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Pseudomonas sp. biofilm development on fresh-cut food equipment surfaces – a growth curve – fitting approach to building a comprehensive tool for studying surface contamination dynamics

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ABSTRACT

The capacity of two primary growth models to describe *Pseudomonas fluorescens* and *Pseudomonas grimontii* biofilms' development was assessed. The Baranyi and the 'Logistic with Breaking Delay' models were applied for biofilms grown in various laboratory and pilot-plant devices, including pipes or mock-ups mimicking vegetables washing tanks in the fresh-cut food industry. An initial short transitional period not described by the growth models was observed during which cells rapidly attached to pilot-plant devices' surfaces. The following observed surface contamination growth patterns were consistent with both growth models. However, only the Baranyi model was relevant to the occurrence of wide variability and/or growth curves with no lag or stationary phases. Both surface design and hydrodynamics in pilot-plant devices strongly influenced biofilm growth curves. Based on fitted parameters, it was possible to differentiate between areas and relate these to design parameters such as sharp corners, welds or specific hydrodynamics as 2D, 3D or near-static flow conditions. 15–25 h lag phases reflecting equilibrium between cell attachment and release were observed for wall shear stresses exceeding 0.5 Pa under dynamic flow conditions. Consequently, flow pattern design improvements by increasing the shear rate would greatly reduce food cross-contamination risk, as cleaning operations could start before any significant biofilm developments.

1. Introduction

Microorganisms have a natural tendency to attach to any kind of surface and under favourable conditions grow and form a slimy matrix composed of extracellular polymeric substances (EPS), forming a biofilm. Therefore, biofilms are of concern in a broad range of areas, specifically in food, environmental and biomedical fields (Lemos et al., 2015; Srey et al., 2013). Biofilms are problematic in many food industry sectors, such as breweries, dairies, poultry or red meat processing factories, as well as fresh-cut industries producing minimal processing of vegetables and fruits (MPV) ready to eat (Brooks and Flint, 2008). Hence, MPV lines are increasingly being recognized as vehicles

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for transmission of human pathogenic bacteria and enteric viruses (Ölmez and Temur, 2010). Besides contamination during primary production, sources of contamination include the washing water, food handlers and equipment surfaces. Particular attention has to be paid to the role of equipment design in the establishment of microbial surface contamination. While no specific hygienic design standards or guidelines specific to equipment used in fresh-cut food processing lines are available, the main hygienic design principles are now well established (EHEDG, 2004). To date, these principles are rarely implemented in the equipment and machineries in use, yet the hygienic quality of MPV products is considered acceptable, as no recent contamination outbreaks have been reported in France. Therefore, it would seem that the hygiene procedures already in use are sufficient in this context. That being said, any change to the daily washing and cleaning procedures towards a more sustainable MPV production *e.g.* lower water and chemical consumption, may increase the presence of unwanted microorganisms and consequently may influence the quality of the final product, at least reducing the shelf-life.

In closed liquid-filled systems, hydrodynamic conditions together with machinery design, are known to alter biofilm dynamics, including formation (Cunault et al., 2015), adhesion (Blel et al., 2010) and detachment of adherent bacteria (Föste et al., 2013). Indeed, turbulent flow has been found to induce the growth of a more compact biofilm (Stoodley et al., 1998) than laminar conditions, with a less porous structure (Vieira et al., 1993), while high wall shear stress conditions result in a decrease in the biofilm's microbial load (Cloete et al., 2003; Tsai, 2005). Despite the importance of these observations in terms of hygienic design, very few works have as yet investigated biofilm dynamics in relation to flow pattern under conditions resembling those encountered in the food industry (Moreira et al., 2013; Simões et al., 2006).

Over recent decades, a lot of attention has been paid to biofilm modelling, especially for biofilm reactors and water distribution systems. Hence many publications have described and/or predicted biomass evolution (formation, equilibrium and removal) and the biological activities of microbial communities, most frequently involving multi-species biofilms (Dukan et al., 1996; Horn et al., 2003). The models developed have linked physical and chemical environmental parameters to the growth characteristics of microbial populations in order to understand and, or predict biomass dynamics or chemical production/consumption. Primary growth models such as the Gompertz, Logistic or the Baranyi models were first defined to describe and explain the cell division of planktonic bacteria as a function of time (Mitchell et al., 2004). Later, these models were also used to identify the growth rate of biofilms in bioreactors, integrating parameters such as mass, COD, or granule sizes (Dukan et al., 1996; Horn et al., 2003; Yang et al., 2004). Conversely, only a few works have used these primary growth models to describe biofilm dynamics and to investigate the role of environmental conditions, namely the modified Gompertz model (Nguyen et al., 2014), Baranyi model (Guillier et al., 2008; Omac et al., 2015), and the Logistic model (Tsai, 2005).

The aim of this study was to evaluate the adequacy and relevance of fitting biofilms growth curves, biofilms being developed in realistic conditions compared to in-use industrial conditions. We consequently used two well-established primary models of biofilms formed under different environmental conditions (flow pattern, surface features, etc.) close to industrial applications. These primary models were applied using two convenient and easy-to-use software tools, bearing in mind that the model and software both had to be usable across the complete dataset. The most suitable model/software had to be identified and parameter variations had to be able to explain the role of both environmental conditions and equipment design. Biofilm growth dynamics were investigated from a micro-scale, using laboratory devices, to the pilot-plant scale, using an experimental rig previously described by Cunault et al. (2015). This rig was designed to reproduce environmental conditions close to those met in tanks used to wash ready-to-eat vegetables. In addition, experiments on a micro-scale allowed a direct observation of the biofilms thereby strengthening the interpretation of the macroscale phenomena observed.

2. Material and methods

2.1. Bacteria strains and growth conditions

Two bacteria of the *Pseudomonas* genus were used, namely *P. grimontii* 13A10, isolated by the French agri-food technical institute ACTALIA from surfaces of fresh cut food industrial equipment after a cleaning operation and *P. fluorescens* PF1 isolated by the French Agency for Food, Environmental and Occupational Health & Safety (ANSES) from cleaning-in-place effluent. These were selected for their ability to grow in diluted lettuce juice and form biofilms at 10 °C, a temperature close to that used in fresh-cut processing conditions. For both strains the growth as biofilm or as planktonic cells were compared.

The t_0 inoculation was performed at 10^6 CFU/mL obtained after an overnight growth in TSB at 30 °C and agitated at 150 rpm. The growth kinetics were then carried out at 10 °C in ten times diluted TSB (Tryptone Soy Broth, Biokar, Beauvais, France) in the four devices tested formerly, namely a pilot rig (planktonic and biofilm), coupons in Petri dish (biofilm), Erlenmeyer (planktonic) and micro-titter plates (biofilm). The ten times diluted TSB was just rich enough to observe significant bacteria growth on surfaces and in surrounding liquid. The characteristics and uses of these devices are described here below. For all devices, the bacteria load was assessed at the following times 0.75 (or 1), 6, 24, 30, 48 and 54 or 72 h. Trials were performed at least in triplicate.

For biofilm counting, after a rinsing step (details are given in the section presenting the growth devices), cotton swabs (Copan, Italy) were used to recover samples surface contamination by performing uniform side-to-side swabbing to completely cover the sample area (details of areas are given in the section presenting the pilot rig). The swabs were previously soaked with peptone water (Biokar, France) diluted ten times with 0.5% TWEEN 80 (Sigma–Aldrich, France). This media prevented osmotic cell damages and facilitated cell detachment from surfaces. Two swabs were used per sample and put into a single container with 10 mL of this elution. The two swabs completely covered the area sampled to recover most of the adhered bacteria. They were then subjected to a three-step treatment consisting of a 0.5 min vortex, a 2.5 min sonication at 40 kHz using an M2800 Branson (VWR, France) and a 0.5 min vortex, in order to release bacteria from the swab and to homogenize the suspension. These suspensions were diluted in peptone water diluted ten times at the appropriate rate. 1 mL was included in TSA (Tryptone Soy Agar, Biokar, France). The Petri dishes were then incubated 48 h at 30 °C prior to a CFU count. The plate-count provided an estimation of the Surface Microbial Load (SML) expressed in Log_{10} (CFU/cm^2).

For the planktonic count, the liquid samples were directly diluted and counted as described for the suspension obtained after swabbing operations for biofilms. The plate-count provided an estimation of the Microbial Load (ML) expressed in Log_{10} (CFU/mL).

2.2. Growth devices

2.2.1. Criteria for the device selection

An original experimental rig designed to mimic the industrial conditions found in industrial washing tanks was used to study biofilm growth under various environmental conditions including at the walls quasi-static and dynamic flowing