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Biofilm formation of *Salmonella* Typhimurium on stainless steel and acrylic surfaces as affected by temperature and pH level

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ABSTRACT

The effect of temperature (28, 37 and 42 °C) and pH (6 and 7) on the biofilm formation capability of *Salmonella* Typhimurium on stainless steel and acrylic was investigated. The rate of biofilm formation increased with increasing temperature and pH, while the number of attached cells after 240 h decreased with increasing temperature and was not different between pH 6 and 7. The surface hydrophobicity of bacterial cells was not significantly ($p > 0.05$) different among tested conditions. Electron-donating/accepting properties changed with pH and temperature, although these changes did not correlate with the ability to form biofilms under respective conditions. Attachment of *S.* Typhimurium showed a preference for stainless steel compared to acrylic surfaces under all conditions tested. The results suggest that salmonellae were less adherent to acrylic than to stainless steel surfaces; thus, acrylic-type surfaces should be considered for use in the food industry over stainless steel where applicable. The rate of biofilm formation increased at higher temperatures and pH levels within the tested ranges. Hurdle technology using lower temperatures reduced pH may help delay biofilm formation on food contact surfaces contaminated with *S.* Typhimurium.

1. Introduction

Salmonella spp. is one of the most important foodborne pathogens, worldwide. More than 95% of cases caused by these bacteria are foodborne and these infections account for ca. 30% of deaths resulting from foodborne illnesses (Hohmann, 2001). Of the approximately 2500 Gram-negative *Salmonella* serovars, *Salmonella* Typhimurium is the most frequently isolated serotype, which accounts for about 35% of reported human isolates (Wilmes-Riesenberg, Bearson, Foster, & Curtiss, 1996).

Within nature as well as in food systems, bacteria generally exist in one of two states: planktonic cells, freely existing in bulk solution, and sessile cells, as a composite, such as when attached to a surface as part of a biofilm. The ability to attach to and subsequently detach from surfaces is a characteristic of all microorganisms. Although attachment is advantageous, and perhaps necessary for their survival in the natural environment, the ability of microorganisms to adhere to surfaces to form biofilms poses a significant risk to the food industry. Product spoilage, reduced production efficiency, corrosion, unpleasant odours, unsightliness, infection, biofouling and equipment failure are examples of the detrimental effects of biofilms. Moreover, several studies have shown that bacteria in biofilms exhibit increased resistance to antimicrobial treatments and sanitizing procedures when compared with planktonic cells (Chavant, Gaillard-Martinie, & Hebraud, 2004; Furukawa, Akiyoshi, O'Toole, Ogihara, & Morinaga, 2010; Joseph, Otta, Karunasagar, & Karunasagar, 2001; Somers, Schoeni, & Wong, 1994). Such microbial biofilms are not easily removed during normal cleaning procedures in food processing environs and could promote risk for cross contamination and post-processing contamination.

The formation of biofilm is a complex phenomenon influenced by several factors, including the chemical and physical properties of the cell surface and the attachment surface, and the composition of surrounding medium (Frank, 2001). Bacterial attachment to surfaces or other cells can be seen as a physicochemical process controlled by various forces including van der Waals, electrostatic, steric, hydrophilic/hydrophobic and osmotic interactions (Kumar & Anand, 1998). The bacterial cell surface, which interfaces with its surroundings, directly influences biofilm formation. Several structures that protrude from or cover the cell surface (e.g., flagella, fimbrae, pilli, curli, surface lipopolysaccharides, etc.), shape the physicochemical surface properties of bacterial cells, and determine the interactions between the bacterial surface and the

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attachment surface (Van Houdt & Michiels, 2010). These structures may be affected by environmental factors such as temperature or pH and can result in a modification of the physiochemical properties of cell surfaces, subsequently affecting bacterial attachment and biofilm formation.

It has been reported that biofilm formation by *Listeria* spp., *Salmonella* spp. and *Staphylococcus aureus* was greatly affected by growing temperatures ranging from 4 to 45 °C (Gorski, Palumbo, & Mandrell, 2003; Herald & Zottola, 1988; Mai & Conner, 2007; Norwood & Gilmour, 2001; Peel, Donachie, & Shaw, 1988; Smoot & Pierson, 1998a). In some studies, biofilm formation increased with elevated temperatures (Mai & Conner, 2007; Smoot & Pierson, 1998a) while in another study reported that sub-optimal growing temperatures appeared to enhance biofilm production (Rode, Langsrud, Holck, & Moretro, 2007). Data regarding pH changes and biofilm formation, however, are more scarce. *Pseudomonas fragi* showed maximum adhesion to stainless steel surfaces at pH ranges of 7–8, which is optimal for its cell metabolism (Stanley, 1983), while other studies demonstrated that biofilm formation of *Listeria monocytogenes*, *Serratia liquefaciens*, *Shigella boydii*, *S. aureus*, *S.* Enteritidis, and *Bacillus cereus* was induced at acidic conditions (Rode et al., 2007; Xu, Lee, & Ahn, 2010).

Overall, the effect of temperature and pH on biofilm formation remains ambiguous and may vary greatly with species, attachment surfaces, and other environmental factors such as nutrient availability. Understanding the characteristics of biofilm formation is essential for preventing their formation, and possibly reducing health risks related to biofilm-forming foodborne pathogens. The objective of this research was to determine the effects of food-related stress conditions, namely temperature and pH, on the biofilm-forming capability of *S.* Typhimurium on stainless steel and acrylic surfaces. In addition, changes in cell surface hydrophobicity were determined by the Microbial Adherence to Solvent (MATS) assay to see if varying temperature and pH directly influences bacterial attachment.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Salmonella Typhimurium (ATCC14028, animal tissue isolate) was purchased from the American Type Culture Collection (Manassas, VA, USA) and stock cultures were maintained at −80 °C in 20% glycerol solution. Prior to use, the frozen culture was activated in trypticase soy broth (TSB, Oxoid, Hampshire, UK) at 37 °C with two consecutive transfers after an 18-h incubation periods. Following incubation, the culture was centrifuged at 10,000 × *g* for 10 min at 4 °C and washed twice in phosphate buffered saline (PBS, pH7.3). Cell suspensions were prepared by adjusting the OD_{600} to ca. 0.4–0.5, which is equivalent to 10^8 CFU/ml.

2.2. Biofilm formation

S. Typhimurium biofilm formation was investigated under varying pH conditions (viz., pH 6, acidified with 0.52 g/ml acetic acid or pH 7, unacidified) and temperatures (28, 37 or 42 °C) using a coupon method. The working cell suspension was prepared by diluting the standardized cell suspension in TSB at pH 6 or 7 to achieve the final concentration of ca. 10^4 CFU/ml.

Acrylic and stainless steel coupons were used to develop the biofilm. Prior to use, coupons (1 × 2 × 0.2 cm) were soaked in a 10% (v/v) bleach solution for 15 min, followed by soaking in detergent (Teepol Multipurpose Detergent, Supply Trade Ltd., Kent, UK) overnight. Coupons were rinsed twice with tap water and finally rinsed with distilled water. All coupons were sterilized at 121 °C for 15 min before use to achieve sterility. Two sterile coupons were then transferred to a Petri dish (diameter 94 mm) filled with 25 ml of a working cell suspension and incubated at 28, 37 or 42 °C for varying lengths of time under static conditions. Following incubation, each Petri dish was removed from the incubator and populations of cells attached to coupons were enumerated, as described below.

2.3. Enumeration of the attached and planktonic cells

To enumerate the planktonic cells after each period, one ml of the cell suspension was pipetted from the Petri dish, diluted with 1 g/L peptone saline water (PSW, 1 g of proteose peptone, 8.5 g of NaCl) and plated on trypticase soy agar (TSA, Oxoid) and incubated at 37 °C for 24 h. To enumerate attached cells, detachment of attached cells from the coupons was performed by the bead vortexing method, which is considered the most suitable method for removal of attached bacteria (Lindsay & von Holy, 1997). Each coupon bearing attached cells was carefully removed from the growth medium with sterile forceps, gently tapped it against the side of the Petri dish to remove excess liquid droplets, and rinsed twice with sterile PBS to remove any loosely-attached cells. Each coupon was then transferred to a sterile test tube containing 9 ml of 1 g/L PSW, 20–25 ea of sterile glass beads (diameter 0.4–0.5 mm) and subsequently vortexed for 3 min in order to detach cells from the coupon. After vortexing, the suspension was diluted with 1 g/L PSW, plated on TSA and incubated at 37 °C for 24 h.

2.4. Attachment kinetics and biofilm formation index

In order to elucidate the effects of temperature and pH on biofilm formation, the kinetic parameters for adhesion were estimated according to the modified Gompertz equation using OriginLab (version 8.5.1., OriginLab Corporation, MA, USA):

$$\log\frac{N_t}{N_i} = C\cdot e^{-e^{-k(t-m)}}$$

where N_t is the number of attached cells at time t (log CFU/cm^2), N_i is the initial number of attached cells at growth phase (log CFU/cm^2), C is the total amount of biofilm that formed after the first hour (log CFU/cm^2), m is the time required to reach the maximum biofilm formation rate (h), k is the formation rate at time m (1/h).

The biofilm index (BI) was calculated by normalizing the numbers of sessile cells with the number of planktonic cells at the same time point. Normalization would eliminate the effect of varying planktonic growth rates, providing a more objective view of the biofilm-forming ability of *Salmonella* under suboptimal growth conditions.

$$BI = \frac{\text{Attached cells}(\log(cfu\ cm^{-2}))}{\text{Planktonic cells}(\log(cfu\ cm^{-2}))}$$

2.5. Microbial adherence to solvent (MATS) assay

Following the 24-h incubation period, cells were harvested by centrifugation at 10,000 × *g* for 10 min at 4 °C and washed twice with 8.77 g/L of NaCl. The cell suspension was prepared in 8.77 g/L of NaCl at a population of ca. 10^8 CFU/ml (OD_{600} = 0.4–0.5). Next, 1.2 ml of washed cells was vortexed for 60 s with 0.2 ml of solvent. The mixture was allowed to stand for 15 min to ensure that the two phases were completely separated before three aliquots of 300 μl of the aqueous phase was removed and the absorbance at 400 nm was