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Use of electrical resistance tomography (ERT) for the detection of biofilm disruption mediated by biosurfactants.

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ABSTRACT

Inline measurement of biofilms could provide a valuable technology for water, food and bioprocessing industries to improve quality control and avoid contamination. This study presents the first use of electrical resistance tomography (ERT) to detect the removal of biofilms in a pipe. It also tests the effectiveness of sophorolipids and rhamnolipids for the disruption of Bacillus subtilis BBK006 biofilms in an industrial setting. Biofilms were grown on the inner side of a section of 1.5" test pipe for 5 days using nutrient broth as the culture medium. After the medium was removed the test pipe was incorporated into a cleaning test circuit for the biofilm disruption experiment, where water, sophorolipids (0.4 g L^{-1}) or rhamnolipids (0.4g L^{-1}) solutions were pumped through respectively for 30 minutes. ERT was used as an indirect evaluation of the film disruption progression. A cleaning parameter was identified based on zonal boundary averages which successfully measured the extent of biofilm removal.

Keywords: ERT; tomography; sophorolipids; rhamnolipids; biofilm; Bacillus**.**

1. Introduction

Biosurfactants are amphiphilic compounds produced on living surfaces, mostly on microbial cells or excreted extracellular hydrophobic and hydrophilic moieties. Their global production was about 17 million tons in 2000, with a market value worth USD 1,735.5 million in 2011 (Silva et al., 2014). Several industrial applications including detergents, agricultural chemicals and textiles, have been reported. More recently, biosurfactants have been considered as promising candidates for the inhibition of microbial biofilms (De Rienzo & Martin, 2016).

Microbial biofilms can be found on almost all natural and artificial surfaces that are submerged in or exposed to an aqueous solution. Given sufficient resources for growth, biofilms may grow

rapidly into macroscopic scales even in very hostile environments. Although studies are made to exploit some of the beneficial side of the biofilm growth, such as fermentation, effluent processing, and microbial fuel cell development etc., in most cases, biofilms are shown to be detrimental. They can result in clogging, equipment failure, corrosion or contamination in an industrial production line (e.g. Cunault et al., 2015). There has been continuous and focused research recently into sourcing new generations of bacterial dispersal agents to develop more effective bacterial inhibition strategies as complete mechanical removal by hydrodynamic forces alone can be challenging (e.g. Paul et al., 2012; Lemos et al., 2015). Novel inline and non-destructive characterization techniques are in demand for insitu measurement of biofilms (Janknecht & Melo, 2003; Valladares Linares et al., 2016; Wang et al., 2018).

We have reported before that pre-formed biofilms by different microorganisms can be disrupted by biosurfactants (De Rienzo et al., 2015; De Rienzo & Martin, 2016). The investigations also demonstrated the effect of mono and di-rhamnolipids against biofilms formed by mixed cultures under dynamic flow conditions. The current work uses two techniques to evaluate the effect of rhamnolipids and sophorolipids on the attachment and disruption of biofilm cells produced by a mixed culture: offline direct SEM analysis of an inner pipe surface combined with inline electrical resistance tomography (ERT).

ERT is an industrially proven non-intrusive imaging technique, which is commonly used to monitor processes requiring both temporal and special resolutions. The system typically consists of a ring of electrodes evenly spaced around the circumference of a tank or pipe (i.e., domain). Alternating electrical currents are injected into the fluid inside the domain. A cross sectional map of the electrical conductivity, which relates to the material composition inside the domain, can then be reconstructed based on the voltages measured on the boundary

electrodes. ERT has been successfully used to monitor and control a wide range of engineering processes, including mixing (Rodgers & Kowalski, 2010), separations (Kowalski et al., 2010) and more recently inline monitoring of cleaning in place (CIP) (Henningsson et al. 2007; Sharifi & Young, 2013; Hou et al., 2016). However, to the authors' best knowledge, the current investigation represents the first attempt to use the ERT method for monitoring the biofilm disruption process.

Materials and methods

2.1 Bacteria strains and culture conditions

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^{-1} glucose, 1% w v^{-1} yeast extract, 0.1% w v^{-1} 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.

Burkholderia thailandensis E264 was maintained on nutrient agar slants at 4 °C, each slant was used to obtain a bacterial suspension, with the optical density (570 nm) adjusted to give 10^7 CFU mL^{-1} for each of the strains used. The standard medium for the production of rhamnolipids by B. thailandensis E264 was nutrient broth (NB) (8 g L^{-1}), with glycerol (20 g L^{-1}). For the biofilm experiments Bacillus subtilis BBK006 and Staphylococcus sp. were stored in nutrient broth plus 20 % glycerol at −80 °C, and used when needed.

2.2 Production of rhamnolipids (RLs)

An Electrolab FerMac 360 fermentation unit was used to perform batch cultivation of B. thailandensis E264. The microorganisms used in this study was aerobically (0.5 vvm)

incubated in nutrient broth, at 30 °C, during 120 h at 400 rpm. Rhamnolipids were extracted using a foam fractionation system (De Rienzo et al., 2016).

2.3 Production of sophorolipids (SLs)

A crude sophorolipid mixture was obtained as the settled product from fed batch cultivation of C. bombicola ATCC 22214, operated without the use of antifoam (Shah et al., 2005) 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^{-1}.

2.4 The test pipe section, ERT electrodes, and growth of biofilm

In this investigation, the biofilm growth and disruption experiments were all conducted on a section of 1.5" nominal sized transparent acrylic pipe. The test pipe section was also fitted with a single plane of 16 electrodes for the ERT measurements. The electrodes were all made of M5 threaded stainless steel rods. They were installed at equal interval around the pipe circumference, with one end flush with the inner surface of the test pipe. Such a configuration ensured that the ERT electrodes were in good electrical contact all the time with the process fluid flowing inside the test pipe section. Note that the electrodes also provided a source of active surfaces for the micro-organisms and biofilms to grow.

For the biofilm growth, the inner side of the circular test pipe section was first inoculated with 50 mL of nutrient broth containing 10^7 colony forming units (CFU) from an overnight culture of each microorganism. It was then incubated at 30 °C for 5 days to allow cells to adhere, with a daily change of the medium to maintain the bacteria viability. Afterwards, the medium was removed, and the test pipe section was ready to be inserted into the cleaning test circuit for the disruption experiments.

2.5 Cleaning test circuit, disruption experiments, and ERT measurements

The test circuit consisted of a 50 L stainless steel cleaning liquid holding tank, a 1.5" lobe pump (Fristam FKL 25, USA) to pump the cleaning liquid, a Coriolis meter (Micro Motion R100, USA) to monitor and regulate the flowrate, and 1.5" pipework including the test pipe section. In particular, the test pipe section was mounted upright, with the cleaning liquid flowing upwards. This was to ensure that the test pipe was fully filled with the cleaning liquid all the time, so that steady ERT signals could be collected throughout a film disruption experiment.

A control ERT pipe section (ERT plane 1, P1) was mounted immediately below the test ERT pipe section (ERT plane 2, P2) in the vertical length of the loop such that the control section was upstream of the biofilm test section. Further images and details of the ERT pipe sections and the ERT methodology were reported by Hou et al., 2016.

Each disruption experiment comprised of three stages, 1) pre rinse, in which clean tap water was loaded into the holding tank and pumped through the test pipe section at a flowrate of 60 kg h^{-1} for around 30 minutes; 2) biosurfactant solution wash, in which an aqueous solution containing 0.4g L^{-1} of either rhamnolipid or sophorolipid was loaded into the holding tank and pumped through the test pipe section at 60 kg h^{-1} for around 30 minutes; and 3) post rinse, in which clean tap water was loaded into the holding tank again and pumped through the test pipe section at 60kg h^{-1} for at least 15 minutes. This flow rate equated to an average velocity of 0.015 m s^{-1} with Re = 560. For laminar flow the friction factor was 0.029 and thus the wall shear stress 0.0031 Pa.

An ITS P2000 ERT system with the ITS P2+ software (version 7.3) (Industrial Tomography Systems, Manchester, UK) was employed to monitor the film disruption process. The instrument permits only the adjacent current injection pattern. The optimum alternating injection current was found to be 5 mA at 9600 Hz. For a single circular 16 electrode plane, this led to 104 independent boundary voltage measurements. The circular domain was then discretised evenly into 316 square pixels by the built-in software, and the conductivity value within each pixel was 'recovered' using a sensitivity coefficient linear back-projection algorithm, based on the current injection magnitude and boundary voltage measurements.

2.6 Scanning electron microscopy (SEM) sample preparation

Stain steel coupons (M8) with adhered cells after each treatment were rinsed with PBS 1X (8.0 g of NaCl, 0.2 g of KCl, 1.4 g of $Na_2HPO_4 \cdot 2H_2O$, and 0.2 g of KH_2PO_4 per liter, pH 7.2). and the cells were 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 and subsequently the cells were observed under SEM.

3. Results and discussion

3.1 Cell attachment to the steel M8

The untreated cells as well as those treated with sophorolipids and rhamnolipids were examined by SEM to visualize the disruptive effect of biosurfactants on the biofilms formed by mixed cultures. Figure 1A shows the cellular structures attached to the steel M8 after 5 days of growing, without being in contact with any disruptive molecule (control). From the microorganisms consortium were detected on SEM images, extracellular polymeric substance (EPS) and several monolayers of cells. After treatment with sophorolipids only monolayers of

cells are observed (Figure 1B) and there is a visible loss of the EPS, however in the presence of rhamnolipids the number of cells is higher (Figure 1C), and the persistence of biofilms is apparent.

In this study, sophorolipids induced disruption on mature biofilms indicating a possible interaction of sophorolipids with the cellular membrane, increasing permeability and a further removal of EPS, regardless of the shear stress and the flow conditions, which is consistent with previous studies (De Rienzo et al., 2016). The effect of rhamnolipids, under the conditions tested in this study, appear less remarkable. Rhamnolipids have been reported as a disruptive molecule against B. subtilis BBK006, under flow conditions (De Rienzo et al., 2016), however the maturity of biofilms used in that study was 48 h.

3.2 Electrical Resistivity Tomography

Figure 2 shows selected tomograms for the control plane (P1) and biofilm plane (P2) for both the sophorolipid and rhamnolipid wash cycles. The tomograms show 2D conductivity maps relative to the first frame (with flowing clean water) which was taken as the reference point. P1 tomograms remained largely constant over the wash cycles as was expected. P2 tomograms showed a relative increase in conductivity at the edge and in the core over the wash cycles. An increase in conductivity at the edge was indicative of biofilm removal and hence increased conductance in that region. Increases in core conductivity are indicative of associated linear back-projection reconstruction errors (Stephenson et al. 2008).

Mean conductivities for the two-pixel boundary zone and remaining core zone were calculated and these are also presented in Figure 2. The cleaning profile in Figure 2 A shows a rapid

deviation of the biofilm P2 from the control P1 which was indicative of biofilm removal in the first water rinse phase. Figure 2 B shows that the biofilm P1 and control P2 zonal mean conductivities are closely matched over the first water-rinse phase and starts to deviate during the rhamnolipid wash phase.

A degree of cleaning parameter, ϕ, was defined as the difference between boundary zone mean conductivity for the biofilm plane P2, $\sigma_{2,\ \text{edge}}$, and that of the control plane P1, $\sigma_{1,\ \text{edge}}$, normalised by the core zone mean conductivity of the control plane P1, $\sigma_{1,\ \text{core}}$, such that:

$$\phi = \left(\sigma_{2,\text{edge}} - \sigma_{1,\text{edge}}\right) \Big/ \sigma_{1,\text{core}} \qquad (1)$$

Figure 3 presents the degree of cleaning for the two rinse-wash-rinse cycles. It is clear that the biofilm in the first case was mainly cleaned during the initial rinse phase where a rapid increase was seen over the first 300 s and little further increase was measured over the sophorolipid solution wash phase. In contrast, the biofilm in the second case was not cleaned in the initial rinse phase, but started to become cleaned by the rhamnolipid solution wash phase which then continued into the final water rinse phase.

These findings corroborate the biofilm images in Figure 1, although the difference in cleaning between the two cycles appears to be due to irreproducibility of the biofilm creation than in the cleaning effectiveness of the different biosurfactant solutions.

4. Conclusions

For the first time ERT has been shown to be an effective tool for the monitoring of the presence of microorganism biofilms residents inside a pipe or vessel, information that could be useful for studies of control processes within the water or food industries for example. A cleaning parameter was identified based on zonal boundary averages which successfully measured the extent of biofilm removal.

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Figures

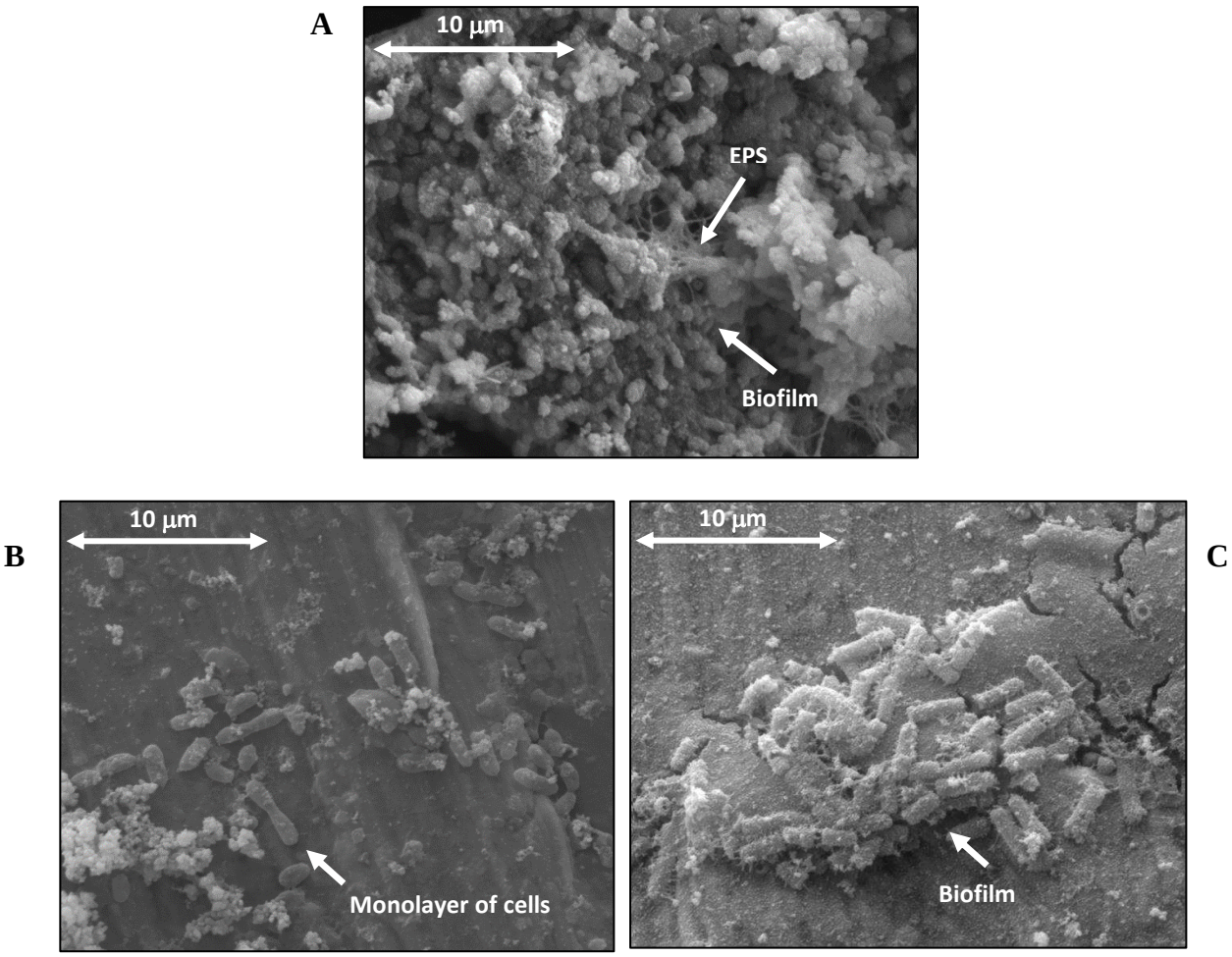

Figure 1. Scanning electron micrographs showing attachment and biofilm formation **A**. Untreated cells (control) showing the EPS substance within the mixed culture. **B**. Surface following sophorolipid 0.4 g/L rinse-wash-rinse cycle, showing dispersed mono layers of cells. **C**. Surface following rhamnolipid 0.4g/L rinse-wash-rinse cycle, showing less thick biofilms in comparison with the control.

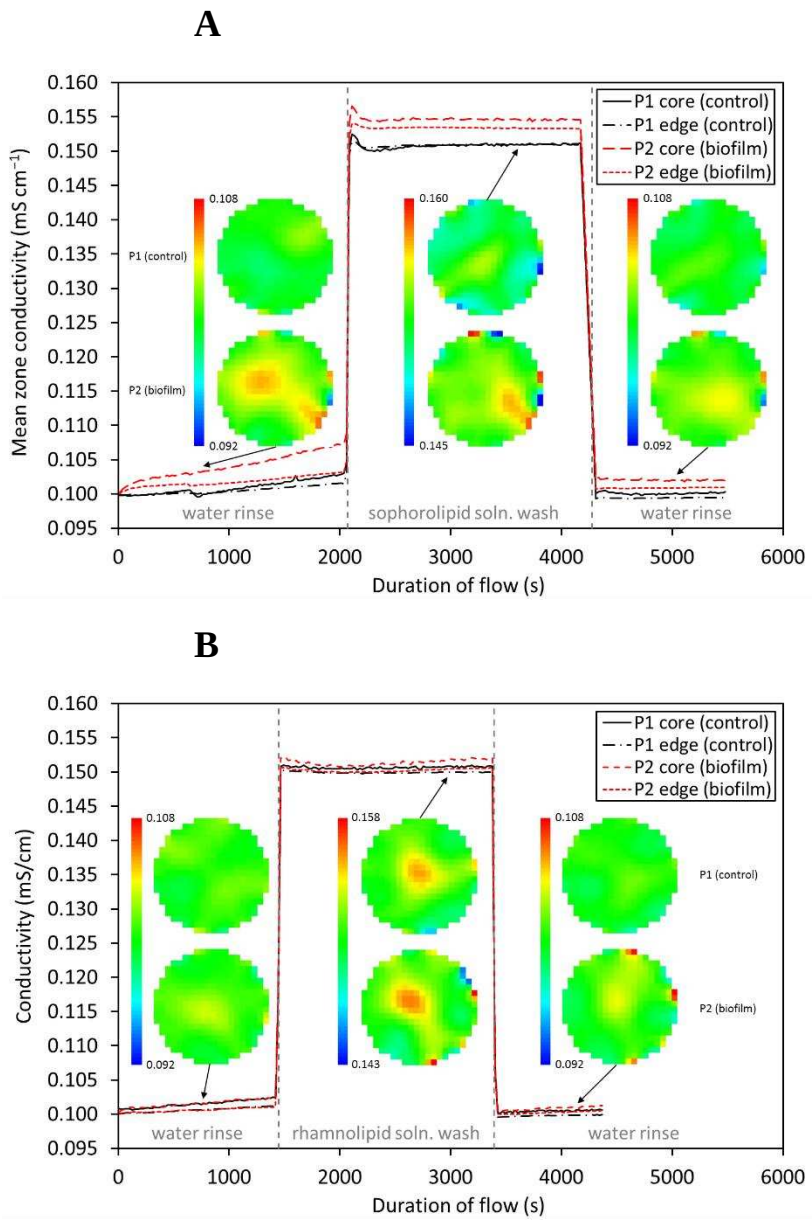

Figure 2. Mean tomogram conductivity over rinse-wash-rinse cycle with selected tomograms. **A.** Sophorolipids 0.4 g/L wash. **B.** Rhamnolipids 0.4 g/L wash.

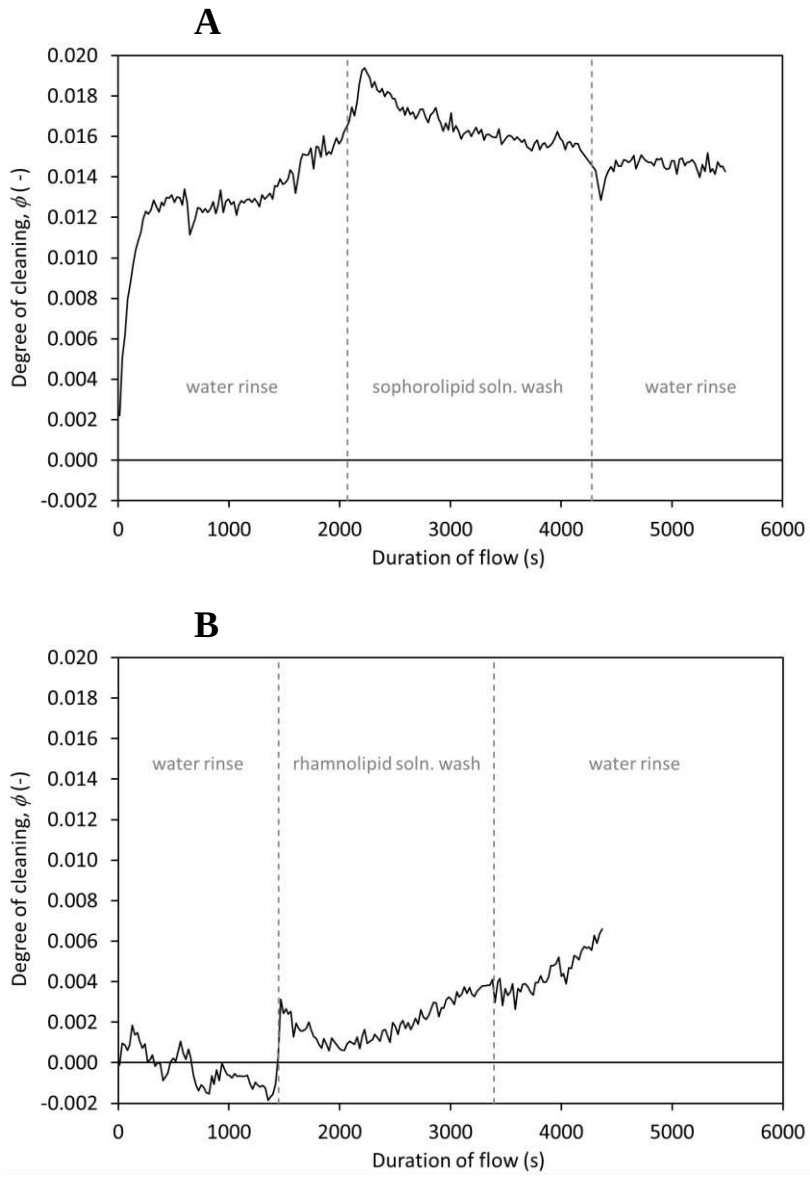

Figure 3. Degree of cleaning over rinse-wash-rinse cycle. **A.** Sophorolipids 0.4 g/L wash. **B.** Rhamnolipids 0.4 g/L wash.