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RESEARCH NOTE

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Comparison of Gamma Irradiation and Sodium Hypochlorite Treatments to Inactivate *Staphylococcus aureus* and *Pseudomonas aeruginosa* Biofilms on Stainless Steel Surfaces

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Abstract Biofilm formation on various surfaces is a well-known phenomenon and it has caused pollution problems, health and safety hazards, and substantial economic loss in many areas including the food industry. In the present study, Gamma irradiation at a dose of 2.0 kGy reduced the bacterial counts of *Staphylococcus aureus* and *Pseudomonas aeruginosa* suspensions by 6.7 and >6.5 log CFU/mL, respectively, and 30 ppm of sodium hypochlorite effectively reduced the counts of both bacterial suspensions to below the limit of detection (<2 log CFU/cm^2). However, in bacterial biofilms attached to stainless steel, gamma irradiation at a dose of 2.0 kGy reduced the counts of *S. aureus* attached for 1 hr and overnight by ≥5.1 and 5.0 log CFU/cm^2, respectively. Gamma irradiation at a dose of 1.0 kGy reduced the counts of *P. aeruginosa* counts to below the limit of detection (<2 log CFU/cm^2). On the contrary, *S. aureus* and *P. aeruginosa* cells attached to stainless steel chips were difficult to eliminate using sodium hypochlorite. Four hundred ppm of sodium hypochlorite reduced the counts of *S. aureus* and *P. aeruginosa* attached for 1 hr by 2.5 and 3.3 log CFU/cm^2, respectively.

Keywords: biofilm, bacterial suspension, gamma irradiation, sodium hypochlorite, stainless steel chip

Introduction

It has been well documented that most surfaces could be colonized by bacterial biofilms. Microbial communities are readily developed on various surfaces under aqueous environments (1) and biofilm formation involves a series of complex processes in which organic and inorganic molecules and microbial cells are transported and attached to the surface (2). After initial attachment of bacterial cells, irreversible adhesion is facilitated by the production of extracellular polymeric substances (EPS) (3).

The biofouling and biodeterioration of surface materials such as metals and their alloys are readily caused by biofilm formation and these are potential pollution problems, health and safety hazards, and may cause substantial economic loss in many industrial areas (2, 4, 5). Biofilm formation in food industry environments also has been extensively studied and these biofilms may serve as a contamination source of pathogenic and spoilage bacteria (6-9).

Recently, biofilm studies have concentrated on a wide range of materials such as packaging materials (10, 11), polymeric composites (12), orthopedic metals (13), prosthetic graft materials (14), biomaterial surfaces (15), space station candidate materials (16), etc. Of these various materials, stainless steel is one of the most commonly used materials in many areas and has been extensively studied with respect to bacterial adhesion on metallic implant materials (13) and food processing surface materials (18) and for testing various disinfectants on biofilms (19).

Staphylococcus aureus, a widespread, opportunistic pathogen, is one of a large range of microorganisms that can cause food-borne illnesses. It is known that when *S. aureus* is introduced into a food processing plant, it can persist in biofilms for long periods of time and can cause cross-contamination from one point to another (20). *Psuedomonas aeruginosa* is a Gram negative bacterium and an opportunistic pathogen that infects immuno-compromised patients, and is a dominant spoilage microorganism under aerobic conditions (21, 22). This microorganism also can compromise food safety through biofilms developed in packaging materials such as polyethylene (23, 24).

Chlorine compounds are widely used as chemical disinfectants and have been studied as a means of inactivating microorganisms in biofilms (18, 19, 25). Sodium hypochlorite is the best example of a chlorine compound used as a disinfectant and its bactericidal effect is based on penetration of the chemical and its oxidative action on essential enzymes in the cell (26, 27). In general, the effectiveness of sodium hypochlorite is reduced by organic materials and it has an inherent corrosive effect on metals. Until now, fumigation with volatile compounds such as ethylene oxide, methyl bromide, and phosphine has been widely used to decontaminate and disinfest various subjects. However, fumigation is being banned or phased out due to its potential toxicity and environmental concerns (28, 29). For this reason, radiation sterilization utilizing ionizing radiation has been extensively used for disinfection and decontamination as an alternative to fumigation. Typically, gamma rays from a cobalt-60 isotope source or machine-generated accelerated electrons

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are used. Gamma irradiation is the most widely accepted form of radiation sterilization and is used when materials are sensitive to the high temperature of autoclaving. The bactericidal effect of gamma irradiation is dependent on oxygen and hydroxyl radicals, as these radicals damage biological structures. It is a simple, rapid, and effective method of sterilization (30).

The present study was conducted to evaluate the bactericidal effects of sodium hypochlorite and gamma irradiation on *S. aureus* and *P. aeruginosa* biofilms attached to stainless steel chips.

Materials and Methods

Bacterial suspension preparation and bacterial attachment to stainless steel chips Test microorganisms were obtained from Korean Collection for Type Cultures (KCTC). *S. aureus* (KCTC 1636) and *P. aeruginosa* (KCTC 1916) were inoculated in trypticase soy broth (Difco Laboratories, Detroit, MI, USA), respectively, and incubated in a gyratory water bath shaker (New Brunswick Scientific, Edison, NJ, USA) at 37°C at 100 rpm overnight. Stainless steel chips (1 cm^2) were cleaned with a neutral detergent, dried in a drying oven at 70°C, and sterilized by autoclaving at 121°C for 15 min. The triplicate sterile stainless steel chips were transferred into a Petri dish (100×15 mm) and 0.2 mL of each bacterial suspension (approximately 10^8-10^9 CFU/mL) was inoculated onto the stainless steel chips for 1 hr or overnight at ambient temperature. After inoculation, the stainless steel chips were gently rinsed with 1 mL of 0.1% sterile peptone water (Difco Laboratories) twice and dried in a laminar flow hood.

Gamma irradiation The inoculated stainless steel chips were transferred to a sterile test tube with a polypropylene closure and irradiated with a dose rate of 7 kGy per hour of gamma ray to obtain 0.5, 1.0, and 2.0 kGy by using a Co-60 gamma ray irradiating facility (IR-79; Nordion Interntional Ltd., Ontario, Canada, 100 kCi). The bacterial suspension (10^8-10^9 CFU/mL) was also irradiated at the same doses for the comparison with biofilm on stainless steel chips. The radiation dose was validated by using 5-mm diameter alanine dosimeters (Bruker Instrument, Rheinstten, Germany) and free radical was measured by using an EMS 104 EPR Analyzer (Bruker Instrument). The actual doses were within±2% of the target doses.

Sodium hypochlorite treatment and total available chlorine content determination A commercial bleach product was purchased from a local grocery market and its sodium hypochlorite content was approximately 5.3%. The sodium hypochlorite solution was diluted to prepare working solutions containing 30, 50, 100, 200, and 400 ppm of total available chlorine contents. The total available chlorine content was determined by the iodometric titration method (31). The each working solutions were freshly prepared on each experimental day and maintained in amber bottles with screwed closures. The working solution was dropped onto the inoculated stainless steel chips for 1 min at ambient temperature. Also, 1 mL of bacterial solution (approximately 10^7-10^8) was mixed with 9 mL of working solutions for 1 min to evaluate the bactericidal effect of sodium hypochlorite on pure bacterial culture.

Microbial analysis of biofilms and bacterial solutions After gamma irradiation and sodium hypochlorite treatments, the stainless steel chips were sonicated in 0.1% sterile peptone water for 3 min and vortexed for 2 min to detach bacterial cells firmly attached. Then, the detached bacterial solution was serially diluted and 0.1 mL aliquots of from appropriate dilutions were plated onto plate count agar (Difco Laboratories) for enumerations of both *S. aureus* and *P. aeruginosa*. The plates in duplicate were incubated at 37°C for 48 hr. The bacterial counts of *S. aureus* and *P. aeruginosa* were expressed as mean log CFU/cm^2 for cells in biofilms and mean log CFU/mL for bacterial suspensions.

Statistical analysis Each mean was acquired from 3 replicate experiments conducted on separate days. Microbial count data was analyzed using an analysis of the variance (ANOVA) and the general linear models (GLM) procedure of a Statistical Package for Social Science 10.05 (32). Fisher's least significant difference test was used to separate treatment means at a significance of $p<0.05$.

Results and Discussion

Inactivation of *S. aureus* and *P. aeruginosa* biofilms by gamma irradiation Gamma irradiation reduced the bacterial counts of *S. aureus* and *P. aeruginosa* in both biofilms and in suspension (Table 1). The bacterial attachment rates of the two test microorganisms were significantly different even when similar inoculum levels (9.14 and 8.86 log CFU/mL) were applied. The 1 hr- and overnight-inoculated bacterial cell counts were 7.06 and 8.03 log CFU/cm^2 in *S. aureus* biofilms and 4.59 and 7.50 log CFU/cm^2 in *P. aeruginosa* biofilms, respectively (Table 1). Similarly, Sommer *et al.* (33) reported that adherent populations of *Psuedomonas fluorescens* was proportional to bacterial suspension concentrations and Sinde and Carballo (34) found that more *Listeria monocytogenes* attached to various surface materials than *Salmonella* spp. *P. aeruginosa* cells both in biofilms and bacterial suspensions were reduced to below the limit of detection (2 log CFU/cm^2) by 2 kGy irradiation while *S. aureus* cells were effectively reduced to 3 log CFU/cm^2 by 2 kGy irradiation (Table 1). Bacterial counts of the two test microorganisms also indicated that *S. aureus* cells were more resistant to gamma irradiation than *P. aeruginosa* cells (Table 1). These results were similar to those published by Tsuji (35) who reported that the radiation D-value of *S. aureus* was slightly higher than that of *P. aeruginosa*.

Several studies reported that bacterial cells in biofilms were more resistant to various disinfecting methods than those in culture suspension. These disinfecting methods include sodium hypochlorite and benzalkonium chloride exposure (36), antibiotics such as tobramycin and cephalexin (37), trisodium phosphate (38), etc. The present study also showed that *S. aureus* and *P. aeruginosa* cells in biofilms were more resistant to gamma irradiation than those in

culture suspension (Table 1).

Even though gamma irradiation is an effective means to eliminate microbial contamination from various substances, gamma irradiation for microbial decontamination in biofilms has not been studied. Recently, Niemira and Solomon (39) reported that gamma irradiation effectively reduces the populations of both planktonic and biofilm-associated *Salmonellae* and the antimicrobial efficacy of gamma irradiation is preserved or enhanced in treatment of biofilm-associated bacteria. In this study, bacterial counts of both *S. aureus* and *P. aeruginosa* cells in biofilms were higher than those in culture suspension (Table 1). However, we concluded that gamma irradiation can effectively eliminate bacterial cells in biofilms even if these cells are less sensitive than cells in suspension to gamma irradiation. It is likely that bacterial cells in suspension were inactivated by the primary action of oxygen and hydroxyl radicals formed by water radiolysis as well as direct damage of ionizing energy. In addition, it was suggested that the microstructural polysaccharides of biofilm may serve a protective role for biofilm-associated pathogens (40).

Inactivation of *S. aureus* and *P. aeruginosa* biofilms by sodium hypochlorite *S. aureus* and *P. aeruginosa* cells in bacterial suspensions were considerably inactivated by sodium hypochlorite and the lowest concentration of sodium hypochlorite (30 ppm) reduced the bacterial counts to below the limit of detection (1 log CFU/mL; Table 2). However, the bacterial cells in biofilms were very resistant and even at concentrations of 400 ppm, the highest concentration used in this study, sodium hypochlorite could not effectively eliminate the microbial population (Table 2). These results are similar to those reported in other studies. For example, Luppens *et al.* (36) found that *S. aureus* cells in biofilms were 600 times more resistant to hypochlorite than planktonic cells. Joseph *et al.* (41) also stated that biofilm cells of *Salmonella* were much more resistant to sanitizers compared to planktonic cells and the bacterial resistance to treatment with sanitizers varied depending on the surfaces.

The different antimicrobial efficacy of sodium hypochlorite between cells in biofilm and bacterial suspension is considered to be caused by the limited penetration of sanitizers into biofilms (42, 43). The cells which are associated with the biofilms have advantages in growth and survival over planktonic cells and these advantages result from the formation of an EPS matrix that surrounds the biofilm, protects it from attack by sanitizer, and supplies it with nutrients (44). Niemira (40) suggested that these polysaccharide elements may protect biofilm-associated pathogens. de Beer *et al.* (43) stated that the loss of antimicrobial efficiency might be attributed either to metabolic change or to EPS that react with free chlorine or prevent its diffusion through biofilms.

In this study, *S. aureus* and *P. aeruginosa* cells in

Table 1. Reductions in the bacterial counts of *S. aureus* and *P. aeruginosa* in biofilms and suspension by gamma irradiation

Radiation dose (kGy)	*S. aureus*			Radiation dose (kGy)	*P. aeruginosa*		
	Biofilm		Bacterial suspension		Biofilm		Bacterial suspension
	1hr-Inoc.[1)]	ON-Inoc.[2)]			1hr-Inoc.	ON-Inoc.	
0	7.06[a3)]	8.03[a]	9.14[a]	0	4.59[a]	7.50[a]	8.86[a]
0.5	6.31[b]	6.83[b]	6.27[b]	0.5	2.23[b]	3.41[b]	4.03[b]
1	4.38[c]	5.22[c]	4.63[c]	1	ND	2.11[c]	2.45[c]
2	ND[4)]	3.03[d]	2.44[d]	2	ND	ND	ND

[1)]Biofilm formed by inoculation for 1 hr.
[2)]Biofilm formed by inoculation overnight.
[3)]Means with different letters are significantly different ($p<0.05$).
[4)]Not detected (<2 log CFU/cm^2 or log CFU/mL).

Table 2. Reductions in the bacterial counts of *S. aureus* and *P. aeruginosa* in biofilms and suspension by sodium hypochlorite

Sodium hypochlorite (ppm)	*S. aureus*		Sodium hypochlorite (ppm)	*P. aeruginosa*	
	Biofilm 1hr-Inoc.[1)]	Bacterial suspension		Biofilm 1hr-Inoc.	Bacterial suspension
0	5.56[a2)]	5.36	0	5.17[a]	5.18
30	4.99[ab]	ND[3)]	30	4.00[b]	ND
50	4.51[c]	ND	50	3.49[c]	ND
100	4.42[cd]	ND	100	3.18[cd]	ND
200	3.50[e]	ND	200	3.04[d]	ND
400	3.09[f]	ND	400	2.91[de]	ND

[1)]Biofilm formed by inoculation for 1 hr.
[2)]Means with different letters are significantly different ($p<0.05$).
[3)]Not detected (<2 log CFU/cm^2 or log CFU/mL).

biofilms were barely inactivated by one of the most widely used sanitizers, sodium hypochlorite in agreement with other studies. Gibson *et al.* (45) stated that it is essential to break up and remove the EPS matrix in order to inactivate harmful microorganisms associated with biofilms. Our findings show that gamma irradiation is an efficient means to eliminate microorganisms protected by EPS in biofilms since this ionizing energy readily penetrate through biofilms and kill bacterial cells. For these reasons, we concluded that bacterial cells in biofilms were more resistant to exposure to sanitizing agents than bacterial cells in suspension. Consequently, biofilms may cause serious problems such as potential pollution, health and safety hazards, and substantial economic loss in many industrial areas. Fortunately, bacterial cells in biofilms were effectively eliminated by gamma irradiation and gamma radiation could be used as an effective biofilm sanitizing agent. Further studies for the potential use of gamma irradiation as a biofilm sanitizer should be conducted in food processing facilities.

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