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Biofilm removal of *Pseudomonas* strains using hot water sanitation

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Abstract. Biofilm formation of *Pseudomonas aeruginosa* and *Pseudomonas stutzeri* and biofilm removal efficacy of hot water were examined. Both strains show different biofilm formation in BH broth and mineral water, but only a slight difference could be observed. Both *Pseudomonas* strain grew better in BH broth than in nutrient-poor medium. *Pseudomonas stutzeri* grow better in mineral water than *Pseudomonas aeruginosa*. There was no difference in attachment concerning the surface quality of the stainless steel coupons, irrespective of the growth medium. Hot water treatment reduced the number of planktonic cells with 4.5–6 log cycles, the number of adhered cells with 4.2–4.9 log cycles, and the number of biofilm cells with 2–3 log cycles, which was not sufficient to eliminate biofilms from the surface.

1 Introduction

The first report on biofilms was presented more then 60 years ago (*Zobell*, 1943). Biofilms are matrices of microorganisms embedded in their own microbial-originated extracellular polymeric substances (EPSs) attached to a solid surface or substratum. Biofilms are a concern in a broad range of areas, causing slime problems, reducing heat transfer efficiency in heat exchangers and condensers, great hygienic and financial concerns in the food, environmental

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and biomedical fields (*Sihorkar* and *Vyas*, 2001; *Beczner*, 2001; *Maukonen et al.*, 2003). Biofilms are particularly problematic in food industry (*Frank et al.*, 2003; *Jessen and Lammert*, 2003). Biofilm bacteria are different from their planktonic counterparts (*Sauer et al.*, 2002). The attachment of bacteria to surfaces results in an increased (up to 1000 times higher) resistance to antimicrobial agents (*Stewart et al.*, 2000), and this makes their elimination from food processing facilities a big challenge (*Simoes et al.*, 2006).

Pathogenic bacteria are also able to form biofilms representing potential health risks (*Armon et al.*, 1997). *Pseudomonas aeruginosa* is a human opportunistic pathogen able to form biofilms on different biotic and abiotic surfaces, e.g. in water systems. It has been emerging as a primary source of nosocomial infections (*Kipnis et al.*, 2006), including infections of artificial implants, contact lenses, urinary cathetersacheal tubes (*Davey* and *O'Toole*, 2000). More than 60% of hospital-acquired infections are biofilm-related (*Ebrey et al.*, 2004). *Pseudomonas stutzeri* can be isolated from soil and surface water. Its presence in food and food environments is not regulated, but because of its good biofilm-producing ability it easily colonizes in pipes, heat-exchangers, air-conditioners, etc.; therefore, its growth should be controlled by sanitation procedures.

Bacteria associated with biofilms are much more difficult to kill and remove from surfaces than planktonic organisms. Biocides and disinfectants have been the principal weapons used to control unwanted biofilms. To kill and remove biofilm cells, cleaning procedures must break up or dissolve the EPS matrix of the biofilms to give the disinfectants access to the viable cells (*Simões et al.*, 2006). The removal of a mature biofilm most often requires extensive mechanical action, such as scrubbing or scraping in conjunction with the use of cleaning and sanitizing agents. However, care should be taken because some brushes and scrapers may be abrasive and leave scratches on surfaces, which promote biofilm formation. Disinfectants are less effective in the presence of organic material. Interfering organic substances, pH, temperature, water hardness, chemical inhibitors, concentration, and contact time generally control disinfectant efficacy (*Bremer et al.*, 2002). Chemicals must be rinsed off from surfaces, leaving no toxic residues. Hot water sanitation provides the green strategy for biofilm control. It has some advantages over chemicals: no chemical residues, no corrosion, and it can get to hard-to-reach areas. However, this practice is not advisable in some industries (e.g. dairy) because it aids in the formation of the conditioning layer by denaturing proteins and increasing the adhesion properties of the equipment.

The purpose of this study was to evaluate the removing and inactivating

effect of hot water sanitation on *Pseudomonas aerugonisa* and *Pseudomonas stutzeri* biofilms on stainless steel surface.

2 Materials and methods

2.1 Cultures

A culture collection strain of *Pseudomonas aeruginosa* (ATCC 9027) and a water source isolate of Pseudomonas stutzeri was used in the investigations. Strains were stored on agar slants at 4 °C. Strains were grown on BHI agar [brain extract, heart extract, and peptones 17.5 g; glucose 2.0 g; yeast extract 2.5 g; sodium chloride 5.0 g; di-sodium hydrogen phosphate 2.5 g; agar-agar 15.0 g; distilled water 1.0 l) (Merck 113825 0500)] at $30 \pm 2°C$ for 24–48 hrs.

2.2 Biofilm formation

Stainless steel coupons (30×9 mm) were used in the experiments. Stainless steel 75× was applied. Two types of stainless steel surfaces were used: stainless steel with smooth surface (SS) and stainless steel with matt surface (MS). A dilution of 106 cells ml^{-1} was prepared from each strain in 80 ml growth medium [BHI broth or still mineral water (MW)] in Petri-dishes (diam. 18 cm). Metal coupons were immersed and left in the growth medium containing the test microorganisms for biofilm formation. Twenty-four, 48, and 168 hours old biofilms of the strains were developed.

2.3 Hot water sanitation

After 24, 48, and 168 hours of biofilm formation, the coupons were rinsed with sterile water to remove unattached cells, and placed into hot water for 1 min, and rinsed with sterile water. The number of viable cells was counted as described in section 2.4. Other hot-water-treated coupons were then placed into sterile Petri dishes and poured with melted TGE agar (glucose 1.0 g; yeast extract 2.5 g; peptone 5.0 g; agar-agar 15.0 g; distilled water 1.0 l) (to detect any survival cells) as well as painted with acridine orange for microscopic investigations. Untreated coupons served as controls.

2.4 Detection of viable cell counts

Coupons were removed after 24, 48, and 168 hours from the Petri dishes containing BH or still mineral water, and rinsed with sterile water to remove

unattached cells. Coupons were placed into test tubes containing sterile glass beads and 10 ml sterile diluent. Tubes were vortexed for 2 minutes to remove attached cells from the surface of the coupons. Appropriate dilutions of these diluents were pour-plated and incubated at 30 °C for 24–48 hours. All measurements were carried out in duplicates.

2.5 Microscopic investigations

Coupons were investigated with epifluorescent microscope (Olympus BH-2, Olympus Optical Gmbh, Germany) after acridine orange (AO, 0.02 g/100 ml water) staining for 2 minutes.

2.6 Heat treatment of planktonic cells

A dilution of 10^6 cells ml^{-1} was prepared from each strain in 6 ml BH broth transferred into a 85 °C water bath for 1 minute (measuring the core temperature). The number of survival cells was determined with pour plating.

2.7 Heat treatment of cells on stainless steel surface

Volume of 0.1 ml of 106 cells ml^{-1} test cultures was spot-inoculated on the small stainless steel coupons. The surface was air-dried at room temperature. After drying, coupons were placed into Petri-dishes containing 85 °C water. Exposure time was 1 minute. Control coupons without heat treatment were also prepared as controls.

3 Results and discussions

3.1 Biofilm formation

Our studies were carried out under static conditions because they are as common as surfaces subjected to continuous liquid flow. Both strains showed biofilm formation on both stainless steel types although its quantity did not increase significantly with time (Figure 1).

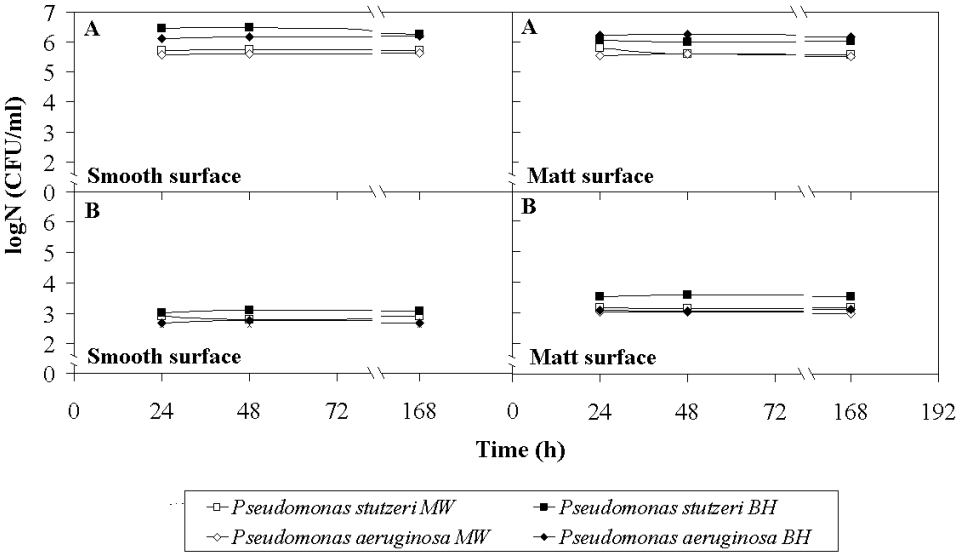
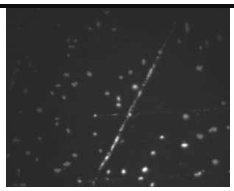
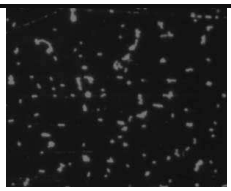
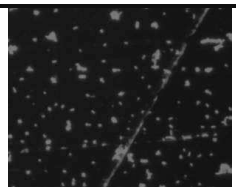
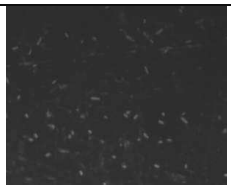
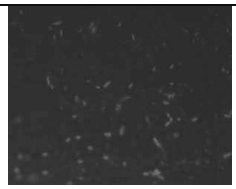
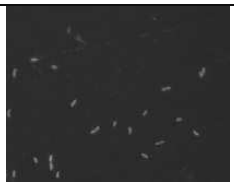
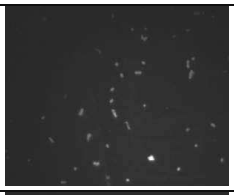
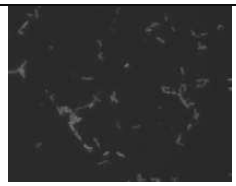
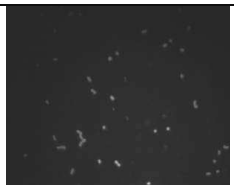
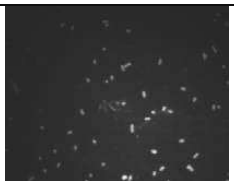
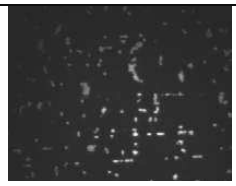


Figure 1: Viable number of irreversibly attached cells without (A) or with (B) hot sanitation treatment

Mays (2000) found similar degree of biofilm formation in bottled mineral water. Results showed that biofilm production was influenced by the growth medium used. Both strains grew better in BH broth compared to mineral water although many publications show that nutrient limitation enhances the attachment of cells to surfaces (*Poulsen*, 1999; *Ryu et al.*, 2004). On smooth surfaces, biofilm production of *P. stutzeri* in BH broth was higher compared to *P. aeruginosa* but the difference was not significant. *P. aeruginosa* produced higher amount of biofilm on matt surface in BH broth compared to mineral water while there was no significant difference in the case of *P. stutzeri*. The reason for that could be the water source origin of the *P. stutzeri* strain (Figure 1). This natural milieu provided good atmosphere for the adaptation of *P. stutzeri* cells to the harsh, low-nutrient-containing environment, and resulted in better biofilm formation ability.

The biofilm formation of the examined *Pseudomonas* strains was not affected by the surface quality of stainless steel coupons although sometimes significant attachments of bacteria to the cracks of stainless steel surfaces could also be seen. Some representative images obtained from microscopic observations of biofilm development during 6 days are shown in Table 1.

Table 1: Biofilm formation of Pseudomonas strains on stainless steel

	24 h	48 h	168 h
Ps. aeruginosa in BH			
Ps. aeruginosa in MW			
Ps. stutzeri in BH			
Ps. stutzeri in MW			

3.2 Hot water sanitation

Planctonic cells of *P. aeruginosa* showed a 6-log cycle and *P. stutzeri* a 4.5 reduction after hot water treatment. It was very effective against both strains in liquid medium. When *Pseudomonas aerugonisa* or *stutzeri* were adhered to stainless steel surface (cells attached to the surface reversible), the reduction was milder: 4.9 for *P. aeruginosa* and 4.2 for *P. stutzeri*, respectively. Biofilm cells showed the greatest resistance (3.2 log cycle reduction) against 85 °C water treatment, as it was expected (Figure 2).

It is well known that attached cells are much more resistant to antimicrobial treatments than their planktonic counterpart (*Norwood* and *Gilmour*, 2000; *González-Fandos et al.*, 2005; *Pap* and *Kiskó*, 2008). Reduction in mineral

water was more remarkable in the case of both *Pseudomonas* strains, which indicates a higher amount of glicocalix production in nutrient-poor medium (mineral water). In agreement with our work, other studies also showed that nutrient limitations result in higher glicocalix production, increasing the resistance of biofilm cells (*Brown* and *Hunter*, 1977; *Wrangstadh et al.*, 1986; *Kim* and *Frank* 1994; *Ryu et al.*, 2004).

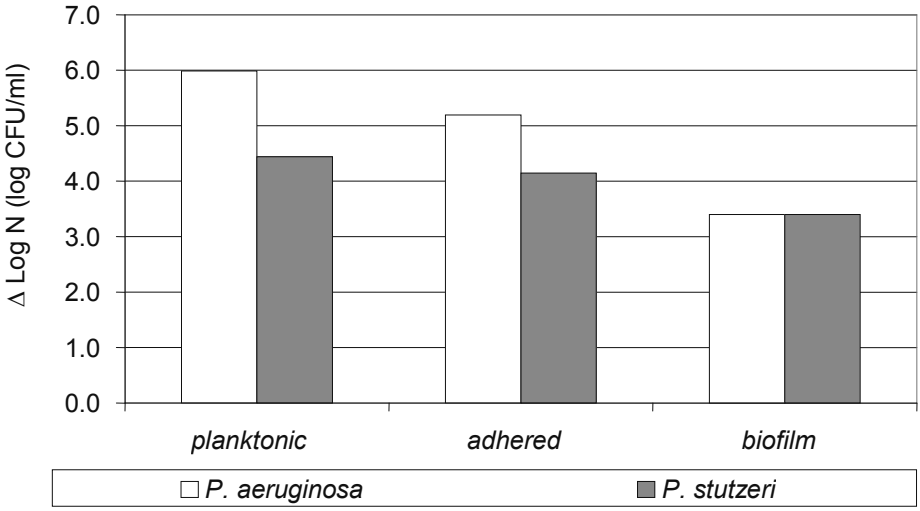


Figure 2: Heat inactivation effect of 85 °C hot water on planktonic, reversible adhered and irreversible attached (biofilm) cells

There was significant difference in heat resistance regarding stainless steel quality (Figure 3). Both *Pseudomonas* strains showed higher reduction on smooth surface. On matt surfaces, biofilm cells of *P. stutzeri* proved to be the most resistant.

Hot water treatment proved to be insufficient to destroy and remove *Pseudomonas* biofilms. A combination of heat and mechanical treatment or chemicals might be more successful. Results of *Jessen* and *Lammert* (2003) also suggested that individually applied disinfection treatments are not sufficient – they need to be used in combinations.

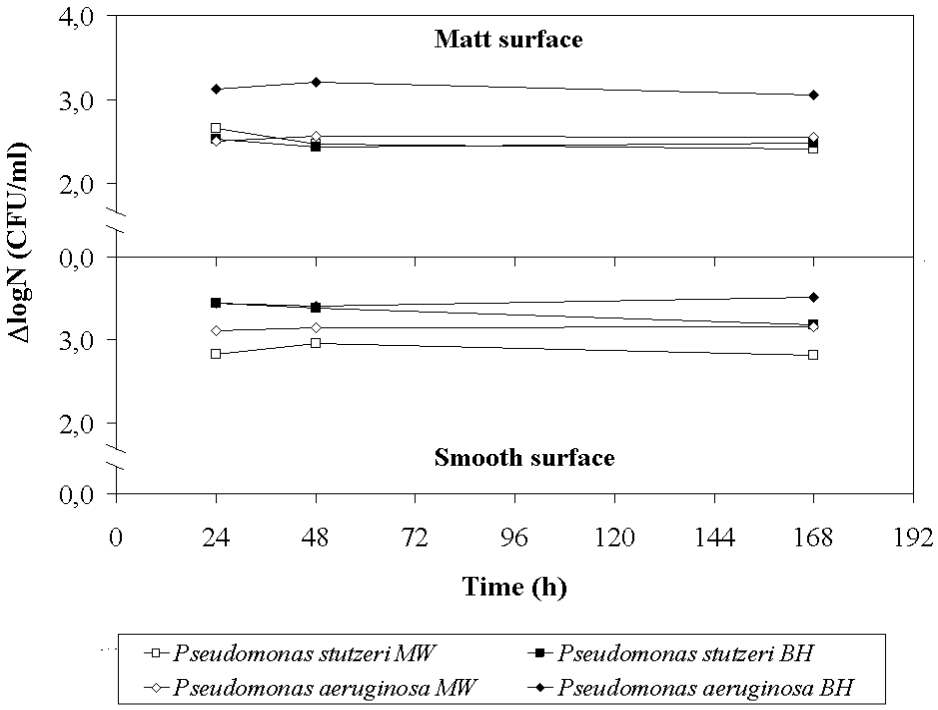


Figure 3: Heat inactivation effect of 85 °C hot water on biofilm cells on different quality stainless steel surfaces

4 Conclusions

Some equipment is designed to be sanitized by hot water or steam. It is successfully used in specific brewery, vinery, or dairy applications. Our results showed that although hot water sanitation reduced the number of *Pseudomonas* biofilm cells by about 3-log cycles, it left surviving cells, which enable further growth of biofilm. Therefore, it can not be considered an effective sanitation procedure unless its application is accompanied by further mechanical or chemical sanitation.

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