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Research Note

Inactivation of *Staphylococcus aureus* Biofilms on Food Contact Surfaces by Superheated Steam Treatment

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ABSTRACT

The objective of this study was to compare the inactivation efficacy of saturated steam (SS) and superheated steam (SHS) on *Staphylococcus aureus* biofilms on food contact surfaces, including type 304 stainless steel coupons with No. 4 finish (STS No. 4), type 304 stainless steel coupons with 2B finish (STS 2B), high-density polyethylene (HDPE), and polypropylene (PP). In addition, the effects of the surface characteristics on the inactivation efficacy were evaluated. Biofilms were formed on each food contact coupon surface using a three-strain cocktail of *S. aureus*. Five-day-old biofilms on STS No. 4, STS 2B, HDPE, and PP coupons were treated with SS at 100°C and SHS at 125 and 150°C for 2, 4, 7, 10, 15, and 20 s. Among all coupon types, SHS was more effective than SS in inactivating the *S. aureus* biofilms. *S. aureus* biofilms on steel coupons were more susceptible to most SS and SHS treatments than the biofilms on plastic coupons. *S. aureus* biofilms on HDPE and PP coupons were reduced by 4.00 and 5.22 log CFU per coupon, respectively, after SS treatment (100°C) for 20 s. SS treatment for 20 s reduced the amount of *S. aureus* biofilm on STS No. 4 and STS 2B coupons to below the detection limit. With SHS treatment (150°C), *S. aureus* biofilms on HDPE and PP needed 15 s to be inactivated to below the detection limit, while steel coupons only needed 10 s. The results of this study suggest that SHS treatment has potential as a biofilm control intervention for the food industry.

HIGHLIGHTS

- SHS was more effective than SS for inactivating biofilm cells of *S. aureus*.
- Biofilms on steel coupons were more susceptible than those on plastic coupons.
- The thermal conductivity of the coupon was an important factor in SHS treatment.
- Biofilm; Saturated steam; *Staphylococcus aureus*; Superheated steam

Key words: Biofilm; Saturated steam; *Staphylococcus aureus*; Superheated steam

Bacteria commonly form resistant survival structures by adhering to surfaces and forming biofilms consisting of hydrated extracellular polymeric substances *(8, 35)*. Microbial biofilms on food processing facility surfaces may contain a considerable number of pathogenic microorganisms and lead to potential hygienic problems in the food processing industry, because pathogens in biofilms could be transmitted to food *(4)*.

Staphylococcus aureus is a pathogenic bacterium causing a range of diseases, from food poisoning to severe infection, and is an adaptable organism that can live in various environments as biofilms *(13, 26)*. *S. aureus* has been frequently found on food processing plant surfaces and has been responsible for outbreaks related to the consumption of fresh and processed foods *(6, 19)*.

Treatment with various sanitizers, including chlorine, chlorine dioxide gas, essential oils, peracetic acid, ozone, hydrogen peroxide, and quaternary ammonium chloride, has been evaluated to inactivate or remove biofilm cells of foodborne pathogens from food processing surfaces *(10, 21, 25, 27, 31, 34)*. However, sanitizer treatments have limited effectiveness against biofilm cells because of their greater resistance to environmental stresses and sanitizers compared with their planktonic counterparts *(11)*. In addition, various methods, such as cold plasma *(5, 18)* and bacteriophage treatment *(28),* have been evaluated for inactivation and removal of biofilms from food processing surfaces, but these methods are difficult to implement and are only applicable to small areas *(16)*.

Superheated steam (SHS) is defined as steam that is given additional heat to raise its temperature above the

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saturation temperature at a constant pressure *(7)*. SHS transfers a larger amount of heat to the subject of treatment when SHS condenses on treated surfaces, which rapidly increases the surface temperature *(14, 30)*. SHS pasteurization is a time-saving and nonpolluting technology in terms of avoiding the use of chemical compounds *(24, 32)*. In addition, SHS can penetrate cavities and crevices effectively, which may provide protection for surface-attached microorganisms *(17)*.

Ban et al. *(3)* reported that SHS treatment effectively reduced populations of biofilm cells of *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* on polyvinyl chloride and stainless steel surfaces and reduced the disinfection time compared with saturated steam (SS) treatment *(3)*. However, the inactivation of biofilm cells of *S. aureus* by SHS has not been studied. Therefore, the purpose of this study was to compare the effectiveness of SS and SHS for inactivating biofilm cells of *S. aureus* on various food contact surfaces and to evaluate the effect of surface characteristics on the inactivation efficacy.

MATERIALS AND METHODS

Bacterial cultures and cell suspension. Three strains of *S. aureus* (ATCC 25923, ATCC 27213, and ATCC 29213) were obtained from the bacterial culture collection of the School of Food Science, Seoul National University (Seoul, South Korea). Each strain (maintained as –80°C frozen stocks) was streaked for isolation onto tryptic soy agar (Difco, BD, Sparks, MD) and incubated at 37°C for 24 h. A single colony of each strain was inoculated into 5 mL of tryptic soy broth (TSB; Difco) and incubated as described earlier. Cells of each strain were collected by centrifugation at 4,000 × *g* for 20 min at 4°C and washed three times with phosphate-buffered saline (PBS, pH 7.4; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2PO_4, and 1.8 mM KH_2PO_4). The final pellets of each of the strains were resuspended in sterile PBS and combined to produce a mixed culture cocktail corresponding to approximately 10^7 to 10^8 CFU/mL.

Preparation of coupons. Coupons (5 by 2 cm) made from type 304 stainless steel with No. 4 finish (STS No. 4), type 304 stainless steel with 2B finish (STS 2B), high-density polyethylene (HDPE), and polypropylene (PP) were used. STS No. 4 and STS 2B coupons were immersed in 70% ethanol for 60 min to disinfect the surface and were rinsed with sterile distilled water before sterilizing by autoclaving at 121°C for 20 min. The HDPE and PP coupons were sterilized by immersion in 70% ethanol at 80°C for 20 min.

Biofilm formation. The biofilm formation method was adapted and modified from Ban and Kang *(1)*. Each prepared coupon was immersed in a sterile 50-mL conical centrifuge tube (Falcon, BD Biosciences, San Jose, CA) containing a 30-mL suspension of *S. aureus* in PBS (ca. 10^7 to 10^8 CFU/mL). Coupons in the bacterial cell suspensions were incubated at 4°C for 24 h to facilitate the attachment of cells. Each coupon was removed with sterile forceps, immersed in 1,300 mL of sterile distilled water (22 ± 2°C), and gently stirred for 5 s. Seven coupons with the same steam treatment used the same distilled water. The rinsed coupons were deposited in 50-mL conical centrifuge tubes containing 30 mL of TSB and then maturated at 25°C for 5 days. The maturation temperature was chosen based on the determination of a well-formed *S. aureus* biofilm from a previous study *(20)*.

SS and SHS treatments. The coupons were removed from the tubes and rinsed as previously described and then exposed to SS and SHS on both sides for 2, 4, 7, 10, 15, or 20 s. The distance between the coupons and the steam generator nozzle was 7 cm. SS treatments were performed at 100°C, while SHS treatments were performed at 125 or 150°C. The temperature was measured with a K-type Teflon-coated thermocouple (AZ Instrument Co. Ltd., Sha Tin, Hong Kong). The probe was located at the nozzle hole, and the steam temperature range during the experiments was controlled within 5°C.

Bacterial enumeration. After SS and SHS treatment, the coupons were transferred into sterile 50-mL conical centrifuge tubes containing 30 mL of sterile peptone water (Difco) and 3 g of sterile glass beads (425 to 600 μm; Sigma, St. Louis, MO) and then agitated with a benchtop vortex mixer set at the maximum speed for 1 min. Cell suspensions in the tubes were 10-fold serially diluted in peptone water, and then 0.1 mL of the undiluted cell suspension or diluents was spread plated onto Baird-Parker agar (MB cell, Kisan Biotech Co., Ltd., Seoul, Korea) to enumerate the number of *S. aureus* biofilm cells detached from the coupon surfaces. When low bacterial numbers were anticipated, 250-μL portions of the undiluted cell suspension were plated onto four plates of the medium. The plates were incubated at 37°C for 24 to 48 h, and the colonies were counted.

Surface hydrophobicity and roughness measurement. The water contact angle measurement was used to evaluate the surface hydrophobicity of the coupons *(23)*. The water contact angle was measured by the sessile drop method using a contact angle goniometer (DSA 100, Krüss, Hamburg, Germany) equipped with a camera. Using a microliter syringe and a 0.5-mm-diameter needle, 3 μL of distilled water was deposited onto the coupon surfaces at room temperature (22 ± 2°C). Contact angle measurements were conducted for less than 30 s to avoid changes in the tested surface. Ten data points were taken for each sample ($n = 10$). White light scanning interferometry was used to measure the surface roughness of the coupons *(23)*. Samples were directly mounted on the stage of a noncontact three-dimensional surface profiler (NanoView-E1000, NanoSystem, Daejeon, South Korea). Topographic images of areas 125 by 95 μm were acquired from each sample. Height profiles were expressed in the three-dimensional topographic images with the color scale. The Ra (arithmetic mean roughness) and Rq (root mean square roughness) values were calculated from 10 scan areas (125 by 95 μm) of each sample using a software package (NanoMap version 2.5.17.0, NanoSystem).

Thermal conductivity and thermal diffusivity measurement. A laser flash technique was used to measure thermal conductivity and thermal diffusivity. Samples (1 by 1 mm square, 1 mm thick) were prepared from each material, and the heat capacity of the samples was evaluated using differential scanning calorimetry to calculate thermal conductivity and diffusivity. For a given geometric sample, applied laser heat was propagated from the top to the bottom surface of the material (LFA 447 Nanoflash, Netzsch, Selb, Germany). Five measurements of each sample were taken.

Statistical analysis. Microbial reductions are provided as the means ± standard deviations of three independent determinations

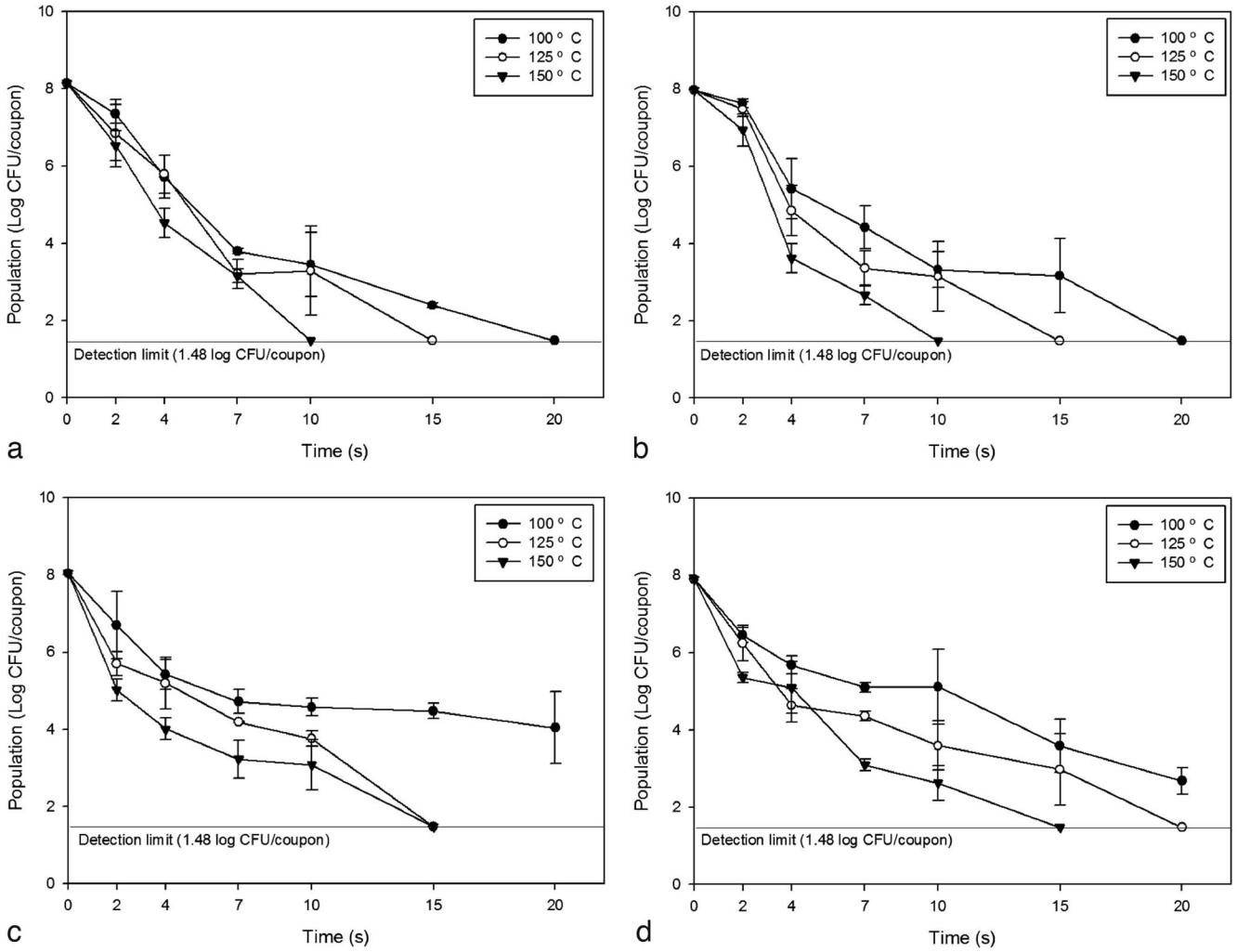

FIGURE 1. *Populations of* S. aureus *biofilm cells on (a) STS No. 4, (b) STS 2B, (c) HDPE, and (d) PP coupons after treatment with saturated steam (SS) at 100°C (●) and superheated steam (SHS) at 125°C (○) and 150°C (▼) for 2 to 20 s.*

with duplicate samples for each trial. Surface roughness, hydrophobicity, thermal conductivity, and diffusivity were conducted in triplicate, with each measurement repeated three times. Data were analyzed by analysis of variance using the Statistical Analysis System (SAS Institute, Cary, NC) and separation of means by Duncan's multiple range test at a probability level of $P < 0.05$.

RESULTS AND DISCUSSION

Biofilms have emerged as a problem in the food industry because of their ability to form on food and food contact surfaces under various conditions when surface bacteria are not properly removed *(15)*. If biofilms contain pathogenic bacteria, cross-contamination from food processing could develop into a food poisoning outbreak. Therefore, effective methods to control pathogens in biofilms are needed, and SHS is suitable, because it can inactivate pathogens by rapidly transferring temperature to the surfaces through its high latent heat *(3)*.

The survival of *S. aureus* on the four types of coupons after SS and SHS treatment is shown in Figure 1. For all types of coupons, SHS heated to a higher temperature required less time to reduce *S. aureus* to below the detection limit. In addition, the *S. aureus* biofilms on both types of stainless steel coupons were more susceptible than plastic coupons to most steam treatments. For SS treatment, *S. aureus* biofilm cell numbers on HDPE coupons were reduced by 1.39 to 4.00 log CFU per coupon and those on PP coupons were reduced by 1.45 to 5.22 log CFU per coupon over the range of treatment times. For stainless steel coupons, STS No. 4 and STS 2B coupons attained 0.79- to 5.75-log and 0.35- to 4.81-log reductions after 5 to 15 s, respectively, and were reduced to below the detection limit after 20 s. SHS treatment (125°C) reduced biofilm populations to below the detection limit within 15 s except on PP coupons, which experienced a 4.93-log reduction. When treated from 2 to 10 s, reductions of *S. aureus* biofilms on HDPE coupons were 2.35 to 4.17 log CFU per coupon, those on PP coupons were 1.66 to 4.30 log CFU per coupon, those on STS No. 4 were 1.30 to 4.85 log CFU per coupon, and those on STS 2B were 0.50 to 5.34 log CFU per coupon. With the 150°C SHS treatment, biofilm cells on plastic coupons needed 15 s to be inactivated to below the detection limit, while steel coupons needed 10 s. Across the range of treatment conditions, 3.03- to 4.67-log and 2.55- to 5.27-log reductions were achieved with HDPE and PP

TABLE 1. *Surface hydrophobicity, thermal conductivity, and diffusivity of four types of coupons*[a]

Coupon	Water contact angle (°)	Diffusivity (mm^2/s)	Conductivity (W/m·K)
STS No. 4	85.6 ± 1.5 A	4.126 ± 0.010 A	16.153 ± 0.039 A
STS 2B	96.8 ± 1.8 B	4.105 ± 0.011 B	16.046 ± 0.045 B
HDPE	96.6 ± 2.8 B	0.157 ± 0.000 C	0.441 ± 0.001 C
PP	86.9 ± 1.7 A	0.106 ± 0.000 D	0.223 ± 0.001 D

[a] The surface hydrophobicity parameter is the water contact angle (°), and if the water contact angle is higher, then the surface is more hydrophobic. The data represent mean ± standard deviation. Values followed by the same letter in the same column are not significantly different ($P > 0.05$).

coupons, respectively, after 2 to 10 s. For STS No. 4 coupons and STS 2B coupons, 1.62- to 4.99-log and 1.04- to 5.31-log reductions were achieved, respectively, with 2 to 7 s of treatment. There have been several studies involving steam treatment to reduce bacteria on surfaces. Song et al. reported that 5 s of steam treatment inactivated *S. aureus* biofilms on polycarbonate and that it was easier to inactivate bacterial biofilms formed on stainless steel than on polycarbonate *(29)*. In addition, Park and Kang *(22)* revealed that *S. aureus* biofilms on stainless steel were more susceptible than polyvinyl chloride when treated with steam. These investigations show similar results compared with this study.

Table 1 shows the surface hydrophobicity, thermal conductivity, and thermal diffusivity of four types of coupons. The water contact angles of STS No. 4 and STS 2B coupons were 85.6 and 96.8°, respectively ($P < 0.05$). Although STS 2B and HDPE were more hydrophobic than STS No. 4 and PP, there were no differences in the numbers of initial bacteria and microbicidal effects according to hydrophobicity differences. In general, hydrophobicity is considered related to the formation of biofilms, but this same tendency is not shown for all bacteria *(3)*. In a previous study, *S. aureus* biofilm formation was more pronounced on hydrophobic (polypropylene) surfaces than on hydrophilic (stainless steel) surfaces *(20)*. However, no significant difference between *S. aureus* biofilm formation on stainless steel and its formation on polypropylene surfaces was reported *(9)*. There was a negative linear relationship between surface roughness and efficacy of inactivation using acidic electrolyzed water and peroxyacetic acid *(33)*. There was no significant difference ($P > 0.05$) in roughness between the coupons in our study. Goulter-Thorsen et al. *(12)* also reported that there was no significant difference of surface roughness between STS 2B and STS No. 4 as measured by atomic force microscopy. Therefore, the effect of roughness on the inactivation of *S. aureus* biofilm cells by steam could not be elucidated from the present study (data not shown).

The thermal conductivity of STS No. 4 and STS 2B, which is 4.126 and 4.105, respectively, is significantly ($P < 0.05$) higher than that of HDPE and PP, which is 0.157 and 0.106, respectively. Unlike the surface hydrophobicity and roughness, thermal conductivity and thermal diffusivity shown in Table 1 correlated with the reduction tendency of *S. aureus* in biofilms when subjected to steam treatment. When thermal conductivity and diffusivity were higher, it was easier to reduce *S. aureus* biofilms with steam treatment. This could be interpreted in two ways. First, if thermal conductivity was high, when one side was exposed to steam, the other side heated relatively quickly; therefore, treating both sides of the coupon increased its effectiveness. In addition, heat was transmitted through areas where the steam was weak; therefore, a coupon with high thermal conductivity could effectively undergo fairly uniform corner-to-corner bacterial inactivation. Other research has revealed that biofilms formed on coupons of different thermal conductivity are shown to be affected differently, and biofilms on surfaces of higher thermal conductivity, such as stainless steel, are easier to control with steam treatment *(2, 3)*.

In this study, the inactivation effects of SHS treatment against *S. aureus* biofilms were investigated with respect to various material properties, such as surface hydrophobicity, roughness, and thermal conductivity. There was not enough evidence to demonstrate that surface hydrophobicity and roughness affected steam treatment efficacy against *S. aureus* biofilms on surfaces; however, thermal conductivity was an important factor. The results of the present study suggest that SHS treatment has potential for use as a control intervention for pathogenic biofilms by the food industry.

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