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Multiple Roles of Biosurfactants in Biofilms

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Abstract: Microbial growth and biofilms formation are a continuous source of contamination on most surfaces with biological, inanimate, natural or man-made. The use of chemical surfactants in daily practice to control growth, presence or adhesion of microorganisms and ultimately the formation of biofilms and biofouling is therefore becoming essential. Synthetic surfactants are, however, not preferred or ideal and biologically derived surface active biosurfactants (BSs) molecules produced mainly by microorganisms are therefore becoming attractive and sought by many industries. The search for innovative and interesting BS molecules that have effective antimicrobial activities and to use as innovative alternatives to chemical surfactants with added antimicrobial value among many other advantages has been ongoing for some time. This review discusses the various roles of BS molecules in association with biofilm formation. Recent updates on several mechanisms involved in biofilm development and control are presented vide this article.

Keywords: Antibacterial, antibiofilm, biosurfactants, bactericidal, disruption.

1. BIOSURFACTANT: BROAD PROSPECTIVE MOLECULES

A wide range of microbes' produces unique metabolic molecules namely biosurfactants (BS) that possess amphiphilic properties which reduces surface tension (SFT) and interfacial tension (IFT) of liquid media. BS molecules with lower critical micelle concentrations (CMC) and contact angle (CA) have many useful potential applications in a wide range of industries [1]. In addition, to all the above motioned features, rheological properties like viscoelasticity of BS has to be explored when considering employing BS compounds for various industrial application purposes. Viscoelasticity represents the viscosity as well as elasticity property while undergoing deformation. When stress is applied, many naturally available substances display a shear flow and strain linearly. After removal of stress, the elastic materials rapidly regain their original state. However, BS shows a shear thinning performance therefore demonstrating a thixotropic behavior which is a typical characteristic of a weak viscoelastic gel [2]. Like other soft materials microbial biofilm also exhibit viscoelastic characteristic which is a time-dependent response to imposed automatic perturbation [3]. Whenever there is an adsorption of one liquid into another medium, there is a remarkable change in the viscoelastic property of BS like other physical properties. Olofsson *et al.* [4] showed that different surfaces affect both the initial adhesion of organisms and the viscoelastic properties of the interaction between the surfaces and adhered bacteria. Emulsification, wetting, foaming, properties of BS are very useful for commercial purposes. Characteristics like biodegradability, low-toxicity, production from cheap renewable raw materials and biocompatibility makes these types of compounds attractive towards various industrial applications. To date a huge number of high and low molecular weight BS with diverse chemical nature has been reported produced by microorganisms. Fig. (**1**) represents few basic structure of well known BSs that has been reported in the literature frequently. Other important properties of BS including tolerance at wide ranges of pH, temperature and salinity is also one of the reasons for displaying preferences for BS use instead of synthetic, chemical surfactants by some industries [5, 6].

BSs molecules have become an important area of interest for many researchers due to their effectiveness in various fields facilitated by their novelty and both structural and functional diversity. A remarkable property for many BSs is their varying antimicrobial activity e.g. the inhibition of colonization of pathogens on various surfaces. Two important properties such as bactericidal and bacteriostatic effects are significant to act as multi-target agents against a wide range of microorganisms [7]. Microbiologically sensitive surroundings need to be free from microorganisms. Bacteria growing in biofilm formation remain a significant challenge in biomedical field especially growing on abiotic material such as catheters and prosthesis, as they tend to be more tolerant/resistant towards antimicrobial treatments. Biofilms formation immediately starts in the body once a biomedical device has been planted within its niche. Frequent replacement of the implanted biomaterials from the body of a patient are often uncomfortable, costly, time consuming and may lead to damage of the cellular tissue. Biofilm development and infection can be limited by preventing microbial adhesion to the surfaces of medical devices [8-11].

Some of the important aspects of BS in relation to bacterial cells are highlighted in Fig. (**2**). It is important to note that a variety of BSs have been explored for their antibiofilm activity. Surfactants may affect the development of flagella, suggesting changes in the attachment capability of bacteria [12]. Bacteria also exhibit varied strategies to defend themselves from environmental attack and aid their own survival. Adhesion by bacteria to the surfaces results in energy saving and therefore, organisms try to shield themselves and protect their ecological niche. The main factors that play an important role in the interference of bacterial adhesion are the initial bacterial hydrophobicity, the concentration and type of BS [13]. It is

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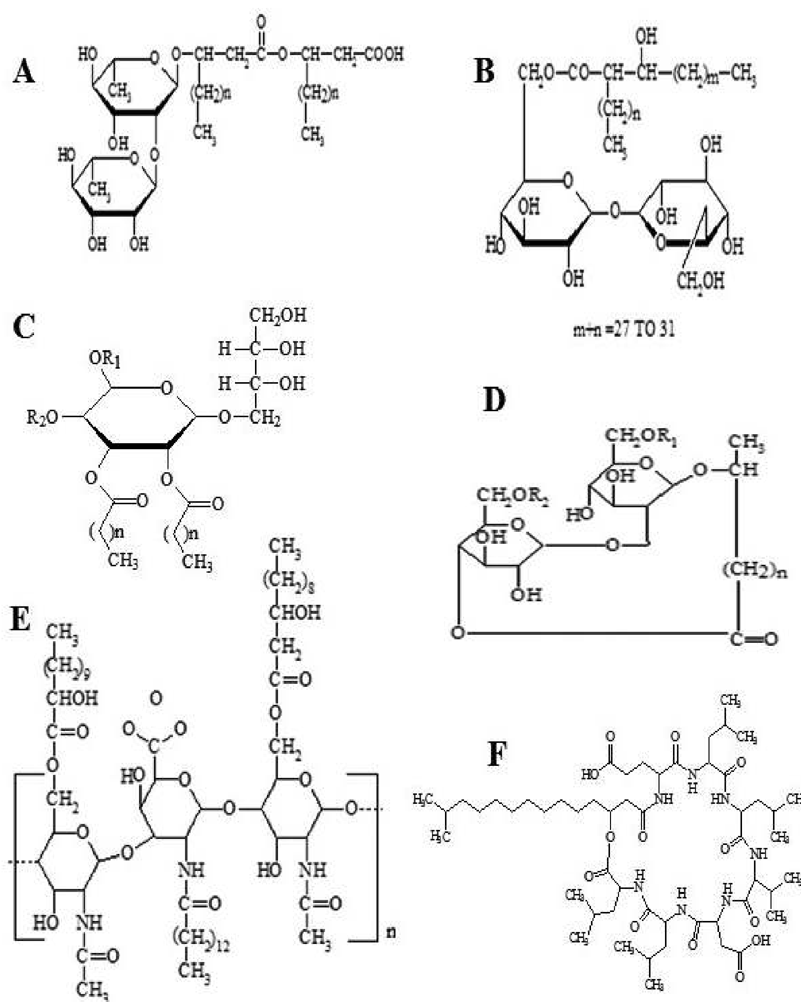

Fig. (1). The basic structures of four main types of low molecular weight glycolipids : (A- Dirhamnolipid; B-Trehalose monomycolates; C-Mannosylerythritol lipids; D-Lactonic Sophorolipid) and Two high molecular weight structure of cyclic lipopeptide (E- Emulsan; F- Surfactin).

common to note that the type of BS which may be active against a Gram-negative strain (like *E. coli*) may invariably be ineffective against the Gram-positive strain (example *S. aureus*) and vice a versa [14]. Antimicrobial action of BS molecules towards pathogenic organisms has drawn much attention by many researchers [15]. A great change in bacterial surface hydrophobicity affected by BS molecule consequently varies the adhesion of organisms on solid surfaces. Therefore, such types of BS molecules are representative candidates towards the development of antibiofilm agents [1]. Intensive efforts are directed towards exploring new novel antimicrobial agents to combat increasing antibiotic resistance by bacteria and innovative approaches are essential to fight microbial infections.

2. BIOFILM FORMATION BY VARIOUS MICROORGANISMS

Biofilms are complex aggregation of microorganisms that grows on various solid surfaces [16]. The concept of biofilms was first illustrated by Antonie van Leeuwenhoek but, the actual biofilm forming process was not revealed until much later. Several researches have demonstrated the universal occurrence of biofilm forming microorganisms in different aquatic and industrial water bodies. An illustration of the overall process of biofilm formation process during bacterial colonization is shown in Fig. (**3**). Currently it is well documented that biofilms represent a heterogeneous structures of microbial cells imbedded into an exopolysaccharide (EPS) phase [17] and it is also suggested that the resistance demonstrated by antimicrobial agents is thoroughly related to the intrinsic three dimensional organizations of cells in this exopolymeric matrix.

Microbes can form colonies on biotic or abiotic surfaces which are represented as a single, small to large communities of multiple-species. The formation of biofilm is one of the significant means for survival of microorganisms in their surrounding environment [18-20]. Those microbes which form biofilm around them are comparatively more resistant to antimicrobial agents. When the microbes are in the planktonic form they are comparably less tolerant to these antibiotics. When organisms are in a planktonic form around us, a simple disinfection process could be sufficient for the removal of these attached microorganisms from biotic and abiotic surfaces. Generally, disinfection at regular interval is one of approaches used in many processes.

A lot of health related diseases occur due to the formation of biofilm by pathogenic microorganisms. The formation of biofilms in and on human body parts and, other food and health related material can be a very serious issue. Various biomedical devices used during the care and treatment of patients needs to be free from opportunistic pathogens. Some of infections such as endocarditis and cystic fibrosis are directly associated with biofilms. With the development of more advanced technologies, it is possible for us to understand the intra and inter-cellular processes of bacteria communities that oversee the overall bacterial physiological conditions. Subsequently, cell to cell interactions results in formation of extracellu-

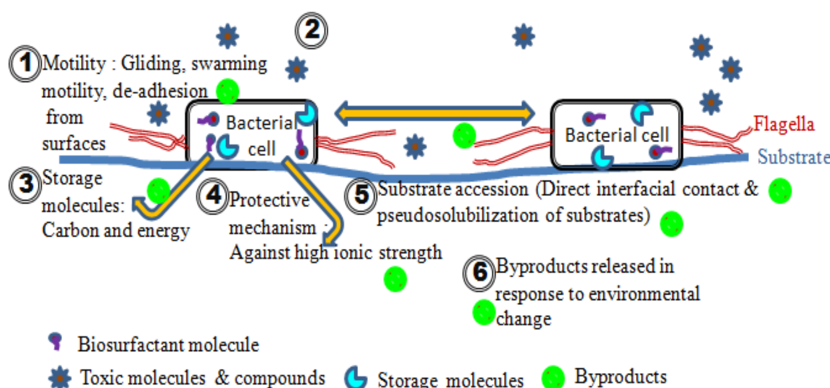

Fig. (2). Importance of biosurfactant molecule for bacterial cell.

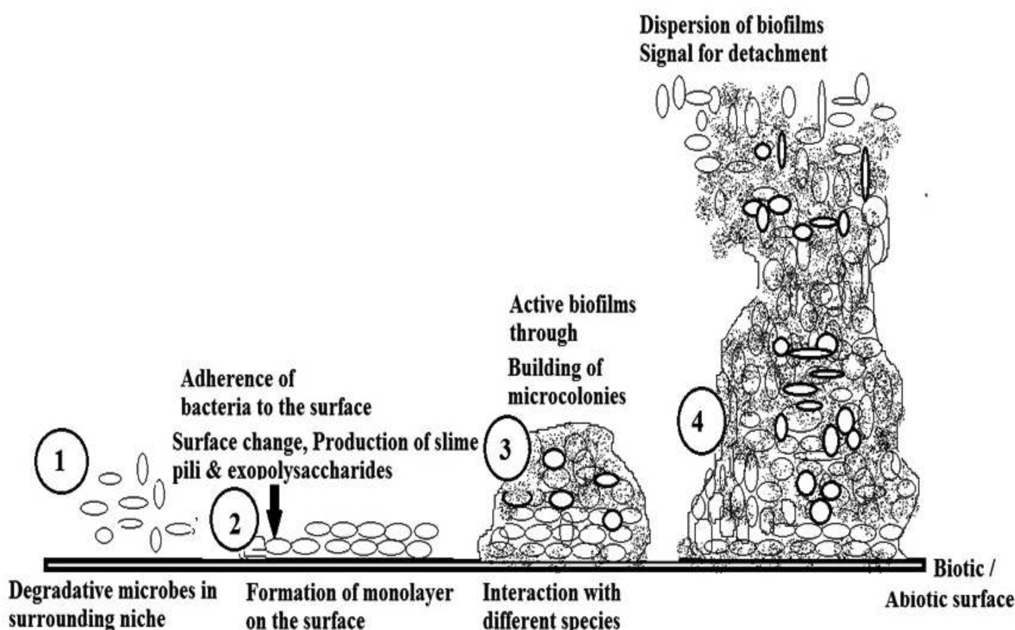

Fig. (3). The overall mechanisms of formation of biofilm by microorganisms.

lar complex polysaccharide based matrices resulting in the formation of biofilm structures [21]. To combat bacterial infections we therefore need to understand the mechanism of resistance development and other aspects such as the molecular biology, biochemistry, physiology, nutritional requirement necessary for their growth and survival in their environments. There is an urgent requirement for novel antibiofilm compounds which can prevent the growth and accumulation of biofilm forming pathogenic microorganisms on the surfaces of various devices and host system. Initial biofilm formation process can be inhibited and ultimately one can get rid of dangerous forms of biofilms. When surfaces are preconditioned with BS, the formation of biofilms can be prevented. The use of chemical antimicrobial agents to control adhesion of microorganisms and ultimately the formation of biofilms has become routine practice. Due to the effect of surfactant molecules, cell membranes are disrupted extensively leading to the lysis of cells, which increase the permeability causing leakage of cell metabolites. This ultimately alters the physical membrane structure and disturbs protein confirmation. Therefore, some of the important membrane functions like generation of energy and transport are severely affected [22].

Bacillus subtilis is one of the most explored Gram-positive bacterium systems for genes, proteins, and molecular mechanisms responsible for formation of biofilms. D´ıaz *et al.* [23] suggested that among Gram-positive bacteria species-specific molecular mechanisms are involved in biofilm formation. When *Staphylococcus epidermidis* was reported it was thought of as a safe organism however quite some time ago this organism was identified as an opportunistic pathogen especially on prosthetic cardiac valves [24, 25] and various orthopedic appliances [26]. Generally the *S. epidermidis* does not produce slime; however, Christensen *et al.* [27] showed that adherence of *S. epidermidis* to medical devices is mediated through the production of slime and at the same time it is also the main factor responsible for its infections. In biofilm communities where multi-species are involved, a number of complex reactions take place which influence its' overall characteristics. Studies on multispecies interactions in biofilm environment however, remain mostly superficial [28]. A very limited efficacy of existing antibiofilm solutions (based on planktonic bacterial physiology) is reported; therefore we need to explore more suitable alternatives to conventional therapies [29].

To some extent, studies including the interactions and resources used by bacteria to flourish in complex biofilm communities have encouraged researchers to propose alternatives to conventional antibiotics used against pathogens [30]. There is some supporting evidence from Qin *et al.* [31] relating the disruption of staphylococcal biofilms through the bactericidal effects. When an organism

community is considered, several bacterial species often coexist and contend for resources available in the surrounding environment. *Pseudomonas aeruginosa* the opportunistic pathogen make use of extracellular products for their interaction with the nosocomial biofilm forming pathogen namely *S. epidermidis*. Qin and collaborators [31] suggested that the quorum-sensing-controlled factors from *P. aeruginosa* supernatant (polysaccharides) inhibit the growth of *S. epidermidis* in planktonic as well as biofilm forms. *P. aeruginosa* extracellular products are important as microbial competition factors that overcome competition with *S. epidermidis*. Such observations may provide clues for the development of a novel strategy for controlling *S. epidermidis* biofilms.

3. MICROBIAL BIOSURFACTANT AS ANTIBIOFILM AGENTS

There is a range of properties shared by BS molecule that may affect their interactions and association with biofilms. Some of the different roles conferred by BS molecule that may interfere with biofilm formation by microorganisms are represented diagrammatically in Fig. (**4**).

3.1. Alteration of Cell Surface Properties

Traditionally bacterial population is divided into the two main groups of Gram positive and Gram-negative which are based on cell-envelope organization. Gram negative bacterial outer membrane which is composed of lipopolysaccharides (LPS), lipoproteins and phospholipids where hydrophobic interactions are involved in linking the peptidoglycan layer [32]. Number of porins and efflux pump are also embedded in the LPS layer [33]. Makin and Beveridge, [34] suggested that the quantity and type of LPS shows a profound effect on the interactions of the microbial cell with its environment. Denyer and Maillard, [35] reported the presence of four major outer membrane proteins (OMPs) including (OprF, OprP, OprB, OprD) and two minor (OprC, OprE) in the membrane of *P. aeruginosa.* Several chemical agents, permeabilizing agents modifies the outer membrane of Gram-negative bacteria resulting the changes in surface properties and membrane permeability and hydrophobicity. Alterations in the membrane due to change in the composition of membrane fatty acids are considered to be one of the most imperative adaptive mechanisms in bacteria [36].

Rhamnolipid (RHL) BS potentials for industrial and environmental applications is the subject of many literature reports. Few researchers however have discussed its interaction of and effects on bacterial surfaces and membrane active properties [37-39]. Such changes in the lipid and fatty acid composition of the bacterial cell membrane are due to effect of interaction with BS have been reported [40]. Sotirova *et al.* [40] worked towards usage of antimicrobial properties of methyl (MTS) and ethyl (ETS) esters of thiosulfonic acid alone and in combination with RHL-BS for their ability in disrupting the normal physiological functions of pathogenic microorganisms' viz., *P. aeruginosa, B. subtilis, Alcaligenes faecalis, and Rhizopus ngtricans.* Sotirova *et al.* [40] reported the combination of RHL with thiosulfonic esters has a synergistic effect towards decreasing the bactericidal and fungicidal concentrations of MTS and ETS. The same group of researchers [41] has previously reported the interaction of RHL-BS with bacterial cells affecting the change in outer membrane proteins of *P. aeruginosa*. RHL-BS obtained from *Pseudomonas* sp. PS-17 has been used to demonstrate the effect on the cell surface structures of *Pseudomonas aeruginosa* NBIMCC 1390. Note worthy observation was put forward by these researchers stating that concentrations of RHLs below and above CMC can provoke a multi-component response of the bacterial cells without any influence on growth and viability. Concentration above CMC value reduces total cellular LPS content of 22% subsequently increasing the cell hydrophobicity to 31% adherence. However, at concentration below CMC value the LPS components of the bacterial outer membrane are not affected instead change in OMP compositions were observed. It was concluded that BS can affect the cell surface morphology drastically which is totally dependent on concentration. The release of LPS from the cell surface may be due to solubilization of the outer

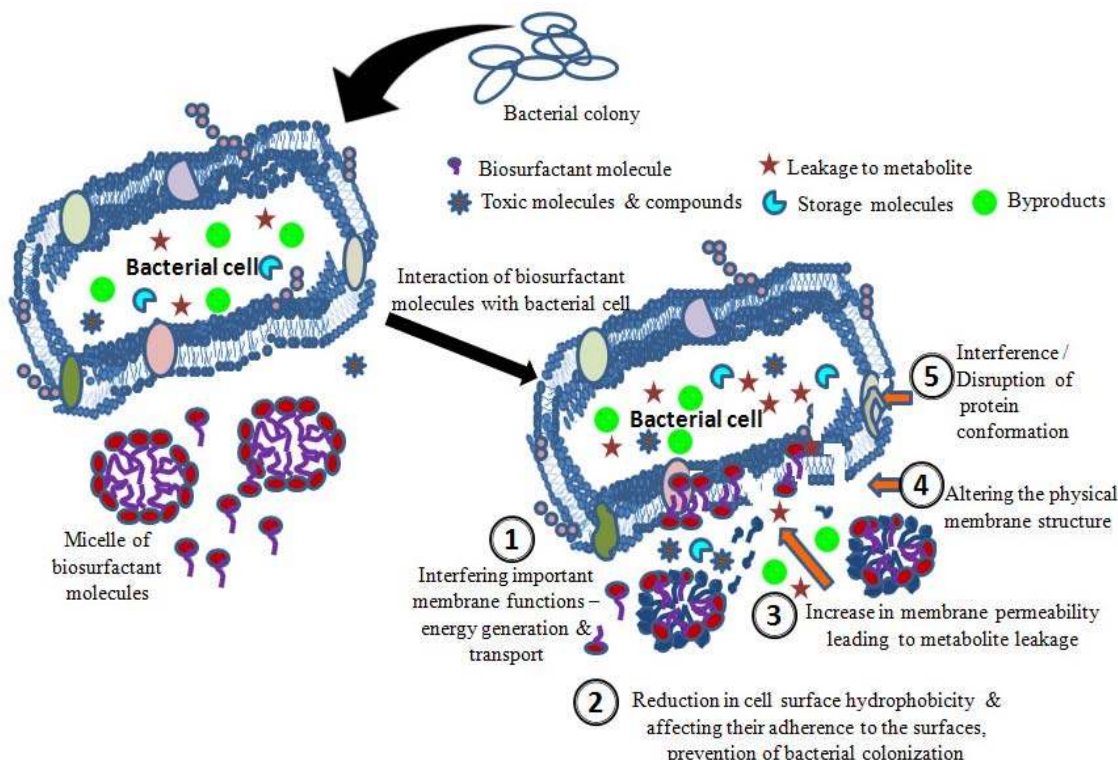

Fig. (4). Different roles of biosurfactant molecule for interference of biofilm formation by microorganisms.

membrane by binding of aggregated BS to the membrane followed by removal of the LPS component. This has been supported by authors from the evidence revealed from analysis of the OMP profiles disclosing the decrease in the amount of major proteins like Opr F, Opr D, Opr J and Opr M.

Quite a few surface active molecules, (chemically/biologically originated) alter the cell surface properties [42] which are reflected from the following examples. Purified RHL-BS affect the hydrophobicity of octadecane-degrading cell and the differences in rates of octadecane biodegradation was analysed. Rhamnolipids increases the cell hydrophobicity of the slow degraders where as hydrophobicity of the fast degraders was unaffected. Of course the change in the hydrophobicity was dependent on the concentration of RHL [43]. Rhamnolipid molecule induces the removal of LPS from *Pseudomonas aeruginosa* and ultimately affects the bacterial cell surface properties and interaction with hydrophobic substrates through the enhancement of cell surface hydrophobicity. On similar lines, Al-Tahhan *et al.* [44] observation supported that the loss of LPS in *P. aeruginosa* strains after treating with RHL (low concentrations) resulting increase in the cell surface hydrophobicity. Limited information was available on the interaction of biologically originated surface active molecules with bacterial cells. Kaczorek *et al.* [45] observed the different way of surfactant in modification of cell surface properties. Surface properties of *Stenotrophomonas maltophilia* were investigated to determine the influence of hydrocarbons and surfactants on surface and enzymatic characteristics. There is much change in the fatty acids profiles which thereby facilitated the adherence of cells to hydrophobic compounds. RHLs BS increases the cell permeability and inhibit the activity of the bacterial cells due to the efflux of intracellular protein, ions and other intra-cellular components, which ultimately lead to the cell death. Such properties allow the use of BS as an additive in antibiotic formulations and other antimicrobial compounds in order to enhance the effectiveness of chemotherapeutic agents [46].

3.2. Reduction in Adherence Abilities to Surfaces, Prevention of Bacterial Colonization

Surfactin and RHL, both BS types are able to prevent attachment of biofilm formed by various pathogens [47]. BS produced by *Bacillus* species inhibits adhesion of biofilms probably through binding to the cell surface or its components and therefore altering the hydrophobicity of the outer membrane [48, 49]. Such effect would promote dispersal of preformed biofilm as reported by Mireles *et al.* [47] using surfactin on *Salmonella enterica* which inhibited adhesion to polyvinyl chloride (PVC) without inhibiting the growth. Rivardo *et al.* [14] also reported anti-adhesion activity against biofilms by lipopeptide BS produced by *B. subtilis* and *B. licheniformis.* The two lipopeptide type BS inhibited adhesion of biofilm of pathogens selectively against Gram –ve *E.coli* CFT073 and Gram +ve *S. aureus* ATCC 29213 with decrease of 97% and 90%, respectively. Cyclic lipopeptide BS isolated from *Bacillus amyloliquefaciens* AR2 inhibits *C. albicans* biofilm by altering the cell surface hydrophobicity or hampers germ tube formation. There is also a reduction of the mRNA expression of hyphae specific genes – HWP1 and ASL3. About 46–100% of the biofilm formation is inhibited by tested BS. Interesting report from Dusane *et al.* [50] documented that RHLs molecules exhibit biofilm disruption activity of biofilms formed by ascomycetous dimorphic fungus viz., *Yarrowia lipolytica.*

Harshey *et al.* [51] reported a cyclic lipopeptides (surfactin) obtained from *B. subtilis* with the capacity to inhibit biofilm formation by coating medical and industrial objects. Surfactin is very stable at higher temperature treatment retaining biofilm inhibiting properties after storing surfactin-baked catheters (One hour at 60°C) or for 5 days at room temperature. There are number of reports for surfactin molecules eradicating the adhesion of biofilm producers. There are difference in the activity depending upon the producing strain and the concentration of BS compound. Some of the reports available in literature have also tested the properties of mixture of surfactin compound with other compound such as fengycin (1: 1) against planktonic *E. coli* [52]. Work by Mireles *et al.* [47] indicated that there was complete extermination of *Salmonella enteric* and *E. coli*, biofilm adhesion on urinary catheters surface pre-coated with 5 µg/ml of surfactin whereas absolutely no effect was detected against *P. aeruginosa*.

Similar to *Bacillus* strains, variety of *Pseudomonas* species has been extensively explored for BS possessing antiadhesive activities. Work demonstrated by Rodrigues *et al.* [9] suggest that RHLs obtained from *P. aeruginosa* DS10-129 hold down bacterial adhesion of *S. epidermidis, Streptococcus salivarius* isolated from explanted voice prostheses to silicone rubber. Hence these RHLs have potential for the development of bio-detergent solution for cleansing purpose of prostheses. Therefore it can prolong the lifetime of this material which would be useful to the laryngectomized patients. Coating of polytetrafluoroethylene (PTFE) surface with RHLs BS has ability to reduce *Listeria monocytogenes* attachment [53]. D´ıaz *et al.* [23] recently reported that sophorolipids type BS containing acid/lactonic content are efficient bactericidal agent which can bring out the cell death of planktonic cells of some Gram positive and Gram negative bacteria. This research group had compared the antimicrobial activity of sophorolipid BS with conventional antimicrobial compounds having bacteriostatic effects. Sophorolipids also disrupts biofilms at concentration is > 5% (v/v). Thus, authors concluded that sophorolipids molecules are promising bactericidal agents biomedical application point of view due to their antimicrobial properties, and potential use to prevent adhesion and biofilm disruption.

One of the best explored probiotic strains for BS production is *Lactobacilli* species. Velraeds *et al.* [54] studied 15 *Lactobacilli* strains and showed that the isolates produce BS in the mid-exponential and stationary growth phases. Inhibition of initial adhesion (99%) of uropathogenic strains like *Enterococcus faecalis* 1131 on glass surface in a parallel-plate flow chamber was successfully demonstrated by the BS from few *lactobacilli* strains. Subsequently Velraeds *et al.* [55] reported on surlactin, a BS from Lactobacillus sp. which decreased the initial adhesion of *E. faecalis* 1131 on a hydrophilic and a hydrophobic substratum in a parallel-plate flow chamber system. They carried out studies by using phosphate-buffered saline and pooled human urine as a suspending fluid. Such studies were highly significant for the development of antiadhesive biologic coatings for catheter materials. It is important to note that the efficiency of BS's under investigation are often affected by the hydrophobicity of the substrate, the suspending fluid, and uropathogen present in the system. Velraeds *et al.* [56] has contributed towards utilization of surlactin' from *L. acidophilus* RC14 BS to inhibit the initial adhesion of various uropathogenic bacteria on medical devices like silicone rubber using parallel-plate flow chamber in filter-sterilised pooled human urine. Other piece of work contributed by the same research group [57] confirmed that adhesion and growth of naturally occurring uropathogens from human urine is discouraged by BS produced from *Lactobacilli* strains. It should be kept in mind that efficiency and variations in this type of activity is dependent on strains of *Lactobacilli* present in human system and for few BS, it is also affected/related to sex. Therefore, clinically it is very important to reduce bacterial adhesion and wipe out the biofilm population for removal of bacterial colonization from biomedical devices surfaces used in urinary tract infections.

Sambanthamoorthy *et al.* [58] evaluated antimicrobial, anti-adhesive and anti-biofilm abilities of cell bound BS produced by two strains *L. jensenii* and *L. rhamnosus* against clinical Multidrug Resistant (MDR) strains of *Acinetobacter baumannii, Escherichia coli,* and *S. aureus*. The cell associated BS bears anti-adhesive and anti-biofilm abilities against *A. baumannii, E. coli* and *S. aureus* and showed that BS can be used as an alternative therapeutic ap-

proach for the prevention and/or treatment of hospital-acquired infections. *L. acidophilus* produces BS which has ability to inhibit biofilm formed by *S. aureus* and *S. epidermidis*. Today several techniques like, confocal laser scanning microscopy and image PHLIP analysis are routinely used for evaluation of bacterial initial adhesion and biofilm formation. Their report also suggests that all different activities are dependent on strain as well as dose or concentration. The dispersion or disruption of biofilm morphology can be hastened by addition of BS. This contribution by Walencka *et al.* [59] highlighted the use of *L. acidophilus*-derived surfactants in inhibiting bacterial deposition rate and biofilm development, their maturation. This activity is believed to be achieved due to the influence on the cell-surface hydrophobicity of *staphylococci* without affecting their growth.

Much reliable approach to prolong the lifespan of voice prostheses is available from the work on BS produced by *Lactococcus lactis* and *Streptococcus thermophilus* that reduces the microbial numbers on prostheses [60]. BS produced by *Lactobacillus* sp. CV8LAC inhibited the adhesion of two *C. albicans* pathogenic biofilm producer strains (CA-2894 and DSMZ 11225 planktonic cell [61]. The authors observed that the planktonic cells of *C. albicans* are not inhibited and therefore they concluded that the compound has anti-biofilm potential rather than antimicrobial activity. Pradhan *et al.* [62] reported a *Lysinibacillus fusiformis* S9 for glycolipid type BS production and explored its inhibition of biofilm formation potential against *E. coli* and *S. mutans*. Authors had an interesting observation that that the concentration of 40 g ml-1 the BS does not show any bactericidal activity but constrain the formation of biofilm entirely. This was the first report available on *L. fusiformis* ability to produce glycolipid type of BS capable of inhibiting biofilms of pathogenic bacteria on hydrophilic (glass) as well as hydrophobic (catheter tubing) surfaces. In this way we can prevent biofouling of biomedical surfaces without any toxicity to biological system.

An interesting contribution by Kanmani and collaborators, [63] investigating EPS production from marine *S. phocae* PI80; they examined the chemical nature, antibiofilm, antioxidant activity and functional properties of EPS and reported excellent emulsifying and flocculating activity which are much similar to xanthan gum, gelatin and guar gum. EPS was also suitable for inhibiting biofilm formation and therefore could be used as food grade adjunct. Kiran *et al.* [64] investigated the antibiofilm activity of a glycolipid BS isolated from the marine *Brevibacterium casei* MSA19 against pathogenic biofilms. The BS has a broad spectrum of antimicrobial activity and proved to be bacteriostatic and disrupts biofilm formation under dynamic conditions, which was consistent against mixed pathogenic biofilm forming bacteria. Glycolipid type BS produced by a tropical marine strain of *S. marcescens* displayed several activities like antimicrobial, anti-adhesive and biofilm disruption against selected pathogenic and marine biofouling microorganisms [65]. Such glycolipid BS therefore is useful materials for development of novel antibiofilm agents.

3.3. Recent Advances of Nanoscale Approaches Towards Reduction in Colonization of Pathogens

Nanotechnology-Nanoscience based recent approaches are attracting researches around the globe interested in controlling biofilms in medical and healthcare applications. Nanoscale materials are providing alternative solutions towards inhibition of biofilms on living and non living surfaces. Researchers are aware with the different microbial behavior in planktonic and during biofilm formation. This knowledge has been found to be essential to tackle the biofilm formation by pathogens in smarter ways. Several efforts have been taken continuously for developing novel mechanisms to eliminate microbial biofilm from medical devices.

In this regard, Singh *et al.* [66] studied the quantitative characterization of the influence nanostructured surfaces on bacterial adhesion and biofilm formation. Using supersonic cluster beam deposition, they produced nanostructured titania thin films with controlled and reproducible nanoscale morphology and characterized the adhesions of *Escherichia coli* and *Staphylococcus aureus* on nanostructured titania surfaces. They observed that the increase of surface roughness improves protein adsorption, which in turn downplays bacterial adhesion and biofilm formation. As roughness increases up to about 20 nm, bacterial adhesion and biofilm formation are enhanced; the further increase of roughness causes a significant decrease of bacterial adhesion and inhibits biofilm formation. They interpret the observed trend in bacterial adhesion as the combined effect of passivation and flattening effects induced by morphology-dependent protein adsorption. The same group further showed that *S. aureus* and *E. coli* interaction with nanostructured surfaces shows an increase in adhesion and biofilm formation with increasing nanoscale morphological properties [67].

Learning lessons from efficient natural processes to design smart fabrics, mimicking natural phenomena also were reviewed for the synthesis of nanoparticles for antimicrobial purposes. Singh *et al.* reviewed nature's design and subsequent parallel advances in biomimetic materials and polymer sciences, with combining interdisciplinary engineering principles to mimic nature inspired designs of nanostructures materials into fabrics in textile industries to combat microbial infections [68]. The same group [69] further emphasis on the systematic evaluation of nanomaterial toxicity in primary cells derived from vital organs and the need to develop an international consortium for a materialomics database was encouraged.

Not only has the design of smart nanostructures, nanoparticles also showed interesting applications for inhibiting the growth of microorganisms and biofilm. Silver nanoparticles were shown to affect the microbial cell at many different levels viz., bacterial wall integrity, synthesis of proteins and DNA through slow release of Ag^+ ions [70] leading to effective contact with and elimination of microorganisms and biofilms [71-74]. Usage of metallic NPs offers better opportunities to avert adhesion of pathogens and subsequently formation of biofilm. Bacterial biofilms on catheters formed by *E. coli*, *S. aureus*, *P. aeruginosa*, S. *epidermidis*, *C. albicans* has been controlled using NPs of silica containing nitric oxide [75,76]. Rai and coworkers [77] also controlled biofilm formation with the help of silver NPSs.

It is also reported in literature that TiO_2 and EDTA nanoparticles can affect biofilms formed by *Candida albicans.* Haghighi *et al.* [78] proposed to use coating of TiO_2 nanoparticle on medical devices in order to treat and or prevent the fungal biofilms. Different NPSs formulations have been used as antimicrobial sprays especially silicon compounds and organic quaternary ammonium salts. He *et al*. [79] synthesized NPs having different charged surfaces so that during ionic physical interaction they bind to microbial cells and subsequently remove them from the surfaces. This ionic interaction approach has proved to be able to inhibit as well as elimination of biofilm forming organisms. Few years' back the use of superparamagnetic iron oxide (γ-Fe_2O_3) NPs was employed effectively to treat biofilm [80]. Taylor and Webster [80] put forward their observation that the generated hydroxyl radicals depolymerize the polysaccharides, leads breakage in DNA and ultimately results in the inactivation of enzymes which are an important component for the EPS matrix. The superparamagnetic iron oxide possesses tremendous potential for leakage of cell membranes of planktonic cells.

Our research group Gholap *et al.* [81] developed an antibiofilm NPS agent with an ability to inhibit quorum sensing mediated biofilm formation in pathogenic organisms. The prepared a nanocomposite, CdTe–TiO_2 has been proved as effective impeder of bacterial growth and biofilm formation (Fig. **5**). About 57% biofilm growth is inhibited with this nanocomposite and therefore can be used as antibacterial agent against Gram positive and Gram negative organisms. This antibacterial activity of nanocoposite on bacte-

ria is due to the generation of reactive oxygen species inside the cells leading them to its rupture. Therefore, TiO_2 or CdTe sensitized TiO_2 has great potential as an antibiofilm agent and has promising applications in photocatalytic destruction. The same research group Patil *et al.* [82] synthesized quantum dots conjugated zinc oxide nanostructures (ZnO/CdTe) an anti-biofilm agent. The nanocomposite impedes biofilms due to photocatalytical action on the cell biofilm surfaces (Fig. **6**). The use of hydrothermal method for ZnO/CdTe nano-structures array synthesis are advantageous since working at low growth temperature and usage of inexpensive material used in fabrication. While using NPs in treatment of microbial biofilm, certain concepts needs to be considered; foremost is the long term release of the active agent, the increased solubility and bioavailability of the agent with reduction in aggregation and finally improving its effectiveness [83]. It is very important that in addition to the conventional approaches, novel concepts are adopted to fight against biofilms and towards increasing the high quality of food safeties and health [84].

3.4. Altering the Physical Membrane Structure and Increase in Membrane Permeability Leading to Metabolite Leakage

Banat *et al.* [1] has put forward a hypothesis that BS molecules play a leading role towards development and maintaining biofilms, may be partly through the maintenance of water channels through the biofilm. This involves the enhanced movement of nutrient and exchange of gases which ultimately results in the dissociation of parts of the biofilm into planktonic mobile forms. Surfactin a cyclic lipopeptide based antibiotic disturbs the integrity of the cytoplasmic membrane's phospholipid composition and its physical properties by three different ways [85]. These include interacting with lipid membrane as (a) Mobile cation carrier [86, 87] (b) Formation of cationic channels [88, 89] (c) Destruction of membrane through the detergent effect [90]. Lipopeptide type BS molecules form selective cationic channels in lipid bilayer membranes and thus are involved in the functional channel-formation of target cells [91, 92].

The main reason for antimicrobial effect of the BS are believed to be due to their adhering property to the cell surfaces leading to weakening in the integrity of cell membrane and therefore disruption in the nutrition cycle [93]. Another reason is due to the structure of BSs, where among polar and non polar portion of the molecules, the fatty acids moiety of BS get inserted into the cell membrane causing an increase in the size of the cell membrane and considerable changes in the cells. Depending upon the type of BS, membrane integrity is affected in different ways. It may happen that

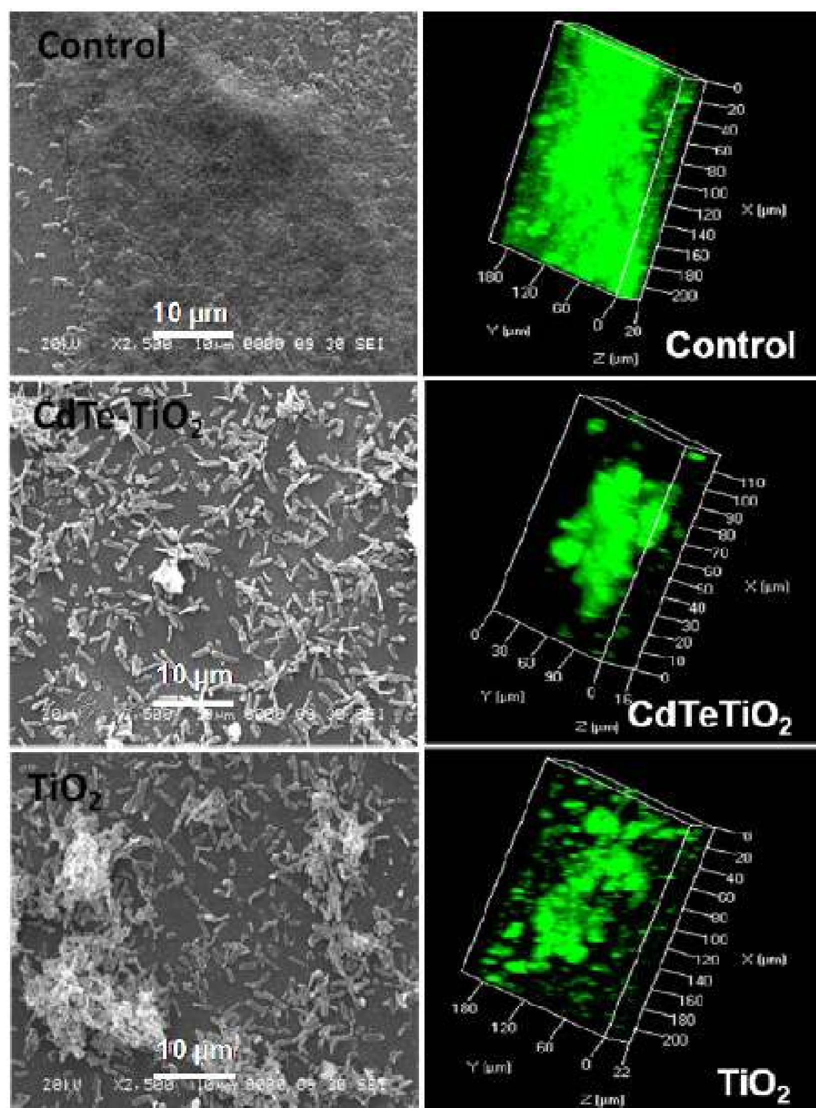

Fig. (5). SEM and CLSM images of the biofilm (*Pseudomonas aeruginosa*) and its inhibition in presence of CdTe–TiO_2 nanocomposites - Adopted from Gholap *et al.* 2013 [81].

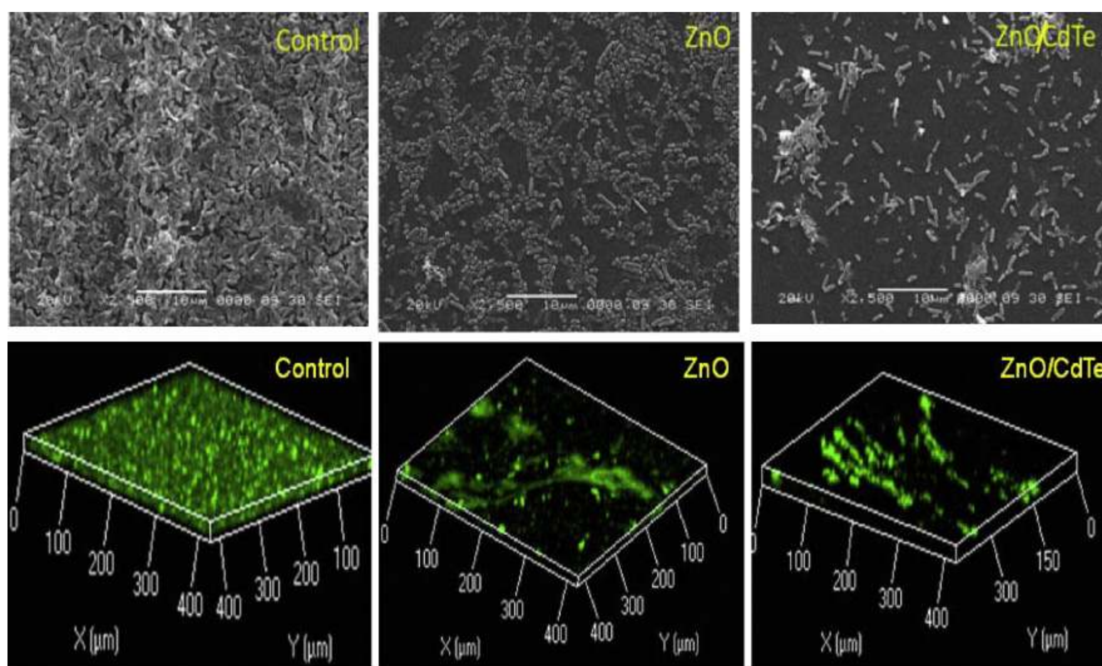

Fig. (6). SEM and CLSM images of the biofilm (*Pseudomonas aeruginosa*) and its inhibition in presence of ZnO and ZnO/CdTe nanostructure - Adopted from Patil *et al,* 2015 [82].

the shorter acyl tails get inserted into the cell membrane disturbing the cytoskeletal elements and the plasma membrane. Such dislodges the membrane away from the cytoplasmic contents [94]. Other methods of actions for membrane disruptions by lipopeptide type BS may be through accumulating intra membranous particles in the cells increasing electrical conductance of the membrane Thimon *et al.* [95]. In contrast, Carrillo *et al.* [96] suggested that the lipopeptide BS increases the permeability of membrane though the interaction with the cell membrane phospholipids

3.5. Interference of Protein Conformation, Important Membrane Functions

At lower CMC values, the potential gentle action of BS on non growing cells and neutral effect on the growth of microbial strains offer several applications in environmental bioremediation as well as biomedicine fields. BS affects the permeability of bacterial cells which is obviously dependant on its concentration. This effect is demonstrated by Sotirova *et al.* [97] on Gram negative cells such as *P. aeruginosa*, *E. coli*, and Gram positive *B. subtilis* in vivo and in vitro conditions. BS has neutral or detrimental effect on the growth of Gram-positive strains. When media supplemented with BS it does not affect the growth of Gram-negative. The BS shows higher permeability with *B. subtilis* (a Gram positive bacterium) as compared with *Pseudomonas aeruginosa* (a Gram negative bacterium). Scanning-electron microscopy analysis indicates that the BS PS does not exhibit disruptive action on resting cells however it is detrimental on growing cells of *B. subtilis*. Ortiz *et al.* [98] investigated the molecular interaction of trehalose based glycolipid BSs isolated from *Rhodococcus* sp. on the membranes composed of phosphatidylethanolamines of different acyl chain length and saturation. The BS under studies has capacity to get incorporated into phosphatidylethanolamine bilayers and affect all structural properties. Authors had proved this using different techniques like differential scanning calorimetry (DSC), small and wide angle X-ray diffraction and infrared spectroscopy (IR). Trehalose lipid molecule intercalates between the phospholipids ones, and can disturb the phospholipid palisade. It is also important to note that BS does not affect the macroscopic bilayer organization of saturated phosphatidylethanolamines. There is a good miscibility between trehalose lipid and saturated phosphatidylethanolamines. BS also increases the hydrocarbon chain conformational disorder and also has a significant dehydrating effect of the interfacial region of the saturated phosphatidylethanolamines.

Ortiz *et al.* [99] also carried out studies on bacterial trehalose lipid of Rhodococcus sp. to observe its interaction with dimyristoylphosphatidylserine membranes using same techniques. Trehalose lipid enhances the fluidity of the phosphatidylserine acyl chains and changes the local environment of the polar head group. There is also a decrease in the hydration of the interfacial region of the bilayer. The BS also makes it possible to affect the thermotropic transition of dimyristoylphosphatidyserine in the presence of calcium. The authors proposed that the BS is incorporated into the phosphatidylserine bilayers and produces structural perturbation that may affect the functional properties of the membrane. Other techniques like Fourier transform infra red spectroscopy and fluorescence polarization has been utilized to observe the effect of bacterial di-RHL on phospholipid membranes composed of 1, 2-Dipalmitoyl-sn-Glycero-3-Phosphocholine (DPPC). Sánchez *et al.* [100] showed that the BS alters both the acyl chain and the interfacial region of the bilayer. Such type of molecules has great applications for making their membrane more hydrophobic in nature and, thereby becoming more susceptible to hydrophobic antibiotics.

Other interesting properties of a succinoyl trehalose lipid isolated from *Rhodococcus* sp. has shown its ability to have a great tendency of partitioning into phospholipid membranes [101]. Zaragoza *et al.* [101] used human red blood cells (RBC) as an experimental model. Trehalose lipid causes the swelling of erythrocytes followed by hemolysis at concentrations well below its critical micellar concentration. In presence of trehalose lipid, K^+ release precedes that of hemoglobin. Osmotic protectants of the appropriate size added to the external medium make it possible to avoid hemolysis. Trehalose lipid lyse the human erythrocytes by the colloid-osmotic mechanism possibly through the formation of enhanced permeability domains, or "pores" enriched in the BS within the erythrocyte membrane. Most of the concepts are apparent through the scanning electron microscopy indicating that trehalose lipid-induce spherocytosis and echinocytosis of RBC.

Other glycolipid namely di-RHL from the crude RHL BS induces leakage in cellular internal contents, which was evident by the carboxyfluorescein, in phosphatidylcholine unilamellar vesicles at a concentration lesser than its CMC [102]. Lysophosphatidylcholine, the cone-shaped lipids in the membrane, accelerates leakage, other lipids like phosphatidylethanolamine, decreases the leakage rate. When cholesterol concentration is high it protects the membrane against di-RHL-induced leakage. Di-RHL can also cause hemolysis of human erythrocytes through a lytic mechanism. In addition to these effects, BS also alters the usual disc shape of erythrocytes into that of spheroechinocytes which was observed through scanning electron microscopy [102].

3.6. Quorum Sensing (QS)

Quorum sensing is a system of stimulate and response correlated to population density. Different groups of organisms use the mechanisms of quorum sensing for coordination purpose. Expression of gene is controlled according to the density of their population present at that time in their environment. Various activities including development of biofilm formation, bioluminescence production, antibiotic resistance, sporulation, plasmid conjugal transfer, virulence, antibiotic synthesis and secretion of enzyme are well coordinated through cell-to-cell communication commonly known as QS. The phenomenon of QS may take place within a single species of bacteria and even among diverse species. It is also possible to control different processes in host system. In simple ways we can say that QS served a simple indicator of population density or the cell's dispersion rate into the immediate environment. The development of biofilms and QS are closely interconnected mechanisms. During the formation of biofilms a cooperative group behavior of bacteria is involved. The bacterial population becomes embedded in a self-produced complex extracellular matrix. QS mainly activates the dispersion process of biofilm [103]. Generally low molecular weight molecules like acyl homoserine lactones (AHLs), furanosyl borate diesters (AI2), cis-unsaturated fatty acids (DSF family signals) and peptides are considered to be the QS molecules.

3.7. Biofilm Detachment

The life cycle of biofilm includes certain phases (i) Initial adhesion, (ii) microcolony formation, (iii) Maturation of biofilms (iv) finally detachment (Fig. **2**). Bacteria need active mechanisms to leave biofilm and return back to the planktonic (free-living) state. This fourth phase has not been focuses and explained much in the literature. Detachment of biofilm process is also a regulated energetic one where certain proteins, genes are expressed. Boles *et al.* [104] put forward that variants of discovered *P. aeruginosa* display accelerated detachment of biofilm. There is a spontaneous raise of hyper-detaching variants from biofilm at a high frequency, and leads vigorous detachment under unusual biofilm growth conditions. Further Boles *et al.* [105] showed that the detachment mechanism of variants requires the BS RHLs. This has been observed in case of wild type strains of *Pseudomonas* species. Under this condition there is rapid restoration of antibiotic sensitivity to separating bacteria. RHLs attack directly on the biofilm matrix and disrupt the components, possibly incorporating the matrix into micelles. RHLs may disrupt cell surface structures which assist biofilms in adhesion process.

Al-Tahhan *et al.* [44] has shown that RHLs play a role in releasing lipopolysaccharide from *P. aeruginosa*. Other surface appendages may face the same situation. The overall cumulative effect on biofilm and matrix persuade disruption and finally cell detachment. Comparable work from Davey *et al.* [106] suggested the working of similar mechanism in maintaining the fluid channels that surround biofilm structures. Conversely here, RHLs may act on cell–surface rather than cell–cell or cell–matrix interactions. Is has been suggested by Davey *et al.* [106] and Klausen *et al.* [107, 108] that in case of *P. aeruginosa* surface-associated motility and BS production both play crucial role structural development of biofilms. Two separate groups of researchers [106, 109] reported similar observation of mutants of *P. aeruginosa rhlA* forms colony on a flat biofilm. However both of Pang *et al.* [110] disagree with the opinion of Davey *et al.* [106] who documented that BS molecules are essential in the initial phase of microcolonies formation, nevertheless contributes towards maintenance of the channels between the microcolonies once they are formed. The RHLs molecule promotes swarming motility and alters cell surface charge which mediates detachment of biofilm. In short we can claim that RHLs are the compounds that can act against broad spectrum of microbes [47]. Using this approach it is easy therefore very much possible to disrupt the established biofilms. Due to the action of RHLs, several cavities are formed within the centre of biofilm structures. In case of variants, central hollowing pattern that is also observed in aged wild-type biofilms. Similarly biofilm interior detachment has been observed for *S. aureus* [111] and in the oral pathogen like *Actinobacillus actinomycetemcomitans* where detachment is mediated by the enzymatic action of n-acetylglucosaminidase instead of surfactant [112]. This indicates that the central hollowing detachment pattern possibly is common across species, in spite of having working the different operative detachment mechanisms.

4. METHODS INVOLVED IN INVESTIGATING THE BIOFILM FORMED BY MICROORGANISMS

Differences between planktonic and biofilms cell behavior, characteristics and general states within microbial culture are well known and reported in literature. Planktonic (free living cells) behavior of diverse populations of cells has also been studied. Therefore, different methodologies are used in order to analyze their interactions with antimicrobial agents. One typical laboratory method may not be suitable for use to study all types of biofilms. Initially biofilm assays were very analogous to the experimental set up for planktonic cells. The output of this work tried to represent BS as weak compounds in comparison with conventional antimicrobial agents. Soon after, as research progressed it became apparent obvious that these tests were not reflecting the efficacy of BS accurately. Currently experiment set up in situ conditions offer a more representative picture [1]. Various methods suitable for evaluating BSs effects on biofilm disruption along with their advantages and limitations are listed in Table **1**. Most of the methods pre-coat the surface with a known amount of BS overlaid with microbial biofilm [113]. Several methods are currently being used by researchers in order to quantify the growth of bacteria in the presence or absence of various antimicrobial compounds.

4.1. Microtiter Plate Static Biofilm Model

This was the first method specially developed for quantification of biofilm. The attachment of microbes to abiotic surfaces is quantified with the help of this assay. For the first time Christensen *et al.* [114] studied the attachment of *Staphylococcus aureus* a coagulase-negative bacterium to the plastic surface. Since then it has been used regularly to study many other species. Short incubation period (1–2 h), longer incubation period (**≈**20 h) assists the analysis of initial attachment of bacteria to surfaces and biofilm formation respectively. It is a high-throughput technique for understanding the different parameters however less well suitable for biofilms possessing antimicrobial resistance properties. It is a challenging task to visualize the biofilms microscopically since live cells and matrix material both are stained by the dye. Several authors have utilized this assay in different ways and observed some kind of interaction of BS molecules with the microorganisms within the biofilm. Some contributions on this aspect are summarized in Table **2**.

4.2. Tube Assay

This technique was developed by modification of the standard method of Christensen *et al.* [114] to test biofilm production. In

Table 1. Summary for different methods used for studying biofilm formed by the microorganisms.

Name of the Method	Quantitative/Qualitative	Advantage	Limitations
Crystal violet (CV) assay	Quantitative	This method is very inexpensive and accurate results can be obtained by conducting the experiment for several times.	Low reproducibility. Staining of living, dead and biofilm matrix by CV does not provide any information on the actual number of living bacteria and therefore cannot evaluate the anti-biofilm efficacy of antimicrobial agents.
Microtiter plate static biofilm model or Tissue culture plate method (TCP)	Quantitative	It measures rapidly the relative biomass levels. High-throughput, screening for mutants defective in attachment or evaluation of the effects of different treatments on attachment or biofilm formation. Well discrimination between weak and biofilm negative isolates.	Less suitable for studies of biofilm structure, antimicrobial resistance properties.
Flow cell system	Qualitative	Formation of biofilm at the biologically relevant surfaces like host–pathogen inters actions. Therefore, biologically relevant mimic of the *in vivo* situation.	It has limited experimental throughput due to difficulty in collecting biomass of biofilm.
Bioluminescent assay	Quantitative	It is rapid, simple, time saving, more convenient than the microscopic technique and suitable for automation. Bacterial attachment on different irregular surfaces can be studied. Live bacteria- metabolic activity depending on their ATP molecules are detected.	Unable to detect the adherence accurately.
Tube biofilm reactors	Quantitative	Accumulated mass of large biofilms can be collected by scraping from the tubing.	High-throughput analysis cannot be done.
Rotating disk reactors (RDR)		Shear strength formed by the freshwater communities of bacteria on biofilms formation can be analysed.	Analysis of only one strain or mutant at a time per reactor. More number of strains is not possible with this system. Difficulty in sample taking; temperature control conditions are dependent on external devices.
Concentric cylinder reactors (CCR)	Quantitative	Accommodates large numbers of coupons or chips and allows dose–response killing relationships accurately determined from a single biofilm.	
Microscopic techniques	Qualitative	Scanning electron microscopy (SEM) gives high-magnification images of how the single bacteria are located and interact within the biofilm. The initial attachment and dispersion of bacteria on the mineral surfaces. Transmission electron microscopy (TEM), reveal greater detail precise location of the iron precipitation on cell surfaces.	Confocal laser microscopy (CLSM) with restricted magnification, SEM requires dehydration of the samples during preparation.
Air-liquid interface coverslip assay	Quantitative	Straight forward approach for quantifying biofilms on biotic surfaces. It is a robust method and gives same results obtained with other biofilm assays.	It hampers progress of the experiment.
Colony Biofilms	Qualitative	Analysis of bacteria in contact with antimicrobial agents.	Live, non-destructive image of biofilm development cannot be carried out.
Congo red agar method (CRA)	Qualitative	Rapid, sensitive, reproducible. The colonies remain viable on the medium.	Non reliable method, possibility of false positive results.
Safranine (SAF)	Quantitative	It detects both live and dead bacteria, and the biofilm matrix.	Time sensitive, Aspects related with cell viability during different stages of the biofilm formation cannot be studied.
Borosilicate test tubes (TT)	Qualitative	This test correlates well with TCP test but cannot discriminate biofilm formation capacity.	Possibility of false positive and cannot be recommended as general screening test for identification of biofilm-producers.
Rresazurin (RES)	Quantitative	Determination of viable cells depending on the reduction of the non fluorescent RES to the fluorescent resorufin. This proportionally reflects the amount of metabolically active cells.	Time sensitive, Aspects related with cell viability during different stages of the biofilm formation cannot be studied.

Table 2. Summary for the few contributions along with biofilm formation using Microtiter plate static biofilm model.

Organism	Work Contributed Towards	References
Salmonella typhimurium	Determination of antibiotic resistance pattern and biofilm formation.	[115]
Staphylococcus aureus, Staphylococcus epidermidis	Development of new antimicrobial agents against multidrug-resistant infections and biofilm-associated diseases.	[116]
C. krusei, *C. guilliermondii*	First time a comprehensive study regarding adhesion to polystyrene was performed which is significant for species virulence attributes.	[117]
Enterococcus faecalis, *E. faecium*	Investigations for possible associations between virulence profiles and biofilm formation in clinical urinary tract infection (UTI) isolates.	[118]
Staphylococcus aureus	To test the efficacy of octenidine hydrochloride (OH) to inhibit biofilm synthesis and inactivation of fully-formed staphylococcal biofilm on different matrices with and without serum protein.	[119]
Enterobacter cloacae	For determination of the effects of growth medium, temperature, and incubation time on biofilm formation.	[120]
Candida albicans	Monitoring Formation and Antifungal Susceptibility Testing.	[121]
Candida albicans, *C. parapsilosis*, and *C. glabrata*	For enhancement of the detection of drug susceptibility differences among strains. To make it possible for high-throughput screening of antifungal compounds.	[122]
Acinetobacter baumanni (ATCC 19606), *Pseudomonas aeruginosa* (ATCC 27853)	To determine the probable relationship between colonization of different medical devices by various bacteria and the differences in biofilm formation under different conditions.	[123]
Pseudomonas aeruginosa	Formation of biofilm formation.	[113]
Pseudomonas aeruginosa	To test the antimicrobial susceptibility testing.	[124]
Staphylococcus epidermidis	Biofilm production in 96-well plates is affected to low oxygen content and should be considered in the interpretation of experimental work using this biofilm model.	[125]
Rhodococcus erythropolis	First report on use of Microtitre assay for the production and utilization of Hydroxy-biphenyl.	[126]
Escherichia coli	The attachment promotion and disruption by indolic compounds.	[127, 128]
Staphylococcus aureus	Identification of mutants deficient in surface attachment.	[129]
Staphylococcus aureus	Effects of surface properties on bacterial attachment to the surfaces.	[130]
Staphylococcus epidermidis	The attachment promotion and disruption by indolic compounds for and evaluating the role of salt and ethanol stress on biofilm formation.	[131]
Bacillus subtilis	Identification of mutants deficient in surface attachment.	[132]
Vibrio cholerae	Identification of mutants deficient in surface attachment.	[133]
Pseudomonas fluorescens	Effect of nutrients in the medium on attachment to polyvinyl chloride.	[134]
Pseudomonas fluorescens, Pseudomonas aeruginosa	Identification of genetic requirements involved in surface attachment.	[134, 135]

summary 2 ml of trypticase soy broth (TSB; Difco Laboratories, Detroit, MI, USA) in 12 x 75 mm borosilicate test tubes (Corning, Tewksbury, MA, USA) were inoculated with a loopful of microorganisms from overnight culture plates and incubated for 48 hours at 37°C, after which the contents were decanted and washed with PBS (pH 7.3) and left to dry at room temperature. Afterward, the tubes were stained with 4% solution of crystal violet (Merck, Darmstadt, Germany). Each tube was then gently rotated to ensure uniform staining and then the contents were gently decanted. The tubes were placed upside down to drain and then observed for biofilm formation which was considered positive when a visible film lined the wall and bottom of the tubes. Ring formation at the liquid interface was not regarded as indicative of biofilm formation. The results were scored visually as 0-absent, 1-weak, 2-moderate, 3-strong.

4.3. Congo Red Assay (CRA) Method

This technique is mainly based on the morphological cultural characteristic of biofilm-forming bacteria on medium supplemented with Congo red. The medium preparation is brain heart infusion broth (BHI) containing 37 g/l, sucrose 50 g/l, agar 10 g/l and Congo red 0.8 g/l. Medium is sterilized (121°C for 15 minutes) after add-

ing all the components without Congo red stain (used as a strong aqueous solution) which was separated from the rest of the medium components and supplemented to the agar when the temperature reached 55°C. After preparation of agar plates, they are inoculated and incubated for 24 hrs at 37°C. Results are interpreted based on the biofilm producers giving black colour colonies with a dry crystalline consistency where red colour colonies developed as the organisms was taken to indicate biofilm production whereas non-biofilm-producing strains develop red colonies [136]. There is an interaction between Congo red dye and certain polysaccharides that can form colored complexes which is helpful for phenotypic characterization of biofilm production. Freeman *et al.* [137] proposed that biofilm producers form black colonies on Congo red agar plates, whereas non-producers form red colonies. The Congo red dye directly interacts with certain polysaccharides, forming colored complexes [138]. A five-color reference scale can be used to determine color variations by the colonies accurately. Those isolates showing two tones like black, bright black (BB) and dry-opaque black (OB), are classified as biofilm producers. The others showing red, pink and bordeaux colonies are classified as negative one. In few cases, the colour variations are also seen like red and bordeaux subcolonies in the center of black colonies (BB) after 48 h of culture. These colonies are removed and can be subcultured for isolation of the producing and non-producing variants.

4.4. Flow Cell System

This system has a chamber where biofilm forming bacteria are grown and are usually attached to a cover slip overlying the chamber. Some of the important specifications need to be followed while working with this system for growth of biofilms are collection of fresh, optimized biomass, tube biofilms and spin-disc reactors. The main advantage of working with this system is biofilms can be visualized at real-time. Small continuous-flow systems is provided with a viewing port that permits direct observation of the biofilm without any troublesome to the community. Fresh medium is entered in the system, passed through the cell, and waste is collected. There are no provision that medium is not recycled through the flow cell. A number of descriptions of flow cell and related techniques have been reported. However, these experiments are not suitable for high-throughput analysis. Pamp and Tim Tolker-Nielsen, [109] used flow cell technology and enhanced confocal laser scanning microscopy and suggested that the BSs produced by *P. aeruginosa* play supplementary roles in development of biofilm. The authors have presented genetic evidence for endorsing the microcolony formation in the initial phase. The later phase, migration-dependent structural development is also resulted in this complex reaction cycle. The *rhlA* mutants of *P. aeruginosa* deficient in synthesis of BSs, cannot accomplish the task of microcolonies formation in the initial phase. Authors proved the concepts by using two and three color-coded mixed-strains of *P. aeruginosa* for the experimental work.

4.5. Tube Biofilm Reactors

This system works similar to flow cells reactor where biofilms that are build up under flow can be investigated. The distinction between these two methodologies is that in the tube reactor where biofilms are grown on the interior surface of silicone tubing, instead of on a cover slip attached to a chamber. Huge growth of biofilms can be observed on the tube biofilm that can enables the accumulation of a large biofilm mass which can be collected effortlessly by just scraping from the tubing. An important prospective of this system is allowing estimation of the effect of antibacterial agents on biofilms through counting of colony forming units. This purpose can be achieved by analysis before and after exposure to treatment with the tested agent. In addition to these, biofilm mass can be studied for biochemical and gene expression purposes. Another most important aspect that is phenotypic diversification also can be analyzed [139].

4.6. Rotating Disk Reactors (RDR)

In this system biofilms are grown under shear stress and can be examined for the efficacy of biocide. The RDR system consists of a circular disk which is made in such a way that it permits the integration of removable coupons or chips which can be flushed with surface of the disk. Zelver *et al.* [140]; Ramey and Parsek, [141] suggested that the disks can be made from a variety of materials depending upon the subject of matter under studies. There is a star-head magnet where the disk is attached and this is placed in a 1 L glass side-arm reactor vessel. This reactor vessel is placed on the top of magnetic stirrer where adjustable rotational speed is provided through the generation of a liquid shear force across the surface of the disk. Different shear strength can be produced through the speed of rotation and as well as the diameter of the cylinders. Another one important feature of this system is maintaining the flow of medium through the reactor regulated by pump. Reservoir system is available from where medium is pumped out and dripped down slowly into the reactor vessel. It is very much possible to remove coupons from the RDR so that growth or viability can be monitored aseptically and bacteria can be plated for relative viable cell counts at various intervals of growth. Therapeutic efficacy evaluation can be accomplished with bioactive molecules / chemicals by introducing them in the reaction vessel or distributing by the continuous flow in the medium.

4.7. Concentric Cylinder Reactors (CCR)

This system is provided with two concentric cylinders. Sample under test is applied on the removable slides which are attached with the internal walls of the outer static cylinder. Medium is fed through the inter cylinder space and then inner cylinder starts rotating. After inoculation, biofilms starts developing on the walls of the cylinder [142]. This methodology assists the role of hydrodynamic conditions on biofilms and different shear stress conditions can be observed. At the same time is also allows to test different shear and periodical sampling can be easily carried out. The problem working with this system is that only per experiment only one surface material is tested. Another issue is that the system is non availability of enough sampling surface area and difficulty in sampling process.

4.8. Microscopic Techniques

Due to the rich emergence of several techniques like phase contrast microscopy (PCM), fluorescence imaging microscopy (FIM), scanning electron microscopy (SEM), transmission electron microscopy (TEM), confocal laser microscopy (CLSM), atomic force microscope (AFM) reasonably superior level of biofilms have been explored the microscopic world. High quality resolution of microscopic biofilms has been achieved and advancement towards in these areas is also opening wide gates to understand biofilms thoroughly. Image analysis of Cellular and sub-cellular components had always tried to answer my biological queries. Initially, electron microscopy was the choice to observe microbial biofilms under high resolution. Three-dimensional structure of biofilms has been successively revealed by several researchers. Few researchers [143-145], demonstrated microscopic biofilm formation. The method can be performed by slight variation in the assay described by Jeong and Frank, [143] and Blackman and Frank, [144]. The experimental set up use stainless steel surfaces and PVC. Both materials need proper cleaning treatments. Stainless still surfaces are cleaned and placed in 12- by 1.5-cm tubes for sterilization by autoclaving. Sterilized tube with broth (10 ml to immerse completely both surfaces) and inoculated with culture. Further slides are removed from the growth medium and washed with distilled water to remove any loosely attached cells, and are fixed with 95% ethanol for 45 s. Djordjevic *et al.* [145] used to assay for formation and analysis of biofilm from *Listeria monocytogenes.* Then biofilm formation can be observed under the microscope.

4.9. Colony Biofilms

Generally a colony of biofilm is developed on a semi-permeable polycarbonate filter (GE) which is placed on a suitable medium. Here filter is provided as a surface so that transfer of the surface-grown biofilm is expedient from one medium source to another one. It is possible to observe the growth of biofilm formation at the air–surface interface. In this case there is no absolute necessity of submersing the biofilm in liquid medium. Within short time span, depending upon the kind of growth, biofilm can form huge colonization. Experimental work demonstrated by Borriello *et al.* [146], Werner, *et al.* [147]; Stewart *et al.* [148] showed that after 48 h of growth colony biofilm formed by *P. aeruginosa*, is approximately 150–300 µm thick whereas *S. epidermidis* produces about 100 µm thick. It is important to note that colony biofilms exhibit a typical stratified profile comparable to flow cell biofilms at an anaerobic conditions. The interior colony biofilm is dominated by lysed cells and at the colony–air interface where more oxygen is available, the exponential phase cells are found [147].

5. INDUSTRIAL PROSPECTUS OF BIOSURFACTANT MOLECULES IN BIOFILM DISRUPTION:

Bio-medical device always need always be free from organisms. Many different cleaning compounds available today in the market may not necessarily be effective as biofilm disruptors [149]. It is very important to note the comment given by Fracchia *et al.* [150] excessive disinfection treatment may yield less efficiency. Most of the devices which are used in hospitals are disposable, i.e. there are no issues of development of biofilms on those devices. In case of some reusable medical devices like surgical instruments, endoscopes, biofilms have no possibility to develop when the devices are sterilized / disinfected after their usage. One of the possible opportunities to develop biofilms is though longer contact of organisms on the medical device which can get attached irreversibly [151]. There is mounting interest in the potential of BS molecule in medically-related applications including the formation and disruption of bacterial biofilm [9-11]. Some antibiofilm activity of BS produced by different microorganisms against pathogenic microorganisms is given in the summary of Table **3**.

Among all reported BS, rhanmonolipid types BS have shown great potential applications. RHLs are reported involved in maintaining the structural characteristics of biofilm and have accepted useful application to prevent biofilm formation on surfaces of various biomedical devices [157]. Other issue like diffusion of nutrients and gases to the cells within the biofilm needs to be considered. Like RHLs, sophorolipid type glycolipids also wok as biofilm disruptors at different concentrations. Studies conducted by D´ıaz *et al.* [23] showed the inhibition of *Cupriavidus necator* ATCC 17699, Gram positive *B. subtilis* BBK006 by sophorolipids (concentrations of 5% v/v) possessing bactericidal effect. At the same concentration single or mixture of colonies are also disrupted. The authors suggested the use of SPs as adjuvants to other antimicrobial against for disruption of biofilm. Two well know BS, viz., surfactin (*B. subtilis*) and RHLs (*P. aeruginosa*) lowers the adhesion and disrupts biofilms of food-borne pathogenic bacteria [165]. Their studies included *S. aureus*, *Listeria monocytogenes* and *Salmonella Enteritidis* on polystyrene surface. Both BS works effectively in controlling the attachment as well as disruption of individual and mixed culture biofilms of the food-borne pathogens. Biofilms formed by candida spp. are one of the most dangerous one. Gomes *et al.* [165] examined the effect of BS isolated from *Pseudomonas aeruginosa* DSVP20 for disruption of *C. albicans* biofilm. Time to time we have seen that microscopic analyses by using SEM and CLSM gives a very clear cut visualization of disruptive effect of BS on biofilms thus proved to be effectiveness of BS for therapeutic purposes. Some of the commercial BS based formulations that are used to inhibit biofilm disruption are listed in the Table **4**.

Table 3. Antibiofilm activity of biosurfactant produced by different microorganisms against pathogenic microorganisms.

Name of the Organism Producing Biosurfactant	Name / Type of Biosurfactant Produces	Antibiofilm Activity Against	References
Lactobacillus brevis CV8LAC	Not mentioned	Initial deposition of *Candida albicans* on medical devices	[152]
Pseudomonas aeruginosa MA01	Rhamnolipid	*S. aureus, B. subtilis, P. aeruginosa, K. pneumoniae*	[153]
Candida bombicola ATCC 22214	Sophorolipids	*Staphylococcus aureus* ATCC 9144, *Cupriavidus necator* ATCC 17699 and *Bacillus subtilis*	[23]
Lactobacillus jensenii Lactobacillus rhamnosus	Not mentioned	*Acinetobacter baumannii, Escherichia coli, Staphylococcus aureus*	[58]
Coral Associated Bacteria (CAB)	Lipopeptide	*Pseudomonas aeruginosa* ATCC10145	[154]
Lysinibacillus fusiformis S9.	Glycolipid	*Escherichia coli* and *Streptococcus mutans*	[62]
Bacillus sp. strain SW9	Lipopeptide	Different strains of bacteria	[155]
Bacillus tequilensis	Lipopeptide	*Escherichia coli*, *Streptococcus mutans*	[156]
Robinia pseudocacis/ Nerium oleander	Not mentioned	*Candida albicans*	[157]
Pseudomonas aeruginosa	Glycolipid	*Yarrowia sp.*	[50]
Candida lypolytica	Rufisan	*Streptococcus sp.*	[158]
Serratia marcescens	Glycolipid	*Candida albicans* and *Pseudomonas aeruginosa* and the marine biofouling bacterium *Bacillus pumilus*	[65]
Candida sphaerica	Mixed BS- Lunasan	*Pseudomonas aeruginosa, S.agalactae*	[159]
Bacillus strain	Lipopeptide	Different Gram positive and negative bacteria	[160]

(Table 3) Contd….

Name of the Organism Producing Biosurfactant	Name / Type of Biosurfactant Produces	Antibiofilm Activity Against	References
Actinobacterium Brevibacterium casei MSA19	Glycolipid	*Candida albicans* FC1, *Escherichia coli* MTCC 2939, *Proteus mirabilis* PC1, *Hemolytic Streptococcus* PC2, *Pseudomonas aeruginosa* MTCC 2453, *Klebsiella pneumoniae* PC3, *Vibrio parahaemolyticus* MTCC 451, Vibrio harveyi MTCC 3438, *Vibrio alginolyticus* MTCC 4439, *Vibrio alcaligenes* MTCC 4442, *Vibrio vulnificus* MTCC 1145, *Thalassomona* ssp. MMD12, *Alteromonas sp*. MMD16, *Pseudoalteromonas* sp. MMD18, *Pseudoalteromonas sp*. MMD19, *Ruegeria* sp. MMD27	[64]
Pseudomonas aeruginosa	Glycolipid	*Bacillus pumilus*	[161]
Lactobacillus paracasei A20	Not mentioned	Different strains of bacteria, yeast, filamentous fungi	[162]
Arctic bacterium *Pseudomonas fluorescens* BD5	Lipopeptide Pseudofactin II	*Escherichia coli*, *Enterococcus faecalis*, *Proteus mirabilis*, *candida* spp.	[163]
Bacillus subtilis, *B. licheniformis*	Lipopeptide-Fengycin	*Escherichia coli*, *S. entrica*	[164]
Pseudomonas aeruginosa DS10-129	Rhamnolipid	*Staphylococcus epidermidis* GB 9/6, *Strep. salivarius* GB 24/9, *Staphylococcus aureus* GB 2/1 and *C. tropicalis* GB 9/9	[9]
Pseudomonas putida strain PCL1445	Lipopeptide putisolvin I & II	Different *Pseudomonas* strains	[48]
Lactococcus lactis / *Streptococcus thermophilus*	Mixed BS- Lunasan	*Staphylococcus, Streptococcus, Rothia, Candida sp.*	[60]
Bacillus subtilis	Lipopeptide	*S. entrica*	[47]
Lactobacillus fermentum B54	Lipopeptide	Urophathogens	[57]
L. acidophilus RC14 and *L. fermentum* B54 *L. casei* subsp. *rhamnosus* 36 & ATCC 7469	Protein rich BS, High polysaccharide and phosphate contents.	Uropathogenic *Enterococcus faecalis*	[54]

Table 4. Commercial available biosurfactant based formulation to inhibit biofilm disruption.

Source Organism	Commercial Available Biosurfactant	Industrial Prospectus as Biofilm Disruption Against	References
Lipopeptide based biosurfactants			
Bacillus spp.	Polymyxin	*Pseudonomas aeruginosa*	[166]
	Lipopeptide – Polymyxins Colistin (Polymyxins E)	Gram negative bacteria	[167]
	Neosporin, Polymyxin B (Supplied as polymyxin B sulphate) Polymyxin + Trimethoprim neomycin + Bacitracin (Triple antibiotic ointment	Wide range of bacterial population	[79]
	Polymyxin D1 Polymyxin D1 + Fusaricidin + Surfactin	Mixed biofilm population	[168]

(Table 4) Contd….

Source Organism	Commercial Available Biosurfactant	Industrial Prospectus as Biofilm Disruption Against	References
Fengycin type biosurfactants			
Pseudomonas putida	Cyclic lipopeptide Putisolvin I & Putisolvin II	Dispersal agent- against other Pseudomonas strains	[48]
Bacillus subtilis & *B. licheniformis*	Fengycin like Lipopeptide	Gram positive bacteria - *S. aureus*	[169]
	Fengycin like Lipopeptide	Gram negative bacteria - *E. coli*	[14]
Pseudofactin type biosurfactants			
Pseudomonas fluores-cens	Cyclic lipodepsipeptide Pseudofactin	*E. coli, S. epidermidis, E. faecalis, E. hirae, Proteus mirabilis*	[163]
Surfactin type biosurfactants			
Bacillus subtilis	Surfactin	Salmonella spp.	[47]
Bacillus cereus	Lipopeptide complexes	Disruption of biofilm	[156]
Surfactant complex			
Bacillus licheniformis	Lipopeptides (In combination with other disruptors/inhibitors)	Uropathogen *E. coli*	[164]
Paenibacillus polymyxa	Polymyxin D1+ Fusaricidin B + Surfactin	Disruption of biofilm - *E. coli, S. aureus*	[168-170]
Glycolipid type biosurfactants			
Pseudomonas aerugi-nosa	Rhamnolipid	*Bordetella bronchiseptica*	[171]
Pseudomonas aerugi-nosa	Rhanmonolipid biofungicin (Product developed by Jeneil Biosurfactant cor-poration)	Prevent crop attack by pathogenic fungi	[172]
Sophorolipid type biosurfactants			
Candida sphaerica	Lunasan	*Pseudonomas aeruginosa, Streptococcus sanguis, S. agalacitae,*	[159]
Candida lypolytica	Rufisan	*S. aureus , Streptococcus sanguis, S. mutans*	[158]
Candida spp.	Sophorolipid	*E. coli*	[173]
Candida bombicola	Sophorolipid	*Vibrio cholerae*	[174]

6. FUTURE PROSPECTUS

Broad views on research areas including food, water and environment, clinical, pharmaceutical microbiology are assisting to explore more on biofilm perceptions. Knowledge on expression of particular genes in organisms associated with biofilm formation is also playing a supporting role to remediate biofilm colonization of several biomedical devices. One of the smart ways to handle the biofilm issues are exploring solutions to the emergence of resistance of biofilm towards antimicrobial compounds and in development of chronic diseases. Industries need to lift these approaches in order to develop newer paths to prevent formation and control of biofilms. Noteworthy accomplishments can be achieved by acquiring more comprehensive knowledge on how biofilm phenotypes are different from the planktonic phenotype. Wide ranges of multi-resistant bacteria genera or fungi have been found to be inhibited through promising activity of BS molecules. Therefore, opportunities are offered by BS based pharmaceutical formulations for substantiate future medicine. Till today, lipopeptide and glycolipid based BS has shown exciting applications in large scale commercial grade products. Some of the newly reported BS like Lunasan, Rufisan can accelerate the development of new formulations to fulfill the specific requirement for the development of effective formulations. We need to explore more innovative BS molecules for antibiofilm activity and can be tried out for synergistic activity with other antibiofilm agents for preparation of different but effective combinations.

CONCLUSION

Due to public health related issues the terms biofilms, BS, antimicrobial agents, antibiotic resistance are very significant. As prevention is always better than cure, we need to look for effective molecules to tackle the issue of biofilms caused by pathogens. More effective strategies for controlling biofilms formation and effective treatment would unquestionably offer us healthier environment and may positively contribute towards the reduction of antimicrobial resistance development through acting as adjuvants to may antibiotics reducing their effective doses and accelerating microbial elimi-

nation. Different methodologies involved in the cultivation of microorganisms would assist the researchers to choose suitable method required for intended purpose. High through put analyses can be achieved through microtitre and peg biofilm techniques. The flow cell device is very much helpful to observe biofilm formation microscopically. High possibility is expected to recover the biofilm for downstream biochemical analyses from the tube biofilm technique. We can assess the biofilm susceptibility to antimicrobials with peg biofilms, colony biofilms and the rotating disk, and concentric cylinder reactors. The current research is being focused on preventing the surface colonization rather than overall bacterial robustness. Development of more effective antibiofilm agents sounds to be more promising technology.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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