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Journal:	*FEMS Microbiology Letters*
Manuscript ID	FEMSLE-16-06-0417.R2
Manuscript Type:	Minireview
Date Submitted by the Author:	n/a
Complete List of Authors:	Elshikh, Mohamed; University of Ulster Faculty of Life and Health Sciences, School of Biomedical Sciences Marchant, Roger; University of Ulster, School of Biomedical Sciences Banat, Ibrahim; University of Ulster, School of Biomedical Sciences
Keywords:	Biosurfactant, Bioactives, Oral hygiene, Dental plaque, Medical devices, Medical devices, Oral formulation
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254x254mm (72 x 72 DPI)

Biosurfactants: Promising bioactive molecules for oral-related health applications.

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Key words: Biosurfactant, Bioactive molecules, Oral hygiene, Oral formulation, Dental plaque, Medical devices

ABSTRACT

Biosurfactants are naturally-produced molecules that demonstrate potentially useful properties such as the ability to reduce surface tensions between different phases. Besides having similar properties to their artificial chemical counterparts, they are regarded as environmental friendly, biodegradable and less toxic, which make them desirable candidates for downstream applications. The structure-activity related properties of the biosurfactants which are directly correlated with potency of the biosurfactants as antimicrobial agents, the ability of the biosurfactants to alter surface energies and their ability to increase bioavailability are particularly what attract researchers to exploit their potential use in the oral-related health applications. Current research into biosurfactant indicates significant future potential for use in cosmetic and therapeutic oral hygiene product formulations and related medical device treatments.

INTRODUCTION

Naturally occurring surfactants, often referred to as biosurfactants, are amphipathic in nature (one molecule composed of two moieties; one hydrophilic and the other hydrophobic). This unique structural arrangement is directly related to the surface activities of these molecules, such as reducing the surface tension between different interface systems and their ability to form emulsions (Banat *et al.* 2010). The main classification of biosurfactants into two sub-groups is based on their molecular weight: compounds of low molecular weight, such as rhamnolipids, sophorolipids, lipopeptides and trehalolipids while other compounds such as lipoprotein and polymeric surfactants which comprise the high molecular weight groups (Fracchia *et al.* 2012). Due to the structure-activity related properties of biosurfactants there is an increasing interest in exploring the biomedical activities of these compounds such as antiviral, antimicrobial, antifungal and anticancer properties (Singh and Cameotra 2004; Fracchia *et al.* 2014, 2015, Callaghan *et al*. 2016).

Synthetic surfactants from non-renewable resources constitute the major component of an estimated more than 13 million tonnes annual worldwide market (Marchant and Banat 2012a). To achieve a more competitive role for biosurfactants in this enormous market it will be necessary to improve scale up production conditions and explore new applications for environmentally sustainable and less damaging biosurfactants which can positively contribute towards reducing reliance on the synthetic surfactants (Marchant and Banat 2012b). In this review we attempt to briefly assess the progress that has been achieved in applying biosurfactants in the area of oral-related health applications.

Oral-related health and hygiene issues

The oral cavity harbours a wide and diverse spectrum of microorganisms (Wright *et al.* 2013). The natural presence of an oral microflora is important for normal survival of the host (Marsh 2000). Various mechanisms have been identified as being responsible for this contribution towards normal status of health in the individual. Primarily, the balance of populations within the oral microflora is carefully maintained. Therefore, populations present as a minor component are prevented from proliferating and disrupting the normal microflora, an example of which would be when use of antibiotics leads to the overgrowth of yeasts (Marsh 2010). Caries which arises from the disruption of the oral microflora balance can potentially be avoided through diet control and the use of fluoride enriched products (Bowden 2000). Furthermore, oral hygiene should be maintained through regular use of oral hygiene products. Such practices can provide control of plaque growth and inhibit bacterial proliferation associated with dental disease and can be achieved without eradicating beneficial bacteria or disrupting the microbial balance (Marsh *et al*. 2015; Marsh 2010).

Microbes can be in a planktonic or biofilm state. Planktonic microorganisms are less difficult to deal with since they are loosely attached to oral tissue or dental surfaces, whereas fungal or bacterial oral biofilm (dental plaque) provides protection for the resident microorganisms which makes them less susceptible to hygienic practices and bioactive ingredients of the products used (Socransky and Haffajee 2002). Under normal physiological conditions saliva conditioning film forms on the dental substratum, known as acquired pellicle which is mainly characterised by a protein rich content that allows bacterial adhesion through specific receptors (Kreth *et al.* 2009). This is followed by co-aggregation and co-adhesion of different types of initial colonisers (e.g. *Streptococci* and members of the *Actinomyces* family) on the dental surface, which may lead to an irreversible adhesion where

microorganisms surround themselves with an extracellular polymeric substances (EPS) as a scaffold for the biofilm (Kolenbranderet *et al*. 2010), which is mainly composed of polysaccharides, proteins, nucleic acids and lipids (Flemming H-C *et al*. 2010). Biofilm development also involves the presence of bridging colonisers (such as *Fusobacteria*), which play an important role in co-adhesion and coaggregation processes, with cell surface proteins providing multi-binding points for such microorganisms (He *et al.* 2012).

Normally the oral cavity environment provides protective mechanisms that maintain the normal physiological status, an example of which would be the secretion of natural peptides such as α-defensins, β-defensins (Tao *et al.*2005). This innate protection is also partly related to the presence of biosurfactant-releasing microorganisms such as *Streptococcus mitis,* which effectively discourages the adherence of *Streptococcus mutans*, an important cariogenic bacterium (Reid *et al.* 2011). A similar important role in the innate maintenance of oral health is also carried out by some *Lactobacillus* species (Haukioja 2010) some of which are known to be biosurfactant producers (Satpute *et al.* 2016b).

Potential Biosurfactant applications for oral health and hygiene

Several reports have demonstrated the ability of biosurfactants to inhibit and/or kill microorganisms, for example with natural lipopeptides (Cochrane *et al.* 2015; Banat *et al.* 2014), rhamnolipids (Díaz De Rienzo *et al.* 2016a,b) and Sophorolipids (Díaz de Rienzo *et al.* 2014, 2016c). It has also been reported that they are able to alter a substrate's surface energy (Busscher and van der Mei 1997) and increase surface area (Rodrigues *et al.* 2006), which would make biosurfactants important potential candidates for oral health-related applications. A direct application of the latter properties is adjuvant delivery of antibiotics using biosurfactants, resulting in a reduced therapeutic dosage of antibiotics (Quinn *et al.* 2013). In this context, sophorolipids demonstrated potency when delivered in a pharmaceutical formulation where they acted synergistically with antibiotics such as Tetracycline and Cefaclor showing an increase in the permeability of the antibiotics across the outer membrane of the bacteria (Joshi-Navare *et al.* 2003). It must be noted, however that in this study the sophorolipids used were not highly purified and the results should be treated cautiously. In a closely related area, biosurfactant properties (such as the ability to inhibit microorganisms and alter surface energy) have been shown to be of vital importance in controlling biofilm formation and proliferation (Satpute *et al.* 2016a). This was demonstrated when voice prostheses, prone to colonisation by oral bacteria and fungi (Bertl *et al.* 2013), were treated with biosurfactants (Rodrigues *et al.* 2006). A summary account of biosurfactant performance and effects in oral-related applications is presented in Table 1.

This search has clearly demonstrated that the application of biosurfactants in oral-related health is still at an early stage. However, published work in this area is promising and developing. Prior to looking at the pros and cons of the available research, it should be recognised and appreciated that working in biosurfactant research is a complex multidisciplinary area of work. There is a substantial requirement for analytical method development and production optimisation, which may be challenging and muddled by some research groups. This is something that has been noted and commented upon by other authors (Marchant *et al.* 2014). An overview examination of related published work reveals that a substantial portion of the working this field is related to biosurfactants from *Bacillus* strains producing glycoprotein-type biosurfactants.

A major problem in many studies is the lack of evidence of complete purity or characterisation of the biosurfactant active fractions used, as in the case of the work carried out by Tahmourespour (2011), Savabi (2014)

and Salehi (2014) and their co-workers when they investigated the gene expression *gtfB* and *gtfC* of *S. mutans*, which are directly involved in biofilm matrix formation. In principle, these studies are unique in being the first to investigate oral-related bacterial gene expression, after treatment with biosurfactant. However, the studies did not specify the relationship between the applied concentration of biosurfactant fraction used in the study and the minimum inhibitory concentration of that fraction against *S.mutans*, which is an important factor to understand and interpret the data obtained. The biosurfactant applied in these studies was characterised by Fourier Transform Infrared Spectroscopy (FTIR), and described as "protein-like" biosurfactant. However, FTIR as a stand-alone technique is very limited for the purpose of characterisation (Barth 2007). It is worth mentioning that Nuclear Magnetic Resonance spectroscopy (NMR) should be used for full characterisation of macromolecules such as lipoprotein. These protocols have been reviewed in detail by Twine and colleagues (2010). Also techniques such as one-dimension H^1-NMR or C^{13}-NMR are frequently used to characterise low molecular weight molecules, such as lipopeptide acting against *S. mutans* biofilm, for example as reported by Pradhan and colleagues (2013). However, such techniques should be used alongside other techniques such as HPLC-MS and in all cases compounds with high purity are needed especially in the case of NMR characterisation, as data obtained from crude extract experiments are not always meaningful.

In a relatively close area, Rodrigues and co-workers investigated a biosurfactant-type described as glycoprotein from the probiotic bacteria *Lactococcuslactis*53 and *Streptococcus thermophiles* A. The active fraction of the biosurfactant produced demonstrated antimicrobial properties against a number of orally-related species (e.g. *Streptococcus salivarius* GB 24/9). Interestingly, when these fractions were used to precondition an artificial model voice prosthesis, it became evident that microorganisms were discouraged from forming a biofilm on the treated surface. Rodrigues and her colleagues (2006) used an expanded characterisation approach for the biosurfactant fractions, where Hydrophobic Interaction Chromatography (HIC) and X-ray Photoelectron Spectroscopy were used for characterisation purpose together with FTIR.Antimicrobial and antiadhesive activities of the biosurfactant crude extract were investigated against several pathogens, some of which were oral-related opportunistic microorganisms.

In another study, Cochis and his colleagues (2012) demonstrated the antimicrobial and antibiofilm efficacy of biosurfactant produced by endophytes, selected from the plants *Robinia pseudoacacia* and *Nerium oleander* against *Candida albicans*, grown on elastomer silicon or denture disks. Good efficacy was shown at a low concentration of 78.12 μg ml^{-1}, which has demonstrated a low toxicity as well. However, biosurfactant characterisation details were not mentioned. In a similar study Rufino and co-workers (2011) reported the production of the biosurfactant Rufisan from the yeast *Candida lipolytica* UCP 0988, although characterisation details were not mentioned. This biosurfactant showed efficacy as an antimicrobial and antibiofilm agent for a wide spectrum of pathogens, including orally-related microorganisms. Dusane et al (2012) investigated the effect of rhamnolipids on *Yarrowia lipolytica* biofilm formation on different surfaces, where rhamnolipids caused an inhibition of *Yarrowia lipolytica* biofilm production on plastic and glass by 50% and 67% respectively. The report does not provide insight into the chemical composition of the rhamnolipids used.

In the same context, Busscher's research group carried out interesting work in this field. An example of this is the early report of the investigation of the of adhesion of some *Candida* strains, isolated from naturally colonized voice prostheses, to silicone rubber with and without a salivary conditioning film in the absence and presence of an adhering biosurfactant-releasing by the dairy isolate *Streptococcus thermophiles* B (Busscher *et al.* 1997). The same research group has also concluded in another study that the biosurfactant-releasing and naturally

oral cavity dwelling *S. mitis* can discourage the adhesion of *S. mutans.* The released biosurfactant from *S. mitis* was not fully characterised. However, based on preliminary analysis it was described as a rhamnolipid-like biosurfactant. When *S. mitis* was deposited on a glass surface (1- 4% surface coverage) in the presence of *S. mutans*, it was found that the adhesion of *S. mutans* was drastically inhibited at 20 mg ml^{-1}. A reduction in *S. mutans* growth on a glass surface coated with biosurfactant released from *S. mitis* was also reported by van Hoogomoed and colleagues (2000). Interaction studies between dental surfaces coated with *S. mitis* biosurfactant and *S. mutans* using Atomic Force Microscopy (AFM), showed a remarkable increase in repulsion when *S. mutans* cells approached the dental surface (van Hoogomoed *et al.* 2006). Much of what is cited in this review deals with biosurfactants produced by Lactic Acid Bacteria (LAB). Beside their production of biosurfactants, these bacteria produce other organic substances such as bacteriocins. These help to maintain oral health for instance, through competing with pathogenic species for niches and nutrients, a topic that has been reviewed elsewhere (Badet and Thebaud 2008).

In regards to oral cavity LAB secreting biosurfactants, the available literature does not appear to discuss the molecular pathways of synthesis of these surfactants, but rather emphasises the role they play in discouraging adhesion of pathogens (Satpute *et al.* 2016). Similarly, it is not fully clear why these bacteria produce biosurfactants in the oral cavity. However, by analogy to the role of rhamnolipids (mainly secreted by *Pseodomonas aeruginsa*) in the formation of biofilm structure which supports adhesion to surfaces (Davey *et al*. 2003); the secretion of biosurfactants from LAB, therefore may be carried out as an aid to help microorganisms that secrete them to adhere to the surface, which consequently helps to maintain the health of the host. In a direct application of biosurfactant in oral hygiene products a patented Emulsan formulation has shown a potent effect in plaque and caries control. It is thought that Emulsan inhibits the adhesion of *S.mutans* through the lectin-specific interaction with the galactose or galactosamine on the *S.mutans* cell surface (Eigen and Simone 1988). It is worth mentioning that these observations have not been scientifically reviewed.

Biosurfactants antimicrobial mode of action

Understanding how an antimicrobial agent works, improves the prospects for application of the agents. Synthetic antimicrobials have been well investigated, including synthetic surfactants. The latter, in general terms, were found to compromise microbial cell surface integrity. Chlorhexidine, for example, an antimicrobial surfactant, was found to causes damage to the outer cell layer followed by penetration of the cell wall or outer membrane through passive diffusion, which leads to the coagulation and leakage of intracellular constituents. It is also evident from studies that only at high concentration does inactivation of ATPase occur, which suggests membrane disruption rather than enzymatic inactivation are associated with its mode of action (McDonnell and Russell1999).

Biosurfactants are thought to follow a similar pattern in their antimicrobial mode of action. When in-vitro interactions between the antimicrobial lipopeptide surfactin (Carrillo *et al.* 2003) and trehalose biosurfactants (Ortiz *et al.* 2009) and the phospholipid bilayer were studied by Differential Scanning Calorimetry (DSC) and Fourier Transform Infrared Spectroscopy (FTIR), it was found that these interactions resulted in the incorporation of trehalose into the lipid membrane. This distorts the system's native properties, with deformities leading to an increase in fluidity of the phosphatidylserine acyl chains and a decrease in the hydration of interfacial regions of the lipid bilayer. NMR interaction studies between deptomycin and the lipid membrane revealed similar findings, such as membrane conformational changes and membrane pore formation (Scott *et al.* 2007).

Biosurfactants may also affect the interaction of the microorganism with different surfaces through altering the hydrophobicity of the microorganism's cell surface. In a detailed and informative review by Neu (1996), it was concluded that microorganism-surface interaction may happen through the biosurfactant anchoring by the hydrophobic or hydrophilic moiety, depending on the nature of the cell surface. Furthermore, the way the biosurfactants arrange themselves upon adsorption on surfaces may alter the surface nature depending on which part of the biosurfactant is extended to the exterior environment. Figure 1 illustrates these theoretical interactions along with the antimicrobial action of biosurfactant on the microbial surface. More recent reports still support this understanding, for example of the effects observed on *Pseudomonas aeruginosa* NBIMCC 1390, when treated with a concentration of rhamnolipids above their Critical Micelle Concentration (CMC) (which caused an increase in cell hydrophobicity, related to a reduction in the total LPS). Rhamnolipids were also effective below their CMC concentration as they caused a notable reduction in the bacterial major outer membrane proteins (OMP) (Sotirova *et al.* 2009).

CONCLUSION

Research findings in this area reveal that biosurfactants have the potential to be applied in different oral-related areas. Biosurfactant properties such as: antimicrobial activity whether biocidal or biostatic and against both Gram positive and Gram negative bacteria, emulsion-forming capability to create either stable or metastable microemulsions and the ability to increase bioavailability of hydrophobic compounds, which results in a reduction of the effective concentrations of hydrophobic active ingredients in addition to some anticancer activities towards some cell lines, make biosurfactants potential candidates in cosmetic or therapeutic oral hygiene products and also oral-related medical devices. However, to optimise the applicability of these compounds, significant efforts are required to enhance the quality of research. Refining the research aspects in this area may attract sceptical industrial collaborators. If we are attempting to assign bioactivity to biosurfactants it is imperative that high purity, single molecule species preparations are used to ensure that the observed effects are not due to a random contaminant, which may vary from one production batch to another. This could be of great therapeutic value on its own and also if chemical modification of naturally available complex congeners is required to enhance the efficacy or to reduce potential toxicity. Furthermore, biosurfactants sterilization methods require further evidence-based understanding.

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Table 1 – Summary Account of Biosurfactants in Oral-related Applications

BS Type	BS Producer	Main Findings	References
Uncharacterised -glycoprotein-type biosurfactants	*Lactobacillus casei (ATCC39392)*	Study investigates effect of biosurfactant-type on *Streptococcus mutans* gene expression of glucosyltransferase (gtfB and gtfC) and fructosyltransferase (ftf) genes (important genes for biofilm matrix formation). All genes found to have been dramatically down regulated upon the application of the biosurfactant.	Savabi *et al.* 2014
Uncharacterised -glycoprotein-type biosurfactants	*Lactobacillus acidophilus* DSM 20079	Experimentally identical to the above study, a similar conclusion was reached.	Tahmourespour *et al.* 2011
Uncharacterised -glycoprotein-type biosurfactants	*Lactobacillus reuteri* (DSM20016)	Experimentally identical to the above study, a similar conclusion was reached.	Salehi *et al.* 2014
Uncharacterised -glycoprotein-type biosurfactants	*Lactobacillus fermentum* ATCC 9338	Experimentally identical to the above, a similar conclusion was reached.	Tahmourespour *et al.* 2011
Biosurfactant-type	*Streptococcus mitis*	Upon coating enamel surface with biosurfactant from natural oral cavity resident bacterium, *Streptococcus mitis*, the surface became less attractive to *S. mutans* cells as repulsive forces increased.	van Hoogmoed *et al.* 2006
Uncharacterised	*Candida lipolytica* UCP0988	Study showed the ability of the biosurfactant to induce noticeable inhibition of several *Lactobacillus* strain biofilm and *Streptococcus mutans* HG985 at different concentrations.	Rufino *et al.* 2011
Uncharacterised	*Lactobacilluspa racasei* A20	Antimicrobial and anti-adhesive activities of the biosurfactant crude extract were investigated against several pathogens; some of those are oral-related opportunistics. The study investigated concentrations between 25 and 50 mg ml^{-1}, which demonstrated good efficacy in vitro.	Gudina *et al.* 2010
Partially characterised - glycoprotein	*Lactococcus lactis* 53 and *Streptococcus thermophilus* A	Biofilms were grown on preconditioned voice prostheses, with biosurfactants obtained from the probiotic bacteria *Lactococcus lactis* 53 and *Streptococcus thermophilus* A, in an artificial throat model. Both biosurfactants greatly reduced microbial numbers on prostheses and also induced a decrease in the airflow resistance that occurs on voice prostheses after biofilm formation.	Rodrigues *et al.* 2004
Partially characterised - glycoprotein	*Lactococcus lactis* 53	Study demonstrated efficacy of the extracted biosurfactant (previously described in Rodrigues et al, 2004) against microorganism isolated from an explanted voice prosthesis.	Rodrigues *et al.* 2006
Mixture of Glycosidic residue and Rhamnolipidlike mixture	*Streptococcus mitis*	Study demonstrated how the *S. mitis* biosurfactant-releasing species significantly inhibited growth of *S. mutans* on a glass surface.	van Hoogmoed *et al.* 2000
Uncharacterised biosurfactant	*Robinia Pseudoacacia* (AC5 and AC7) and *Nerium oleander* (OC5)	Prevention of biofilm formation of silicon and acrylic resin for dental prostheses	Cochis *et al,* 2012
Lipopeptide	*Bacillus tequilensis*	Inhibition of *S. mutans* biofilm at concentration of 50µg/ml at different hydrophobic and hydrophilic surfaces	Pradhan *et al.* 2003
Uncharacterised Rhamnolipid	*Pseudomonas aeruginosa*	50% and 67% inhibition of fungal *Yarrowia lipolytica* biofilm formation on 96 well plate surface and glass surface	Dusane *et al.* 2012

		respectively, in a pre-coating experiment.	
Emulsan	Unidentified (usually produced by *Acinetobacter calcoaceticus*)	Control of dental plaque and caries	US 4737359 A. Patent

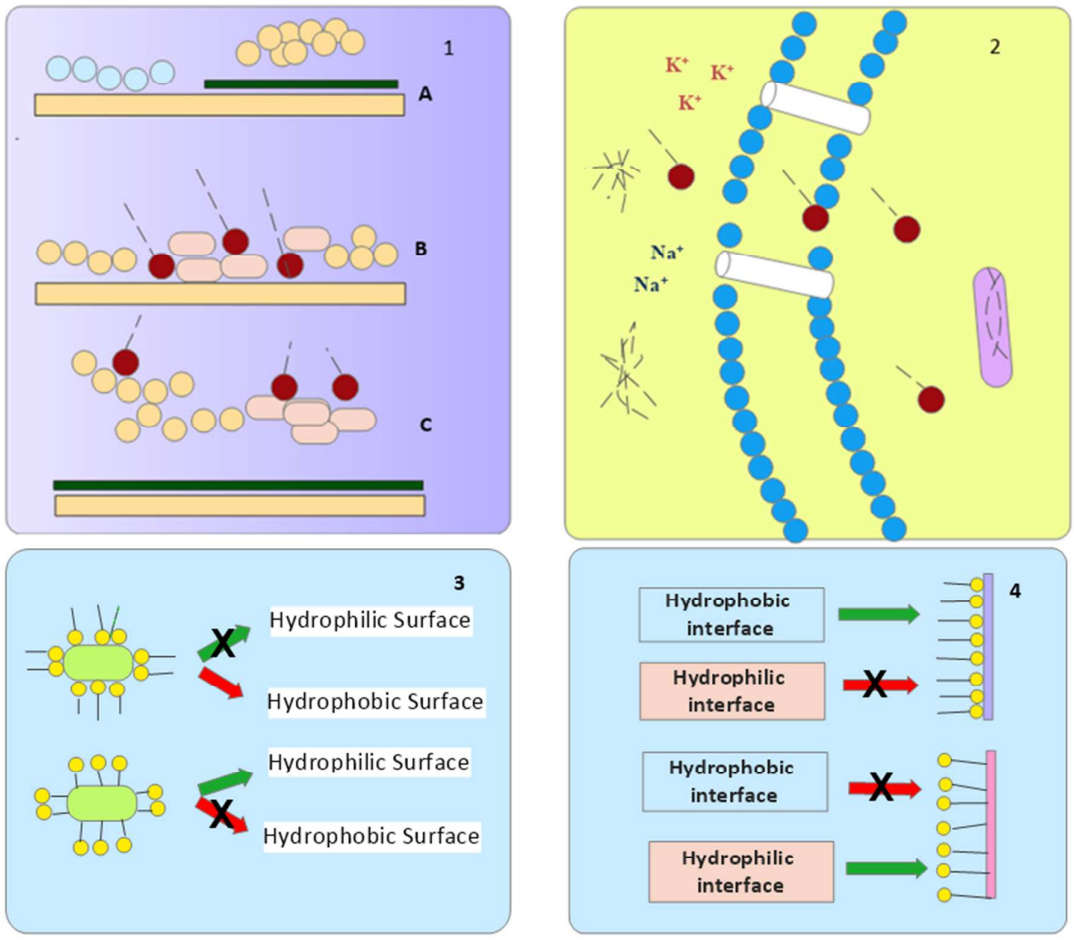

Fig 1: **(1)** Represents the mechanisms through which biosurfactants can affect the inhibition of microorganism growth on surfaces. **(A1)** demonstrates how, in natural settings, when a microorganism secrets biosurfactant as a means of survival it may discourage other pathogens from adhering to the surface. The two coloured shapes (Circular and rod) representing two different types of microorganism and the thinner line above the base (surface) represents the secreted biosurfactant. **(B1)** represents the ability of the biosurfactant to disrupt biofilm through affecting the cell surface. The circular and rod shapes represent different types of microorganism and the circular shape attached to a straight line represents biosurfactant showing an amphiphilic structure with a head (circular) and tail (straight line), (this applies to all figures). **(C1)** Illustrates the ability of the surface-coated with biosurfactant to inhibit microorganisms. **(B1)** and **(C1)** are further explained by **(3)** and **(4). (2)** Represents

the direct antimicrobial effect of biosurfactant on the microorganism. The bacterial cell membrane that has been disrupted by a biosurfactant, it also shows the disruption of ion flow and passage of large molecules to the exterior environment as a result of membrane disruption. **(3)** and **(4)** adopted from (Neu 1996). In **(3)** orientation of the biosurfactant through which it may anchor into the microorganism cell surface and may enhance or inhibit the adhesion depending upon the interaction between the biosurfactant-influenced cell surface and the surface under investigation. In **(4),** upon applying the biosurfactant to surfaces, the biosurfactant may adhere using the hydrophobic moiety or the hydrophilic moiety depending on the surface type, this as a result will influence the type of interaction between the coated surface and the microorganism. In both **(3)** and **(4)**, the unfavourable route of adhesion has been cross marked.