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Two-phase kinetics of biofilm removal during CIP. Respective roles of mechanical and chemical effects on the detachment of single cells vs cell clusters from a *Pseudomonas fluorescens* biofilm

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ABSTRACT

The objective of this study was to investigate the roles of mechanical and chemical actions on the detachment kinetics during CIP of biofilms of *Pseudomonas fluorescens* at a pilot-plant scale.

Biofilms were grown 48 h at 20 °C in diluted milk. Shear stress conditions ranged from 0 (static) to 20 Pa and NaOH concentrations from 0.1 to 0.5% w/w. A simple two-phase model was applied to model the removal kinetics and compare detachment parameters.

A quick removal of the biofilm during the first phase (4–5 log decrease in three minutes) was followed by a second phase of slow removal of the cells and small aggregates still present at the surface (0–2 log decrease). Hydrodynamics was responsible for removal of both biofilm and single cells while chemicals mainly disrupted biofilm clusters during the first phase. No complete biofilm removal was observed, suggesting a significant role of the interaction forces between bacteria and substrata in the CIP efficiency.

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1. Introduction

In agro-food industrial environments, surfaces have been reported to be contaminated by a range of microorganisms, including pathogenic and spoilage bacteria (Srey et al., 2013). Once introduced, many bacteria are able to persist on the contaminated surfaces or even to grow if environmental conditions are suitable. These adherent microorganisms may in turn cross-contaminate food products in contact (Reij and Den Aantrekker, 2004). Hence, 80% of all microbial diseases, including foodborne illnesses, would be caused by microorganisms in biofilms (National Institutes of Health, USA, 1997), and foodborne illness would cost up to $144 billion a year (Scharff, 2012).

Adherent bacteria are often found in the form of biofilms, defined as complex microbiological ecosystems formed by single or multiple species associated with a matrix of self-produced polymers. Theses biofilms could be found on every surface in direct contact or not with food, even after hygiene procedures. Indeed, it is widely accepted that there is hardly a material that does not allow biofilm formation (Faille and Carpentier, 2009), including stainless steel and polymers. Moreover, the remarkable resistance of biofilms to antimicrobial compounds and thermal treatments would account for their persistence in food-processing environments. Hence, cleaning is the key procedure for the hygienic control of food processing lines and environment. Bacterial adhesion is governed by physicochemical interactions, e.g. Van der Waals, Lewis acid/base or electrostatic. Therefore, for a cleaning procedure to be effective, the physicochemical interactions must be overcome by the mechanical action of the flowing fluid, in particular due to the mean wall shear stress and its fluctuations (Blel et al., 2010; Faille et al., 2013) and the chemical action due to the detergents and surfactants (Faille et al., 2013; Marchant and Banat, 2012). But it is now widely accepted that routine cleaning in place procedures (cleaning of plants or pipeline circuits without dismantling or opening of the equipment, CIP) may not remove all of the bacterial surface contamination (Brooks and Flint, 2008). It is thus of utmost importance to improve our understanding of the removal kinetics of biofilms and the underlying phenomena. Such an approach has been previously achieved on food soils to improve the efficiency of cleaning in place procedures, e.g. by investigating the relative role of temperature and chemicals (Peng et al., 2002), time of treatment (Lelièvre et al., 2002), as well as of mechanical action through

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hydrodynamics (Blel et al., 2008, 2007; Jensen et al., 2007).

An issue is the modelling of cleaning kinetics taking into account the various parameters governing the cleaning process (time, temperature, nature of detergents as well as other parameters such as the physico-chemistry and topography of the substratum, and the physicochemical properties of the soil). In biology, information has been reported in the literature on the modelling of detachment curves with a first order kinetics, e.g. on bacteria (Eginton et al., 1998; Midelet et al., 2006), on yeasts (Demilly et al., 2006) and on *Dictyostelium discoideum*, a unicellular eukaryote (Décavé et al., 2002). However, other authors (Herrera et al., 2007; Lelièvre et al., 2002) suggested that the removal phenomenon was not simply exponential.

The purpose of this study was the investigation of the removal kinetics of a bacterial biofilm through the modeling of detachment curves resulting from mechanical and/or chemical action. In order to access this information, the detachment of a *P. fluorescens* biofilm was followed by enumeration of the residual CFU at different times. These measurements were carried out under various shear stresses during a rinsing procedure (mechanical detachment), and in static conditions in the presence of increasing concentrations of NaOH (chemical detachment). Detachment curves were compared to those obtained during a classical CIP procedure. In order to confirm hypothesis about underlying phenomena, microscopic observations were also performed.

2. Material and methods

2.1. Bacterial strain and biofilm formation

The *Pseudomonas fluorescens* D3-276 strain was selected for the ability to form biofilms highly resistant to rinsing and cleaning procedures (Lequette et al., 2010). This strain was recovered from a dairy product by the University of Cornell, Department of Food Science, Ithaca, New York 14853, USA (Kathryn J. Boor, kjb4@cornell.edu).

Overnight cultures at 30 °C were first diluted at a final concentration of around 10^5 CFU ml^{-1} in reconstituted skimmed milk diluted 1/10 in distilled water (Cora, Villeneuve d'Ascq, France). *P. fluorescens* D3-276 was then allowed to form biofilms on the inner surface of stainless steel pipes (304L, 2B finish, Ra = 0.3 μm) of 0.20 m in length and 0.0237 m in inner diameter. Twenty four pipes were filled with the *P. fluorescens* suspension in skimmed milk diluted 1/10 and incubated at 20 °C under gentle shaking on a rotating shaker (8 rpm) in order to avoid any localized sedimentation inside the pipes. After 24 h the growth medium was drained and replaced by sterile diluted skimmed milk and pipes were further incubated for 24 h. Pipes were then drained before further experiments. In order to observe biofilms (epifluorescence microscopy), the same two-step procedure was perform to contaminate stainless steel coupons (316L, 2B finish) of 1.5 × 4.5 cm^2. After soiling, coupons were inserted in square flow cells (Jullien et al., 2008). Contaminated square flow cells and pipes were then subjected to the various detachment procedures described below.

2.2. Mechanical vs chemical detachment of Pseudomonas biofilms

After 48 h of biofilm formation, contaminated pipes or square flow cells were drained and installed in the pilot rig (Le Gentil et al., 2010). An entry pipe which length was chosen to generate a fully developed flow was inserted upstream the test section ($L/D_h = 25$). The rig was first rinsed with softened water for 3 min at room temperature, without recirculation to flush out residual growth medium and loosely attached cells. Three pipes were then removed to identify the so-called "initial level of contamination". In a set of experiments, contaminated pipes were subjected to rinsing procedures, in order to investigate the influence of the shear stress (mechanical action) on the kinetics of biofilm removal. Softened water (water devoid in calcium and magnesium ions) at 40 °C was circulated at wall shear stresses (WSS) of 0.144 Pa, 1.556 Pa, 4.502 Pa, and 19.99 Pa, in turbulent flow regime (Re = 4740–75187). The mean wall shear stress values were obtained experimentally from power loss measurements using differential pressure sensors Shlumberger, type 8D. It was previously shown at PIHM that working with flowing water at 40 °C did not lead to biofilm inactivation and therefore allow to identify the potential role of the only mechanical effect on their detachment. For each flow condition, sets of 3 pipes were removed after 1, 3, 5, 10, 15, 20 and 30 min and further analyzed. In parallel, some contaminated pipes were subjected to a CIP procedure with NaOH 0.5% at 60 °C at a wall shear stress of 0.144 Pa (Re = 4246). Sets of 3 pipes were removed and analyzed as above.

Between trials, the loop was cleaned with NaOH 2% (w/w) at 80 °C for 60 min and the flow cells and the coupons were sterilized at 180 °C using a dry heat oven during one hour.

The influence of NaOH concentration was investigated by filling the contaminated tubes (after rinsing with softened water) with NaOH at 0.10%, 0.25%, 0.35%, and 0.50% at 60 °C (temperature considered to be sufficient to induce a reasonable activity on various soils, e.g. bacterial spores, biofilms or food residues) and with water at 40 °C as control. Tubes were then placed in heat chambers to maintain the temperature constant.

For each NaOH concentration, sets of 3 pipes were removed after 3, 5, 10, 15, 20 and 30 min incubation in static condition, rinsed in softened water to remove NaOH and potentially disrupted structures, and further analyzed.

2.3. Biofilm enumeration and observation

In order to quantify the number of CFU on the pipe surfaces, bacteria were detached by scraping the internal pipe surfaces with a piston ring (using the same principle as syringe piston, and which detachment efficiency has been previously validated (data not shown)). The piston ring was then placed in saline supplemented with 0.5% Tween 80 and subjected to a sonication step for 2.5 min in an ultrasonic bath (Branson 2510, 40 kHz). Detached cells were plated on nutrient agar and counted after 48 h incubation at 30 °C. The absence of residual surface contamination following the use of piston rings was checked using the agar molding method previously described (Bénézech et al., 2002).

The spatial distribution and structure of the biofilms were observed on coupons by epifluorescence after staining with Acridine Orange 0.01% for 10 min. Biofilms were observed with an epifluorescence microscope (Olympus BH2-RFL microscope, Tokyo, Japan) at a magnification of ×1000.

Trials were performed in triplicate.

2.4. Kinetics modeling

Inspection of the kinetic profiles suggested that a two-phase model (equation (1)) could be used to fit the data. The fitting was performed using GInaFiT, a freeware tool for assessing non-log-linear microbial survivor curves (Geeraerd et al., 2005). This two phase model was the most appropriate amongst the GInafit list. According to our experimental data (data not shown), the two other potential options were the single phase or the Weibull models. However fitting operations returned very low R^2 compared to the biphasic model. Moreover, the biphasic model parameters could be easily related to the observed phenomena, as shown below.

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