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Effect of sanitizer combined with steam heating on the inactivation of foodborne pathogens in a biofilm on stainless steel

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ABSTRACT

The combined effect of chemical sanitizers including sodium hypochlorite, hydrogen peroxide, iodophor, and benzalkonium chloride with steam heating on the inactivation of biofilms formed by *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* on stainless steel was investigated. Six day old biofilms, comprised of a mixture of three strains each of three foodborne pathogens, were produced on stainless steel coupons at 25 °C and treated with each sanitizer alone (for 5, 15, and 30 s), steam alone (for 5, 10, and 20 s), and the combination. There was a synergistic effect of sanitizer and steam on the viability of biofilm cells of the three pathogens as evidenced by plating counts and imaging. The combination treatment achieved an additional 0.01 to 2.78 log reduction compared to the sum of each individual treatment. The most effective combination for reducing levels of biofilm cells was the combination of steam and iodophor; steam for 20 s and merely 20 ppm iodophor for 30 s reduced cell numbers to below the detection limit (<1.48 log CFU/coupon). These results suggest that the combination treatment of sanitizer with steam can be applied to control foodborne pathogens biofilm cells in food processing facilities as a potential intervention.

1. Introduction

Escherichia coli O157:H7, *Salmonella* Typhimurium, *and Listeria monocytogenes* are major pathogens implicated in numerous foodborne outbreaks. *E. coli* O157:H7 is an important pathogen capable of causing bloody diarrhea (hemorrhagic colitis) and renal failure (hemolytic uremic syndrome) in humans (Doyle, 1991). Salmonellosis caused by Salmonella results in diarrhea, abdominal pain, fever, chills, nausea, and vomiting (Baird-Parker, 1990). The principal symptoms of *L. monocytogenes* infection are abortion, neonatal death, septicemia, and meningitis (Farber and Peterkin, 1991). Contamination with these pathogens in food-processing environments and food-processing lines may be a frequent and important cause of outbreaks of food-borne disease (Reij and Den Aantrekker, 2004).

Improper cleaning and disinfection of food contact surfaces contributes to soil buildup, and, in the presence of water, facilitates the development of bacterial biofilms which may include pathogenic microorganisms (Chmielewski and Frank, 2003). A biofilm is a sessile bacterial community of microbial cells that is irreversibly associated (not removed by gentle rinsing) with a surface and enclosed in extracellular polymeric substances (Costerton, 1995; Donlan, 2002). Such biofilms can lead to potential hygiene problems by concomitant bacterial transmission to food products (Shi and Zhu, 2009).

Many researchers have studied the effectiveness of sanitizers used in the food industry against food-borne pathogens, including chlorine and chlorine derivatives, iodophors, quaternary ammonium compounds (QAC) and hydrogen peroxide (HP) (Greene et al., 1993; Peng et al., 2002; Joseph et al., 2001). However, individual sanitizer treatments used in many studies showed little effect on cells in a biofilm even when long time exposure times were utilized (Joseph et al., 2001; Chmielewski and Frank, 2003). Furthermore, bacteria within a biofilm matrix have decreased sensitivity to disinfectants compared to planktonic cells, and the resistance of biofilm bacteria typically increases with age (Bower et al., 1996; Costerton et al., 1999).

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To improve the ability of killing and removing biofilm organisms from food processing facilities, combination treatments of sanitizers with other methods may be useful. It is expected that the use of combined factors will have greater effectiveness at inactivating microorganisms than the use of any single factor alone. Many researchers have evaluated combinations of sanitizers with other cleaning methods such as, ultrasonication, heat, and other sanitizers to eradicate or inhibit foodborne pathogens (Berrang et al., 2008; Scouten and Beuchat, 2002; Jin and Lee, 2007).

Steam treatment is a rapid method of heating that has previously been studied for inactivating foodborne pathogens on foods and biofilms in food processing environments (Chang et al., 2010; Ban et al., 2012). The main advantage of steam treatment is the large amount of heat transferred to the food or material when steam condenses, which rapidly increases surface temperature (James et al., 2000). Steam at 100 °C has a greater heat capacity than the same amount of water at that temperature (James and James, 1997), and is able to effectively penetrate cavities, crevices, and feather follicles that may provide protection for surface-attached bacteria (Morgan et al., 1996). However, to date, no studies have investigated the combination of steam and sanitizers such as sodium hypochlorite (SHC), iodophor, benzalkonium chloride (BKC; a kind of QAC), and HP for reducing biofilms.

Therefore, the objective of this study was to determine and compare the effectiveness of individual treatments (steam and sanitizers) and the combination of steam and sanitizers for reducing foodborne pathogenic biofilm cells on stainless steel.

2. Materials and methods

2.1. Bacterial strains and culture preparation

Three strains each of *E. coli* O157:H7 (ATCC 35150, ATCC 43889, ATCC 43890), *S.* Typhimurium (ATCC 19585, ATCC 43971, DT 104), and *L. monocytogenes* (ATCC 15315, ATCC 19114, ATCC 19115) were obtained from the bacterial culture collection of Seoul National University (Seoul, Korea) and used in this study. Stock cultures were stored at −80 °C in 0.7 ml of tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD, USA) and 0.3 ml of 50% glycerol. Working cultures were maintained on tryptic soy agar (TSA; Difco) slants at 4 °C and were subcultured monthly. Each strain of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* was grown in 10 ml of TSB at 37 °C for 24 h. Culture cocktails of each pathogen species were prepared individually as follows: the three strains of each pathogen species were combined and cells were collected by centrifugation at 5000 × g at 4 °C for 15 min and washed three times with phosphate-buffered saline (PBS, pH 7.4; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4). The final pellets of each pathogen species were resuspended in sterile PBS, corresponding to approximately 10^7–10^8 colony-forming units (CFU)/ml.

2.2. Preparation of stainless steel coupons

Stainless steel coupons (type 316, 5 × 2 × 0.1 cm^3, bright annealed) were used in this study. Coupons were immersed in 70% ethanol for 10 min to disinfect the surface, and rinsed with sterile distilled water. Washed stainless steel coupons were autoclaved at 121 °C for 15 min in covered glass beakers before use.

2.3. Biofilm formation

Prepared sterile stainless steel coupons were transferred to sterile 50 ml conical centrifuge tubes (SPL Lifesciences, Pocheon, Korea) containing separate 30-ml cell suspensions in PBS of the 3-strain culture cocktail for each pathogen (ca. 10^7–10^8 CFU/ml). Conical centrifuge tubes containing coupons were incubated at 4 °C for 24 h to facilitate attachment of cells. After incubation, coupons were aseptically removed with sterile forceps, immersed in 300 ml of sterile distilled water (22 ± 2 °C), and gently stirred for 5 s. Rinsed coupons were deposited in 50 ml conical centrifuge tubes containing 30 ml of TSB, and then incubated at 25 °C for 6 days. This method was adapted from Kim et al. (2006).

2.4. Sanitizer preparation

The sanitizers tested were SHC (Yuhan Co., Incheon, Korea), HP (Junsei Chemical Co., Tokyo, Japan), BKC (3 M, USA), and iodophor (Namkang Co., Incheon, Korea). The concentrations of SHC, BKC, and iodophor were 20, 50, and 100 ppm, and HP was 0.5, 1, and 2%. The sanitizers were all diluted according to manufacturers' instructions with sterile distilled water to the target concentration; the concentration of free chlorine was quantified using a HI 95771 Chlorine Ultra High Range Meter (Hanna Instruments, Ann Arbor, MI, USA). The solutions were prepared on the day experiments were performed.

2.5. Combination treatment of sanitizer and steam

The steam generation system included a water inlet tube, a steam outlet tube, an electrical resistance heater, and an intelligent power module (IPM; IPM-1W-0050; Dain Technologies, Daegu, Korea). Water was converted into steam by heating with an electrical resistance heater in the SS generator. During these experiments, the steam temperature was controlled automatically by means of a temperature sensor and IPM in the steam generators.

Coupons were removed with sterile forceps, rinsed for 5 s in 300 ml of sterile distilled water (22 ± 2 °C), then immersed in each type of sanitizer for 5, 15, and 30 s. Then they were treated with steam on both sides for 5, 10, and 20 s, respectively, while maintaining an absolute pressure of 143 kPa. During these experiments, the distance between the coupons and the steam outlet was set at 40 mm. Coupons treated with steam alone or immersed into each sanitizer alone were used as controls.

2.6. Bacterial enumeration

After treatment, stainless steel coupons were transferred to sterile 50-ml conical centrifuge tubes containing 30 ml of PBS and 3 g of sterile glass beads (425–600 μm; Sigma–Aldrich, St. Louis, MO, USA) and then agitated with a benchtop vortex mixer set at maximum speed for 1 min. Immediately after vortexing, cell suspensions were tenfold serially diluted in buffered peptone water (BPW; Difco), and 0.1 ml of undiluted cell suspension or dilutions were spread-plated onto Sorbitol MacConkey Agar (SMAC; Difco), Xylose Lysine Desoxycholate Agar (XLD; Difco), or Oxford Agar Base (OAB; Difco) with antimicrobic supplement (Difco) to enumerate the numbers of *E. coli* O157:H7, *S.* Typhimurium, or *L. monocytogenes* biofilm cells, respectively, attached to the surfaces of stainless steel coupons. When low bacterial numbers were anticipated, 250 μl of undiluted cell suspension was plated onto four plates of each respective medium. The plates were incubated at 37 °C for 24–48 h. After incubation, colonies were counted. The detection limit for three pathogens by direct plating was 1.48 log CFU/coupon.

2.7. Confocal laser scanning microscopy

In order to examine cell membrane integrity, a BacLight Live/Dead bacterial viability kit (L-7012, Molecular Probes, USA) was used. This kit includes SYTO9 and propidium iodide (PI) to differentiate between cells with intact membranes (live) and damaged