Microbial biofilms: biosurfactants as antibiofilm agents

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Abstract

Current microbial inhibition strategies based on planktonic bacterial physiology have been known to have limited efficacy on the growth of biofilms communities. This problem can be exacerbated by the emergence of increasingly resistant clinical strains. All aspects of biofilm measurement, monitoring, dispersal, control and inhibition are becoming issues of increasing importance. Biosurfactants have merited renewed interest in both clinical and hygienic sectors due to their potential to disperse microbial biofilms in addition to many other advantages. The dispersal properties of biosurfactants have been shown to rival that of conventional inhibitory agents against bacterial and yeasts biofilms. This makes them suitable candidates for use in new generations of microbial dispersal agents and for use as adjuvants for existing microbial suppression or eradication strategies. In this review we explore aspects of biofilms characteristics and examine the contribution of biologically derived surface-active agents (biosurfactants) to the disruption or inhibition of microbial biofilms.

Introduction

Microorganisms in general gravitate towards solid surfaces forming biofilms as a strategy to protect themselves from environmental challenges. Such deposition and subsequent biofilm formation is a phenomenon that happens naturally and is usually part of the microorganisms' strategy to protect themselves from external toxic factors (Pereira et al. 2007). They have the ability to sense their own cell density, communicate and behave as a population through cell to cell signalling, a phenomenon known as quorum-sensing (Liu et al. 2012). This behaviour has been documented for some time in microbial biofilm formations (Davies 2003) and is dependent on the nutritional/environment and the maturation stage of development of the microorganisms. Microbial biofilms represent a distinct bacterial physiology characterised by a multicellular phenotype that is fundamentally different from planktonic bacteria. They have been implicated in chronic and recalcitrant healthcare associated infections (Dowd et al. 2008), the dissemination of community acquired diseases (Stewart et al. 2012), effective hygienic processing, increased failure rate of anti-infective therapy (Bueno, 2014) and marine water and electronics environments (Lourenco et al. 2011). Biofilms that are composed of one species are relatively rare in the majority of the natural environment; rather microorganisms tend to be found in complex multispecies communities associated with surfaces (Stoodley et al. 2002).

Until recently the differences between planktonic and biofilm physiologies seemed inconsequential. Standard bacterial inhibition tests were almost exclusively based on planktonic bacterial physiology and not the biofilm physiology even though these conditions were not readily observed in the natural environment. The standard planktonic bacterial physiology is typically exemplified by free-living single bacteria with optimal nutrition, gas exchange and agitation (typically 250rpm) (Bueno 2014; Kotulova and Slobodnikova, 2010).

In contrast, the biofilm physiology has multicellular differentiation, multicellular communication, internal architecture and rudimentary fluid transport systems (Girard et al. 2010; Leis et al. 2005). More importantly for in-vitro testing procedures, biofilms have variable levels of nutrients, gas exchange, little or no agitation and therefore slower growth.

This difference in bacterial physiology can be critical especially in clinical situations where there is a higher production of virulence factors in pathogens such as *Pseudomonas aeruginosa* (Croda-García et al. 2011). In the biofilm physiology these pathognes can be one to three orders of magnitude more resistant to dispersal/inhibition by conventional chemotherapy than their planktonic counterparts of the same species (Girard et al. 2010; Olson et al. 2002; Sepandj et al. 2004). This has been demonstrated in recent experiments on biofilm formation during peritoneal dialysis, where all the antibiotics tested were effective in laboratory MIC tests but (with the exception of gentamicin) lost their efficacy against Staphylococcus aureus biofilms (Girard et al. 2010). Globally, methicillin resistant *Staphylococcus aureus* (MRSA) is a serious problem due to limited efficacy of antibiotic options, hospital hygiene and the resistance of biofilm associated clinical strains (Samadi et al. 2012). Some biofilms also undergo phenotypic change as a result of chemotherapy resulting in increased resistance.

New insights into biofilm physiology have now enabled researchers to design more effective bacterial inhibition/dispersal strategies. There are two main inhibitory strategies, based on the formulation of new antibiofilm compounds and the construction of biofilm resistant surfaces (Villa and Cappitelli, 2013).

Some of the most promising candidates for the inhibition of bacterial biofilms have come from biological surface-active agents (biosurfactants) (Kiran et al. 2010; Pradhan et al. 2013). Many of these have been reported to have anti-adhesive, antimicrobial and biofilm disruption properties (Rodrigues et al., 2006a,b,c; Rodrigues et al., 2007). Enzymatically synthesized surfactants such as lauryl glucose have also been reported to be effective against fungal and bacterial biofilms (Dusane et al. 2010).

Biosurfactants are a heterogeneous group of amphiphilic compounds produced mainly by microorganisms that accumulate at the interface between liquid phases and therefore reduce surface and interfacial tension. They have been recognised for some time in a diverse array of potential applications in a wide range of industries including agriculture, food, cosmetic, pharmaceutical and petroleum industries (Banat et al. 2010). The surface

and interfacial tension reducing properties of surfactants provide excellent detergency, emulsification, foaming and dispersing traits, making them some of the most versatile products in chemical processes (Desai and Banat 1997). They are highly sought after molecules due to their specificity, low toxicity, high biodegradability, widespread applicability and effectiveness at extremes of pH and temperature (Muthusamy et al. 2008).

Several strands of research have demonstrated that under certain testing conditions biosurfactants can be more effective than many traditional biofilm inhibition and or disruption strategies (Epstein et al. 2011). There have been many reviews of biosurfactants and their potential applications in environmental and biomedical related areas (Neu 1996; Banat et al. 2010; Banat et al. 2000). There has been however renewed interest in biosurfactants in relation to healthcare associated infections (Krasowska 2010). In addition, the rapid pace of advances in biofilm inhibition, control/disruption and the emergence of biofilms as potential reservoirs for the dissemination of disease have necessitated a review of the current state of the art on biofilms measurements and potentially effective biosurfactants against microbial biofilms.

To our knowledge the area of biofilms and role of biosurfactants within is becoming an increasingly important topic of research yet has not been the subject of a review article. In this review therefore we examine biofilms characteristics, monitoring and quantification and the main classes of current biosurfactants in use, their contribution to the dispersal or inhibition of biofilms, their scope and efficiency, quantification of this dispersal/inhibition and the sources and limitations of their uses.

The nature and functions of biosurfactants

Biosurfactants are amphiphilic compounds of biological origin containing a hydrophilic region (polar or non-polar) and a hydrophobic region (lipid or fatty acid). The hydrophilic group is the base of the International Union of Pure & Applied Chemistry nomenclature i.e. those biosurfactants containing rhamnose are described as rhamnolipids; while those containing sophorose are sophorolipids and those generally containing a carbohydrate moiety including

the previously mentioned two types are described as glycolipids. Other lipopeptide biosurfactants contain a lipophilic hydrocarbon chain described as hydrophobic and a polar or hydrophilic part which is usually composed of a string of amino acids.

Function

Biosurfactants have been identified in many biological processes as the components of cellular metabolism, motion and defence. They are found in great abundance in bacteria, in biofilms, as quorum sensing molecules, lubricants, promoting the uptake of poorly soluble substrates, as immune modulators, virulence factors, secondary metabolites and antimicrobial compounds (Fracchia et al. 2012). In a review by Neu (1996) it has also been proposed that biosurfactants act as important molecules for interfacial processes, conditioning the microbial cell surface, interfaces and surfaces with which the microorganisms interact. These biosurfactants can be found in greater concentrations in the layers of cells associated with movement and hydration although they can have an intracellular location.

Biosurfactants' also have important roles in the dissolution and accessibility of oil molecules especially for oil degrading microorganisms; adhesion to hydrocarbons as a result of the emulsification of water-insoluble substrate compounds; the de-adhesion from interfaces; facilitating the in gliding of bacteria through wetting interfaces. Such surface active molecules can also have a role in enhancing the interaction between microorganisms and all the natural organic hydrophobic compounds interfaces including plant and animal derived polymeric compounds and microbial exopolysaccharides (Neu 1996). The role of bacterial biosurfactants has been extensively studied in *Pseudomonas* where they are known to promote colonisation and migration-dependent structural development (Pamp and Tolker-Nielsen 2007).

Other roles for biosurfactants including biocidal activity have been reported. This is mainly related to the effects of the lipidic moiety against eucaryotic cells. This has also been reported to lead to toxicity, lysis, pyrogenicity, mitogenicity and immunogenicity among other

effects (Wicken and Knox 1980). Lysis of red blood cells has been used as a selection criterion for microorganisms producing biosurfactants (Satpute et al. 2009). Finally human derived biosurfactants have recently received increased attention because of their role in immunity and defence (Gakhar et al. 2010).

Measurements of biosurfactants physical properties

There are many methods employed to test physico-chemical properties of biosurfactants. These are very important for base line comparisons. The standard tests are based on the physical properties of biosurfactants such as measurement of reduced surface tension. Other tests measure the critical micelle concentration (CMC) which is the concentration of surfactants above which micelle formation occurs. The CMC for example of sodium dodecyl sulphate in water (with no other additives or salts) at 25°C and, atmospheric pressure, is 8x10-3mol/l. The emulsification index (E24 or EI24) is another method used to characterize a biosurfactants' ability to form a stable emulsion with a hydrophobic phase. The hydrophilic phase in this instance is usually water which, can be mixed with Kerosene and the biosurfactant, shaken vigorously and allowed to stand for 24 hours. The percentage emulsion of the water solution in kerosene is reported as the E24 or EI24 (Desai and Banat 1997). Other characterisation methods in use are the oscillating jets and the maximum bubble pressures measured in the presence of the surface active compounds.

Conditions for monitoring biofilm formation

There is no standard laboratory method for quantifying biofilms though there are preferred methods.. In the past, planktonic bacterial inhibition assays have had to have strictly defined experimental criteria in order to reduce variation in results and increase confidence in antibiotic comparisons. However, these tests do not adequately represent different bacterial growth physiologies such as that in biofilms. The first biofilm tests were very similar to these planktonic experiments and created the impression that biosurfactants were weak counterparts of conventional inhibitory agents. Later, research into biofilm inhibition showed

that these tests did not give an accurate reflection of the efficacy of biosurfactants. Today's biosurfactant tests are more accurate and try to represent the in-situ environment as much as possible. Many of these tests are based on pre-coating a surface with a known amount of biosurfactant overlaid with microbial biofilm (O'Toole 2011). This can be alternated with overlaying pre-existing biofilms with the test substance.

Since the biofilm physiology is distinct from the planktonic physiology, biofilm experimental conditions have had to be adjusted accordingly. In terms of temperature the biofilm cultivation is carrier out at the optimal temperature for biofilm growth of a particular species which may not be the same as the optimal temperature for planktonic growth, this could mean that biofilm cultivation may be at 20°C (even for clinical specimens) whilst others may be at 10°C in the cases of some environmental biofilms (Quinn et al. 2012).

In terms of nutrition, it is common practice for biofilms to be cultivated in a dilution of the media that is used for planktonic cultivation, this is usually ½ to 1/5th of standard concentrations reflecting the sub-optimal conditions of biofilm growth, however this practice is not universally applicable (Stepanovic et al. 2004).

Since biofilms also grow slower that optimised planktonic conditions, the typical cultivation period for biofilms can vary from 4 hours to 3-4 days or even 7-10 days in the case of slower growing environmental biofilms (Quinn et al. 2012; Stepanovic et al. 2007). Agitation considerations are equally important. In the earliest biofilm growth assays it was thought that environments of high sheer stress were necessary. However more recent research has shown that environments of high agitation are not necessary for all biofilm growth and these growth conditions can be considered strain specific. Rather biofilm tests are typically conducted in almost static environments or environments of minimum perturbation (O'Toole 2011; Stepanovic et al. 2007).

The standard inoculation density of microorganisms also differs greatly from standard planktonic tests. For planktonic MIC tests organisms are seeded at a density of $1x10^6$ /ml of fresh cells taken from the logarithmic stage of growth. In biofilm cultivation seeding densities are typically a 1/100 dilution of a stationary phase culture (McLaughlin and Hoogewerf 2006;

Quinn et al. 2012). Some researchers use biofilm induction agents such as high glucose or alcohol to aid biofilm formation but these may add unknown variables to the assay making the final biofilm data difficult to interpret.

Recently, Lourenco and co-workers (2014) published the results of an initiative to establish “minimum information about a biofilm experiments” (MIABiE) which is a project partly funded by EU grants to find a scientifically adequate procedures to document biofilm-related data. They asserted that this could be achieved through ensuring a set of minimum information that should be reported to guarantee the independent verification and interpretation of experimental results in a way that would allow their integration with biofilm related information generated by other fields.

Surfaces for the quantification of biofilm growth

The physiochemical properties of substrates used for biosurfactant evaluations can affect the nature of biofilm adhesion, the subsequent biofilm architecture in the case of monocultures or the selection of the microbial species which colonise in the case of mixed and environmental biofilms. Biofilms also express different repertoires of proteins or adhesion characteristics depending on the surface characteristics of the substrate they are attached to (Stoodley et al. 2002). Hence the choice of surface for biofilm cultivation is very important and must be taken into account even when comparing the results of inhibitory tests. .

The different surfaces used in biofilm tests range from animate/inanimate, rough/smooth, hydrophobic/ hydrophilic and liquid/air/liquid etc. Laboratory cultivation of biofilms can be conducted on many surfaces including glass, plastic, metal, silicone and tissue models (O'Toole 2011). In more comprehensive assessments of the inhibition potential biosurfactants can be applied to a broad range of surfaces especially in clinical environments. Research into the efficacy of Pseudofactin II (a newly characterised biosurfactant) used many different surfaces such as glass, polystyrene and silicone to cultivate biofilms in combination with different bacterial strains in order to demonstrate its

wide efficacy (Janek et al. 2010 & 2012). In other research on biofilms of *Salmonella*, investigators used PVC and silicone (urethral catheters) as biofilm substrates to demonstrate the applicability of biosurfactants in the reduction of biofilm formation/attachment (Mireles et al. 2001).

Quantification of biofilm inhibition/dispersal

The Calgary Biofilm Device

One of the first devices employed to measure biofilm inhibition/dispersal was the Calgary biofilm device (CBD) (Olson et al. 2002). This technique is widely used in flow tests for microbial biofilms (Rivardo et al. 2009; Girard et al. 2010; Rivardo et al. 2011). The cultivation chamber consists of a 96-well plate together with a lid that contains 96 peg projections (Figure 1). These pegs provide a maximum surface area for the growth of biofilms. The CBD has a typical seeding density of $1x10^4$ to $1x10^6$ bacteria per well or McFarland standard 1, a cultivation speed of ≥10rpm and an incubation period of 4-24 h depending on species and conditions (Girard et al. 2010).

Microbial biofilms are cultivated on test pegs projecting into a growth media, removed after a given time, washed and then inserted into wells containing an inhibitory/test substance. Mature biofilms can be subsequently detached from the pegs by ultrasonic treatment. The detached microbes can be enumerated by standard cultivation techniques or quantified by measuring their optical density at 650nm. The amount of bacterial inhibition of the biofilms is referred to as the minimal biofilm eradication concentration (MBEC). The MBEC represents the lowest dilution of inhibitory substance. If cultivation conditions require a greater circulation of media the lid of this plate can be modified to accommodate 12 media channels into which the 96 pins are extended.. In this manner 96 pins can be simultaneously exposed to a given culture (Ceri et al 1999)

Although the CBD was a welcome departure from planktonic based testing regimes and a step towards a more accurate portrayal of biofilm physiology; the method still relied on the final detection of viable planktonic microorganisms rather than directly measuring the

whole biofilm biomass. It also assumes that bacteria from viable biofilms can immediately rejuvenate on agar or directly into culture broth. This is an important point in biofilm physiology since studies on the resuscitation of bacterial cells have shown that some microorganisms may still be viable in the biofilm but not immediately cultivatable especially after prolonged chemotherapy (Rollet et al. 2009). This is also important when considering the negative impacts of the selective pressure of chemotherapy on biofilm forming pathogens. In some cases it has been shown that severe chemotherapy can induce a viable but dormant pathogen that can resuscitate in more favourable conditions to contribute to the chronic character of a biofilm infection (Zhang 2014).

Finally, the CBD measures the amount of cells in a biofilm and not the biofilm biomass i.e. the biofilm + extra polymeric substances (EPS). However biofilm substances that are not cells can constitute a significant proportion of biofilms (Decho 2013).

Biofilm growth within flow-through devices

Biofilms can be analysed under flow conditions by a variety of methods including the CBD. However another flow system currently used to test biofilms is the BioFlux 200 system (Fluxion Biosciences Inc., South San Francisco, CA) (Benoit et al. 2010; Ding et al 2014; Chabane et al. 2014).One of the benefits of such a system is that it is amenable to real-time analysis of the biofilm through automated image acquisition within specialized multi-well plates. In order to cultivate biofilms, microfluidic channels are primed with the culture medium at a specific rate. Each channel is seeded with an overnight culture with a cell density of 10^7 CFU. The biofilms are subsequently incubated at specific time and temperature levels in order for the bacterial cells to adhere. Once the biofilms have formed, planktonic cells are removed, and washed. The biofilm growth can then be recorded using a phase contrast or fluorescence microscope (Ding et al. 2014).

In-vitro Biofilm formation in a 8 well chamber

Another variation of biofilm chamber growth is the use of an 8-well chamber slide. This method uses 200µL aliquots of mid-logarithmic cells diluted in fresh medium (1:2500 (v/v)). The medium can be replaced every 12h if the biofilm takes longer than 24h to grow or as needed to maintain bacterial viability (O'Toole 2011).

The resulting biofilms can be visualized by aspirating the medium and washing with saline. The viability of the biofilm cells is typically assessed by the addition of BacLight Live/Dead stain (O'Toole 2011). Additionally, EPS or pili in the biofilms can be visualized under SEM by dehydrating the sample in a graded series of alcohols and adding hexamethyldisilazane (HMDS) (Araujo et al 2003).

Crystal violet quantification of biofilm growth

One of the most commonly used methods to assess the effectiveness of biosurfactants and biofilm inhibitory agents is the crystal violet quantification of biofilm growth (O'Toole 2011). The technique involves the cultivation of a microbial biofilm in a 96-well (high bind PVC) plate, a rinsing step and final staining with 1% crystal violet. Biofilms are quantified by assessing the proportion of crystal violet bound to the biofilm biomass in control and test cultivations. The surfaces of high-bind 96 well plates were originally designed for ELISA tests and hence contain organically compatible high protein binding plastic (other types of PVC have different binding properties). This type of surface allows the binding of large molecules with ionic groups or large hydrophobic regions and permits a wide diversity of bacteria to form biofilms.

The advantages of this method of biofilm quantification is that dispersal/inhibition can be measured directly in-situ rather than extrapolated from viable planktonic microorganisms. The crystal violet stains the total biofilm biomass which includes EPS and extracellular proteins rather than just its component cells. There may be some variability in the results obtained from this test but this can be rectified by a higher number of replicates which is afforded by the 96-well plate.

Quantification of biofilm inhibition by direct analysis

One of the simplest methods of biofilm quantification is by direct measurements of bacterial viability as directly proportional to biofilm dispersal (Rodrigues et al. 2004). This technique does not measure total biofilm biomass or biofilm adhesion, however it is a useful validation step for other methods. This quantification becomes more problematic for mixed bacterial populations and viable but non-cultivatable microorganisms.

Bacterial Viability Quantification

There are several viability dyes that are used to quantify biofilm. Most of these are based on DNA binding. These include two of the most widespread fluorescent dyes, propidium iodide which binds to DNA when the cell nuclear membrane is damaged fluorescing red and syto 9 green which binds to DNA when the nuclear membrane is intact (Lehtinen et al. 2004). In the case of biofilms this quantification can be complicated by extracellular DNA but this might only apply in very dense biofilms.

Digital quantification

Fluorescent stains are easily quantified by digital technologies. This makes it easier to assess biofilm growth/dispersal. As mentioned above although this technique may be directly quantitative for bacterial monolayers or biofilms of several layers thick, there are still technical issues however with proportional measurements of complex multi-layered biofilms with all the associated dead spaces and channels.

Other microscopic quantification

Scanning electron microscopy (SEM) has proved to be a useful technique for pictorial representations of biofilms, however the preparation methods involved including successive dehydrations in alcohol and gold sputtering can fundamentally alter the composition and biofilm architecture of biofilms. More promising results have recently been obtained by the use of cryo-SEM (Alhede et al. 2012). As previously stated, the biofilm substrates used in

microscopic techniques have to be quite robust such as glass; however this may also have a role in determining the formation of the biofilm and cannot always be used in direct comparisons to the same biofilms growth on plastic or silicone.

Biosurfactants as antibiofilm molecules

One of the most common questions posed on the effects of biosurfactants on biofilms is why are there still biofilms when biosurfactants are powerful molecules mostly leading to biofilm inhibition? The current hypothesis is that surface active molecules play a major role in the development and maintenance of biofilms partly through the maintenance of water channels through the biofilm which enhances nutrients movements and gaseous exchange, and which ultimately leads to the dissociation of parts of the biofilm into planktonic mobile forms (Marchant and Banat 2012). However the current focus of research is the ability of biosurfactants to disrupt established biofilms and prevention of the development of new ones. Although there are diverse arrays of biosurfactants, this review focuses on those in current use or known for the ability to disrupt biofilms in-vitro (Table 1).

Lipopeptides biosurfactant as disruptor molecules

Lipopeptides are one of the largest groups of biosurfactants that can effectively disperse microbial biofilms. These generally referred to by their group name although they can be composed of three or more varieties of homologous or congeners molecules. This group includes surfactins, polymixins, fengycins and fusaricidins (Krupovic et al. 2007; Pecci et al. 2010; Raza et al. 2009; Rivardo et al. 2009). Structurally lipopeptides are composed of a hydrophilic peptide attached to hydrophobic lipid or fatty acid. The peptides can either be aliphatic, branched or cyclic. Similarly, the lipids chains can vary in length and conformations ensuring a wide diversity of structures. Many of the current lipopeptides reported to inhibit/disperse biofilms originate from *Bacillus* or *Paenibacillus* (Kim et al. 2009; Price et al. 2007; Quinn et al. 2012).

Polymyxins

Polymyxins, are a class of non-ribosomally synthesized cyclic lipopeptides. They are generally produced as secondary metabolites of *Bacillus* or similar species (Price et al. 2007). Their typical structure is that of a cyclic polypeptide attached to a fatty acid tail. They can also contain exotic bacterial amino acids such as 2, 4, diaminobutyric acid (DAB) (Figure 2A). Polymyxins are known have a limited clinical spectrum of inhibition in the treatment of Gram negative infections. There are several commercially available formulations of polymyxins including Colistin (polymyxin E) (Falagas and Kasiakou 2005), Neosporin and Polymyxin B which can be supplied as polymyxin B sulphate (a mixture of polymixins) (He et al. 2010). Polymyxin can also be combined with trimethoprim for eye conditions (Polytrim) and with neomycin and bacitracin to make triple antibiotic ointment Neosporin.

Polymyxins are the last drug of choice in some infections and are often prescribed with caution due to fears of their toxicity however this estimation has been reappraised in the light of more rigorous testing (Arnold et al. 2007). Polymyxins are prescribed intestinally or topically as cream or powders in most cases of multi drug resistant *Acinetobacter baumannii*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* (Falagas and Kasiakou 2006; Milletli Sezgin et al. 2012). Polymyxin has been reported to reduce biofilms of *Pseudomonas aeruginosa* at concentrations of 20µg/ml by 99% in a 12 hour time period and almost completely over 24 hours (Jass and Lappin-Scott 1996). However these results are based on the viability of bacteria and not their dispersal; although it was noted that bacterial cells displayed an altered morphology. Polymyxin E (colistin) is recommended as an early aggressive therapy to delay the onset of chronic P. aeruginosa infection (which frequently forms biofilms) or intermittent colonization in cystic fibrosis patients, a combination of oral ciprofloxacin with colistin inhalation (Doring et al. 2000).

Polymyxin D1 has been shown to be effective against mixed bacterial biofilms, however our earlier work has shown this compound was found in combination with fusaricidin and surfactin in undefined ratio's (Quinn et al. 2012). This complex of biosurfactants was also reported to inhibit the formation of biofilms of both Gram positive

bacteria such as *Staphylococcus aureus, Streptococcus bovis, Bacillus subtilis and Micrococcus luteus* and Gram negative bacteria such as *Pseudomonas aeruginosa*. Most interestingly the biosurfactants were able to inhibit the formation of mixed species biofilms such as self-assembling marine biofilm (SAMB) in co-incubation assays by 99.3% and disrupt previously established mixed SAMB by 72.4% (Quinn et al. 2012).

The mechanism of action of polymyxins on bacterial biofilms remains largely undefined. However the mechanism of action on planktonic bacteria is proposed to be related to their high affinity for lipopolysaccharide (LPS) (Domingues et al. 2012). This induces LPS aggregation increasing the surface charge of LPS leading to internalization and binding to the bacterial phosphatidylglycerol-rich membrane leaflets which in turn induces leakage of cellular contents (Domingues et al. 2012).

Fengycin-like lipopeptides

Fengycin-like lipopeptides are derived from *Bacillus subtilis* and *Bacillus licheniformis.* These are cyclic lipopeptides containing 8-10 amino acids linked to a beta hydroxy fatty acid (Figure 2B). Fengycin-like peptides have also been reported to be involved in the inhibition of biofilms (Xu et al. 2013) causing up to 90% dispersion of Gram positive *S. aureus* biofilms and up to 97% dispersion of Gram negative *E. coli* biofilm (Rivardo et al. 2009) .

Putisolvin

Putisolvin is a cyclic lipodepsipeptide isolated from *Pseudomonas putida*. This has been characterised in two forms, putisolvin I and putisolvin II. This biosurfactant has a four member cyclic peptide; the valine residue in putisolvin I being substituted by a leucine or isoleucine in putisolvin II. (Figure 2C) (Dubern et al. 2006). Although Putisolvin is involved in biofilm formation by *Pseudomonas putida* these surfactants have also been shown to be effective dispersal agents in pre- and post-addition to biofilms of other *Pseudomonas* sp. strains (Kuiper et al. 2004).

Pseudofactin

Pseudofactin is a cyclic lipodepsipeptide derived from P. fluorescens. The structure of Pseudofactin is based on that of a palmitic acid attached to the terminal amino group of an eight amino acid peptide chain. The C-terminal carboxylic group of the last amino acid forms a lactone with the hydroxyl of third amino acid which is a threonine (Figure 2D). Pseudofactin II has been reported to be 36-90% effective against the adhesion of five species of bacterial biofilms on glass, polystyrene and silicone substrates. These strains include *Enterococcus faecalis*, *Escherichia coli, Staphylococcus epidermidis, Enterococcus hirae* and *Proteus mirabilis*. Similar inhibition of adhesion (92-99%) was reported on yeast biofilms of *Candida albicans* at concentrations of 0.5 mg/ml (Janek et al. 2012).

Pseudofactin has been documented to produce an effective dispersal of 26-70% on pre-existing biofilms grown on untreated surfaces and has been shown to cause a marked inhibition of the initial adhesion of *E. hirae, E. coli, E. faecalis* and *C. albicans* to silicone urethral catheters. Total growth inhibition of *S. epidermidis* has been observed at the highest concentration tested (0.5 mg/ml), which causes a partial (18-37%) inhibition of other bacteria, a 8-9% inhibition of *C. albicans* yeast growth and a 99% prevention of adhesion (Janek et al. 2012).

Surfactin

Surfactins are one of the most powerful biosurfactants originally isolated from *Bacillus subtilis* and consist of a cyclic peptide heptamer connected to a 13-15 carbon, beta-hydroxy fatty acid chain (Figure 3A). Unfortunately surfactins can also be indiscriminately cytotoxic with haemolytic activities due to its interactions with cellular membranes (D'Auria et al. 2013). They have been reported to inhibit the growth of biofilms of *Salmonella* sp. cultivated on PVC microtitre wells and urethral catheters (Mireles et al. 2001). They have been observed to cause a rippling effect in lipid bilayers perhaps indicating a clue to the mechanism of biosurfactant action or biofilm permeability or integrity (Brasseur et al. 2007)

most likely through the formation of some kind of channels within the biofilm increasing penetrability.

Complexes of lipopeptides

Although many lipopeptides have been characterised for experimental purposes as pure compounds they are in fact usually associated with groups of similar compounds. This is reflected in their availability as minimally purified preparations. Siram and co-workers (2011) reported on one such complex of lipopeptide biosurfactants produced by a heavy metal tolerant strain of *Bacillus cereus.* This surfactant effectively dispersed biofilms at an active dose of 0.150µg and was noted to be very tolerant of fluxes in pH, temperature and NaCl, in addition to being resistant to high levels of iron, lead and zinc whilst maintaining antimicrobial and biofilm dispersal activity. Another complex of surfactants isolated from *Paenibacillus polymyxa*. PPE was found to consist of polymyxin D1, fusaricidin B and traces of surfactin (Deng et al. 2011; Quinn et al. 2012) (Figure 3 B & C).

A preparation containing 2mg/ml of such lipopeptides tested in one of our laboratories inhibited (87-98%) the formation of many Gram positive bacterial biofilms such as *Staphylococcus aureus*, *Streptococcus bovis*, *Micrococcus luteus*, *Bacillus subtilis* and also some Gram negative bacteria such as *Pseudomonas aeruginosa* (Quinn et al. 2012). More uniquely in terms of biofilm experiments, this combination of lipopeptides was effective against mixed environmental strains' biofilms formation (99% inhibition) and up to 74% in pre-established biofilms.

Synergy of lipopeptides with other inhibitors

Lipopeptide biosurfactants have been combined with conventional antibiotics in an effort to produce synergistic inhibition effects. Lipopeptides isolated from *Bacillus licheniformis* (strain V9T14) were reported by Rivardo and co-workers (2011) to have a synergistic effect against a mature 24-h uropathogenic *E. coli* (CFT073) biofilms when combined with ciprofloxacin, cefazolin, piperacillin, ceftriaxone, ampicillin, tobramycin and

trimethoprim/sulfamethoxazole. They concluded that some combinations led to total eradication of biofilm; however the antibiotics on their own had poor inhibitory activity (Rivardo et al 2011).

Glycolipid biosurfactants as antibiofilm molecules

Glycolipids consist of a carbohydrate attached to aliphatic or hydroxy-aliphatic acid. These are one of the most studied groups of biosurfactants in other fields although they are underrepresented as agents of biofilm dispersal.

Rhamnolipids

Rhamnolipids consist of di- or mono-rhamnose sugars attached to a fatty acid chain (Figure 3 D & E). Originally isolated from *Pseudomonas aeruginosa,* analogues are also produced by isolates of *Burkholderia* (Costa et al. 2011), *Renibacterium salmoninarum, Cellulomonas cellulans, Nocardioides* and *Tetragenococcus koreensis* (Abdel-Mawgoud et al. 2010). Rhamnolipids have been reported as a potential replacement to chemical surfactants for many uses in the oil and petroleum industries and in use for the bioremediation of oil contaminated environments (Marchant and Banat 2012a & b). They are frequently cited as inhibitors of bacterial growth although their capacity to inhibit biofilms however has not been as extensively documented.

Rhamnolipids are involved in biofilm formation in *Pseudomonads* sp. through the promotion of motility, the inhibition of attachment and degradation of the matrix maintaining channels throughout the biofilm for movement of water and oxygen (Boles et al. 2005; Davey et al. 2003). These biosurfactants were previously reported as antibacterial against *Staphylococcus aureus, Mycobacterium sp, Bacillus sp, Serratia marsecens*, *Enterobacter aerogenes*, *Klebsilella pneumonia* and against fungi such as *Chaetomium globosum, Aureobacidium pullulans*, *Gliocladium virens*, *Botryhs cinerea* and *Rhizoclonia solanii* (Benincasa et al. 2004; Haba et al. 2003).

Rhamnolipids have also been shown to be effective against biofilms of *Bordetella bronchiseptica* (Irie et al. 2005). The mechanism of biofilm inhibition is thought to be by the detachment of cells; however some unattached cells may still be viable. They have been reported to disrupt pre-formed biofilms such as *Bacillus pumilus* from the marine environment (on polystyrene microplates) resulting in a dispersal at sub-MIC concentrations and confirming ability to remove pre-formed biofilms (Dusane et al. 2010). This was corroborated by scanning electron microscopy which showed that rhamnolipids removed biofilm-matrix components (Dusane et al. 2010). The effects of rhamnolipids on pre-formed biofilms of *P. aeruginosa* PAO1 generated in our laboratory can be seen here in Figure 4.

Rhamnolipids were also been tested on devices such as voice prostheses and have been noted to reduce the initial deposition rates of biofilm after 4h (Rodrigues et al. 2006a). A maximum reduction of adhesion (≈66%) was observed when the surfaces such as silicone rubber had been preconditioned with rhamnolipids using biofilms of *Streptococcus salivarius* and *Candida tropicalis*. The number of cells adhering after 4h was reduced to ≈48% for *Staphylococcus epidermidis*, *Streptococcus salivarius*, *Staphylococcus aureus* and *C. tropicalis* in comparison to controls. This group managed to optimise the actions of this biosurfactant on the detachment of microorganisms adhering to silicone rubber by perfusing the flow chamber with a biosurfactant containing solution followed by passage at the liquid-air interface. By this method they were able to achieve a high detachment (96%) for most of the microbial cells.

Rhamnolipids have also been shown to be effective dispersal agents for fungi disrupting pre-formed biofilms of *Yarrowia lipolytica* on glass surfaces by ≈67% which was more effective in comparison to the surfactants cetyl-trimethyl ammonium bromide (CTAB) and sodium dodecyl sulphate (SDS) (Dusane et al. 2012).

It is important to note that although rhamnolipids can effectively disrupt biofilm formation and integrity which we observed through phase contrast microscopy where thick dense cellular biofilm (Figure 4A) of microcolonies structures on glass coverslips stained with crystal violet was much reduced in after treatment with rhamnolipids biosurfactants

(Figure 4B). These molecules are also known to be extracellular virulence factors and related to the pathogenesis (infection procedure) in *Pseudomonas aeruginosa*. It has been noted that rhamnolipids are also linked to increased lung epithelial permeability, rapid necrotic killing of polymorphonuclear leukocytes and the malfunction of normal tracheal ciliary motion in the respiratory system of infected patients (Read et al. 1992).

Sophorolipids

Sophorose lipids are typical glycolipids biosurfactants consisting of a dimer of sophorose suger and a long-chain fatty acid that are produced by yeasts belonging to the genus *Candida*.

The synergy between sophorolipids and antibiotics has been studied as potential strategy to disrupt biofilms using The LIVE/DEAD BacLight Bacterial Viability Kits as a method for detection . This method employs two nucleic acid stains — the green-fluorescent SYTO 9® stain and the red-fluorescent propidium iodide stain. These stains differ in their ability to penetrate healthy bacterial cells. When used alone, SYTO 9 stain labels both live and dead bacteria. In contrast, propidium iodide penetrates only bacteria with damaged membranes, reducing SYTO 9 fluorescence when both dyes are present. Thus, live bacteria with intact membranes fluoresce green, while dead bacteria with damaged membranes fluoresce red. Joshi-Navare and Prabhune (2013) reported the the effect of sophorolipds in the disruption of biofilms from *Escherichia coli.* Figure 5 illustrated the examination of cells of *Bacillus subtitlis* attached to coverslips after 48h and stained with LIVE/DEAD BacLight showing the presence of individual bacteria, small clusters of cells (microcolonies), and extended areas of the glass surface covered with large numbers of microcolonies of active cells (Figure 5A), as well as, those which their membrane was damage due to the effect of sophorolipids 5% (v/v) concentration after 30min of treatment (Figure 5B).

Other Glycolipids as antibiofilm molecules

Dusane et al. (2012) reported a glycolipid based on glucose and palmitic acid produced by a tropical marine *Serratia marcescens* was effective in inhibiting biofilms of the marine biofouling bacterium *Bacillus pumilus* and the adhesion of *C. albicans* and *P. aeruginosa* PAO1. This effect was also observed with preformed biofilms of these cultures on microtitre plate tests. Other complexes of glycolipids from *Brevibacterium casei* MSA19 have been reported to disrupt and significantly inhibit individual and mixed culture biofilms of human and fish at concentrations of 30 mg/ml (Kiran et al. 2010).

Antibiofilm glycolipids have also been isolated from *Lactobacillus* (Tahmourespour et al. 2011; Zakaria Gomaa 2013). In this case *L. paracasei* A20 produced biosurfactants that inhibit Gram-positive and Gram-negative bacteria, yeasts and filamentous fungi (Gudina et al. 2010). The biosurfactant also showed anti-adhesive activity against pathogenic *Candida albicans, Escherichia coli, Staphylococcus aureus, Staphylococcus epidermidis* and *Streptococcus agalactiae*. Glycolipids derived from plants have also been reported to inhibit biofilms. These include a novel hydroxyproline rich glycopeptide from the pericarp of *Datura stramonium* known as datucin which is also reported to eradicate biofilms of antifungal resistant *Candida albicans* (Mandal 2012).

Complex surfactant mixtures

Biosurfactants are seldom found in pure form or isolation and are often associated together with isomers or congeners that share similar physiochemical characteristics which makes the process of purification either exhaustive or uneconomical. However these complexes of biosurfactants may have the advantage of a broader applicability than pure compounds. The same is true of complexes of compounds in other environments; this can be illustrated by the large diversity of antimicrobial peptides and surfactants found on the skin of amphibians (Bevins and Zasloff 1990). Similarly in innate human defence, antimicrobial peptides such as human beta defensins 1 , 2 and 3 and related human neutrophil peptides (Ganz et al. 1985) are found in homogenous groups. .

Combinations of biosurfactants have also been extracted from *Robinia pseudoacacia* and *Nerium oleander*. These secretions inhibited attachment of biofilms of *Candida albicans* on silicon and denture prosthesis at concentrations of 78µg/ml and 156µg/ml (Cochis et al. 2012). Other biosurfactants obtained from probiotic bacteria *Lactococcus lactis* 53 and *Streptococcus thermophilus* greatly reduced microbial numbers on preconditioned voice prostheses in an artificial throat model and induced a decrease in the airflow resistance that occurs on voice prostheses after biofilm formation (Gakhar et al. 2010).

Biosurfactants from fungi

Biosurfactants that inhibit biofilms have been found in fungi such as *Candida bombicola.* This produces sophorolipids that inhibit biofilms of *V. cholerae* (Mukherji and Prabhune 2014). Other strains of yeast such as *Candida sphaerica* have also been reported to produce biosurfactants such as Iunasan (Luna et al. 2011). This inhibits the adhesion of *Pseudomonas aeruginosa*, *Streptococcus agalactiae* and *Streptococcus sanguis* to levels between 80-92%. Similarly rufisan from *Candida lypolytica* inhibits biofilm formation at concentrations greater or equal to 0.75 µg/ml against *S. aureus*, *Streptococcus agalactae*, *S. mutans* NS (Rufino et al. 2011).

Mammalian surface active secretions

From a chemotherapeutical perspective, the most interesting groups of biosurfactants are those produced by humans. Not much is known about these molecules however it has recently been reported that PLUNC ("Palate, lung, nasal epithelium clone") protein has anti-biofilm activity (Gakhar et al. 2010). These molecules are mainly produced as a secretory product of epithelia lining the airways tubes within mammals including humans. They are evolutionarily related to the lipid transfer/lipopolysaccharide binding protein (LT/LBP) family. PLUNC are believed to have novel biologically relevant surface active properties as they significantly reduce surface tension at the air-liquid interface within aqueous solutions they

also inhibited biofilm formation in the airways colonising potential pathogen *Pseudomonas aeruginosa in vitro* at physiologically relevant concentrations (Gakhar et al. 2010).

Conclusions

It has been acknowledged that microbial biofilms lie at the heart of many recalcitrant patient infections in the clinical environment, the dissemination of airborne pathogens and the fouling of industrial surfaces. These problems are increasingly exacerbated by the rise of resistant biofilm populations and the paucity of alternative eradication solutions. Biosurfactants represent an emerging therapy which has inherent anti-bacterial, fungal and viral properties with an ability to effectively disperse or disrupt such biofilms. Their use therefore either on their own or as adjuvants to other antimicrobial chemotherapies may represent a potential way forward in tackling biofilms in the future.

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Table 1 Selected biosurfactants reported in literature with antibiofilm/microbial activities.

Biosurfactant class	Name	Source	Reference	Effectiveness
Lipopeptide	Putisolvin I & II	*Pseudomonas putida*	Kuiper et al. 2004	Biofilm inhibition of *Pseudomonas* spp.
Lipopeptide	Pseudofactin II		Janek et al. 2010	Effective against *E. coli Enterococcus faecalis Proteus mirabilis* and *Candida sp.*
Lipopeptide	NS	*Bacillus subtilis*	Mireles et al. 2001	Biofilm inhibition of *S. entrica* on urethral catheter
Lipopeptide	Fengycin	*B. subtilis & B. licheniformis*	Rivardo et al. 2009	Inhibition of pathogenic *E. coli & S. entrica*
Lipopeptide	NS	Heavy metal tolerant strain of *Bacillus*	Sriram et al. 2011	Inhibits Gram positive and negative bacteria and fungi
Lipopeptide	NS	*Bacillus* sp. strain SW9	Wu et al. 2013	Inhibits biofilm formation in a wide range of bacteria
Lipopeptide	NS	*Bacillus tequilensis*	Pradhan et al. 2013	Biofilm inhibition of *E. coli & Streptococcus mutans*
Lipopeptide	L. fermentum B54	*Lactobacillus*	Velraeds et al. 2000	Inhibits uropathogens
Glycolipids	NS	*Brevibacterium casei.*	Kiran et al. 2010	Inhibits mixed pathogenic biofilm bacteria
Mixture of biosurfactants	Lunasan	*Candida sphaerica*	Luna et al. 2011	Inhibition of *P. aeruginosa* and *S. agalactae*

NS	NS	*Lactobacillus paracasei* A20	Gudina et al. 2010	Biofilm inhibition for a range of bacteria, yeasts & filamentous fungi.
Glycolipid	Rhamnolipid	*P. aeruginosa*	Rodrigues et al. 2006b	Inhibits biofilms in *S. aureus* *Candida tropicalis*
Glycolipid	Rhamnolipid	*P. aeruginosa*	Dusane et al. 2010	Inhibits *B. pumulus*
Mixed biosurfactants		*Lactococcus lactis /Strep thermophilus*	Rodrigues et al. 2004	Effective against *Staphylococcus, Streptococcus , Rothia and Candida sp*.
NS	NS	*Robinia pseudocacia/ Nerium oleander*	Cochis et al. 2012	Effective against *C. albicans*
Glycolipids	Rhamnolipid	*P. aeruginosa*	Dusane et al. 2012	Effective against *Yarrowia sp.*
NS	Rufisan	*Candida lypolytica*	Rufino et al. 2011	Effective against *Streptococcus sp*
Glycolipid	Glucose + palmitic acid	*Serratia Marsecens*	Dusane et al. 2011	Effective against *C. albicans , P. aeruginosa* and *B. pumilus*

NS= Not specified

Figures legends

Figure 1. Calgary Biofilm Device (CBD) measures the minimum biofilm eradication concentration (MBEC). (1) Biofilms cultivated on pegs in 1/10th Muller Hinton broth (2) Pegs rinsed with PBS (3) Pegs exposed to test substances in new wells (4) Pegs rinsed in PBS (5) Biofilm removed by sonicating pegs into sterile media (6) Remaining viable bacteria in wells is proportional to the biofilm biomass.

Figure 2. Biosurfactants: (**A**) Polymyxin B2,. **(B)** Fengycin-like peptide, **(C)** Putisolvin II and. **(D)** Pseudofactin II:

Figure 3. Biosurfactants : **(A)** Surfactin, **(B)** - Polymyxin D1, (**C**) Fusaricidin B1,. **(D)** Rhamnolipids: mono rhamnolipid, (l-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate)(RL-1) and (**E)** di-rhamnolipid, (l-rhamnosyl l-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate (RL-2), DAB = diaminobutyric acid.

Figure 4. Representative images depicting the effect of rhamnolipids on pre-formed biofilms of *P. aeru*ginosa PAO1 on cover slips. Cells were stained with crystal violet 1%, and observed using a phase contrast microscope at 40x. (**A**) *P. aeruginosa* PAO1 Biofilms after 48h. (**B**) After 30min treatment with rhamnolipids (5%) v/v on 48h biofilms.

Figure 5. Biofilm formation by *Bacillus subtilis* BBK006 on coverslips. Cells were stained with Syto9®, and observed using a fluorescence microscope at 40x. The bar represent 100µm . (**A**) *Bacillus subtilis* BBK006 biofilms after 48h as a control. (**B**) After 30min treatment in the presence of Sophorolipids 5% v/v on 48h preformed biofilms.

Figure 1

Biofims cultivated in wells with pegs

Biofilms mature

Non-adherant bacteria rinsed off

Biofims incubated with test substance

Rinse off inhibitor

Sonicate pegs into media growth = biofilm survival

Figure 2

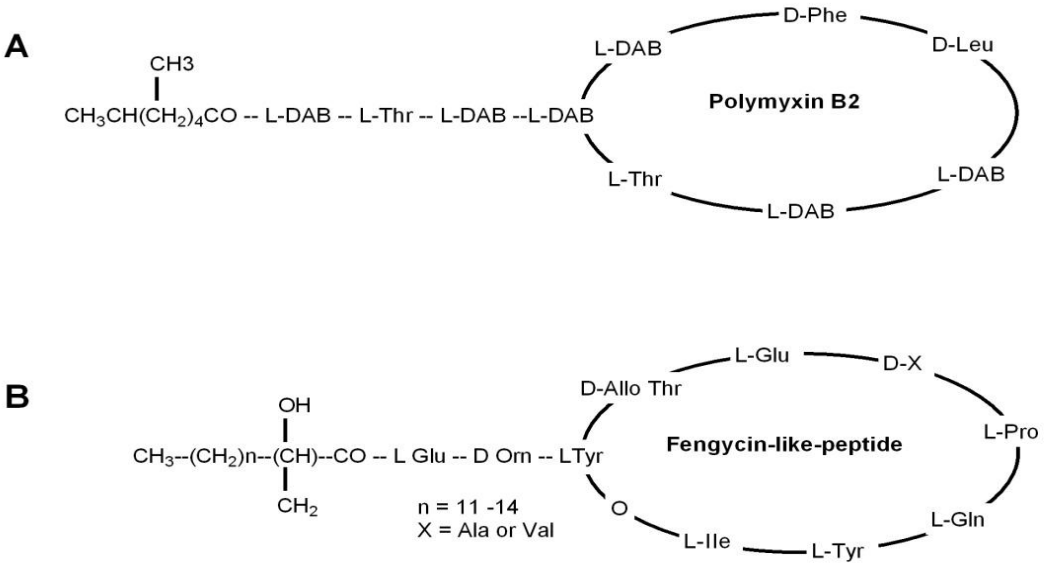
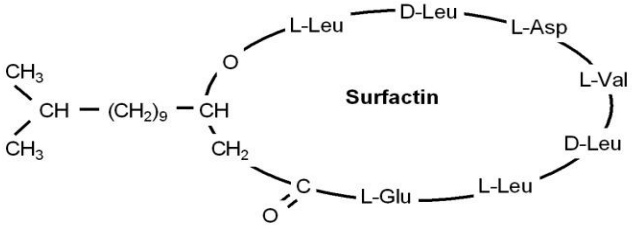
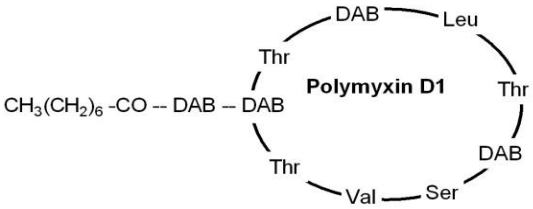

A
CH3
CH3CH(CH2)4CO -- L-DAB -- L-Thr -- L-DAB --L-DAB
D-Phe
L-DAB
D-Leu
Polymyxin B2
L-Thr
L-DAB
L-DAB
B
OH
CH3--(CH2)n--(CH)--CO -- L Glu -- D Orn -- LTyr
CH2
n = 11 -14
X = Ala or Val
L-Glu
D-X
D-Allo Thr
L-Pro
Fengycin-like-peptide
O
L-Gln
L-Ile
L-Tyr
C
CH3(CH2)4 --CO--Leu--Glu --Leu--Ile--Gln--Ser--Val--Ile--Ser
Leu
Putisolvin II
Xle
CH2
O
C
O
Ser
D
CH3(CH2)14-- CO -- Gly -- Ser -- Thr
Leu
Leu
Pseudofactin II
Ser
O
Leu
Leu

Figure 3

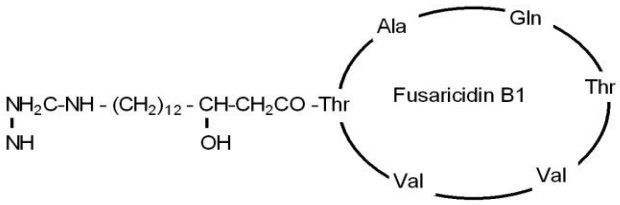
A



B



C



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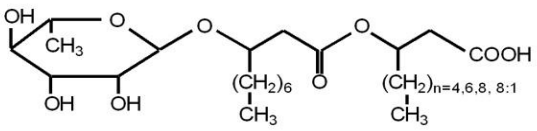

E

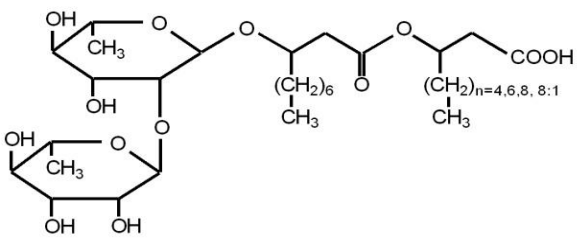

Figure 4

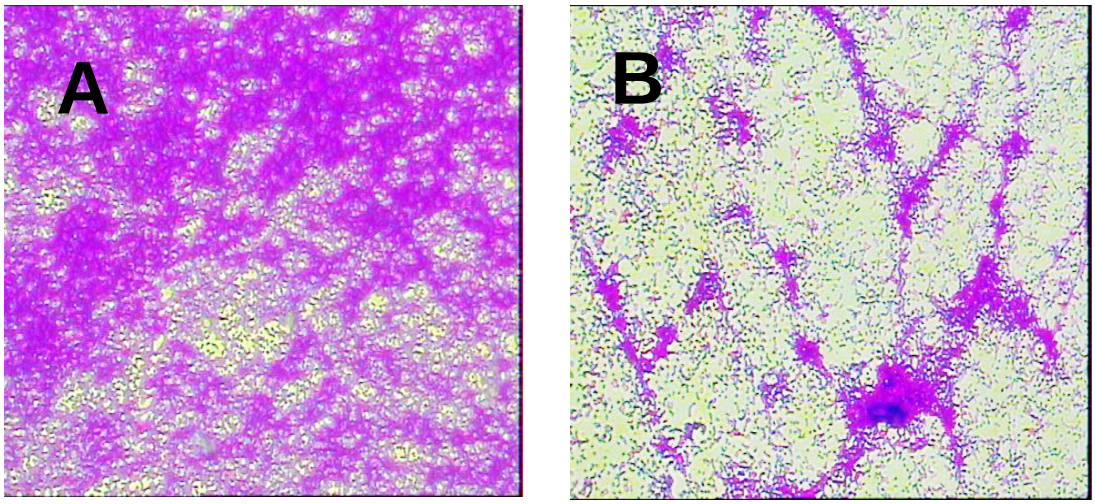

Figure 5