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Pseudomonas aeruginosa biofilm disruption using microbial surfactants

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Running title: Surfactants Biofilm Disruption

Abstract

Aims: To establish the ability of the rhamnolipids biosurfactants from *Pseudomonas aeruginosa*, in the presence and absence of caprylic acid and ascorbic acid, to disrupt bacterial biofilms, compared with the anionic alkyl sulfate surfactant Sodium dodecyl sulfate (SDS).

Methods and Results: *P. aeruginosa* ATCC 15442 biofilms were disrupted by rhamnolipids at concentrations between 0.5 and 0.4g/L and with SDS at 0.8g/L. The combination of rhamnolipids 0.4g/L and caprylic acid at 0.1 g/L showed a remarkable effect on biofilm disruption and cell killing. After 30 min of treatment most of the biofilm was disrupted and cell viability was significantly reduced. Neither caprylic acid nor ascorbic acid has any effect on biofilm disruption at 0.1g/L. SDS is an effective antimicrobial agent, however in the presence of caprylic acid its effect was neutralised.

Conclusions: The results show that rhamnolipids at low concentration in the presence of caprylic acid are promising molecules for inhibition/disruption of biofilms formed by *P. aeruginosa* ATCC 15442.

Significance and Impact of the Study: The disruption of biofilms has major significance in many industrial and domestic cleaning applications and in medical situations.

Introduction

Bacteria are able to sense their environment, process information, and react accordingly; however, their ability to sense their own cell density, to communicate with each other, and to behave as a population instead of individual cells has only recently been understood (Seshadri 2011). This phenomenon, called quorum-sensing (QS), has been described in many gram negative and gram positive bacteria. QS activities have been documented in biofilms for

some time (McLean *et al*. 1997; Davies *et al*. 1998;), although the magnitude of their role in biofilms depends on the nutritional environment (Shrout *et al*. 2006). During the process of biofilm development and maturation, surface-attached cells will aggregate into microcolonies that are surrounded by regions of few cells which are referred to as water channels (Davey and O'Toole 2000; Sauer *et al*. 2002). In the majority of natural environments, monospecies biofilms are relatively rare, rather micro-organisms are associated with surfaces in complex multispecies communities (James *et al*. 1995; Stoodley *et al*. 2002).

Biosurfactants of microbial origin are reported to have anti-adhesive and biofilm disruption abilities (Boles *et al*. 2005; Irie *et al*. 2005; Rodrigues *et al*. 2007; Diaz De R *et al*. 2015; Banat *et al*. 2015). Fungal and bacterial biofilms have also been shown to be disrupted by enzymatically synthesized surfactants such as lauroyl glucose (Dusane *et al*. 2010). In recent years, rhamnolipids derived from *Pseudomonas aeruginosa* have emerged as an important group of biosurfactants with a number of applications and as a result they have also been produced on a commercial scale (Banat *et al*. 2010; Marchant and Banat 2012a,b). Rhamnolipids play a role in the swarming process, acting both as surface wetting agents and as chemotaxis stimuli. In swarming but not swimming, rhamnolipids function as chemo-attractants whereas the chemically related, hydroxy alkanoic acids, which are metabolic precursors of the rhamnolipids, function as chemo-repellents (Tremblay *et al*. 2007).

Swarming can be blocked by branched chain fatty acids, which presumably compete with rhamnolipids (Inoue *et al*. 2008). Rhamnolipids are also important for the formation of water channels in mature biofilms as shown by Davey *et al*. (2003). Overproduction of rhamnolipids caused an inhibition of biofilm formation, blocked cellular aggregation, and also blocked secondary colonization onto preformed biofilms (Dusane *et al*. 2010) and

increased planktonic bacteria (Davey *et al*. 2003). Rhamnolipids have also been associated with cell dispersal from biofilms (Boles *et al*. 2005; Pamp and Tolker-Nielsen 2007). Limited information is available on the evaluation of biosurfactants against bacterial biofilms. The aim of this study was to determine the effect of rhamnolipids biosurfactants against bacterial biofilms in the presence of adjuvant compounds and compare their antimicrobial action with SDS, caprylic acid and ascorbic acid under static and flow/mobile continuous conditions. All the biosurfactants/chemical surfactants/adjuvants were tested against *P. aeruginosa* ATCC 15442.

Materials and methods

Microorganisms and Culture conditions

Pseudomonas aeruginosa ATCC 15442 was maintained in nutrient broth plus 200g/L glycerol at −20°C. Bacterial growth from a nutrient agar slant incubated for 24h at 30°C was used to obtain a bacterial suspension with an optical density at 570nm adjusted to give 10^8 cfu/mL.

Rhamnolipids Characteristics

The rhamnolipids containing 100 g/L mono-rhamnolipids ($C_{26}H_{48}O_9$, Molecular Weight (MW): 504, Critical Micelle Concentration (CMC):20mg/L at neutral pH) and 100g/L dirhamnolipids ($C_{32}H_{58}O_{13}$, MW: 650, CMC: 1.5×10^{-4} 30 mg/L at neutral pH) was obtained from Jeneil Biosurfactant Co. (Saukville, Wisconsin).

Growth and determination of the viability of Biofilms on coverslips

P. aeruginosa ATCC 15442 was grown overnight and diluted 100-fold with tryptic soy broth (TSB) 50% following which 2 mL samples were dispensed in triplicate to fill the 12 well plates, biofilms were formed on sterile, glass coverslips (18mm x 18mm) which were put into the 12 well plates (vertically) and incubated at 30°C for 48h (Diaz De Rienzo *et al*. 2015).

After a period of 48h, planktonic cells were removed and fresh medium (TSB 50%) was added containing different treatments (Phosphate-buffered saline (PBS) buffer 1X, rhamnolipids 0.4g/L, caprylic acid 0.1g/L and rhamnolipids 0.4g/L + caprylic acid 0.1g/L together). The plates were incubated at 30°C for 24h. The planktonic cells were discarded after the incubation time and the biofilms were stained with LIVE/DEAD® BacLight™ Bacterial Viability Kit (ThermoFisher Scientific), and the structure was observed in a fluorescence microscope with a 40x objective.

Growth of biofilm in flow cells.

Biofilms of *P. aeruginosa* ATCC 15442 were allowed to form in the flow cell system. The system comprised a flow cell that served as a growth chamber for the biofilm. The flow cell was supplied with nutrients and oxygen from a medium flask containing 50% TSB via a peristaltic pump (mL/h/channel) and spent medium was collected in a waste container. A bubble trapping device confined air bubbles from the tubing which otherwise could disrupt the biofilm structure in the flow cell. After 48 h of incubation at 30°C the medium was replaced with different treatments (Phosphate-buffered saline (PBS) buffer 1X, rhamnolipids 0.4g/L, caprylic acid 0.1g/L and rhamnolipids 0.1g/L + caprylic acid 0.1g/L together) for 30 min. After treatment the cells were stained with LIVE/DEAD® BacLight™ Bacterial Viability Kit and observed using a Leica SP5 inverted confocal microscope, providing highly detailed 3D information about developing microbial biofilms using FiJi (Schindelin *et al.* 2012).

Biofilm growth on the BioFlux flowthrough device.

To analyze biofilm formation under flow conditions, the BioFlux 200 system (Fluxion Biosciences Inc., South San Francisco, CA) was used which allows automated image acquisition within specialized multi-well plates. To grow biofilms, the microfluidic channels

(depth, 75µm; width, 350µm) were primed with TSB (50%) at 279µL/min. Channels were seeded with 10^7 CFU from an overnight culture of *P. aeruginosa* ATCC 15442. The plate was then incubated at 30°C for 48h to allow cells to adhere. After biofilms had formed, planktonic cells were removed, and PBS 1X (as control) and different treatments (rhamnolipids 0.4g/L, caprylic acid 0.1g/L, rhamnolipids 0.4g/L + caprylic acid 0.1g/L together, SDS 0.8g/L, SDS 0.8g/L + caprylic acid 0.2g/L together) were added to the input wells at a flow rate of 279µL/h for 30 min. The results were recorded with images provided to a microscope Evon (10x objective) (17% Light)

Growth of microtiter plate "static" biofilms.

P. aeruginosa ATCC 15442 was grown overnight as previously described and diluted 100-fold with TSB 50% following which a 100µL sample of each diluted culture was dispensed (four replicates) to 96 well plates, biofilms were formed in a fresh microtiter plate after 48h of incubation at 30°C. After a period of 48h, planktonic cells were removed and fresh medium (TSB 50%) was added containing different treatments (Table 1). The plates were incubated at 30°C for another 24h. The planktonic cells were discarded after the incubation time and the biofilms were stained with 10g/L aqueous crystal violet for 10 min, washed with deionized water, and resuspended in 200µL of 95% ethanol. The optical density at 600nm was recorded using a Filter-based multi-mode microplate reader loaded with the program FLUOstar Omega, by BMG LABTECH®.

Statistical analysis

Statistical analysis was carried out using the SPSS 19.0 program. To determine the significant differences between the results obtained with the different biosurfactants a one way ANOVA was performed together with a Dunnett Multiple Comparison test.

Results

Effect of different treatments on Biofilms of *Pseudomonas aeruginosa* ATCC 15442 on coverslips

Biofilm formation was evaluated microscopically after 48h. Fluorescence microscopy examination of cells attached to coverslips and stained with LIVE/DEAD® BacLight™ Bacterial Viability Kit 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 1A). In agreement with previous studies (Davey *et al.* 2003; Schooling *et al*. 2004) we are able to confirm here that *P. aeruginosa* ATCC 15442 biofilm cells are sensitive, to some extent, to rhamnolipids as seen by the reduction in the number of active cells after exposure and the appearance of some inactive reddish brown fluorescing cells (Fig 1B). The presence of an adjuvant i.e. caprylic acid was also investigated. Figure 1C shows that there is no observable effect with caprylic acid alone while when added together with the rhamnolipids a noticeable effect was observed on the viability of the biofilm dispersion (Fig. 1D). In the present experiments, the combination of rhamnolipids and caprylic acid at lower concentration (0.4 and 0.1g/L respectively) showed an effect on biofilm disruption of *P. aeruginosa* ATCC 15442 as well as a reduction in cell viability (Fig. 1D).

Effect of different treatments on Biofilms of *Pseudomonas aeruginosa* ATCC 15442 in flow cells

To illustrate that biofilm formation and disruption can be mediated by rhamnolipids and caprylic acid, the cells were then stained with LIVE/DEAD® BacLight™ Bacterial Viability Kit and confocal microscopy was used. Biofilm was grown for 2 days in continuous flow mode in the flow-cell channel. Prior to the addition of each treatment, a well-developed biofilm was observed (Fig. 2A), the thickness of the biofilm was about 40µm. After 30 min contact with rhamnolipids (0.4g/L) and rhamnolipids plus caprylic acid (0.04

and 0.1g/L respectively), the structure of the biofilm was visibly affected (Fig. 2B and 2C) showing an important part of the biofilm compromised by damaged cells. The possible synergistic effect between rhamnolipids and caprylic acid was evaluated through the Bioflux device as well as using static conditions.

Effect of different treatments on pre-formed biofilms by *Pseudomonas aeruginosa* ATCC 15442 in the BioFlux flowthrough device.

Studies have been directed toward utilizing metal-binding chelators that have the capability to inhibit bacterial growth by disrupting surface adherence and preventing biofilm formation. In this study, the effect of rhamnolipids, caprylic acid and SDS on biofilms of *P. aeruginosa* ATCC 15442 was determined under Bioflux flowthrough conditions (Fig. 3.). Under these conditions, the effect of caprylic acid together with rhamnolipids was confirmed. Phase contrast images before and after treatment for each set of experiments, showed that even when the thickness of the preformed biofilm is different the treatments have different impacts on the biofilm dispersal. When a preformed biofilm of *P. aeruginosa* is treated with a solution of PBS 1X (Fig. 3B), there is no apparent effect, while when it is treated with rhamnolipids alone (Fig. 3D) or in combination with caprylic acid (Fig. 3H) the disruption is appreciable, a similar effect is evident in the presence of SDS (Fig. 3J). Interestingly, there seems to be an inhibitory effect on biofilm disruption when the SDS is in a mixture with caprylic acid (Fig. 3L).

Evaluation of the growth of microtiter plate biofilms

Biofilms were grown in 96-well microtiter plates for 48 h, treated with different treatments (Table 1) in quadruplicate and stained with crystal violet 10g/L. Optical density values were significantly different for the biofilm in the presence of the different treatments with respect to the control (Fig. 4). The highest impact was in the presence of the rhamnolipids plus

caprylic acid. In the presence of ascorbic acid on its own or together with rhamnolipids, no changes were detected; which could indicate that once the biofilm is formed the chelator effect of the ascorbic acid is insignificant, and its capacity to inhibit microbial growth is through disrupting the surface adherence and not after the cells are already attached to the surface. This data supports the results obtained through the Bioflux device, indicating a possible synergistic effect between rhamnolipids and caprylic acid as biofilm disruptor.

Discussion

Under the experimental conditions used in this study we have been able to show that *Pseudomonas aeruginosa* ATCC 15442 was able to form biofilms similar to those described for other *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* strains (Dunne 2002). It is now generally recognized that biofilms are heterogeneous structures (McAuliffe *et al.* 2004) which display a high degree of resistance to antibiotic and biocide treatments (Govan and Deretic 1996) and this resistance to antimicrobial agents is intimately related to the inherent three dimensional organization of cells and exopolymeric matrix which result from multifactorial processes.

The possible effects of surfactant molecules, both chemical surfactants and biosurfactants, on bacterial cells have been extensively reported. Bai *et al.* (1997) had previously associated rhamnolipids 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 *et al.* (2001) demonstrated that a range of surfactants (rhamnolipids, 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.

The results we have obtained demonstrating the inhibitory effect of rhamnolipids alone on biofilm architecture are similar to those reported by Davey and coworkers (2003), although different media, strains and means of growing biofilms were used, similar conclusions were drawn. Their main conclusions were that rhamnolipids (in a similar concentration range of 162.5 mg/L to that used in this study) interfered with both cell–cell interactions and cell–substratum interactions. Both of these conclusions are in agreement with the observations reported here. However, the effect of caprylic acid has not been previously reported. Caprylic acid is the common name for the eight-carbon saturated fatty acid called octanoic acid. Caprylic acid is used commercially in the production of esters used in perfumery and also in the manufacture of dyes (Beare-Rogers *et al*. 2001). Caprylic acid is also used in the treatment of some bacterial infections. Due to its relatively short chain length it has no difficulty in penetrating fatty cell wall membranes, hence its effectiveness in combating certain lipid-coated bacteria, such as *Staphylococcus aureus* and various species of *Streptococcus* (Nair *et al*. 2005).

The efficiency of biofilm disruption when rhamnolipids and caprylic acid are used together may be a result of a synergistic effect between them. First the rhamnolipids could be involved in the removal of extracellular polymeric substances (EPS) and destruction of microcolonies, which accords with the results obtained by Schooling *et al*. (2004). Once the overall biofilm environment is altered by the activity of the surface active agent (rhamnolipids) cells are more sensitive to the intervention of the caprylic acid which is able to pass through the cell membrane causing the death of the cell. It has also been demonstrated that the cis-2-docenoic acid, can induce cell detachment from biofilms; interestingly, cis-2-decenoic acid displays biofilm-dispersing effects on both Gram positive and Gram negative bacteria, suggesting that short and mono-unsaturated fatty acids act as "broad spectrum" signal molecules (Davies and Marques 2009). In summary, biosurfactants,

either alone or in combination with antimicrobial agents, can be used to remove or significantly reduce detrimental biofilms.

As suggested above the removal of EPS, could be beneficial in the treatment of biofilms. Such removal of the EPS matrix has been reported when *P. aeruginosa* was treated with mono-rhamnolipids. The authors observed that the outer cell membrane lipopolysaccharide protein complexes were removed by the rhamnolipids (Al-Tahhan *et al*. 2000). Interestingly, the removal of the EPS cover is known to enhance the effect of antimicrobial agents and disinfectants. The EPS removal in the present case was probably aided by the surfactant activity of the rhamnolipids. Apparently, pre-treatment of biofilms for 30 min would make them significantly more susceptible to chemicals such as caprylic acid.

Biofilm dispersal can occur as a consequence of mechanical breakage of biofilms due to flow or shear stresses. Often, however, dispersal is induced by the biofilm itself in response to environmental cues, such as changes in nutrient availability (Sauer *et al*. 2004; Gjermansen *et al*. 2005). There are a lot of molecules involved in the processes of microbial adherence, biofilm formation, and bacterial growth; metallic cations are essential for all these process. In this context we have investigated the use of ascorbic acid alone and in combination with rhamnolipids in the disruption of biofilms. It is clear from our results that ascorbic acid does not significantly affect established biofilm and that any benefit would only accrue from disrupting the initial stages of cell to surface adherence. These results are in agreement with those of Oosterhof *et al*. (2003), who demonstrated that ascorbic acid is unable to remove the EPS involved in biofilm formation on voice prostheses used to rehabilitate speech in laryngectomized patients, which results in the same high flow resistance impeding speech.

The anionic surfactant SDS, a common ingredient in dental cleaning agents, exhibits bactericidal activity against numerous oral bacteria (Drake *et al*. 1992; Wade and Addy 1992). SDS is thought to kill bacteria by penetrating the cytoplasmic membrane and causing cell lysis (Adair *et al*. 1979). The SDS has a clear effect on *P. aeruginosa* ATCC 15442 biofilm disruption at 0.8g/L (Fig 3J) in comparison to those treated with PBS 1X, SDS has also been shown to kill planktonic *Aggregatibacter actinomycetemcomitans* cells at a minimum inhibitory concentration (MIC) of 0.1g/L (Drake *et al*. 1992; Wade and Addy 1992). Since SDS causes protein unfolding at concentrations above its critical micelle concentration (Otzen 2002), these findings suggest that SDS mediated biofilm detachment results from the denaturation of proteinaceous matrix adhesions. Interestingly, when the SDS is present with caprylic acid (Fig. 3L) no effect was detected. Apparently the caprylic acid is capable of masking the action of SDS, shielding the contact between the SDS and the cell membrane, and it may act as a diffusion barrier that prevents SDS from entering the biofilm, consequently allowing the maintenance of the biofilm structure. Thus, the mechanisms by which dispersal is mediated are numerous, complex and not fully characterized, but this work provides a good approach to discover the possible mode of interaction between surfactants and small and long chain acids in the maintenance/disruption of biofilms formed by *P. aeruginosa* ATCC 15442.

We therefore conclude that rhamnolipids were effective antimicrobial agents in the presence of caprylic acid at pH 5 as they induced cell death and disruption of biofilm better than conventional antimicrobials such as SDS (at the concentration used in this study). The results show that rhamnolipids are promising molecules for commercial applications in a wide range of products. However, there is a need for the development of suitable, economic production systems to provide the quantities of biosurfactant required for high volume consumer products.

Conflicts of Interest

The authors declare no conflict of interest related to this work which has already been fully available to Unilever.

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Figure Legends

Fig. 1. Biofilm formation by *P. aeruginosa* ATCC 15442 on coverslips. Cells were stained with LIVE/DEAD® BacLight™ Bacterial Viability Kit and observed using a fluorescence microscope at 40×. The bar represents 20µm for all images. (**A**) Control. (**B**) In the presence of rhamnolipids 0.4g/L. (**C**) In the presence of caprylic acid 0.1g/L. (**D**) In the presence of rhamnolipid (0.4g/L) and caprylic acid (0.1g/L).

Fig.2. Effect of rhamnolipids in the presence and absence of caprylic acid on biofilm disruption by *P. aeruginosa* ATCC 15442 in flow cells. The biofilm formation under control conditions remains stable after 30min in the presence of PBS 1X (A) showing a thickness of about 40µm. In presence of rhamnolipids the cell viability is affected (B), however after 30min there are still live cells. In the presence of both molecules: rhamnolipids and caprylic acid the

effect is more accentuated (C), where an important proportion of the cells are damaged and scattered. Cells were stained with LIVE/DEAD® BacLight™ Bacterial Viability Kit. The images were analized using FiJi, with the volume viewer 2.01., a scale of 4.0 was used for images (**A**) and (**B**), and 1.76 for (C). The green color is an indicator of live cells, and the red is an indicator of those cells whose membranes have been compromised/damage. The bar represents 10 μm for images (**A**) and (**B**), and 120 μm for (**C**).

Fig3. Biofilm formation and development in a BioFlux channel by *P. aeruginosa* ATCC 15442. Each phase contrast image on the left hand side represents an individual experiment treated with a specific biosurfactant/surfactant (right hand side images). (A) Control (48h Biofilm). (B) In the presence of PBS 1X. (C) Control (48h Biofilm). (D) In the presence of rhamnolipids 0.4g/L. (E) Control (48h Biofilm). (F) In the presence of caprylic acid 0.1g/L. (G). Control (48h Biofilm). (H) In the presence of rhamnolipids (0.4g/L) and caprylic acid (0.1g/L). (I) Control (48h Biofilm). (J) In the presence of SDS 0.8g/L. (K) Control (48h Biofilm). (L) In the presence of SDS (0.8g/L) and caprylic acid (0.2g/L).

Fig. 4. Antimicrobial activity of rhamnolipids, caprylic acid and ascorbic acid against *P. aeruginosa* ATCC 15442 biofilms on static conditions. All the treatments applied are as follows: **1**. Control (No treatment), **2**. Rhamnolipids 5g/L, **3**. Rhamnolipids 4g/L, **4**. Rhamnolipids 1g/L, **5**. Rhamnolipids 0.8g/L, **6**. Rhamnolipids 0.5g/L, **7**. Rhamnolipids 0.4g/L, **8**. Caprylic acid 8g/L, **9**. Caprylic acid 1g/L, **10**. Caprylic acid 0.8g/L, **11**. Caprylic acid 0.2g/L, **12**. Caprylic acid 0.1g/L, **13**. Rhamnolipids 4g/L-Caprylic acid 1g/L, **14**. Rhamnolipids 0.8g/L-Caprylic acid 0.2g/L, **15**. Rhamnolipids 0.4g/L-Caprylic acid 0.1g/L, **16.** Ascorbic acid 8g/L, **17**. Ascorbic acid 1g/L, **18**. Ascorbic acid 0.8g/L, **19**. Ascorbic acid 0.2g/L. **A, B. C** and **D** correspond to the analogue effect shown using the same treatment in the Bioflux Device. Each percentage value represents g/100mL. Error bars are standard deviation from 8 independent experiments. $p < 0.05$ (***) value was taken to indicate statistical significance, Dunnett's test.

Table 1. Biosurfactant/Adjuvant combinations used for biofilm disruption

TREATMENT	CONCENTRATION (g/L)
Rhamnolipids	10.0
Rhamnolipids	5.0
Rhamnolipids	4.0
Rhamnolipids	1.0
Rhamnolipids	0.8
Rhamnolipids	0.5
Rhamnolipids	0.4
Caprylic acid*	10.0
Caprylic acid*	8.0
Caprylic acid*	1.0
Caprylic acid*	0.8
Caprylic acid*	0.2
Caprylic acid*	0.1
Rhamnolipids/ Caprylic acid	4.0/1.0
Rhamnolipids/ Caprylic acid	0.8/0.2
Rhamnolipids/ Caprylic acid	0.4/0.1
Abscobic acid*	8.0
Abscobic acid*	1.0
Abscobic acid*	0.8
Abscobic acid*	0.2
Abscobic acid*	0.1
Rhamnolipids/ Ascorbic acid	4/1
Rhamnolipids/ Ascorbic acid	0.8/0.2
Rhamnolipids/ Ascorbic acid	0.4/0.1
SDS *	0.8
SDS/Caprylic acid	0.8/0.2
SDS/Ascorbic acid	0.8/0.2

*Chemicals supplied by Sigma-Aldrich Co. LLC. (United Kingdom)

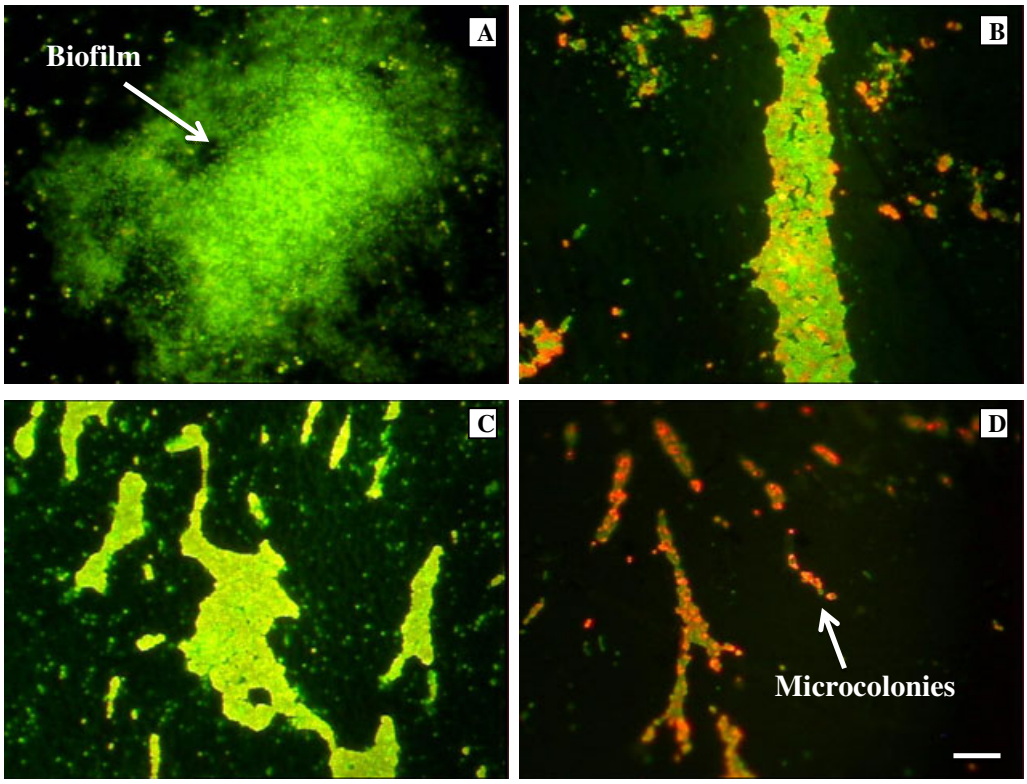

Fig. 1

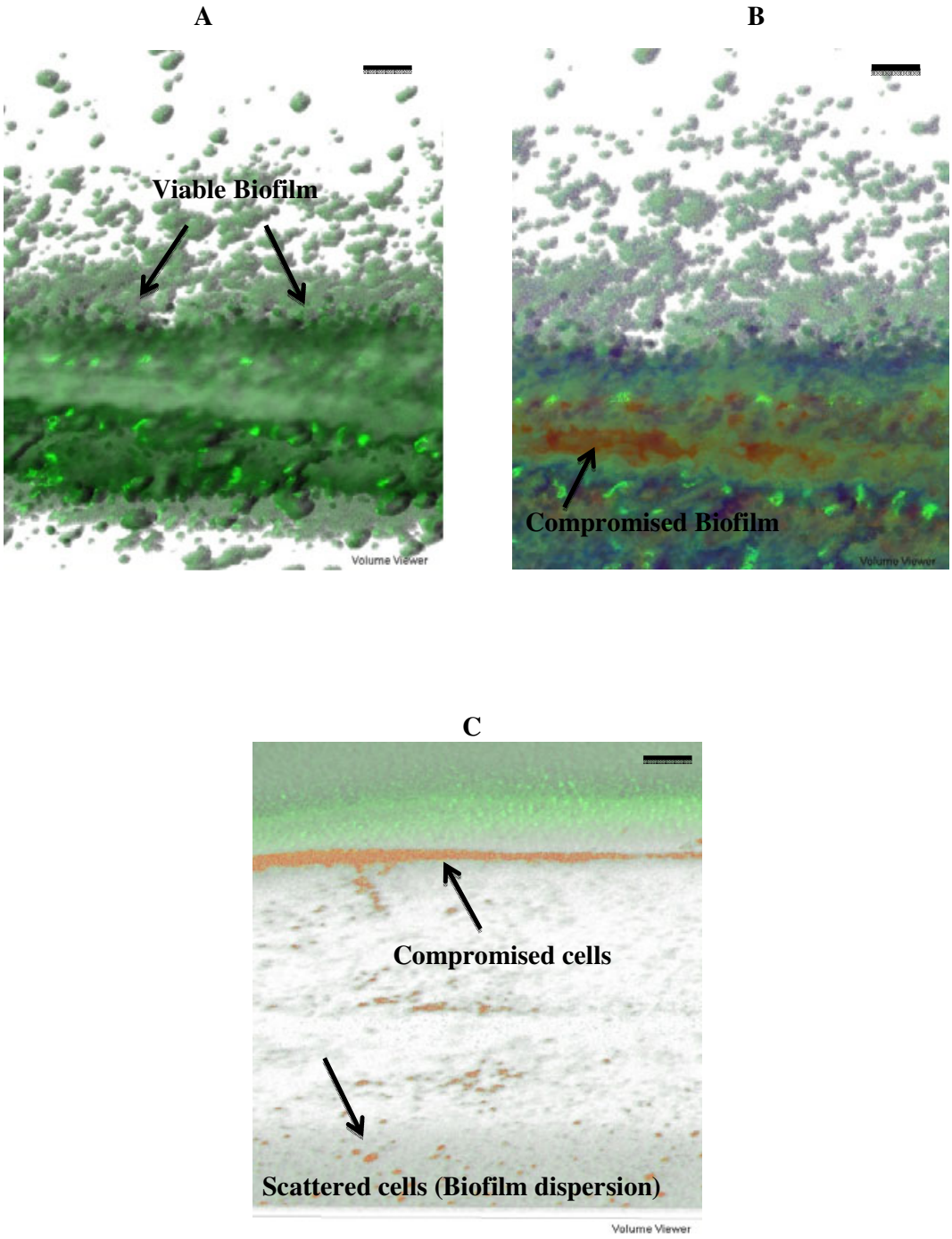

Fig. 2

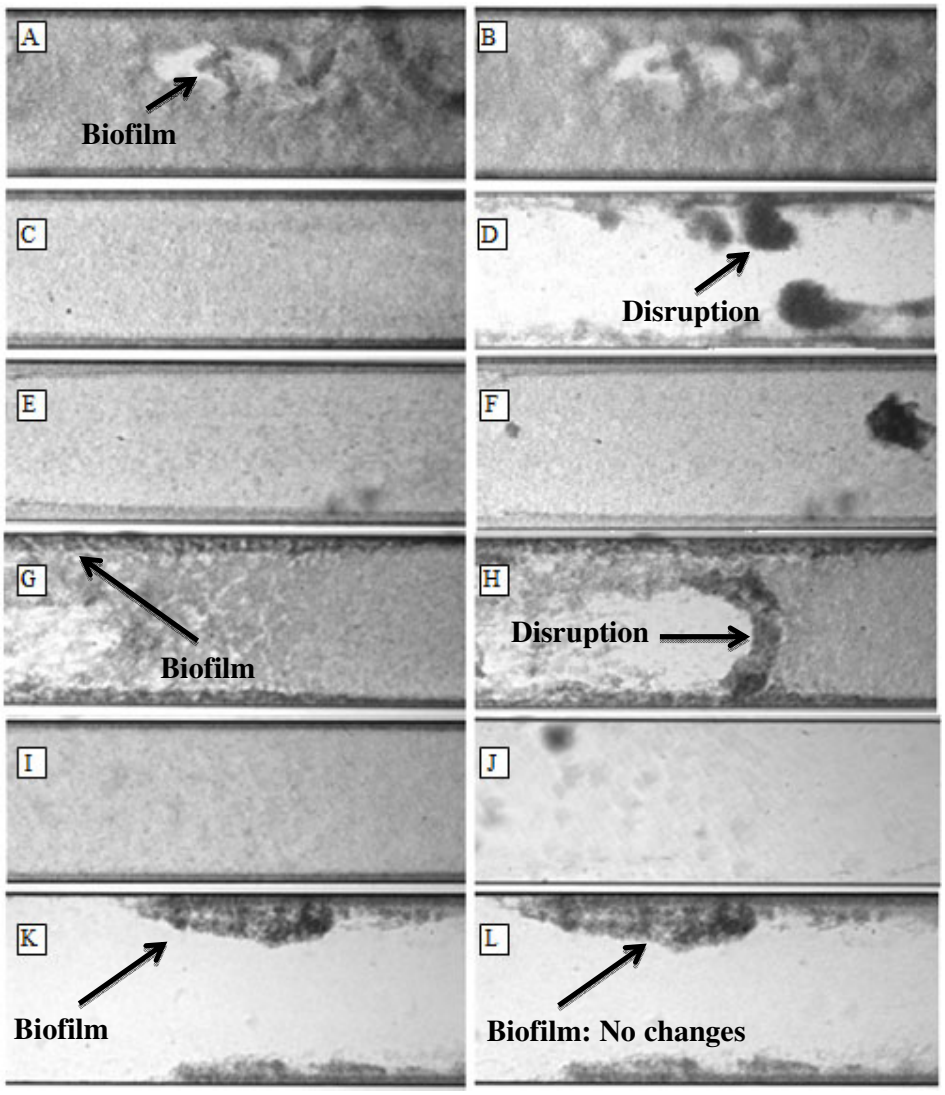

Fig. 3

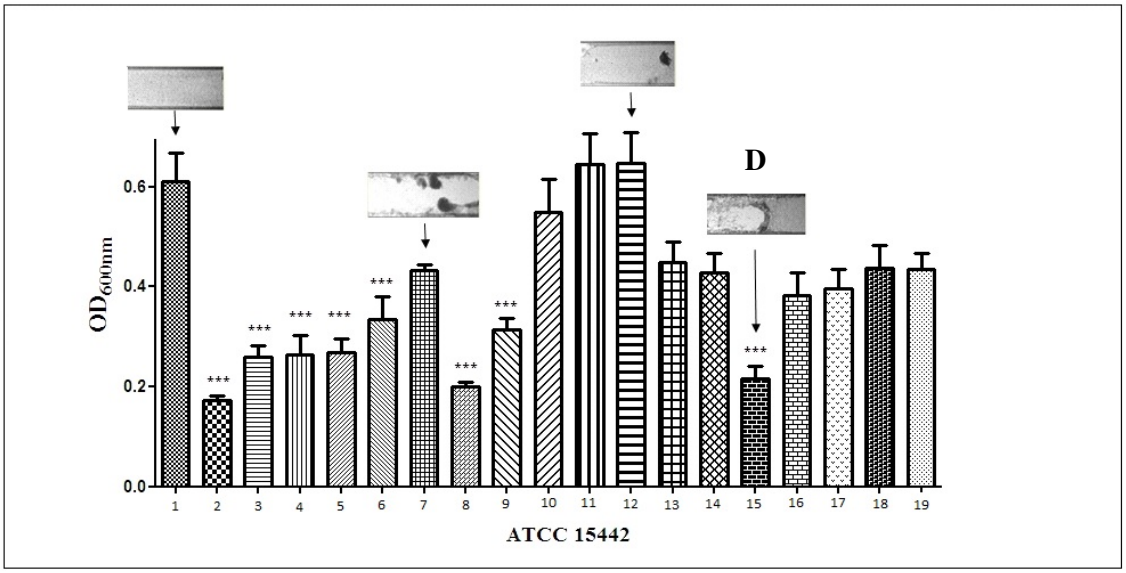

Fig. 4