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Effect of surface conditioning with cellular extracts on *Escherichia coli* adhesion and initial biofilm formation

J.M.R. Moreira[a], L.C. Gomes[a], K.A. Whitehead[b], S. Lynch[c], L.A. Tetlow[b], F.J. Mergulhão[a,*]

[a] LEPABE – Department of Chemical Engineering, Faculty of Engineering, University of Porto, Portugal
[b] School of Healthcare Science, Manchester Metropolitan University, UK
[c] School of Computing, Mathematics and Digital Technology, Manchester Metropolitan University, UK

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ABSTRACT

Bacterial adhesion and subsequent biofilm formation start with surface conditioning by molecules originating from the surrounding medium and from cell lysis. Different cell extracts e.g. total cell extract (TCE), cytoplasm with cellular debris (CCDE) and periplasmic extract (PE) were tested in agitated 96-well microtiter plates and in a flow cell. Crystal violet assay demonstrated that a polystyrene substratum conditioned with TCE or CCDE decreased initial biofilm formation, however cell adhesion generally increased when PE was used. These results were dependent on conditioning film concentration. Using a parallel plate flow chamber, the use of optimal conditioning film concentrations resulted in all the different cellular extracts reducing biofilm formation. Multifractal analysis was used to generate quantitative data on the number of cell clusters. Surface conditioning with cellular components affected the amount and clustering of bacteria on polystyrene surfaces and their propensity to induce biofilm formation. To the best of our knowledge, this is the first study addressing the effect of cellular surface conditioning of cellular compartments on *Escherichia coli* adhesion and initial biofilm formation. This work leads to a greater understanding of the factors that influence biofilm formation under flow conditions which are prevalent in food industry.

1. Introduction

The contamination of food contact surfaces by residual organic materials and food-borne microorganisms in the food processing industries has been an ever-persistent issue. Organic material on a surface will affect cell–substratum interactions, and will introduce additional cell-organic material and organic material–substratum interactions (Whitehead and Verran, 2015). Such biofouling may result in increasing or decreasing cell viability and surface cleanability. Retained organic material on a surface may also impede the recovery of cells or may protect cells from cleaning agents (Bernbom et al., 2011; Whitehead et al., 2009b). Bacterial adhesion and subsequent biofilm develop-

Abbreviations: BSA, bovine serum albumin; CCDE, cytoplasm with cellular debris; D_q, fractal dimensions; DAPI, 4′,6-diamidino-2-phenylindole; EPS, extracellular polymeric substances; OD, optical density; PE, periplasmic extract; PPFC, parallel plate flow chamber; SD, standard deviation; SEM, scanning electron microscopy; TCE, total cell extract; ΔG, free energy of interaction; γ^+, electron acceptor parameter; γ^-, electron donor parameter; γ^{AB}, Lewis acid–base component of the surface free energy; γ^{LW}, Lifshitz–van der Waals component of the surface free energy; γ^{Tot}, total surface free energy; θ, contact angle; S_a, average surface roughness; S_{pv}, value of the peak to valley surface roughness; S_q, root mean square of surface roughness.

* *Corresponding author at*: Department of Chemical Engineering, Faculty of Engineering, University of Porto, Rua Dr. Roberto Frias s/n, Porto 4200-465, Portugal. Fax: +351 5081449.
E-mail address: filipem@fe.up.pt (F.J. Mergulhão).

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ment start with surface conditioning with molecules originating from the surrounding medium, since these molecules will diffuse faster than bacterial cells due to their reduced size (Bruinsma et al., 2001). The formation of conditioning layers may take from few seconds to some minutes (Garrido et al., 2014). Following surface conditioning, bacteria will become attached, adhered and then retained on a surface (Whitehead and Verran, 2015). It is at this point that proliferation of bacteria may result in biofilm formation. Since the nature of the initial surface chemistry will influence bacterial attachment and retention, it may be speculated that the type of conditioning film present will in turn affect biofilm formation (Whitehead and Verran, 2015).

Biofilms are microbial communities attached to surfaces and surrounded by a matrix of extracellular polymeric substances (EPS). Conditioning agents may be components of the culture medium or cell-derived components originating from cell lysis such as individual molecules or cellular debris fragments (Renner and Weibel, 2011). A wide variety of polymers can be found in the biofilm matrix, but the major components are proteins and polysaccharides, whereas lipids and nucleic acids are found in minor quantities (Ras et al., 2011). These compounds are excreted by the microbial population, but can also result from natural cell lysis and from hydrolytic or cleaning activities (Ras et al., 2011). Although cell adhesion is usually affected by the properties of the original unconditioned surface, it can also be altered by the formation of a conditioning film (Hwang et al., 2012, 2013). The effects of conditioning films on bacterial adhesion have been widely discussed in the literature with contradictory findings, probably due to the diversity of environmental conditions that the surfaces were exposed to (Hwang et al., 2013; Lorite et al., 2011). For instance, it was demonstrated that the presence of alginate and other organic matter in conditioning films significantly increased initial bacterial adhesion onto glass surfaces at low ionic strength, nonetheless adhesion was not enhanced by bovine serum albumin (BSA) conditioning (Hwang et al., 2012). Recently, Ribeiro et al. (2017) observed higher adhesion of *Bacillus cereus* spores when using whole milk as conditioning matrix compared to the unconditioned stainless steel surface (4.93 versus 3.01 log CFU cm^{-2}), suggesting an interaction between milk fat and bacteria on the surface. In opposition, it has also been shown that preconditioning of stainless steel with aqueous cod muscle extract significantly impeded bacterial adhesion (Bernbom et al., 2009). Hamadi et al. (2014) studied the adhesion of *Staphylococcus aureus* to stainless steel treated with three types of milk and found that they reduced bacterial attachment. Moreover, the lowest and the highest adhesion values were obtained when the surface was treated by the semi-skimmed milk and skimmed milk, respectively, showing that adhesion is affected by fat content (Hamadi et al., 2014). Recently, it was found that adsorption of alginate and albumin onto aluminum coatings inhibited *Escherichia coli* adhesion by altering the surface hydrophobicity (He et al., 2015). This conditioning layer also enhanced the anti-corrosion performance of the coatings (He et al., 2015). Additionally, other authors have shown that protein films reduced the bacterial adhesion to different surfaces (Chapman et al., 2001; Dat et al., 2014; Merghni et al., 2016; Robitaille et al., 2014).

Quantification of the percentage coverage of retained cells in a monolayer following epifluorescence microscopy has been carried out for some time (Whitehead and Verran, 2007). However, the development of a quantifiable method to determine the percentage coverage of both conditioning film and cells on a surface has proved to be more difficult, but can be achieved using certain staining methods (Whitehead et al., 2009b). Multifractal analysis has been used to obtain numerical data on the effect of surface properties on cell dispersion, density and clustering (Wickens et al., 2014). In this work, multifractal analysis has been used in order to demonstrate the level of cell/biofilm clustering and the overall percentage coverage of bacteria and biofilms on the surfaces following conditioning with cellular extracts. Understanding, comparing and quantifying how the patterns of cellular adhesion and biofilm formation occur across a surface is imperative for designing hygienic surfaces in the future.

Despite the investigation of surface conditioning effects on cell adhesion, there are few studies analyzing the impact of cellular surface conditioning components on biofilm formation. Further, this work used the determination of the upper bacterial/biofilm percentage coverage to quantify the number of clusters present. The present study evaluates the effect of cellular components as surface conditioning agents and quantifies their impact on initial biofilm formation under flow conditions.

2. Material and methods

2.1. Bacteria and culture conditions

E. coli JM109(DE3) from Promega (USA) was used in this study because it has shown a good biofilm forming capacity in a diversity of platforms operated at different shear stresses (Gomes et al., 2014; Moreira et al., 2015b; Teodósio et al., 2011). Additionally, it was shown that its biofilm formation was similar to other *E. coli* strains which are often used for antimicrobial susceptibility tests (Gomes et al., 2014). The strain was grown overnight at 30 °C and 120 rpm after the inoculation of 500 μl of a glycerol stock kept at −80 °C in 0.2 l of culture medium (Teodósio et al., 2011). This medium consisted of 5.5 $g\,l^{-1}$ glucose, 2.5 $g\,l^{-1}$ peptone, 1.25 $g\,l^{-1}$ yeast extract in phosphate buffer (1.88 $g\,l^{-1}$ KH_2PO_4 and 2.60 $g\,l^{-1}$ Na_2HPO_4), pH 7.0. All medium components were purchased from Merck KGaA (Germany). Cells were then centrifuged (3202×*g*, 10 min) and washed twice with citrate buffer 0.05 $mol\,l^{-1}$, pH 5.0 (Moreira et al., 2015b). The pellet was resuspended in citrate buffer and the cellular suspension was adjusted to a final concentration of 7.6×10^7 cell ml^{-1} determined by optical density at 610 nm ($OD_{610\,nm} = 0.1$). This cell suspension was used for initial biofilm formation assays in 96-well microtiter plates and in a parallel plate flow chamber (PPFC).

2.2. Conditioning agents

Three different types of cellular extracts were tested as conditioning agents: total cell extract (TCE), cytoplasm with cellular debris (CCDE) and periplasmic extract (PE). Cells from an overnight culture were harvested by centrifugation and washed twice with distilled water. The pellet was concentrated and re-suspended in water to reach a cell concentration of 30.4×10^8 cell ml^{-1} ($OD_{610\,nm} = 4.0$). This cell suspension was then divided in two parts, one part for the preparation of PE and CCDE, and another to prepare the TCE. The TCE was obtained by subjecting this cellular suspension ($OD_{610\,nm} = 4.0$) to four cycles of freezing (at −80 °C) and thawing (in a water bath at 30 °C). To prepare the PE (Mergulhão et al., 2001), the initial suspension ($OD_{610\,nm} = 4.0$) was centrifuged again, re-suspended in a sucrose solution (20% sucrose, 0.3 $mol\,l^{-1}$ Tris–HCl pH 8.0, 1 $mmol\,l^{-1}$ EDTA) and incubated at room temperature for 15 min. This suspension was centrifuged (6000×*g*, 10 min), and the pellet was re-suspended in ice-cold water and incubated for 15 min on ice. This mixture was centrifuged (12,000×*g*, 10 min) and the PE was obtained in the supernatant, whereas the pellet was re-suspended in ice-cold water and the cells were disrupted by sonication (Sonopuls HD 2070, Bandelin Electronics, Germany) (7 cycles of 30 s at 20 Hz) yielding the CCDE. The TCE, CCDE and PE that were obtained from a cell suspension with an $OD_{610\,nm} = 4.0$ were further diluted in water to recreate cell suspensions with cellular concentrations of (0.38, 0.76, 3.04, 6.08, 12.2 and 24.3) × 10^8 cell ml^{-1}.