Sophorolipid biosurfactants: Possible uses as antibacterial and antibiofilm agent.

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ABSTRACT

Biosurfactants are amphipathic, surface-active molecules of microbial origin which accumulate at interfaces reducing interfacial tension and leading to the formation of aggregated micellular structures in solution. Some biosurfactants have been reported to have antimicrobial properties, the ability to prevent adhesion and to disrupt biofilm formation. We investigated antimicrobial properties and biofilm disruption using sophorolipids at different concentrations. Growth of Gram negative *Cupriavidus necator* ATCC 17699 and Gram positive *Bacillus subtilis* BBK006 were inhibited by sophorolipids at concentrations of 5% v/v with a bactericidal effect. Sophorolipids (5% v/v) were also able to disrupt biofilms formed by single and mixed cultures of *Bacillus subtilis* BBK006 and *Staphylococcus aureus* ATCC 9144 under static and flow conditions, as was observed by scanning electron microscopy. The results indicated that sophorolipids may be promising compounds for use in biomedical application as adjuvants to other antimicrobial against some pathogens through inhibition of growth and/or biofilm disruption.

1. INTRODUCTION

Biosurfactants are amphiphilic compounds produced on living surfaces, mostly on microbial cells or excreted extracellular hydrophobic and hydrophilic moieties, with the ability to accumulate and partition between fluid phases, thus reducing surface and interfacial tension at the surface and interface respectively [1]. Surfactants are widely used in industrial, agricultural, food, cosmetic and pharmaceutical applications; however the majority of surfactants are derived from petro- or oleochemicals and have the potential to cause environmental toxicology problems due to the recalcitrant and persistent nature of these substances [2].

The advantages associated with the use of microbially produced biosurfactants over their chemical counterparts include; lower toxicity, higher biodegradability, a wider range of effectiveness at different environmental conditions such as pH, temperature and high ionic strength, in addition to biocompatibility. These advantages allow applications of biosurfactants in cosmetic, pharmaceutical and food additives industries [3]. Biosurfactants are classified according to their chemical structure and their microbial origin. The main classes of biosurfactants are glycolipids, phospholipids, polymeric compounds and lipopeptides. In this work we focus on Sophorolipids (SL), a type of glycolipid. Sophorolipids are mainly produced by yeasts such as *Candida bombicola* and are composed of a dimeric sugar linked by a glycosidic bond to a hydroxyl fatty acid [4]. The fatty acid structure and carbon chain length may vary depending on the carbon source used to produce a given sophorolipid.

Biosurfactants have long been reported as molecules with potential applications in environmental and biomedical related areas [5, 6, 7]. There is renewed interest mounting in the use of biosurfactants in healthcare associated infections [8] and in particular the rapid advances in biofilm inhibition, control or disruption involving their use. Previous studies have shown that adsorption of biosurfactants to a solid surface can modify its hydrophobicity, affecting the adhesion process and consequently biofilm formation [4]. Most studies regarding anti-adhesive properties of biosurfactants were carried out using pure cultures of microorganisms and in the absence of culture medium. However it is known that mixed cultures are predominantly found in biofilms and that the presence of nutrients can affect the adhesion of single and mixed cultures cells [9]. In this work we tested the ability of SL to compromise cell membranes and inhibit growth of Gram positive *Bacillus subtilis* BBK006 and Gram negative *Cupriavidus necator* ATCC 17699 bacteria. We also studied the disruption of biofilm formation in *Bacillus subtilis* BBK006, as a single culture as well as in mixed cultures of *Staphylococcus aureus* ATCC 9144 and *Bacillus subtilis* BBK006, to evaluate possible potential use in the health care industry.

2. MATERIALS AND METHODS

2.1 Microorganisms and Media

Candida bombicola ATCC 22214 was stored in nutrient broth with 20% glycerol at −80°C. The standard medium for the production of sophorolipids was glucose/yeast extract/urea (GYU) medium (10% w/v glucose, 1% w/v yeast extract, 0.1% w/v urea). The fermentation medium contained the same growth medium, with rapeseed oil, as a second carbon source, being fed at regular intervals to induce sophorolipid production. For the antimicrobial assays *Staphylococcus aureus* ATCC 9144, *Cupriavidus necator* ATCC 17699 and *Bacillus subtilis* BBK006 were stored in nutrient broth plus 20% glycerol at −80°C, and used when needed.

2.2 Culture Conditions

Cupriavidus necator ATCC 17699, *Bacillus subtilis* BBK006 and *Staphylococcus aureus* ATCC 9144 grown on nutrient agar slants and incubated for 24 h at 30°C were used to obtain a bacterial suspension, with the optical density (570 nm) adjusted to give 10^7 CFU/ml for each of the strains used.

2.3 Production of Sophorolipids

A crude SL (S1) mixture was obtained as the settled product from fed batch cultivation of *C. bombicola* ATCC 22214, operated without the use of antifoam, according to Shah et al [10], feeding glucose and rapeseed oil rather than waste frying oil. The dry matter content of the crude mixture sophorolipid was adjusted to 45% v/v and contained a mixture of acidic and lactonic congeners of sophorolipids (data not shown). Residual fatty acids were less than 1% of the total dry sophorolipid mass. As a comparison commercially available SL (S2) were used as obtained from Soliance (Reims, France) under the brand name Sopholiance, the main

difference between this and the crude S1 mixture being the lack of C18:1 lactonic form and the presence of mainly acidic sophorolipids (data not shown).

2.4 Determination of the minimum inhibitory concentration (MIC) of sophorolipids

One mL of each culture (adjusted to give 10^7 CFU/ml) was inoculated into a 250 ml Erlenmeyer flask containing 50 ml of LB broth, following which a 100 µl sample of each diluted culture was dispensed (eight replicates) to fill a 96 well Oxoplate OP96C® for antimicrobial assays, where S1 and S2 were applied at 5% v/v.

OxoPlate OP96C (PreSens, Regensburg, Germany) contains oxygen-sensitive particles PSLi-Pt-1 (Opto-Sense, Wörth, Germany), which consist of small polystyrene particles. The sensor has a thickness of about 10 µm and is fixed at the bottom of each well of a 96-flat bottom-well plate (Greiner, Frickenhausen, Germany). The oxygen concentration in each well was measured for 21 h at 20 min intervals. Fluorescence of each well was measured in dual kinetic mode (BMG Labtech GmbH, Germany). Filter pair 1 (544/650nm) detects fluorescence of the indicator dye. The second filter pair (544/590 nm) measures fluorescence of the reference dye.

All experiments were repeated on independent days. Oxygen concentration as percentage air saturation was calculated for each well by using the following equation:

$$pO_2 = \frac{\left(\frac{k_0}{I_R} - 1\right)}{\left(\frac{k_0}{I_R} - 1\right)} \times 100 \qquad \{1\}$$

Where $_R$ is the fluorescence intensity ratio at the oxygen concentration [O_2]. A two-point calibration at [O_2] 0 and at [O_2]=[O_2]*, where [O2]* is the saturation concentration, is sufficient. The Intensity ratios I_R were calculated for each individual well by dividing the intensity of the indicator dye by the intensity of the reference dye. The constant k_0 is defined as the mean of the IR's of at least four wells filled with calibration 0. Analogously, k_{100} is defined as the mean of the IR's of at least four wells filled with cal 100.

MIC values were determined by measuring the OD at 570 nm and comparing to those cultures where biosurfactant was added. All the biosurfactants were added from time 0 (min) to evaluate inhibition *in vivo.*

2.5 Growth and determination of the viability/disruption of biofilms on coverslips.

Cupriavidus necator ATCC 17699 and *Bacillus subtilis* BBK006 were grown overnight and diluted 100-fold with nutrient broth 50% w/v, following which 2 ml samples were dispensed in triplicate to fill a 12 well plate, with biofilms formed on sterile, glass coverslips (18mm x 18mm) which were put into the 12 well plates (vertically) and were incubated at 30°C for 48 h. After this period the plates were washed three times and the biosurfactant treatment was applied with three replicates, for a period of 30 min (at 200 rpm). Positive and negative controls were added, using MES (2-(*N*-morpholino) ethanesulfonic acid) and PBS buffer. Biofilms were then stained with Syto®9 and the structure was observed with a fluorescence microscope at 40X magnification.

2.6 In vitro Biofilm formation using an eight well chamber

An overnight culture of *Staphylococcus aureus* ATCC 9144, *Bacillus subtilis* BBK006 was adjusted to OD_{490} 0.65 and was diluted 1:6 and incubated at 30°C with 5% CO_2 for approximately 3h in order to reach the mid-log phase. Once the mid-log phase was reached, the cells were diluted 1:2500 in fresh nutrient broth and 200 µl were placed in each well and incubated for 24h with a change of medium every 12 h to maintain bacterial viability. To visualize the biofilms the medium was aspirated and the resident biofilm was washed twice with PBS 1X. The viability of the cells was analysed using 200 µl of BacLight Live/Dead stain in each well. The disruption of the biofilms was analysed using SEM, where the cells are dehydrated in graded alcohols (50%, 65%, 80%, 95% and 100%) and after the final dehydratation step ethanol is replaced with hexamethyldisilazane (HMDS) in ratios of (1:1), (1:2), (1:3) and 100%, after which period the samples were left overnight for the solvent to evaporate [11] and subsequently the biofilms were observed under SEM.

3. RESULTS

3.1 Effect of MIC of sophorolipids on planktonic cells of *Cupriavidus necator* ATCC 17699 and *Bacillus subtilis* BBK006.

Surfactants of both biological and chemical origin are usually characterized by the formation of aggregated structures such as micelles, their critical micelle concentration (CMC) and their foaming and detergent abilities [12, 13, 14]. The Minimum Inhibitory Concentration (MIC) is the lowest concentration of a compound that inhibits bacterial growth. Lang and co-workers [15] reported some biosurfactant antimicrobial activity towards *B. subtilis, S. epidermis* and *P. acnes* at low MIC concentrations (<1.6 mM). Figure 1 shows the antimicrobial effect of sophorolipids at concentrations higher than 5% (v/v) during the first 3h of growth cells of *Cupriavidus necator* ATCC 17699 and *Bacillus subtilis* BBK006, (higher than those required to inhibit the grown of other Gram positive and Gram negative bacterial cells reported earlier [15,16]). However there is a resistance shown by the cells after approximately 3h of time indicating a possible bacteriostatic effect of sophorolipids on *Cupriavidus necator* ATCC 17699 and *Bacillus subtilis* BBK006, as non-pathogenic models of study.

3.2 Biofilm formation by *Bacillus subtilis* BBK006 on glass coverslips: "static conditions"

The behaviour of planktonic cells and organized structures (biofilms) is different when they face stressful environmental conditions. The study model selected for this study was *Bacillus subtilis* BBK006, due to the inability of *Cupriavidus necator* ATCC 17699 to develop stable biofilms. *Bacillus subtilis* biofilms were evaluated microscopically after 48h of incubation. Fluorescence microscopy examination of cells attached to coverslips and stained with

Syto®9 showed 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 (Fig 2A). *Bacillus subtilis* BBK006 was able to form biofilms like those observed for other *Pseudomonas aeruginosa*, *Staphylococcus epidermidis* strains [17]. The biofilm formed was treated with sophorolipid sample S1 (which was selected as no significant differences were observed to S2 sample on planktonic cells). In agreement with previous studies [18, 19] were are able to confirm here that *Bacillus subtilis* BBK006 biofilm cells are sensitive to some extent to sophorolipids (S1) which was seen by the reduction of number of active cells upon exposure and the appearance of some inactive reddish-brown fluorescing cells (Fig 2B). The results of the present study indicate that sophorolipids have the potential to be used for biofilm disruption and removal. This is in agreement with the data shown by Shah et al. [4], who reported sophorolipids having significant antibacterial activities especially against Gram positive bacteria.

3.3 Effect of S1 on pre-formed biofilms by *Bacillus subtilis* BBK006 and mixed cultures within the eight well chamber: "flow conditions".

In this experiment, sophorolipids at 5% v/v induced disruption on mature maximal biofilms of *Bacillus subtilis* BBK006 and a mixed culture of *Bacillus subtilis* BBK006 and *Staphylococcus aureus* ATCC 9144. The untreated cells as well as those treated with S1 (5% v/v) were examined by SEM to visualize the disruptive effect of sophorolipids on the biofilms (Figure 3). The SEM photomicrographs of the control (Figures 3A, 3C and 3E) and treated (3B, 3D and 3F) biofilms show the changes in cell morphology. In the control the cells form different layers of growth and the extracellular polymeric substance (EPS) are visible (Figure 3E). After treatment with sophorolipid only monolayers of cells are observed and there is a visible loss of the EPS and a release of the cytoplasmic content (Figure 3F), this

effect is also supported by results reported by Kim et al [20] and Dengle-Pulate et al [21] where *Bacillus subtilis* cells surfaces (after treatment) were not only distributed in monolayers but also disrupted with the outpouring of their cytoplasmic contents, indicating that SL causes the release of an intracellular enzyme malate dehydrogenase that interacts with SL increasing the permeability.

4. DISCUSION

Biosurfactants are amphiphilic compounds produced by microorganisms that reduce surface and interfacial tension. They have been recognised for some time in potential applications in a wide range of industries including agriculture, food, cosmetic, pharmaceutical and petroleum industries [6]. 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 [22]. The current hypothesis is that surface-active molecules like biosurfactants play a major role in the development and maintenance of biofilms, partly through the maintenance of water channels through the biofilm which enhance nutrient movement and gaseous exchange which leads to the dissociation of parts of the biofilm into planktonic mobile forms [5]. 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 [23].

Recently studies [4, 5, 24] reported the use of biosurfactants as antimicrobial molecules, however due to the differences between planktonic and biofilm physiologies affected by these kind of compounds, this work aimed to evaluating the impact of sophorolipids on cells present in both forms/environments, a behavioural variation that so far seems inconsequential. Standard bacterial inhibition tests are almost exclusively based on planktonic bacterial physiology and not the biofilm physiology, even though these conditions are 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 250 rpm) [24, 25]. In contrast, the biofilm physiology has multicellular differentiation, multicellular communication, internal architecture and rudimentary fluid transport systems [26, 27]. Shah et al [4] reported on the antibacterial activity of SLs in various carbohydrate-containing media against a selection of Gram-positive

and Gram-negative bacteria, in our study we selected *Cupriavidus necator* ATCC 17699 and *Bacillus subtilis* BBK006 as model microorganisms.

Cupriavidus necator was selected as suitable gram-negative versatile PHB-producing bacterium extensively studied and commonly used for its ability to accumulate up to 90% of its dry weight as PHB, the first discovered PHA [28, 29]. The extraction of PHAs using organic solvents is the most common used technique [30], however there is a need for using green and cheap technologies to recover polyhydroxyalkanoates (PHAs) from microbial biomass for the development of a reliable and sustainable production chain [31]. The importance of the use of sophorolipids is thought to be as a novel molecule proposed to extract PHAs from *Cupriavidus necator*. This however has to be further investigated.

Bacillus subtilis on the other hand was selected as one of the most studied Gram-positive bacteria in terms of the elucidation of the genes, proteins, and molecular mechanisms involved in biofilm formation. However we note that among Gram-positive bacteria, the molecular mechanisms of biofilm formation appear to be species-specific. Several systems are in use to quantify bacterial growth in the presence or absence of these compounds and to study planktonic behaviour of diverse populations of cells [5]. Here we used a fluorescence assay system, called Oxoplates®, that quantifies the oxygen concentration in the growth medium to evaluate the effect of S1 and S2 on planktonic cells of *Cupriavidus necator* ATCC 17699 and *Bacillus subtilis* BBK006. Using this system a minimum number of cells were required to consume a threshold amount of oxygen before they were detected in the system. All the results presented in Figure 1 are beyond this threshold (high inoculum density), consequently consumption of oxygen was detected immediately and the growth medium was essentially free of oxygen after 2h, in absence of treatment (Figure 1A).

In the presence of sophorolipids at 5% v/v we detected similar kinetics of bacterial growth inhibition for S1 and S2, where after addition of the treatment the oxygen concentration

increased (Figure 1B). This increase is attributable to the enhanced diffusion of atmospheric oxygen into the wells after cell death, which is an indication that we might be dealing with a bactericidal compound. The mechanism for bioactivity of biosurfactants is suggested to be associated with their intercalation into target cell membranes, demonstrating in this case that sophorolipids are effective against *Cupriavidus necator* ATCC 17699 and *Bacillus subtilis* BBK006, and that at the concentration tested sophorolipids are comparable to conventional antimicrobials used in agriculture and healthcare industry [32], as well as synthetic surfactant as SDS for the extraction of PHA [33].

The deposition of microorganisms on solid surfaces, and subsequent biofilm formation is a phenomenon that happens naturally and is part of microorganisms' strategy to protect themselves from external toxic factors [34]. The inability to form biofilms by *Cupriavidus necator* ATCC 17699 led us to focus on the formation of biofilms by *Bacillus subtilis* BBK006 as a model of a gram positive bacteria (most of them can cause various infections including hospital-acquired infections), which is best known for its ability to become competent and undergo sporulation in response to starvation and high population densities [35]. These biofilms are difficult to treat due to their resistance to antibiotics and biocides [4, 37]. Interestingly the surfactant produced by *Streptococcus thermophilus* has also been shown to be effective industrially for the control of fouling of heat exchanger plates in pasteurizers [38].

In this study biofilm formation of *Bacillus subtilis* BBK006 was evaluated microscopically after 48 h of incubation. Fluorescence microscopy examination of cells attached to coverslips and stained with Syto®9 showed 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 (Fig 2A). *Bacillus subtilis* BBK006 was able to form biofilms like those observed for strains of *Pseudomonas aeruginosa* and *Staphylococcus epidermidis*

[39]. In agreement with previous studies [40, 41] we are able to confirm here that *Bacillus subtilis* BBK006 biofilm cells are sensitive to some extent to sophorolipids as seen by the reduction of number of active cells upon exposure and the appearance of some inactive reddish-brown fluorescing cells (Fig 2B). These results indicate that sophorolipids have the potential to be used for efficient removal of detrimental biofilms.

It is now generally recognized that biofilms are heterogeneous structures [42] and that the appearance of specific biofilm functions such as resistance to antimicrobial agents is intimately related to the inherent three dimensional organizations of cells and exopolymeric matrix which result from multifactorial processes. Bai and co-workers [43] had previously associated biosurfactants with an enhanced transport of bacteria through soil columns, achieved through steric hindrance of the contact between bacterium and surface and an increase in the negative surface charge density of the soil. Mireles and co-workers [37] demonstrated that a range of surfactants (rhamnolipid, surfactin, Tween 80 and sodium dodecyl sulphate) brought about dissolution of *Salmonella enterica* biofilms, which reflects the diversity in the nature and recalcitrance of biofilms produced. These observations were similar to those reported by Davey and co-workers [44], although different media, strains and means for growing biofilms were used, similar conclusions were drawn as in the present study. However we note that concentrations evaluated need to be optimized to be considering as a focus point for further scale-up of production and future to biotechnological applications. The development of a biofilm mainly constitutes a survival strategy for bacteria providing a protective environment safe from stresses such as microbicide action and can thus lead to significant health-care problems. Using a model of study for biofilm resistance we used a mixed culture biofilm of *Staphylococcus aureus* ATCC 9144 and *Bacillus subtilis* BBK006 to test the effects of sophorolipids.

Understanding the complex way that bacteria (as single or mixed culture) colonize and build specialized structures like biofilms and formulating new strategies to deal with their formation or facilitate their disruption through removal or killing are current issues in medical and industrial microbiology. One of the possible solutions for this global problem is the appropriate use of antimicrobial combinations [45]. In this report, sophorolipids (S1) at 5% v/v induced disruption on mature maximal biofilms of *Bacillus subtilis* BBK006 and a mixed culture between *Bacillus subtilis* BBK006 and *Staphylococcus aureus* ATCC 9144. *Bacillus subtilis* cells treated with sophorolipids were disrupted with the outpouring of their cytoplasmic contents, likely due to the release of an intracellular enzyme; malate dehydrogenase indicating the interaction of sophorolipids with the cellular membrane and increased permeability [21]. This is true for either Gram-positive or mixed cultures, despite the fact that most bacterial biofilms display resistance against antimicrobials such as antibiotics and various host immune responses [46]. Sophorolipids are biologically produced compounds from yeasts strains and are generally regarded as being biocompatible and safe for human use while having significant disruption of biofilms produced by different microorganisms [47].

Although the mechanism of action of biosurfactants on biofilm disruption is not well known, a generalized activity of altering charge-charge properties is hypothesized [44], which may decrease the chances for bacteria to acquire antibiotic resistance due to spontaneous mutations. Further studies on the action of different natural sophorolipids, alone or as adjuvants in combination with other compounds such as antibiotics or enzymes is of great importance. Such combination may play an important role on the stability of the EPS during biofilm formation [40, 48] which can lead to new approaches to combat the establishment or disrupt biofilms formed by different bacterial species. It is also important to take into account that the combinations treatments may behave differently for some species.

5. CONCLUSIONS

Sophorolipids were effective as a bactericidal agent regardless of their acid/lactonic content, able to induce cell death of planktonic cells of a representative Gram positive and Gram negative bacteria comparable to conventional antimicrobials which had bacteriostatic effects. Sophorolipids were able as to disrupt biofilms at concentrations over than 5% (v/v). The results show that sophorolipids are promising bactericidal molecules for biomedical technological applications in industrial systems and need to be studied in detail at large scale systems and in conjunction with animal tissue models.

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7. REFERENCES

[1] Marchant R, Banat IM. Biosurfactants: a sustainable replacement for chemical surfactants? Biotechnol Lett 2012a; 34:1597-1605.

[2] Makkar RS, Rockne KJ. Comparison of synthetic surfactants and biosurfactants in enhancing biodegradation of polycyclic aromatic hydrocarbon. Environ Toxicol Chem 2003; 22: 2280-2292.

[3] Fakruddin Md. Biosurfactant: Production and Application. J Pet Environ Biotechnol 2012; 3:4.

[4] Shah V, Badia D, Ratsep P. Sophorolipids having enhanced antibacterial activity Antimicrob. Agents Chemother 2007; 51(1): 397-400.

[5] Banat IM, Diaz De R MA, Quinn GA. Microbial biofilms: biosurfactants as antibiofilm agents. Appl Microbiol Biot 2014; 98: 9915-9929.

[6] Banat IM, Franzetti A, Gandolfi I, Bestetti G, Martinotti MG, Fracchia L, Smyth TJP Marchant R. Microbial biosurfactants production, applications and future potential. Appl Microbiol Biot 2010; 87(2): 427-444.

[7] Diaz MA, Urdaneta I, Dorta B, Banat IM, Blazquez ML, Gonzalez F, Muñoz JA, Ballester A. Metal Removal from Contaminated Soils Through Bioleaching with Oxidizing Bacteria and Rhamnolipid Biosurfactants. Soil & Sediment Contamination 2015 24 (1): 16-29.

[8] Krasowska A. Biomedical activity of biosurfactants. Postepy Hig Med Dosw 2010; 64:310-313.

[9] Zezzi do Valle Gomes M, Nitschke M. Evaluation of rhamnolipid and surfactin to reduce the adhesion and remove biofilms of individual and mixed cultures of food pathogenic bacteria Food Control 2012; 25: 441-447.

[10] Shah V, Doncel GF, Seyoum T, Eaton KM, Zalenskaya I, Hagver R, Azim A, Gross R. Sophorolipids: novel glycolipid preventive agentsfor conception and sexual transmission. Antimicrob. Agents Chemother 2005; 49:4093–4100.

[11] Araujo JC, Téran FC, Oliveira RA, Nour EA, Montenegro MA, Campos JR, Vazoller RF. Comparison of hexamethyldisilazane and critical point drying treatments for SEM analysis of anaerobic biofilms and granular sludge. J Electron Microsc (Tokyo)

[12] Chen ML, Penfold J, Thomas RK, Smyth TJP, Perfumo A, Marchant R, Banat IM, Stevenson P, Parry A, Tucker I, Grillo I. Solution self-assembly and adsorption at the air-water interface of the mono and di-rhamnose rhamnolipids and their mixtures. Langmuir 2010a; 26 (23): 18281-18292.

[13] Chen ML, Penfold J, Thomas RK, Smyth TJP, Perfumo A, Marchant R, Banat IM, Stevenson P, Parry A, Tucker I, Grillo I. Mixing behaviour of the biosurfactant, rhamnolipid, with a conventional anionic surfactant, sodium dodecyl benzene sulfonate. Langmuir 2010b; 26 (23): 17958-17968.

[14] Penfold J, Chen M, Thomas RK, Dong C, Smyth TJP, Perfumo A, Marchant R, Banat IM, Stevenson P, Parry A, Tucker I, Grillo I. Solution self-assembly of the sophorolipid biosurfactant and its mixture with anionic surfactant sodium dodecyl benzene sulfonate. Langmuir 2011; 27 (14): 8867-8877.

[15] Lang S, Katsiwela E, Wagner F. Antimicrobial effects of biosurfactants. Fat. Sci. Technol 1989; 91*:* 363–66.

[16] Dusane DH, Nancharaiah YV, Zinjarde SS, Venugopalan VP. Rhamnolipid mediated disruption of marine *Bacillus pumilus* biofilms. Colloid surf B 2010; 81: 242-48.

[17] Dunne WM Jr. Bacterial adhesion: seen any good biofilms lately? Clin microbial rev 2002; 15(2), 155-166.

[18] Joshi-Navare K, Prabhune A. A biosurfactant sophorolipid acts in synergy with antibiotics to enhance their efficiency BioMed Res Int 2013; 1-8.

[19] Jing HU, Xiao-hui Z, Chun-yu Z, Feng-qing H, Jing H. Inhibitory effect and mechanisms of sophorolipids against *Staphylococcus aureus*. Food Sci 2012; 33:33–36.

[20] Kim KJ, Yoo D, Kim Y, Lee B, Shin D, Kim EK. Characteristics of sophorolipids as an antimicrobial agent. J Microbiol Biotechnol 2002; 12:235-41.

[21] Dengle-Pulate V, Chandorkar P, Bhagwat S, Prabhune AA. Antimicrobial and SEM Studies of Sophorolipids Synthesized Using Lauryl Alcohol. J Surfact Deterg 2014; 17:543-552.

[22] Desai JD, Banat IM. Microbial production of surfactants and their commercial potential. Microbiol Mol Biol Rev 1997; 61:47–64.

[23] Epstein AK, Pokroy B, Seminara A, Aizenberg J. Bacterial biofilm shows persistent resistance to liquid wetting and gas penetration. Proc Natl Acad Sci USA 2011;108:995–1000.

[24] Bueno J. Anti-biofilm drug susceptibility testing methods: looking for new strategies against resistance mechanism. J Microbial Biochem Technol 2014; S3:004. doi:10.4172/1948-5948.S3-004.

[25] Kotulova D, Slobodnikova L. Susceptibility of *Staphylococcus aureus* biofilms to vancomycin, gentamicin and rifampin. Epidemiol Mikrobiol Imunol 2010; 59:80–87.

[26] Girard LP, Ceri H, Gibb AP, Olson M, Sepandj F. MIC versus MBEC to determine the antibiotic sensitivity of *Staphylococcus aureus* in peritoneal dialysis peritonitis. Perit Dial Int 2010; 30:652–656.

[27] Leis AP, Schlicher S, Franke H, Strathmann M. Optically transparent porous medium for nondestructive studies of microbial biofilm architecture and transport dynamics. Appl Environ Microbio 2005; l71:4801–4808.

[28] Arun A, Murrugappan R, Ravindran A, Veeramanikandan V, Balaji S. Utilization of various industrial wastes for the production of poly-β-hydroxy butyrate (PHB) by *Alcaligenes eutrophus*. Afr J Biotechnol 2006; 5(17): 1524-1527.

[29] Sichwart S, Hetzler S, Bröker D, Steinbüchel A. Extension of the Substrate Utilization Range of *Ralstonia eutropha* Strain H16 by Metabolic Engineering To Include Mannose and Glucose. APPL ENVIRON MICROB 2011; 77. 4:1325-1334.

[30] Gumel AM, Annuar MSM, Chisti Y. Recent advances in the production, recovery and applications of polyhydroxyalkanoates. J Polym Environ 2013; 21: 580-605.

[31] Samorì C, Basaglia M, Casella S, Favaro L, Galletti P, Giorgini L, Marchi D, Mazzocchetti L, Torri C, Tagliavini E. Dimethyl carbonate and switchable anionic surfactants: two effective tools for the extraction of polyhydroxyalkanoates from microbial biomass. Green Chem 2014; DOI: 10.1039/c4gc01821d.

[32] Davey ME, Caiazza NC, O'Toole GA. Rhamnolipid surfactant production affects biofilm architecture in *Pseudomonas aeruginosa* PAO1. J Bacteriol 2003; 185:1027-1036.

[33] Kim M, Cho K, Ryu H, Lee E, Chang Y. Recovery of poly (3-hydroxybutyrate) from high cell density culture of *Ralstonia eutropha* by direct addition of sodium dodecyl sulfate. Biotechnol Lett 2003; 25: 55-59.

[34] Pereira MO, Machado I, Simões M, Vieira MJ. Preventing biofilm formation using surfactants. BiofilmClub© 2007; 167-74.

[35] Lemon, KP, Earl, AM, Vlamakis, H, Aguilar C, Kolter R. Biofilm Development with an Emphasis on *Bacillus subtilis*. Curr Top Microbiol Immunol 2008; 322: 1–16.

[36] Dusane DH, Nancharaiah YV, Zinjarde SS, Venugopalan VP. Rhamnolipid mediated disruption of marine *Bacillus pumilus* biofilms. Colloid surf B 2010; 81: 242-48.

[37] Mireles JR, Toguchi A, Harshey RM. *Salmonella enteric serovar typhimurium* swarming mutants with altered biofilm forming abilities: Surfactin inhibits biofilm formation. J. Bacteriol 2001; 183: 5848.

[38] Mireles JR, Toguchi A, Harshey RM. *Salmonella enteric serovar typhimurium* swarming mutants with altered biofilm forming abilities: Surfactin inhibits biofilm formation. J. Bacteriol 2001; 183: 5848.

[39] Busscher HJ, van der Kuij-Booij M, van der Mei HC. Biosurfactants from thermophilic dairy streptococci and their potential role in the fouling control of heat exchanger plates J. Ind. Microbiol. Biotechnol 1996; 16: 15.

[40] Joshi-Navare K, Prabhune A. A biosurfactant sophorolipid acts in synergy with antibiotics to enhance their efficiency BioMed Res Int 2013; 1-8.

[41] Jing HU, Xiao-hui Z, Chun-yu Z, Feng-qing H, Jing H. Inhibitory effect and mechanisms of sophorolipids against *Staphylococcus aureus*. Food Sci 2012; 33:33–36.

[42] McAuliffe L, Kokotovic B, Ayling RD, Nicholas RA. Molecular epidemiological analysis of Mycoplasma bovis isolates from the United Kingdom shows two genetically distinct clusters. J Clin Microbiol 2004; 42(10): 4556-4565.

[43] Bai GY, Brusseau ML, Miller RM. Biosurfactant enhanced removal of residual hydrocarbon from soil. J Cont Hydrol 1997; 25, 157.

[44] Davey ME, Caiazza NC, O'Toole GA. Rhamnolipid surfactant production affects biofilm architecture in *Pseudomonas aeruginosa* PAO1. J Bacteriol 2003; 185:1027-1036.

[45] Menichetti, F. Current and emerging serious Gram-positive infections. Clin Microbiol Infect 2005; 11(3): 22-28.

[46] Dengle-Pulate V, Chandorkar P, Bhagwat S, Prabhune AA. Antimicrobial and SEM Studies of Sophorolipids Synthesized Using Lauryl Alcohol. J Surfact Deterg 2014; 17:543-552.

[47] Irie Y, O'Toole GA, Yuk MH. *Pseudomonas aeruginosa* rhamnolipids disperse *Bordetella bronchiseptica* biofilms. FEMS Microbiol. Lett 2005; 250:237-43.

[48] Meers P, Neville M, Malinin V, Scotto AW, Sardaryan G, Kurumunda R, Mackinson C, James G, Fisher S, Perkins WR. Biofilm penetration, triggered release and in vivo activity of inhaled liposomal amikacin in chronic *Pseudomonas aeruginosa* lung infections. J antimicrob chemoth 2008; 61(4): 859-868