has shown antimicrobial properties [61]. The adsorption of biosurfactants to a solid surface can modify its hydrophobicity affecting the adhesion process and consequently the biofilm formation. A 0.1% surfactin solution was reported to reduce significantly the adhesion of *L. monocytogenes* and *E. sakazakii* to stainless steel and polypropylene [62]. The pre-conditioning of polystyrene with 0.1% solution of surfactin reduces by 84% the adhesion of *L. monocytogenes* ATCC 7644 whereas rhamnolipids at 0.75% reduced the adhesion of *L. monocytogenes* ATCC 15313 by 82% [63]. However, the antiadhesive properties of biosurfactants shows to be dependant on the surface and microorganism involved as well as the temperature, and the type and concentration of the surfactant. The time of contact and the concentration of biosurfactant have influenced the ability to disrupt the biofilms [64].

#### 4.5. Nanoparticles

Biofilm formation can be prevented by designing surfaces that can restrict bacterial colonization and biofilm formation. The “nano-functionalization” of surfaces by coating, impregnation, or embedding nanomaterials can inhibit bacterial adhesion and biofilm formation [65].

Many of the nanoparticles with antibacterial properties also exhibit antibiofilm activity. Magnesium fluoride nanoparticles have antimicrobial activity and are able to prevent the biofilm formation of common pathogens such as *E. coli* and *S. aureus* [66]. The catheters modified with magnesium fluoride nanoparticles are able to restrict the biofilm formation of these bacteria significantly [67]. Glass surfaces coated with zinc oxide nanoparticles are able to produce reactive oxygen species (ROS) that interfere with *E. coli* and *S. aureus* biofilm formation [68]. In case of the nitric oxide (NO)-releasing nanoparticles, the rapid diffusion properties of NO may result in enhanced penetration into the biofilm matrix and thus improve efficacy against biofilm embedded bacteria. Its antibiofilm activity is broad-spectrum and is most effective against gram-negative species. Its ability to eradicate both *P. aeruginosa* and *E. coli* ( $\geq 99.999\%$  biofilm killing) is promising since gram-negative bacteria are generally more invasive than gram positive infections [69].

It is notable that magnetic NPs have considerable capability to penetrate into biofilms, using external magnetic fields [70]. Among various types of NPs, superparamagnetic iron oxide NPs (SPIONs) with different surface coatings (e.g., gold and silver) show highest antibacterial activity against biofilms. On adding SPION solution to *P. aeruginosa* biofilms, a dramatic disintegration of the bacterial cell membrane was observed. This inactivation was largely due to the thermal effect. Local heating of a specific area is also possible using this method, and the heating temperature can be easily adjusted by controlling the concentration of the SPION solution. Therefore, hyperthermia using magnetic nanoparticles holds promise as an effective tool for inactivating the bacterial biofilm. SPIONs are considerably more powerful because not only do they generate heat in the specific area where the AC magnetic field is applied but also chemicals such as antimicrobial agents which are considered as hazardous waste are not needed [71]. Thus, nanoparticles offer a means of new line of research in combating biofilms.

#### 4.6. Phytochemicals and functional foods

There are a number of bioactive compounds with activity against bacterial adhesion and many of these are found in natural foods and beverages, such as cranberry, tea, coffee, wine and milk. For example, the green tea catechin, epigallocatechin-3-gallate inhibited the adhesion of periodontopathogenic bacteria *P. gingivalis* to buccal epithelial cells [72]. The polyphenols at different concentrations from various types of grapes and pomace, are capable of inhibiting *S. mutans* glucosyl transferases (GTFs), a bacterial enzyme involved in EPS production and biofilm growth [73]. Shiitake mushroom aqueous extract can inhibit *S. mutans* adhesion to hydroxyapatite and biofilm formation, and also co-aggregation between pairs of *Fusobacterium nucleatum* and *S. mutans*, and *F. nucleatum* and *Neisseria subflava* [74]. Analysis of the mode of action and the morphogenetic effects of this mixture of compounds from the aqueous extracts of the edible mushroom in *Prevotella intermedia* has shown that DNA synthesis is the main target in bacteria which may contribute to antibiofilm activity [75]. Proteins from milk such as bovine and human caseins and lactoferrin, inhibit initial attachment of cariogenic mutans streptococci to HA coated with saliva or purified saliva host ligands [76]. Discovering new anti-adhesive compounds from natural products derived from food and beverages can lead to the development of safe antibiofilm strategies [77].

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