INFLUENCE OF MATERIAL TYPE AND SURFACE BENZALKONIUM CHLORIDE PRECONDITIONING ON BIOFILM FORMATION AND ACTIVITY

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ABSTRACT

This study investigates the potential of benzalkonium chloride (BC), a cationic surfactant, on the prevention of biofilm formation on stainless steel ASI 316 and silicone rubber, two distinct surfaces currently used on food processing facilities. The surfaces were preconditioned with several concentration of BC for 30 min. Treated surfaces were characterized by the sessile drop method, demonstrating that surfactant pre-treatment increased the hydrophobicity of the surfaces, this increase being a function of BC concentration increase applied for preconditioning. In order to ascertain the preventive effect in biofilm formation, the treated surfaces where inserted in a chemostat continuously inoculated with P. fluorescens in the exponential phase of growth, being the biofilm allowed to grow for 6 days. The results showed that BC preconditioning did not prevent or impair biofilm formation. In fact, biofilms developed on the treated surfaces presented higher biomass and respiratory activity than the ones formed on the untreated surfaces, this phenomenon being more evident for silicone than for stainless steel and for surfaces treated with higher BC concentrations. Scanning electron microscopy and biochemical analysis reveal that the difference of surface type and surface preconditioning, by itself, gave rise to the formation of structural and biochemical distinct biofilms. The overall results suggest that preconditioning of stainless steel and silicone rubber surfaces with BC allowed the formation of biofilms with more recalcitrant properties than the ones found on untreated surfaces.

INTRODUCTION

Microorganism's deposition on solid surfaces, and consequent biofilm formation, are phenomena that happen naturally but are also microorganism's strategies to protect themselves from external toxic factors. Bacterial adhesion to surfaces and subsequent biofilm formation are well noticed phenomena in almost all of the industrial areas. Food processing environments congregate a wide range of characteristics that favours the proliferation of microrganisms and thus biofilm formation. Biofilm microorganisms can cause serious problems in this type of industry since they often present reduced susceptibility to the action of antimicrobial agents than their liquid suspended counterparts. Biofilm occurrence is often combated with the increase of the frequency of the cleaning and disinfection (sanitation) programmes and the use of increased doses of sanitation products. The sanitation formulations usually encompasses chemical products with marked biocidal and surfactant properties. Surfactants are added to increase the washing effect of the sanitation practices. If these practices are not effective, microrganisms and product residues can remain in the equipment surface at concentrations that may affect the quality and safety of the food product (Gibson *et al*., 1999). Also, those products residues and remaining microrganisms can

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contribute to the re-growth of biofilms with persistent characteristics to the sanitation products (Gilbert and McBain, 2003). In recent years, there is growing concern about the increased number of reports noticing phenomena of bacterial adaptation and resistance to those biocides and surfactants. In order to overcome this drawback, it is crucial to comprehend all the parameters that can contribute to the prevalence of biocide and surfactant resistance. One of the factors that can contribute to that understanding it is to establish whether pre-contact of bacteria with a chemical can contribute to their reduced susceptibility to that product.

The aim of the present study is to ascertain when the type of material and the preconditioning of the adhesion surfaces can alter the physiological and morphological properties of *P. fluoresces* biofilms, and to investigate whether these two factors have impact on the prevalence of biofilm resistance to chemical treatment.

MATERIAL AND METHODS

Microrganism and Culture Conditions

Pseudomonas fluorescens ATCC 13525^{T} was the microorganism used to produce biofilm. This bacterium was maintained in nutrient agar plates and, when required, fresh liquid cultures were prepared to implement the biofilm formation assays. The growth conditions were 27 ± 1 ºC, pH 7, and glucose as the carbon source. The bacterial planktonic culture was grown in a 0.5 l chemostat, continuously fed with a sterile concentrated nutrient solution (5 g/l glucose, 2.5 g/l peptone and 1.25 g/l yeast extract, in phosphate buffer at pH 7), at a flow rate of 10 ml/h. The bacteria were let to grow in that fermenter as a batch for approximately one day (to reach the steady state) before the beginning of the continuous feeding process.

Biofilm Formation

Biofilms were developed on metal and rubber coupons placed in a well stirred continuous 3 l glass reactor at 27 ± 1 ºC, suitable aerated and magnetically agitated. This reactor was continuously inoculated with bacteria coming from the chemostat and fed with 0.97 l/h of a sterile nutrient solution consisting of 40 mg glucose l^{-1}, 20 mg peptone l^{-1} and 10 mg yeast extract l^{-1} prepared in phosphate buffer pH 7. Twelve slides of ASI 316 stainless steel and twelve slides made of silicone rubber were placed within the reactor contained the bacterial suspension during 6 days for biofilm growth. The slides were degreased, rinsed twice with water and sterilised before they were hung in the reactor through a device that was suitable fitted in the reactor and could be removed for biofilm sampling. Prior to each experiment all system components were sterilised by autoclaving at 120 ºC and 1 atm for 25 min. The experiments were repeated in three different occasions by performing three independent biofilm formation experiments.

Antimicrobial Chemical

Benzalkonium chloride (BC), a cationic surfactant, obtained from Calbiochem (CMC of 5.00 mM; Cat. No. 198901) was the antimicrobial chemical used to preconditioning the coupons, before biofilm formation. Before each experiment, BC solutions were prepared to the required concentration, with sterile distilled water.

Surfaces Preconditioning

Biofilms were formed on stainless steel and silicone rubber coupons (2 cm x 2 cm and 1 mm thick) treated or not with the surfactant. The treatment, denominated preconditioning,

consisted on the pre-contact of all the coupons with BC solutions of 0,0625 mM, 0,125 mM, 0,25 mM, and 0,5 mM mM, for 30 min.

Biofilm Characterization

The biofilm that covered the slides was completely scraped off and resuspended into 20 ml of phosphate buffer pH 7. These biofilm suspensions were used to assess the cellular respiratory activity of the biofilm through oxygen uptake rates due to glucose oxidation and afterwards biofilm mass.

Respiratory Activity Assessment

The respiratory activity of the biofilm was evaluated by measuring oxygen uptake rates due to glucose consumption in a biological oxygen monitor (BOM) in short-term assays. The assays were performed in a Yellow Springs Instruments BOM (Model 53) and the procedure used was described elsewhere (Simões et al., 2005). Briefly, the biofilm suspensions were placed in the temperature-controlled vessels of the BOM (T = 27 ºC ± 1ºC), each one contained a dissolved oxygen (DO) probe connected to a DO meter. Once inside the vessel, the samples were aerated for 30 min to ensure oxygen saturation. After that, vessels were closed and the decrease of the oxygen concentration was monitored over time. The initial linear decrease observed corresponded to the endogenous respiration rate. To determine the oxygen uptake due to substrate oxidation, a small volume (50 μl) of a glucose solution (100 mg/l) was injected within each vessel. The slope of the initial linear decrease in the DO concentration, after glucose injection, corresponded to the total respiration rate. The difference between the two respiration rates gives the oxygen uptake rate due to the glucose oxidation. This respiratory activity was expressed in mg of O_2 per g dry biofilm mass per minute.

Biofilm Mass Quantification

The dry biofilm mass was assessed by the determination of the total volatile solids (TVS) of the homogenised biofilm suspensions, according to the Standard Methods (1989), method number 2490 A-D. The dry biofilm mass accumulated on the adhesion surfaces was expressed in g of TVS per cm^2 of surface area of the coupons.

Scanning Electron Microscopy Observations

During the experiments, the superficial structure of the biofilms formed on distinct materials (stainless steel and silicone rubber), untreated and preconditioning, were observed by scanning electron microscopy (SEM). The SEM inspections always comprised the observation of at least 15 fields of each biofilm-covered coupon. Prior to SEM observations, the biofilm samples were gradually dehydrated in an absolute ethanol series to 100% (15 min each in 10, 25, 40, 50, 70, 80, 90 and 100% v/v), and dried in a desiccator for 3 d. SEM observations were documented through the acquisition of representative microphotographs.

Physico-chemical Characterization of The Adhesion Surfaces

The surface tension of the coupons, preconditioning or not, was determined by the sessile drop contact angle measurements as described by Busscher (1984). The measurements were carried out at 27 ± 1 ºC, using water, formamide and β-bromonaphtalene as reference liquids (Merck). The determination of contact angles was performed automatically with the aid of an image analysis system (G2/G40) installed in the OCAA 15 plus, Dataphysics.

The total surface tension and the relative contributions of Lifshits-van de Waals (LW) and electron donor and electron acceptor parameters of the Lewis-acid base (AB) interactions were calculated using the approach of van Oss *et al*. (1988). The surface hydrophobicity of

both type material coupons (stainless steel and silicone rubber) was calculated with the surface tension values, using the method proposed by van Oss and Giese (1995). According to these authors, the hydrophobicity of a solid can be evaluated as the free energy of interaction between the molecules of its surface when immersed in water (w), represented as ΔG_{sws}^{tot}. This expresses the degree to which the attraction of the solid molecules to water is greater (hydrophilicity) or smaller (hydrophobicity) than the attraction that water molecules have to each other. Thus, when the global free energy of interaction between the molecules of the solid surfaces immersed in water is repulsive ($\Delta G_{sws}^{tot} > 0$) the solid is considered hydrophilic. On the contrary, the more negative ΔG_{sws}^{tot} is, the higher is the solid hydrophobicity.

RESULTS AND DISCUSSION

The aim of this work was to investigate whether the type of material of the adhesion surfaces and the preconditioning, with a range of concentrations of BC, a cationic surfactant widely included in sanitation formulations of food facilities, could have any effect in the physiological (mass accumulation and respiratory activity) and morphological properties of *P. fluorescens* biofilms.

The data related with biofilm mass accumulated on the metal and silicone coupons, untreated and preconditioning with several BC concentrations, are depicted in Figure 1.

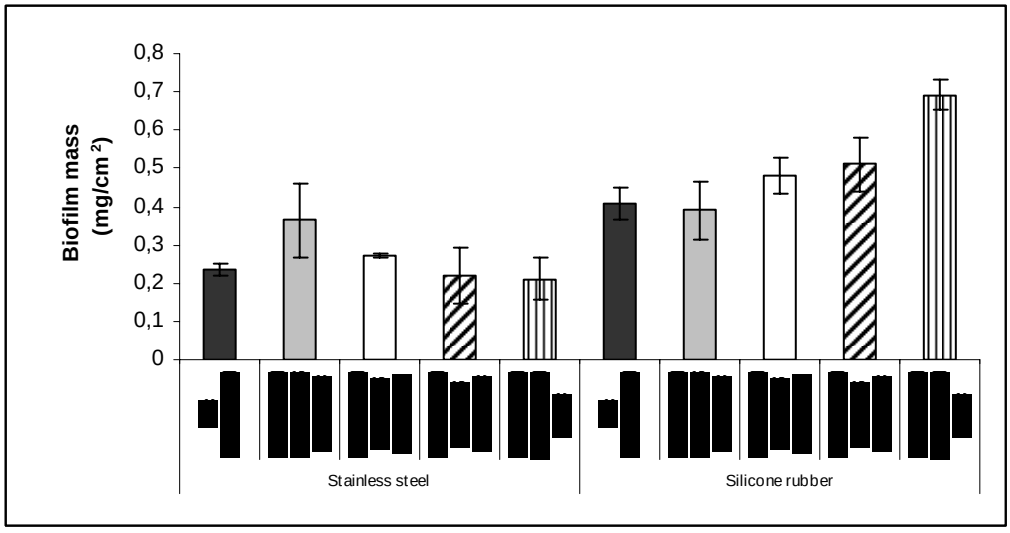

Figure 1 Biofilm mass adhered to the stainless steel and silicone rubber coupons without and after preconditioning with several concentrations of BC (bars represent the standard deviation).

This figure showed that biofilms formed on the silicone coupons have clearly more mass than the ones grown on the metal surfaces, independently of surfaces being or not preconditioned. The preconditioning of the surfaces causes different behaviours in terms of biofilm accumulation according to the type of the material of the coupon. In fact, the preconditioning of the stainless steel coupons seemed not to significant amend biomass accumulation since the biofilm mass observed on the treated coupons are rather similar to the one monitored on the untreated surface. Conversely, the amount of biofilm mass formed on the silicone coupons increase with the preconditioning of the surfaces, this amount increase being a function of BC concentration. Based on the results from Figure 1, it can be concluded that the

preconditioning of the adhesion surfaces appears to favour biofilm accumulation. This event it is unexpected since BC it is a chemical with marked surfactant properties and previous works (Meylheuc *et al*., 2001) have demonstrated that the adsorption of a biosurfactant to a stainless steel surface reduced the adhesion of *L. monocytogenes* on that metal surface.

Concerning biofilm respiratory activity, Figure 2 indicated that the preconditioning of the adhesion surfaces gives rise to biofilms clearly more actives than the ones formed on the untreated coupons. Furthermore, for both material types, the respiratory activity of the *P. fluorescens* biofilms increased gradually with the increase of BC concentration used in the preconditioning.

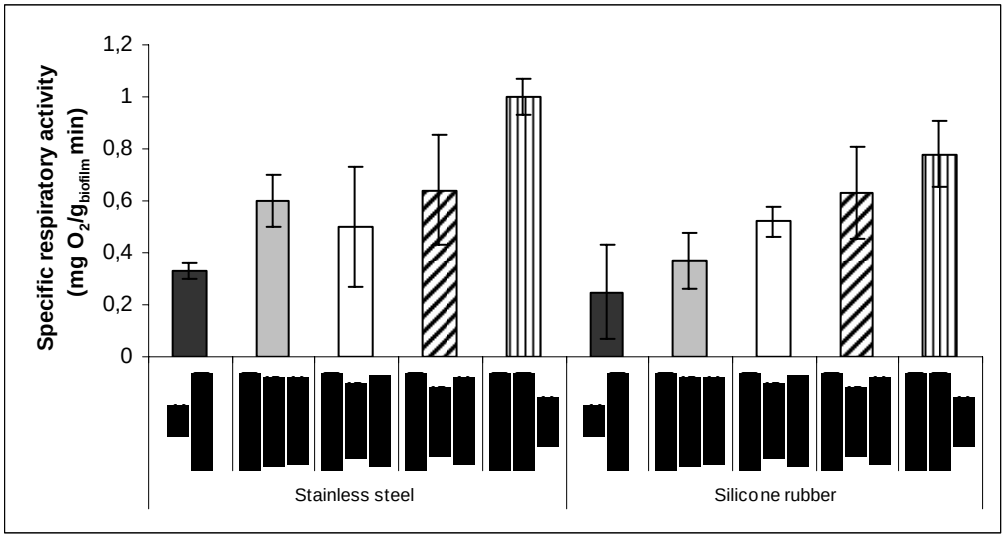

Figure 2 Respiratory activity of the biofilms formed on the stainless steel and silicone rubber coupons without and after preconditioning with several concentrations of BC (bars represent the standard deviation).

Figure 2 also highlighted that the respiratory activity of the biofilms formed on the stainless steel coupons is, in general, higher than the one monitored in the biofilms accumulated on the silicone coupons, regardless the fact of the surfaces being or not preconditioned. This fact assumes special interest because biofilms formed on the metal coupons presented noticeable less mass than the ones formed on the silicone (Figure 1). This evidence emphasised the role of the type of material of the coupons in biofilm physiology, since silicone, contrary to what happens with stainless steel, seems to induce biofilms with more mass but less active.

The possible alterations of the biofilms superficial structure induced by the type of material of the adhesion surface and by the preconditioning of the surfaces were investigated by SEM observation (Figure 3).

SEM photomicrographs showed that the *P.fluorescens* biofilms formed on the metal coupons (Figure 3 AI and AII) seem to present more cells and less amount of extracellular polymeric substances (EPS). In fact, the biofilm polymeric matrix is more noticeable in the biofilms formed on the silicone untreated or preconditioned. These SEM observations may help to explain the fact that biofilms formed on the stainless steel coupons are more actives (Figure 2), even if they have lower mass (Figure 1). The major respiratory activity of the biofilms that covered stainless steel can be due to the existence of more bacterial cells in the entire amount of biofilm mass, as Figure 3 appears to demonstrate. Likewise, the higher amount of mass and

lower activity of the biofilms formed on the silicone can be a consequence of the major quantity of EPS.

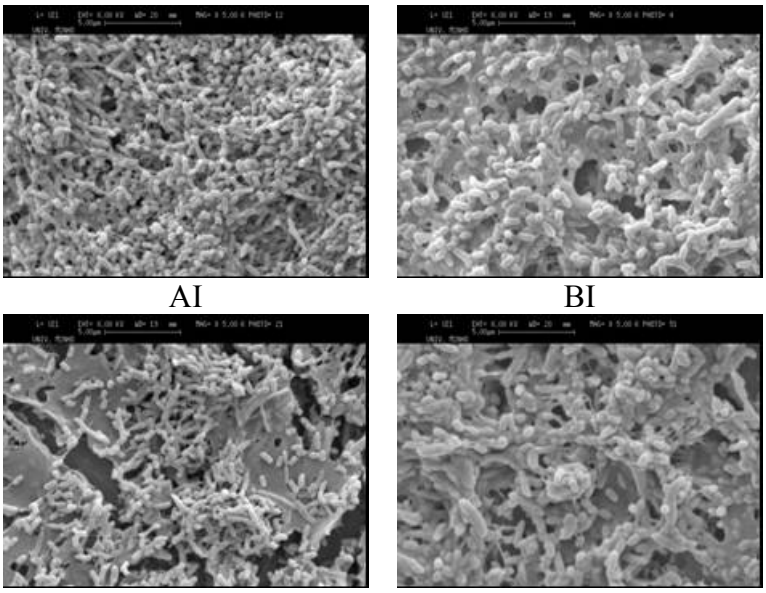

Figure 3 SEM photomicrographs of *P. fluorescens* biofilms formed on the stainless steel (A) and silicone rubber (B) coupons untreated (I) and after preconditioning of the coupons with 0,0625 mM of BC (II). X 5000 magnification; bar = 5 µm

Figure 3 also showed that the size of the cells embedded in the biofilms formed on the silicone coupons is apparently higher than the cells of the biofilms formed on the steel. These evidences, noticed in both, untreated and BC treated surfaces, can be an indication of the role of the type of the material not only in the physiologic characteristics (as Figure 1 and 2 depicted) but also in their morphology.
Based on the Figure 3 it can be concluded that the type of material of the adhesion surfaces has greater impact in biofilm structure and morphology than the preconditioning of the surfaces.

In order to comprehend the mechanisms of adhesion of the bacteria to the coupons (stainless steel and silicone rubber), the free energy of interaction (that determines the hydrophobicity of the surfaces) of both material types was determined. Table 1 showed that both materials present negative hydrophobicities ($\Delta G_{sws}^{tot} < 0$), sign of the hydrophobic character of both surfaces (van Oss and Giese, 1995), even though silicone rubber presents a more marked hydrophobic feature. With the preconditioning, both materials maintain or reinforce (in the case of stainless steel) their hydrophobic character. In the thermodynamic point of view, the initial adhesion of the *P. fluorescens* (a bacterium clearly hydrophilic because $\Delta G_{sws}^{tot} = 25$ mJ/m^2) to the metal and rubber coupons is favoured by the surface preconditioning.

Table 1 Free energy of interaction (hydrophobicity - ΔG_{sws}^{tot}) of the surface of the stainless steel and silicone rubber coupons, without and after BC preconditioning

Preconditioning	Hydrophobicity - ΔG_{sws}^{tot} (mJ/m^2)	
([BC] mM)	stainless steel	silicone rubber
without	-10.7	-92.3
0.0156	-47.4	-79.9
0.0625	-56.2	-57.8
0.125	-35.7	-65.5
0.25	-40.2	-57.4

CONCLUSIONS

This preliminary study has showed that the BC preconditioning of the surfaces did not prevent or impair biofilm formation on the coupons, no matter what kind of material type, since higher extent of biofilm mass was monitored in the treated surfaces. Furthermore, for both types of material, preconditioning induced significant increase of the biofilm respiratory activity. Thus, it can be concluded that the previous adsorption of BC to steel and silicone coupons favoured biofilm physiology, in terms of mass and activity, which thereby could augment its resistance to sanitation. This evidence represents a drawback in a future cleaning programme. The overall results also showed that the type of material of the adhesion surfaces plays an important role in the physiological properties and morphology of the biofilms, highlighting the need of suitable choosing the material more representative of the real environments.

REFERENCES

APHA., AWWA., WPCF., 1989, Standard methods for the examination of water and wastewater, 17th edition, Clesceri, L.S., Greenberg, A.E. and Trussel, R. R., (eds) (American Public Health Association), Washington DC, USA.

Busscher, H. J., 1984. Surface free energies and the adhesion of oral bacteria. *Ph*D Thesis, Universidade de Groningen, Holanda.

Gibson, H., Taylor, J. H., Hall, K. E. and Holah, J. T., 1999, Effectiveness of cleaning techniques used in the food industry in terms of the removal of bacterial biofilms. Journal of Applied Microbiology 87: 41-48.

Gilbert, P. and McBain, A. J., 2003. Potential impact of increased use of biocides in consumer products on prevalence of antibiotic resistance. Clinical Microbiology Reviews 16: 189-208.

Meylheuc, T., van Oss, C. J. and Bellon-Fontain, M-N., 2001. Adsorption of biosurfactant on solid surfaces and consequences regarding the bioadhesion of *Listeria monocytogenes LO 28*. Journal of Applied Microbiology 91: 822-832.

Simões, M., Pereira, M.O. and Vieira, M.J., 2005, Validation of respirometry as short-term method to assess the toxic effect of a biocide. Biofouling 21: 9-17.

van Oss, C. J. and Giese, R. F., 1995. The hydrophilicity and hydrophobicity of clay minerals. Clay minerals. 43: 474-477.

van Oss, C. J.; Good, R. J. e Chaudhury, M. K. (1988) *Additive and nonadditive surface tension components and the interpretation of contact angles*. Langmuir. 4, 884-891